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# THESE

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Par **Ahmed SHEIKH HASSAN**
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**Sujet**

## Microparticules à libération prolongée et réduisant la libération intiale prématurée

## Prolonged release microparticles able to reduce the initial burst effect

**MEMBRES DU JURY**

**Juges :**

| | |
|---|---|
| **Professeur Philippe Maincent** (Faculté de Pharmacie, Nancy) | **Directeur de thèse** |
| **Docteur Anne Sapin** (Faculté de Pharmacie, Nancy) | **Co-directeur de thèse** |
| **Professeur Alf Lamprecht** (Faculté de Pharmacie, Besançon) | **Examinateur** |
| **Professeur Pierre Leroy** (Faculté de Pharmacie, Nancy) | **Examinateur** |

**Rapporteurs :**
**Professeur Hatem Fessi** (Faculté de Pharmacie, Lyon)
**Professeur Marie-Christine Andry** (Faculté de Pharmacie, Reims)

**« L’essentiel est invisible pour les yeux... »**

*Antoine de Saint Exupery*

*A mon dieu le miséricordieux*
*A tous ceux  qui travaillent  pour guider l'humanité vers le chemin de la Vérité*
*A mes parents*

***Tribute to Dr. Nathalie Ubrich***

***From her friends of the laboratory***

*Nathalie Ubrich was only 48 years old when she passed away on 24 April 2007. She died in the middle of her young research career without being able to achieve all she wanted to do.*

*After obtaining her Ph.D. at the University of Nancy in 1994, she joined our laboratory in 1996 as a free-lance researcher and was later appointed officially to a research position in the INSERM (French medical research organization). Throughout this period, she worked for the success of the laboratory and the students she was supervising (graduate students and trainees from all over Europe through Erasmus exchanges). Many publications reflect the tremendous amount of work that our small team (one professor and two assistant professors) was able to achieve while she was part of it. As well as working hard and teaching the students in the laboratory, she was always looking for new challenges and ideas by carefully reading and analysing the scientific literature.*

*Nathalie showed perfect team spirit. She could not think of research without thinking of collaboration to expand our network or to share experience and fruit of the results with all those involved, without forgetting the technicians and students.*

*She was like this before her illness was discovered 2.5 years ago. Afterwards, she continued to behave in same way but more intensely and taking on more responsibility. She used to tell us that she has so many things to finish before it was too late. She never complained about her illness. On the contrary, she did not want to talk about it. She wanted to prove that she could defeat this invisible enemy. Many people in the Faculty did not know that she was ill and would never have been able to guess. She never missed a working day except when her treatment lasted for a whole day and then she was there the next morning, pushing and encouraging everybody.*

*More than anything, she wanted us to continue and to have a fruitful research activity.*

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# TABLE DES MATIERES

# INTRODUCTION GENERALE

Les études sur les formes multiparticulaires injectables ont débuté dans les années 80 mais il a fallu attendre les années 90 pour voir les autorités de santé allouer les premières autorisations de mise sur le marché. Le tableau suivant, étendu également aux formes monolithiques, fait le point sur les médicaments aujourd'hui disponibles.

| Forme | Type | Nom | Principe actif | Approbation |
|---|---|---|---|---|
| **Microsphères** | LP | Lupron depot (US)<br>Enantone LP (EU) | Leuprolide<br>Leuproréline | 1989 (US)<br>1996 (EU) |
| | LP | Sandostatin LAR (US)<br>Sandostatine LP (EU) | Octreotide | 1998 (US)<br>1995 (EU) |
| | LP | Neutropin depot (US) | Somatropine | 1999 (US) |
| | LP | Decapeptyl LP (EU)<br>Trelstar depot (US) | Triptoreline | 1995 (EU)<br>2000 (US |
| | LP | Risperdal Consta (US, EU) | Risperidone | 2003 (US, EU) |
| **Liposomes** | V | Daunoxome (US, EU) | Daunorubicine | 1996 (US, EU) |
| | V | Ambisome (US, EU) | Amphotericine B | 1997 (US)<br>1998 (EU) |
| | V, LP | Depocyt (US, EU) | Cytarabine | 1999 (US)<br>2001 (EU) |
| | V | Myocet (EU) | Doxorubicine | 2000 (EU) |
| | V | Caelyx (EU), Doxil (US) | Doxorubicine | 1995 (US, EU) |
| **Complexes lipidiques** | V | Ambelcet (US, EU) | Amphotericine B | 1995 (US, EU) |
| | V | Amphotec (EU) | Amphotericine B | 1997 (EU) |
| | V | Visudyne (US) | Verteporfine | 2000 (US) |
| **Implants** | LP | Norplant (US, EU) | Levonorgestrel | 1990 (US)<br>1994 (EU) |
| | V, LP | Gliadel (US, EU) | Carmustine | 1996 (US)<br>1998 (EU) |
| | LP | Zoladex (EU, US) | Goséréline | 1995 (EU)<br>1998 (US) |
| | LP | Viadur (US) | Leuprolide | 2000 (US) |
| | LP | Implanon (EU) | Etonogetrel | 1999 (EU) |

Formes parentérales à libération prolongée (LP), hors suspensions, ou à distribution modifiée (V, vecteurs)
États unis (US), Europe (EU). Sources FDA/CDER, données US et AFFSAPS, données EU.

On peut ainsi remarquer que les principes actifs incorporés correspondent à des domaines thérapeutiques bien particuliers : cancérologie, antibiothérapie, hormonothérapie, psychiatrie.

Bien que limitées en nombre, toutes ces spécialités ont trouvé une place importante dans l'arsenal thérapeutique et de nombreuses autres molécules sont aujourd'hui développées avec des systèmes multiparticulaires.

Outre une prolongation d'action remarquable des principes actifs incorporés permettant une amélioration de la qualité de vie des patients, des formes pharmaceutiques comme les

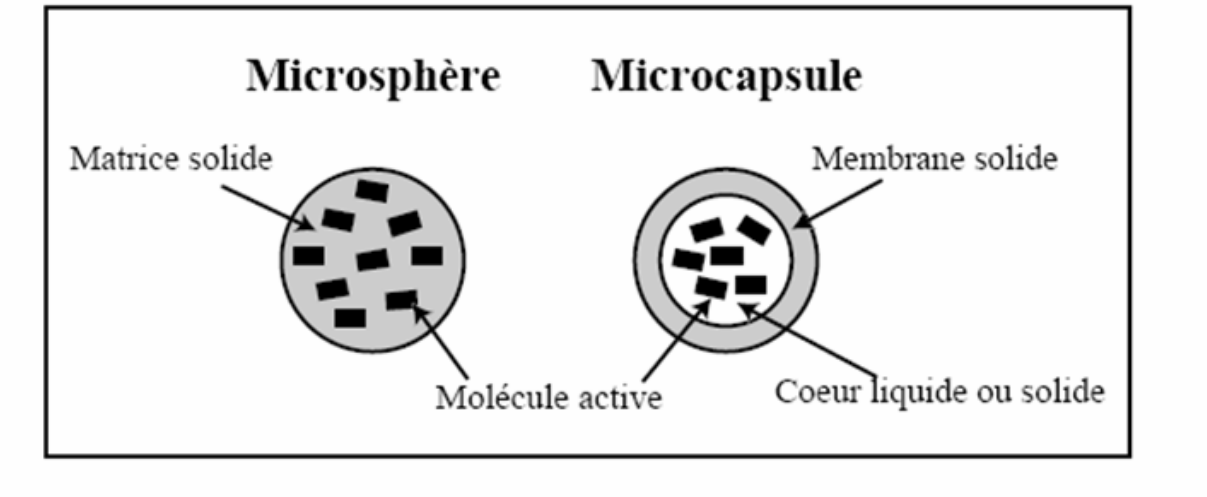
liposomes peuvent aussi répondre à des objectifs plus ambitieux tels le ciblage des principes actifs ou au minimum une réduction de la toxicité systémique des molécules considérées.

Les formes galéniques qui seront étudiées pendant ce travail correspondent aux nano- et microparticules polymériques. Les nano- et microparticules sont des systèmes dont la taille est comprise entre 10 et 1000 nm (nanoparticules) et de 1 à 100 µm (microparticules). Elles sont constituées d'un matériau capable de retenir les molécules actives par séquestration ou adsorption. Les matériaux enrobants utilisés sont des polymères d'origine naturelle ou synthétique voire des lipides.

Selon la nature de ces systèmes, on peut distinguer :

- les systèmes matriciels (nano- et microsphères) : dans ce cas, la particule est constituée d'un réseau polymère ou lipidique continu formant une matrice dans laquelle se trouve dispersée ou dissoute l'espèce que l'on souhaite encapsuler.
- les systèmes réservoirs (nano- et microcapsules) : dans ce cas, la particule est constituée d'un coeur liquide (plus ou moins visqueux) ou solide contenant l'espèce encapsulée, entouré d'une écorce solide et continue de matériau enrobant.

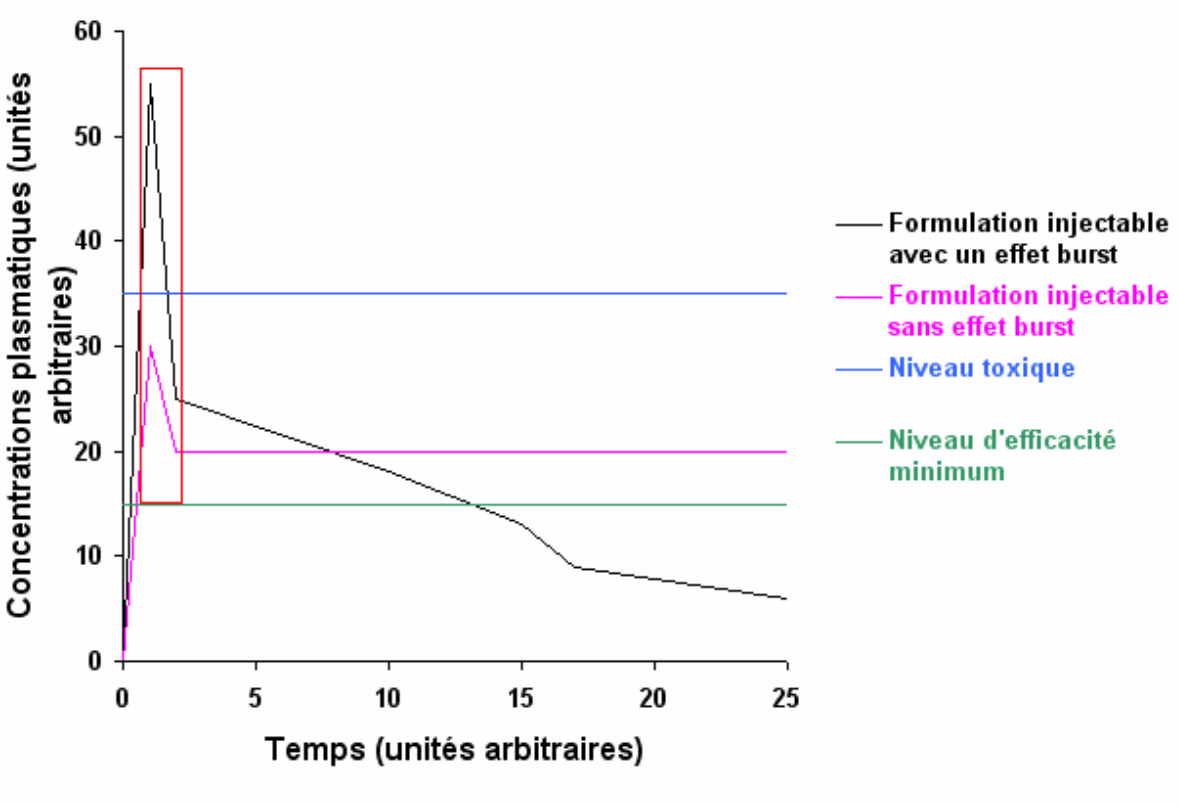
Dans les deux types de systèmes, l'espèce encapsulée peut aussi être adsorbée à la surface de la matrice. La figure ci-dessous décrit la structure schématique (matricielle ou réservoir) dans le cas des microparticules.




La cinétique de libération des principes actifs encapsulés est un élément critique des formes multiparticulaires dispersées. En effet, ces formes doivent être capables de libérer, selon une cinétique constante préalablement définie et de façon reproductible, le principe actif encapsulé. Or certaines de ces formes ont été mises au point pour délivrer de façon constante le principe actif pendant plusieurs mois. Aujourd'hui certains laboratoires pharmaceutiques cherchent à développer des formes multiparticulaires susceptibles de libérer leur principe actif

pendant une période pouvant s'étendre sur une année entière. Dans ce type de mise au point, les 2 principaux paramètres à prendre en compte sont la nature et les propriétés physico-chimiques du polymère mais aussi la masse de principe actif à incorporer qui sera d'autant plus importante que la libération *in vivo* doit être longue. D'autres paramètres comme la solubilité aqueuse du principe actif ou le mode de préparation doivent évidemment être également considérés.

Un des problèmes majeurs des formes multiparticulaires, avant que la cinétique de libération ne devienne constante et assure ainsi une imprégnation régulière de l'organisme (du type perfusion intraveineuse), est la libération initiale d'une partie du principe actif encapsulé. Cette libération initiale peut avoir des conséquences cliniques importantes pouvant expliquer une toxicité initiale liée à une concentration trop élevée du principe actif dans l'organisme. Cette libération initiale rapide et éventuellement importante est appelée effet « burst » ou « burst effect » en anglais. La figure ci-après reproduit schématiquement les conséquences d'une libération initiale trop importante.




De plus, en dehors des problèmes toxiques éventuels, la libération initiale d'une quantité importante de principe actif peut diminuer la quantité encapsulée. En conséquence, cela peut aussi entraîner des modifications de libération dans les dernières semaines de la vie des

systèmes multiparticulaires soit en ralentissant la cinétique de libération du principe actif soit en abaissant sa concentration sanguine.

Cette libération initiale importante est considérée comme un problème majeur des formes multiparticulaires injectables et de nombreuses recherches, tant académiques qu'industrielles, ont été initiées pour essayer de la ralentir au maximum voire de totalement la faire disparaître.

Dans ce travail, nous proposons une voie originale pour chercher à maîtriser le phénomène du « burst ». Il s'agit en effet d'associer les potentialités de deux systèmes multiparticulaires, à savoir les nano- et microparticules. L'idée à la base de ce travail est d'encapsuler des nanoparticules chargées en principe actif dans des microparticules. Globalement l'échelle de taille est de 1 à 100 entre les nanoparticules et les microparticules ce qui permet d'envisager théoriquement une encapsulation des premières dans les secondes. L'hypothèse de départ est que le principe actif étant encapsulé dans un système polymère à l'intérieur d'une autre structure polymère, sa libération initiale sera fortement ralentie et qu'il sera ainsi possible de diminuer fortement ou de faire disparaître le « burst ». En outre ce système double, appelé aussi microparticules composites, devrait pouvoir assurer une libération largement ralentie par rapport aux microparticules simples (sans nanoparticules à l'intérieur) et éventuellement permettre des libérations sur des périodes encore plus prolongées.

Pour visualiser les potentialités de ce concept, nous avons d'abord conduit une première étude *in vitro* avec comme objectif de démontrer la possibilité pour des nanoparticules d'un premier polymère biodégradable [poly(ε-caprolactone)] d'être encapsulées dans la matrice de microparticules fabriquées avec un second polymère non biodégradable [éthylcellulose ou un copolymère d'esters d'acide acrylique et d'acide méthacrylique (Eudragit® RS)]. Deux principes actifs correspondant soit à une petite molécule lipophile (ibuprofène) soit à une molécule peptidique hydrophile (acétate de triptoréline) ont été choisis comme modèles pour démontrer la faisabilité du concept. Ce travail initial a fait l'objet d'un premier article qui a été publié dans International Journal of Pharmaceutics en 2007.

Une fois la démonstration de l'encapsulation de nanoparticules dans des microparticules validée, il était important de vérifier si les microparticules composites étaient également capables de réduire l'effet « burst » *in vivo*. Il est évident que ces systèmes étant destinés à la voie parentérale, les polymères utilisés pour la fabrication des nanoparticules et des microparticules devront être biodégradables ce qui nous a conduit à incorporer des nanoparticules de poly(ε-caprolactone) dans des microparticules à base de copolymère d'acide lactique et glycolique.

Dans cette deuxième partie de notre recherche, nous avons sélectionné à nouveau l'ibuprofène qui correspond à une molécule de faible masse moléculaire et donc plus susceptible de subir une importante libération initiale. Pour réaliser les dosages plasmatiques avec suffisamment de précision, nous avons été amenés à améliorer les techniques analytiques de l'ibuprofène actuellement décrites. Nous avons ainsi mis au point et validé une technique permettant une limite de quantification beaucoup plus basse. Notre technique analytique a fait l'objet d'une deuxième publication actuellement sous presse dans la revue Drug Development and Industrial Pharmacy. La deuxième molécule était toujours de nature peptidique puisqu'il s'agit de l'insuline. En effet des difficultés d'approvisionnement et de dosage nous ont conduits à remplacer l'acétate de triptoréline.

Les travaux relatifs à ces 2 molécules sont présentés sous forme d'articles et sont actuellement soumis à European Journal of Pharmacy and Biopharmaceutics pour l'ibuprofène et Journal of Controlled Release pour l'insuline.

La présentation de mes travaux personnels est précédée par l'analyse des travaux antérieurs où seront décrits les mécanismes physico-chimiques qui sont à l'origine du « burst » après administration parentérale de différentes formes multiparticulaires ainsi que des solutions proposées par différents groupes pour y remédier.

Une discussion générale sur l'ensemble des résultats obtenus *in vitro* et *in vivo* dans le cas des microparticules composites permettra de faire une analyse d'ensemble des différents résultats.

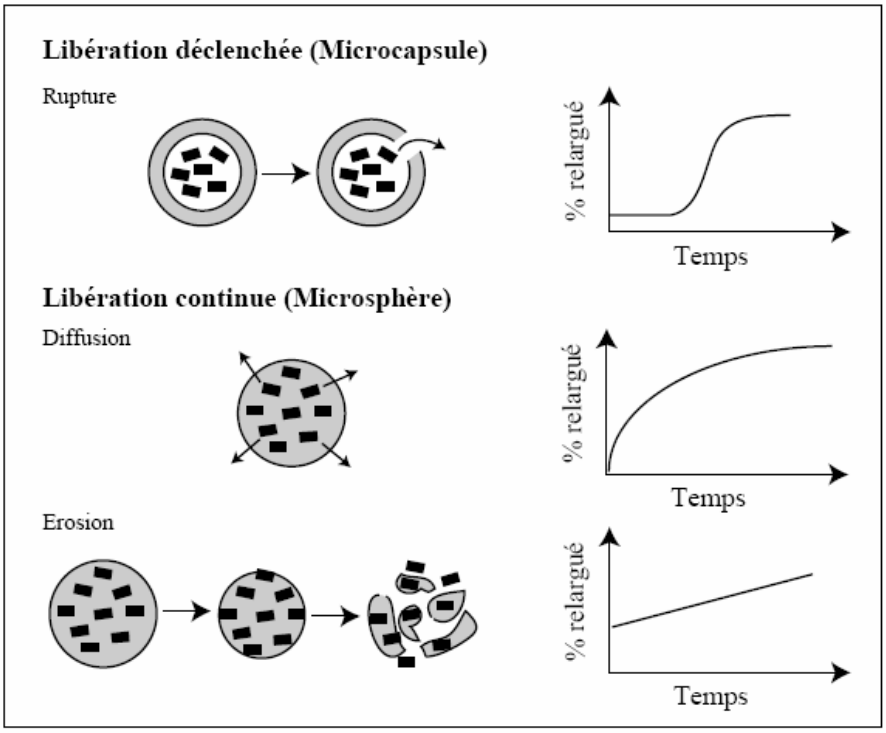
# REVUE BIBLIOGRAPHIQUE

## Initial burst release from polymeric nano/microparticles

Depuis quelques années, un des axes principaux de la recherche dans le domaine de la microencapsulation concerne les vecteurs nano et microparticulaires de nature polymérique ou lipidique.

Ces systèmes sont nombreux et variés aussi bien dans leur composition que dans leur mode d'obtention et les paramètres qui gouvernent leurs propriétés physicochimiques sont tout aussi nombreuses. Nous nous attacherons donc à présenter uniquement leurs différents paramètres gouvernant leurs propriétés de libération des principes actifs.

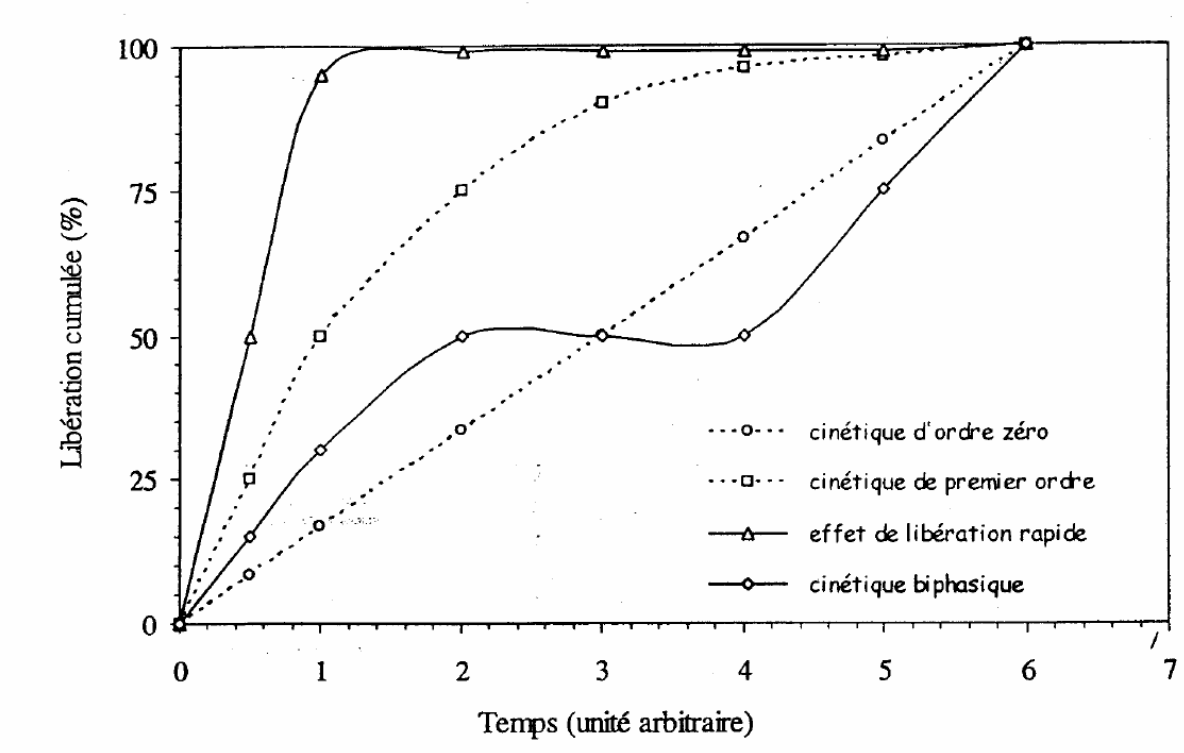
En fonction de l'application visée, les nano/microparticules sont conçues pour libérer le principe actif, soit de manière déclenchée, soit de manière continue (figure ci-dessous). Les systèmes à libération déclenchée sont généralement des microcapsules formées d'une membrane de faible perméabilité qui vont libérer brutalement leur contenu par éclatement de cette membrane. La rupture est alors déclenchée, soit par une pression (mécanique ou osmotique), une variation de température, une variation de pH ou encore la dégradation enzymatique de la membrane. Les systèmes à libération continue sont majoritairement des microsphères. Les mécanismes mis en jeu sont, soit la diffusion de l'espèce à travers la matrice, soit la dégradation (érosion) ou la dissolution de la matrice ou encore un couplage des deux (Shah et al., 1992).




**Représentation schématique des différents modes de relargage des microparticules et allure des cinétiques de relargage (D'après Kreuter, 1994)**

Les profils de la figure précédente représantent l'allure générale des différents modes de relargage des microparticules (Kreuter, 1994). Cependant, les cinétiques de libération de l'espèce encapsulée sont avant tout déterminées par les caractéristiques physicochimiques du système. En plus des mécanismes généraux, il existe d'autres voies par lesquelles la vitesse de libération du principe actif peu être augmentée. Par exemple, l'application d'un champ magnétique externe sur des particules contenant une sonde magnétique permet de cibler le site de la libération du principe actif. Les ultrasons peuvent également accentuer l'érosion de la matrice de polymère, accélérant ainsi la libération.

La libération des principes actifs étant gouvernée par de multiples paramètres, il en résulte divers profils de libération cumulée au cours du temps dans le cas de systèmes biodégradables et qui sont schématisés dans la figure ci-dessous :




- l'effet de libération rapide, « burst effect», caractérise une libération immédiate du principe actif. Le vecteur n'est donc pas efficace pour une libération contrôlée/prolongée après l'injection. Ce type de profil de libération est observé généralement dans les cas de principe actifs adsorbés en surface des particules, lorsque les interactions principe actif-polymère ne sont pas très fortes, dans le cas d'une distribution inhomogéne du principe actif dans la matrice ou encore dans le cas de particules poreuses. le principe actif est alors libéré avant que la matrice ne se dégrade.
- Une cinétique d'ordre zéro reflète une libération idéale et totalement contrôlée.

- La cinétique de premier ordre correspond à la majorité des profils observés lors de la libération de principes actifs encapsulés dans les nano/microparticules. Au début de la libération la vitesse est rapide (diffusion du principe actif encapsulé proche de la surface) puis se ralentit (diffusion plus lente au travers de la matrice).
- La cinétique biphasique reflète un mode de libération généralement dépendant de la dégradation de la matrice. Dans un premier temps, la cinétique de libération correspond à la diffusion du principe actif au proche voisinage de la surface. Dans un second temps, il faut attendre la dégradation de la matrice pour que la totalité de principe actif soit libérée.

Les caractéristiques physicochimiques de système particulaires sont fonction du type de polymère ou de lipide utilisé mais aussi du processus de fabrication. Plus de 200 types de formulation sont actuellement citées dans la littérature et chacune d'elle a une influence sur l'arrangement de la microparticule mais aussi son taux d'encapsulation (Pinto Reis *et al*., 2006; Couvreur *et al.*, 1995).

Le lien établi entre la particule et la molécule active influence aussi sa cinétique de libération.

La molécule active peut être insérée dans le système au cours du processus de fabrication ou après formation de la particule. Dans le premier cas, un couplage covalent entre la molécule et le polymère peut s'établir. La molécule peut se trouver sous forme de solution solide ou dispersée au sein de la matrice. Dans le deuxième cas, après fabrication de la particule, la molécule active est adsorbée à sa surface. Elle peut alors rester à la surface ou diffuser vers la matrice et former une solution solide (Kreuter, 1994).

Les principaux paramètres qui gouvernent les cinétiques de libération de nano/microparticules sont :

- les paramètres externes tels que la température, le pH, la présence d'enzyme dans le milieu,
- les paramètres intrinsèques de principes actifs tels que sa solubilité, poids moléculaire et sa température d'ébullition/fusion,
- les paramètres intrinsèques à la matrice tels que la taille de la particule, l'épaisseur de la membrane, la structure chimique du polymère, sa masse moléculaire, sa cristallinité et sa porosité.

# Initial burst release from polymeric nano/microparticles

## 1- Definition of initial burst release

Over the years of controlled release research, different systems, ranging from coated tablets and gels to biodegradable microspheres and osmotic systems, have been explored experimentally and computationally to get predesigned release profiles. In many of the controlled release formulations, immediately upon placement in the release medium, an initial large bolus of drug is released before the release rate reaches a stable profile. This phenomenon is typically referred to as 'burst release'. As shown in fig. 1, burst release leads to higher initial drug delivery and also reduces the effective lifetime of the device. Because burst release happens in a very short time compared to the entire release process, it has not been specifically investigated in most published reports, and it has been ignored in most mathematical models. However, among the plethora of controlled release publications, burst phenomena have often been observed and studied (Atkins *et al*., 1993; Ficek *et al.,* 1993; Shively *et al*., 1995; Patil *et al.,* 1996; Narasimham and Langer 1997 (a); Brazel and Peppas 1999; Huang and Brazel 2001). Several researchers have observed burst release without giving advanced explanations (Atkins *et al*., 1993; Ficek *et al.,* 1993; Shively *et al.,* 1995; Brazel and Peppas 1999); some tried to find the mechanisms of burst and prevent it technologically (Patil *et al*., 1996; Huang and Brazel 2001; Yeo and Park 2004) and some have made an effort to include burst in models to simulate the release process (Narasimham and Langer 1997 (a); Brazel and Peppas 1999; Huang and Brazel 2001). At the opposite end of spectrum, burst release has been used to deliver drugs at high release rates as part of the drug administration strategy (Setterstrom *et al*., 1984).

Normally short in duration, burst release is worth thorough study due to the high release rates that can be reached in the initial stages after activation. The burst effect can be viewed from two perspectives: it is often regarded as a negative consequence of creating long-term controlled release devices, or, in certain situations, rapid release or high initial rates of delivery may be desirable (Table 1).

Burst release may be the optimal mechanism of delivery in several instances. One of the current difficulties with burst release is that it is unpredictable, and even when the burst is desired, the amount of burst cannot be significantly controlled. It has been shown that many drugs need to be administered at varying rates, and for some drugs, such as those used at the

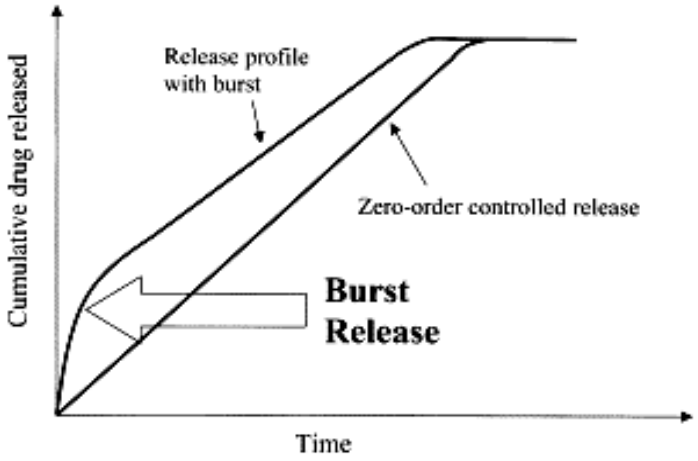



***Figure 1.*** Schematic showing the burst effect in a zero-order drug delivery system. (From Huang and Brazel 2001)

| Favorable burst release situations | Negative burst release effects |
|---|---|
| 1-Wound treatment (burst release followed by a diminishing need for drug ) | 1-Local or systemic toxicity (high drug concentrations) |
| 2-Encapsulated flavors | 2-Short half-life of drugs *in vivo* (rapid loss in activity) |
| 3-Targeted delivery ( triggered burst release) | 3-Economically and therapeutical wasteful of drug |
| 4-Pulsatile release | 4-Shortened release profile; requires more frequent dosing |

***Table I.*** Applications where burst release may be advantageous or detrimental (from Huang and Brazel 2001).

beginning of wound treatment, an initial burst provides immediate relief followed by prolonged release to promote gradual healing (Setterstrom *et al.*, 1984). Food companies also have an interest in the development of burst release systems: coatings are desired to protect flavors and aromas during processing and storage, but must allow rapid release when the product is consumed (Madene *et al*., 2006). Recent advances in the ability to target specific cells and organs, through either surface modification or implantation, attempt to locate a specific delivery, and either burst or prolonged release may be desired at that site, after the coating has served its purpose of sequestering the drug to protect it from denaturation and first-pass metabolism. In several pulsatile delivery processes, burst release may also be a goal,

so that the active agent can be delivered rapidly upon changes in environmental conditions that trigger the release.

Most of the published work related to burst release has been carried out in the pharmaceutical field, because of the important roles of drug intital burst release; both favorable and unfavorable, researchers have begun to focus on the study of its mechanism. One of the most important questions in developing controlled release devices is to know how to predict when burst release will occur and quantify its effects a priori. Answers have focused on ways to prevent it from occurring in parenteral controlled release formulations because the drug released in this period is not available for prolonged release and may lead to drug concentrations near or above the toxic level *in vivo* (Shively *et al*., 1995 and Jeong *et al*., 2000) which is real challenge in medications with "narrow therapeutic index" drugs (include monoamine oxidase (MAO) inhibitors, warfarin, oral hypoglycaemic agents, proteins, digoxin, opiate narcoties and many cancer drugs). Any drug released during the burst stage may also be metabolized and excreted without being effectively utilized (Thote *et al.,* 2005) so its problem in medications with "short half-life" or "high-alert" drugs. Even if no harm is done during the burst release, this amount of drug is essentially wasted, and the ineffective drug usage may have therapeutic and economic effects.

Burst release generaly concerns low molecular weight drugs which are more likely to have burst release profiles due to their molecular size and osmotic pressures which accentuate the concentration gradient. (Huang and Brazel 2001; Yeo and Park 2004).

## 2- Mechanisms of burst release from controlled release devices

Among various delivery systems (liposomes, emulsions, gel, lipid particles…etc), only polymeric devices (nano/microparticles) will be described in the following parts. Two different types of polymeric delivery systems can be formulated: reservoir systems, about which burst release has been explored and defined more explicitly, and matrix systems.

### 2.1. Burst release from polymeric reservoir systems

Polymeric reservoir drug delivery devices (microcapsule, nanocapsule) consist in an inert membrane enclosing the active agent, which, upon activation, diffuses through the membrane at a finite, controllable rate (Baker 1987). These systems are especially good at achieving zero-order, or constant, drug delivery, although there is a risk of dose dumping due to minor flaws in capsule coatings that lead to significant burst release even prior to patient administration. Burst release has been observed in membrane reservoir systems, and credited

to the storage effect (Baker 1987). This happens when reservoir systems are stored for some time prior to use, and the agent saturates the entire membrane enclosing the drug reservoir. When placed in a release medium, the agent that has diffused to the surface of the membrane is released immediately, causing a burst effect. Because in the case of capsule systems, only few parameters contribute to burst effect, the amount of drug released with an initial burst, $M_t$, from these systems is estimated by:

$$M_t = \frac{DC_o}{l}\left(t + \frac{l^2}{6D}\right)$$

Where $D$ is the drug diffusion coefficient, $C_o$ is the drug concentration on the inside of the membrane, and $l$ is the membrane thickness (Baker 1987), with a given burst of $C_o l/6$, but the release profile during burst stage ($t>0$) was not predictable.

## 2.2. Burst release from polymeric matrix systems

Matrix drug delivery systems (nanospheres, microspheres) where the drug is dispersed in a porous network. These systems include both swellable and non-swellable matrices. Mechanisms of burst release in matrix systems based on particle-forming directly in tissue or medium ("*in situ* systems") are different from those of matrix systems like nano/microspheres prepared before administration.

### 2.2.1 *In situ* forming systems

Burst release is observed in these systems because the polymer particles do not set immediately, causing some drug not to be successfully encapsulated, thus allowing free drug to 'release' in a burst. These systems based on injectable polymer have the benefit of localizing internal treatment in a minimally invasive manner. In one such system, an initial burst of naltrexone from an injectable formulation based on poly (lactide-co-glycolide) (PLGA) was attributed to slow implant gel formation (Shively *et al*., 1995).

### 2.2.2 Systems prepared before administration

Drug release from the matrix system during the initial release stage depends on how successfully the diffusion of water into the particles happened and on diffusional escape of the drug through channels existing in the polymer matrix during the first few minutes, hours or days. The degree of initial burst from the matrix systems depends on the ability of the

polymer matrix to encapsulate the drug, thereby making it unavailable for immediate diffusion (Mehta *et al.,* 1996).

Moreover, in the case of biodegradable polymeric systems such as [poly-lactic acid (PLA), poly (ε-caprolactone) (PCL) and PLGA] drug burst release depends at first on diffusion-based process through polymeric matrix whereas the subsequent release is a function of the polymer degradation ranging from weeks to months (depending on degradation mechanism: hydrolysis, enzymatic reaction…etc), so anthore burst effect may happened after polymer degradation.

Thus, it is in these polymeric matrix systems that the burst release is the most difficult to control, to predict and to mathematicaly modelise. We will try to summarize in the following part the factors affecting the drug release and particularly this initial burst release.

## 3-Factors affecting the drug release rate from polymeric particles (including drug initial burst release)

Controlled release is an attainable and desirable characteristic for drug delivery systems. The factors affecting the drug release rate revolve around the structure of the matrix where the drug is contained and the chemical properties associated with both the polymer and the drug. A drug encapsulated in a slowly degrading matrix provides the opportunity for slower release effects, but polymer degradation is not the only mechanism for the release of a drug. The drug release is also diffusion controlled as the drug can travel through the pores formed during sphere hardening. In some cases, drugs containing nucleophilic groups can cause increased chain scission of the polymer matrix, which also increases the rate of drug expulsion. Polymer molecular weight, drug distribution, polymer blending, crystallinity, and other factors are important in manipulating release profiles. The most desirable release profile would show a constant release rate with time. However, in many cases release profiles are more complicated and often contain two main expulsion processes: the first being an initial burst of expelled medication from the sphere surface; the second, a usually more constant stage with release rates depending on diffusion and degradation (Le Corre *et al.,* 1994; Ghaderi *et al*., 1996 and Mogi *et al*., 2000). An example showing the initial burst and linear release by Yang *et al.* 2000 (b) is shown in fig. 2.

Some researchers have been able to achieve a relatively constant release after the initial burst, some have been able to achieve close to zero-order kinetics without a significant burst effect, and others have obtained even more complex but adjustable profiles depending on the desired application (Narayani and Rao, 1996; Makino *et al*., 2000; Yang *et al*., 2000 (b); Berkland *et*

*al*., 2002 and Kakish *et al.*, 2002). In the following discussion, the factors responsible for different release profiles (and more precisely burst release) will be discussed in terms of physical and chemical properties of the microsphere.

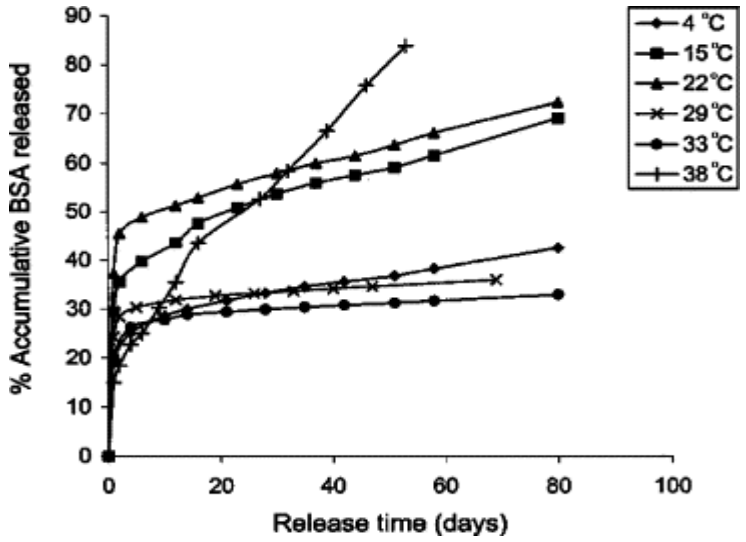



***Figure 2.*** Release profiles of bovine serum albumin from PLA and PLGA microspheres as presented by Yang *et al*. 2000(b). Microspheres formed at lower temperatures show a fast burst process followed by slow continued release. Microspheres formed at the highest temperature exhibit the fastest release rate.

### 3.1. Drug properties affecting the burst release

The chemical and physical structure [drug form salt or molecular (acid or base), solubility, crystallinity, charge and moleculare wheigt (Mw)] of various drug molecules can deeply affect the burst effect in controlled release systems. In this part we will focalise on how drug solubility and Mw influence burst release effect.

The solubility of drugs as well as their partition coefficients affect the driving forces for release, and can lead to rapid release due to thermodynamic imbalances (Huang and Brazel 2001; Faisant *et al.*, 2006). This effect has been observed by a number of researchers, including Narasimhan and Langer (1997 a, b) who studied release of sodium salicylate and bovine serum albumin (BSA) from hemispherical devices and developed a model capable or predicting burst release based on solute solubility differences and diffusivities.

In the current trends of conclusion studies, the burst effect has been noted to occur especially in systems encapsulating:

- Small Mw lipophilic solutes for example, molecular form of acidic [ibuprofen (Thompson *et al.*, 2007)] or basic drugs [papaverine (Jeong *et al.*, 2003)].

- Small Mw hydrophilic solutes for exemple, salt of acidic [sodium diclofenac (Lin *et al*., 2000, a)] or basic drugs [procaine hydrochloride (Govender *et al*., 1999)] and peptide drugs [triptorelin (Nicoli *et al*., 2001)].
- High Mw hydrophilic drugs for example proteins (immunoglobulin G, α-interferon) or polysaccharide like heparin (Tan *et al*., 2004).

The specific mechanisms for burst release may be radically different in these drugs:

- Small hydrophilic Mw solutes are often highly soluble in aqueous systems and can pass easily through the porous structure of particles even prior to swelling (Govender *et al*., 1999).
- For Small lipophilic Mw solutes, burst release has been affected in generally by the presence of drug crystals on the surface of matrix (because of drug matrix intracrion) and the internal morphology of matrix (porosity, desity) (Mao *et al.*, 2007).
- High Mw hydrophilic drugs burst release observed from controlled delivery systems is often attributed to their heterogeneous distribution inside the polymeric matrix (Yeo and Park 2004) [microspheres with homogeneous protein distribution present a reduced burst release (Wischke *et al.*, 2006)]. But in case of drug surface adhesion and adsorption as for DNA plasmid, burst release effect happened because of the bad distribution of drug onto particle surface or bad interaction with matrix (bad cationic and anionic intraction) (Luo *et al*., 1999).

Finaly, the solubility of the drug may change a lot with the modification of medium parameters of *in vitro* test (pH, temperature, agitation rate, enzyme, surfactant and ionic strength) or with different administation routes *in vivo*. So, burst release effect will realy change as showen by Faisant *et al*., 2006 who noticed that the high increased permeability of the matrix at 37 °C would allow a faster diffusion of small lipophilic drugs molecules through the matrix and so contribute to the observed burst release.

### 3.2 Matrix physical and chemical properties affecting the burst release

In the next part, we will study three principal polymers characteristics which controlled drug release from polymeric particles: polymer's Mw, hydrophilicity and concentration.

#### 3.2.1. Polymer molecular weight (Mw)

Drug initial burst release from particles is affected by the Mw of the polymer used. In general, low Mw polymers lead to a high burst release of the encapsulated drug (Yang *et al.*, 2001). This observation could be partly explained by the fact that the low Mw polymer is more soluble in the organic solvent (when emulsion techniques are used) and undergoes slow solidification to produce more porous microparticles. On the other hand, it can also be

attributed to the smaller size (generally obtained when low Mw polymer used to produced particles) which provides more surface area for drug diffusion (Yang *et al*., 2001). Indeed, the use of low Mw polymers generate a low viscosity of the organic phase (simple/double emulsion techniques) resulting in small size particles whereas larger size and denser internal structure particles are obtained with high Mw polymers (Mao *et al*., 2007).

Moreover, when biodegradable polymers are used, drug release depends on the degradation of particles which also shows a clear dependence on the Mw of the polymer. In spheres initially containing low Mw chains, water can be easily incorporated into polymer matrix and contributes to fast degradation of particles and drug release with high burst. For spheres made from high Mw polymers, the polymer degradation is much slower for long periods of time.

Makino *et al*., (2000) showed pulsatile drug release in high Mw PLGAs microspheres as shown in fig. 3. At lower Mw (19,000), a relatively constant release profile was obtained; increasing the Mw to 44 000 and 74 000 decreased the linearity of release (Makino *et al*., 2000). The rate of drug release from particles containing higher Mw polymers was initially high, followed by a decrease which was then followed again by an increase. The two-stage release profile suggested the presence of two dominating release mechanisms in high Mw polymers. Drug diffusion and polymer degradation are the main release mechanisms (happened in same time) of microspheres containing low Mw polymers after the initial burst stage (Park *et al*., 1994). But, microspheres containing high Mw polymers likely undergo initial slow drug release due to diffusion, followed by the main drug release due to degradation. Blending two polymers of different Mw allows the manipulation of the timing associated with the degradation release. Le Corre *et al*. (1994) combined a low Mw PLA (Mw = 2000) and a higher Mw PLA (Mw = 9000) to obtain an extra degree of controlled release.

### 3.2.2. Polymer hydrophilicity

In general, hydrophilic polymers [gelatine, alginate, chitosan, polyethylene oxide (polyox) and carbopol] result in high initial burst release rates. These hydrophilic swellable polymers used for particles favor water uptake and consequently induce a faster drug release. However, Efentakis *et al,*. (2007) noticed that carbopol matrix demonstrated greater maximum expansion and lower drug release than the polyox matrices (especially with theophylline, a lipophilic drug model) due to a smaller degree of erosion of carbopol.

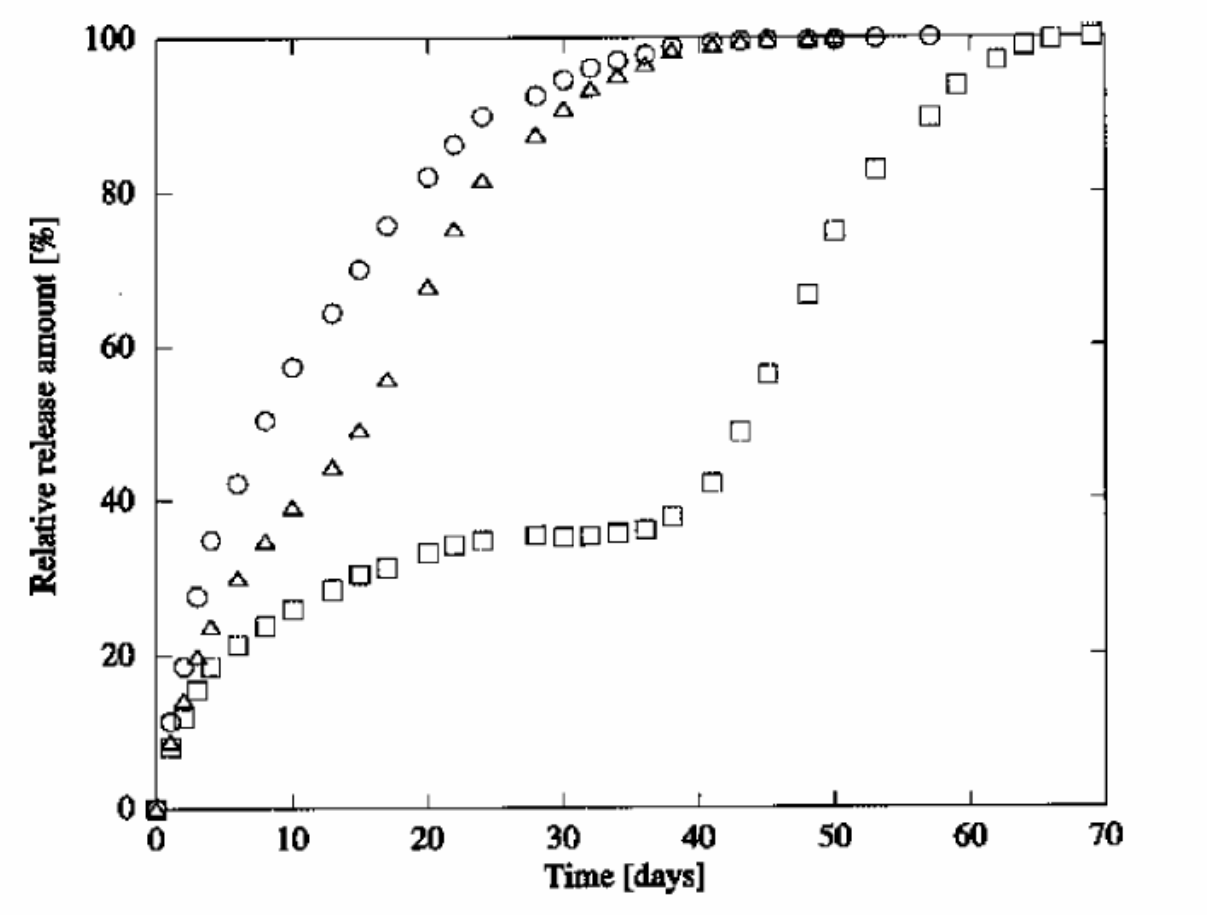



***Figure 3.*** Drug release from PLGA microspheres manufactured with polymers of different Mw; the pulsatile release character increases with Mw. Circles, Mw = 19000; triangles, Mw = 44,000; squares, Mw = 74,000. (From Makino *et al*., 2000)

Efentakis *et al.,* (2007) concluded that almost all hydrophilic polymers used for drug delivery systems exhibited burst release which mainly depends on the particle size (or layer thickness) and erosion speed / rate.

On the contrary, hydrophobic polymers (Eudragit® RS, ethylcellulose, PLGA, PCL, PLA) have less pronounced drug burst release than hydrophilic ones; this is probably due to the slow diffusion of water into the lipophilic matrix.

Despite, the slow diffusion of water into the hydrophobic particles, drug burst release can still be observed. It depends on different polymers characteristics such as crystallinity degree, Mw and chemical function (nature, numbers, charge), which in turn affects final particles properties such as water permeability, matrix porosity, particle size and density (Mehta *et al*., 1996).

Devrim and Canefe (2006) mentioned that ibuprofen release rates from Eudragit RS microspheres were slower than from Eudragit RL microspheres, because Eudragit RL has

more content in quaternary ammonium groups and therefore becomes more water permeable. O'Hagan *et al*., (1994) and Uchida *et al*., (1995) noticed that the extent of initial ovalbumin release was relatively high when PLGA with relatively high glycolide content (50/50) was used. The hydrophilic moieties (glycolide or quaternary ammonium groups) contained in polymers, facilitated water uptake from the release medium, and resulted in a higher initial burst.

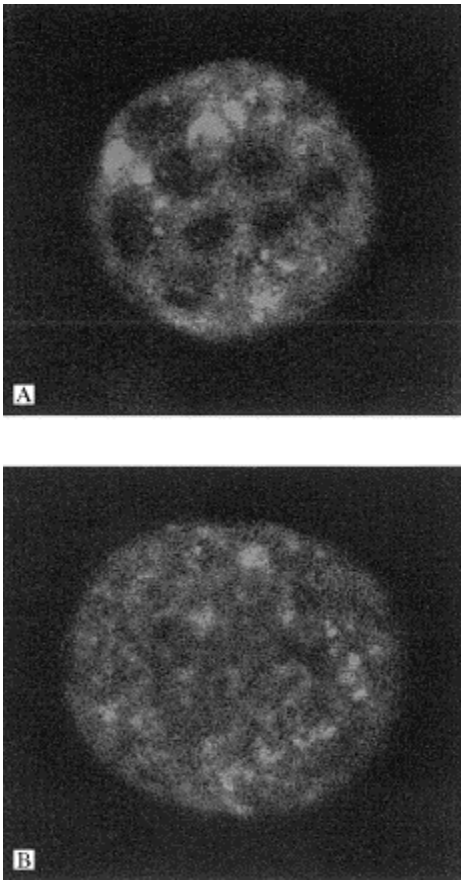
Furthermore, hydrolysis and biodegradation of polymers decrease with increasing of polymer hydrophobicity, for example, microspheres formulated with relativly hydrophilic PLGA (with free carboxilic groups) have faster degradation and higher burst release than particle formulated with the end-capped PLGA one, in which carboxilic groups are esterified (O'Hagan *et al.,* 1994).

### 3.2.3. Polymer concentration

A low polymer concentration results in small size and high internal porosity particles leading to a high initial burst release (Mao *et al*., 2007). A dilute dispersed phase with low viscosity (organic phase) can introduce more water from the continuous phase and/or the internal water phase and create water pores or channels before it completly solidifies (simple/double emulsion techniques). Once the microparticles are dried, the water channels become hollow holes through which drugs can be burst-released [Yang *et al*., 2000(a)]. High porosity of the microparticles made of a low concentration polymer solution was observed using confocal microscopy fig. 4 (a) (Yang *et al*., 2001). Due to their high porosity, the microparticles resulted in high and steep drug release profile fig. 4 (b). On the other hand, Mao *et al.* (2007) noticed that ABT627 (a synthesized chemotherapeutic agent for the treatment of prostate cancer) loading increased considerably when PLGA concentration increased from 5% to 10% and that PLGA microparticles size increased at the same time with increasing polymer concentration. This can be explained by the increased viscosity of the organic phase and the denser internal particles structure obtained, limiting the drug loss during the evaporation/extraction process (Mao *et al*., 2007).

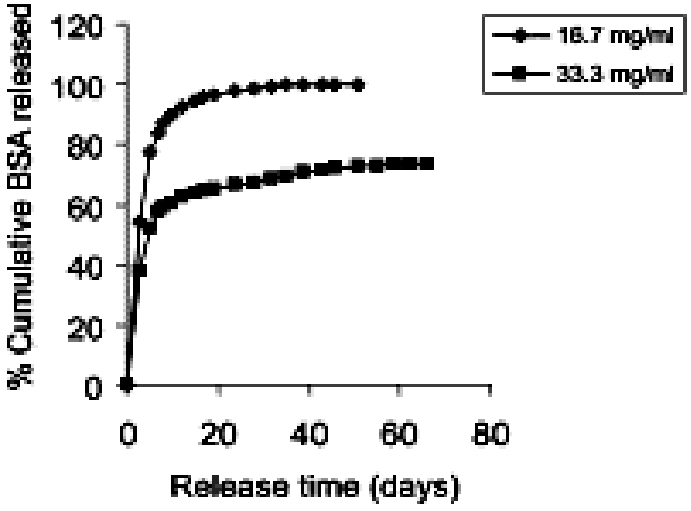
Thus, it can be concluded that two reasons could be responsible for the lower burst release with increasing polymer concentration. One is the increase in particle size which means a decrease in the exchanging surface area. The other is the high viscosity of the organic solutions (decrease of porosity, increase of matrix density) which restricted the diffusion of the drug substance out of the matrix (Mao *et al*., 2007; Yeo and Park 2004; Schlicher *et al*., 1997).

***Figure 4.*** Confocal laser scanning microscope images of microspheres fabricated at different polymer concentrations. A: 16.7 mg/ml, B: 33.3 mg/ml. PCL Mw 80 000. (Yang *et al*., 2001).




(a)

Effect of polymer concentration on BSA release profiles. PCL Mw 80 000. (Yang *et al*., 2001).



(b)

### 3.3 Drug/Matrix interaction implicated in burst release

There are a lot of kinds of drug/matrix intractions which can influence drug encapsulation efficiency, stability and release. So, in this section we will discuss the principal factors implicated in drug/matrix interactions that can modify drug release. As example we will focalise on proteins/matrix interaction and how such interactions modify proteins burst release.

#### 3.3.1. Crystallinity of drug/polymer and interactions

Crystallinity in microspheres has been usually investigated by differential scanning calorimetry (DSC) or X-ray diffraction (XRD) studies. DSC can detect phase transitions including the melting of crystalline regions, whereas XRD directly detects the crystallinity properties of a material. Using these 2 methods, Yuksel *et al.* (1996) investigated drug crystallinity and drug-polymer interactions. They observed that although a physical mixture of the drug and polymer exhibited crystallinity, the drug was amorphous after dispersion in the microspheres. Attempts to crystallize the drug inside the microspheres by annealing above the polymer's glass transition temperature (Tg), and by heat–cool cycles were unsuccessful showing a true molecular dispersion of the drug. In the same study it was shown that molecular dispersions may be more favorable than particulate dispersions (drug formed a particulate dispersion inside particles matrix which resulting in the presence of drug crystallinity) for drug delivery since the drug was released more readily from a microsphere system than from a particulate form at pH 7.4. The polymer matrix likely disturbs drug crystallinity and initiates rate-controlled delivery with higher drug delivery efficiencies (Yuksel *et al*., 1996).

In accordance, Le Corre *et al*. (1997) observed the crystallinity of a lipophilic drug in polymer microspheres by DSC. Usually, drug has been found to be molecularly dispersed inside a polymer matrix and crystallinity is not observed (Le Corre *et al*., 1997 and Guyot and Fawaz, 1998). However, Le Corre *et al.* (1997) also observed that the relatively highly loaded drug was present in a particulate dispersion instead of a molecular dispersion, which is possibly due to its lack of solubility in the polymer matrix.

Considering polymer crystallinity, Edlund and Albertsson (2000 a, b) suggested that matrix degradation occurred first in the amorphous microsphere regions followed by a slower degradation in the crystalline regions. This suggests that the crystallinity in the polymer

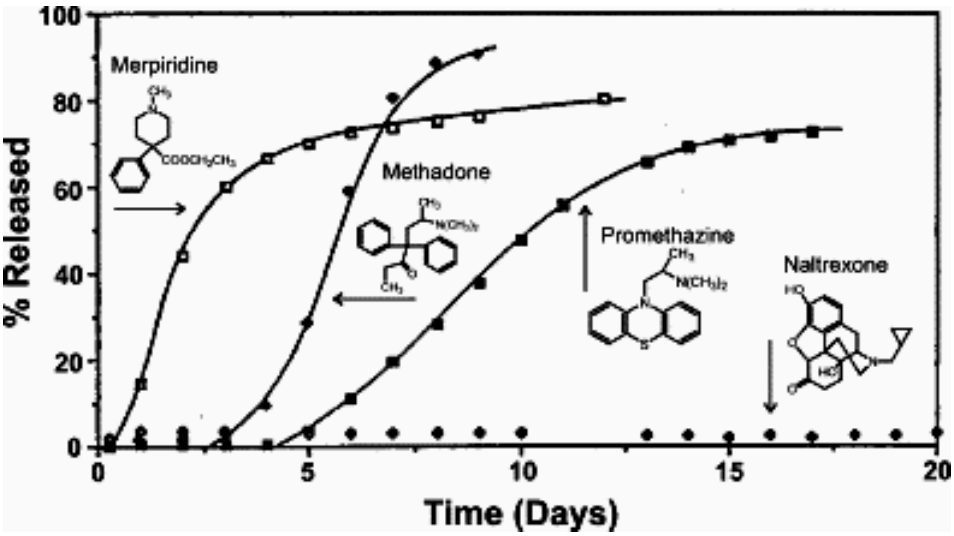
chains can affect the degradation rate and then drug release. Furthermore, at the beginning of microsphere degradation, the degree of crystallinity actually increased slightly. This was attributed to the crystallization of partly degraded chains and the preferential degradation of amorphous regions.

Izumikawa *et al*. (1991) studied polymer crystallinity and drug crystallinity employing PLA microspheres loaded with progesterone. At low drug loading (5%), XRD and DSC showed that the polymer dominates the crystalline properties of the microsphere and no crystallinity arose from the drug although the drug was dispersed in the microspheres. At high drug loading (30%), crystallinity was dependent on the organic solvent removal process; at slow solvent removal rates, microspheres crystallinity was observed from both the drug and polymer but fast removal resulted in amorphous spheres. At high concentrations and slow solvent removal, the drug formed a particulate dispersion resulting in the presence of drug crystallinity. However, the faster solvent removal rate may have resulted in amorphous spheres by not giving the drug and polymer molecules adequate time to crystallize. Release profiles suggest that more amorphous microspheres release the drug less rapidly than crystalline spheres. Therefore, the lack of polymer crystallinity suggests better drug dispersion and increased drug–polymer interactions and decreases the possibility of burst release. The drug release rate can be tailored by manipulating the degree of matrix crystallinity. A reduced matrix crystallinity is favorable when slow and reduce initial burst release is desired.

### 3.3.2. Drug induced polymer chain scission

It is obvious that the nature of drug/polymer interaction influences the drug release and especially burst release. In some cases the drug employed can induce polymer chain scission through nucleophilic degradation. Typically this is observed in medications containing amines whose nitrogen atom is nucleophilic, just like the oxygen atom in water. Cha and Pitt (1989) reported that sterically available amines increased the rate of polymer degradation. PLLA was loaded with different amine-containing drugs, the polymer Mw in spheres containing the most active amine decreased more rapidly and to a greater extent throughout the release process (Fig. 5). In the case of a less active, sterically hindered tertiary amine, polymer degradation was not significant nor was drug release, unless it was co-loaded with another drug capable of causing polymer chain scission. Other groups have also considered chain scission when reporting their results (Cha and Pitt, 1989; Le Corre *et al*., 1997 and Tuncay *et al.*, 2000).

***Figure 5.*** Tertiary amine containing drugs loaded into a PLLA microsphere matrix as reported by Cha and Pitt (1989). Drugs with less sterically available amines are released more slowly from the matrix.




### 3.3.3. Drug distribution in polymeric matrix

How the drug is distributed in the medium (matrix medium) can modify its release profile (Kakish *et al*., 2002). Drug release begins at the microsphere surface followed by release from the inner layers of the sphere; therefore the diffusional distance between the initial drug location inside the sphere affects the release profile (Lee *et al*., 1986). Heterogenously dispersed drug in the nano/microparticles matrix can increase the initial burst effect.

Kakish *et al*. (2002) have successfully modified the drug distribution in microspheres to obtain constant drug release. The microspheres were modified by stirring dry spheres in an ethanol–water mixture followed by freeze-drying. The resulting distribution is believed to be characterized by an increase in drug concentration toward the center of the microspheres thus resulting in a more constant release rate. Kakish *et al*. (2002) obtained a system with a relatively constant release rate over 10h. Their microsphere treatment methods significantly improved controlled release when compared to untreated microspheres whose drug release rate decreases with time.

### 3.3.4 Protein (as drug example)/polymer interaction

In many cases, proteins delivered from controlled release systems present burst release; the initial release increases with increasing proteins loading (O'Hagan *et al*., 1994; Huang and Brazel 2001; Yeo and Park 2004).

There are two possible explanations for the effect of protein loading. First, the elution of surface associated protein creates water-filled channels that allow subsequent elution of the proteins located inside the microparticles. By facilitating formation of these channels, high protein loadings lead to high initial bursts (Hora *et al.,* 1990). Alternatively, a large protein concentration gradient between the microparticles and the release medium may promote the high initial bursts (Yang *et al*., 2001). However, there are different opinions: The concentration gradient does not influence the protein release until the onset of polymer degradation (Sah *et al.,* 1994). Interaction between protein and polymer contributes to increasing encapsulation efficiency (Boury *et al.,* 1997). Proteins are generally capable of ionic interactions and are better encapsulated within PLGA that carry free carboxylic end groups than the end-capped ones (Yeo and Park 2004). On the other hand, if hydrophobic interaction is a dominant force between the protein and the polymer, relatively hydrophobic end-capped PLGA are more advantageous in increasing encapsulation efficiency (Mehta *et al*., 1996). For example, encapsulation efficiencies of more than 60% were achieved for salmon calcitonin (sCT) microparticles despite the high solubility of sCT in the continuous phase (Jeyanthi *et al*., 1997). This is attributed to the strong affinity of sCT to hydrophobic polymers such as PLGA. On the other hand, such interactions between protein and polymer can limit protein release from the microparticles (Park *et al*., 1998; Kim and Park, 1999). In certain cases, a co-encapsulated excipient can mediate the interaction between protein and polymer (Johansen *et al*., 1998). Encapsulation efficiency increased when gammahydroxypropylcyclodextrin (g-HPCD) was co-encapsulated with tetanus toxoid in PLGA microparticles. It is supposed that the g-HPCD increased the interaction by accommodating amino acid side groups of the toxoid into its cavity and simultaneously interacting with PLGA through van der Waals and hydrogen bonding forces. Similarly, release of cyclosporin A from PLGA microparticles was affected by copolymer composition (Lee *et al*., 2002 b). Higher lactide content delayed the drug release because of the stronger hydrophobic interaction between the polymer and the drug.

Nicoli *et al*., (2001) prepared triptorelin-loaded nanospheres with three different PLGA co-polymers. The encapsulation efficiency reached the maximum value (82 %) when PLGA rich in free carboxylic groups was employed. The release profiles obtained with this co-polymer were characterized by the absence of burst effect (fig. 6). This behavior as well as the high encapsulation efficiency was explained by an ionic interaction occurring between the peptide and the co-polymer. This supports the already expressed theory that the release of peptides

and proteins from PLGA nanospheres is governed by the affinity of the encapsulated molecule versus the polymer.

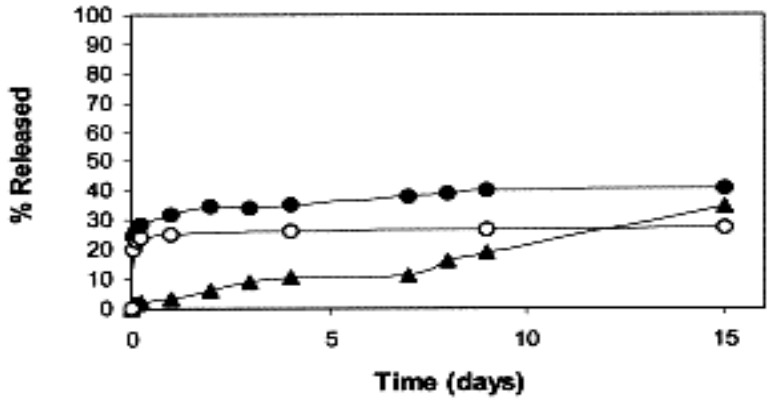



***Figure 6***. Release kinetics of triptorelin from different PLGA nanospheres: PLGA with more lipophilic group 75/25 (open circles) PLGA lower molecular weight 752 75/25 (closed circles); PLGA characterized by a high presence of free carboxyl groups, 50/50 (closed triangles). (From Nicoli S et al., 2001)

### 3.4. Manufacturing process affecting burst release

The most commonly used methods (using preformed polymers) of polymeric particles encompass simple or double emulsion (w/o, $w_1/o/w_2$) solvent extraction or evaporation, coacervation and spray-drying (Jain *et al.*, 1998; Tamber *et al*., 2005). Tamber *et al.*, (2005) described the conventional microparticles preparation methods for the formulation of microsphere for antigen delivery (Fig. 7). Each of these methods employs a similar first step, where an aqueous drug solution is emulsified in an organic polymer solution to form a water-in-oil dispersion ($w_1/o$). If appropriate, the drug may also be dispersed as solid powder in the organic polymer solution (s/o/w), or codissolved in a common solvent with the polymer (o/w). The solution or dispersion is then processed according to one of the mentioned microencapsulation methods.

In solvent extraction or evaporation, the drug solution or $w_1/o$ emulsion is further dispersed, in one or two steps, into a larger aqueous volume containing a suitable emulsifier, commonly poly (vinyl alcohol) to form a $w_1/o/w_2$. Polymer hardening and particles formation is induced by solvent extraction into the $w_2$-phase. Solvent extraction may be facilitated either by the use of a cosolvent in the $w_2$-phase, such as an alcohol or acetone, or by evaporation of the solvent under atmospheric or reduced pressure. At the end of the procedure, the solidified particles are harvested, washed and dried.

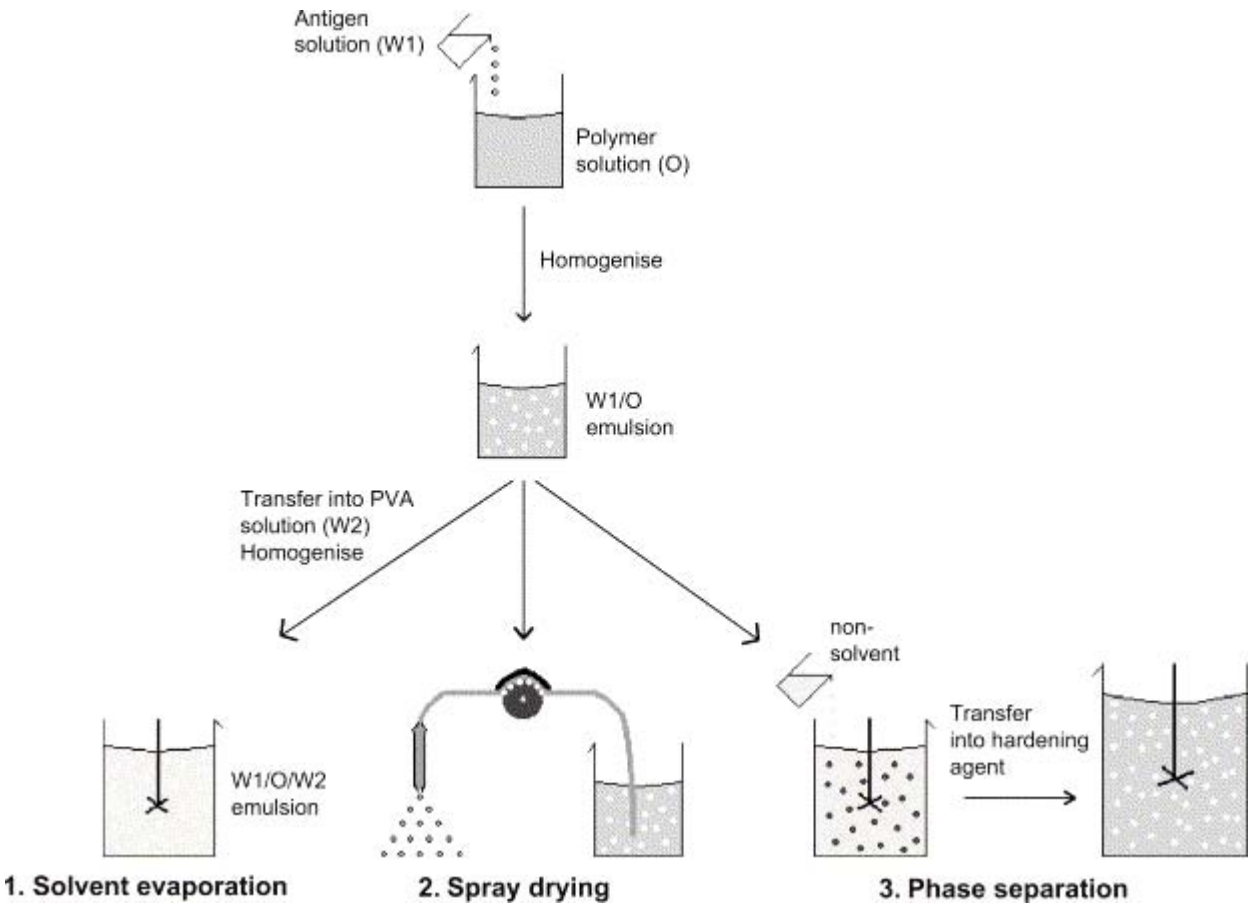



***Figure 7***. Conventional microparticles preparation methods. An aqueous antigen solution is dispersed into an organic polymer solution by ultrasonication or homogenisation (w1/o emulsion). The w1/o emulsion is processed further by the specific methods to prepare antigen containing micropaticles: (1) Solvent extraction or evaporation; (2) Spray-drying; (3) Polymer phase separation. In the final stages before drying and storage, the microspheres are collected and washed with water to remove nonencapsulated antigen. (From Tamber *et al.*, 2005)

Coacervation, also called polymer phase separation, involves several stages of polymer desolvation and hardening during which the solid particles are formed. To the drug solution or w1/o emulsion, an organic nonsolvent for the polymer and proteinaceous compound is added. The nonsolvent induces polymer phase separation into a coacervate phase, engulfing the proteinaceous compound, and a continuous phase. The polymer solvent is then gradually extracted from the coacervate phase, yielding polymer-rich and physically quite stable coacervate droplets. The two-phase system is then transferred into a large volume of an organic hardening agent (*e.g.*, alkanes) miscible only with the polymer solvent and nonsolvent. Here, the solid particles are formed by rapid and efficient extraction of the remaining polymer solvent from the coacervate droplets. The particles are harvested, washed

with a suitable volatile nonsolvent for the polymer to remove residual coacervation liquids, and dried (Thomasin *et al*., 1996; Thomasin *et al*., 1998 a, b).

Spray-drying offers an attractive and relatively simple alternative to the previous two methods. Here, the drug solution or w1/o emulsion is atomised in a flow of drying air at slightly elevated temperature. The organic solvent is rapidly vaporised leaving behind solid particles that are separated from the drying air in a cyclone and collected in a deposition chamber (Bodmeier and McGinity, 1988; Gander *et al*., 1996).

Among the other methods using preformed polymers and could lead to polymeric particle nanoprecipitation, salting-out and techniques based on supercritical or compressed fluid can be mentioned.

All these processes influence the surface/internal morphology (porosity, size, surface and density) of the final polymeric particles prepared and homogeneity of drug in the particles. When initial burst release is mainly due to drug diffusion through the preformed water channels, the solidification rate / speed of the dispersed phase is a critical factor that influences the drug initial release. Therefore, most formulation parameters and process which affect solidification of particles are involved in control of the initial burst as well. In the next paragraph we will thus be exploring which formulation and process parameters control particles size and porosity.

### 3.4.1 Manufacturing process and nano/microparticle size

As known from Fick's law, the release profiles (including initial burst effect) are dependent on the size of the particles; the rate of drug release was found to decrease with increasing microparticle size (Akhtar and Lewis, 1997; Sansdrap and Moes, 1997 and Bezemer *et al.*, 2000 ; Klose *et al*., 2006).

In the double emulsion technique, particles size can be affected by the polymer concentration, temperature process, organique phase viscosity, stirring rate in the emulsion step, and amount of emulsifier employed.

Considering the effect of polymer concentration, it has often been reported that increasing the polymer concentration increases microsphere size (Lin and Vasavada, 2000). In another study, Yang *et al.* (2000a, 2000b) scanning electron microscopy (SEM) was used to show that microsphere size was temperature dependent ; lower and higher temperatures produced larger microspheres (and consequently a lower initial burst release) whereas intermediate temperatures produced smaller spheres (and then a higher initial burst release). Once again, different mechanisms dominated microsphere formation at different temperatures. At lower

temperatures, the solution's higher viscosity resulted in the formation of larger particles; this has also been confirmed by other researchers (Jeyanthi *et al*., 1997). Larger particles were also obtained at higher temperatures due to the higher rate of solvent evaporation which resulted in higher solvent flow pressure moving more material from the microsphere center outward (Yang *et al*., 2000 a,b).

Jalil and Nixon (1990) studied the variation of microsphere size with respect to the stirring rate and the influence of the emulsifier in the second emulsion step. It was shown that microsphere size decreased with increasing stirring rate since increased stirring (increase of shear force) results in the formation of finer emulsions. The authors employed a sorbitan ester as an emulsifier and reported a sharp drop in diameter when the sorbitan ester concentration was increased from 1 to 2%. Little change in diameter size was reported by increasing emulsifier concentration beyond 2%. It is possible that in this particular case, emulsifier packing was optimum at 2% concentration and that no more emulsifier could be adsorbed at the sphere surface above this concentration (Jalil and Nixon, 1990).

### 3.4.2 Manufacturing process and nano/microparticle porosity

The microsphere porosity is determined during hardening as the organic solvent evaporates during preparation. As already mentioned, particles porosity can be controlled by changes in particles preparation technique since differences in porosity do affect release kinetics (Jeyanthi *et al.*, 1996 ; Yang *et al*., 2000 a, b; Klose *et al.,* 2006). This is noticeable in a study by Yang *et al.* (2000 a, b) where a highly porous matrix released a drug at a considerably higher rate than its non-porous counterpart. Other researchers also reported that particles porosity affected the release profile in similar ways (Yuksel *et al*., 1996 ; Chung *et al*., 2001). Therefore, when preparing microspheres, it should be kept in mind that increasing the number of pores should increase the release rate. Thus, particles porosity is the main factor which can amplify initial burst effect. Scanning electron microscopy showed that PLGA microspheres with more porous and dimpled surface structure have a larger initial burst release but, microspheres that displayed a relatively smooth and compact surface showed the lowest burst release (Zheng *et al.* 2006). This is attributed to the leaching which occurs at the outer wall of the microspheres as it becomes hydrated (Ghaderi *et al*., 1996). This can be minimized by supporting the formation of a non-porous outer microspheres skin which can be controlled by manufacturing temperature (Yang *et al.*, 2000b).

The first w/o emulsion can be used to control the microsphere pores. Crotts and Park (1995) have shown that microspheres porosity increased with the water content in the first emulsion.

When the w1/o system contained less water, porosity decreased; the formation of spheres by skipping the first w/o-step resulted in microspheres with a non-porous outer skin and a monolithic inner composition. At low water contents the inner core contained hollow structures and a non-porous skin. Porosity was observed throughout the particle at higher water content. In a related study, Tuncay *et al.* (2000) also found that the employment of methanol instead of water in the first emulsion phase may reduce the surface porosity of the microspheres.

Li *et al.* (1995) determined the effects of varying the amount of water in the second emulsion or continuous phase on porosity. The continuous phase containing the largest amount of water resulted in faster polymer precipitation and therefore less porous spheres were formed. In another study Jeyanthi *et al*. (1996) obtained spheres containing a uniform honeycomb structure with no hollow core by continuously adding water to the continuous phase of the dispersion up to 1.5 times the initial volume. Continuous dilution up to 2.5 times the initial volume resulted in similar microspheres but with larger pores. The larger pores at increased dilution were explained by an increased rate of solvent removal with increasing water content (higher solvent flow exit pressures) which occurs in coordination with a faster hardening rate (Ghaderi *et al.*, 1996 ; Jeyanthi *et al.*, 1996).

The rate at which the solvent is removed from the microspheres is dependent on temperature, pressure, and the amount of water in the final emulsion phase and can be directly related to sphere porosity (Izumikawa *et al.*, 1991; Jeyanthi *et al*., 1996 ; Yang *et al*., 2000(b)). Yang et al. (2000 a, b) have systematically increased sphere preparation temperature; their results suggested that skin porosity tends to decrease with increasing temperature until a limiting value. Therefore, a relatively high microspheres formation temperature resulted in rapid hardening of the outer wall and low porosity. However, very high temperatures resulted in a highly porous skin and inner core due to the very rapid solvent evaporation. Other researchers also showed that high evaporation rates resulted in more porous spheres (Izumikawa *et al*., 1991 and Chung *et al*., 2001).

Jeyanthi *et al.* (1996) have investigated microspheres porosity at variable temperatures; solvent removal was well correlated to preparation temperature and the solvent was removed more rapidly at higher temperatures. Quick solvent leaching from the soft microspheres formed hard microspheres with hollow inner cores and thin outer walls, thus showing how the high flow pressure of the evaporating solvent increases porosity. By varying the ramping conditions the authors were able to create microspheres with a thicker outer wall and a smaller core whose porosity was controllable by the ramping steps (Jeyanthi *et al.*, 1996).

### 3.4.3 Spray-drying method and the w/o/w emulsion methods

Bittner *et al.* (1998) presented a comparative study of recombinant human erythropoietin (EPO) or FITC-dextran microencapsulation using the spray-drying method and the w/o/w technique using PLGA as polymer.

As expected, microsphere morphology and particle size strongly depended on the production process. Microsphere size produced by w/o/w is 4 times larger than microsphere prepared by spray-drying but, w/o/w particles showed higher porosity than spray-drying particles. These pores were most likely caused by dissolution of surface located drug particles into the water during microsphere formation and by the internal aqueous phase. On the other hand, a number of deformed particles could be observed amongst the microspheres prepared by spray-drying. This collapsed structure is caused by the rapid evaporation of the organic solvent.

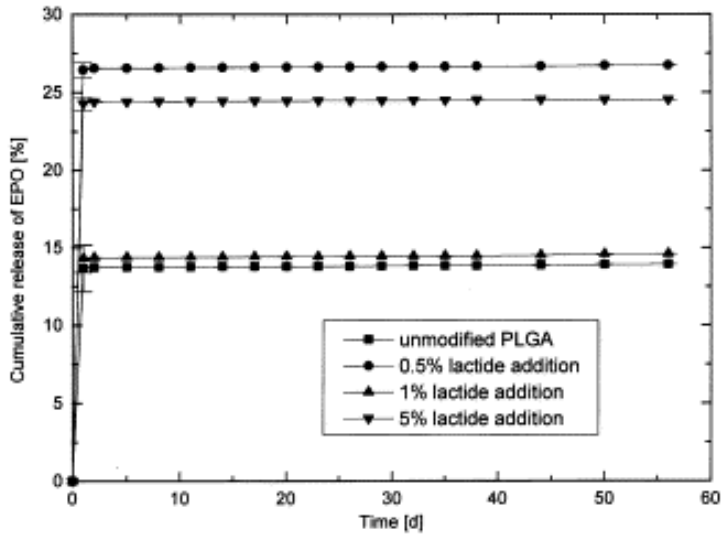
The spray-drying method was found to have a number of advantages compared to the w/o/w double-emulsion technique. The content of residual dichloromethane in the final product was significantly lower in case of the microspheres prepared by spray-drying. Concerning EPO loaded microspheres, spray-drying yielded higher encapsulation efficiencies. Although the microspheres obtained by spray-drying are subjected to intensive mechanical and thermal stress during the preparation, the amount of aggregates of EPO in PLGA microspheres were not increased compared to the w/o/w technique. Thus, the spray-drying process is favorable for the incorporation of hydrophilic drugs such as polypeptides or proteins in polymers. But relatively low microparticles yield obtained by spray-drying (80 % yield for w/o/w but only 40 % for spray-drying). Such a yield was clearly influenced by the geometry of the equipment and cannot be improved by optimization of the process conditions.

The *in vitro* release of EPO from PLGA microspheres was influenced in the initial release phase by the encapsulation technique. Concerning the microspheres prepared by the w/o/w double-emulsion technique, the relatively high initial release (compared to spray-drying particles) of EPO can be interpreted by the porous inner structure of the PLGA matrix (Fig. 8 A, B). As mentioned before, an increase in the number of pores inside the particles was accompanied by an increase in the initial burst. Moreover, the diffusion of EPO from the microsphere surface at early times is facilitated and the initial drug release is increased. But, interactions between EPO and the polymer and formation of insoluble EPO aggregates during the initial release phase have most likely contributed to the unfavorable *in vitro* release profiles shown in fig.8 (A) only one release phase could be observed coresponding to the initial burst. These aggregates are no longer available for release. Also, spray-dried microspheres showed a similar release behavior (Fig. 8 B) and the spray-drying process did

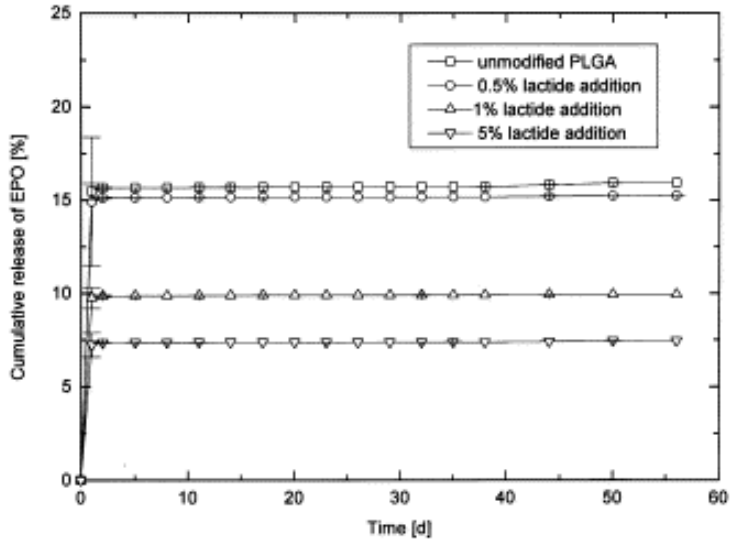
not change this general pattern. The amount of EPO released after this initial burst was negligible. On the other hand, FITC-dextran loaded microspheres produced by spray-dried microspheres present initial FITC-dextran burst release 10 times more than microspheres produced by w/o/w technique (Fig. 8C). This may be explained by the larger particles size obtained by w/o/w technique (4 times more than particles obtained by spray-dried).

***Figure 8.*** Release profile of EPO from the poly (lactide-co-glycolide) microspheres prepared by the w/o/w double-emulsion technique (A) or spray-dried (B) over a period of 50 days (From Bittner *et al*., 1998).

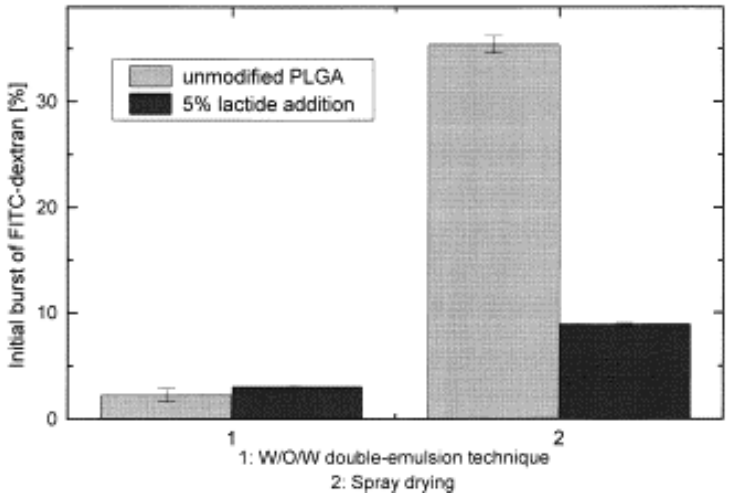
**A**




**B**




**C**




### 3.4.4 Solid-in-oil-in-water (s/o/w) emulsion

The use of solid-in-oil-in-water (s/o/w) technique is an excellent alternative to the water-in-oil-in-water (w/o/w) technique, which is still mainly used in the encapsulation of drug in polymeric particles.

Lamprecht *et al.,* (2000) compared two processes w/o/w and s/o/w for the manufacturing of PCL, PLA and PLGA microparticles loaded sulfasalazine (hydrophilic drug) and betamethasone (lipophilic drug) in the same microparticles formulation. They found better drug (for two drugs) encapsulation efficiency when s /o/w-method used. Moreover, controlled release of sulfasalazine and betamethasone from microparticles prepared by the s/o/w-method and a pronounced burst release of sulfasalazine was observed from microparticles prepared by the w/o/w-method (during the *in vitro* release studies). The decrease of burst release observed permits to conclude that the s/o/w-technique appears more adapted for the simultaneous encapsulation of both sulfasalazine and betamethasone in the same microparticles for the treatment of inflammatory bowel disease.

Also, the results of Castellanos *et al*. (2001) demonstrated that the encapsulation procedure of s/o/w did not cause detrimental structural perturbations in BSA and the microspheres obtained showed a decrease of initial burst release of about 20 %, a sustained release over a period of about 19 days, and a cumulative release of at least 90 % of the encapsulated BSA. On the other hand, Yamaguchi *et al.* (2002) have demonstrated that when the crystalline insulin was dispersed in methylene chloride as (s/o) inner dispersion, most of the insulin molecules were inlaid on the surface of the PLGA microparticles. Consequently, insulin–loaded PLGA microparticles exhibited a high initial burst.

### 3.4.5 Phase separation [water/oil/oil (w/o/o)]

Li *et al.*, (2000) encapsulated Glucose oxidase as a model protein within poly-DL-lactide–poly(ethylene glycol) (PELA) microspheres to investigate the effect of the preparation method ($w_1/o/w_2$ emulsion extraction/evaporation or phase separation techniques $w/o_1/o_2$) on the protein released and stability during *in vitro* assay. They show that microspheres with larger size were obtained from the $w/o_1/o_2$ technique (silicon oil as the second oil phase). The different size of microspheres prepared by the two methods may result from the surface tension between the polymer organic solution phase and the external aqueous ($w_2$) or oil ($o_2$) phase in the emulsion system. In the $w_1/o/w_2$ formulation, PELA solution in more polar solvents (*e.g.*dichloromethane/ethylene acetate) had a lower surface tension with the external aqueous phase than silicon oil, leading to form microspheres with smaller size. Moreovre, in the phase separation system, the external oil phase had a higher transfer resistance of protein from the internal aqueous phase, which led to larger protein entrapment and loading efficiency than $w_1/o/w_2$ emulsion. But, 70– 80% of entrapped protein dispersed at or near the surface of microspheres prepared from the phase separation technique, while the surface protein content of microspheres from the $w_1/o/w_2$ emulsion process was 20–30%. The exposure of these protein molecules located at the surface to various unfavorable factors (the mechanical stirring and the contact with organic solvents) may promote deactivation during the microencapsulation procedure. Thus, high burst release and lower activity retention of protein was obtained for microspheres prepared by the phase separation process, despite a higher protein entrapment being achieved.

### 3.4.6 Drying and storage methods

Heterogeneous drug distribution in matrix and consequently increase of burst release can happen because of migration of drugs during drying and storage steps (Huang and Brazel, 2001). According to Huang and Brazel (2001), the mechanism by which drugs are released requires dissolution of the drugs followed by diffusion through the swelling porous structure. In the same manner, the diffusion and migration of drugs may occur during the drying process as water (or residual organic solvent) moves to the polymeric matrix surfaces and evaporates. Drugs may diffuse by convection with water, leaving an uneven drug distribution across the polymeric matrix, with higher concentrations at the surface (Fig. 9). Such phenomena happens during the air or vacuum drying process, in particular; water flows to the matrix surfaces before evaporation. Igartua *et al.*, (1997) proposed that convection-induced drug migration can be reduced by freeze-drying the microparticles and confirmed a lower burst

release when microparticles were freeze-dried instead of air-dried. In another example, the burst release was almost entirely eliminated by freeze-drying the microparticles (Wang *et al.*, 1991). The authors attribute this result to the effectiveness of drying; however, suppressed migration of drug during the drying process can also be a possibility.

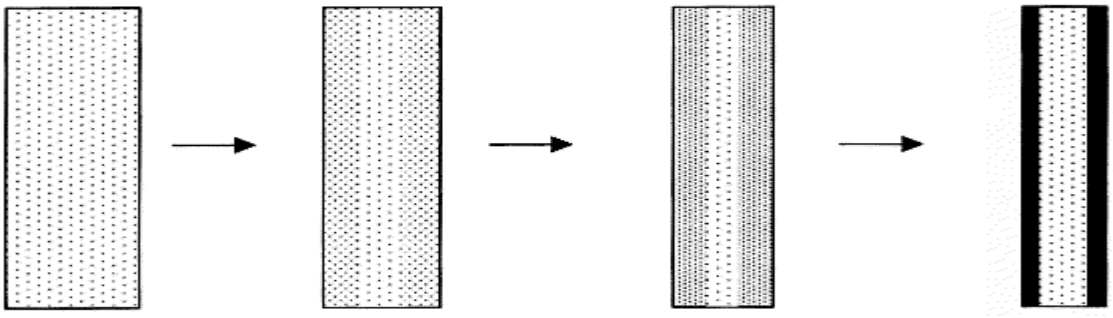


***Figure 9.*** the diffusion and migration of drugs occur during the drying process as water moves to the gel surfaces and evaporates. Drugs diffuse by convection with the water, leaving an uneven drug distribution across the gel, with higher concentrations at the surface (From Huang and Brazel 2001)

On the other hand, the freeze-drying process is not always effective in preventing the burst release. The volume expansion of encapsulated water during the freezing stage can cause additional cracks in the microparticles and induce a large initial burst (Kim and Park 2004; Cleland *et al. 1997*). Indeed, Kim and Park (2004) noticed that freezing and subsequent drying processes of the embryonic PLGA microspheres resulted in much increased extent of burst release, suggesting that the initial burst release was primarily caused by the rapid diffusion of fluorescein isothiocyanate (FITC)-labelled dextran through the microporous channels. Confocal microscopic analysis revealed that the freeze-drying process generated water-escaping micro-channels, through which the encapsulated molecules were presumably dumped out. In conclusion; Kim and Park (2004) noticed that vacuum-drying was a good alternative choice in reducing the initial burst, compared to freeze-drying (Fig. 9).

Furthermore, Cleland *et al.* (1997) compared the impact of different drying process (lyophilization, vacuum drying, and nitrogen drying) on the initial burst of rgp120 (a recombinant form of the surface glycoprotein of HIV-1) from PLGA microsphere immediately after production (wet microparticles). The wet microparticles showed a lower initial burst than the dried microparticles. The extent of the initial burst from the dried microparticles followed of, lyophilization > vacuum > nitrogen drying, respectively. The removal of entrapped water droplets by evaporation during the drying step obviously caused

cracks and pores in the polymer matrix and, upon hydratation of the microparticles, these cracks and pores even increased in size and number. However, microparticle drying by lyophilization caused more cracks and pores compared to other techniques.

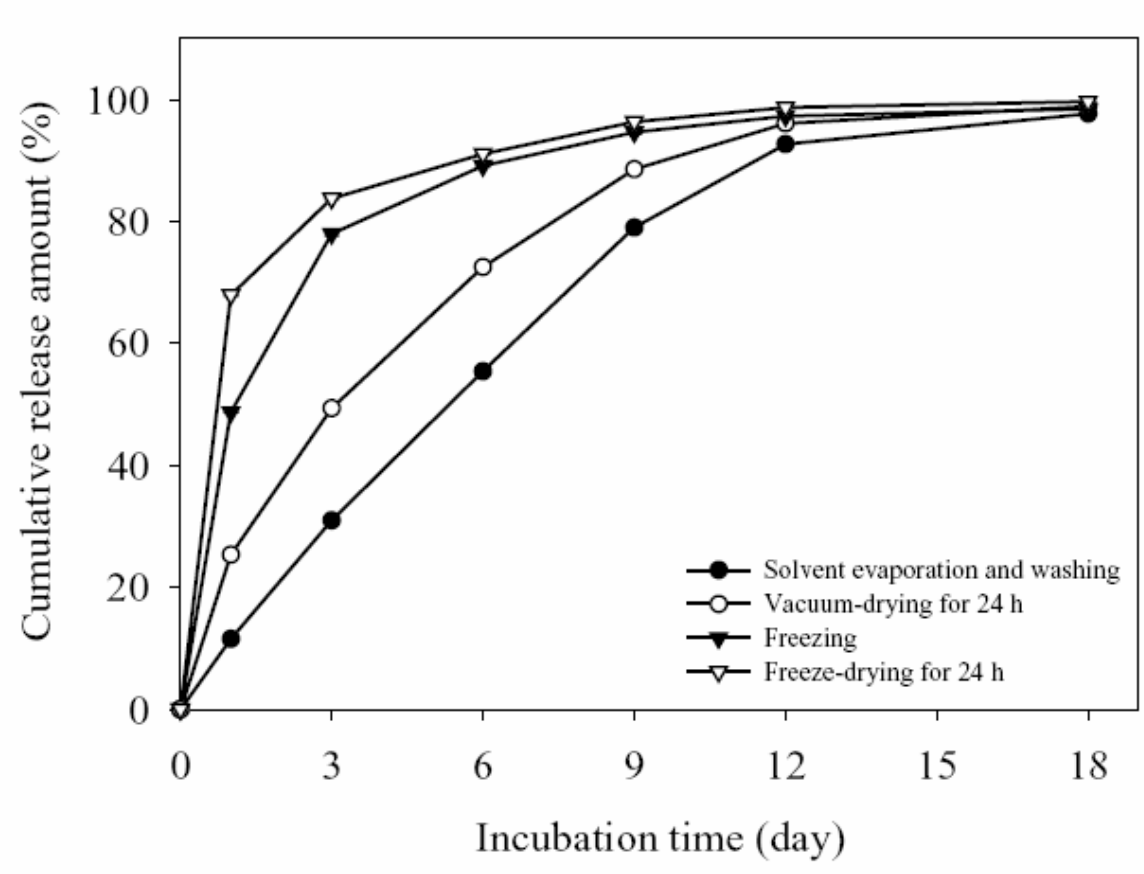



***Figure 9.*** Release profiles of FITC-dextran loaded PLGA microspheres treated with different processes (n=3). (From Kim *et al.*, 2004)

This could be explaining as follows: during lyophilization, the microparticles are frozen and excess water is removed by sublimation. The formation of ice crystals within the microparticles may contribute to a higher cracking or a complete fracture of the microparticles.

Some researchers proposed that a low polymer concentration induced low polymeric matrix density. This could allow movement of drug towards the surface of microspheres easily during the drying process as well as forming larger pores where diffusion is not controlled by the polymer. The increase of polymer concentration in the matrix at the beginning of preparation gave a denser structure of particles, thus the migration of drug during drying and storage became more difficult. (Kim and Park 2004; Cleland *et al*. 1997).

### 3.4.7. Sterilization methods

The traditional methods of sterilization of medical devices include the use of dry or moist heat, chemicals (ethylene oxide) or radiation. Steam sterilization by autoclaving at 121°C is the most widely employed method today but might induce hydrolysis and/or melting of the polymer matrix (Nair, 1995). Chemical sterilization with ethylene oxide gas offers the advantage of effective treatment at ambient temperature and is useful for hydrolytically unstable polymers. Nevertheless, its popularity is decreasing due to the well-known toxicity and flammability of ethylene oxide. Gas residues may, being a strong alkylating agent, react with functional groups on the polymer surface thereby altering its biological properties. To ensure the complete removal of trace residues before use, the specimens must be degassed for 2–5 days, depending on the porosity (Nair, 1995).

High-energy radiation sterilization (with β-rays or γ-rays) has the advantages of high efficiency, negligible thermal effects, and allows for packaging prior to treatment. Polymers exhibiting high heats of polymerization tend to cross-link upon radiation, indicating an apparent increase in mechanical stability with increasing radiation doses. However, radiation of polymers with low heats of polymerization induces degradation by random chain scission (rather than cross-linking) affecting the bulk and surface properties and in turn the physicochemical properties. Radiation of polymers may also cause free-radical formation, gas evolution, additive leakage and discoloration (Nair, 1995; Bruck and Mueller, 1988; and Edlund *et al.*, 2000(a)). The radiation sterilization of biodegradable polymer matrices may induce degradation reactions that may continue upon storage, affecting the erosion and drug release performance (Edlund *et al.,* 2000(a)). The susceptibility to radiation of the incorporated therapeutic agent must also be considered. In fact, γ-rays are a form of electromagnetic radiation characterized by high penetration into matter but at a very low dose rate (kGy/h). On the contrary, β-rays are a form of corpuscular radiation characterized by low penetration into matter, but at a very high dose rate (kGy/s) (Sintzel *et al.,* 1997; Montanari *et al.,* 2003). These peculiarities of β- and γ-rays can modify the performance of irradiated drug delivery systems. For the same administered dose, β-ray treatment may cause an overheating of the material while γ-ray treatment could prolong the peroxidative radiolitic mechanism due to the exposure time (Woo and Sandford, 2002; Montanari *et al.,* 2003).

The effects of γ-irradiation on drug loaded polymeric microspheres, drug profil release and burst release were debated by a number of papers, and controversial results are reported, depending on the active ingredient used (Montanari *et al.*, 2003; Volland *et al*., 1994).

However, little information is available at the moment on the effects of β-irradiated onto microparticulate polymeric systems.

We can summarize the effects of γ-irradiation on drug burst effect by the work of Lee *et al*., (2002 a) who discusses the effects of γ-irradiation on double-walled microsphere [poly (L-lactic acid) (PLLA) shells and PLGA cores] etanidazole (highly water-soluble) loaded. They found that typical therapeutic irradiation dosage of 50 Gy was found to be too mild to have noticeable effects on the drug release profiles, whereas, sterilization dosages of 25 kGy, lowered the glass transition temperatures and crystalline melting point of polymers, indirectly indicating a decrease in molecular weight and crystallinity. This accelerated degradation of the polymer, hence releasing properties of the drug and consequently increasing the drug initial burst release.

Also, γ-irradiation can affect drug itself. As example, γ-irradiation of insulin-like growth factor (rhIGF-I) causes its aggregation and an increased his burst effect *in vitro* release experiments (Carrascosa *et al.*, 2003)

## 4. Methods to prevent drug initial burst release from polymeric nano/microparticles:

Several attempts have been made so far to prevent or minimize the burst effect in a wide range of polymer/drug systems. A system for controlled release would be optimal, if it could be produced in a single step to include a high drug loading and would have no burst release. Several approaches have been undertaken to reduce the initial burst, such as modification of formulation or preparation conditions, or as surface drug extraction, surface modification, additional coating, …. etc. However, these techniques involve additional costly steps, result in reduced drug loading percentages or require the introduction of additional materials. In the next section, we present the main attempts to reduce the burst release from polymeric nano/microparticles.

### 4.1. Drug pretreatment:

Gèze et al. (1999) tried to reduce initial burst from 5-iodo-2'-deoxyuridine (IdUrd) loaded PLGA microspheres by modifying the drug grinding conditions. IdUrd particle size reduction has been performed using spray-drying or ball milling, followed by drug loaded in microspheres (with o/w emulsion/extraction method). Spray-drying significantly reduced drug (IdUrd) particle size with a change of the initial crystalline form to an amorphous one (led to a IdUrd high initial burst from microsphere *in vitro*); conversely, ball milling did not affect the initial IdUrd crystallinity [low drug initial burst release was noticed especially with

particle size 15.3 μm (8.7 %) compared to 19 % for (19.5 μm) milled drug]. Thus, the crystalline lowest drug particle size (15.3 μm milling by ball) loaded in 40–50 μm PLGA microspheres has reduced significantly burst effect (Fig. 10).

This study outlined the importance of the milling process and the complex dependence of the milling parameters. Although the initial stage could be controlled by drug size, the rest of the release depends strongly on polymer degradation.

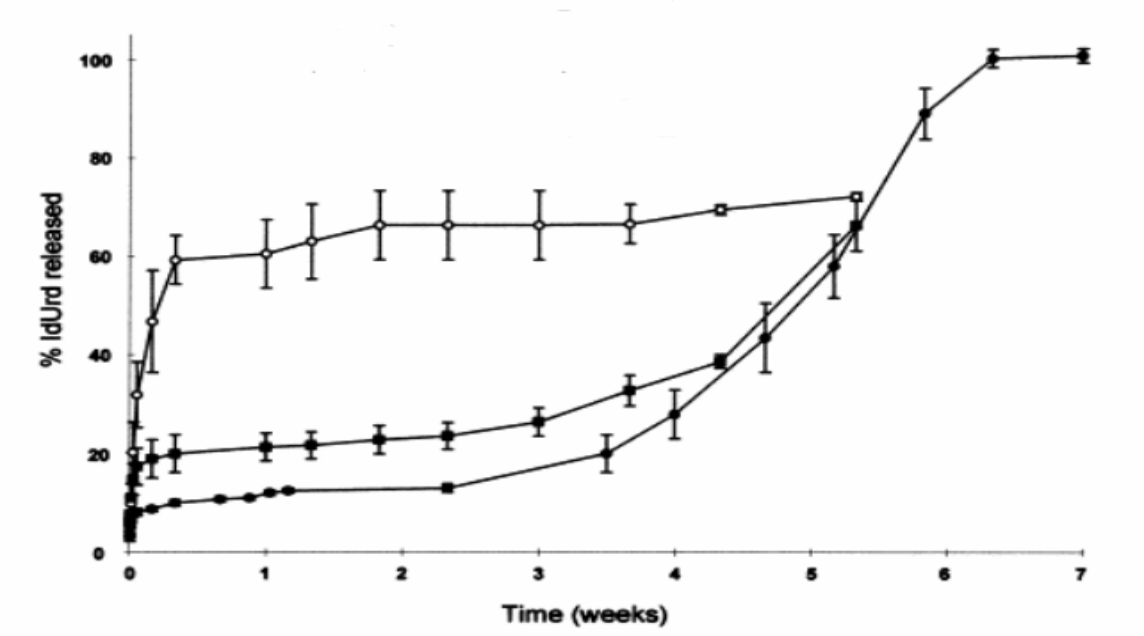



***Figure 10***: IdUrd release profiles from microspheres prepared after IdUrd size reduction according to: ○, 11.5 μm (spray-dried); ▪, 19.5 μm (ball milled); •, 15.3 μm (ball milled). (Gèze *et al., 1999*).

Other authors produced recombinant human growth hormone (rhGH) (Constantino *et al.,* 2004) or BSA (Constantino *et al.,* 2000) powder in the form of a reversible zinc complex by spray-freeze drying [powder smallest particle size (*e.g.*, sub-micron), was achieved]. Then encapsulated in PLGA microspheres using a non-aqueous cryogenic, non-aqueous process followed. It was found that the reduction in particle size of the encapsulated protein lowered the initial release both *in vitro* and *in vivo*, presumably due to a lower fraction of material with access to the microsphere surface. Hence, judicious selection of process variables to reduce the particle size of rhGH or BSA is one strategy that can be used to minimize initial release of the microencapsulated protein.

**4.2. Optimal polymers blends**

In general, reduced burst release was noticed with the use of lipopilic and high Mw polymers, because of the solidification of the particles is more rapid which may result in a viscous layer at the surface of the particles droplet and inhibition of the diffusion of drug towards the external surface which gave particles with high drug loading and good drug homogeneity. Moreovre, the use of lipopilic and high Mw polymers often leads to nano/microparticles with a large size.

On the other hand, high lipopilic polymers such as PCL and poly (D,L-lactic acid) (PDLLA high Mw) have high christalinity structure which stop drug solubility or distribution in nano/microparticles matrix and generally produced particles with deposition of drug on the microspheres surface. So, in order to increase the amorphous state of PDLLA (high Mw) for example, PDLLA (high Mw) matrices have been modified by the addition of a lower Mw PDLLA fraction (Bodmeier *et al*., 1989 ; Bain *et al*., 1999), resulting in enhanced drug release (without burst release effect) and polymer degradation rates restricted to the higher Mw PDLLA fraction alone. Also, Liggins and Burt (2004) have demonstrated that paclitaxel (used to treat lung, ovarian, breast cancer) release from 100 Kg/mol poly-L,L-lactide (PLLA) microspheres (high Mw) show high burst release (due to deposition of paclitaxel near the surface of the microspheres). But when paclitaxel was loaded into low Mw (1K g/mol) PLLA microspheres, the same burst release effect was observed (microsphere with high porosity). Thus, they prepared paclitaxel microsphere from blend of low Mw PLLA (1K g/mol) and high Mw PLLA (100 K g/mol) and the burst effect was reduced.

Edlund and Albertsson (2000 b) have provided an important comparison of a blend system with the corresponding copolymer system involving PLLA (cristalyne) and poly (1, 5-dioxepan-2-one) (PDXO) (amorphous). Microspheres made from the linear co-polymers Poly(L-LA-*co*-DXO) were porous and larger than those made from the blends. The release of a hydrophilic drug (timolol maleate) was slower for the blend PLLA-PDXO microspheres (Fig. 11). P (L-LA-co-DXO) demonstrated higher moisture sensitivity than a PLLA-PDXO blend of corresponding composition. The more crystalline and dense morphology of blend PLLA-PDXO microspheres was considered good in reducing burst effect release and improving the storage stability (Edlung and Albertsson, 2000b).

Saravanan M *et al.* (2003) reduced ibuprofen burst effect from ethylcellulose microspheres by developping ethylcellulose/polystyrene microspheres. Ethylcellulose/polystyrene (ratio of 80:20) microspheres showed prolonged ibuprofen release and less burst effect when

compared to microspheres prepared with ethylcellulose alone (microspheres lipophilicity increased).

As already discussed in part 3.2.1., the physical blend of two different polymers (with differences in hydrophilicity, crystallinity, charge or mechanism of degradation) can affect the release profiles of polymer microspheres.

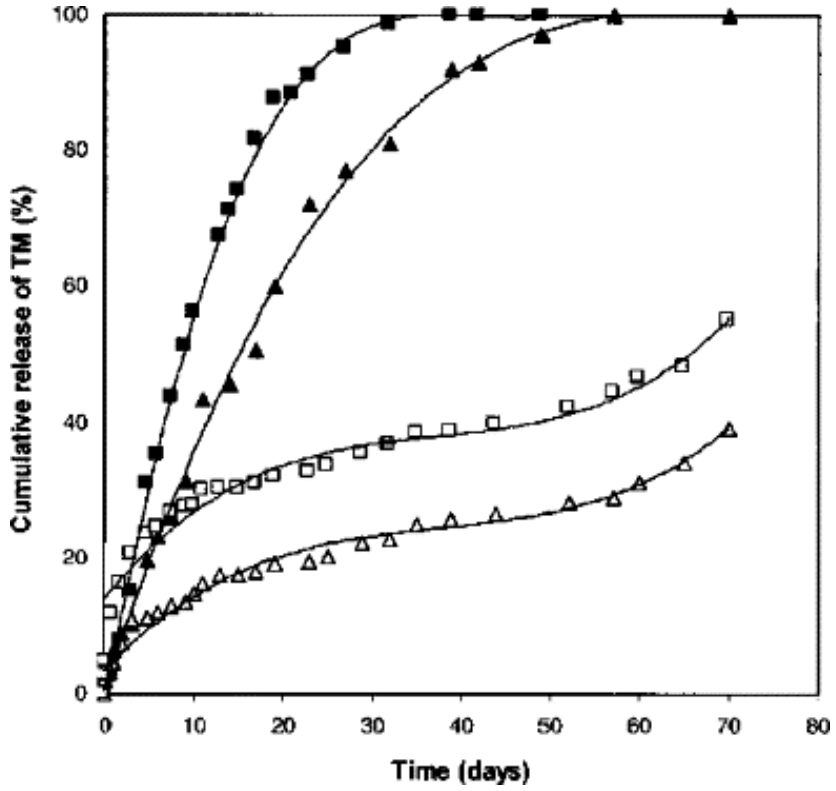



***Figure 11.*** Drug release from a blend system versus its copolymer system. Empty symbols represent the blend systems and the solid symbols represent the copolymer systems. The consistencies of the trend are shown by the two sets of data; the triangles and the squares represent PLLA–PDXO ratios of a 90:10 and 70:30, respectively. (From Edlund and Albertsson, 2000 b).

**4.3. Blend of different particles size**

By mixing microspheres of different sizes, it is possible to obtain another degree of controlling release. More importantly, linear, zero-order kinetics is obtainable by combining the proper formulation of microsphere sizes (Namur *et al*., 2006). For instance, Narayani and Rao (1996) employing gelatin microspheres with differents sizes (from 1 to 35 μm) successfully achieved zero-order drug release. Furthermore, in detailed studies, Berkland *et*

*al*. (2001, 2002), have also obtained a zero-order release by mixing microspheres of different sizes; sphere size was well controlled by fabricating spheres using the spray-drying technique.

### 4.4. Optimal formulation and preparation conditions

Choices of technique and formulation process with a view to limiting the burst effect depend on drug and polymer nature but also on the application (oral, pulmonary or parenteral administration). Each parameter exposed in the following part has to fit to each drug and each encapsulation study. Nevertheless, some explanations and efficient modifications are now known to limit burst effect.

Interestingly, the initial burst release was markedly changed by both additives and processing parameters Zheng *et al.* (2006) summarized the effects of additives and combined processing parameters on BSA release *in vitro* from PLGA microspheres (prepared by the double-emulsion solvent extraction/evaporation method). Initial burst release was accelerated by hydrophilic additives inside microspheres matrix (beta-cyclodextrin, hydroxypropyl-beta-cyclodextrin, various PEG, and sorbitol) but was delayed by hydrophobic additives (glycerol in the oil phase). In addition, burst release was delayed by operating microspheres preparation at high/low temperature or by adding hydrophilic additives such as sugar/inorganic salt in the external aqueous phase.

#### 4.4.1. Optimal choice of solvent or co-solvent used

The importance of the solvent type or mixed solvent (co-solvent) system was demonstrated by Park *et al*. (1998). Lysozyme-loaded PLGA microparticles were prepared using the oil in water (o/w) single emulsion technique. Here, the authors used a co-solvent system (DMSO, methylene chloride) varying the ratio of the component solvents. DMSO was used for solubilization of lysozyme and PLGA, and methylene chloride was used for generation of emulsion drops as well as solubilization of PLGA. Encapsulation efficiency increased, and initial burst decreased as the volume fraction of DMSO in the co-solvent system increased because particle size increased. Overall, results indicate that the presence of DMSO increased the hydrophilicity of the solvent system and allowed fast extraction of the solvent into the continuous phase and fast polymer precipitation which led to higher encapsulation efficiency, larger particle size and decrease of initial burst release.

In another study, praziquantel (treatment for human schistosomiasis) PLGA nanoparticles were made according to an emulsion-solvent evaporation method (Mainardes and Evangelista, 2005). Two organic solvents were separately utilized as the dispersed phase:

methylene chloride (poor soluble solvent) and ethyl acetate (partly soluble solvent). Smaller particle size was obtained when ethyl acetate was used. It could be explained by the obtention of smaller droplets in emulsion formulated with ethyl acetate when compared to the emulsion obtained with methylene chloride. The interfacial tension between methylene chloride and water is higher than the tension between ethyl acetate and water (Li *et al.,* 2000). The smaller nanoparticle size obtained with ethyl acetate resulted in faster drug release and large initial burst release.

On the other hand, ethyl acetate showed high drug loading and low initial burst effect when emulsion-solvent diffusion technique was used (fast polymer precipitation) in place of emulsion-solvent evaporation technique (Meng *et al*., 2003). Indeed, Meng *et al*., 2003 found rapid solidification of the microparticles (fast diffusion rate of ethyl acetate from embryonic microparticles to outer aqueous phase) which led to high drug loading, lower burst effect and a slower drug release of the microparticles.

Thus, to obtain particles with high drug loading and low initial burst effect it will be better i) to use a poor soluble solvent as methylene chloride [according to the USP XXIII requirement the residual methylene chloride presented in microspheres should be below the limit of 500 ppm (Bitz & Doelker 1996)] in an emulsion-solvent evaporation method, and ii) to use a partly soluble solvent as ethyl acetate (which is also less toxic) in an emulsion-solvent diffusion method.

### 4.4.2. Control of preparation temperature

Preparation temperature is a parameter influencing the evaporation or extraction speed of the solvent; consequently it can influence the morphology of the particles and the drug release kinetics. It has been reported that microparticles formulated by w/o/w process at 5 or 42°C have the lowest initial burst release of BSA, in contrast to microparticles prepared at 33°C, which showed the highest initial burst (Yang *et al*., 2000 a) (fig. 12 et 13).

In another study, Cleland *et al*., 1997 noticed that the kinematic viscosity of the polymer (PLGA) solution was increased, by lowering the temperature of the first emulsion to 0°C. A more viscous polymer solution is broken up with more difficulty into smaller droplet which often leads to particles with a large size. So, this change in the kinematic viscosity significantly reduced the initial burst release.

### 4.4.3. Emulsion stability improvement

In all emulsions techniques [solvent extraction/evaporation (o/w; w/o/w; s/o/w); spray-drying; phase separation (s/o/o; s/o/o/o)] used to obtain particles, emulsion stability is the most important factor which should be taken in consideration to obtain good particles morphology and drug ditrubution in the matrix. In this section we will focus on w/o/w emulsions.

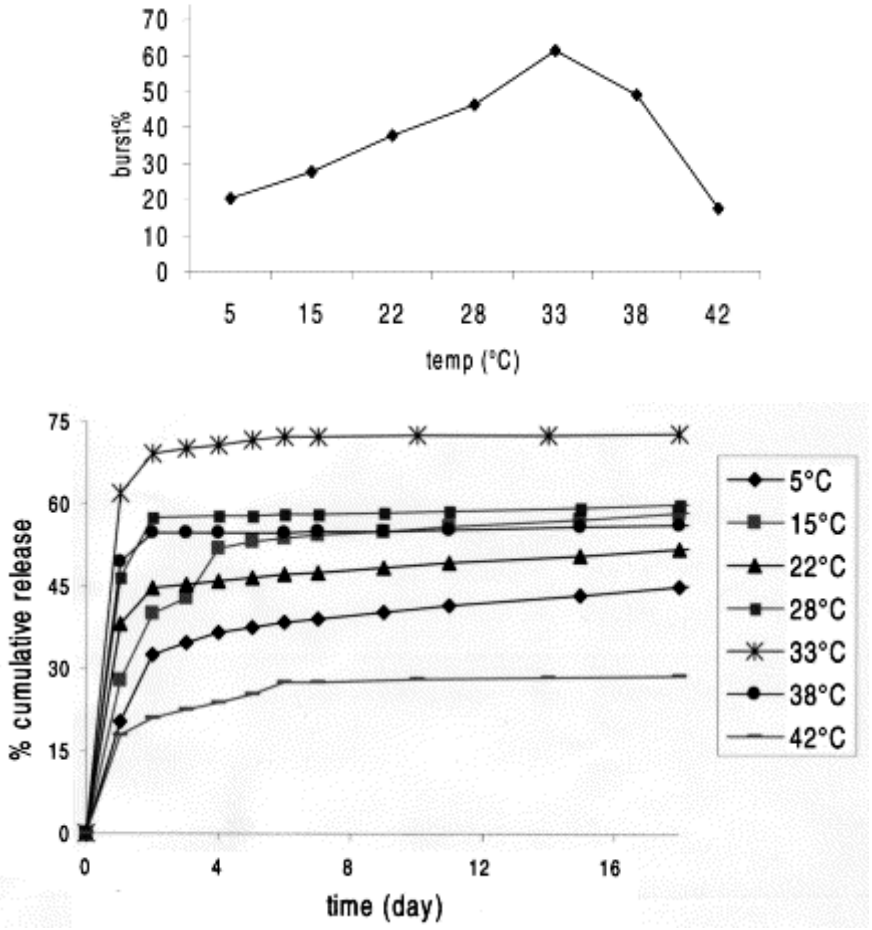



***Figure 13.*** *In vitro* release profiles of BSA release of PLGA microspheres. The microspheres were prepared using the w/o/w technique. (From Yang *et al*., 2000 a)

- ✓ **Primary water in oil ($w_1$/o) emulsion**

In the double emulsion-solvent evaporation/extraction method w/o/w, stability of the primary (water (w1) in oil (o), $w_1$/o) emulsion is a critical factor for efficient internalization of the active ingredient. When the primary emulsion is unstable, the drug distribution in final polymeric matrix is not homogeneous because the internal aqueous phase ($w_1$) tends to merge

with the neighboring aqueous continuous phase ($w_2$). In this case, burst release can be important.

Stability of the primary emulsion ($w_1$/o) can be enhanced by including emulsifying agents such as PVA (Yeo and Park 2004). A high concentration of PVA included in the internal water phase led to an increase in the encapsulation efficiency and a decrease in initial burst (Yang *et al.,* 2001) of the encapsulated drug model (BSA). This was found because BSA was uniformly distributed within the microparticles. This indicates that emulsifying agents such as PVA in internal aqueous phase (w1) contributed to stabilization of the primary emulsion and limit burst release. In addition, adding phosphate buffer in the internal aqueous phase ($w_1$) results in a decrease of drug loading and an increase of drug burst release (Mao *et al*., 2007) which may destabilise the emulsion.

Good stability of emulsion (w/o or o/w) can be also achieved by generating a fine primary emulsion (Sah *et al*., 1995; Yan *et al*., 1994). The fine primary emulsion was obtained with the help of a high energy homogenization method such as sonication (Yan *et al*., 1994) or high shear rate mixing (Sah *et al*., 1995). This stable and fine primary emulsion (w1/o) gave good homogeneous drug distribution in the matrix [demonstrated by confocal microscopy (Yan *et al*., 1994) or SEM (Sah *et al.,* 1995)]. However, the use of a high energy homogenization can be responsible for protein denaturation (Mao *et al*., 2007; Zhang *et al*. 2005).

Finally, Stability of the $w_1$/o influenced by the volume and the viscosity of the internal aqueous phase. High volume and high viscosity of the internal phase are not preferable in achieving homogeneity drug in the matrix system. Moreover, high internal volume can produce high porosity particles (Yeo and Park 2004). It seems to be preferable to formulate microspheres with low volume and low viscosity of the internal aqueous phase ($w_1$) to avoid important burst release.

- ✓ **Second emulsion oil in water (o/$w_2$) emulsion**

The stabiliy of o/w2 influenced by the type and concentration of the surfactant. Zhang *et al.,* (2005) optimized the double emulsion process in ordre to decrease the burst release of BSA from PLGA microspheres. As far as surfactant type was concerned (in the outside phase), a diminished burst was found when poly (vinyl pyrrolidone) or Tween 80 was used rather than PVA. But, a low microsphere yield was noticed when Tween 80 was used as surfactant in the outer aqueous phase.

The particle size decreases with increasing surfactant concentration in the continuous phase, thus, small particles have generally high burst release and low drug loading (Hsu *et al*., 1999; Yang *et al*., 2001). It is due to the increased of the continuous phase viscosity which increase shear stress.

### 4.4.4. Use of additives molecules

It is well known that additives are able to modify the release rate of drugs from microspheres. The effect of these substances can be important (Urata *et al*., 1999 and Sansdrap and Moes, 1998) and depend on the nature of the substance added (Table 2).

- ✓ Hydrophilic additives:

Classically, higher drug burst release is obtained when hydrophilic additives are added in the nano/microparticles matrix, because they are known to increase porosity of nano/microspheres, thus causing an increase in permeability to mass transport. But, some authors motined different results, for example, Wang *et al*. (2004) in a surprising study mentioned the significant reduction in initial burst release of octreotide acetate (a highly water-soluble peptide) from PLGA microspheres by the co-encapsulation of a small amount of glucose (hydrophilic excipient) dissolved in the inner water phase. They conclude that the effect of glucose on initial burst is determined by two opposite factors:

- increased initial burst due to increased osmotic pressure during encapsulation and drug release
- Decreased initial burst due to decreased permeability of microspheres (denser periphery microspheres).

However, it was observed that the addition of salt (such as sodium chloride) in the external aqueous phase (to generate a higher osmotic pressure) reduced the initial burst and revealed microparticles which displayed a smooth surface without pores and a spherical, absolutely dense and compact shape (Jiang *et al.*, 2002; Freytag *et al*., 2000). Addition of salt or sugar into the continuous phase contributes to suppress the initial burst (Jiang *et al*., 2002). According to the authors, the initial burst decreased by 46 % and 27 % by addition of NaCl and sucrose, respectively. Here, the presence of salt or sugar increased the osmotic pressure across the polymer phase, which was in essence a semi-permeable membrane in the semisolid state. This increase in osmotic pressure prevented influx of the continuous phase into the dispersed phase and reduced the formation of water channels which could have led to a high

initial burst. On the other hand, in a different study on insulin-loaded PLA microparticles, the osmotic pressure gradient induced by the addition of NaCl to the external aqueous phase (0.5 % PVA solution) led to diffusion of the w1 phase into the $w_2$ phase, resulting to low insulin encapsulation efficiency (Uchida *et al.,* 1997). This decrease may be due to high concentration of NaCl used 10 % (w/v) compared to 3.6 % (w/v) used by Jiang *et al* (2002). Going back to our discussion on the initial burst, the difference between NaCl and sucrose in their capabilities of reducing the initial burst seems due to their different contributions to the continuous phase (Jiang *et al.,* 2002). In addition to osmotic pressure, NaCl increases the polarity of the continuous phase (increase interfacial tension between organic phase and aqueous phase).

Conjugate of PEG to therapeutic protein increase structural and functional stability of protein in solution (Kim *et al*., 2002). Kim *et al*., 2002 demonstrated that PEG-conjugated (pegylated) lysozyme, compared with native lysozyme, exhibited enhanced physical stability during a w/o emulsification step required in the formulation of PLGA microspheres. In addition, Kim *et al*., 2002 Pegylated recombinant human epidermal growth factor rhEGF (conjugated with N-hydroxysuccimide-derivatized methoxy-PEG of Mw 2000 and 5000) and show that pegylated-rhEGF loaded PLGA microspheres exhibit a greatly reduced initial burst release of 29 % at day 1, compared with that of rhEGF loaded microspheres (66 %). The reduced burst release for pegylated rhEGF can be attributed to increased hydrodynamic volume of rhEGF owing to pegylation and to more even distribution of pegylated rhEGF within the matrix of microspheres. Pegylated rhEGF, becoming less aggregated during the homogenization process was expected to be more homogeneously loaded within PLGA microspheres than native rhEGF that was more prone to aggregate in response to a shear-induced emulsification process. On the other hand, more physically stable pegylated rhEGF might be more slowly diffused out due to the increased molecular size due to the presence of PEGchains on the surface of rhEGF.

Furthemore, addition of L-arginine from concentration of 0 to 4 % (w/w) into the internal water phase remarkably reduced the *in vivo* and *in vitro* initial burst release of the drug (TAK-029, a water-soluble GPIIb/IIIa antagonist, non-ionic) from the microspheres prepared by the w/o/w emulsion solvent extraction/evaporation method (Takada *et al.,* 1997). These authors supposed that the reduced initial burst of microspheres with high contents of arginine results probably from hydrophobic diffusion barriers surrounding the drug core portions, which are formed by an ionic interaction between positively charged amino groups of arginine and negatively charged carboxylic acid groups of PLGA. They also mention that the Tg of the

polymeric microparticles was raised with an increasing amount of L-arginine, suggesting the formation of hydrophobic barriers or of a more rigid structure of the polymer matrix. This was confirmed by SEM studies, where the cross-section of the microspheres revealed a porous structure for microparticles without L-arginine and a rigid inner structure for microparticles with L-arginine. The hydrophobic diffusion barriers are supposed to cause a delay of water penetration, hydration and swelling of the microparticles.

Finally, Yamaguchi *et al.* (2002) have demonstrated that when the crystalline insulin was dispersed in methylene chloride as (s/o) inner dispersion, most of the insulin molecules were inlaid on the surface of the PLGA microparticles. Consequently, insulin–loaded PLGA microparticles exhibited a high initial burst. Furthermore, the addition of glycerol or water to the primary methylene chloride dispersion (s/o) resulted in drastically suppressed initial burst release. This phenomenon theoretically occurs because the insulin and PLGA molecules, having amphiphilic properties, converge to the interface between the hydrophilic additive and dichloromethane. Hence, the insulin molecules are heterogeneously located inside of PLGA microparticles but not on the surface and would be gradually released following PLGA hydrolytic decomposition. As an additional effect of glycerol, the initial burst was further suppressed due to the decrease of the glass transition temperature of PLGA from 42.5 to 36.7°C. Since the annealing of PLGA molecules took place at around 37°C, the porous structure of microspheres immediately disappeared after immersion in release medium or subcutaneous administration. The insulin diffusion through the water-filled pores was therefore effectively prevented. Wang *et al*. (2002) also reported that the PLGA microparticles surface pores disappeared after 24h incubation in the release medium.

- ✓ **Hydrophobic additives**

Among hydrophobic additives, fluorosilicone oil (FsiO) has been shown to diminish the rate of diffusion of a guanosine derivative (ganciclovir) from PLGA microspheres and delayed the penetration of watre into the microspheres, thus burst release effect reduced (Herrero-Vanrell *et al*., 2000).

Fernández-Carballido *et al.,* (2004), reported that the addition of Labrafil in the PLGA microspheres (loaded with ibuprofen) modified the release profiles of the drug by controlling its release rate and reducing its initial burst (fig. 14). Labrafil, a PEG derivative and non-ionic amphiphilic excipient, is used as a co-surfactant in pharmaceutical systems such as microemulsions. Although Labrafil is a PEG derivative, the presence of fatty acids (mainly

oleic acid) in the non-ionic amphiphilic compound granted hydrophobic properties to the molecule. Furthermore, Labrafil is soluble in methylene chloride and may be dissolved in the organic solvent at the same time with ibuprofen which results more homogeneous ibuprofen distribution into the particles matrix. For this reason, they consider that Labrafil, contrary to PEG, promotes an obstruction of channels, resulting in a slow release rate of the drug.

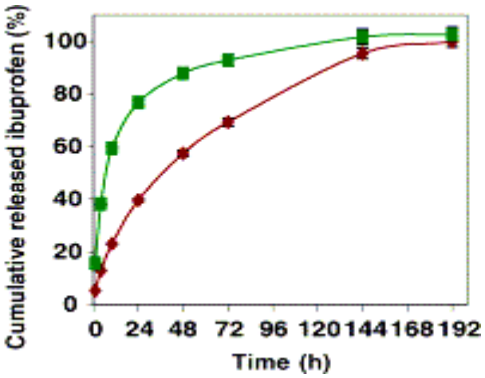



***Figure 14.*** Cumulative percentage of ibuprofen released from microspheres made from Resomer 504 PLGA (Mw=48,000 Da), without Labrafil (▪) and with Labrafil (♦). (From Fernández-Carballido *et al*., 2004).

Furthermore, Zhao *et al* (2007) reported that doxorubicin release profiles from PLGA nanoparticles with vitamin E-PEG conjugates [D-α-tocopheryl PEG succinate (TPGS)] or vitamin $D_3$-PEG conjugates [cholecalciferol PEG succinate (CPGS)], had a lower initial burst and more sustained release pattern than PLGA nanoparticles without TPGS or CPGS. PLGA prepared nanoparticles by a dialysis method [TPGS /CPGS, doxorubicin and PLGA were dissolved in the dimethyl sulfoxide (DMSO)]. Since the initial burst release can be attributed to doxorubicin molecules located near the surface or on the surface of the nanoparticles, it was suggested that the addition of TPGS or CPGS during the nanoparticle formation may introduce a hydrophilic layer on the surface of the nanoparticles which forms a double-wall configuration and subsequently delay the drug release.

Another study described the addition of cyclic DL-lactide dimers (in organic phase) during the microparticles preparation which also affected the *in vitro* release profiles of FITC-dextran from PLGA microspheres. The DL-lactide remarkably reduced the initial burst release of FITC-dextran. This reduction of burst *in vitro* can be explained by a significant decrease of the glass transition temperature caused by monomers inside the spray-dried microspheres which plasticise the polymer matrix. At early time points of the *in vitro* release

process, pores which have already been preformed are blocked and the release rate is strongly reduced. (Bittner *et al*., 1998) (Fig. 15).

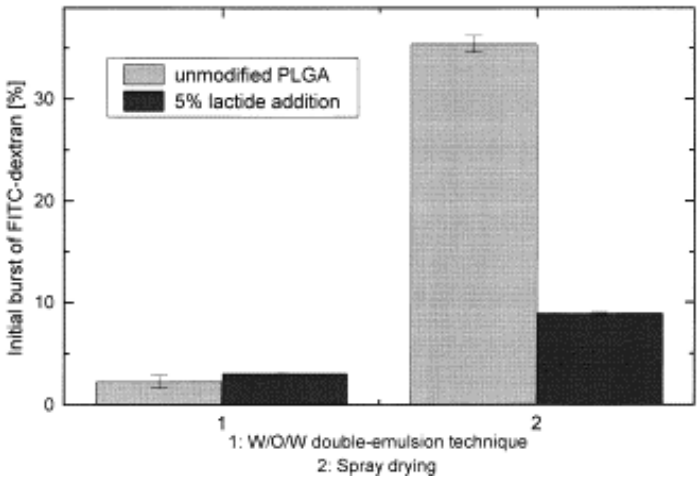



***Figure 15.*** Initial release of FITC-dextran from poly (lactide-co-glycolide) microspheres prepared by spray-drying and by the w/o/w double-emulsion technique within the first 24 h. Spray-drying (1), w/o/w technique (2) (from Bittner *et al*. 1998).

Other studies added oleate complex during the microspheres preparation. Choi and Park (2000) reported that microparticles prepared by the o/w single emulsion method with leuprolide-oleate complex showed a significantly suppressed initial burst release of leuprolide (approximatelty 10% after 24h) in contrast to that prepared with free leuprolide. This is due to the much lower aqueous solubility of the hydrophobically modified leuprolide in the complexation.

The following table summarizes the means, discussed so far, able to achieve a reduction in burst release.

| Additives | Formulation | Burst effect | Explanation / Action | Authors |
|---|---|---|---|---|
| **Hydrophilic additives :** | | | | |
| Salt (such as sodium chloride ) | Double emulsion w/o/w evaporation / extraction or o/w | Added in w2=> decrease burst | Osmotic pressure gradient and increases the polarity of the continuous phase (particle with less porosity and highly dense structure) | Yeo & Park 2004 |
| | | Added in w1 => Increase burst | Led to diffusion of the w1 phase into the w2 phase (bad drug distribution in matrix ) | |
| Sugar (such as glucose, fructose and sorbitol) | Double emulsion w/o/w evaporation/ extraction or o/w | Added in w1 => increased initial burst | Due to increased osmotic pressure during encapsulation and drug release and increase porosity | Wang *et al.*, 2004 |
| | | Added in w2 => decreased initial burst | Due to decreased permeability of microspheres (denser periphery microspheres). | |
| β-cyclodextrin, hydroxyl -propy-β-cyclodextrin, methyl-β-cyclodextrin | Double emulsion w/o/w evaporation/ extraction | Added in w1=> increased initial burst | Increase porosity of microspheres, causing an increase in permeability to mass transport. | Trapani *et al*., 2003 |
| | s/o/w or spray-drying emulsion | Co-lyophilization with proteins prior to encapsulation => decreased initial burst | Due to complexed with proteins, preservation of their stability and their good ditribution in microspheres | Castellanos *et al*., 2006<br>De Rosa *et al*., 2005 |
| Poly(ethylene-glycol) 400 | Double emulsion w/o/w evaporation/extraction or o/w | Added to the internal aqueous phase (or mixed with drug powder) => reduced initial burst (especially peptide and proteins) | Limited the penetration of drug in the interfacial film of the primary w/o emulsion. Consequently, they stabilized the emulsion and reducing the drug contact with the organic phase | Pean *et al.*, 1999<br>Graves *et al.*, 2005 |

| Additives | Formulation | Burst effect | Explanation / Action | Authors |
|---|---|---|---|---|
| **Hydrophilic additives :** | | | | |
| Gelatin or hydroxypropyl methylcellulose | o/w solvent evaporation method | Dispersed into oil phase => Increased burst release | Increase water uptake of microspheres | Martínez-Sancho *et al.*, 2003 |
| L-arginine | s/o/w emulsion solvent extraction / evaporation method | Added into an oil phase => reduced drug initial burst release | Ionic interaction between positively charged amino groups of arginine and negatively charged carboxylic acid groups of PLGA. Hydrophobic diffusion barriers surrounding the drug core portions delayed water penetration, hydration and swelling of the microparticles. The Tg of the polymeric microparticles was raised (formation of hydrophobic barriers) | Takada *et al.*, 1997 |
| **Hydrophobic additives :** | | | | |
| Phosphatidylcholine | Double emulsion w/o/w evaporation/ extraction or o/w | Added in into an oil phase=> reduced the initial burst | Increase resistant water uptake of microspheres | Chung *et al.*, 2006 |
| Fluorosilicone oil | s/o/o phase separation | Dispersed in acetone solution, and emulsified in silicone oil => reduced the initial burst | Delayed the penetration of water into the microspheres | Herrero-Vanrell *et al.*, 2000 |
| Isopropyl myristate or vitamin E | o/w solvent evaporation method | Added in into an oil phase => reduced initial burst release | Decrease water uptake of microspheres | Martínez-Sancho *et al.*, 2003 |
| Labrafil | o/w emulsion solvent evaporation method | Dissolved in the organic solvent the same time than drug and polymer => reduced the initial burst | Homogeneously drug distributed into the particles and obstruction of particles channels | Fernández-Carballido *et al.*, 2004 |

| Additives | Formulation | Burst effect | Explanation / Action | Authors |
|---|---|---|---|---|
| **Hydrophobic additives :** | | | | |
| Vitamin E or D-PEG conjugates | Dialysis method | Dissolved with drug in the organic phase which was subsequently transferred into a dialysis membrane => reduced the initial burst. | Hydrophilic layer on the surface of the particles to form a double-wall configuration. | Zhao *et al.*, 2007 |

Table 2: Additives used in nano/microparticles and their action on burst release.

## 4.5. New techniques

### 4.5.1. Solid-in-water-in-oil-in-water (s/w/o/w) emulsion

Lee *et al.* (2007) proposed a new method (s/w/o/w) to maintaining protein stability during protein encapsulation and release which was also able to reduce protein initial burst release. The following part compares the conventional multi-emulsion w/o/w and s/o/w methods.

In the emulsion step, fine solid lysozyme lyophilized with cyclodextrin derivative (Sulfobutyl ether β-cyclodextrin sodium salt (Captisol®) (SBE-CD)) and PEG were incorporated into aqueous microdroplets comprising viscous polysaccharides solution and then were coated with a mixture of PLGA (Fig. 17). Polysaccharides such as starch and hyaluronate formulate an internal highly viscous aqueous solution that may delay certain interactions between proteins and organic solvent. In addition, viscous polysaccharides may have a favorable influence on protein stability after microspheres hydration, reducing the passive diffusion of the metabolites and the acids released by PLGA, and minimizing initial burst.

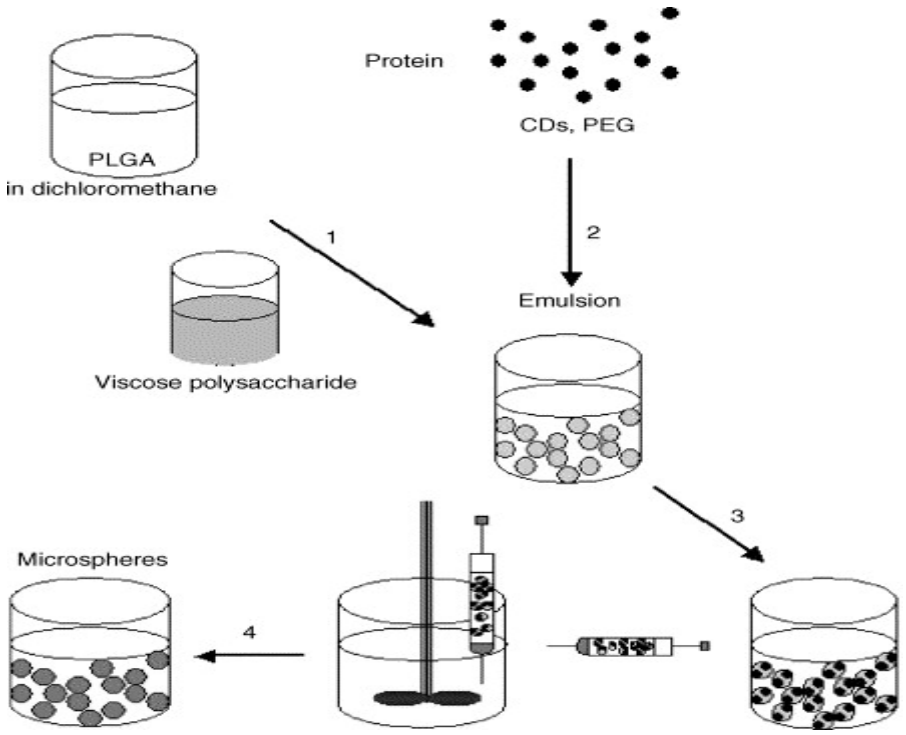



***Figure 17.*** Procedure of microsphere manufacturing by the novel viscous s/w/o/w method. (From Lee *et al.*, 2007)

Lysozyme release profiles and kinetics from lysozyme-encapsulated microspheres were evaluated under *in vitro* condition and presented in Fig. 18. The microspheres manufactured by the viscous s/w/o/w method followed nearly zero-order kinetics with minimal initial burst and had a broad protein drug distribution in the microspheres (as visualized by confocal

microscopy). However, the s/o/w method showed decreased total drug release, probably due to exacerbated release by protein denaturation during microsphere fabrication and protein degradation during incubation at 37°C (Abgar *et al.*, 2001). The w/o/w method induced drug burst release of about 50% for 2 days. It is thought that in the w/o/w method, much (hydrophilic) protein was incorporated next to the surface area of microspheres, which is ascribed as a typical cause of initial burst (Yeo and Park, 2004).

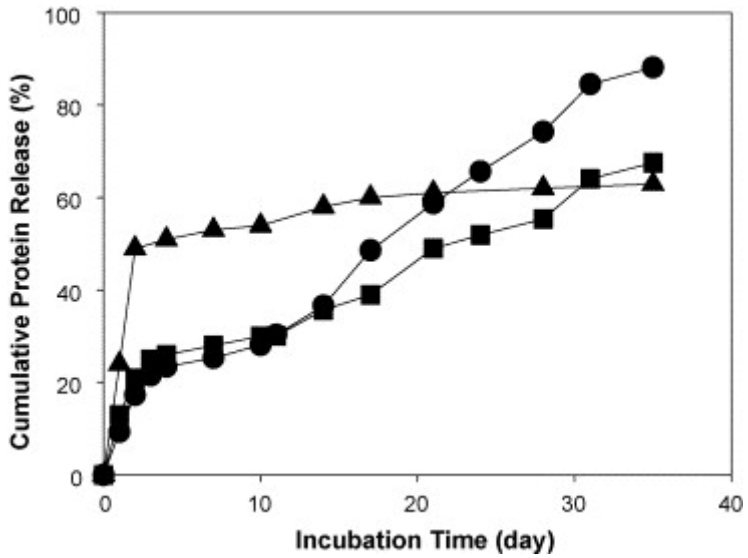



***Figure 18.*** The cumulative lysozyme release (wt. %) from s/w/o/w (●), s/o/w (■), w/o/w (▲) microspheres prepared by different manufacturing methods. Average values from triplicate experiment were plotted. (From Lee *et al.*, 2007)

In addition, this means that the viscous s/w/o/w method provides a safe strategy for microsphere manufacturing and has promising properties, involving the preservation of protein bioactivity, the inhibition of protein denaturation or agglomeration, and long-term protein release.

### 4.5.2 Thermoreversible gel-method microspheres

Leo *et al.*, (2006 a) developed a novel strategy for the formulation of biodegradable PLA microspheres as delivery systems for proteins or peptides (Fig. 19). The strategy was based on the exploitation of the gel-sol transition of the thermoreversible Pluronic F127 gel. The gel allowed the formation of the particles without being co-entrapped in the matrix. The microspheres were prepared using the novel technique (thermoreversible gel (TG) -method microspheres) in comparison with microspheres prepared using the classical double emulsion/solvent evaporation method (w/o/w). Two types of BSA, with different water

solubility, were used as model proteins. TG produced smaller size particles compaired to the w/o/w particles; they displayed a reduced initial burst effect and a higher rate in the second release phase that resulted in a quasi-constant profile. The release behavior of the TG particles may be attributable to both the localization of the protein in the particle core and the few pores in the matrix.

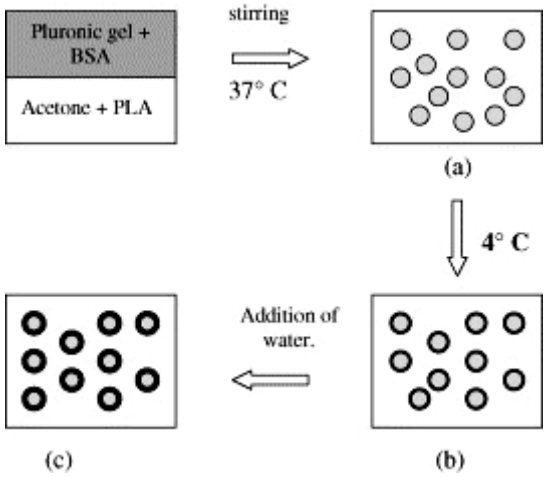



***Figure 19.*** Schematic description of the proposed mechanism of formation of PLA microspheres by the thermoreversible gel-method: (a) formation of the pseudo-emulsion between Pluronic-gel and acetone; (b) formation of the polymer crust upon the gel–sol transition; (c) formation of particles upon the solvent extraction.(Leo *et al*., 2006 a).

## 4.6. Surface modifications of nano/microspheres

### 4.6.1. Surface drug extraction

Extracting the surface drug is a fairly simple approach in order to reduce the initial burst, (Lalla and Snape, 1993; Leo *et al*., 2000). To remove the surface drug fraction from the microspheres a treatment with sodium carbonate solution was used. This method is effective at reducing burst because drug is removed from the outer layers of controlled release devices (Leo *et al.*, 2000). However, implying a loss of drug and consequently a problem of cost of fabrication, this approach has a very limited use.

### 4.6.2. Solvent post-treatment:

Another suggested method of burst prevention in PLGA systems includes the treatment of the wet microparticles with an organic solvent/water mixture or adding organic solvents to the external aqueous phase during the microparticle preparation. This resulted in a reduction of

the pores present on the microsphere surface, as well as of the burst effect (Ahmed *et al.*, 2000).

### 4.6.3. Polymer cross-linking and modification of the matrix

Addition of surfactants, which are amphiphilic molecules, was also used to reduce the burst. Indeed such molecules are located especially at the surface of polymeric particle and may modify surface properties. During the last few years, cross-linking was a method widespread used (Thote *et al.*, 2005).

The biodegradable polymer, PLA was blended with a series of pluronic surfactants (triblock copolymers of ethylenoxide/propylenoxide/ ethylenoxide), to modify the morphology of the matrices. It was found that PLA/pluronic L-101 blend exhibited particularly a significant reduction in the initial burst due to the formation of a less porous surface morphology (Park *et al*., 1992 a, b). It was later on discovered that if the above blend matrices were treated with an aqueous solution of polyethyleneimine (PEI), there was an even more pronounced decrease in the protein burst as well as a significant extension of the release time. The mechanism of this phenomenon was believed to be that PEI chains adsorb onto or diffuse into the polymer matrices; thereby ionically cross-linking protein molecules present near the microparticle surface region and avoid subsequently the burst release (Park *et al*., 1992 b).

Thote *et al*. (2005) reduced dexamethasone (hydrophobic drug) initial burst from PLGA microparticles by crosslinking of the microparticle surface using ethylene glycol dimethacrylate and tri-(ethylene glycol)-dimethacrylate. The surface crosslinking was performed by ultraviolet radiation. Due to surface crosslinking, an additional diffusional resistance was created which prevented easy dissolution of the drug into the release medium and brought about a substantial reduction in the initial burst release. Moreover, the time required for reaching a stationary-state release was also observed to be delayed, prolonging the sustained drug delivery. This concept was further tested with a hydrophilic drug, the sodium salt of dexamethasone phosphate, encapsulated in PLGA polymer microparticles and was observed to reduce the burst release as well.

## 4.7. Particle coating

Another popular method used to prevent the burst release is the surface modification by additional coating steps to provide an outer layer containing no drug (fig. 21 A). Three types of studies could be distinguished.

A coating (based on interaction between anionic and cationic charges) of microparticle with a polycationic molecule has been investigated. In a study on the delivery of bio-macromolecules including proteins and dextrans, Wheatley *et al*. (1991) used alginate (negative charge) beads coated three times with a polycation, either poly (L-lysine HBr) or poly (vinyl amine), to prevent the initial burst release. It was found that increasing the polycation concentration decreased the burst effect and lead to more sustained release profile.

Another simple strategy of coating method consisted of the dipping of microparticles or/ short portion (BSA segment) of implant into a polymer solution in which the microparticles/implant are not soluble *e.g.* an aqueous solution of gelatine for PLGA microparticles or a PLGA solution in acetone for alginate microparticles (Huang *et al.,* 1999; Zhou *et al*., 1998). In these studies coating of microparticles/implant reduced the initial burst release. Zhou *et al.* (2000) used coating/dipping method and proposed a new microsphere delivery system composed of alginate microcores surrounded by biodegradable PLA/PEG, which showed an improved loading efficiency, stability of proteins as well as a significantly reduced initial burst compared to the conventional microspheres. With thicker coatings, there were slower releasing rates, and the release rate can be simply related to the coating thickness. Also, Chiou *et al.* (2001) reported that the post-coating of PLA microspheres with different chitosans reduced the initial burst and controlled the drug release of the microspheres. However, coating with high Mw and high viscosity chitosans reduced the initial burst and controlled the drug release of PLA microspheres more significantly than with low Mw/viscosity chitosans.

An alternative possibility to coat microparticles (microencapsulated microspheres) is the dispersion of preformed microparticles into an organic polymeric coating solution followed by emulsification with an aqueous PVA solution (Göpferich *et al.*, 1994). In a similar method proposed by Ahmed *et al.*, (2002), microparticles were also coated by a two step procedure based on the preparation of the primary (uncoated) microparticles containing a highly water soluble drug (oligonucleotide) by the w/o/w-method followed by an additional coating with the same polymer. During this coating procedure, wet or dry (primary) microparticles were dispersed in peanut oil before dispersing them in the organic coating polymer. The peanut oil served to cover the microparticles surface and therefore reduced or prevented the rapid dissolution of the microparticles in the organic coating solution. After emulsification of organic dispersion containing the microparticles within an aqueous PVA solution, an additional polymeric layer precipitated onto the surface of microparticles. The coated mircoparticles showed a significant reduction of the burst release.

Another approach to coat microparticles (double-walled microspheres) is the formation of a core and a shell upon phase separation (double wall) by mixing two incompatible polymers. The initial burst from such double wall microparticles was observed to be reduced by (Leach, 1999).

Berkland *et al.,* 2004 proposed a method for fabricating uniform double-walled microspheres with controllable size and shell thickness (fig. 20). The method employs multiple concentric nozzles to produce a smooth coaxial jet comprising an annular shell and core material, which is acoustically excited to break up into uniform core–shell droplets. The orientation of the jets, material flow rates, and rate of solvent extraction are controlled to create uniform and well-centered "double-walled" microspheres exhibiting a controllable shell thickness. Double-walled microspheres were fabricated with PLGA as microsphere core and poly[(1,6-bis-carboxyphenoxy) hexane] shell phase. Utilizing this technology, double-walled microsphere maintaining complete and well-centered core encapsulation for double-walled microspheres and uniform surface coating which controlled drug release from these microspheres.

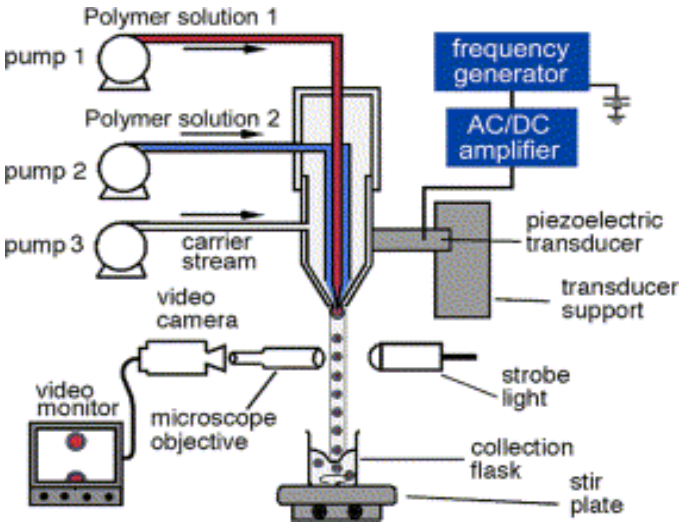



***Figure 20.*** Schematic diagram of precision particle fabrication apparatus used to create uniform double-walled microspheres (from Berkland *et al.,* 2004).

Finally, Wang *et al.* (2007) deposited polysaccharides on drug-loaded microspheres using layer-by-layer self-assembly to produce core-shell microparticles for sustained drug release. They prepared ibuprofen loaded poly (3-hydroxybutyrate-co-3-hydroxyvalerate) microparticles by conventional solvent evaporation. The multilayer shells of chitosan/sodium alginate and poly (diallyldimethylammonium chloride) /sodium poly (styrenesulfonate) were formed on the ibuprofen loaded poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

microparticles using layer-by-layer self-assembly. The *in vitro* release experiments revealed that, as for the microparticles with three chitosan/sodium alginate bilayer shells, the initial burst release of ibuprofen from the microparticles was significantly suppressed and the half release time was prolonged to 62 h with regards to 1h for the microparticles without coverage.

### 4.8. Microparticles incorporation into gels

Lagarce *et al.,* (2005), designed baclofen PLGA microspheres which were then dispersed in chitosan thermosensitive gels (fig 21 B), Pluronic PF-127 gels, carboxymethylcellulose solutions or Ringer lactate solution. The release rate was assessed *in vitro* using a continuous flow cell method and *in vivo* after intrathecal injection in goats. Their results showed that, after microsphere dispersion in a viscous medium, the burst effect was reduced by at least a factor of 2 *in vitro*. *In vivo*, PF-127 gel was found to be the best vehicle to reduce the burst effect by a factor of 10 in cerebrospinal fluid, and by a factor of 2 in plasma.

Moreover, in the work of Ahmed *et al* (2001), microparticles were incorporated into molten monoglycerides or into preformed cubic phases, as well as dispersed into a monoglyceride based three component formulation which formed a cubic phase (ICP) in-situ around the microparticles upon contact with aqueous medium. The microparticles in this approach get entrapped inside the cubic phase matrix which forms an extended diffusional barrier and therefore significantly reduces the initial burst. Sullivan *et al.* (2000) reported that the incorporation of drug –loaded polymeric microparticles into the SABER™ Delivery System [a novel controlled-release technology that uses a high-viscosity base component, such as sucrose acetate isobutyrate (SAIB)] provides an additional barrier to diffusion, thus dramatically reducing the initial burst and extending the duration of release (Okumu et al., 2000). Similarly, Zn-human growth hormone was incorporated into PLGA microspheres. The microspheres were then added to reverse thermal gelation solution (RTG) to suspend the particles and at body temperature (37 °C) the gel formed around the microparticles. As a consequence the initial burst was significantly reduced (Okumu *et al*., 2000; Shih and Zentner, 2000).

### 4.9. Nanoparticles encapsulated in microparticles (composite microparticle)

During the last decade, encapsulation of nanoparticles in microparticles has been investigated in research for differents applications which are summarized in table 3. This encapsulation practice creates a new composite system (fig 21 C). Different techniques to prepare these formulations have been used and are reported in details, in the following part.

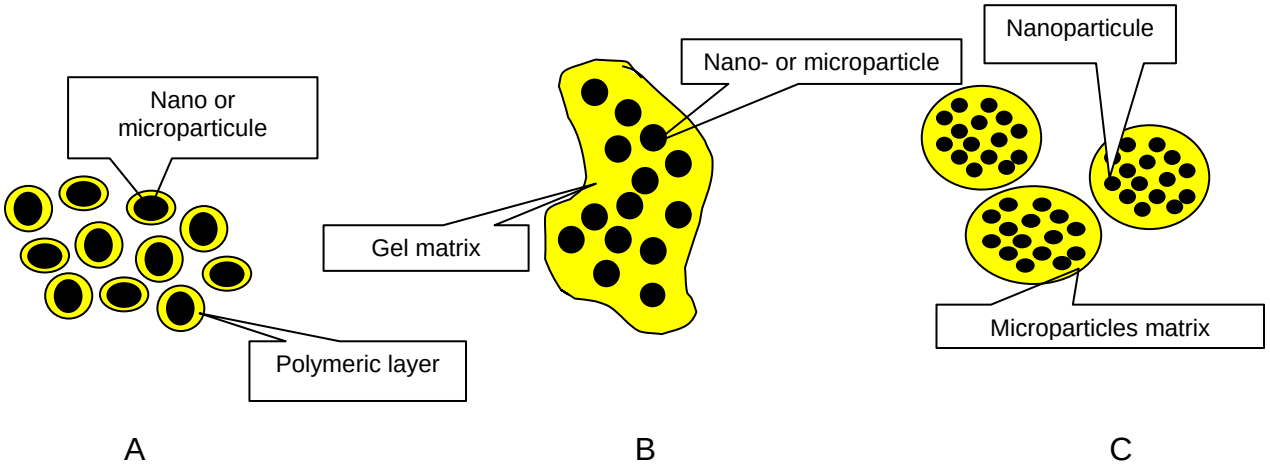



***Figure 21***. (A) Coated nano or microparticles, (B) Nano or microparticles incorporated in gel, (C) Nanoparticles in microparticle.

**4.9.1. Polymeric nanoparticles encapsulated into polymeric microparticles:**

Li *et al.* (1997) developed a novel biodegradable system for prolonged or controlled release of protein/peptide drugs. This new system is a combination of a hydrophilic polymer (gelatin) and a hydrophobic polymer (PLGA).

Neither the hydrophilic nor the hydrophobic system is ideal for protein/peptide drug delivery. Each has its own advantages and disadvantages. As already mentioned, the hydrophilic polymeric systems are biocompatible with the protein/peptide drugs, but have difficulty achieving sustained drug release. When the systems absorb water and swell, protein/peptide molecules will rapidly diffuse out. In contrast, the hydrophobic polymeric systems have the capability of yielding sustained drug release. However, they are incompatible with the water soluble protein/peptide drugs. The hydrophobicity of the polymers may induce unfolding of protein/peptide molecules; therefore, the protein/peptide drugs may lose their biological activity after being loaded in and then released from the hydrophobic polymeric systems (Johnson *et al.*, 1990). To promote the advantages and overcome the disadvantages of both the hydrophilic and the hydrophobic polymeric systems, they combined a hydrophilic system *i.e.* gelatin nanoparticles, encapsulated in a hydrophobic polymeric system, PLGA microspheres. Gelatin nanoparticles (loaded BSA) were prepared by w/o/o-technique. But, the encapsulation of nanoparticle into microparticles was conducted by a phase separation method and a solvent extraction method, separately.

In the preparation of gelatin nanoparticle in PLGA microsphere using a phase separation methods, gelatin nanoparticles (containing BSA) were suspended in the PLGA dissolved in

methylene chloride. Silicon oil was then progressively added to the stirred suspension to form an o/o emulsion. The emulsion was transferred with stirring to a quenching tank containing heptane. The quenching tank was stirred to harden the gelatine nanoparticle-loaded PLGA microspheres.

For the preparation of gelatin nanoparticle-PLGA composite microsphere using solvent extraction methods (s/o/w extraction), the same amount and the same type of BSA-loaded gelatin nanoparticles as described above were suspended in the PLGA solution in methylene chloride (10 % w/w). The suspension was poured into aqueous PVA solution saturated with methylene chloride. The mixture was stirred to produce an o/w emulsion. The emulsion was transferred to a mixture of ice and distilled water (5 °C) to harden the droplets. The water was stirred to extract the solvent from the microspheres containing the BSA-laden gelatin nanoparticles.

The average diameter of the composite microparticle (gelatin nanoparticle in PLGA microsphere) was between 160 and 175 µm. BSA loading efficiency is 93% for the composite prepared by the phase separation method, whereas it is 31% for the composite microsphere prepared by the solvent extraction method. Release experiments show that only 66% of BSA is released from the PLGA composite microsphere in 18 days, which indicates that this new system possesses sustained release characteristics for protein drugs (fig. 22). Also, BSA burst release is reduced (as known BSA has high Mw which show less burst release effect than low Mw protiens or peptides). This new system also demonstrates the capability of preventing protein drugs from integrity loss or denaturation.

In order to complete this study, the same authors combined PLGA and PVA in the phase separation method previously mentioned (Wang and Li,1999). BSA (hydrophilic drug model) was loaded in PVA nanoparticles then encapsulated in PLGA microspheres. The resulting microparticles presented a size ranging from 71 µm to 282 µm (average diameter 180µm). The protein could be released from this microsphere for two months and with good stability.

### 4.9.2. Polymer-free nanoparticles encapsulated into polymeric microparticles:

More recently, stable protein nanostructured particles (based on BSA), produced by spray freezing into liquid (SFL) nitrogen were encapsulated homogenously into microspheres to reduce the burst release over the first 24 hours [Leach WT et al., 2005 (b)]. Upon sonication, these friable highly porous and solid protein particle aggregates broke up into submicron

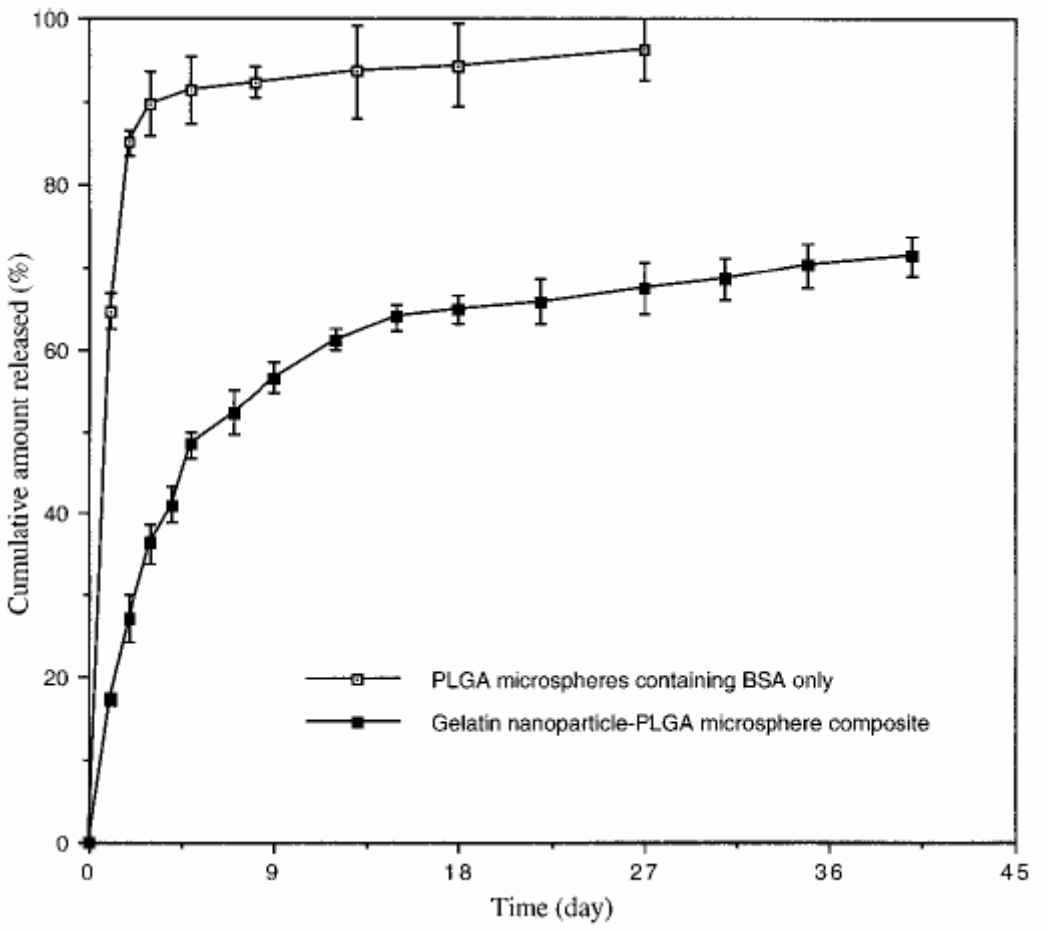



***Figure 22.*** *In vitro* release of BSA from the gelatin nanoparticles-PLGA microsphere composite and the PLGA microspheres containing only BSA, prepared by the phase separation method.

particles. These particles were encapsulated into PLGA and PLA microspheres by solid-in-oil-in-oil (s/o/o) techniques. Reduction of burst release was observed when compared to powder BSA (sieved BSA crystals). According to the authors, this reduction is in relation to the homogeneous dispersion protein nanoparticles into polymeric matrix.

In accordance, upon inspection via confocal microscopy, the SFL powder was found to have a fine morphology and to be homogenously dispersed within the microspheres. In contrast, the sieved crystalline-BSA particles appeared as discrete particles that were large relative to the confines of the microsphere (fig. 23A).

This technique was developped in order to limit protein aggregation and then denaturation, to limit burst effect and to obtain a high loading efficiency.

The same principle was adapted to delivery of another hydrophilic drug, dexamethasone phosphate (Thote and Gupta 2005). Size of drug particles was first reduced by the use of

supercritical antisolvent technique with enhanced mass transfer. The second step was the use of an s/o/o/o phase separation/coacervation technique for microencapsulation (PLGA) of drug nanoparticles.

Once microencapsulated, these nanoparticles provide high encapsulation efficiencies and sustained drug release without initial burst release (fig. 24). Because the complete process is anhydrous, it can be easily extended to produce sustained release formulations of other hydrophilic drugs.

Finally, the following table summarizes the studies carriaed out so far in the case of composite microparticles.

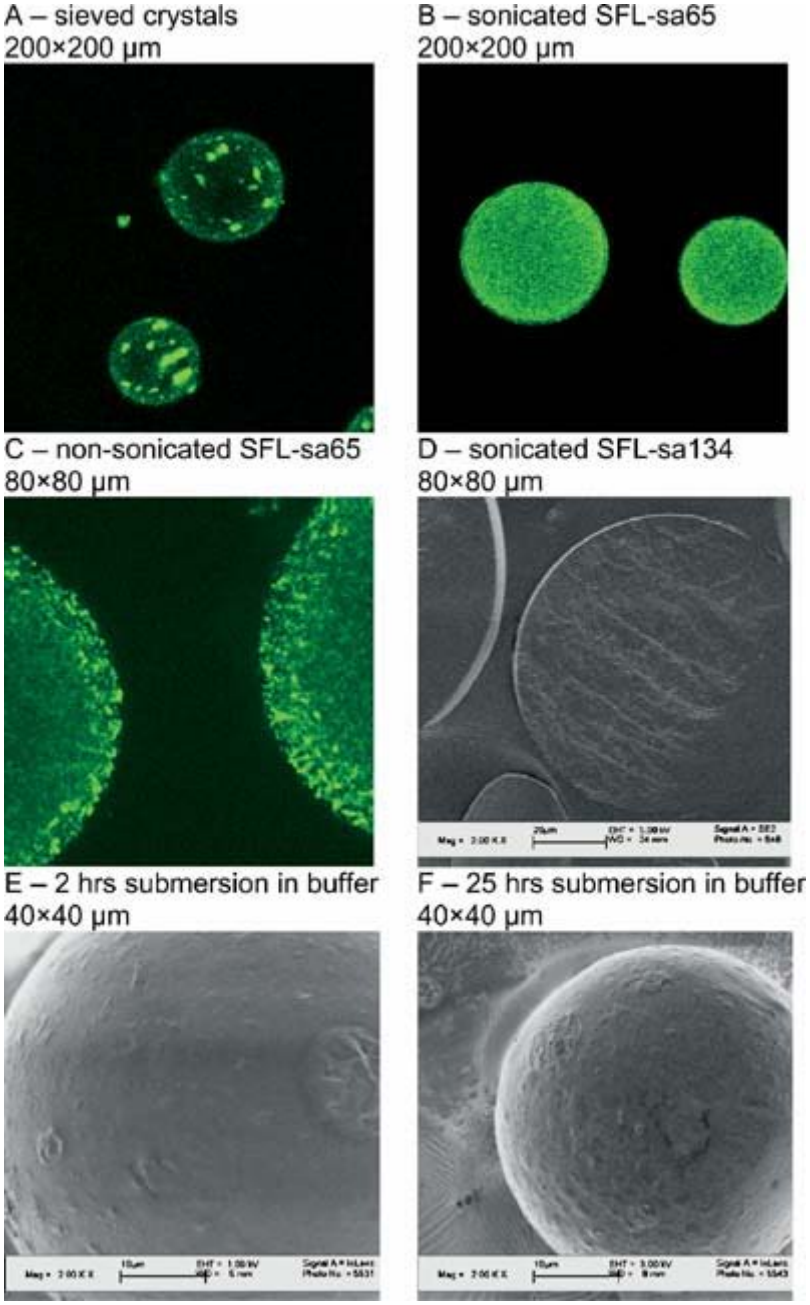



***Figure 23.*** Two-dimensional cross-sectional images of (A) sieved crystalline-BSA loaded PLA microspheres and (B) SFL loaded PLA microspheres were obtained using confocal microscopy (40 × magnifications, 200 × 200 µm view, the fluorescing protein particles show up as bright regions on a dark background). Through graphical manipulation, several flat images taken at 1 µm increments were stacked as transparent plates and interpolated to recreate (C) a transparent 3D slice of SFL loaded PLA microspheres (100× magnification, 80 × 80 µm view). A cross-sectional SEM image shows (D) an SFL-BSA loaded PLA

microsphere. SEM images show the surface of SFL-BSA loaded microspheres after (E) 2 h and (F) 25 h submersion in buffer. (From Leach *et al*., 2005(b))

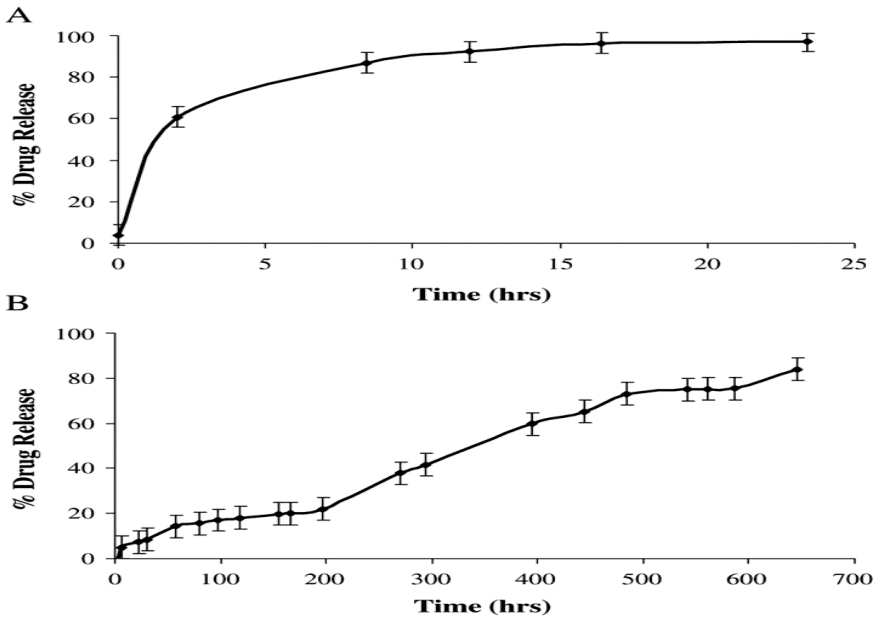



***Figure 24.*** (A) In vitro release profile of dexamethasone phosphate from polylactide-coglycolide microencapsulated drug microparticles as provided by supplie. (B) In vitro release profile of dexamethasone phosphate from polylactide-coglycolide microencapsulated drug nanoparticles obtained via supercritical antisolvent technique with enhanced mass transfer (n = 1).

**Table 3:** Summary of the different studies describing composite microparticles (nanoparticles encapsulated into microparticles)

| Composite microparticle description | Technique of composite microparticle preparation | Application / Aim of the study | Authors |
|---|---|---|---|
| **Polymeric nanoparticles into polymeric microparticle :** | | | |
| 1. Gelatin nanoparticles into poly (ε-caprolactone) microparticles | double emulsion-like s/w/o/w | Gene delivery and transfection in specific regions of the gastrointestinal tract. (Plasmid DNA) / tissue targeting | Bhavsar and Amiji, 2007 |
| 2. chitosan/tripolyphosphate nanoparticles into mannitol microspheres | spray-drying | Transport therapeutic protein-loaded nanoparticles to the lungs owing to their favorable aerodynamic properties / tissue targeting | Grenha *et al.*, 2005 |
| 3. Iron (III) oxide (alpha-Fe(2)O(3)) nanoparticles into poly(methyl methacrylate) microparticles | s/o/w solvent evaporation method | Diagnostic imaging to drug delivery. | Morello *et al.*, 2007 |
| 4. Herein (fluorescein isothiocyanate)/ Iron oxide Fe3O4 nanoparticles into poly (N-isopropylacrylamide) microcapsule | seed precipitation polymerization | Model to study the basic mechanism of chemical separation and purification, as controlled-delivery systems for drugs, and in other related biomedical fields. | Guo *et al.*, 2005 |

| Composite microparticle description | Technique of composite microparticle preparation | Application / Aim of the study | Authors |
|---|---|---|---|
| **Polymeric nanoparticles into polymeric microparticle (suite):** | | | |
| 5. Tacrolimus (FK506) loaded PLGA nanoparticles entrapped into pH-sensitive Eudragit P-4135F microspheres | s/w/o emulsion technique | Drug targeting to the inflammation site of inflammatory bowel disease and drug protection. | Lamprecht *et al.*, 2005 |
| 6.Nanoparticles of water-insoluble pranlukast hemihydrate (PLH) into microparticles of Mannitol | Spray drying | These particles were designed to improve the absorption of PLH and to allow delivery by oral, pulmonary, and injection routes | Mizoe *et al.*, 2007 |
| 7. Poly (epsilon-caprolactone) nanocapsules into silicon dioxide (Aerosil 200) microparticles | Spray-drying | Silica can prevent nanoparticles aggregation within aqueous medium and good stability. Effective technique capable to enhance the shelf life of these nanoparticles. | Tewa-Tagne *et al.*, 2007. |
| 8. Gelatin nanoparticles into PLGA microparticles | phase separation methods (s/o/o technique) or solvent extraction methods (s/o/w) | Prolonged or controlled release of protein/peptide drugs (without burst effect and high drug loading) | Li *et al.*, (1997) |
| 9. Polyvinyl alcohol nanoparticles into PLGA microparticles | phase separation methods (s/o/o technique) | Prolonged or controlled release of protein/peptide drugs (without burst effect and high drug loading) | Wang *et al.*, 1999 |

| Composite microparticle description | Technique of composite microparticle preparation | Application / Aim of the study | Authors |
|---|---|---|---|
| **Free polymer nanoparticles into polymeric microparticle :** | | | |
| 1.Protein nanostructured particle (based on BSA) in PLGA or PLA | (s/o/o) techniques | Prolonged or controlled release of protein/peptide drugs (without burst effect and high drug loading) | Leach *et al.* , 2005 (a, b) |
| 2.dexamethasone phosphate nanostructured particle in PLGA | s/o/o/o phase separation / coacervation technique | Prolonged or controlled release of hydrophilic drug drugs (dexamethasone phosphate) without burst effect and high drug loading | Thote *et al*., 2005 |

## 5. Conclusion

Initial burst is usually undesirable because the drug released in this period is not available for prolonged release, and more importantly, it can result in toxic side effects. In order to prevent the initial burst and gain efficient control over the release rate, it is necessary to understand possible causes of the initial release and relevant formulation parameters. In general, initial burst depends on how efficiently the drug is captured within the microparticles. Drug release out of polymeric devices is dependent on diffusional escape of the drug prior to the onset of polymer degradation (if the the polymer is bidegradable). Poor control over the diffusion during the initial stage results in large initial burst and premature depletion of the drug.

For many applications, small burst quantities may be acceptable, as long as the burst release is predictable. But with increase of medications with "high-alert drugs", "short half-life" or "narrow therapeutic index" drugs burst with small quantities is not acceptable. The total elimination of the burst effect is most likely cost-prohibitive, but a better understanding of the phenomena occurring at the early stages of release may help researchers to quantifiably predict burst release.

In order to control the release profiles efficiently, it is important that the microparticles have appropriate morphological characteristics including drug homogenously dispersed in the polymeric matrix, eliminating “bad” drug/matrix interaction. In addition, polymers should have a desirable degradation profile.

Despite burst effect has been reported in numerous publications, there is still a need for a lot of new researches on methods able to prevent the burst effect and to elucidate the mechanisms of burst release. Understanding of the burst effect during controlled release is still limited because the causes of burst release change with the use of new polymers, drugs, administration way and formulation techniques, but knowledge continues to grow as researchers realize the therapeutic importance of the burst period espcialy for parenteral products.

## 6. References

Abgar S, Vanhoudt J, Aerts T, Clauwaert J, Study of the chaperoning mechanism of bovine lens α-crystallin, a member of the α-small heat shock superfamily, J Biophys, 80 (2001) 1986–1995.

Ahmed AR, Dashevsky A, Bodmeier R, Reduced burst effect in drug release with solvent treated microparticles prepared by the solvent evaporation method.27th international symposium on controlled release of bioactive materials , Paris , France,2000.

Ahmed AR, Dashevsky A, Bodmeier R, Reduced burst release from microparticles by incorporating in cubic phase forming systems .13th international symposium on Microencapsulation, Angers, France, 2001.

Ahmed AR, Dashevsky A, Bodmeier R, Reduction of the burst release of PLGA microparticles by coating with second polymeric layer Proc. 4th World meeting ADRITELE /APGI/APV, Florence, Italy 8-11 April 2002.

Akhtar S and Lewis K, Antisense oligonucleotide delivery to cultured macrophages is improved by incorporation into sustained-release biodegradable polymer microspheres, Int. J. Pharm. 151 (1997) 57–67.

Atkins TW, McCallion RL and Tighe BJ, Incorporation and release of fluorescein isothiocyanate-linked dextrans from a bead-formed macroporous hydrophilic matrix with potential for sustained release, Biomaterials 14 (1993) 16–20.

Bain DF, Munday DL, Smith A, Modulation of rifampicin release from spray-dried microspheres using combinations of poly-(DL-lactide), J Microencapsul. 16 (1999) 369-85.

Baker R : Controlled Release of Biologically Active Agents, Wiley, New York (1987).

Berkland C, Kim K and Pack DW, Fabrication of PLG microspheres with precisely controlled and monodisperse size distributions, J. Control Release 73 (2001) 59–74.

Berkland C, King M, Cox A, Kim K and Pack DW, Precise control of PLG microsphere size provides enhanced control of drug release rate, J. Control Release 82 (2002) 137–147.

Berkland C, Pollauf E, Pack DW, Kim K, Uniform double-walled polymer microspheres of controllable shell thickness, J. Controlled Release 96 (2004) 101-111.

Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Blitterswijk CAV and Feijen J. Microspheres for protein delivery prepared from amphiphilic multiblock copolymers 2. Modulation of release rate. J. Control Release 67 (2000) 249–260.

Bhavsar MD, Amiji MM. Gastrointestinal distribution and *in vivo* gene transfection studies with nanoparticles-in-microsphere oral system (NiMOS), J Control Release 119 (2007) 339-48.

Bittner B, Morlock M, Koll H, Winter G, Kissel T, Recombinant human erythropoietin (rhEPO) loaded poly (lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics, Eur J Pharm Biopharm. 45 (1998) 295-305.

Bitz C & Doelker E, Influence of preparation method on residual solvents in biodegradable microspheres, Int. J.Pharm. 131 (1996) 171–181.

Bodmeier R and McGinity JW, Solvent selection in the preparation of poly(D,L-lactide) microspheres prepared by the solvent evaporation method, Int. J. Pharm. 43 (1988) 179–186.

Bodmeier R, Oh KH, Chen H, The effect of the addition of low molecular weight poly(DL-lactide) on drug release from biodegradable poly(DL-lactide) drug delivery systems, Int. J. Pharm 51 (1989) 1-8.

Bouissou C, Rouse JJ, Price R, van der Walle CF, The influence of surfactant on PLGA microsphere glass transition and water sorption: remodeling the surface morphology to attenuate the burst release, Pharm Res. 23 (2006) 1295-305.

Boury F, Marchais H, Proust JE, Benoit JP, Bovine serum albumin release from poly(alpha-hydroxy acid) microspheres: effects of polymer molecular weight and surface properties. J. Controlled Release, 45 (1997) 75-86.

Brazel CS and Peppas NA, Recent studies and molecular analysis of drug release from swelling-controlled devices, STP Pharma Sci. 9 (1999) 473–485.

Bruck SD and Mueller EP, Radiation sterilization of polymeric implant materials, J. Biomed Mater Res: Appl Biomaterials 22 (1988) 133–144.

Carrascosa C, Espejo L, Torrado S, Torrado JJ, Effect of gamma-sterilization process on PLGA microspheres loaded with insulin-like growth factor-I (IGF-I), J Biomater Appl. 18 (2003) 95-108.

Castellanos IJ, Carrasquillo KG, Lopez JD, Alvarez M, Griebenow K, Encapsulation of bovine serum albumin in poly (lactide-co-glycolide) microspheres by the solid-in-oil-in-water technique, J Pharm Pharmacol. 53 (2001) 167-78

Castellanos IJ, Flores G, Griebenow K, Effect of cyclodextrins on alpha-chymotrypsin stability and loading in PLGA microspheres upon S/O/W encapsulation, J Pharm Sci. 95 (2006) 849-58.

Cha Y and Pitt CG, The acceleration of degradation-controlled drug delivery from polyester microspheres, J. Control Release 8 (1989) 259–265.

Chiou SH, Wu WT, Huang YY, Chung TW, Effects of the characteristics of chitosan on controlling drug release of chitosan coated PLLA microspheres, J Microencapsul. 18 (2001) 613-25.

Choi SH, Park TG, Hydrophobic ion pair formation between leuprolide and sodium oleate for sustained release from biodegradable polymeric microspheres, Int J Pharm. 203 (2000) 193-202.

Chung T-W, Huang Y-Y and Liu Y-Z, Effects of the rate of solvent evaporation on the characteristics of drug loaded PLLA and PDLLA microspheres, Int. J. Pharm. 212 (2001) 161–169.

Chung TW, Tsai YL, Hsieh JH, Tsai WJ, Different ratios of lactide and glycolide in PLGA affect the surface property and protein delivery characteristics of the PLGA microspheres with hydrophobic additives, J Microencapsul. 23 (2006) 15-27.

Cleland JL, Lim A, Barron L, Duenas ET, Powell MF, Development of a single-shot subunit vaccine for HIV-1: Part 4. Optimizing microencapsulation and pulsatile release of MN rgp120 from biodegradable microspheres, J Control Release 47 (1997) 135-150.

Costantino HR, Firouzabadian L, Hogeland K, Wu C, Beganski C, Carrasquillo KG, Córdova M, Griebenow K, Zale SE, Tracy MA, Protein spray-freeze drying, Effect of atomization conditions on particle size and stability, Pharm Res 17 (2000) 1374-1383.

Costantino HR, Johnson OL, Zale SE., Relationship between encapsulated drug particle size and initial release of recombinant human growth hormone from biodegradable microspheres, J Pharm Sci. 93 (2004) 2624-34.

Couvreur P, Dubernet C, Puissieux F, Controlled drug delivery with nanoparticles: current possibilities and future trends, Eur. J. Pharm. Biopharm. 41 (1995) 2-13.

Crotts, G. and Park, T.G., Preparation of porous and nonporous biodegradable polymeric hollow spheres, J. Control Release 35 (1995) 91–105.

De Rosa G, Larobina D, Immacolata La Rotonda M, Musto P, Quaglia F, Ungaro F, How cyclodextrin incorporation affects the properties of protein-loaded PLGA-based microspheres: the case of insulin/hydroxypropyl-beta-cyclodextrin system, J Control Release 102 (2005) 71-83.

Dhoot NO, Wheatley MA, Microencapsulated liposomes in controlled drug delivery: strategies to modulate drug release and eliminate the burst effect. J Pharm Sci. 92 (2003) 679-89.

Edlund U, Albertsson AC, Singh SK, Fogelberg I, Lundgren BO(a), Sterilization, storage stability and *in vivo* biocompatibility of poly (trimethylene carbonate) /poly(adipic anhydride) blends, Biomaterials 21 (2000) 945-55.

Edlund U and Albertsson AC (b), Morphology engineering of a novel poly(L-lactide) /poly(1,5-dioxepane-2-one) microsphere system for controlled drug delivery, J. Polym. Sci.: Part A: Pol. Chem. 38 (2000) 786–796.

Efentakis M, Pagoni I, Vlachou M, Avgoustakis K, Dimensional changes, gel layer evolution and drug release studies in hydrophilic matrices loaded with drugs of different solubility, Int J Pharm. 339 (2007) 66-75.

Faisant N, Akiki J, Siepmann F, Benoit JP, Siepmann J, Effects of the type of release medium on drug release from PLGA-based microparticles: experiment and theory, Int J Pharm. 314 (2006) 189-97.

Fernandez-Carballido A, Herrero-Vanrell R, Molina-Martinez IT, Pastoriza P, Biodegradable ibuprofen-loaded PLGA microspheres for intraarticular administration, Effect of Labrafil addition on release *in vitro*, Int J Pharm. 279 (2004) 33-41.

Ficek BJ and Peppas NA, Novel preparation of poly (vinyl alcohol) microparticles without crosslinking agent for controlled drug delivery of proteins, J. Control. Release 27 (1993) 259–264.

Freytag T, Dashevsky A, Tillman L, Hardee GE, Bodmeier R, Improvement of the encapsulation efficiency of oligonucleotide-containing biodegradable microspheres, J Control Release 69 (2000) 197-207.

Gander B, Johansen P, Nam-Tran H and Merkle HP, Thermodynamic approach to protein microencapsulation into poly(D,L-lactide) by spray drying, Int. J. Pharm. 129 (1996) 51–61.

Gèze A, Venier-Julienne MC, Mathieu D, Filmon R, Phan-Tan-Luu R, Benoit JP, Development of 5-iodo-2'-deoxyuridine milling process to reduce initial burst release from PLGA microparticles, Int J Pharm. 178 (1999) 257-68.

Ghaderi R, Struesson C and Carlfors J, Effect of preparative parameters on the characteristics of poly(D,L-lactide-co-glocolide) microspheres made by the double emulsion method, Int. J. Pharm. 141 (1996) 205–216.

Göpferich A, Alonso MJ, Langer R, Development and characterization of microencapsulated microspheres, Pharm Res. 11 (1994) 1568-74

Govender T, Stolnik S, Garnett MC, Illum L, Davis SS, PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J Control Release. 57 (1999) 171-85.

Graves RL, Makoid MC, Jonnalagadda S, The effect of coencapsulation of bovine insulin with cyclodextrins in ethylcellulose microcapsules. J Microencapsul. 22 (2005) 661-70.

Grenha A, Seijo B, Remunan-Lopez C, Microencapsulated chitosan nanoparticles for lung protein delivery.Eur J Pharm Sci. 25 (2005) 427-37.

Guo J, Yang W, Deng Y, Wang C, Fu S, Organic-dye-coupled magnetic nanoparticles encaged inside thermoresponsive PNIPAM Microcapsules. Small. 1 (2005) 737-43.

Guyot M and Fawaz F, Nifedipine loaded polymeric microspheres: preparation and physical characteristics. Int. J. Pharm. 175 (1998) 61–74.

Hasan AS, Socha M, Lamprecht A, Ghazouani FE, Sapin A, Hoffman M, Maincent P, Ubrich N. Effect of the microencapsulation of nanoparticles on the reduction of burst release. Int J Pharm. 344 (2007) 53-61.

Herrero-Vanrell R, Ramirez L, Fernandez-Carballido A, Refojo MF, Biodegradable PLGA microspheres loaded with ganciclovir for intraocular administration. Encapsulation technique, *in vitro* release profiles, and sterilization process. Pharm Res. 17 (2000) 1323-8.

Hinds KD, Campbell KM, Holland KM, Lewis DH, Piché CA, Schmidt PG, PEGylated insulin in PLGA microparticles. *In vivo* and *in vitro* analysis. J Control Release. 104 (2005) 447-60.

Hora MS, Rana RK, Nunberg JH, Tice TR, Gilley RM and Hudson ME, Release of human serum albumin from PLGA microspheres. Pharm. Res. 7 (1990) 1190-1194.

Huang X and Brazel CS, On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. J Control Release 73 (2001) 121-36.

Huang YY, Chung TW, Tzeng TW, A method using biodegradable polylactides/polyethylene glycol for drug release with reduced initial burst. Int J Pharm. 182 (1999) 93-100.

Hsu Y-Y, Hao T, and Hedley ML, Comparison of process parameters for microencapsulation of plasmid DNA in poly(D,L-lactic-co-glycolic) acid microspheres. J. Drug Targeting 7 (1999) 313-323.

Igartua M, Hernandez RM, Esquisabel A, Gascon AR, Calvo MB, and Pedraz JL, Influence of formulation variables on the in vitro release of albumin from biodegradable microparticulate systems. J. Microencapsulation, 14 (1997) 349-356.

Izumikawa S, Yoshioka S, Aso Y and Takeda Y, Preparation of poly(L-lactide) microspheres of different crystalline morphology on drug release rate. J. Control Release 15 (1991) 133–140.

Jain R, Shah NH, Malick AW and Rhodes CT, Controlled drug delivery by biodegradable poly(ester) devices: different preparative approaches, Drug Dev. Ind. Pharm. 28 (1998) 703–727.

Jalil R and Nixon JR, Microencapsulation using poly (L-lactic acid) II: preparative variables affecting microcapsule properties. J. Microencapsul. **7** (1990) 25–39.

Jeong B, Bae YH and Kim SW, Drug release from biodegradable injectable thermosensitive hydrogel of PEG-PLGA-PEG triblock copolymers. J. Control. Release 63 (2000) 155–163.

Jeong JC, Lee J, Cho K, Effects of crystalline microstructure on drug release behavior of poly (epsilon-caprolactone) microspheres. J Control Release 92 (2003) 249-58.

Jeyanthi R, Thanoo BC, Metha RC and DeLuca PP, Effect of solvent removal technique on the matrix characteristics of polylactide/glcolide microspheres for peptide delivery. J. Control Release 38 (1996) 235–244.

Jeyanthi R, Mehta RC, Thanoo BC, and DeLuca PP, Effect of processing parameters on the properties of peptidecontaining PLGA microspheres. J. Microencapsulation, 14 (1997) 163-174.

Jiang G, Thanoo BC, DeLuca PP, Effect of osmotic pressure in the solvent extraction phase on BSA release profile from PLGA microspheres. Pharm Dev Technol. 7 (2002) 391-9.

Johnson ER; Lanaski LA, Gupta V, Griffin MJ, Gaud HT, Needham TE, Zia H, Stability of atriopeptin III encapsulated in poly(D,L-lactide-co-glycolide) microcapsules. J. Control. Release 17 (1991) 61-67.

Johansen P, Men Y, Audran R, Corradin G, Merkle HP, and Gander B, Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives. Pharm. Res. 15 (1998) 1103-1110.

Kakish HF, Tashtoush B, Ibrahim HG and Najib NM, A novel approach for the preparation of highly loaded polymeric controlled release dosage forms of diltiazem HCl and diclofenac sodium. Eur. J. Pharm. Biopharm. 54 (2002) 75–81.

Kim TH, Park TG. Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism. Biotechnol. Bioeng. 65 (1999) 659-667.

Kim TH, Lee H, Park TG, Pegylated recombinant human epidermal growth factor (rhEGF) for sustained release from biodegradable PLGA microspheres. Biomaterials 23(2002) 2311-7.

Kim TH, Park TG, Critical effect of freezing/freeze-drying on sustained release of FITC-dextran encapsulated within PLGA microspheres. Int J Pharm. 271 (2004) 207-14.

Klose D, Siepmann F, Elkharraz K, Krenzlin S, Siepmann J, How porosity and size affect the drug release mechanisms from PLGA-based microparticles. Int J Pharm.314 (2006) 198-206.

Kreuter J, ‘‘Nanoparticles’’ in: Colloidal Drug Delivery Systems, Kreuter J, Ed., Marcel Dekker, New York 1994, p. 219 -343

Lagarce F, Faisant N, Desfontis JC, Marescaux L, Gautier F, Richard J, Menei P, Benoit JP. Baclofen-loaded microspheres in gel suspensions for intrathecal drug delivery: in vitro and in vivo evaluation. Eur J Pharm Biopharm. 61 (2005) 171-80.

Lalla JK, Sapna K, Biodegradable microspheres of poly(DL-lactic acid) containing piroxicam as a model drug for controlled release via the parenteral route. J. microencapsul. 10 (1993) 449-460.

Lamprecht A, Rodero Torres H, Schäfer U, Lehr CM. Biodegradable microparticles as a two-drug controlled release formulation: a potential treatment of inflammatory bowel disease. J Control Release 69 (2000) 445-54.

Lamprecht A, Yamamoto H, Takeuchi H, Kawashima Y. A pH-sensitive microsphere system for the colon delivery of tacrolimus containing nanoparticles. J Control Release 104 (2005) 337-46.

Leach K, Noh K, Mathiowitz E. Effect of manufacturing conditions on the formation of double-walled polymer microspheres. J Microencapsul. 16 (1999) 153-67.

Leach WT, Simpson DT, Val TN, Yu Z, Lim KT, Park EJ, Williams RO 3rd, Johnston KP (a), Encapsulation of protein nanoparticles into uniform-sized microspheres formed in a spinning oil film.AAPS PharmSciTech. 6 (2005) E605-17.

Leach WT, Simpson DT, Val TN, Anuta EC, Yu Z, Williams RO 3rd, Johnston KP (b), Uniform encapsulation of stable protein nanoparticles produced by spray freezing for the reduction of burst release. J Pharm Sci. 94 (2005) 56-69.

Le Corre P, LeGuevello P, Gajan V, Chevanne F and LeVerge R, Preparation and characterization of bupivacaine-loaded polylactide and polylactide-co-glycolide microspheres. Int. J. Pharm. 107 (1994) 41–49.

Le Corre P, Rytting JH, Gajan V, Chevanne F and Verge RL. In vitro controlled release kinetics of local anasthetics from PDLLA and PLGA microspheres. J. Microencapsul. 14 (1997) 243–255.

Lee PI. Initial concentration distribution as a mechanism for regulating drug release from diffusion controlled and surface errosion controlled matrix systems. J. Control Release 4 (1986) 1–7.

Lee ES, Kwon MJ, Lee H, Kim JJ. Stabilization of protein encapsulated in poly(lactide-co-glycolide) microspheres by novel viscous S/W/O/W method. Int J Pharm. 331 (2007) 27-37.

Lee TH, Wang J, Wang CH (a). Double-walled microspheres for the sustained release of a highly water soluble drug: characterization and irradiation studies. J Control Release 83 (2002) 437-52.

Lee Wk, Park Jy., Yang EH, Suh H, Kim SH, Chung DS, Choi K, Yang C W and Park JS (b). Investigation of the factors influencing the release rates of cyclosporin Aloaded micro- and nanoparticles prepared by high-pressure homogenizer. J Controlled Release 84 (2002) 115-123.

Leo E, Forni F, Bernabei MT. Surface drug removal from ibuprofen-loaded PLA microspheres. Int J Pharm. 196 (2000) 1-9.

Leo E, Ruozi B, Tosi G, Vandelli MA (a). PLA-microparticles formulated by means a thermoreversible gel able to modify protein encapsulation and release without being co-encapsulated. Int J Pharm. 323 (2006) 131-8.

Leo E, Scatturin A, Vighi E, Dalpiaz A (b). Polymeric nanoparticles as drug controlled release systems: a new formulation strategy for drugs with small or large molecular weight. J Nanosci Nanotechnol. 6 (2006) 3070-9.

Li JK, Wang N, Wu XS. A novel biodegradable system based on gelatin nanoparticles and poly (lactic-co-glycolic acid) microspheres for protein and peptide drug delivery. J Pharm Sci. 86 (1997) 891-5

Li X, Zhang Y, Yan R, Jia W, Yuan M, Deng X, Huang Z. Influence of process parameters on the protein stability encapsulated in poly-DL-lactide-poly(ethylene glycol) microspheres. J Control Release 68 (2000) 41-52.

Li W-I, Anderson KW, Mehta RC, DeLuca PP, Prediction of solvent removal profile and effect on properties for peptide-loaded PLGA microspheres prepared by solvent extraction/evaporation method. J. Controlled Release 37 (1995) 199-214.

Liggins RT, Burt HM. Paclitaxel-loaded poly (L-lactic acid) microspheres 3: blending low and high molecular weight polymers to control morphology and drug release. Int J Pharm. 282 (2004) 61-71.

Lin SY, Chen KS, Teng HH, Li MJ (a). *In vitro* degradation and dissolution behaviours of microspheres prepared by three low molecular weight polyesters. J Microencapsul. 17 (2000) 577- 86.

Lin Y-HE and Vasavada RC (b). Studies on microencapsulation of 5-fluorouracil with poly (ortho ester) polymers. J. Microencapsul. 17 (2000) 1–11.

Luan X, Skupin M, Siepmann J, Bodmeier R. Key parameters affecting the initial release (burst) and encapsulation efficiency of peptide-containing poly (lactide-co-glycolide) microparticles. Int J Pharm. 324 (2006) 168-75.

Luo D, Woodrow-Mumford K, Belcheva N, Saltzman WM. Controlled DNA delivery systems. Pharm Res. 16 (1999) 1300-8.

Madene A, Jacquot M, Scher J & Desobry S. Flavour encapsulation and controlled release. International Journal of Food Science & Technology 41(2006) 1-21.

Mainardes RM, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. Int J Pharm. 290 (2005) 137-44.

Mainardes RM, Evangelista RC. Praziquantel-loaded PLGA nanoparticles: preparation and characterization. J Microencapsul. 22 (2005)13-24

Makino K, Mogi T, Ohtake N, Yoshida M, Ando S, Nakajima T and Ohshima H. Pulsatile drug release from poly (lactide-co-glycolide) microspheres: how does the composition of the polymer matrixes affect the time interval between the initial burst and the pulsatile release of drugs? Colloid. Surf. B: Biointerfaces. 19 (2000) 173–179.

Mallard C, Coudane J, Rault I, Vert M. The use of additives to modulate the release of a sparingly water soluble drug entrapped in PLA50 microparticles.J Microencapsul.17 (2000) 81-93.

Mandal TK, Bostanian LA, Graves RA, Chapman SR. Poly(D,L-lactide-co-glycolide) encapsulated poly(vinyl alcohol) hydrogel as a drug delivery system. Pharm Res. 19 (2002) 1713-9.

Martínez-Sancho C, Herrero-Vanrell R, Negro S. Poly(D,L-lactide-co-glycolide) microspheres for long-term intravitreal delivery of aciclovir: influence of fatty and non-fatty additives. J Microencapsul. 20 (2003) 799-810.

Mao S, Shi Y, Li L, Xu J, Schaper A, Kissel T. Effects of process and formulation parameters on characteristics and internal morphology of poly(d,l-lactide-co-glycolide) microspheres formed by the solvent evaporation method. Eur J Pharm Biopharm. 2007 in press.

Mehta RC, Thanoo BC, and DeLuca PP. Peptide containing microspheres from low molecular weight and hydrophilic poly(D,L-lactide-co-glycolide). J. Controlled Release, 41(1996) 249-257.

Meng FT, Ma GH, Qiu W, Su ZG. W/O/W double emulsion technique using ethyl acetate as organic solvent: effects of its diffusion rate on the characteristics of microparticles. J Control Release. 91 (2003) 407-16.

Mi F-L, Lin Y-M, Wu Y-B, Shyu S-S and Tsai Y-H. Chitin/PLGA blend microspheres as a biodegradable drug-delivery system: phase-separation. Biomaterials 23 (2002) 3257–3267.

Mizoe T, Ozeki T, Okada H. Preparation of drug nanoparticle-containing microparticles using a 4-fluid nozzle spray drier for oral, pulmonary, and injection dosage forms. J Control Release 122 (2007) 10-5.

Mogi T, Ohtake N, Yoshida M, Chimura R, Kamaga Y, Ando S, Tsukamoto T, Nakajima T, Uenodan H, Otsuka M, Matsuda Y, Ohshima K and Makino K. Sustained release of 17β-estradiol from poly (lactide-co-glycocide) microspheres *in vitro* and *in vivo*. Colloid Surface B. Biointerfaces 17 (2000) 153–165.

Montanari L, Cilurzo F, Selmin F, Conti B, Genta I, Poletti G, Orsini F, Valvo L. Poly(lactide-co-glycolide) microspheres containing bupivacaine: comparison between gamma and beta irradiation effects. J. Control. Release 90 (2003) 281–290.

Morello AP 3rd, Burrill R, Mathiowitz E. Preparation and characterization of poly (methyl methacrylate) - iron (III) oxide microparticles using a modified solvent evaporation method. J Microencapsul. 24 (2007) 476-91.

Namur JA, Cabral-Albuquerque EC, Quintilio W, Santana MH, Politi MJ, de Araujo PS, Lopes AC, da Costa MH. Poly-lactide-co-glycolide microparticle sizes: a rational factorial design and surface response analysis. J Nanosci Nanotechnol. 6 (2006) 2403-7.

Nair PD. Currently practised sterilization methods-some inadvertent consequences. J Biomater. Appl. 10 (1995) 121–135.

Narasimham B and Langer R (a). On the importance of the burst effect during drug release from polymer films. Polym. Mater. Sci. Eng. Proc. 76 (1997) 558–559.

Narasimhan B and Langer R (b). Zero-order release of micro- and macromolecules from polymeric devices: the role of the burst effect. J. Control. Release 47 (1997) 13– 20.

Narayani R, and Rao KP, Gelatin microsphere cocktails of different sizes for the controlled release of anticancer drugs. Int. J. Pharm. 143 (1996) 255–258.

Nicoli S, Santi P, Couvreur P, Couarraze G, Colombo P, Fattal E. Design of triptorelin loaded nanospheres for transdermal iontophoretic administration. Int J Pharm. 214 (2001) 31-5.

O'Hagan DT, Jeffery H, Davis SS. The preparation and characterization of poly (lactide-co-glycolide) microparticles: III. Microparticle/polymer degradation rates and the in vitro release of a model protein. Int J Pharm. 103 (1994) 37-45.

Okumu FW, Darling S, Dao LN, Fielder PJ, Sullivan SA, Tipton AJ, Cleland JL SABER$^{TM}$ Provides Burst Free Release of Growth Hormone from PLGA Microspheres. Millennial World Congress of Pharmaceutical Sciences, San Francisco, California, April, 16-20, 2000.

Park TG. Degradation of poly (D,L-lactic acid) microspheres: effect of molecular weight. J. Control Release 30 (1994) 161–173.

Park TG, Cohen S, Langer R (a). Poly(L-lactic acid)/pluronic blends: characterization of phase separation behavior, degradation, and morphology and use as protein-releasing matrices. Macromolecules 25 (1992) 116-122.

Park TG, Cohen S, Langer R (b). Controlled protein release from polyethyleneimine-coated poly (L-lactic acid)/pluronic blend matrices.Pharm Res. 9 (1992) 37-9.

Park TG, Lee HY and Nam YS. A new preparation method for protein loaded poly (D,L-lactic-co-glycolic acid) microspheres and protein release mechanism study. J. Controlled Release 55(1998) 181-191.

Patil NS, Dordick JS and Rethwisch DG. Macroporous poly (sucrose acrylate) hydrogel for controlled release of macromolecules. Biomaterials 17 (1996) 2343–2350.

Pean JM, Boury F, Venier-Julienne MC, Menei P, Proust JE, Benoit JP. Why does PEG 400 co-encapsulation improve NGF stability and release from PLGA biodegradablemicrospheres? Pharm. Res. 16 (1999) 1294-1299.

Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine 2 (2006) 8-21.

Sah H, Toddywala R and Chien Y W. The influence of biodegradable microcapsule formulations on the controlled release of a protein. J. Controlled Release, 30(1994) 201-211.

Sah HK, Toddywala R, and Chien YW. Biodegradable microcapsules prepared by a w/o/w technique: effects of shear force to make a primary w/o emulsion on their morphology and protein release. J. Microencapsul. 12 (1995) 59-69.

Saravanan M, Bhaskar K, Srinivasa Rao G, Dhanaraju MD. Ibuprofen-loaded ethylcellulose/polystyrene microspheres: an approach to get prolonged drug release with reduced burst effect and low ethylcellulose content. J Microencapsul. 20 (2003) 289-302.

Sansdrap P and Moes A.J. In vitro evaluation of the hydrolytic degradation of dispersed and aggregated poly(D,L-lactide-co-glycolide) microspheres. J. Control Release 43 (1997) 47–58.

Schlicher EJAM, Postma NS, Zuidema J, Talsma H, Hennink WE. Preparation and characterisation of Poly (d,l-lactic-co-glycolic acid) microspheres containing desferrioxamine. Int J Pharm. 153 (1997) 235-245.

Setterstrom JA, Tice TR, Meyers WE and Vincent JW. Development of encapsulated antibiotics for topical administration to wounds. In: Annual Meeting of the Society for Biomaterials Symposium, San Antonio, TX, USA 7(1984), p. 4.

Shah SS, Cha Y, Pitt CG. Poly(glycolic acid-co-D,L-lactic acid): diffusion or degradation controlled drug delivery, J. Control. Rel. 18 (1992) 261-270.

Shively ML, Coonts BA, Renner WD, Southard JL and Bennet AT. Physicochemical characterization of polymeric injectable implant delivery system. J. Control. Release 33 (1995) 237–243

Shih C, Zentner GM. Drug delivery system based on biodegradable polyester microparticles. US Patent 790276, 2000.

Sintzel MB, Merkli A, Tabatabay C and Gurny R. Influence of irradiation sterilization on polymers used as a drug carriers—a review. Drug Dev. Ind. Pharm. 23 (1997) 857–879.

Sullivan SA, Meador SK, Carraway KM, Tipton AJ, Gibson JW. Incorporation of polymer microparticles into Sucrose Acetate Isobutyrate reduces burst and extends release. 27th Symposium on Controlled Release of Bioactive Materials, July 7-13, 2000, Paris, France.

Takada S, Kurokawa T, Miyazaki K, Iwasa S, Ogawa Y. Utilization of an amorphous form of a water-soluble GPIIb/IIIa antagonist for controlled release from biodegradable microspheres. Pharm Res. 14 (1997) 1146-50.

Tan LP, Venkatraman SS, Sung PF, Wang XT. Effect of plasticization on heparin release from biodegradable matrices. Int J Pharm. 283 (2004) 89-96.

Tamber H, Johansen P, Merkle HP, Gander B. Formulation aspects of biodegradable polymeric microspheres for antigen delivery. Adv Drug Deliv Rev. 57 (2005) 357-76.

Tewa-Tagne P, Briancon S, Fessi H. Spray-dried microparticles containing polymeric nanocapsules: formulation aspects, liquid phase interactions and particles characteristics. Int J Pharm. 325 (2006) 63-74.

Thompson CJ, Hansford D, Higgins S, Rostron C, Hutcheon GA, Munday DL. Evaluation of ibuprofen-loaded microspheres prepared from novel copolyesters. Int J Pharm. 329 (2007) 53-61.

Thomasin C, Corradin G, Men Y, Merkle HP and Gander B. Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response, J. Control. Release 41 (1996) 131–145.

Thomasin C, Merkle HP and Gander B (a). Drug microencapsulation by PLA/PLGA coacervation in the light of thermodynamics. 2. Parameters determining microsphere formation, J. Pharm. Sci. 87 (1998) 269–275.

Thomasin C, Nam-Tran H, Merkle HP and Gander B (b). Drug microencapsulation by PLA/PLGA coacervation in the light of thermodynamics. 1. Overview and theoretical considerations, J. Pharm. Sci. 87 (1998) 259–268.

Thote AJ, Chappell JT Jr, Gupta RB, Kumar R. Reduction in the initial-burst release by surface crosslinking of PLGA microparticles containing hydrophilic or hydrophobic drugs. Drug Dev Ind Pharm. 31 (2005) 43-57.

Thote AJ and Gupta RB. Formation of nanoparticles of a hydrophilic drug using supercritical carbon dioxide and microencapsulation for sustained release. Nanomedicine. 1(2005) 85-90.

Trapani G, Lopedota A, Boghetich G, Latrofa A, Franco M, Sanna E, Liso G. Encapsulation and release of the hypnotic agent zolpidem from biodegradable polymer microparticles containing hydroxypropyl-beta-cyclodextrin. Int J Pharm. 268 (2003) 47-57.

Tuncay M, Calis S, Kas HS, Ercan MT, Peksoy I. and Hincal AA. Diclofenac sodium incorporated PLGA (50:50) microspheres: formulation considerations and *in vitro*/*in vivo* evaluation. Int. J. Pharm. **195** (2000) 179–188.

Uchida T, Nagareya N, Sakakibara S, Konishi Y, Nakai A, Nishikata M, Matsuyama K, Yoshida K. Preparation and characterization of polylactic acid microspheres containing bovine insulin by a w/o/w emulsion solvent evaporation method. Chem Pharm Bull (Tokyo). 45 (1997) 1539-43.

Uchida T, Goto S, Foster TP. Particle size studies for subcutaneous delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin as vaccine formulation, J Pharm Pharmacol. 47 (1995) 556-60.

Urata T, Arimori K, Nakano H. Modification of release rates of cyclosporin A from polyl (L-lactic acid) microspheres by fatty acid esters and in-vivo evaluation of the microspheres. J Control Release 58 (1999) 133-41.

Volland C, Wolff M, Kissel T. The influence of terminal gamma sterilization on captopril containing poly (dl-lactide-co-glycolide) microspheres. J. Control. Release 31 (1994) 293–305.

Wang C, Ye W, Zheng Y, Liu X, Tong Z. Fabrication of drug-loaded biodegradable microcapsules for controlled release by combination of solvent evaporation and layer-by-layer self-assembly. Int J Pharm. 338 (2007) 165-73.

Wang HT, Schmitt E, Flanagan DR, and Linhardt RJ. Influence of formulation methods on the in vitro controlled release of protein from poly (ester) microspheres. J. Controlled Release, 17 (1991) 23-32.

Wang J, Wang BM, Schwendeman SP. Mechanistic evaluation of the glucose-induced reduction in initial burst release of octreotide acetate from poly (D, L-lactide-co-glycolide) microspheres. Biomaterials 25 (2004) 1919-27.

Wang J, Wang BM, Schwendeman SP. Characterization of the initial burst release of a model peptide from poly(D,L-lactide-co-glycolide) microspheres. J Control Release 82 (2002) 289-307.

Wang N, Wu XS, Li JK. A heterogeneously structured composite based on poly(lactic-co-glycolic acid) microspheres and poly(vinyl alcohol) hydrogel nanoparticles for long-term protein drug delivery. Pharm Res. 16 (1999)1430-5.

Wheatley MA, Chang ME, Park E, Langer R. Coated alginate microspheres: Factors influencing the controlled delivery of macromolecules. J. of Appl Polym. Sci., 43 (1991) 2123 – 2135.

Wischke C, Borchert HH. Influence of the primary emulsification procedure on the characteristics of small protein-loaded PLGA microparticles for antigen delivery. J Microencapsul. 23 (2006) 435-48.

Woo L and Sandford CL. Comparison of electron beam irradiation with gamma processing for medical packaging materials. Radiat. Phys. Chem. 63 (2002) 845–850.

Yamaguchi Y, Takenaga M, Kitagawa A, Ogawa Y, Mizushima Y, Igarashi R. Insulin-loaded biodegradable PLGA microcapsules: initial burst release controlled by hydrophilic additives.J Control Release. 81 (2002) 235- 49.

Yan C, Resau JH, Hewetson J, Mest M, Rill WL and Kende M. Characterization and morphological analysis of protein-loaded poly(lactide-co-glycolide) microparticles prepared by water-in-oil-in-water emulsion technique. J. Controlled Release, 32 (1994) 231-241.

Yang YY, Chia HH, Chung TS (a). Effect of preparation temperature on the characteristics and release profiles of PLGA microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. J Control Release.69 (2000) 81-96.

Yang Y-Y, Chung T-S, Bai X-L and Chan W-K, (b). Effect of preparation conditions on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method. Chem. Eng. Sci. 55 (2000) 2223–2236.

Yang YY, Chung TS, Ng NP. Morphology, drug distribution, and *in vitro* release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials, 22 (2001) 231-41.

Yeo Y and Park K. Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res. 27 (2004)1-12.

Yuksel N, Tincer T and Baykara T. Interaction between nicardipine hydrochloride and polymeric microspheres for a controlled release system. Int. J. Pharm. 140 (1996) 145–154.

Zhang JX, Chen D, Wang SJ, Zhu KJ. Optimizing double emulsion process to decrease the burst release of protein from biodegradable polymer microspheres. J Microencapsul. 22 (2005) 413-22.

Zhao HZ, Tan EC, Yung LY. Potential use of cholecalciferol polyethylene glycol succinate as a novel pharmaceutical additive. J Biomed Mater Res A. 2007 Jul 23 (in press).

Zheng CH, Gao JQ, Liang WQ, Yu HY, Zhang YL. Effects of additives and processing parameters on the initial burst release of protein from poly (lactic-co-glycolic acid) microspheres. PDA J Pharm Sci Technol. 60 (2006) 54-9.

Zhou S, Deng X, Li X. Investigation on novel core- coated microspheres protein delivery system. J. control .Rel 75(2000) 27-36.

Zhou T, Lewis H, Foster RE, Schwendeman SP. Development of a multiple-drug delivery implant for intraocular management of proliferative vitreoretinopathy.J Control Release. 55 (1998) 281-9.

# TRAVAUX PERSONNELS

## CHAPITRE I

### Article 1
### Effect of the microencapsulation of nanoparticles on the reduction of burst release

L'objectif de cette première étude était de mettre au point une forme multiparticulaire qui pourrait régler le problème du burst en utilisant des polymères hydrophobes permettant également d'assurer une libération prolongée des principes actifs encapsulés. En effet, les principes actifs encapsulés dans des polymères hydrophobes présentent une libération assez lente avec un effet burst réduit, mais presque toujours présent, bien que la diffusion soit limitée pour plusieurs raisons comme une faible prise en eau, un gonflement au contact d'eau limité ou encore une perméabilité de l'eau à travers le matrice réduite. En conséquence, un contrôle efficace de la diffusion du principe actif, qui représente le principal mécanisme de libération dans les premiers temps à partir des particules, peut permettre d'espérer une réduction significative du burst.
Le caractère innovant de notre travail repose sur une formulation de microparticules contenant en leur sein des nanoparticules à laquelle nous avons donné le nom de microparticules composites. Les deux types de particules ont été fabriqués avec des polymères hydrophobes. Les microparticules ont été préparées selon la technique de double émulsion (eau/huile/eau) en utilisant une suspension aqueuse de nanoparticules comme phase interne aqueuse. Les microparticules résultantes ont fait l'objet d'une analyse complète *in vitro* incluant notamment la mesure de leur taille, leur charge en principe actif et évidemment le profil de libération des principes actifs incorporés. En ce qui concerne les principes actifs modèles, ils ont été choisis en fonction de critères de solubilité aqueuse et de masse moléculaire. La première molécule sélectionnée était l'ibuprofène (hydrophobe) et la seconde l'acétate de triptoréline (peptide hydrophile) ce qui nous a permis de tester l'efficacité de notre formulation pour solutionner le problème du burst sur des molécules aux propriétés largement différentes car les raisons de burst sont multiples et notamment fonction de la solubilité aqueuse du principe actif.

Les résultats des microparticules composites sont ensuite comparés aux résultats de microparticules ne contenant pas de nanoparticules (microparticules simples) et avec les résultats obtenus pour les nanoparticules seules.

L'originalité du travail repose sur le choix adéquat des couples polymères solvants. Il fallait en effet choisir, pour la fabrication des microparticules, un solvant dans lequel les nanoparticules de la phase aqueuse interne ne seraient pas solubilisées. C'est la raison du choix de l'acétate d'éthyle qui s'est révélé être un bon solvant de l'éthylcellulose ou de l'Eudragit® RS qui sont les polymères constitutifs de la matrice des microparticules. En revanche, les nanoparticules de poly(ε-caprolactone) ne sont que très faiblement solubles dans l'acétate d'éthyle.

Les résultats expérimentaux présentés dans l'article démontrent que le burst est fortement réduit, pour les deux types de molécules modèles, *in vitro*. Ce premier travail a donc apporté la validation expérimentale du concept à la base des différents travaux de cette thèse.

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# Effect of the microencapsulation of nanoparticles on the reduction of burst release

A. Sheikh Hasan [a], M. Socha [a], A. Lamprecht [b], F. El Ghazouani [a], A. Sapin [a], M. Hoffman [a], P. Maincent [a,*], N. Ubrich [a]

[a] *EA 3452-Inserm U 734, School of Pharmacy-5, rue A. Lebrun, BP 80403, 54001 Nancy Cedex, France*
[b] *Laboratory of Pharmaceutical Engineering, School of Pharmacy, Place Saint Jacques, 25030 Besançon Cedex, France*

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**Abstract**

The initial burst release is one of the major problems in the development of controlled release formulations including drug-loaded micro- and nanoparticles, especially with low molecular weight drugs. The objective of the present work was to encapsulate, by the W/O/W emulsion, polymeric nanoparticles into polymeric microparticles by using non-water soluble polymers and appropriate organic solvents for the preparation of these composite microparticles. They were characterized in vitro (encapsulation efficiency, mean diameter and release kinetics) and compared with nanoparticles and classical microparticles prepared by the same method. Poly-ε-caprolactone (PCL) dissolved in methylene chloride was used to make nanoparticles, whereas ethylcellulose and Eudragit RS dissolved in ethyl acetate, a non-solvent of poly-ε-caprolactone, were used for the preparation of microparticles. Ibuprofen and triptorelin acetate were chosen as lipophilic and hydrophilic model drugs, respectively. High entrapment efficiencies were obtained with ibuprofen whereas lower amounts of triptorelin acetate were encapsulated, mainly with formulations prepared with poly-ε-caprolactone and Eudragit RS used alone or blended with ethylcellulose. The burst was significantly lower with composite microparticles and may be explained by the slower diffusion of the drugs through the double polymeric wall formed by the nanoparticle matrix followed by another diffusion step through the microparticle polymeric wall.


*Keywords:* Burst release; Nanoparticles; Microparticles; Controlled release

## 1. Introduction

One major persistent problem in the development of injectable polymeric delivery systems is the drug initial burst release of drug which occurs during the first minutes of contact with the external medium (Yeo and Park, 2004). This burst release can be useful for the penetration of a drug, especially in the case of dermal applications, but sustained release is important for active drugs that are toxic at high concentrations or those that need to be present over a prolonged period. Burst release is often observed with microparticulate systems; it is unpredictable and generally difficult to control, but may be prevented by changing the drug distribution within the polymer matrix (Fu et al., 2003) or by developing more sophisticated drug delivery systems. Examples of the latter are liposomes encapsulated inside dextran (Stenekes et al., 2000) and alginate microcapsules allowing the release of the drug in a controlled way and eliminating the burst effect (Dhoot and Wheatley, 2003). A pentamidine-loaded hydrogel has also been microencapsulated within PLGA microparticles using the solvent evaporation technique, leading to a significant reduction of the burst effect (Mandal et al., 2002). Double-walled microspheres (Lee et al., 2002), double-layered minipellets (Maeda et al., 2003) and coated microspheres (Huang et al., 1999; Hurteaux et al., 2005) have all been developed to reduce the initial burst and provide sustained release profiles of the drug. A substantial reduction of the initial burst was also obtained by crosslinking the microparticle surface, thus creating an additional barrier to diffusion which prevented easy dissolution of the drug in the external medium (Thote et al., 2005). Microparticles prepared with blends of polymers characterized by different viscosity, molecular weight and swelling properties may also modify the

* Corresponding author. Tel.: +33 3 83 68 22 96; fax: +33 3 83 68 23 01.
*E-mail address:* philippe.maincent@pharma.uhp-nancy.fr (P. Maincent).

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release of the drug compared with microparticles prepared from a single polymer. Indeed, the release of ibuprofen from microspheres prepared with a blend of ethylcellulose and polystyrene was prolonged over 24 h with a reduced burst compared with microspheres prepared with ethylcellulose alone (Saravanan et al., 2003). The use of surfactants (Bouissou et al., 2006) or hydrophilic additives such as glycerol (Yamaguchi et al., 2002) added to the organic dispersion of methylene chloride containing PLGA and crystalline insulin results in a drastically reduced initial burst of insulin, which is preferentially located inside microcapsules rather than at the surface. Moreover, the addition of glycerol decreased both the glass transition temperature of PLGA and the porosity of microspheres: thus the diffusion of insulin was effectively prevented. The microencapsulation of lipid (Lee et al., 2003) or polymeric nanoparticles prepared from hydrophilic polymers (Grenha et al., 2005; Bhavsar et al., 2006) can lead to the controlled release of the encapsulated drug. Nanostructured protein particles produced by spray-freezing and encapsulated within lactide/glycolide copolymer and poly(lactic acid) microspheres decreased the burst release and maintained high structural integrity (38%) of the encapsulated protein (Leach et al., 2005).

The goal of our study was to encapsulate, by the double emulsion method, polymeric nanoparticles within polymeric microparticles by using non-water soluble polymers and an appropriate organic solvent for the preparation of these composite microparticles, thus preventing the dissolution of the nanoparticulate suspension used as internal phase. Poly-ε-caprolactone (PCL) dissolved in methylene chloride was used for the manufacture of nanoparticles, whereas ethylcellulose and Eudragit® RS dissolved in ethyl acetate, a non-solvent of PCL, were used for the preparation of microparticles. Ibuprofen and triptorelin acetate were chosen as lipophilic and hydrophilic model drugs, respectively. Microparticles containing nanoparticles, called composite microparticles, were characterized in vitro (encapsulation efficiency, mean diameter, release kinetics) and compared with nanoparticles and simple microparticles prepared by the same double emulsion method.

## 2. Materials and methods

### 2.1. Materials

Ibuprofen [(R, S)-2(4-isobutylphenyl) propionic acid] (batch number 450025) generously supplied by Knoll Pharma Chemicals (Nottingham, UK) and triptorelin acetate, a gift of Debiopharm (Lausanne, Switzerland), were used as model drugs. Poly(ε-caprolactone) (MW 40,000 Da) was supplied by Aldrich, USA. An acrylic polycationic nonbiodegradable polymer (copolymers of acrylic and methacrylic acide esters with a low content of quaternary ammonium groups (0.5–0.8%) (4.48–6.77% ammonium methacrylate units by dry weight)) Eudragit® RS PO (MW 150,000 Da) (RS) and ethylcellulose powder (Ethocel viscosity 4) were donated by Röhm Pharma polymers, Degussa (Darmstadt, Germany) and The Dow Chemical Company (Michigan, USA), respectively.

Poly(vinyl alcohol) (PVA) (MW 30 kDa, 88% hydrolyzed) was supplied by Sigma–Aldrich (St. Louis, Missouri, USA). Ethyl acetate (water solubility = 8.3 g/100 ml at 20 °C)) was purchased from Fluka Chemie GmbH (Switzerland). Methylene chloride (water solubility = 1.3 g/100 ml at 20 °C) was supplied by Prolabo (Paris, France). Acetonitrile and orthophosphoric acid were obtained from Carlo-Erba (Val de Reuil, France) and Prolabo (Paris, France), respectively. All other chemicals were of analytical grade and used without further purification.

### 2.2. Methods

#### 2.2.1. Preparation of particles

*2.2.1.1. Nanoparticles (NP).* Ibuprofen or triptorelin acetate-loaded PCL NP were prepared by the W/O/W solvent evaporation method (Hoffart et al., 2002). Briefly, 1 ml of aqueous internal phase was emulsified for 15 s in 5 ml of methylene chloride (containing 125 mg of PCL) using an ultrasound probe (Vibra cell 72,434, BioBlock Scientific, Strasbourg, France) at 50 W output. This primary emulsion was poured into 40 ml of a 0.1% PVA aqueous solution and sonicated again with the same ultrasound probe for 1 min under the same conditions in order to create the water in oil-in-water emulsion. Three to four milliliters of NP suspension were obtained after solvent evaporation under reduced pressure (Rotavapor, Heidolph, Germany). Nanoparticles were separated from the bulk suspension by centrifugation (Biofuge Stratos, Heraeus Instruments, Germany) at 42,000 × *g* for 20 min. The supernatant was kept for drug assay as described later and the sedimented nanoparticles were redispersed in 3 ml of purified water before freeze-drying. After lyophilization, the dried nanoparticles were resuspended in 2 ml of purified water shortly before preparing the composite microparticles.

Due to the lipophilic nature of ibuprofen, 50 mg of the drug were dissolved in the organic phase but, in order to keep the same preparation method for both drugs (W/O/W emulsion), 1 ml of purified water was used as the aqueous internal phase in the case of ibuprofen-loaded NP. On the other hand, since triptorelin acetate is water soluble, 1 ml of a triptorelin acetate aqueous solution (2.5 mg/ml) was used as the internal phase in the case of triptorelin acetate-loaded NP.

Blank nanoparticles were prepared under the same conditions without drug.

*2.2.1.2. Microparticles (MP).* Microparticles containing either ibuprofen or triptorelin acetate PCL NP (so-called composite microparticles) were prepared by the W/O/W solvent extraction method (Freytag et al., 2000). In the first step (W/O emulsion), the PCL NP suspension (2 ml) used as the internal aqueous phase was emulsified (ultrasound probe at 50 W output for 15 s) in an organic solution of polymer in ethyl acetate (5 ml). The polymers (250 mg) dissolved in ethyl acetate were (i) Eudragit® RS, (ii) ethylcellulose and (iii) a 1/1 blend of Eudragit® RS and ethylcellulose.

The primary emulsion was poured into 20 ml of 0.1% PVA aqueous solution in order to obtain a W/O/W pre-emulsion. After magnetically stirring for 1 min (1000 rpm) at room temperature, this pre-emulsion was added to 400 ml of a 0.1% PVA aque-

ous solution and stirred mechanically (three-bladed propeller, 1600 rpm) for 10 min to form the final W/O/W emulsion.

Upon solvent extraction, the polymers precipitated and the microparticle cores solidified.

Microparticles were collected by filtration (Millipore® Type: 0.45 μm nitrate cellulose for ibuprofen MP and cellulose acetate for triptorelin acetate MP) and dried at room temperature for 24 h.

Blank composite microparticles (with blank PCL NP) and simple microparticles (without PCL NP but containing one or the other drug) were prepared under the same conditions.

*2.2.2. Mean diameter and zeta potential*

*2.2.2.1. Microparticles.* Mean diameter and size distribution of MP were analyzed by laser diffraction in a particle size analyzer (Mastersizer S, Malvern Instruments, France). Each sample was measured in triplicate.

*2.2.2.2. Nanoparticles.* The mean diameter of NP and their surface potential were evaluated with a Malvern Zetasizer 3000 HSA (Malvern Instruments, France) using, respectively, photon correlation spectroscopy and electrophoretic mobility. Nanoparticles were diluted in 0.001 M NaCl prior to zeta potential measurements. The results were all normalized with respect to a polystyrene standard suspension (Malvern Instruments). Each sample was measured in triplicate.

*2.2.3. Determination of drug content*

*2.2.3.1. Ibuprofen.* The amount of ibuprofen entrapped within polymeric particles was determined spectrophotometrically at 222 nm (UV-160 1PC, UV-visible spectrophotometer, Shimadzu, Kyoto, Japan) by measuring the amount of non-entrapped ibuprofen in the external aqueous solution (indirect method) which was recovered after filtration and washing of microparticles. In the case of nanoparticles, the external aqueous solution was obtained after centrifugation of the colloidal suspension for 20 min at 42,000 × g. A standard calibration curve was performed with the ibuprofen solution (aqueous solution of 0.1% PVA with 1% acetone). The established linearity range was 2–10 μg/ml ($r > 0.99$).

In order to validate the indirect assay for routine purposes, the results were compared with those obtained after measuring the amount of ibuprofen within Eudragit® RS microparticles (without PCL NP) directly by an established but slightly modified HPLC method (Fernandez-Carballido et al., 2004).

Briefly, 20 mg of particles were accurately weighed and dissolved in 20 ml of mobile phase (water/acetonitrile: 40/60 acidified with orthophosphoric acid pH 2.7). Fifty microliters of this solution were injected into the HPLC system (Model Shimadzu HPLC 10A vp, Shimadzu, Japan) with UV detection (SPD-10 A VP, Shimadzu, Japan). The separation was achieved by using a reversed phase column (Uptisphere ODB, 3 mm i.d., 150 mm long, 12 nm porosity, 5 μm particle size, Interchim, France). The detection wavelength was set at 264 nm. The flow rate of the mobile phase was 0.8 ml/min. Under these conditions, the polymer did not interfere with the drug at this specific wavelength. The ibuprofen calibration curve was linear from 1 to 100 μg/ml ($r = 0.999$).

*2.2.3.2. Triptorelin acetate.* Triptorelin acetate content was determined by an established reversed phase HPLC method previously described (Schuetz et al., 2005). The separation was achieved by using a $C_{18}$ Whatman Partisphere WVS column (4.6 mm i.d., 125 mm long, 5 μm particles size, Interchim, France). The detection wavelength was set at 278 nm. The triptorelin acetate curve was linear from 1 to 500 μg/ml ($r = 0.999$) in an aqueous solution of PVA 0.1% and NaCl 0.1 M. The amount of triptorelin acetate entrapped within polymeric particles was also determined by two methods. In the indirect method, the amount of non-entrapped triptorelin acetate in the external aqueous solution was measured by injecting 20 μl directly into the HPLC system (Model Shimadzu HPLC 10A VP, Shimadzu, Japan) with UV detection (SD-10 A VP, Shimadzu, Japan). In the direct method, the triptorelin acetate amount was directly determined after extraction from the particles followed by injecting 20 μl of the extract directly into the HPLC system. To extract the drug, about 10 mg of particles were accurately weighed and dissolved in 0.5 ml of methylene chloride. Triptorelin acetate was extracted 6 times (vortex 10 min) from the organic phase with 2 ml of an aqueous solution of Tween 80 (0.1%), followed by a 20 min centrifugation at 800 × g.

*2.2.4. In vitro drug release from both nanoparticles and microparticles*

Fifty milligrams of freeze-dried or dried ibuprofen or triptorelin acetate loaded particles were suspended in 20 ml of saline phosphate buffer ($KH_2PO_4$ 0.0044 M, $Na_2HPO_4 \cdot 0.0451$ M, NaCl 0.1 M, pH 7.4 adjusted by orthophosphoric acid) or NaCl 0.1 M, respectively. The particles suspension was gently stirred (200 rpm) at 37 °C into a water bath. One milliliter of suspension was withdrawn at appropriate intervals (5, 15, 30, 45 min, 1, 2, 3, 4, 5, 6, 8, 24 h) and filtered with a 0.22 μm nitrate cellulose filter (Millipore®) in the case of ibuprofen. For triptorelin acetate, due to established filter adsorption during the preliminary trials, each sample was centrifuged at 42,000 × g for 10 min. The filtrate (ibuprofen) or the supernatant (triptorelin acetate) was replaced by 1 ml of fresh buffer. The amount of ibuprofen in the release medium was determined by UV at 222 nm as previously described. For triptorelin acetate, the HPLC method described previously was used. Each particle batch was analyzed in triplicate.

## 3. Results and discussion

Burst release is a critical problem with currently marketed injectable microparticles, especially when slow release for a few weeks or months is required. The encapsulation of nanoparticles within microparticles as an alternative way to decrease the burst has been proposed by only a few groups (Lee et al., 2003; Sheikh Hasan et al., 2004; Grenha et al., 2005; Leach et al., 2005; Bhavsar et al., 2006). For example, Bhavsar et al. encapsulated gelatin nanoparticles in poly(ε-caprolactone) microparticles; whereas Grenha et al. encapsulated chitosan

nanoparticles within mannitol or lactose microparticles: however, these hydrophilic polymers used for nanoparticles might favor water uptake by the microparticles and consequently induce a faster release and/or a faster hydrolysis of the non-soluble polyester polymer, as was shown in the case of the PCL microparticles prepared by Bhavsar et al. Furthermore, Grenha et al. observed a very high release of their model drug (insulin) after 20 min in a dissolution test, although their insulin nanoparticles were incorporated within mannitol microparticles. In order to prevent such rapid release, we have prepared composite microparticles based on non-water soluble polymers such as PCL, ethylcellulose and Eudragit® RS. Since the double emulsion technique was used for the preparation of composite microparticles, our original approach consisted in using a polymer for the nanoparticles (PCL) which is insoluble in the organic solvent used to manufacture the microparticles in the second step of the double emulsion method. Indeed, PCL was dissolved in methylene chloride because of the low boiling point and poor water solubility of this solvent. Microparticles were prepared by dissolving the two other polymers (ethylcellulose, Eudragit® RS and 1/1 blend of each polymer) in ethyl acetate which is a poor solvent for PCL: in our conditions of microparticle preparation the maximum solubility of PCL added directly to ethyl acetate is 5%. Therefore, it was possible to use the PCL nanoparticle suspension as the internal aqueous phase in the preparation of the composite microparticles.

In order to test the effect on burst release, two model drugs differing in their water solubility were selected. One was a peptide drug. Indeed, most of the currently marketed injectable microparticles are peptide dosage forms for which a reduction of the burst would be highly desirable. Triptorelin acetate is a decapeptide (MW = 1311.5) which is highly water soluble. Consequently, the only way to obtain sufficiently high incorporation in nanoparticles to use the W/O/W emulsion technique (Nicoli et al., 2001). Ibuprofen was the second model drug; with contrasting properties since it is very poorly water soluble (35.89 μg/ml) substance with a low molecular weight (MW = 206.3). Due to the very low aqueous solubility of ibuprofen, it would have been possible to prepare ibuprofen nanoparticles according to the simpler O/W emulsion technique. However, with a view to comparing the drug release with that of triptorelin acetate, ibuprofen nanoparticles and control simple microparticles were also prepared by the double emulsion technique. In this case, ibuprofen was dissolved in the organic phase and plain water (1 ml) was used as the internal aqueous phase.

Tables 1–3 summarize the main physicochemical parameters (mean diameter, zeta potential and encapsulation efficiency) of the three types of particles prepared with the various polymers.

Table 1
Mean diameter, drug encapsulation efficiency and zeta potential of blank or ibuprofen and triptorelin acetate loaded PCL nanoparticles ($n = 3 \pm$ S.D.)

| | Blank NP | Ibuprofen NP | Triptorelin acetate NP |
|---|---|---|---|
| Mean diameter (nm) | 355 ± 6.0 | 341 ± 9.0 | 380 ± 5.0 |
| Zeta potential (mV) | −3.1 ± 2.9 | +15.1 ± 5.5 | +19.8 ± 12.4 |
| Encapsulation efficiency (%) | – | 95 ± 2.0 | 38 ± 7.0 |

Both drug-loaded NP preparations had diameters ranging from 340 to 380 nm (Table 1). Blank nanoparticles were similar. Unloaded NP showed a slightly negative zeta potential which became positive after the incorporation of either drug. Due to its low water solubility, ibuprofen was very efficiently incorporated into PCL nanoparticles (mean encapsulation efficiency 95%) (Table 1). On the other hand, the encapsulation efficiency was only 38% for triptorelin acetate nanoparticles. This is probably the result of its higher water solubility; it would diffuse more readily through the aqueous continuous phase of the second emulsion before polymer precipitation. For both triptorelin acetate and ibuprofen, the average diameter of PCL nanoparticles obtained by the double emulsion technique was in the usual range for this polymer and this method of preparation. Indeed, Hoffart et al. (2002) obtained nanoparticle diameter ranging from 380 to 500 nm with the same polymer and preparation method. With the same double emulsion technique and also using methylene chloride, Nicoli et al. (2001) obtained triptorelin acetate nanoparticles in a slightly larger range (574–743 nm) when the polymer was various types of PLGA.

Simple microparticles are very small whatever the drug or the polymers used, except ibuprofen-loaded MP prepared with ethylcellulose (Table 2). Indeed, the diameter of all MP was close to 15 μm whereas ibuprofen-loaded MP prepared with ethylcellulose were larger (136 μm). In addition, simple blank ethylcellulose MP are slightly larger (32 μm) than blank MP prepared with Eudragit® RS alone or blended with ethylcellulose. The difference in encapsulation efficiency of the two drugs in simple ethylcellulose MP is not significant. Indeed, similar encapsulation efficiency (Table 2) was observed for ibuprofen and triptorelin encapsulated inside ethylcellulose MP (89 and 87%, respectively). A more significant difference between the two drugs was obtained after encapsulation in MP prepared with Eudragit® RS and the blend of EC and Eudragit® RS (around 80% for ibuprofen and 60% for triptorelin). The encapsulation

Table 2
Mean diameter and drug encapsulation efficiency of blank, ibuprofen (IBU) and triptorelin acetate (TRP) simple microparticles (SMP) prepared with ethylcellulose (EC), Eudragit® RS (RS) and a blend 50/50 of ethylcellulose and Eudragit® RS (EC/RS) ($n = 3 \pm$ S.D.)

| Polymers | Mean diameter (μm) | | | Encapsulation efficiency (%) | | |
|---|---|---|---|---|---|---|
| | Blank SMP | IBU SMP | TRP SMP | Blank SMP | IBU SMP | TRP SMP |
| EC | 32 ± 5 | 136 ± 28 | 20 ± 5 | – | 89 ± 5 | 87 ± 8 |
| RS/EC | 13 ± 4 | 18 ± 5 | 16 ± 3 | – | 80 ± 8 | 61 ± 4 |
| RS | 15 ± 2 | 14 ± 7 | 10 ± 5 | – | 79 ± 5 | 65 ± 7 |

Table 3
Mean diameter and drug encapsulation efficiency of blank, ibuprofen (IBU) and triptorelin acetate (TRP) composite microparticles (CMP) prepared with ethylcellulose (EC), Eudragit® RS (RS) and a blend 50/50 of ethylcellulose and Eudragit® RS (EC/RS) ($n = 3 \pm$ S.D.)

| Polymers | Mean diameter (μm) | | | Encapsulation efficiency (%) | | |
|---|---|---|---|---|---|---|
| | Blank CMP | IBU CMP | TRP CMP | Blank CMP | IBU CMP | TRP CMP |
| EC | 83 ± 10 | 161 ± 35 | 27 ± 6 | – | 87 ± 3 | 89 ± 8 |
| RS/EC | 23 ± 7 | 68 ± 18 | 24 ± 3 | – | 85 ± 4 | 66 ± 8 |
| RS | 16 ± 2 | 45 ± 9 | 21 ± 8 | – | 74 ± 5 | 59 ± 7 |

efficiency of triptorelin acetate was much higher in MP prepared with the two polymers used alone or blended than in NP prepared with PCL.

The PCL nanoparticle suspension of each drug was directly used as the internal aqueous phase in the preparation of the composite microparticles. Three types of composite microparticles were manufactured with either ethylcellulose, Eudragit® RS or the blend (50/50) of these 2 polymers. Composite microparticles had a larger diameter than simple microparticles (Table 3). This effect was more marked for ibuprofen composite microparticles. Encapsulation efficiencies remained fairly high and did not show major differences compared with simple microparticles. The high encapsulation efficiency of ibuprofen could again be explained by the lipophilic nature of the drug which has no affinity for the external aqueous phase, compared with the hydrophilic triptorelin acetate. The similar encapsulation efficiency observed with ethylcellulose simple and composite microparticles may be explained by the overall viscosity of the system. Indeed, it was observed (although not measured) that the solution of ethylcellulose in ethyl acetate was more viscous than with Eudragit® RS or with the blend ethylcellulose/Eudragit® RS. It would consequently be more difficult for the hydrophilic drug present in the internal dispersed phase to diffuse into the outer water phase (Bodmeier and McGinity, 1988). The triptorelin acetate encapsulation efficiency was higher in simple and composite microparticles than in nanoparticles: this is probably the effect of a faster precipitation of polymers after solvent (ethyl acetate) extraction during microparticle manufacturing compared with solvent evaporation (methylene chloride) during NP preparation.In order to demonstrate conclusively that nanoparticles were effectively entrapped in the microparticles, we performed the following experiment with unloaded micro- and nanoparticles. Blank composite EC/RS microparticles (containing PCL nanoparticles), blank simple EC microparticles, blank simple Eudragit® RS microparticles and PCL nanoparticles were dispersed in ethyl acetate. It should be noted that ethylcellulose and Eudragit® RS are soluble in ethyl acetate while the PCL polymer is not (or very slightly as stated above). Therefore, if PCL nanoparticles had really been encapsulated, the resulting suspension in ethyl acetate should have been very turbid, as should the blank PCL nanoparticle suspension dispersed in ethyl acetate. Fig. 1 clearly displays a similar turbidity in ethyl acetate of both PCL nanoparticles and composite microparticles showing the dissolution of ethylcellulose and Eudragit RS but the presence of PCL nanoparticles. In contrast, blank simple EC microparticles and blank simple RS microparticles are totally dissolved in ethyl acetate and produce a clear solution. Consequently, Fig. 1 shows that the manufacturing process allows the encapsulation of PCL nanoparticles within microparticles and demonstrates the composite character of these microparticles.

Most nano- and microparticulate systems are characterized by an initial burst which is generally difficult to control. Such a burst may be due to a number of phenomena, including (i) heterogenous drug distribution including surface-associated drug (Huang and Brazel, 2001); (ii) temperature, which affects drug distribution and morphology of particles (Guiziou et al., 1996); (iii) the physico-chemical nature of the polymeric matrix (Fernandez-Carballido et al., 2004; Thompson et al., 2007) and (iv) porosity of particles which is generally higher for the solvent evaporation method (methylene chloride) than for the extraction method (ethyl acetate) (Yeo and Park, 2004). There are two ways to determine whether the burst is controlled: by an in vitro dissolution test or by an in vivo approach after subcutaneous or intramuscular administration. As a first approach, we have used the in vitro dissolution test. These tests were carried out under sink conditions, in phosphate buffer (pH 7.4) and NaCl 0.1 M for ibuprofen and triptorelin acetate, respectively. The resulting release profiles are presented for each drug (ibuprofen or triptorelin acetate) and type of polymer (ethylcellulose, ethylcellulose/Eudragit RS and Eudragit RS). Fig. 2A–C displays the release profiles of ibuprofen from ethylcellulose, ethylcellu-

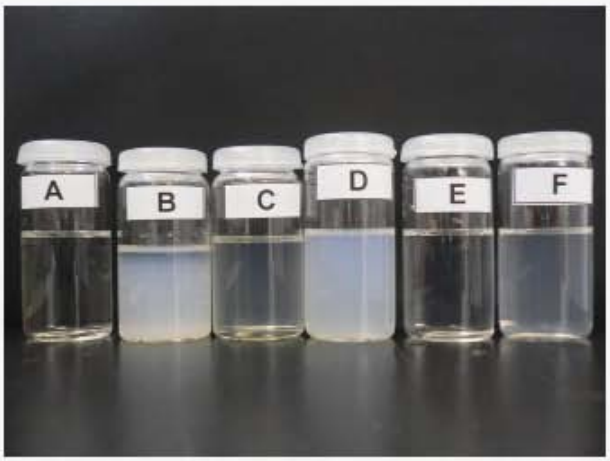



Fig. 1. Picture showing the macroscopic solubility in ethyl acetate of different nano- and microparticles prepared with different polymers: (A) ethyl acetate, (B) blank PCL nanoparticles, (C) EC/Eudragit® RS microparticles, (D) composite EC/Eudragit® RS microparticles, (E) simple Eudragit® RS microparticles, (F) simple EC microparticles.

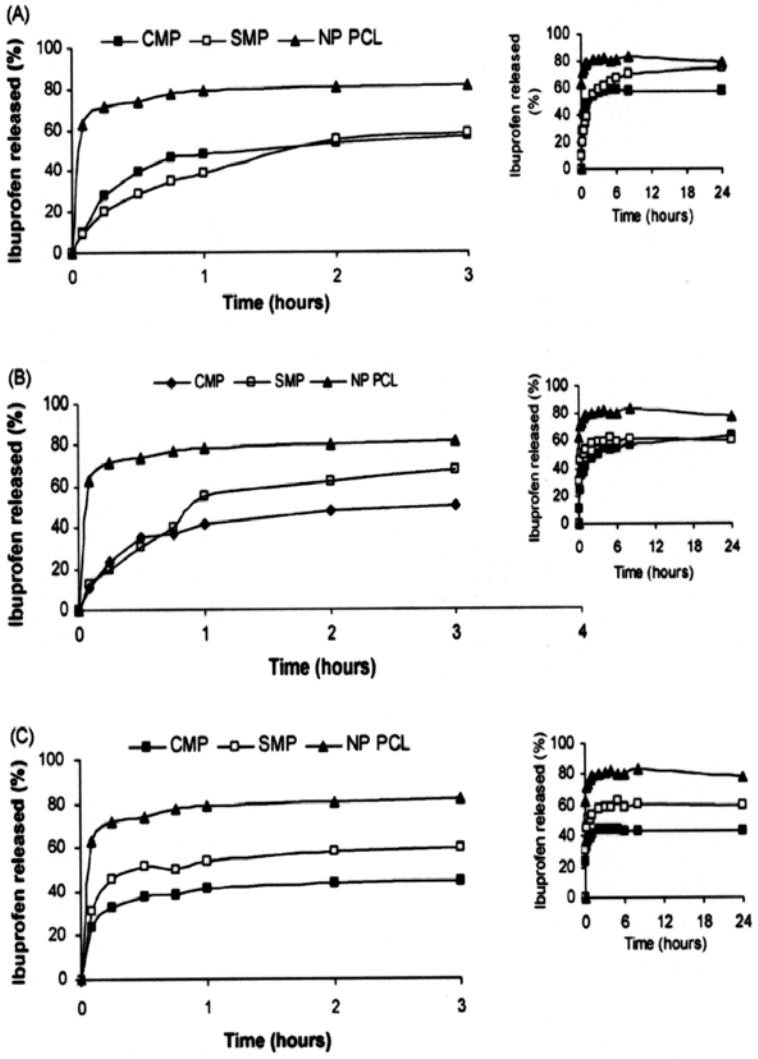



Fig. 2. Release kinetics of ibuprofen from PCL nanoparticles (solid triangles), simple (SMP, open squares) and composite microparticles (CMP, solid squares) prepared with (A) ethylcellulose, (B) Eudragit RS/ethylcellulose (50/50) and (C) Eudragit RS. Experiments were performed in phosphate buffer pH 7.4 at 37 °C. Data shown as mean ± S.D. ($n = 3$).

lose/Eudragit RS and Eudragit RS microparticles, respectively. Fig. 3A–C displays the release profiles of triptorelin acetate from ethylcellulose, ethylcellulose/Eudragit RS and Eudragit RS microparticles, respectively.

PCL-loaded nanoparticles of each drug displayed an immediate and important initial drug release in the first 15 min, followed by a plateau of around 80 and 70% at 24 h for ibuprofen and triptorelin acetate nanoparticles, respectively (Table 4). This immediate high release may be due to the small diameter of nanoparticles leading to a large exchange surface and probably to a more porous structure owing to the solvent evaporation method, favoring the release of the encapsulated drugs. Indeed, it has been already demonstrated that the slow precipitation of microparticles after solvent evaporation leads to more porous particles compared to the fast polymer precipitation obtained after solvent extraction (Jiang et al., 2002; Wang et al., 1991). This principle can be applied to the PCL nanoparticles which were prepared by solvent evaporation. It has also to be noted that, in the case of triptorelin acetate nanoparticles, the dissolution experiments were carried out in NaCl 0.1 M since the peptide was only slightly soluble in the PBS (solubility <0.25 mg/ml) used for ibuprofen. The higher ibuprofen release in PBS may be explained by its higher solubility in this buffer (3800 μg/ml) than in water (35.89 μg/ml). Indeed, ibuprofen is an acid ($pK_a$ 4.5) and has better solubility in water with increasing pH (Levis et al., 2003). Not all the encapsulated ibuprofen and triptorelin acetate was released, since a plateau (around 80 and 70%, respectively) was obtained after 30 min and was stable up to 24 h (the end of the dissolution test). Similar behavior has already been observed with triptorelin acetate PLGA nanoparticles (Nicoli et al., 2001).

Although not all the encapsulated drug was released in 24 h, the dissolution test was limited to this time because the aim of this research was to demonstrate the influence of the encapsulation of nanoparticles within microparticles on the initial burst release. The initial burst and the dissolution profiles were

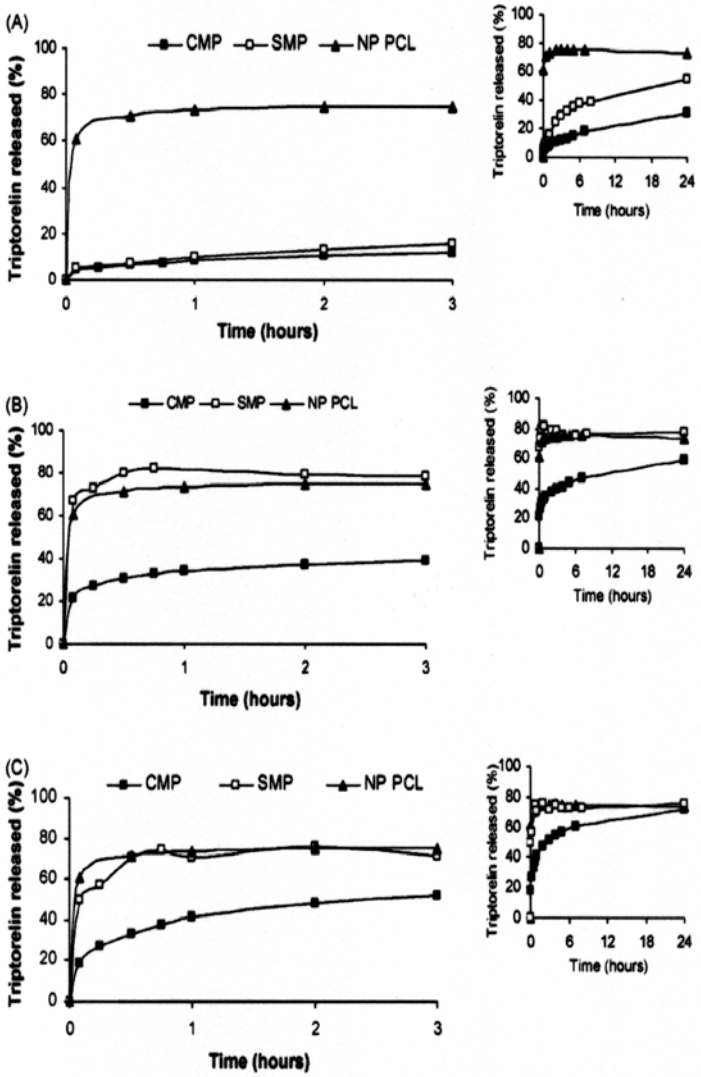



Fig. 3. Release kinetics of triptorelin acetate from PCL nanoparticles (solid triangles), simple (SMP, open squares) and composite microparticles (CMP, solid squares) prepared with (A) ethylcellulose, (B) Eudragit RS/ethylcellulose (50/50) and (C) Eudragit RS. Experiments were performed in NaCl 0.1 M at 37 °C. Data shown as mean ± S.D. ($n = 3$).

very different with all types of microparticles compared with nanoparticles, as shown in Figs. 2 and 3. The burst release was determined after 15 min, together with the percentage released after 24 h, as shown in Table 4.

For ibuprofen, the burst was higher for Eudragit® RS microparticles than for microparticles prepared with ethylcellulose or the blend of polymers (45.8 ± 1.2% when compared to 19.9 ± 5.3 and 19.8 ± 3.3%, respectively). This could be

Table 4
Mean percentage of ibuprofen (IBU) or triptorelin acetate (TRP) released after 15 min and 24 h from NP, simple and composite MP prepared with the different polymers used alone or blended ($n = 3 \pm$ S.D.)

| Formulations | Polymers | IBU released (%) | | TRP released (%) | |
|---|---|---|---|---|---|
| | | 15 min | 24 h | 15 min | 24 h |
| NP | PCL | 71.7 ± 12.1 | 78.3 ± 4.5 | 71.0 ± 11.2 | 73.5 ± 10.0 |
| Simple MP | RS | 45.8 ± 1.2 | 59.9 ± 1.4 | 56.9 ± 3.2 | 76.0 ± 2.4 |
| | EC | 19.9 ± 5.3 | 75.3 ± 4.8 | 7.1 ± 4.3 | 54.9 ± 3.6 |
| | EC/RS | 19.8 ± 3.3 | 58.2 ± 0.5 | 73.0 ± 6.0 | 77.3 ± 5.2 |
| Composite MP | RS | 32.9 ± 6.5 | 43.4 ± 4.2 | 26.7 ± 2.2 | 72.0 ± 2.3 |
| | EC | 27.7 ± 9.7 | 57.3 ± 8.0 | 5.4 ± 7.8 | 31.5 ± 5.8 |
| | EC/RS | 23.8 ± 3.7 | 63.8 ± 4.4 | 27.0 ± 6.1 | 59.5 ± 3.2 |

due to the physicochemical properties of Eudragit® which is a more hydrophilic polymer owing to its quaternary ammonium groups that favor water uptake and drug diffusion towards the dissolution medium (Huang et al., 2006). However, composite microparticles tend to reduce the initial burst effect especially for microparticles prepared with ethylcellulose used alone or blended with Eudragit® RS (27.7 and 23.8%, respectively, compared with 32.9% for Eudragit® RS alone composite microparticles). This can be explained by the rather lipophilic properties of ibuprofen does not favor its dissolution in aqueous media, but also to the high encapsulation ratio of PCL ibuprofen nanoparticles. Encapsulation of nanoparticles into microparticles also had a strong effect on the dissolution profile, especially for the ethylcellulose and Eudragit® RS/ethylcellulose microparticles. Indeed for the latter two types of microparticle, the total ibuprofen released was much lower after 24 h and a plateau of around 50% was obtained as early as 2 h. The presence of EC in the matrix of microparticles conferred a slower and more progressive release of ibuprofen during the time of the experiment. This could be explained by different interactions between the drug and either Eudragit® RS or ethylcellulose polymers.

For triptorelin acetate, the burst was the lowest for both the composite and the simple ethylcellulose microparticles compared with Eudragit® RS or blended microparticles (Fig. 3A) but the drug was more slowly released from the composite than from the simple microparticles in the 24 h period of the dissolution test (54.9 ± 3.6% versus 31.5 ± 5.8%). As for ibuprofen, except for composite Eudragit® RS microparticles which displayed a reduced burst, there was no major difference in terms of burst reduction between simple and composite microparticles for ethylcellulose and the blend of ethylcellulose and Eudragit® RS (from 54.9 to 31.5% and from 77.3 to 59.5%, respectively). Again, the burst was higher for Eudragit® RS microparticles compared with ethylcellulose or polymers blended microparticles. On the other hand, the effect of reducing the burst is much more marked with triptorelin acetate. Indeed, due its high hydrophilicity, this compound has a natural tendency to diffuse very rapidly towards the surrounding aqueous phase. Therefore, any mechanism which is able to restrict this diffusion of triptorelin acetate towards water would be easily observed. The influence of nanoparticle encapsulation in microparticles is obvious for each of the three types of microspheres. As a first observation, it can be noticed that simple microparticles prepared with Eudragit® RS (alone or blended with ethylcellulose) allowed a very fast release of triptorelin acetate. Basically the release profile was the same as for the triptorelin acetate PCL nanoparticles. It is only with simple ethylcellulose microparticles that the triptorelin acetate profile was intermediate between nanoparticles and composite microparticles when taking into account the whole 24 h of the experiment. This is probably due to the slow diffusion of water into the lipophilic EC matrix. However, in terms of burst reduction there is no difference between simple and composite ethylcellulose microparticles since the burst was under 10% in both cases (Table 4). When PCL triptorelin acetate nanoparticles were encapsulated in microparticles, there was a large decrease in the burst. Again, this decrease is much more marked than that for ibuprofen due to the hydrophilic nature of the drug. Therefore, the advantage of encapsulating nanoparticles in microparticles (composite microparticles) has been definitely demonstrated for hydrophilic drugs. The decrease in the burst is greater for ethylcellulose than for Eudragit® RS due to the lipophilic nature of ethylcellulose which slows down the entrance of water in the polymeric matrix. On the other hand, Eudragit® RS is more permeable to water which facilitates drug release from the encapsulated PCL nanoparticles.

## 4. Conclusion

More generally, the differences observed with the composite microparticles may be explained by the heterogeneous composition of the polymeric matrix. Indeed, in order to be released into the external dissolution medium, both drugs have to diffuse first through the PCL nanoparticles followed by another diffusion step through the ethylcellulose or Eudragit® RS matrix. The diffusion pathway takes longer for ethylcellulose due to the hydrophobicity of this polymer. The overall dissolution profiles show the potential of composite microparticles to dramatically change the burst effect and the release profile of drugs in vitro. This concept remains to be verified after subcutaneous or intramuscular administration, in this case using biodegradable polymers.

## References

Bhavsar, M.D., Tiwari, S.B., Amiji, M.M., 2006. Formulation optimization for the nanoparticles-in-microsphere hybrid oral delivery system using factorial design. J. Control Rel. 110, 422–430.

Bodmeier, R., McGinity, J.W., 1988. Solvent selection in the preparation of poly(DL-Lactide) microspheres prepared by the solvent evaporation method. Int. J. Pharm. 43, 179–186.

Bouissou, C., Rouse, J.J., Price, R., van der Walle, C.F., 2006. The influence of surfactant on PLGA microsphere glass transition and water sorption: remodeling the surface morphology to attenuate the burst release. Pharm. Res. 23, 1295–1305.

Dhoot, N.O., Wheatley, M.A., 2003. Microencapsulated liposomes in controlled drug delivery: strategies to modulate drug release and eliminate the burst effect. J. Pharm. Sci. 92, 679–689.

Fernandez-Carballido, A., Herrero-Vanrell, R., Molina-Martinez, I.T., Pastoriza, P., 2004. Biodegradable ibuprofen-loaded PLGA microspheres for intraarticular administration. Effect of Labrafil addition on release in vitro. Int. J. Pharm. 279, 33–41.

Freytag, T., Dashevsky, A., Tillman, L., Hardee, G.E., Bodmeier, R., 2000. Improvement of the encapsulation efficiency of oligonucleotide-containing biodegradable microspheres. J. Control Rel. 69, 197–207.

Fu, K., Harrell, R., Zinski, K., Um, C., Jaklenec, A., Frazier, J., Lotan, N., Burke, P., Klibanov, A.M., Langer, R., 2003. A potential approach for decreasing the burst effect of protein from PLGA microspheres. J. Pharm. Sci. 92, 1582–1591.

Grenha, A., Seijo, B., Remunan-Lopez, C., 2005. Microencapsulated chitosan nanoparticles for lung protein delivery. Eur. J. Pharm. Sci. 25, 427–437.

Guiziou, B., Armstrong, D.J., Elliott, P.N., Ford, J.L., Rostron, C., 1996. Investigation of in-vitro release characteristics of NSAID-loaded polylactic acid microspheres. J. Microencapsul. 13, 701–708.

Hoffart, V., Ubrich, N., Simonin, C., Babak, V., Vigneron, C., Hoffman, M., Lecompte, T., Maincent, P., 2002. Low molecular weight heparin-loaded polymeric nanoparticles: formulation, characterization, and release characteristics. Drug Dev. Ind. Pharm. 28, 1091–2000.

Huang, X., Brazel, C., 2001. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. J. Control Rel. 73, 121–136.

Huang, Y.Y., Chung, T.W., Tzeng, T.W., 1999. A method using biodegradable polylactides/polyethylene glycol for drug release with reduced initial burst. Int. J. Pharm. 182, 93–100.

Huang, J., Wigent, R.J., Schwartz, J.B., 2006. Nifedipine molecular dispersion in microparticles of ammonio methacrylate copolymer and ethylcellulose binary blends for controlled drug delivery: effect of matrix compostion. Drug Dev. Ind. Pharm. 32, 1185–1197.

Hurteaux, R., Edwards-Levy, F., Laurent-Maquin, D., Levy, M.C., 2005. Coating alginate microspheres with a serum albumin-alginate membrane: application to the encapsulation of a peptide. Eur. J. Pharm. Sci. 24, 187–197.

Jiang, G., Thanoo, B.C., DeLuca, P.P., 2002. Effect of osmotic pressure in the solvent extraction phase on BSA release profile from PLGA microspheres. Pharm. Dev. Technol. 7, 391–399.

Leach, W.T., Simpson, D.T., Val, T.N., Anuta, E.C., Yu, Z., Williams III, R.O., Johnston, K.P., 2005. Uniform encapsulation of stable protein nanoparticles produced by spray freezing for the reduction of burst release. J. Pharm. Sci. 94, 56–69.

Lee, T.H., Wang, J., Wang, C.H., 2002. Double-walled microspheres for the sustained release of a highly water soluble drug: characterization and irradiation studies. J. Control Rel. 83, 437–452.

Lee, K.E., Cho, S.H., Lee, H.B., Jeong, S.Y., Yuk, S.H., 2003. Microencapsulation of lipid nanoparticles containing lipophilic drug. J. Microencapsul. 20, 489–496.

Levis, K.A., Lane, M.E., Corrigan, O.I., 2003. Effect of buffer media composition on the solubility and effective permeability coefficient of ibuprofen. Int. J. Pharm. 253, 49–59.

Maeda, H., Nakagawa, T., Adachi, N., Sakai, Y., Yamamoto, T., Matsuoka, A., Sano, A., Satoh, Y., Miyata, T., Fujioka, K., 2003. Design of long-acting formulation of protein drugs with a double-layer structure and its application to rhG-CSF. J. Control Rel. 91, 281–297.

Mandal, T.K., Bostanian, L.A., Graves, R.A., Chapman, S.R., 2002. Poly(D,L-Lactide-Co-Glycolide) encapsulated poly(Vinyl Alcohol) hydrogel as a drug delivery system. Pharm. Res. 19, 1713–1719.

Nicoli, S., Santi, P., Couvreur, P., Couarraze, G., Colombo, P., Fattal, E., 2001. Design of triptorelin loaded nanospheres for transdermal iontophoretic administration. Int. J. Pharm. 214, 31–35.

Saravanan, M., Bhaskar, K., Srinivasa Rao, G., Dhanaraju, M.D., 2003. Ibuprofen-loaded ethylcellulose/polystyrene microspheres: an approach to get prolonged drug release with reduced burst effect and low ethylcellulose content. J. Microencapsul. 20, 289–302.

Schuetz, Y.B., Naik, A., Guy, R.H., Vuaridel, E., Kalia, Y.N., 2005. Trandermal iontophoretic delivery of triptorelin in vitro. J. Pharm. Sci. 94, 2175–2182.

Sheikh Hasan, A., Maincent, P., Lamprecht, A., Rissmann, R., Ubrich, N., 2004. A drug delivery system based on the encapsulation of nanoparticles in microparticles. AAPS J. 6, T3290.

Stenekes, R.J., Loebis, A.E., Fernandes, C.M., Crommelin, D.J., Hennink, W.E., 2000. Controlled release of liposomes from biodegradable dextran microspheres: a novel delivery concept. Pharm. Res. 17, 690–695.

Thompson, C.J., Hansford, D., Higgins, S., Rostron, C., Hutcheon, G.A., Munday, D.L., 2007. Evaluation of ibuprofen-loaded microspheres prepared from novel copolyesters. Int. J. Pharm. 329, 53–61.

Thote, A.J., Chappell, J.T., Gupta, R.B., Kumar, R., 2005. Reduction in the initial-burst release by surface crosslinking of PLGA microparticles containing hydrophilic or hydrophobic drugs. Drug Dev. Ind. Pharm. 31, 43–57.

Wang, H.T., Schmitt, E., Flanagan, D.R., Linhardt, R.J., 1991. Influence of formulation methods on the in vitro controlled release of protein from poly (ester) microspheres. J. Control Rel. 17, 23–31.

Yamaguchi, Y., Takenaga, M., Kitagawa, A., Ogawa, Y., Mizushima, Y., Igarashi, R., 2002. Insulin-loaded biodegradable PLGA microcapsules: initial burst release controlled by hydrophilic additives. J. Control Rel. 81, 235–249.

Yeo, Y., Park, K., 2004. Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch. Pharmacol. Res. 27, 1–12.

# CHAPITRE II

## Article 2
## Simple and sensitive HPLC method with fluorescence detection for the measurement of ibuprofen in rat plasma. Application to a long-lasting dosage form

**Sous presse: Drug Development and Industrial Pharmacy, 2008**

La plupart des techniques analytiques utilisées pour le dosage de l'ibuprofène dans le plasma ont des limites de détection et de quantification assez élevées (entre 200-1000 ng/mL). L'administration sous-cutanée de microparticules d'ibuprofène devait théoriquement conduire à des concentrations plasmatiques faibles qui devaient être de plus suivies pendant des temps prolongés. C'est sur la base de cette présomption que nous avons été amenés à proposer une technique plus sensible destinée à mettre en valeur les capacités de notre système de microparticules composites.

La méthode HPLC que nous avons développée repose sur une détection par fluorescence (limite de quantification 50 ng/mL). Il a en effet été tiré profit des propriétés de fluorescence de l'ibuprofène qui sont fonctions du pH. En effet pour des pH > $pK_a$, la fluorescence de l'ibuprofène augmente énormément par rapport aux pH < $pK_a$.

Généralement, les méthodes de dosage HPLC de l'ibuprofène utilisent des phases mobiles de pH = 2 donc inférieures au pKa (= 4,4) de l'ibuprofène. Dans la nouvelle méthode mise au point, un pH de 6,5 a été choisi. De plus, afin d'améliorer la fluorescence de l'ibuprofène et d'améliorer la limite de détection, de la β-cyclodextrine a été ajoutée dans la phase mobile.

Une fois les conditions expérimentales définitivement mises au point, la nouvelle méthode analytique a fait l'objet d'une validation selon les critères actuellement retenus.

Cette méthode a effectivement permis de doser l'ibuprofène dans le plasma chez le rat suite à l'administration des formes multiparticulaires à libération prolongé d'ibuprofène et de bien mettre en évidence la pharmacocinétique de l'ibuprofène dans le sang. Accessoirement, cette nouvelle méthode pourra être utilisée par d'autres équipes souhaitant tester d'éventuelles autres formes pharmaceutiques avec une précision accrue.

# Simple and sensitive HPLC method with fluorescence detection for the measurement of ibuprofen in rat plasma. Application to a long-lasting dosage form.

**Ahmed Sheikh Hassan[a], Claire Bolzan[a,b], Anne Sapin[a], Nathalie Ubrich[a], Philippe Maincent[a] and Pierre Leroy[b,] ***

[a] *Laboratoire de Pharmacie Galénique et Biopharmacie – EA3452, Inserm U 734 Faculté de Pharmacie, Nancy-University - BP 80403 – F 54001 Nancy Cedex, France*

[b] *Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, UMR 7564 CNRS UHP-Nancy 1 - Faculté de Pharmacie, Nancy-University – BP 80403 – F 54001 Nancy Cedex, France*

*Corresponding author. Tel: +33.3.83.68.21.55; fax: +33.3.83.68.21.54. E-mail address: pierre.leroy@pharma.uhp-nancy.fr

**Abstract**

A simple and sensitive HPLC assay devoted to the measurement of ibuprofen in rat plasma has been developed. Two parameters have been investigated in order to improve ibuprofen detectability using fluorescence detection: variation of mobile phase pH and use of β−cyclodextrin (β−CD). Increasing pH value from 2.5 to 6.5 and adding 5 mM β−CD contributed to an enhancement of the fluorescence signal ($\lambda_{exc}$ = 224 nm; $\lambda_{em}$ = 290 nm) of 2.5 and 1.3-fold, respectively when using standards. In the case of plasma samples, only pH variation significantly lowered detection and quantification limits, down to 10 and 35 ng.mL$^{-1}$, respectively. Full selectivity was obtained with a single step for plasma treatment, i.e. protein precipitation with acidified acetonitrile. The validated method was applied to a pharmacokinetic study of ibuprofen encapsulated in microspheres and subcutaneously administered to rats.

*Keywords:* Ibuprofen; Microspheres; HPLC; Fluorescence; β−Cyclodextrin; pH; Plasma.

**1. Introduction**

Ibuprofen, (±)-2-(p-isobutylphenyl)propionic acid, is a non-steroidal anti-inflammatory (NSAID) drug which is available in a wide variety of pharmaceutical preparations commonly used in the treatment of acute and chronic pain and inflammation, in rheumatoid arthritis and other musculoskeletal disorders. It offers a good tolerability but a relative short plasma half-life (2-3 h), resulting in short pharmacological activity duration. In order to overcome this problem, various ibuprofen prodrugs (Wang et al., 2005; Zhao et al., 2005) and formulations (Borovac et al., 2006; Lamprecht et al., 2004; Fernandez-Carballido et al., 2004) have been proposed.

Numerous assays of ibuprofen in biological fluids have already been reported. Most of them rely upon separative methods, especially HPLC techniques coupled with UV (Espinosa-Mansilla et al., 2006, Kot-Wasik et al., 2006; Sochor et al., 1995; Teng et al., 2003; Zhao et al., 2005), fluorescence detection (Canaparo et al., 2000; Palmgren et al., 2004; Quintana et al., 2006 ; Santos et al., 2005), and mass spectrometry (Kot-Wasik et al., 2006). Capillary electrophoresis has also been recently introduced for ibuprofen measurement but it offers a limited sensitivity for bioanalysis; it has been applied to quality control of pharmaceutical preparations (Hamoudová. & Pospíšilová., 2006) and more recently to enantiomeric pharmacokinetic studies (Główka & Karaźniewicz, 2005). Generally, HPLC methods devoted to pharmacokinetic studies of ibuprofen offer relatively high detection limits, probably due to the high doses administered in therapeutics. However, determination of very low drug levels in

environmental waters (Kot-Wasik et al., 2006; Quintana et al., 2006; Santos et al., 2005) and newly developed pharmaceutical preparations with slow drug release (Lamprecht et al., 2004) imply the enhancement of ibuprofen detectability in dedicated HPLC systems. Furthermore, the determination of low concentrations of ibuprofen might still be of interest to evaluate the pharmacokinetics of the relatively higher doses administered in human in the late phase of elimination, i.e. to get a more precise value of the elimination half-life.

Two ways have previously been investigated to improve the limit of detection (LD) of ibuprofen assays in biological and environmental matrices: *(i)* sample treatment including a concentration step (Kot-Wasik et al., 2006), *(ii)* detection mode, especially fluorescence (Canaparo et al., 2000; Palmgren et al., 2004; Quintana et al., 2006 ; Santos et al., 2005). The first approach relies on off-line solid phase extraction with an evaporation step of the cartridge effluent (a high concentration factor up to 2,000-fold (Santos et al., 2005) can be realized), and hyphenated and automated on-line devices, which also allow important concentration factors of the analyte, thus authorizing low LD values, e.g. 0.36 $ng.mL^{-1}$ of ibuprofen in wastewater (Quintana et al., 2006).

Different authors have used the native fluorescence properties of ibuprofen for its detection in a HPLC system, with a 2.5-times LD improvement *versus* UV spectrophotometric detection, leading to a LD value within 20 $ng.mL^{-1}$, using no sample purification step (Palmgren et al., 2004). On the other hand, cyclodextrins (CDs), the cyclic oligosaccharides consisting of six or more D-(+)-glucopyranose units, are well known to have inclusion complexing properties with guest molecules which possess suitable polarity and dimension. They have been extensively used in pharmaceutical formulations in order to improve water solubility, to prevent degradation and to modify bioavailability of drugs, especially in the field of NSAID drugs (Ravelet et al., 2002; Szejtli, 1998). Moreover, CDs can act as chiral selectors of racemic drugs in separative systems (Główka & Karaźniewicz, 2005), and increase the fluorescence intensity of numerous analytes, since the movement of their fluorophore (generally a planar aromatic ring) is restricted by its inclusion inside the hydrophobic cavity of CDs, as demonstrated for ibuprofen (Hergert & Escandar, 2003; Oh et al., 1998).

In the case of ibuprofen, the binding constant corresponding to its inclusion complex with β−CD has been reported to be equal to 2,600 $M^{-1}$, which is considerably higher than with α−CD and γ−CD: 55 and 59 $M^{-1}$, respectively (Szejtli, 1998). Thus, the cavity size of β−CD seems convenient for the inclusion process of the aromatic part of ibuprofen. Association constants between β−CD and either protonated (IBH) or deprotonated ($IB^-$) form of ibuprofen

have also been calculated: 1,900 and 8,700 $M^{-1}$ for [IBH - β−CD] and [$IB^-$- β−CD], respectively (Manzoori & Amjadi, 2003). These values clearly demonstrate the prevailing role of the carboxylic group and its ionization degree in the stability of the complex. Both complexes have 1:1 stoechiometries.

Lower constant values (in the range 400-700 $M^{-1}$) have been reported for the complex between ibuprofen and 2-hydroxypropyl-β-cyclodextrin (HP-β−CD) which has the main advantages to exhibit higher water solubility and lower toxicity than β−CD (Oh et al., 1998). Upon complexation with CDs, ibuprofen exhibits enhanced fluorescence efficiency without any significant shift of excitation and emission wavelengths (224 and 290 nm, respectively). The fluorescence emission signal of $IB^-$ and IBH is increased about 2 and 4-fold, respectively on β−CD addition and the LD of the corresponding fluorescence method is 30 $ng.mL^{-1}$ (Manzoori & Amjadi, 2003). The previously reported spectroscopic studies describing the influence of pH and CD inclusion complexation on fluorescence properties of ibuprofen have only been carried out in batch solutions (Hergert & Escandar, 2003; Oh et al., 1998). The present work is focused on the optimization of the eluting conditions in a reversed phase (RP) HPLC system, especially with regards to *(i)* pH values of the mobile phase and *(ii)* the use of β−CD, in order to enhance the fluorescence signal and to give rise to a lower LD value of the ibuprofen HPLC assay in rat plasma.

## 2. Experimental

### *2.1. Chemicals and standards*

All chemicals and solvents were of analytical or HPLC reagent grade and were used without further purification. Ibuprofen (batch number 450025) was a gift from Knoll Pharmaceuticals (Nottingham, UK). The ibuprofen stock solution was prepared in methanol at a concentration of 0.1 $mg.mL^{-1}$ and stored at 4°C for a period which does not exceed 3 months. Carprofen, diclofenac, ketoprofen, indomethacin and acetylsalicylic acid were obtained from Sigma (Saint-Quentin Fallavier, France); β-CD hydrate was purchased from Acros Chemicals (Geel, Belgium) and HP-β−CD from Sigma; *D-L* poly(lactic-co-glycolic) acid (PLGA) 50:50 (m/m) Resomer RG 504 S (MW 48,000; viscosity: 0.47 $dL.g^{-1}$) was purchased from Boehringer Ingelheim Inc., Germany. Polyvinylalcohol (PVA, MW 30,000, 88% hydrolyzed) was supplied by Sigma and sorbitan monostearate Span® 60 by Seppic (Paris, France).

*2.2. HPLC system and operating conditions*

The HPLC system (model HPLC 10 A VP, Shimadzu, Champs-sur-Marne, France) consisted of a low-pressure gradient solvent delivery pump, an autosampler, a column oven, a spectrofluorimetric detector (model RF-10A XL), and a data processing software. Both guard (8 x 3-mm i.d.) and analytical (150 x 3-mm i.d.) columns were packed with Uptisphere ODB (porosity: 12 nm; particle size: 5 µm) (Interchim, Montluçon, France). The different mobile phases tested consisted of various mixtures of methanol or acetonitrile and 0.05 M phosphate buffers. They were prepared from a 0.05 M natrium dihydrogenophosphate solution adjusted to pH = 2.5, 4.5 or 6.5 with either a concentrated hydrochloric acid solution or a 40% natrium hydroxide solution. β−CD was added at a final concentration of 5 mM (Table 1). They were filtered through a 0.45 µm filter, degassed before use and run at a flow rate of 0.6 $mL.min^{-1}$, and at a column temperature of 30 °C. Spectrofluorimetric detection was operated at an excitation wavelength of 224 nm and emission wavelength of 290 nm (gain: x 4; sensitivity: medium).

*2.3. Microsphere preparation and characterization*

Microspheres were prepared by the O/W solvent extraction method (Soppimath & Aminabhavi, 2002). Briefly, 50 mg of ibuprofen, 400 mg of PLGA and 200 mg of Span® 60 were dissolved in 20 mL of ethylacetate. The organic solution was poured into 50 mL of 0.1 % (w/v) polyvinylalcohol (PVA) aqueous solution and stirred mechanically (1,000 rpm) using a three-bladed propeller for 30 s at 25 ± 2 °C in order to obtain a pre-emulsion (O/W). This pre-emulsion was added to 2 L of purified water and magnetically stirred at 600 rpm for 10 min, to obtain the final O/W emulsion. Upon solvent extraction during 15 min, the polymer precipitates (due to the good miscibility and fast diffusion of the organic polymer solvent in water) and the microsphere cores solidify. Microspheres were then collected by filtration through a 0.45 µm HA membrane (Millipore) and dried at 25 ± 2 °C for 48 h.

Mean diameter and size distribution of microspheres were analyzed by laser diffraction in a particle size analyzer (Mastersizer S, Malvern Instruments, France). Each sample was measured in triplicate. The amount of ibuprofen entrapped within polymeric particles was determined spectrophotometrically at 222 nm (UV-visible spectrophotometer model UV-160 1PC, Shimadzu , Kyoto, Japan) by measuring the amount of non-entrapped ibuprofen in the

external aqueous solution (indirect method) which was recovered after filtration and washing of microparticles. A standard calibration curve was performed with the ibuprofen solution (aqueous solution of 0.1% PVA with 1% acetone). The established linearity range was 2-10 $\mu g.mL^{-1}$ ($r > 0.998$).

*2.4. Rat treatment and plasma collection and preparation*

An ibuprofen microsphere aqueous suspension or an ibuprofen solution (Pedea®, Orphan Europe SARL, Paris la Défense, France, 5 $mg.mL^{-1}$) was administered subcutaneously at the dose of 1 mg of ibuprofen $kg^{-1}$, to overnight fasted rats (male Sprague–Dawley, 300 ± 20 g; $n \geq 4$). There were 5 groups of 4 rats making a total of 20 rats. Indeed, it was not possible to withdraw blood samples from the same rats at each time. Each rat was only sampled 2 times between 15 min and 10 h. Starting at 24 h, the groups were made larger and there were a minimum of 6 rats per time since there was more time for rats to recover. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (15 $mg.kg^{-1}$). 400 µL of blood were collected by cardiac puncture 15, 30, 45 min and 1, 2, 4, 6, 8, 10, 24, 48, and 80 h after administration, into 1.5-mL polypropylene vials containing 60 µL of 0.129 M sodium citrate solution. After centrifugation at 3,000 g for 10 min at 18°C, the obtained plasma was immediately stored at -20 °C.

The 5-points calibration curve was built by spiking blank (drug-free) plasma samples (180 µL) with ibuprofen (20 µL of ibuprofen standard solutions prepared in mobile phase) to give concentrations ranging from 35 to 200 $ng.mL^{-1}$. Frozen plasma samples (200 µL) were thawed in a water bath at 37 °C and proteins were precipitated by vortex-mixing with 200 µL of a mixture of acetonitrile-1 M HCl (99:1 v/v) for 4 min. After centrifugation at 42,000 *g* for 10 min at 4°C, the supernatant was transferred into HPLC sample vials and a 50-µL volume was injected into the HPLC system.

**3. Results and discussion**

*3.1. HPLC system development for enhancement of fluorescence signal*

Different eluting conditions of ibuprofen were evaluated on a RP column in order to precise the influence of two parameters, i.e. pH of mobile phase and use of CD, on both chromatographic parameters and fluorescence signal of the considered analyte. The corresponding data are summarized in Table 1.

First, three pH values were tested, i.e. 2.5, 4.5 and 6.5, which are below the ibuprofen $pK_a$ (which is equal to 4.41, according to (Palmgren et al., 2004)), close to $pK_a$, and higher than $pK_a$, respectively. Shifting mobile phase pH from 2.5 to 6.5 decreased retention factor (k) and number of theoretical plates (N) of ibuprofen peak, and increased 2.5-fold the signal-to-noise (S/N) ratio of the ibuprofen peak. Thus, a pH value of 6.5 appeared favourable to improve LD of ibuprofen in the RP-HPLC system, as its deprotonated form has a higher fluorescence signal; these data confirm previous ones obtained in batch solutions (Manzoori & Amjadi, 2003).

Second, the addition of β−CD at a concentration of 5 mM to different mobile phases buffered at pH 6.5 was tested. When they contained a high proportion of organic solvent, either acetonitrile (45 %, v/v) or methanol (60 %, v/v), the ibuprofen retention time and fluorescence signal did not change, which demonstrated that the inclusion process of ibuprofen into CD cavity did not occur. When a low content of acetonitrile (20%) was used, the retention time of ibuprofen decreased from 50 to 11.5 min with a peak broadening under addition of β−CD, proving a complex formation between ibuprofen and β−CD. A linear increase of fluorescence, as observed through the variations of the ibuprofen peak area, was also noted (Fig. 1A). The addition of HP-β−CD in the same eluting conditions (20% acetonitrile; [HP-β−CD] = 5 mM) changed ibuprofen retention time from 50 to ca. 13 min but did not increase ibuprofen peak area. Only β−CD was used in further experiments. The apparent formation constant ($K_f$) of the complex between ibuprofen and β−CD was calculated in order to verify that the inclusion process really takes place in the present chromatographic conditions. For that purpose, the following equation was used (Flood et al., 2000; Ravelet et al., 2002): $1/k = [\beta\text{−CD}]\ K_f/k_0 + 1/k_0$

Where k is the retention factor of ibuprofen at a particular concentration of β−CD, and $k_0$ is its retention factor in the absence of β−CD. For a compound with a 1:1 stoichiometry with β−CD, a plot of 1/k vs. [β−CD] yields a straight line which has a slope equal to $K_f/k_0$. The determination coefficient $r^2$ of the regression line was 0.96 (Fig. 1B), and $K_f$ was equal to 875 $M^{-1}$ at 30°C, which is a lower value than those already reported: 2 600 (temperature not indicated) and 7 100 $M^{-1}$ (32°C) according to Szejtli (1998) and Manzoori & Amjadi, (2003), respectively. $K_f$ was presently calculated in the presence of an organic solvent (acetonitrile) in the mobile phase, which modified low energy bonds between ibuprofen and β−CD, thus it can explain the lower $K_f$ value obtained.

The enhancement of the S/N ratio observed when adding β−CD to the mobile phase and using ibuprofen standards was about 30% (Table 1) but this gain was not confirmed when evaluating the LD with real biological samples. As a matter of fact, the slopes of the calibration curves built with plasma samples fortified with ibuprofen standards were 16 139 ± 687 (n = 3) in the absence of β−CD and 19 204 ± 1 090 in the presence of 5 mM β−CD. The resulting LD values were 9 and 10 ng.mL$^{-1}$ with and without addition of β−CD to the mobile phase, respectively, which can be considered as very similar values.

All these data demonstrate the usefulness of varying pH but no influence of using β−CD to enhance florescence signal of ibuprofen in the present HPLC system. Thus further experiments in the present work (including validation process and pharmacokinetics studies) were carried out using the mobile phase without β−CD, i.e. methanol-0.05 M phosphate buffer pH 6.5; 60:40, v/v.

*3.2. Validation of the ibuprofen HPLC assay in rat plasma*

First, selectivity of the overall analytical method was tested versus other NSAIDs, i.e. carprofen, ketoprofen, acetyl salicylic acid, indomethacin and diclofenac, by injecting a 100 ng.mL$^{-1}$ standard solution. No compound was detected within a 1-h elution period. The use of a single step for plasma preparation before injecting into the HPLC system, *i.e*. protein precipitation with acidified acetonitrile, afforded full selectivity *versus* endogenous compounds (three different batches of blank plasma were tested) (Fig. 2).

The stability of ibuprofen plasma extracts was tested over a 15-h period, by injecting every hour a standard and an extract of plasma sample fortified at a concentration of 100 ng.mL$^{-1}$: no significant variation of the ibuprofen peak area was observed (the signal variation observed in the 15-h period was not higher than the RSD value calculated at the same ibuprofen concentration, i.e. 5.6% (Table 2)), thus the HPLC measurements were further run overnight.

Main validation parameters are summarized in Table 2. The resulting LQ of 35 ng.mL$^{-1}$ was lower than values previously obtained in HPLC assays with UV detection (in the range 0.5-1 µg.mL$^{-1}$) (Wang et al., 2005; Zhao et al., 2005) and with fluorescence detection in urine (100 ng.mL$^{-1}$) (Fan et al., 2005) and in wastewater samples (1.6 µg.mL$^{-1}$) (Santos et al., 2005). The present assay appeared to be convenient for pharmacokinetics studies of a long-lasting form.

*3.3. Pharmacokinetics studies*

Most of the analytical techniques used for determining ibuprofen in plasma have relatively high LQ (i.e. in the 200-1,000 $ng.mL^{-1}$ range). Although this is generally acceptable for the high doses of ibuprofen administered in man (200 to 400 mg as a single dose a few times daily), there is a need for more sensitive methods. For instance this is the case when ibuprofen is used as an orphan aqueous solution drug (i.e. Pedea®) to close the patent ductus arteriosus in newborn humans (Aranda & Thomas, 2006). In this later case, the administered dose is 5 $mg.kg^{-1}$. An injectable and long lasting dosage form of ibuprofen has presently been developed after encapsulation of the drug into biodegradable polymers. Such polymers are commonly used in man and some dosage forms are already on the market mainly for the treatment of prostatis cancer when the release of drugs for weeks is needed. The ibuprofen microparticles presently obtained had an average diameter of 31 ± 7 µm (n = 3) and the encapsulation efficiency was 70% with regards to the initial amount of ibuprofen in the preparation. Therefore they could easily pass through a 21 G gauge needle for subcutaneous administration. The ibuprofen plasma concentration profile was followed up to 80 h; at this time the mean ibuprofen concentration was still 49.1 $ng.mL^{-1}$ with the microparticles (Fig. 3). By comparison, plasma concentrations were not detectable after 24 h with the aqueous solution of ibuprofen. $C_{max}$ was dramatically reduced with the microparticles suspension (from 8400 to 2000 $ng.mL^{-1}$ for the solution and suspension, respectively) as expected due to the slow release from the microparticles. This property of the microparticles can also be related to the change in $T_{max}$ which is much earlier for the solution (15 min) than for the suspension (120 min). After $C_{max}$, the ibuprofen plasma concentrations followed a pseudo-plateau until 10 h then decreased slowly till 80 h, which was the final sampling point. The newly ibuprofen assay also allowed to follow significant plasma concentrations to the last sampling point with accuracy. Based on the areas under the curves obtained with both the solution and the microparticles suspension, it was possible to calculate the relative bioavailability of the microparticles with regards to the solution. The relative bioavailability is about 73 % after 24 h, which demonstrated that most of the encapsulated ibuprofen was released into the systemic circulation from the subcutaneous depot. Taking into account the plasma level at 80 h (which is more than the 35 $ng.mL^{-1}$ of the quantification limit) leads to a relative bioavailability of 99%. Such a high figure can be considered as a total biovailability and definitely reflects a very good diffusion of the drug from the microspheres. However, it should be remembered that different animals are sampled at each time which may slightly overestimates the actual 99% bioavailability figure.

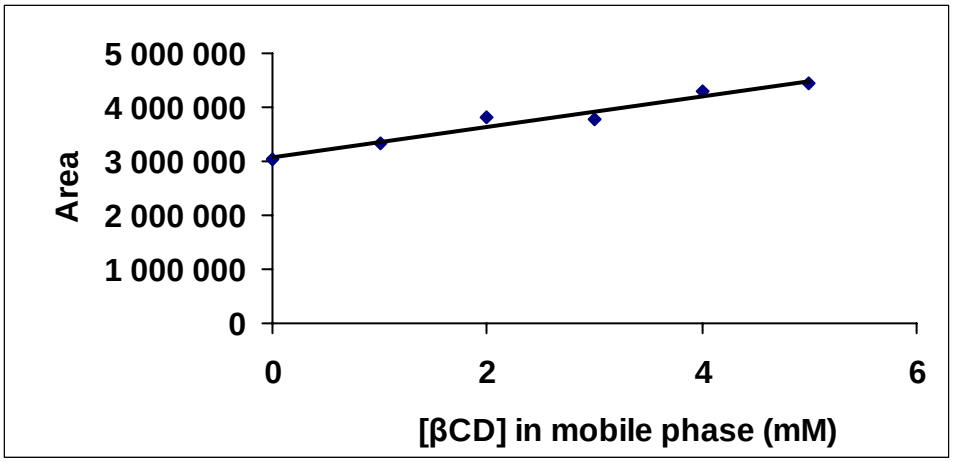
Nevertheless, the goal of developing an injectable slow release dosage form of ibuprofen was demonstrated successful thanks to the new ibuprofen assay.

## 4. Conclusions

A long lasting dosage form was developed using encapsulation of ibuprofen into biodegradable PLGA microspheres. Microspheres characteristics and particularly their mean diameter allowed them to be administered subcutaneously through a 21 G gauge needle. The optimized HPLC method allowed ibuprofen to be monitored during 80 h in rats plasma and demonstrated an almost total drug availability from the microspheres. With regards to the newly developed method, although β−CD addition to the mobile phase has not the expected effect on the improvement of the biological HPLC assay sensitivity, the pH increase has a real benefit. Further sensitivity improvement should be performed through the development of a liquid/solid extraction step including a high concentration factor of the analyte.

Fig. 1. Influence of β−CD concentration added to the mobile phase ($CH_3CN$-phosphate buffer pH 6.5; 20:80, v/v) on the ibuprofen peak area (A) and plot of 1/k vs. β−CD concentration at a column temperature of 30°C (B). Values are the mean of two experimental data.

**A**




**B**

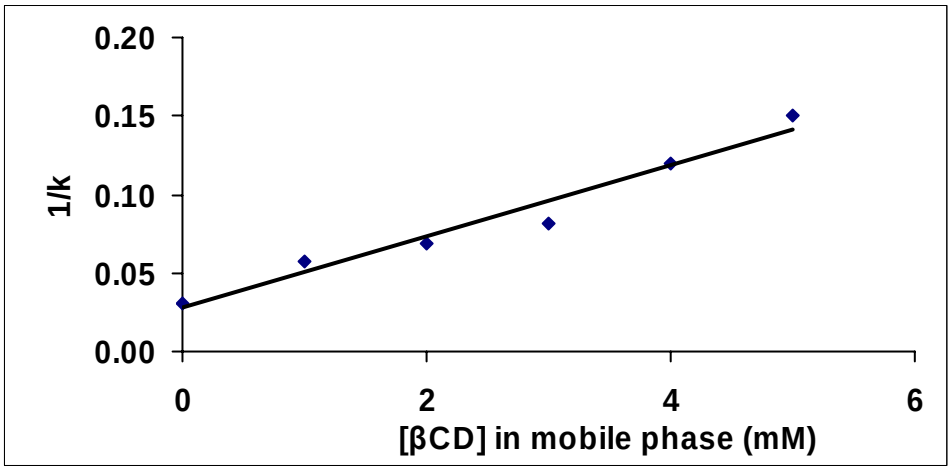


Fig. 2. Typical chromatograms corresponding to samples of rat blank plasma (A), rat plasma fortified with ibuprofen at a concentration of 75 ng.mL$^{-1}$ (B) and rat plasma collected after 48 h of ibuprofen loaded microparticles administration by subcutaneous route (dose; 1 mg.kg$^{-1}$) (C). Eluting conditions: methanol-0.05 M phosphate buffer pH 6.5 (60:40, v/v) at a flow rate of 0.6 mL.min$^{-1}$ and at 30°C.

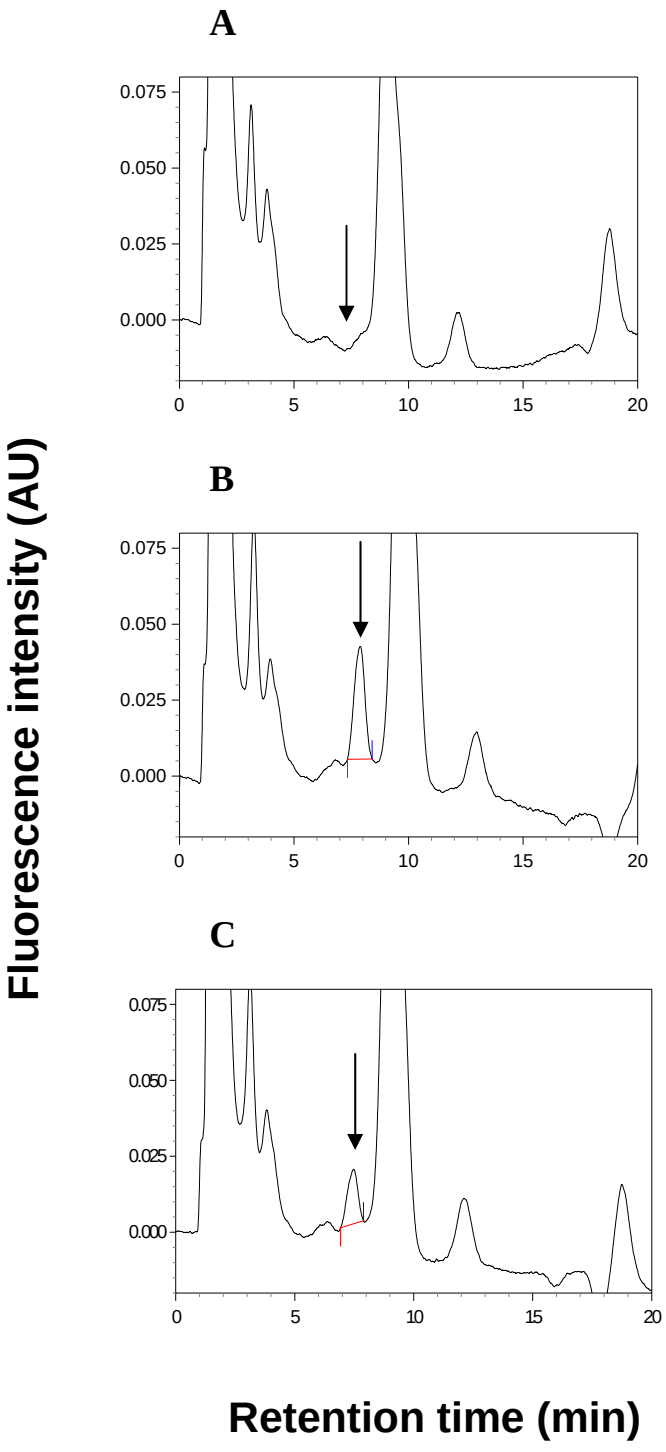


Fig. 3. Profile of ibuprofen concentration in rat plasma after subcutaneous administration of ibuprofen solution (♦), and PLGA microspheres (Δ) (dose: 1 mg.kg$^{-1}$; n = 4 rats).

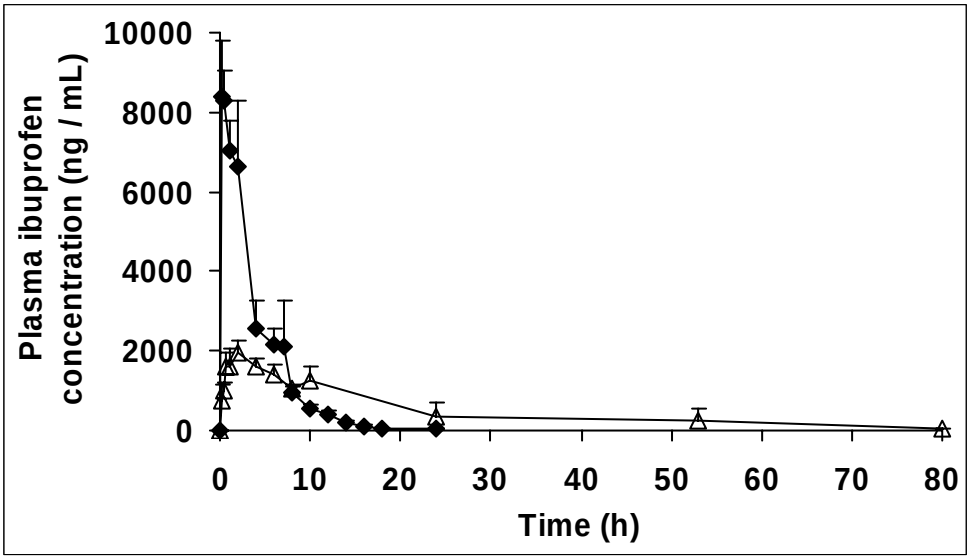


Table 1

Influence of mobile phase pH and β–CD addition on the ibuprofen peak parameters calculated according to European Pharmacopoeia rules).

| **Mobile phase composition** | **k[1]** | **N ($m^{-1}$)[2]** | **S/N[3]** |
|---|---|---|---|
| **Influence of pH buffer** | | | |
| acetonitrile-0.05 M phosphate buffer pH 2.5 (45:55, v/v) | 8.5 | 29 500 | 9.3 |
| acetonitrile-0.05 M phosphate buffer pH 4.5 (45:55, v/v) | 7.3 | 39 950 | 9.3 |
| acetonitrile-0.05 M phosphate buffer pH 6.5 (20:80, v/v) | 27.3 | 36 200 | 25 |
| methanol-0.05 M phosphate buffer pH 6.5 (60:40, v/v) | 4.1 | 8 900 | 23.5 |
| **Influence of β–CD addition** | | | |
| acetonitrile-0.05 M phosphate buffer pH 6.5 (20:80, v/v) | 27.3 | 36 200 | 25 |
| acetonitrile-0.05 M phosphate buffer pH 6.5 + 5 mM β–CD (20:80, v/v) | 6.6 | 8 550 | 32 |

[1] *$t_0$ was measured according to baseline disturbance marking void volume (presently: 1.5 min)*

[2] *$5.54\ (tr/w_{h/2})^2$*

[3] *calculated at an ibuprofen concentration of 100 $ng.mL^{-1}$*

Table 2

Main validation parameters for the HPLC assay of ibuprofen using mobile phase: methanol-0.05 M phosphate buffer pH 6.5 (60:40, v/v) and plasma samples fortified with standard solutions.

| | |
|---|---|
| Slope ± SD | 16 139 ± 687 |
| Intercept ± SD | 1 254 ± 55 154 |
| Determination coefficient $r^2$ | 0.9948 |
| LD* ($ng.mL^{-1}$)<br>LQ* ($ng.mL^{-1}$) | 10<br>35 |
| Repeatability: RSD (%; n = 3) at:<br>50 $ng.mL^{-1}$<br>100 $ng.mL^{-1}$<br>200 $ng.mL^{-1}$ | <br>4.9<br>5.6<br>4.9 |
| Recovery: (%; n = 3) at:<br>50 $ng.mL^{-1}$<br>100 $ng.mL^{-1}$<br>200 $ng.mL^{-1}$ | <br>84 ± 3<br>83 ± 3<br>90 ± 2 |

*LD and LQ values were calculated as follows:

- LD = (mean value of intercept + 3 SD of the intercept) / mean value of the slope

- LQ = (mean value of intercept + 10 SD of the intercept) / mean value of the slope

**References:**

Aranda, J.V., & Thomas, R. (2006). Systematic Review: Intravenous Ibuprofen in Preterm Newborns. *Seminars in Perinatology*, 30, 114-120.

Borovac, T., Pelage, J.P., Kasselouri, A., Prognon, P., Guiffant, G., & Laurent, A. (2006). Release of ibuprofen from beads for embolization: in vitro and in vivo studies. *J. Control. Release*, 115, 266-274.

Canaparo, R., Muntoni, E., Zara, G.P., Pepa, C.D., Berno, E., Costa, M., & Eandi, M. (2000). Determination of Ibuprofen in human plasma by high-performance liquid chromatography: Validation and application in pharmacokinetic study. *Biomed. Chromatogr*., 14, 219-226.

Espinosa-Mansilla, A., Muñoz de la Peña, A., González Gómez, D., & Cañada-Cañada, F. (2006). HPLC determination of ciprofloxacin, cloxacillin, and ibuprofen drugs in human urine samples. *J. Sep. Sci.*, 29, 1969-1976.

Fan, Y., Feng, Y.Q., Da, S.L., & Wang, Z.H. (2005). In-tube solid phase microextraction using a β-cyclodextrin coated capillary coupled to high performance liquid chromatography for determination of non-steroidal anti-inflammatory drugs in urine samples. *Talanta*, 65, 111-117.

Flood, K.G., Reynolds, E.R., & Snow, N.H. (2000). Characterization of inclusion complexes of betamethasone-related steroids with cyclodextrins using high-performance liquid chromatography. *J. Chromatogr. A*, 903, 49-65.

Fernandez-Carballido, A., Herrero-Vanrell, R., Molina-Martinez, I.T., & Pastoriza, P. (2004). Biodegradable ibuprofen - loaded PLGA microspheres for intraarticular administration effect of Labrafil addition on release in vitro. *Int. J. Pharm*., 279, 33-41.

Główka, F.K., & Karaźniewicz, M. (2005). High performance capillary electrophoresis method for determination of ibuprofen enantiomers in human serum and urine. *Anal. Chim. Acta.*, 540, 95-102.

Hamoudová, R., & Pospíšilová, M. (2006). Determination of ibuprofen and flurbiprofen in pharmaceuticals by capillary zone electrophoresis. *J. Pharm. Biomed. Anal.*, 41, 1463-1467.

Hergert, L.A., & Escandar, G.M. (2003). Spectrofluorimetric study of the β-cyclodextrin-ibuprofen complex and determination of ibuprofen in pharmaceutical preparations and serum. *Talanta*, 60, 235-246.

Kot-Wasik, A., Debska, J., Wasik, A., & Namiesnik, J. (2006). Determination of non-steroidal anti-inflammatory drugs in natural waters using off-line and on-line SPE followed by LC coupled with DAD-MS. *Chromatographia*., 64, 13-21.

Lamprecht, A., Saumet, J.L., Roux, J., & Benoit, J.P. (2004). Lipid nanocarriers as drug delivery system for ibuprofen in pain treatment. *Int. J. Pharm.*, 278, 407-414.

Manzoori, J.L., & Amjadi, M. (2003). Spectrofluorimetric study of host-guest complexation of ibuprofen with β-cyclodextrin and its analytical application. *Spectrochim. Acta part A.* , 59, 909-916.

Oh, I., Lee, M.-Y., Lee, Y.-B., Shin, S.-C., & Park, I. (1998). Spectroscopic characterization of ibuprofen/2-hydroxypropyl-β-cyclodextrin inclusion complex. *Int. J. Pharm*., 175, 215-223.

Palmgren, J.J., Monkkonen, J., Jukkola, E., Niva, S., & Auriola, S. (2004). Characterization of Caco-2 cell monolayer drug transport properties by cassette dosing using UV/fluorescence HPLC. *Eur. J. Pharm. Biopharm*., 57, 319-328.

Quintana, J.B., Miro, M., Estela, J.M., & Cerdà, V. (2006). Automated on-line renewable solid-phase extraction-liquid chromatography exploiting multisyringe flow injection-bead injection lab-on-valve analysis. *Anal. Chem*., 78, 2832-2840.

Ravelet, C., Geze, A., Villet, A., Grosset, C., Ravel, A., Wouessidjewe, D., & Peyrin, E. (2002). Chromatographic determination of the association constants between nimesulide and native and modified β-cyclodextrins. *J. Pharm. Biomed. Anal*., 29, 425-430.

Santos, J.L., Aparicio, I., Alonso, E.E., & Callejon, M. (2005). Simultaneous determination of pharmaceutically active compounds in wastewater samples by solid phase extraction and high-performance liquid chromatography with diode array and fluorescence detectors. *Anal. Chim. Acta.*, 550, 116-122.

Sochor, J., Klimes, J., Sedlacek, J., & Zahradnicek, M. (1995). Determination of ibuprofen in erythrocytes and plasma by high performance liquid chromatography. *J. Pharm. Biomed. Anal*., 13, 899-903.

Soppimath, K.S., & Aminabhavi, T.M. (2002). Ethyl acetate as a dispersing solvent in the production of poly (DL-lactide-co-glycolide) microspheres: Effect of process parameters and polymer type. *J. Microencapsul.*, 19, 281-292.

Szejtli, J. (1998). Introduction and general overview of cyclodextrin chemistry. *Chem. Rev.,* 98, 1743-1753.

Teng, X.W., Wang, S.W.J., & Davies, N.M. (2003). Stereospecific high - performance liquid chromatographic analysis of ibuprofen in rat serum. *J. Chromatogr. B.*, 796, 225-231.

Wang, P., Qi, M., Liu, L., & Fang, L. (2005). Determination of ibuprofen in dog plasma by liquid chromatography and application in pharmacokinetic studies of an ibuprofen prodrug in dogs. *J. Pharm. Biomed. Anal*., 38, 714-723.

Zhao, X., Chen, D., Li, K., & Wang, D. (2005). Sensitive liquid chromatographic assay for the simultaneous determination of ibuprofen and its prodrug, ibuprofen eugenol ester, in rat plasma. *Yakugaku Zasshi*, 125, 733-737.

# CHAPITRE III

## Article 3
## Composite microparticles with *in vivo* reduction of the burst release effect

**Article soumis au European Journal of Pharmaceutics and Biopharmaceutics**

Les microparticules composites préparées dans le premier article ont démontré leur efficacité pour réduire le burst *in vitro* (tampon PBS, pH = 7,4, 37°C) que ce soit pour un principe actif hydrophobe (ibubrofène) ou hydrophile (acétate de triptoréline). Une fois le concept démontré *in vitro,* il était important de l'évaluer *in vivo* et de démontrer l'existence d'une éventuelle corrélation *in vitro/in vivo*. La première étude *in vivo* a été conduite avec l'ibuprofène.

Les microparticules utilisées aujourd'hui en thérapeutique sont administrées par voie sous-cutanée ou intramusculaire. Un effet burst a été démontré pour les principes actifs encapsulés par ces deux voies. Les microparticules composites d'ibuprofène ont été administrées par voie sous-cutanée en choisissant le rat comme modèle animal. L'ibuprofène a été dosé dans le plasma des rats selon la technique mise au point précédemment et qui a permis de diminuer la limite de quantification de cette molécule. Les résultats obtenus suite à l'administration des microparticules composites ont été comparés avec les résultats obtenus après l'injection sous-cutanée d'une solution d'ibuprofène, de nanoparticules et de microparticules simples (ne contenant pas de nanoparticules).

La dose d'ibuprofène administrée a été calquée sur celle administrée chez l'homme. Les modifications les plus importantes avec les microparticules composites de la première étude résident dans le choix des polymères. En effet, l'administration *in vivo* exigeait de rendre la formulation de microparticules composites compatible avec la voie sous cutané. En conséquence, les polymères non biodégradables initiaux (Eudragit® RS et éthylcellulose) ont été remplacés par le copolymère biodégradable le plus utilisé (copolymère d'acide lactique et glycolique) dans les formes multiparticulaires actuellement sur le marché. Ce copolymère biodégradable présente aussi l'avantage d'être soluble dans l'acétate d'éthyle ce qui ne remet pas en question la fabrication des microparticules selon le procédé mis au point dans le premier travail *in vitro*. De plus, il a été nécessaire d'ajouter du Span 60 pour optimiser la formulation.

L'ibuprofène est une molécule faiblement hydrosoluble et donc moins susceptible de provoquer un burst important. Quoi qu'il en soit, les nanoparticules d'ibuprofène administrées par voie sous-cutanée ont montré un effet burst important qui est largement limité par les microparticules simples et plus encore par les microparticules composites. Les résultats obtenus chez le rat ont ainsi permis de confirmer l'intérêt du concept proposé pour des applications *in vivo*.

# Composite microparticles with in vivo reduction of the burst release effect

A. Sheikh Hasan[1], A. Sapin[1], P. Leroy[2], A. Lamprecht[3], E. Emond[1], F. El Ghazouani[1], N. Ubrich[1], P. Maincent[1*]

[1]Laboratoire de Pharmacy Galénique, Faculty of Pharmacy, Nancy University, 5, rue A. Lebrun, BP 80403, 54001 Nancy, France

Article soumis au European Journal of Pharmaceutics and Biopharmaceutics

[2]Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, Faculty of Pharmacy, Nancy University, BP 80403, Nancy, 54001, France

[3]Laboratory of Pharmaceutical Engineering, Faculty of Pharmacy, Place Saint Jacques, 25030 Besançon (France)

Dedicated to Dr N. Ubrich who died on April 24, 2007.

To European Journal of Pharmaceutics and Biopharmaceutics

Corresponding author:

P. MAINCENT
EA 3452
Faculty of Pharmacy
5, rue A. Lebrun, BP 80403
54001 Nancy Cedex (France)

Phone : +33 3 83 68 22 96
Fax : +33 3 83 68 23 01
E-mail : maincent@pharma.uhp-nancy.fr

## Abstract

The aim of this study was to develop microparticles containing nanoparticles (composite microparticles) for prolonged drug delivery with reduced burst effect *in vitro* and *in vivo*. Such composite microparticles were prepared with hydrophobic and biodegradable polymers [Poly (ε-caprolactone), poly (lactic-co-glycolic) acid]. Ibuprofen was chosen as the model drug and microparticles were prepared by the solvent extraction technique with ethyl acetate as the solvent. Nano/microparticles and an ibuprofen solution (Pedea®) were administered subcutaneously at the dose of 1 mg of ibuprofen per kg to overnight fasted rats (male Wistar). Composite microparticles showed prolonged ibuprofen release and less burst effect when compared to simple microparticles (without nanoparticles inside) or nanoparticles both *in vitro* (PBS buffer) and *in vivo*. Moreover, ibuprofen was still detected in the plasma after 96 h with composite microparticles. Consequently, it has been demonstrated that composite microparticles were able to reduce burst release and prolong the release of ibuprofen for a long period of time.

*Keywords*, composite microparticles, ibuprofen, burst effect, *in vitro, in vivo,* Poly (ε-caprolactone), poly (lactic-co-glycolic) acid.

## 1. Introduction

Various drug delivery systems, such as liposomes, micelles, emulsions and micro/nanoparticles have broad applications in controlled and/or targeted delivery. Several biodegradable polymers [such as poly(glycolic acid) (PLA), poly(lactide-co-glycolide) (PLGA), poly (ε-caprolactone) (PCL), poly(3-hydroxybutyrate), gelatin, alginate…] are popularly used for this purpose. The most popular techniques for preparing polymer micro/nanoparticles include solvent evaporation/extraction, emulsion polymerization, interfacial polycondensation and spray drying.

Drug release from micro/nanoparticles depends on various factors including nature of polymer, physicochemical properties of drug and/or formulation factors. It has to be pointed out that the composition and process manufacturing of micro/nanoparticles, not mentioning the particle size, can strongly affect the underlying release mechanisms [1].

In many controlled release formulations, immediately upon placement in the release medium, an initial large bolus of drug is released before the release rate reaches a stable profile. This phenomenon, typically referred to as ‘burst release’, may have dramatic consequences in case of low therapeutic index drugs.

It is well known that the oil in water (O/W) or water in oil in water (W/O/W) emulsion-solvent evaporation technique is a complex process in which the organic solvent generates pores in the micro/nanoparticles structure during its evaporation/extraction [2]. Furthermore the accumulation, through partitioning, of drug crystals on the surface of microspheres or the adsorption of drug crystals onto the surface during the encapsulation process generally produces burst release of the drug after administration [3-5].

The degree of burst release generally depends upon the nature of the polymer, drug nature (molecular weight), polymer: drug ratio [6-8] and/or the relative affinities of the drug for the polymer and the aqueous phase [4, 9].

Multiple approaches have attempted to alleviate the burst by varying the polymer chemistry [10, 11], adding excipients to the polymer phase [12-14], utilizing new polymers [4, 15, 16] or encapsulating particulate forms of the drugs in to microparticles [17, 18].

Attempts to reduce the burst effect have mainly involved macromolecules or hydrophilic drugs because burst control is one of the major challenges of parenteral administration. Although usually not so important as the burst release of hydrophilic drugs, attempts to control the burst with lipophilic drugs [14, 19] may also be of interest in order to decrease the toxic side effects of high potent drugs or to prolong the drug release time *in vivo*.

Non-steroidal anti-inflammatory drugs (NSAIDs) are poorly water-soluble drugs and solvent evaporation techniques are generally used for their microencapsulation [20]. However, during microencapsulation, a partial crystallisation of the NSAIDs in the dispersing phase and/or on the microspheres surface may occur [4, 20, 21]. These free NSAIDs crystals are undesired because their release is not controlled by the polymer matrix. Therefore, various strategies have been tested in order to prevent NSAIDs crystallisation during the solvent evaporation process [22] or to remove the free NSAIDs crystals from the microparticles surface after their preparation [19, 20].

One promising method for reducing the release rate and suppressing the initial burst consists of encapsulating nanoparticles directly inside microparticles [21, 23, 24]. Encapsulating of nanoparticles inside microparticles has already been used with different goals such as i) intestinal mucosal delivery plasmid DNA [25], ii) targeting drugs to the inflammation site of inflammatory bowel disease and/or prevention of premature uptake or degradation of nanoparticles during their passage through the small intestine [26] and iii) for lung protein delivery [27]. Moreover, the idea of encapsulating nanoparticles into microparticles was also used to reduce the burst effect *in vitro* with different hydrophilic drug models such as

dexamethasone sodium salt (low molecular weight drug) [18] and macromolecules i.e. bovine serum albumin (high molecular weight) [23, 24].

Encapsulation of NSAIDs into nanoparticles followed by their encapsulation in microparticles could be of great help to reduce the undesired burst effect with NSAIDs (lipophilic drugs). Therefore, we have previously successfully encapsulated ibuprofen loaded PCL nanoparticles inside ethyl cellulose/ Eudragit RS polymeric microparticles [21] and demonstrated a control of the burst effect.

Ibuprofen is indicated for the relief of mild to moderate pain and inflammation. To the best of our knowledge, there is no ibuprofen injectable sustained release dosage form on the pharmaceutical market. Based on the indications of ibuprofen, this would certainly be of interest. For instance, the intra-articular administration of ibuprofen in the management of chronic rheumatoid arthritis could be an alternative to corticosteroid administration, avoiding their devastating effects [14]. An ibuprofen intravenous solution has been marketed in Europe under the trade name of Pedea® for the treatment of ductus arteriosus in newborns [28]; this dosage form is a conventional aqueous solution allowing a fast therapeutic activity after intravenous administration. However, previous studies have indicated that ibuprofen shows both a high initial burst when formulated as nanoparticles [29] or microparticles [4] and a short half-life in biological medium (blood [30], synovial liquid[14], ocular[31]). Thus, controlling a potential ibuprofen burst effect would improve the therapeutic effect, prolong the biological activity, control the drug release rate and decrease the administration frequency. However, both types of polymers used in our last formulation [21] (ibuprofen loaded PCL nanoparticles inside ethyl cellulose/ Eudragit RS microparticles) cannot be used for parenteral administration due to their non biodegradable status. But, biodegradable polymers should be used instead of the 2 latter ones for subcutaneous administration. So in the following research work, we have prepared ibuprofen nanoparticles of a biodegradable polymer (PCL) which was then encapsulated in (PLGA) biodegradable microparticles with a view to reduce their burst effect and prolonging the ibuprofen blood residence time after subcutaneous injection. Such nanoparticles in microparticles are called composite microparticles.

## 2. Materials

The acid form of ibuprofen [(R, S)-2(4-isobutylphenyl) propionic acid] (batch number 450025) generously supplied by Knoll Pharma Chemicals (Nottingham, UK), was used as the

model drug. Poly (ε-caprolactone) ($M_W$ 40,000 Da) and *D-L* poly (lactic-co-glycolic) acid 50:50 (m/m) Resomer® RG 504S end-capped ($M_W$ 48,000; viscosity: 0.47 dL/g) were purchased from Aldrich (USA) and Boehringer Ingelheim (Germany) respectively.
Polyvinylalcohol (PVA, $M_W$ 30,000, 88% hydrolyzed) was supplied by Sigma and sorbitan monostearate Span® 60 by Seppic (Paris, France). Ethyl acetate (water solubility = 8.3 g/100 mL at 20°C) was purchased from Fluka Chemie GmbH (Switzerland). Methylene chloride (water solubility = 1.3 g/100 mL at 20°C) was supplied by Prolabo (Paris, France). Acetonitrile and orthophosphoric acid were obtained from Carlo-Erba (Val de Reuil, France) and Prolabo (Paris, France), respectively. All other chemicals were of analytical grade and used without further purification.

## 3. Methods

### *3.1. Preparation of particles*

#### *3.1.1. Nanoparticles*

Ibuprofen loaded PCL nanoparticles were prepared by the W/O/W solvent evaporation method [32]. Briefly, 1 mL of aqueous internal phase was emulsified for 15 s in 5 mL of methylene chloride (containing 125 mg of PCL and 50 mg ibuprofen ) with the help of an ultrasound probe (Vibra cell 72 434, BioBlock Scientific, Strasbourg, France) at 80W output. This primary emulsion was poured into 40 mL of a 0.1% PVA aqueous solution and sonicated again with the same ultrasound probe for 1 min in the same conditions in order to create the W/O/W emulsion. Three mL (± 1 mL) of nanoparticles suspension were obtained after solvent evaporation under reduced pressure (Rotavapor, Heidolph, Germany).
Nanoparticles were separated from the bulk suspension by centrifugation (Biofuge Stratos, Heraeus Instruments, Germany) at 42,000 *x g* for 20 min. The supernatant was kept for drug assay according to the methods described later and the sedimented nanoparticles were then redispersed in 3 mL of purified water before freeze-drying. After lyophilization, a dry powder of nanoparticles was obtained. The nanoparticles preparation method was slightly modified for manufacturing the composite microparticles. Indeed, the only difference was that the solvent evaporation process was continued till 1.5 mL (± 0.5 mL) of nanoparticles was obtained: this suspension was used directly (without freeze-drying) as the internal aqueous phase in the preparation of the composite microparticles. Due to the hydrophobic nature of ibuprofen, an O/W technique could have been used to prepare nanoparticles. However, with a

view to comparing the dosage forms, a same preparation method (W/O/W emulsion) was used for all formulations *i.e.* PCL nanoparticles, PLGA simple microparticles (without nanoparticles) and composite microparticles (microparticles incorporating nanoparticles). This is the reason why 1 mL of purified water was used as the aqueous internal phase in the case of ibuprofen loaded nanoparticles. Blank nanoparticles were prepared under the same conditions but without drug in the organic phase.

*3.1.2. Microparticles*

Microparticles containing ibuprofen PCL nanoparticles (so-called composite microparticles) were prepared by the W/O/W solvent extraction method [33]. In the first step (W/O emulsion), the PCL nanoparticles suspension (1.5 mL) was used as the internal aqueous phase which was emulsified (ultrasound probe at 80 W output for 15 s) in the organic solution of ethyl acetate (20 mL) containing PLGA (400 mg) and Span 60 (200 mg).

This primary emulsion was poured into 50 ml of 0.1% PVA aqueous solution in order to obtain a W/O/W pre-emulsion. After magnetically stirring for 30 s (1000 rpm) at room temperature, this pre-emulsion was added to 2 L of purified water and stirred mechanically (three-bladded propeller, 600 rpm) for 10 min to form the final W/O/W emulsion.

Upon solvent extraction, the polymers precipitated and the microparticles cores solidified. Microparticles were collected by filtration (Millipore® Type: 0.45 µm nitrate cellulose) and freeze-dried.

Blank PLGA composite microparticles (with blank PCL nanoparticles) and PLGA simple microparticles (with or without ibuprofen) were prepared according to the same conditions.

*3.2. Mean diameter and zeta potential*

*3.2.1. Nanoparticles*

The mean diameter of nanoparticles and their surface potential were evaluated with a Malvern Zetasizer 3000 HSA (Malvern Instruments, France) using respectively photon correlation spectroscopy and electrophoretic mobility. Nanoparticles were diluted in NaCl 0.001 M prior to zeta potential measurements. Each sample was measured in triplicate.

*3.2.2. Microparticles*

Mean diameter and size distribution of microparticles were analyzed by laser diffraction in a particle size analyzer (Mastersizer S, Malvern Instruments, France). Each sample was measured in triplicate.

*3.3. Determination of ibuprofen content*

The amount of ibuprofen entrapped within polymeric particles was determined spectrophotometrically at 222 nm (UV-160 1PC, UV-visible spectrophotometer, Shimadzu , Kyoto, Japan) by measuring the amount of non-entrapped ibuprofen in the external aqueous solution (indirect method) which was recovered after filtration and washing of microparticles. In the case of nanoparticles, the external aqueous solution was obtained after centrifugation of the colloidal suspension for 20 min at 42,000 *x g*. A standard calibration curve was performed with the ibuprofen solution (aqueous solution of 0.1% PVA with 1% acetone). The established linearity range was 2-10 µg/mL ($r > 0.998$).

In order to validate the indirect assay method for routine purposes, the results have been compared with those obtained after measuring the ibuprofen amount directly into PLGA simple microparticles and PLGA composite microparticles according to an established but slightly modified HPLC method [14].

Briefly, 10 mg of particles were accurately weighed and dissolved in 20 mL acetonitrile. Then 50 µL of this later solution were injected into the HPLC system (Shimadzu HPLC 10A vp, Shimadzu, Kyoto, Japan) with UV detection (SPD-10 A VP, Shimadzu, Kyoto, Japan ) and a data processing software (model Class VP). The separation was achieved by using a reversed phase column (Uptisphere ODB, 3 mm i.d., 150 mm long, 12 nm porosity, 5 µm particle size, Interchim, France). The detection wavelength was set at 222 nm. The flow rate of the mobile phase (water/acetonitrile: 40/60 acidified with orthophosphoric acid pH 2.7) was 0.8 mL/min. The ibuprofen curve was linear from 1 to 100 µg/mL ($r = 0.999$).

*3.4. In vitro drug release from nanoparticles and microparticles*

Fifty mg of freeze-dried loaded particles were suspended in 20 mL of saline phosphate buffer ($KH_2PO_4$ 0.0044 M, $Na_2HPO_4$ 0.0451M, NaCl 0.1 M, pH 7.4 adjusted by $H_3PO_4$). Dissolution studies were carried out under sink conditions (ibuprofen solubility in saline phosphate buffer is 3.8 mg/mL). The particles suspension was stirred (200 rpm) at 37°C into a water bath. One milliliter of suspension was withdrawn at appropriate intervals (5, 15, 30, 45 min, 1, 2, 3, 4, 5,

6, 8, 24 h) and filtered through a 0.22 µm nitrate cellulose filter (Millipore®). The filtrate was replaced by 1 mL of fresh buffer. Released ibuprofen was determined by UV spectrophotometry at 222 nm as previously described. Each particle batch was analyzed in triplicate.

*3.5. In vivo studies of ibuprofen-loaded particles in rats*

*3.5.1. Treatment and plasma collection*

An ibuprofen PCL nanoparticles, PLGA simple microparticles and PLGA composite microparticles aqueous suspension or an ibuprofen solution (Pedea®, Orphan Europe SARL, Paris la Défense, France, 5 mg/mL) was administered subcutaneously at the dose of 1 mg of ibuprofen per kg to overnight fasted rats (male wistar, 300 ± 20 g; n = 4). The administration volume was 300 ± 50 µL in a 2.25 % (m/v) carboxymethylcellulose aqueous solution. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (15 mg/kg). It has to be noticed that animals were divided in 5 groups of 3 rats each so that only 2 blood samples per animal were collected for 24 h for each formulation. For the following times, blood was sampled from two groups leading to 6 samples at each time.

Blood samples (400 µL) were collected by cardiac puncture 15, 30, 45 min and 1, 2, 4, 6, 8, 10, 24, 48, 72 and 96 h after administration into 1.5 mL polypropylene vials containing 60 µL of 0.129 M sodium citrate solution. After centrifugation at 3,000 *x g* for 10 min at 18°C, the plasma was immediately stored at -20 °C.

*3.5. 2. Ibuprofen plasma assay*

The same HPLC technique (cf. 3.3), but with little changes aimed to increase the sensitivity of the method, was used to measure ibuprofen in plasma [42]. Indeed the detection was carried out with a spectrofluorimetric detector (model RF-10A XL, Shimadzu). The mobile phase consisted of a mixture of methanol (60 %) and 0.05 M phosphate buffer pH = 6.5 (40 %) filtered through a 0.45 µm filter, degassed before use and run at a flow rate of 0.6 mL/min. Spectrofluorimetric detection was operated at an excitation wavelength of 224 nm and emission wavelength of 290 nm. The 5 points calibration curve was built by spiking blank (drug-free) plasma samples (180 µL) with ibuprofen (20 µL of ibuprofen standard solutions prepared in mobile phase). Frozen plasma samples (200 µL) were thawed in a water bath at 37 °C and proteins were precipitated by vortex-mixing with 200 µL of a mixture of acetonitrile/1 M HCl (99:1 v/v) for 4 min. After centrifugation at 42,000 *x g* for 10 min at

4°C, the supernatant was transferred into HPLC sample vials and a 50 µL volume was injected into the HPLC system. The ibuprofen calibration curve was linear from 50 to 200 ng /mL (r = 0.999) in plasma.

*3.5.3. Relative bioavailability after subcutaneous administration of the different dosage forms of ibuprofen*

Ibuprofen-loaded particles were administered subcutaneously (1mg/kg) to overnight fasted rats. As a reference of immediate dosage form, an ibuprofen solution (Pedea®) was administered subcutaneously at the same dose (1mg/kg) in a second group of rats. The areas under the curves (AUC) of the concentration-time profiles were calculated with the linear trapezoidal method. The relative bioavailability was calculated by the ratio of the respective AUC corrected by the administered doses. Cmax and Tmax were also observed as the kinetic parameters of absorption.

*3.5.4. Statistical analysis*

Results are presented as means ± standard deviation (SD). Multiple mean comparisons were performed by Kruskal-Wallis Test, followed by the Student –Newman-Keals test for group x group comparisons. A p value < 0.05 was considered significant.

## 4. Results

Tables 1 and 2 summarize the main physicochemical parameters (mean diameter, zeta potential and encapsulation efficiency) of the three types of ibuprofen dosage forms prepared with the various polymers. Both unloaded PCL nanoparticles and ibuprofen loaded PCL nanoparticles have a diameter around 350 nm with almost no charge. The encapsulation efficiency is high (95 ± 2 %) (Table 1).

Ibuprofen loaded simple and composite microparticles are larger than unloaded ones. Indeed, the diameter of all ibuprofen-loaded microparticles is close to 35 µm whereas unloaded microparticles exhibit a smaller size (25 µm). Composite microparticles have almost the same diameter as simple microparticles for both ibuprofen loaded and unloaded ones. The ibuprofen encapsulation efficiency in simple microparticles (84 ± 3 %) and composite microparticles (89 ± 2 %) is less than PCL nanoparticles (95 ± 2 %) but still relatively high (Table 2).

Fig. 1 displays the release profiles of ibuprofen from ibuprofen bulk powder, PCL nanoparticles, PLGA simple microparticles and PLGA composite microparticles. Ibuprofen bulk powder was completely dissolved at the first sampling point (i.e. 100% at 5 min). Ibuprofen loaded PCL nanoparticles display an important drug release (86 ± 7 %) in the first 15 min corresponding to a significant ibuprofen initial burst effect followed by a plateau up to 24 h (Table 3). Different release profiles were obtained with PLGA simple and PLGA composite microparticles. The initial ibuprofen burst release was more important (21 % after 15 min) with PLGA simple microparticles than PLGA composite microparticles (9 % after 15 min) with a progressive and controlled release profile up to 24 h for both types of microparticles (62 % and 39 % after 24 h for simple and composite microparticles, respectively). Moreover, a common trend for all tested microparticles was the non-complete release of ibuprofen in 24 h as well as a controlled burst effect release with PLGA composite microparticles and, to a lower extent, with PLGA simple microparticles.

After subcutaneous administration of the ibuprofen solution (Pedea®) and the different multiparticular ibuprofen dosage forms, blood was collected till 96 h. Ibuprofen was only detected in the blood samples (limit of quantification: 50 ng/mL) during 24 h following the ibuprofen solution or ibuprofen PCL nanoparticles subcutaneous administration whereas it was detected for a much longer time (up to 48 h) for PLGA simple microparticles and PLGA composite microparticles. Fig. 2 shows the mean plasma concentrations of ibuprofen after subcutaneous administration (1 mg/kg) of the solution (Pedea®), the ibuprofen-loaded PCL nanoparticles, the PLGA simple and composite microparticles. During the first 8 h, the ibuprofen plasma profiles appeared different with all dosage forms. The subcutaneous administration of ibuprofen solution (Pedea®) gave rise to an immediate peak. The highest mean plasma concentration (Cmax 8691 ng/mL) for the Pedea® ibuprofen solution was observed rapidly (15 min) and then decreased to 961 ng/mL after 8 h (Table 4). Administration of PCL nanoparticles suspension led to a high initial ibuprofen serum level of about 5070 ng/mL after 45 minutes. This initial peak was followed by a gradual decrease in serum ibuprofen which reached 1630 ng/mL after 8 hours. Cmax was much lower and much longer to achieve with PLGA simple microparticles (about 2 h) and PLGA composite microparticles (4 h) dosage forms (Cmax 2033 and 1249 ng/mL respectively). Then, a prolonged plateau, up to 6-8 h, was observed for composite and simple microparticles indicating a constant release rate for ibuprofen (fig. 2B). Plasma concentrations decreased slowly till 48 h where the quantification limit was reached.

The pharmacokinetic parameters obtained from the plasma ibuprofen concentrations are summarized in Table 5. Based on the concentration-time profiles, areas under the ibuprofen concentration curves (AUC) were calculated. Although it is difficult to conclude to a significant difference between median AUC values from different formulations, the AUCs ranked according to the following order: Pedea® > PCL nanoparticles > simple microparticles > composite microparticles. For the first 24 h, the highest relative bioavailability with regards to the Pedea® solution is obtained with the nanoparticles suspension whereas PLGA composite microparticles exhibit the lowest one. Moreover PLGA simple and composite microparticles show continuous ibuprofen release and stable plasma concentration for longer times leading to either 99 and 56 % relative bioavailability after 48 h for simple and composite microparticles respectively.

Results in Table 5 also indicate that the ibuprofen burst release is significantly more reduced considering Tmax ($p = 0.004$) and Cmax ($p = 0.016$) values with PLGA composite microparticles than with both the ibuprofen solution, the PCL nanoparticles suspension and with PLGA simple microparticules *in vivo*. The same result was also observed *in vitro*.

**Table 1:** Mean diameter, drug encapsulation efficiency and zeta potential of unloaded or ibuprofen loaded PCL nanoparticles (n = 3 ± SD).

| | Unloaded PCL nanoparticles | Ibuprofen PCL nanoparticles |
|---|---|---|
| Mean diameter (nm) | 375 ± 6.0 | 341 ± 9.0 |
| Zeta potential (mV) | - 1.3 ± 1.6 | + 2.9 ± 3.9 |
| Encapsulation efficiency (%) | - | 95 ± 2.0 |

**Table 2:** Mean diameter and drug encapsulation efficiency of unloaded, ibuprofen simple microparticles (SMP) and composite microparticles (CMP) (n = 3 ± SD).

| | Mean diameter (µm) | | Encapsulation efficiency (%) | |
|---|---|---|---|---|
| | Unloaded | Ibuprofen | Unloaded | Ibuprofen |
| SMP | 24.3 ± 4,7 | 37.9 ± 2 | - | 84 ± 3 |
| CMP | 27. 9 ± 4 | 33.7 ± 7 | - | 89 ± 2 |

**Table 3:** Mean percentages of ibuprofen released *in vitro* after 15 min and 24 h from bulk powder, PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP) (n = 3 ± SD).

| Formulations | Polymers | Ibuprofen released (%) | |
|---|---|---|---|
| | | 15 min | 24 h |
| Powder | - | 100 | 100 |
| PCL NP | PCL | 86 ± 6.9 | 87.5 ± 3.5 |
| PLGA SMP | PLGA | 21.5 ± 6.1 | 62.2 ± 7.3 |
| PLGA CMP | PCL/PLGA | 8.7 ± 4.1 | 39.5 ± 3.3 |

**Table 4:** Mean ibuprofen plasma concentrations (ng/mL) 15 min and 8 h after subcutaneous injection of Pedea® solution, PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP) in rats at the dose of 1 mg/kg (n ≥ 3 ± SD).

| formulations | Polymers | Ibuprofen plasma concentration (ng/mL) | |
|---|---|---|---|
| | | 15 min | 8 h |
| Ibuprofen solution (Pedea®) | – | 8691 ± 1386 | 961 ± 183 |
| PCL NP | PCL | 2449 ± 932 | 1630 ± 386 |
| PLGA SMP | PLGA | 795 ± 395 | 1119 ±100 |
| PLGA CMP | PLGA (PCL) | 398 ± 51 | 1043 ± 165 |

**Table 5:** Pharmacokinetic parameters of ibuprofen after subcutaneous administration in rats (n ≥ 3) of each formulation: PCL nanoparticles (PCL NP), PLGA simple (SMP) and PLGA composite (CMP) microparticles and ibuprofen marketed solution (Pedea®).

| | Ibuprofen solution | PCL NP | PLGA SMP | PLGA CMP |
|---|---|---|---|---|
| Tmax (h) | 0.25 | 0.75 | 2 | 4 |
| Cmax (ng/mL) | 8691 | 5071 | 2033 | 1249 |
| AUC 0 → 24 | 35950 | 30491 | 26506 | 17955 |
| AUC 0 → 48 | - | - | 35814 | 20151 |
| AUC 0 → 96 | - | - | - | 21450 |
| Rel. F (%) | | | | |
| (0-24h) | 100 | 84 | 73 | 49 |
| (0-48h) | 100 | 84 | 99 | 56 |

Figure 1: Release kinetics of ibuprofen from ibuprofen bulk powder, ibuprofen PCL nanoparticles (PCL NP), ibuprofen PLGA simple microparticles (PLGA SMP), and ibuprofen PLGA composite microparticles (PLGA CMP) for 24 h. Experiments were performed in phosphate buffer pH 7.4 at 37°C under sink conditions. Data shown as mean ± SD (n=3).

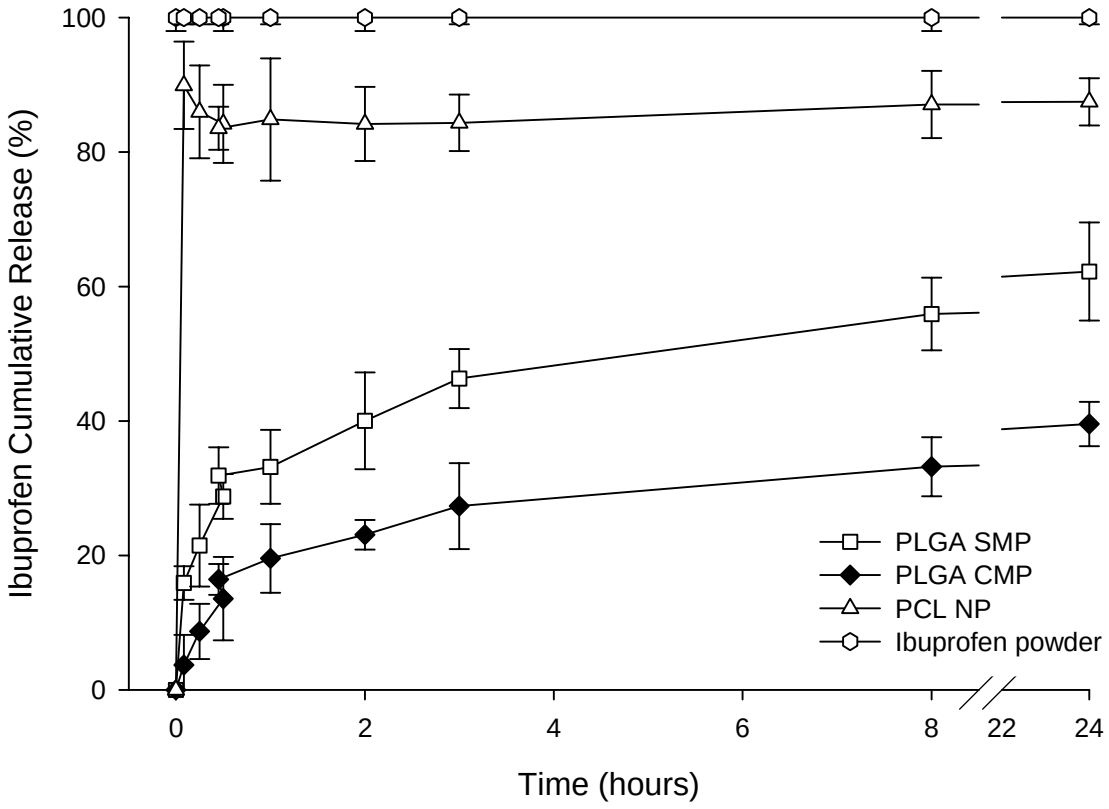
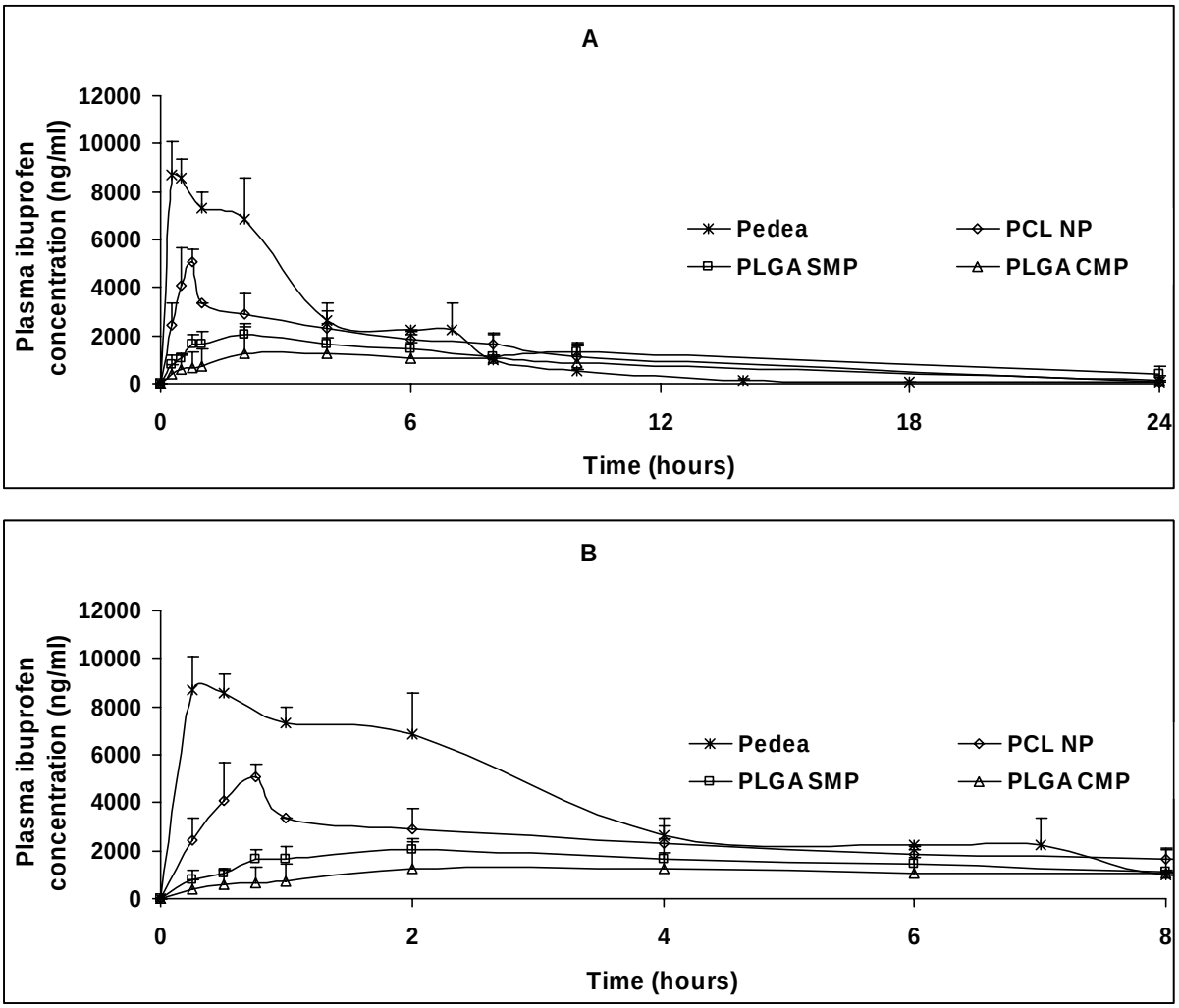


Figure 2: Plasma ibuprofen concentrations as a function of time after subcutaneous administration in rats (1 mg/kg) of various formulations: ibuprofen solution (Pedea®), ibuprofen nanoparticles (PCL NP), ibuprofen PLGA simple microparticles (PLGA SMP), and ibuprofen PLGA composite microparticles (PLGA CMP). Data shown as mean ± SD ($n \geq 3$) either for 24 h (A) or 8 h (B).



## 5. Discussion

Burst release is a critical problem with currently marketed injectable microparticles especially when slow release for a few weeks or months is expected. The encapsulation of nanoparticles into microparticles, as an alternative way to decrease the burst, has been proposed by only a few groups including ourselves for hydrophilic drug [18, 21-24, 34].

As previously reported [21] composite microparticles made with hydrophobic polymers for both microparticles and nanoparticles (such as PLGA, PCL, PLA, Eudragit® RS, ethylcellulose) would certainly have the potential to eliminate or at least decrease the burst effect for hydrophilic and hydrophobic drugs when compared to hydrophilic polymers (such as gelatin, alginate, chitosan). But the encapsulation of a hydrophobic polymer (as nanoparticles) in another hydrophobic polymer (as microparticles) is really challenging with classical microparticles techniques.

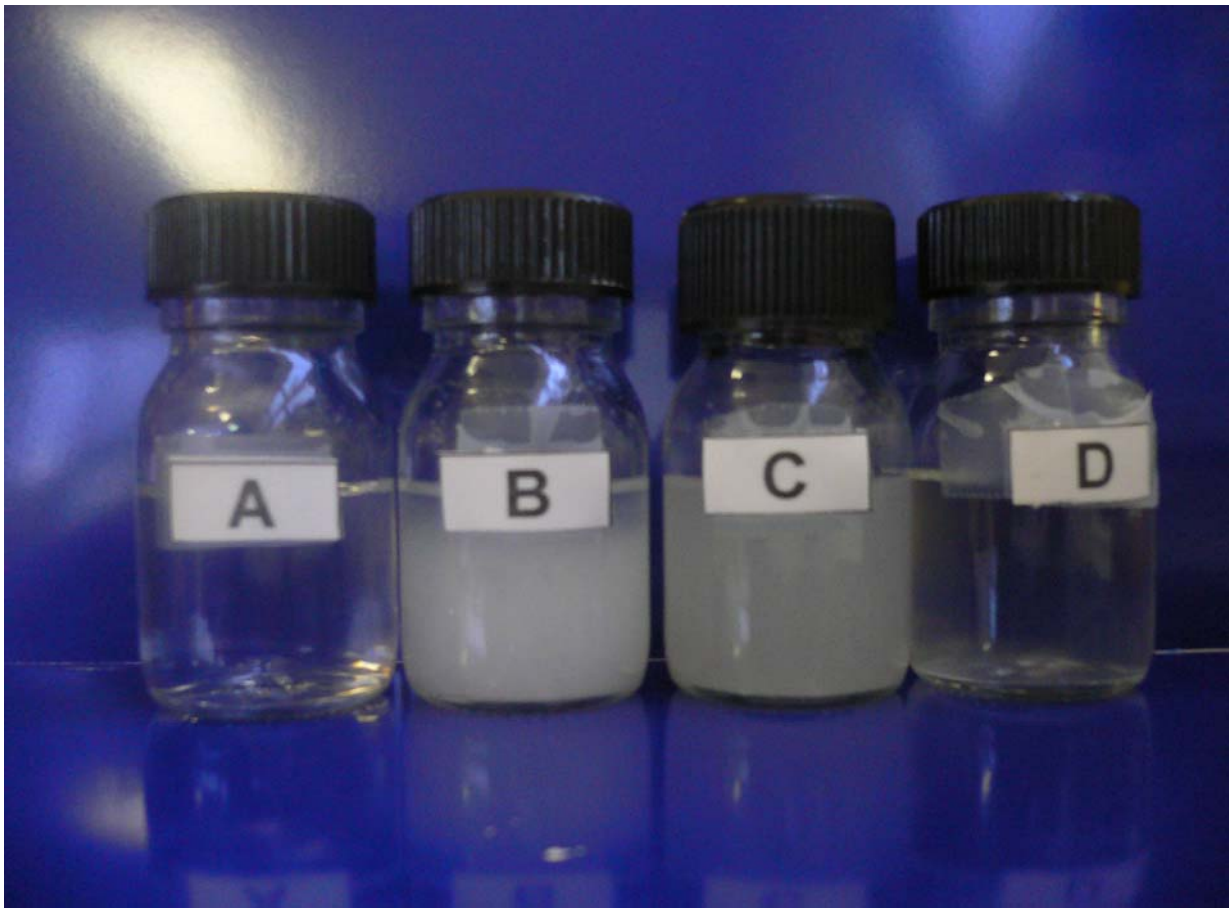
In a previous work, we have already prepared ibuprofen PCL nanoparticles which were incorporated into microparticles made of ethylcellulose, Eudragit® RS or their 1:1 blend. Such composite microparticles were able to control the ibuprofen burst *in vitro* [21]. However, with a view to injecting the composite microparticles subcutaneously, it was mandatory to use two biodegradable polymers. This is why PLGA was selected as the polymer of microparticles. In our already mentioned previous work, the original idea was to use a solvent (ethyl acetate) in which one of the polymers (PCL) was not soluble during the double emulsion process. Replacing the previous non biodegradable polymers (ethylcellulose, Eudragit® RS) by PLGA did not change anything since the latter is also soluble in ethyl acetate. Indeed, microparticles were prepared by dissolving PLGA and span 60 in ethyl acetate which is a poor solvent for PCL. In our conditions of microparticles preparation, the maximum solubility of PCL, when added directly in ethyl acetate, is 5 %. Therefore, it was possible to use the PCL nanoparticles suspension as the internal aqueous phase in the preparation of the composite microparticles since this polymer is mostly not dissolved in ethyl acetate.

Ibuprofen was selected as the model drug for the *in vivo* study. Indeed, i) it corresponds to a small molecule (MW = 206.3) and it is poorly water soluble (35.89 µg/mL [29]), ii) almost all ibuprofen microparticles and nanoparticles show initial burst release [4, 20, 29, 35, 36] and iii) it has a short elimination half-life (about 2 h after oral administration in man [37]). Furthermore, it could be interesting to develop a new injectable and possibly once a day dosage form of ibuprofen for intraarticular administration [14].

Ibuprofen was encapsulated with a high efficiency both in nanoparticles and microparticles (simple or composite). This high encapsulation efficiency of ibuprofen could be explained by the lipophilic nature of the drug which has no or very low affinity for the external aqueous phase. However, the encapsulation efficiency of ibuprofen in PCL nanoparticles (95 %) is higher than in both types of microparticles (84 and 89% for simple and composite microparticles, respectively). This might be due to a solvent effect: indeed, methylene chloride was used during PCL nanoparticles preparation instead of ethyl acetate for the microparticles manufacturing. Ibuprofen is more soluble in methylene chloride (< 800 mg/ml at 4°C) than in ethyl acetate (<300 mg/ml at 4°C) so diffusion of ibuprofen from ethyl acetate to water is probably easier than from methylene chloride. Indeed, this was already reported by Mainardes and Evangelista [38] who noticed that the encapsulation efficiency of praziquantel in PLGA nanoparticles increased when the solvent was methylene chloride compared to ethyl acetate. They attributed this phenomenon to the higher water solubility of ethyl acetate. Thus there is a faster partitioning of ethyl acetate in the external phase accompanied by the polymer precipitation with decreasing drug incorporation into nanoparticles. In addition, it has also to be kept in mind that the volume of the external phase is much smaller during the nanoparticles preparation (40 mL) than during the microparticles preparation (2 liters). The similar encapsulation efficiency observed with PLGA simple and composite microparticles may be explained by the overall fast PLGA precipitation when ethyl acetate is extracted in water [39]. Thus, it would consequently be more difficult for ibuprofen to diffuse towards the outer water phase very differently for the two types of microparticles.

In order to definitely demonstrate that nanoparticles were effectively entrapped in microparticles, we have carried out the following experiment with ibuprofen loaded micro- and nanoparticles. Ibuprofen PLGA composite microparticles, ibuprofen PLGA simple microparticles, ibuprofen PCL nanoparticles and also 50 mg of ibuprofen powder (same quantity used for manufacturing microparticles) were dispersed in ethyl acetate (fig. 3). It has to be remembered that PLGA and span 60 are soluble in ethyl acetate but not the PCL polymer (or very slightly as stated before). Therefore, if PCL nanoparticles have effectively been encapsulated, the resulting suspension in ethyl acetate should be very turbid as should be the ibuprofen PCL nanoparticles suspension also dispersed in ethyl acetate. Fig. 3 clearly displays a similar turbidity in ethyl acetate of both PCL nanoparticles and PLGA composite microparticles showing the dissolution of PLGA and Span 60 but the presence of PCL nanoparticles.

Figure 3: Picture showing the macroscopic solubility in ethyl acetate of different nano- and microparticles prepared with different polymers: (A) ibuprofen powder, (B) ibuprofen loaded PCL nanoparticles, (C) ibuprofen loaded PLGA composite microparticles, (D) ibuprofen loaded PLGA simple microparticles.




At the opposite, ibuprofen PLGA simple microparticles are totally dissolved in ethyl acetate and lead to the same clear solution as the ibuprofen powder. Consequently, fig. 3 shows that the manufacturing process allows the encapsulation of PCL nanoparticles in PLGA composite microparticles and demonstrates the composite character of these microparticles.

There are two ways to verify that the burst is controlled i.e. either by an *in vitro* dissolution test or by an *in vivo* approach for instance after subcutaneous or intramuscular administration. We have used both approaches to verify the potential burst reduction with composite microparticles.

*In vitro*, it was possible to rank the four dosages forms according to the burst; bulk powder > PCL nanoparticles > simple microparticles > composite microparticles (fig. 1 and table 3). It is obvious that the lowest burst was achieved with the composite microparticles. Indeed the

burst at 15 min was almost 3 times more with the simple microparticles and about 10 times more with the PCL nanoparticles and the bulk powder than the composite microparticles. Differential scanning calorimetry was carried out with the 3 multiparticular dosage forms and demonstrated that ibuprofen was dispersed under amorphous form in the polymer matrix (data not shown). This is in favor of fast ibuprofen dissolution in the medium but there is no difference between the 3 dosage forms and this factor is not able to explain the observed burst differences. In the case of PCL nanoparticles, the main factor driving ibuprofen dissolution is the large exchange surface area developed with the outer medium due to the very small diameter of nanoparticles. Since the diameter of both simple microparticles and composite microparticles is relatively close (around 35 µm), this parameter cannot explain the differences observed between the two types of microparticles.

Therefore, other hypotheses have to be taken into account to explain such differences in the burst. For instance, it has been shown for lipophilic drugs (NSAID, nifidepine, dexamethasone, lidocaine) that the burst is mainly dependent on the internal morphology of particles and drug distribution state including surface association [4, 36, 40] which is affected by process and formulation parameters (especially temperature) and drug/matrix interactions. Temperature was the same for preparing the simple microparticles and the composite microparticles so this parameter can also be ruled out. Both types of microparticles being prepared according to the same double emulsion method, this should also not affect the overall porosity. It seems more likely that heterogeneous distribution and physico-chemical nature of the polymer matrix are the 2 main different parameters. Indeed, for composite microparticles it is not only the drug but the PCL nanosuspension which is distributed in the PLGA polymer matrix. Thus, drug distribution could be different between simple and composite microparticles. In addition PCL is a more hydrophobic polymer than PLGA which may also slow down water diffusion in the incorporated nanoparticles. As for the polymer matrix, it is also obvious that, in composite microparticles, there are 2 barriers for the drug to diffuse through. The first barrier is due to the PCL polymer and the second barrier is the outer PLGA matrix. It is reasonable to make the assumption that the double layer of polymers is the main reason to explain the dramatic in vitro burst reduction.

Similar ibuprofen burst reduction has been described by different authors but using different approaches. For instance, Wang *et al.* [5] deposited polysaccharides on ibuprofen-loaded poly(hydroxybutyrate-co-hydroxyvalerate) microspheres using layer-by-layer self-assembly to produce core-shell microparticles. Saravanan *et al.* [41] prepared ibuprofen microspheres with very lipophilic and non biodegradable polymers such as ethylcellulose/polystyrene and

Fernandez-Carballido *et al* [14] added Labrafil® oil (non-ionic amphiphilic excipient) in PLGA microspheres loaded with ibuprofen.

As observed in figure 1, both simple and composite microparticles release ibuprofen very slow since only around 60 and 40% of drug are found in the outer phase after 24 h, respectively. This is another feature of the composite microparticles whose very slow release pattern could also be of interest in the development of a long lasting injectable dosage form.

Although the entire encapsulated drug was not released within 24 h, the dissolution test was limited to this time since the goal of this research work was to demonstrate the influence of the encapsulation of nanoparticles into microparticles on the initial burst release.

Due their small average diameter, the suspension of nanoparticles was easily injected subcutaneously. As for the simple and composite microparticles it was first necessary to disperse them in a relatively viscous aqueous solution prior to injection. The carboxymethylcellulose aqueous suspension of simple and composite microparticles also allowed an easy subcutaneous injection and was characterized by i) a good stability [no sedimentation observed during preparation and injection (2 to 5 min)], ii, good syringability (23 G) and iii) a physiological pH of 7.4. As already observed, the ibuprofen solution and nanoparticles display a fast absorption whereas absorption is much slower for simple and composite microparticles. As well known for matrix systems, diffusion of the drug through the microparticles is the rate limiting step of absorption.

Cmax and Tmax reflect the absorption rate of a drug. Therefore the initial burst is correlated to the values of the 2 latter parameters. In terms of burst effect, the 4 dosage forms can be ranked as: Pedea® solution > PCL nanoparticles > simple microparticles > composite microparticles. There is a 7 fold times difference between the solution and the composite microparticles without mentioning that the Tmax are also very different between the 2 dosage forms (0.25 and 4 h, respectively). The difference in Cmax between the composite microparticles and simple microparticles is less spectacular but is still about 1.6 times. Thus it is confirmed that the microparticle systems, and more particularly the composite microparticles, have a dramatic influence on the initial burst release *in vivo.* Furthermore, the rank order in decreasing Cmax is exactly the same as it is for the ibuprofen dissolved percentage after 15 min in the *in vitro* release test. The same hypotheses, as already discussed for the *in vitro* dissolution results, may explain the observed *in vivo* results. Thus, it has been definitely confirmed that the composite microparticles present a strong potential in reducing the burst effect not only *in vitro* but also *in vivo*.

The *in vivo* study was performed for 96 h in order to evaluate the potential of the 3 multiparticular dosage forms as prolonged release compositions. Unfortunately, and despite the set-up of a new HPLC method with lower detection limits for ibuprofen [42], the ibuprofen plasma concentrations were below the limits of quantification after 18 h and 24 h for the ibuprofen solution and the PCL nanoparticles, respectively. For the simple and composite microparticles, the limit of quantification was reached after 48 h. However for composite microparticles, ibuprofen was still detected in the plasma till 96 h which demonstrates that ibuprofen is released for longer periods of time with composite microparticles. However taking into account the relatively low number of animals and the normal *in vivo* variability, the figures of relative bioavailability for nanoparticles and simple microparticles are relatively close and it could be considered that the bioavailabilities are the same and around 100 %. In the case of composite microparticles, about half of the incorporated ibuprofen has been released in 48 h. This shows the great potential of such dosage forms to act also as long release microparticles. One way to increase or decrease the release rate of drugs from such composite microparticles would be to play on the type of biodegradable polymers used in their manufacturing. Indeed, higher or smaller molecular weights of the 2 types of polymers could lead to tailor made release. Such an approach will be used in the continuation of this work.

## 6. Conclusion

When ibuprofen PCL nanoparticles are encapsulated in microparticles, a strong decrease in the drug burst release is observed. Therefore the advantage of encapsulating nanoparticles in microparticles (composite microparticles) is definitely demonstrated *in vivo* with hydrophobic nature matrix such as PCL and PLGA. The use of slower degrading polymers such as PLA or PLGA or PCL with larger molecular weights could still increase the slow release potential of these formulations.

## References

[1] J. Siepmann, N. Faisant, J. Akiki, J. Richard, J.P. Benoit, Effect of the size of biodegradable microparticles on drug release: experiment and theory, J. Control. Release 96 (2004) 123-134.

[2] S. Sant, V. Nadeau, P. Hildgen, Effect of porosity on the release kinetics of propafenone-loaded PEG-g-PLA nanoparticles. J. Control. Release 107 (2005) 203-14.

[3] R.A. Jain, The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices, Biomaterials 21 (2000) 2475-2490.

[4] C.J. Thompson, D. Hansford, S. Higgins, C. Rostron, G.A. Hutcheon, D.L. Munday, Evaluation of ibuprofen-loaded microspheres prepared from novel copolyesters. Int. J. Pharm. 329 (2007) 53-61.

[5] C. Wang, W. Ye, Y. Zheng, X. Liu, Z. Tong, Fabrication of drug-loaded biodegradable microcapsules for controlled release by combination of solvent evaporation and layer-by-layer self-assembly. Int. J. Pharm. 338 (2007) 165-173.

[6] R. Bodmeier, H. Chen, Preparation and characterization of microspheres containing the anti-inflammatory agents, indomethacin, ibuprofen, and ketoprofen, J. Control. Release 10 (1989) 167-175.

[7] M. Hombreiro-Pérez, J. Siepmann, C. Zinutti, A. Lamprecht, N. Ubrich, M. Hoffman, R. Bodmeier, P. Maincent, Non-degradable microparticles containing a hydrophilic and/or a lipophilic drug: preparation, characterization and drug release modeling, J. Control. Release 88 (2003) 413–428.

[8] E. Karasulu, H. Yeşim Karasulu, G. Ertan, L. Kirilmaz, T. Güneri, Extended release lipophilic indomethacin microspheres: formulation factors and mathematical equations fitted drug release rates, Eur. J. Pharm. Sci. 19 (2003) 99-104.

[9] T.R. Tice and D.R. Cowsar, Biodegradable controlled-release parenteral systems, Pharm. Technol. 11 (1984) 26–36.

[10] H. Okada, One-and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate, Adv. Drug Deliv. Rev. 28 (1997) 43-70.

[11] H. Okada, Y. Doken, Y. Ogawa, H. Toguchi, Preparation of three-month depot injectable microspheres of leuprorelin acetate using biodegradable polymers. Pharm. Res. 11 (1994) 1143-1147.

[12] W. Jiang, S.P. Schwendeman, Stabilization and controlled release of bovine serum albumin encapsulated in poly (D,L-lactide) and poly(ethylene glycol) microsphere blends, Pharm. Res. 18 (2001) 878- 885.

[13] J. Wang, B.W. Wang, S.P. Schwendeman, Mechanistic evaluation of the glucose-induced reduction in initial burst release of octreotide acetate from poly(D,L-lactide-co-glycolide) microspheres, Biomaterials 25 (2004) 1919-1927.

[14] A. Fernandez-Carballido, R. Herrero-Vanrell, I.T. Molina-Martinez, P. Pastoriza, Biodegradable ibuprofen-loaded PLGA microspheres for intraarticular administration. Effect of Labrafil addition on release in vitro, Int. J. Pharm. 279 (2004) 33-41.

[15] J. Hanes, M. Chiba, R. Langer, Degradation of porous poly(anhydride-co-imide) microspheres and implications for controlled macromolecule delivery. Biomaterials 19 (1998) 163-172.

[16] Y.Y. Yang, J.P. Wan, T.S. Chung, P.K. Pallathadka, S. Ng, J. Heller, POE-PEG-POE triblock copolymeric microspheres containing protein: I. preparation and characterization, J. Control. Release 75 (2001)115–128.

[17] I.J. Castellanos, K.G. Carrasquillo, J.D. Lopez, M. Alvarez, K. Griebenow, Encapsulation of bovine serum albumin in poly(lactide-co-glycolide) microspheres by the solid-in-oil-in-water technique.J Pharm Pharmacol 53 (2001) 167-178.

[18] A.J. Thote, R.B. Gupta, Formation of nanoparticles of a hydrophilic drug using supercritical carbon dioxide and microencapsulation for sustained release, Nanomedicine 1(2005) 85-90.

[19] J.K. Lalla, K. Snape, Biodegradable microspheres of poly (D,L -lactic acid) containing piroxicam as a model drug for controlled release via the parenteral route, J. Microencapsul. 10 (1993) 449-460.

[20] E. Leo, F. Forni, M.T. Bernabei, Surface drug removal from ibuprofen-loaded PLA microspheres, Int. J. Pharm. 196 (2000) 1-9.

[21] A. Sheikh Hasan, M. Socha, A. Lamprecht, F.E. Ghazouani, A. Sapin, M. Hoffman, P. Maincent, N. Ubrich, Effect of the microencapsulation of nanoparticles on the reduction of burst release, Int. J. Pharm. 344 (2007) 53-61.

[22] S. Benita, J.P. Benoit, F. Puisieux, C. Thies, Characterisation of drug-loaded poly(D,L-lactide) microspheres. J. Pharm. Sci. 73 (1984) 1721–1724.

[23] J.K. Li, N. Wang, X.S. Wu, A novel biodegradable system based on gelatin nanoparticles and poly(lactic-co-glycolic acid) microspheres for protein and peptide drug delivery, J. Pharm. Sci. 86 (1997) 891-5.

[24] W.T. Leach, D.T. Simpson, T.N. Val, E.C. Anuta, Z. Yu, R.O.3rd Williams, K.P. Johnston, Uniform encapsulation of stable protein nanoparticles produced by spray freezing for the reduction of burst release, J. Pharm. Sci. 94 (2005) 56-69.

[25] M.D. Bhavsar, M.M. Amiji, Gastrointestinal distribution and in vivo gene transfection studies with nanoparticles-in-microsphere oral system (NiMOS), J. Control. Release 119 (2007) 339-48.

[26] A. Lamprecht, H. Yamamoto, H. Takeuchi, Y. Kawashima, A pH-sensitive microsphere system for the colon delivery of tacrolimus containing nanoparticles, J. Control. Release. 104 (2005) 337-46.

[27] A. Grenha, B. Seijo, C. Remunan-Lopez, Microencapsulated chitosan nanoparticles for lung protein delivery, Eur. J. Pharm. Sci. 25 (2005) 427-37.

[28] J.V. Aranda, R. Thomas, Systematic review: intravenous Ibuprofen in preterm newborns, Semin. Perinatol, 30 (2006) 114-20.

[29] B. Jiang, L. Hu, C. Gao, J. Shen, Ibuprofen-loaded nanoparticles prepared by a co-precipitation method and their release properties, Int. J. Pharm. 304 (2005) 220-30.

[30] T. Borovac, J.P. Pelage, A. Kasselouri, P. Prognon, G. Guiffant, A. Laurent, Release of ibuprofen from beads for embolization: in vitro and in vivo studies, J. Control. Release 115 (2006) 266-74.

[31] R. Pignatello, C. Bucolo, P. Ferrara, A. Maltese, A. Puleo, G. Puglisi, Eudragit RS100 nanosuspensions for the ophthalmic controlled delivery of ibuprofen, Eur. J. Pharm. Sci. 16 (2002) 53-61.

[32] V. Hoffart, N. Ubrich, C. Simonin, V. Babak, C. Vigneron, M. Hoffman, T. Lecompte, P. Maincent, Low molecular weight heparin-loaded polymeric nanoparticles: formulation, characterization, and release characteristics, Drug. Dev. Ind. Pharm. 28 (2002) 1091-2000.

[33] T. Freytag, A. Dashevsky, L. Tillman, G.E. Hardee, R. Bodmeier, Improvement of the encapsulation efficiency of oligonucleotide-containing biodegradable microspheres, J. control. Release 69 (2000) 197-207.

[34] N. Wang, X.S. Wu, J.K. Li, A heterogeneously structured composite based on poly(lactic-co-glycolic acid) microspheres and poly(vinyl alcohol) hydrogel nanoparticles for long-term protein drug delivery, Pharm. Res. 16 (1999)1430-5.

[35] N. Bolourtchian, K. Karimi, R. Aboofazeli, Preparation and characterization of ibuprofen microspheres, J. Microencapsul. 22 (2005) 529-38.

[36] K.J. Zhu, Y Li, H.L. Jiang, H. Yasuda, A. Ichimaru, K. Yamamoto, P. Lecomte, R. Jerome R, Preparation, characterization and in vitro release properties of ibuprofen-loaded

microspheres based on polylactide, poly (epsilon-caprolactone) and their copolymers. J. Microencapsul. 22 (2005) 25-36.

[37] P. Karttunen, V. Saano, P. Paronen, P. Peura, M. Vidgren, Pharmacokinetics of ibuprofen in man: a single-dose comparison of two over-the-counter, 200 mg preparations, Int. J. Clin. Pharmacol. Ther. Toxicol. 28 (1990) 251-5.

[38] R.M. Mainardes, R.C. Evangelista, Praziquantel-loaded PLGA nanoparticles: preparation and characterization, J. Microencapsul. 22 (2005) 13-24.

[39] Y. Yeo, K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems, Arch. Pharm. Res. 27 (2004) 1-12.

[40] S. Mao, Y. Shi, L. Li, J. Xu, A. Schaper, T. Kissel, Effects of process and formulation parameters on characteristics and internal morphology of poly(D,L-lactide-co-glycolide) microspheres formed by the solvent evaporation method. Eur. J. Pharm. Biopharm. Article in Press 2007.

[41] M. Saravanan, K. Bhaskar, G. Srinivasa Rao, M.D. Dhanaraju, Ibuprofen-loaded ethylcellulose /polystyrene microspheres: an approach to get prolonged drug release with reduced burst effect and low ethylcellulose content, J. Microencapsul. 20 (2003) 289-302.

[42] A. Sheikh Hassan, C. Bolzan, A. Sapin, N. Ubrich, P. Maincent, P. Leroy, Simple and sensitive HPLC method with fluorescence detection for the measurement of ibuprofen in rat plasma. Application to a long-lasting dosage form, Drug. Dev. Ind. Pharm. 2008, in press.

[43] E.M.E.A., London, 29 July 1999 committee for proprietary medicinal products, note for guidance on quality of modified release products.

# CHAPITRE IV

## Article 4
## *In vivo* reduction of insulin burst effect from microparticles

**Article soumis au Journal of Controlled Release**

Les microparticules composites chargées avec de l'ibuprofène ont montré leur efficacité pour réduire le burst à la fois *in vitro* et *in vivo* ce qui confirme la possibilité d'utiliser notre formulation avec succès dans le cas de principes actifs hydrophobes et notamment dans le cas d'anticancéreux qui présentent un marge thérapeutique étroite.

Toutefois, l'effet burst a plus souvent été décrit dans les cas de principes actifs hydrophiles, comme les peptides et les protéines, que dans celui des principes actifs hydrophobes. Cela s'explique naturellement par la diffusion plus difficile d'un principe actif dans un environnement physiologique hydrophile. C'est pour cette raison que la plupart des recherches visant à réduire le burst avec de nouveaux types de formulation utilisent des principes actifs hydrophiles comme modèles et plus généralement des peptides et/ou protéines.

Les protéines thérapeutiques sont aujourd'hui très utilisées pour le traitement d'un grand nombre de maladies. Cependant, ces protéines quoique très actives ont des durées d'action de quelques heures, une marge thérapeutique étroite et peuvent présenter des toxicités très élevées. Il existe donc un besoin réel de nouveaux systèmes permettant de prolonger leur temps d'action et d'autre part de réduire leur toxicité via l'utilisation de formes galéniques susceptibles de libérer ces peptides/protéines de façon prolongée et sans effet burst par voie parentérale. En effet, cette voie est la voie principale d'administration de toutes les formulations des protéines car leur absorption par les voies orale, pulmonaire et/ou nasale est limitée.

Il était donc important de vérifier également que le concept de microparticules composites permettait non seulement de limiter le burst *in vitro* (comme démontré précédemment pour l'acétate de triptoréline) mais également *in vivo*. Le choix de la molécule modèle s'est porté sur l'insuline en raison d'un approvisionnement et d'un dosage plus faciles. L'insuline est en effet un peptide hydrophile largement utilisé sous forme d'une solution ou suspension sous-cutané ou alors dans des formulations modernes (implants, particules, gels) et présentant en général un effet burst. L'étude *in vivo* a été effectuée après administration sous-cutanée des microparticules chez des rats diabétique de type I (modèle à la streptozotocine). Des prélèvements sanguins réguliers sur plusieurs jours suivis du dosage de l'insuline dans le sérum des ces rats ont permis d'établir la courbe concentration d'insuline dans le sérum en fonction du temps. Les résultats obtenus après l'administration des microparticules composites ont été comparés avec les résultats obtenus après l'injection sous-cutanée des nanoparticules et microparticules simple chargées d'insuline. La biodisponibilité

absolue pour les trois formulations a été calculée par rapport l'injection intraveineuse d'une solution d'insuline.

Comme à chaque fois dans le cas de l'encapsulation d'un nouveau principe actif, ici l'insuline, un certain nombre d'adaptations du procédé ont permis d'obtenir un taux d'encapsulation satisfaisant de façon à limiter la quantité de microparticules polymériques devant être administrées. Une recherche préliminaire de doses à administrer a permis de définir la dose la plus adaptée à notre étude et permettant de ne pas provoquer une hypoglycémie chez les rats diabétique.

Les conclusions sont équivalentes à celles déjà émises dans le cas des microparticules d'ibuprofène. Le système des microparticules composites permet de réduire le burst observé avec les autres formulations d'insuline. Toutefois la différence dans la réduction du burst est non significative par rapport à l'administration de microparticules simples d'insuline. Ces résultats soulignent cependant l'intérêt de notre approche et de l'intérêt thérapeutique du système que nous avons mis au point.

# *In vivo* reduction of insulin burst effect from microparticles

A. Sheikh Hassan[1], A. Sapin[1], C. Damgé[4], P. Leroy[2], A. Lamprecht[3], E. Emond[1], F. El Ghazouani[1], N. Ubrich[1], P. Maincent[1*]

[1]Laboratoire de Pharmacie Galénique, Faculty of Pharmacy, Nancy University, 5, rue A. Lebrun, BP 80403, 54001 Nancy, France

Article soumis au Journal of Controlled Release

[2]Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, Faculty of Pharmacy, Nancy University, BP 80403, 54001 Nancy, France

[3]Laboratory of Pharmaceutical Engineering, Faculty of Pharmacy, Place Saint Jacques, 25030 Besançon Cedex, France

[4]Institut de Physiologie, Faculty of Medicine, 11, Rue Humann, 67000 Strasbourg, France

Dedicated to Dr N. Ubrich who died on April 24, 2007.

To journal of controlled release

Corresponding author:

P. MAINCENT
EA 3452
Faculty of Pharmacy
5, rue A. Lebrun, BP 80403
54001 Nancy Cedex (France)

Phone : +33 3 83 68 22 96
Fax : +33 3 83 68 23 01
E-mail : maincent@pharma.uhp-nancy.fr

## Abstract

The main objective of this work was to evaluate the behaviour of composite microparticles (microparticles containing nanoparticles) for parenteral delivery with a view to reducing the initial burst release of short half-lives proteins (using insulin as a model drug). These composite microparticles were prepared with hydrophobic and biodegradable polymers [Poly (ε-caprolactone), poly (lactic-co-glycolic) acid] by the double emulsion extraction technique. Particles were administered subcutaneously (1IU/kg of insulin) as a single dose to diabetic rats (streptozotocin-induced) and serum insulin levels were monitored. The results show a significant reduction of insulin burst release from composite microparticles both *in vitro* (around 19 % after 15 min.) and *in vivo* (around 582 µU/mL and Tmax 4 h) with a progressive and controlled release profile up to 24 h when compared to simple microparticles (without nanoparticles inside) and nanopaticles. These experiments demonstrate that a desirable insulin injection formulation with negligible burst effect *in vivo* has been developed. Such effect was attributed to the double polymer barrier that the drug has to diffuse through before reaching the external medium either *in vitro* or *in vivo*.

*Keywords:* Insulin; Composite microparticles; Burst release; Subcutaneous delivery; Diabetic rats; Poly (ε-caprolactone); Poly (lactic-co-glycolic) acid.

## 1. Introduction

Bioactive proteins and peptides are a rapidly growing class of therapeutic agents. Injections of proteins have to be renewed frequently because their *in vivo* half-life is generally rather short (not more than a few hours). Therefore, sustained delivery of proteins and peptides is still of major interest for parenteral administrations [1].

However, parenteral peptides or proteins controlled release products based on biodegradable polymers (nano/microparticles and hydrogels) or lipid-based systems (liposome and solid lipid nano/microparticles) often present an inconsistent release profile characterized by a high initial burst effect defined as the initial release of a large bolus of drugs [2-4]. This initial burst phase is then followed by a plateau frequently associated with an incomplete release [4]. In most cases, the burst release corresponds to a significant loss of drug from both therapeutic and economic standpoints. Indeed, high initial drug release is not suitable for parenteral peptides and proteins products because drug released in this period is no longer available for prolonged release and may lead to a high risk of side effects (due to elevated serum levels) especially for drugs with narrow therapeutic index and short half-life. For this reason, one of

the goals of protein microencapsulation is to reduce the initial burst and achieve a constant release rate thereafter.

For many parenteral therapeutic proteins, achieving acceptable high drug content into particles while maintaining satisfactory release kinetics (*i.e.* minimal burst together with acceptable duration), represents a very significant formulation challenge.

Protein release from biodegradable polymeric particles during the initial release stage depends on diffusion escape of the protein through channels existing in the polymer matrix. Given that the time required until the onset of polymer degradation ranges from weeks to months, drug release during the first few days (or hours) depend on how successfully the diffusion is controlled. In most cases, the burst release is due to poor control over the diffusion based release in this stage. The degree of initial burst from the nano/microparticles depends on the ability of the polymer matrix to encapsulate the protein, thereby making it unavailable for immediate diffusion [4] [5]. For this reason, efforts to reduce the initial burst have followed in the same track as those to increase drug encapsulation efficiency.

Initial burst release of peptides and proteins from polymeric matrix may depend on their molecular weight. Peptides and protein with small molecular weights (for example triptorelin, calcitonin) may escape from the polymeric matrices through the pores and cracks that form during the particles manufacturing process [3] [6] [7]. On the other hand, burst release may also occur with high molecular weight proteins (immunoglobulin G [8], Glial cell line-derived neurotrophic factor (GDNF) [9], α-interferon [10]) due to i) their heterogeneous distribution in the matrix ii) their loosely association with the surface or iii) their embedding in the surface layer [3] [4].

In a previous work, we have prepared composite microparticles [11]. These composite microparticles correspond to drug loaded poly(ε-caprolactone) (PCL) nanoparticles encapsulated into Eudragit® RS or ethyl cellulose microparticles. This study showed high encapsulation efficiency and low initial burst effect of triptorelin (hydrophilic drug model peptide). However, the concept of controlling the burst effect by encapsulating nanoparticles into microparticles was only demonstrated in vitro. In addition, the tested microparticle polymers (Eudragit® RS and ethyl cellulose) are not acceptable for parenteral administration (subcutaneous or intramuscular). Thus, we decided to use a biodegradable polymer such as poly (lactic-co-glycolic) acid (PLGA) to replace Eudragit® RS or ethyl cellulose in the microparticles matrix. Furthermore, due to its broader interest and its easier dosage in serum, insulin was chosen as another drug model of peptide. Indeed, patients with type 1 diabetes mellitus depend on external insulin (most commonly injected subcutaneously) for their

treatment. Patients with type 2 diabetes mellitus have insulin resistance which is progressively associated with a reduced production of insulin; thus, some type 2 diabetic patients require insulin when oral antidiabetic drugs become insufficient in controlling blood glucose levels. It is also well known that insulin-dependent patients need a relatively constant basal insulin supply to attain a near-normal physiological pattern of insulin.

In order to achieve blood glucose control in diabetes, intensive insulin treatment is used. This treatment involves the daily injection of one or more doses of intermediate- or long-acting insulin to satisfy basal insulin requirement, as well as injection before each meal. The multiple daily injections regimen leads to poor patients' compliance, pain and even mental stress. Theoretically, injectable polymeric or lipidic delivery systems (microspheres, microcapsules, microemulsion, liposomes, and gel) could be used for controlled release of insulin continuously for a desired period of time [12] [13]. Such a release pattern would satisfy continuous low-level of basal insulin requirement. Basal insulin delivery from these novel systems could partly relieve patients from multiple daily injections. Such formulations would contribute not only to an improvement in the patient's compliance, but also to a reduction of developing additional diabetes complications [14]. In the literature, there are a lot of attempts to prepare satisfactory parenteral sustained insulin formulation without burst release [13, 15-18].

The purpose of the present study was to develop and test in vivo composite microparticles based on biodegradable polymers only, thus adapted to the subcutaneous injection and *in vivo* administration of insulin.

## 2. Materials

Regular and fast human insulin (Actrapid®) was from Novo Nordisk (Bagsvaerd, Denmark). The concentration of the solution was 100 IU/mL of human insulin. Excipients of Actrapid® are metacresol, zinc chloride, glycerol, sodium hydroxide and/or hydrochloric acid and water for injectable preparation. Poly (ε-caprolactone) (*Mw* 40,000 Da) and D-L poly(lactic-co-glycolic) acid 50:50 (m/m) Resomer RG 504S end-capped ($M_w$ 48,000; viscosity: 0.47 dL/g) were purchased from Aldrich, (Saint Quentin Fallavier, France) and Boehringer Ingelheim (Ingelheim, Germany), respectively.

Polyvinylalcohol (PVA, *Mw* 30,000, 88% hydrolyzed) was supplied by Sigma (Saint Quentin Fallavier, France). Ethyl acetate (water solubility = 8.3 g/100 mL at 20°C) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Methylene chloride (water solubility = 1.3 g/100 mL at 20°C) was supplied by Prolabo (Paris, France). Acetonitrile and orthophosphoric

acid were obtained from Carlo-Erba (Val de Reuil, France) and Prolabo (Paris, France), respectively. All other chemicals were of analytical grade and used without further purification.

## 3. Methods

### *3.1. Preparation of particles*

#### *3.1.1. Nanoparticles*

Insulin loaded PCL nanoparticles were prepared by the water in oil in water (W/O/W) solvent evaporation method [19]. Briefly, 1 mL of Actrapid® aqueous solution was emulsified for 15 s in 5 mL of methylene chloride (containing 125 mg of PCL) with the help of an ultrasound probe (Vibra cell 72 434, BioBlock Scientific, Strasbourg, France) at 80W output. This primary emulsion was poured into 40 mL of a 0.1% PVA aqueous solution and sonicated again with the same ultrasound probe for 1min. in the same conditions in order to create the water in oil-in-water (O/W) emulsion. Three mL (± 1 mL) of nanoparticles suspension were obtained after solvent evaporation under reduced pressure.

Nanoparticles were separated from the bulk suspension by centrifugation (Biofuge Stratos; Heraeus Instruments. GmbH&Co., Hanau, Germany) at 42,000 *x g* for 20 min. The supernatant was kept for drug assay according to the methods described later and the sedimented nanoparticles were then redispersed in 3 mL of purified water before freeze-drying. After lyophilization, a dry powder of nanoparticles was obtained. The nanoparticles preparation method was slightly modified for manufacturing of composite microparticles (nanoparticles in microparticles). Indeed, the only difference was that the solvent evaporation process was continued till 1.5 mL (± 0.5 mL) of nanoparticles were obtained: this suspension was used directly (without freeze-drying) as the internal aqueous phase in the preparation of the composite microparticles. Blank nanoparticles were prepared under the same conditions but without drug.

#### *3.1.2. Microparticles*

Microparticles containing insulin PCL nanoparticles (so-called composite microparticles) or not were prepared by the W/O/W solvent extraction method [20]. In the first step (W/O emulsion), the PCL nanoparticles suspension (1.5 ± 0.5 mL as mentioned before) was used as

the internal aqueous phase which was emulsified (ultrasound probe at 80 W output for 15 s) in the organic solution of ethyl acetate (10 mL) containing PLGA (150 mg).
This primary emulsion was poured into 30 mL of 0.1% PVA aqueous solution in order to obtain a W/O/W pre-emulsion. After magnetically stirring for 20 s (600 rpm) at room temperature, this pre-emulsion was added to 1L of purified water and stirred mechanically (three-bladed propeller, 600 rpm) for 10 min. to form the final W/O/W emulsion.
Upon solvent extraction, the polymers precipitated and the microparticles cores solidified. Microparticles were collected by filtration (Millipore® Type: 0.45µm acetate cellulose (low protein adsorption affinity)) and freeze-dried.
Blank PLGA composite microparticles (with blank PCL nanoparticles inside) and PLGA simple microparticles without nanoparticles inside *i.e.* simple microparticles (with or without insulin) were prepared under the same conditions. For insulin loaded simple microparticles, a solution of insulin (Actrapid® 100 IU/mL) was used as the internal aqueous phase.

*3.2 Mean diameter and zeta potential*

Mean diameter and size distribution of microparticles were analyzed by laser diffraction in a particle size analyzer (Mastersizer S, Malvern Instruments, Orsay, France). Each sample was measured in triplicate.
The mean diameter of nanoparticles and their surface potential were evaluated with a Malvern Zetasizer 3000 HSA (Malvern Instruments, Orsay, France) using respectively photon correlation spectroscopy and electrophoretic mobility. Nanoparticles were diluted in NaCl 0.001 M prior to zeta potential measurements. Each sample was measured in triplicate.

*3. 3. Determination of insulin content in the particles*

The amount of insulin entrapped within polymeric particles was determined according to an established but slightly modified HPLC method [21] by measuring the amount of non-entrapped insulin in the external aqueous solution (indirect method) which was recovered after filtration of microparticles. In the case of nanoparticles, the external aqueous solution was obtained after centrifugation of the colloidal suspension for 20 min. at 42,000 *x g*.
Briefly, 50 µL of the external aqueous solution were injected into the HPLC system (Shimadzu HPLC 10A vp, Shimadzu , Champs sur Marne, France) with UV detection (SPD-

10 A VP, Shimadzu, Champs sur Marne, France) and a data processing software (model Class VP).

The separation was achieved by using a C-18 reversed phase column (250 x 4.6 mm, 5 µm, 300 Å - Vydac, Interchim, Montluçon, France) at 40°C. The detection wavelength was set at 214 nm. The flow rate of the mobile phase was 1.2 mL/min [(A) water acidified with 0.1% trifluoroacetic acid; (B) acetonitrile acidified with 0.1% trifluoroacetic acid] changed by gradient way to separate metacresol peak from insulin one. A standard calibration curve was performed with the Actrapid® solution in the 0.1% PVA aqueous solution. The established linearity range was 0.05-2 IU/mL ($r > 0.99$).

*3. 4. In vitro drug release from both nanoparticles and microparticles*

Fifty mg of freeze-dried loaded particles were suspended in 20 mL of saline phosphate buffer ($KH_2PO_4$ 4.4 mM, $Na_2HPO_4$ 45.1 mM, NaCl 0.1 M, pH 7.4 adjusted by orthophosphoric acid). Dissolution studies were carried out under sink conditions. The particles suspension was gently stirred (200 rpm) at 37°C into a water bath. One milliliter of suspension was withdrawn at appropriate intervals (5, 15, 30, 45 min., 1, 2, 3, 4, 5, 6, 8, 24 h) and centrifuged at 42,000 *x g* for 10 min. (due to established insulin filter adsorption during the preliminary trials). Sampling volume was replaced by the same volume of fresh buffer. The amount of released insulin was determined by HPLC coupled with UV detection at 214 nm as previously described. A standard calibration curve was performed with the Actrapid® solution in the aqueous solution of saline phosphate buffer. Each particle batch was analyzed in triplicate.

*3. 5 In vivo studies of insulin-loaded particles in diabetic rats*

*3. 5.1 Animals*

Adult male Wistar rats (300 ± 20 g) (Charles River laboratories, ville, France) were housed in air-conditioned quarters under a photoperiod schedule of 12 h light/12 h dark. They received standard laboratory chow diet (UAR, Villemoisson-sur-Orge, France) and tap water, available ad libitum. All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC).

*3. 5.2. Induction of diabetes*

Diabetes was induced in male Wistar rats by an intravenous injection of streptozotocin (65 mg/kg) in a 10 mM citrate buffer at pH 4.5 as previously described [22]. Rats were considered diabetic when glycemia was higher than 300 mg/dL (about three weeks after streptozotocin treatment). Type 1 diabetic rats provide the best *in vivo* model available for our study as these rats produce very little endogenous insulin; therefore, the observed serum insulin concentrations are purely a result of the insulin delivery system [17].

*3. 5. 3. Treatment and serum collection*

Unloaded composite microparticles (control) and insulin-loaded particles (PCL nanoparticles, PLGA simple microparticles and PLGA composite microparticles) were injected subcutaneously (20 IU/kg) as a single administration (aqueous suspension) to overnight fasted (water at libitum) diabetic rats. As a reference, an insulin aqueous solution (Actrapid® Novo-Nordisk, 100 IU/mL) was administered intravenously in control animals at 1 IU/kg.

The administration volume was 100 µL/100g of rat in a 2.25% carboxymethylcellulose aqueous solution (m/v). Rats were anesthetized (slight anesthesia prior and sequentially from 30 min. to 24 h after administration) by intraperitoneal injection of pentobarbital sodium (15 mg/kg). Animal groups (5 groups of 3 rats each) were randomly done. Blood (400 µL) was collected by cardiac puncture 15, 30, 45 min. and 1, 2, 4, 6, 8, 10, 24, 48, 72 and 96 h after administration into 1.5 mL polypropylene vials. After 30 min. storage at 4°C and centrifugation at 3,000 *x g* for 10 min at 4°C, the obtained serum was immediately stored at -20 °C. Due to the 5 animal group's randomization, only 2 blood samples per animal were collected during the first 24 h for each formulation. For further times, blood was sampled from two groups leading to 6 samples at each time.

*3. 5.4. Insulinemia and absolute bioavailability after oral administration of insulin-loaded nanoparticles*

Insulin (loaded or unloaded) particles (20 IU/kg) were administered subcutaneously to overnight fasted diabetic rats according to the scheme shown before. Aqueous free insulin (1 IU/kg) was intravenously administered in a second group of rats. Blood serum insulin concentrations were measured by radioimmunoassay (Insulin-CT kit from CIS Bio International, Gif-sur-Yvette, France). The areas under the curves (AUC) of the concentration-time profiles were calculated with the linear trapezoidal method. The absolute bioavailability was calculated by the ratio of the respective AUCs corrected by the administered doses.

*3. 5. 5 Statistical analyses*

The results were expressed as mean values ± standard deviation (SD). For the pair-wise comparison, the Mann-Whitney U test was used to investigate statistical differences. The statistical treatment with more than two groups was performed with Kruskal-Wallis test followed by Dunn's test, except when normality and equal variance were passed, it was followed by the Tukey test. In all cases, $p < 0.05$ was considered to be statistically significant.

## 4. Results

Tables 1 and 2 summarize the main physico-chemical parameters (mean diameter, zeta potential and encapsulation efficiency) of nanoparticles and microparticles, respectively. Both unloaded PCL nanoparticles and insulin loaded PCL nanoparticles showed a diameter around 380 nm and beared almost no charge. The encapsulation efficiency was high (80 ± 4%) (table 1).

Insulin loaded simple microparticles (71 ± 7 µm) were smaller than blank ones (92 ± 4 µm), but insulin loaded composite microparticles had the same size as blank ones (table 2). Although it is very difficult to see a general trend, it can be noticed that simple microparticles (either unloaded or loaded with insulin) are somehow larger than composite microparticles. For both types of microparticles, the insulin encapsulation efficiency was high (composite microparticles; 99%, simple microparticles; 90%) (table 2). Therefore it is obvious that nanoparticles (blank and insulin loaded) are very well incorporated in the matrix of composite microparticles.

Fig. 1 displays the *in vitro* release profiles of insulin from the three types of formulations (PCL nanoparticles, PLGA simple microparticles and PLGA composite microparticles). Insulin loaded PCL nanoparticles and PLGA simple microparticles display an important drug release or burst (around 41 % and 36 %, respectively) in the first 15 min. followed by a plateau up to 24 h for PCL nanoparticles (around 50 % at 24h). The same type of profile is observed for PLGA simple microparticles with a maximum release of around 56 % (Table 3). A somehow different release profile was obtained with PLGA composite microparticles formulations. Indeed, the initial insulin burst release was the lowest (around 19 % after 15 min) with a progressive and controlled release profile up to 24 h for PLGA composite

microparticles (around 39 % after 24 h). Moreover, a common trend for all tested particles was the non-complete release of insulin in 24 h.

An *in vivo* study was conducted by injecting subcutaneously the 3 types of polymer particles into diabetic rats. The serum insulin profiles (Fig. 2) show the insulin level in the 3 treated diabetic groups as well as the control group (diabetic rats treated with blank PLGA composite microparticles). In Fig. 2A, the intravenous administration of insulin solution (Actrapid®) (1 IU/kg) gave rise to an immediate peak at the first sampling time. In contrast, serum insulin levels of the control group (diabetic rats treated with blank PLGA composite microparticles) showed a low insulin level around 22 µU/mL throughout the study which is the same as insulin serum level of rats without any treatment. Treatment with insulin PCL nanoparticles led to a high initial insulin serum level of about 2400 µU/mL within 30 min. after particles injection (fig. 2B) which can be related to the 40 % (at 15min.) *in vitro* insulin burst release (table 3 and 4); this initial peak is followed by a very fast decrease in serum insulin which reach the initial basal level after 6 h (fig. 2B). Relatively lower serum insulin levels were initially detected (around 582 µU/mL in 30 min) when PLGA simple microparticles were administered (fig. 2B). Then insulin serum levels increased progressively to a peak value of 959 µU/mL that was reached after 2 h. With later times, a gradual decrease back to initial serum insulin levels was observed after 24 h. On the other hand, in the case of composite microparticles, the increase and decline of insulin serum level was more gradual and there was a steady insulin plateau up to 72 h (fig. 2B). Indeed, during the first 24 h the insulin serum levels following the composite microparticles subcutaneous administration displayed fairly stable concentrations without any remarkable modification. Moreover, insulin serum level decreased slowly and the initial serum basal level was reached after a much longer time (up to 96 h) than with PLGA simple microparticles. Such a profile means a continuous insulin release leading to stable blood concentrations for longer times with remarkably low burst release as already noticed *in vitro.*

The pharmacokinetic parameters derived from the serum concentrations are summarized in Table 5. The highest serum concentration (Cmax 2872 µU/mL) was observed with the PCL nanoparticles after 1 h but only at 2 h for the PLGA simple microparticles (Cmax 959 µU/mL). Finally, the lowest insulin Cmax (435 µU/mL) was obtained at 4 h after PLGA composite microparticles administration. Based on the concentration-time profiles, areas under the insulin concentration curves (AUCs from 0 to 24 h) were calculated and ranked according to the following order: PCL nanoparticles > PLGA simple microparticles = PLGA composite microparticles. However, in case of PLGA composite microparticles, the AUC still

increased up to 96 h. The *in vivo* results show that the highest absolute bioavailability (with regards to the insulin solution) was obtained with the nanoparticles suspension [65 % (0-10 h)] whereas simple microparticles and composite microparticles exhibit only around 35 % (0-24 h). It has to be noted that these results have been calculated between 0 and 24 h although insulin was still detected much later in serum for composite microparticles leading, for instance, to a 48 % absolute bioavailability after 48 h.

Results in table 5 also indicate that the insulin burst release is significantly more reduced with composite microparticles than with both insulin nanoparticles and simple microparticles confirming the results observed *in vitro*.

**Table 1:** Mean diameter, drug encapsulation efficiency and zeta potential of blank or insulin loaded PCL nanoparticles (NP) (n = 3 ± SD).

| | Blank NP | Insulin NP |
|---|---|---|
| Mean diameter (nm) | 375 ± 6.0 | 390 ± 17 |
| Zeta Potential (mV) | - 1.3 ± 1.6 | 0.2 ± 0.6 |
| Encapsulation efficiency (%) | - | 80 ± 4 |

**Table 2:** Mean diameter and drug encapsulation efficiency of blank, insulin simple microparticles (SMP) and composite microparticles (CMP) (n = 3 ± SD).

| | Mean diameter (µm) | | Encapsulation efficiency (%) | |
|---|---|---|---|---|
| | Blank | Insulin | Blank | Insulin |
| SMP | 92 ± 4 | 71 ± 7 | - | 90 ± 3 |
| CMP | 113 ± 2 | 111 ± 4 | - | 99 ± 0.3 |

**Table 3:** Mean percentage (n = 3 ± SD) of insulin released after 15 minutes and 24 hours from PCL NP, PLGA simple (SMP) and PLGA composite (CMP) microparticles in vitro.

| formulations | Polymers | Insulin released (%) | |
|---|---|---|---|
| | | 15 min | 24 h |
| **Insulin solution** | **-** | **95 ± 2** | **30 ± 3** |
| PCL NP | PCL | 40.8 ± 0.1 | 49.8 ± 3.0 |
| PLGA SMP | PLGA | 36.4 ± 2.0 | 56.5 ± 2.0 |
| PLGA CMP | PLGA (PCL) | 19.3 ± 0.2 | 39.4 ± 1.7 |

**Table 4:** Mean insulin serum concentrations (µU/ml) in diabetic rats 30 minutes and 8 hours after an intravenous injection of insulin solution (1 IU/kg) or a subcutaneous injection of polymeric insulin loaded particles (20 IU/kg) (n = 4 ± SD). * statistically different from PCL nanoparticles ($p < 0.05$)

| formulations | Polymers | Insulin serum concentration (µU/ml) | |
|---|---|---|---|
| | | 30 min | 8 h |
| No injection | - | 22 ± 2.2 | 22 ± 2.2 |
| Insulin solution (IV) | - | 262 ± 190 | 16 ± 2 |
| Free insulin loaded CMP (SC) | PLGA (PCL) | 30 ± 5 | 21 ± 6 |
| PCL NP (SC) | PCL | 2 389 ± 280 | 47 ± 7 |
| PLGA SMP (SC) | PLGA | 582 ± 54 | 83 ± 24 |
| PLGA CMP (SC) | PLGA (PCL) | 433 ± 71* | 145 ± 20 |

**Table 5**: Main insulin pharmacokinetic parameters after subcutaneous administration to rats (n = 3 ± SD) of different formulations: insulin solution, PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP). * statistically different from PCL nanoparticles ($p < 0.05$).

| | Insulin solution IV | PCL NP SC | PLGA SMP SC | PLGA CMP SC |
|---|---|---|---|---|
| Tmax (hours) | 0.033 | 1 | 2 | 4 |
| Cmax (µIU/mL) | 5 109 ± 467 | 2 872 ± 366 | 959 ± 128* | 435 ± 21* |
| Mean AUC | | | | |
| AUC 0 → 1 | 662 | - | - | - |
| AUC 0 → 10 | 662 | 8 704 | 3 832 | 3 096* |
| AUC 0 → 24 | 662 | 8 704 | 4 680 | 4 463 |
| Rel. F (%) | | | | |
| (0-10h) | 100 | 65.8 | 28.9 | 23.4 |
| (0-24h) | 100 | 65.8 | 35.4 | 33.7 |
| (0-48h) | 100 | 65.8 | 35.4 | 48 |

**Figure 1:**

Release profiles of insulin [mean cumulative release (%) versus time (hours)]from PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP) in pH 7.4 phosphate buffer under sink conditions (n=3 ± SD).

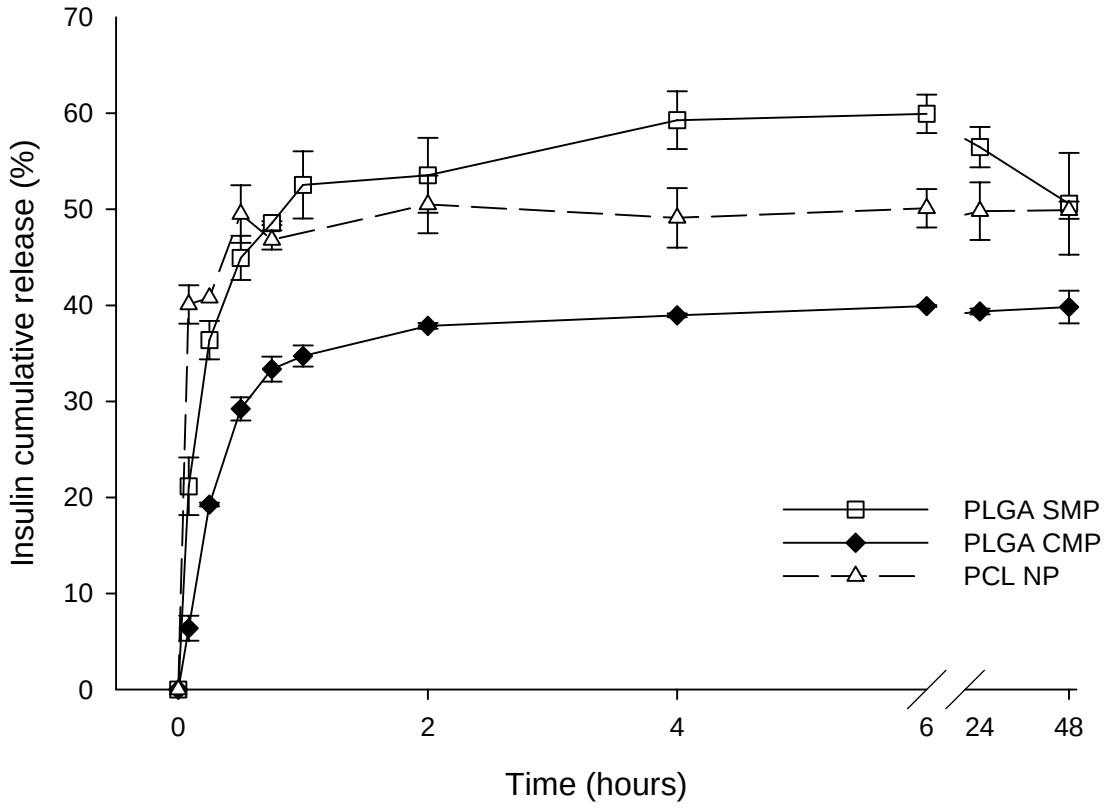
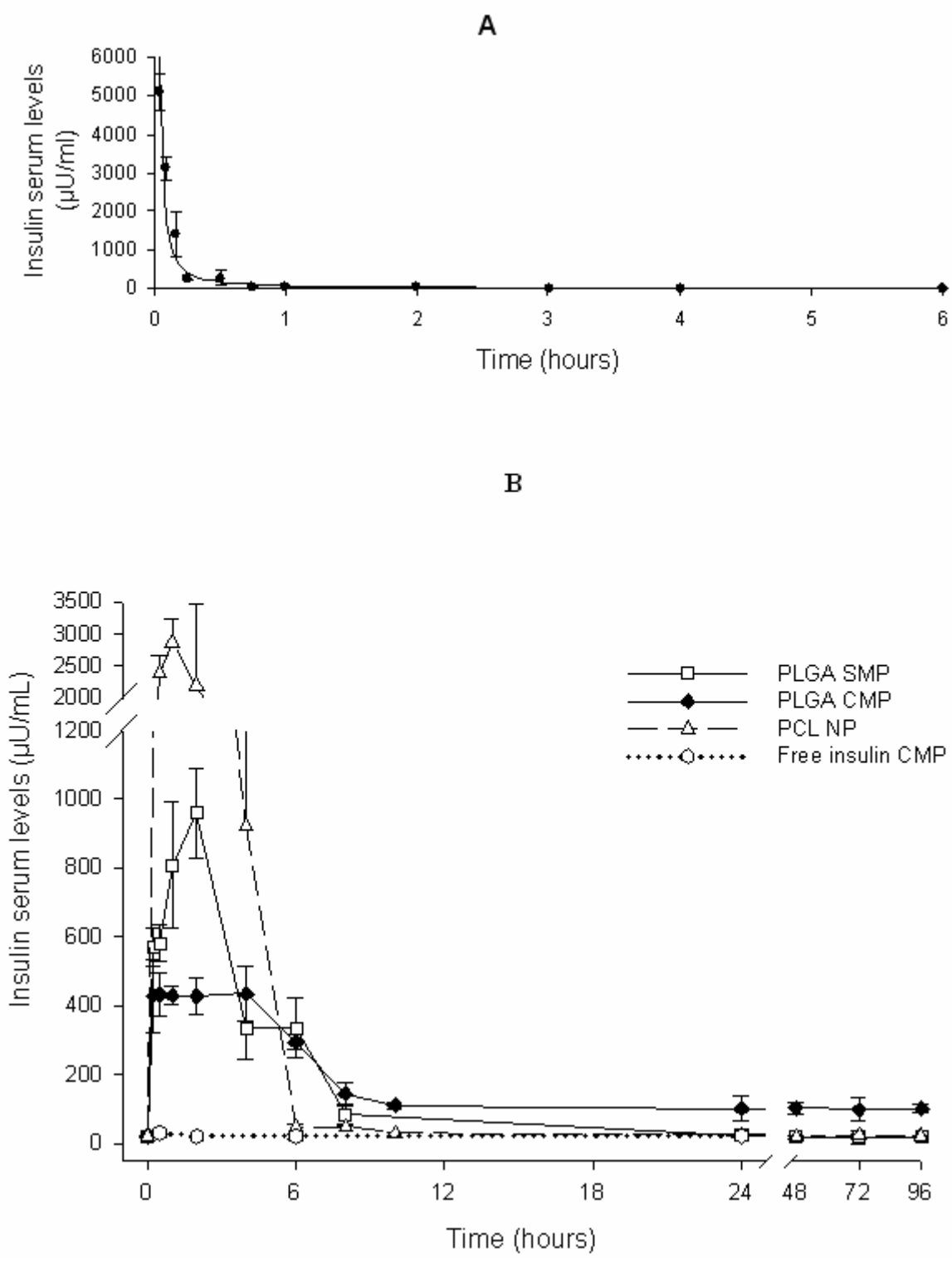


**Figure 2**.

Mean insulin serum concentration versus time (hours) after intravenous injection of 1 IU/kg of insulin as a solution (A) or after subcutaneous injection of 20 IU/kg of insulin [PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP) (B)] to rats (n = 3 ± SD).



## 5. Discussion

Recent improvements in insulin formulations and delivery, including ultra-fast acting and intermediate to "long-acting" (once a day) injections have allowed the development of basal-bolus insulin administration regimens that better mimic the normal pattern of insulin secretion [23][24]. By combining existing delivery technologies, a simple method was developed to manufacture a unique long-acting insulin formulation that shows promise of providing prolonged basal serum insulin levels.

Optimal sustained release insulin dosage forms for parenteral formulation should have a high insulin loading together with a significant burst reduction. As mentioned before, drug burst release may occur due to high drug water solubility, non homogeneous drug distribution in matrix systems and/or drug adsorption on surface of the different delivery systems. Insulin formulations (nanoparticles, microparticles, liposomes and hydrogels) frequently display an initial burst release. For instance, Reis et al. [25] prepared insulin-loaded alginate-dextran nanospheres which showed a high in vitro burst effect at physiological pH (80% of insulin release after 30 min.) but almost zero at pH 1.2. Such a burst effect (high at pH 7.5 and zero at pH 1.2) may be advantageous in the case of formulations intended for oral or pulmonary administration. At the opposite when insulin formulations are intended for parenteral administration, burst release is a major drawback especially when sustained-release is needed.

Among the different attempts to reduce the insulin burst effect, composite structures were already prepared by combination of different techniques: particles coating [26], nanoparticles microencapsulation [27], gel or insulin /cyclodextrin complex in microparticles [28-30] and gel containing particles (nanoparticles or liposome,) [31] [32]. Due to an homogenous distribution of drug loaded particles (gel, nanoparticles, liposomes) throughout the polymer matrix, microparticles seem to be the most effective to reduce the initial burst. Except for Jiang et al. [30] who showed a successful 10-days glucose suppression in diabetic rats after subcutaneous administration of gel in PLGA composite microspheres, there is very little information on the *in vivo* behaviour of such composite systems.

On the other hand, heterogeneous composite systems based on hydrophilic polymers cannot effectively control the burst effect of insulin [11]. In addition, some composite systems were prepared by combining hydrophobic polymers (PLGA, ethylcellulose) with hydrophilic inner polymers (acryloyl hydroxyethyl starch, cyclodextrin complex, gelatin). Such systems were shown to stabilize the entrapped insulin and to incorporate insulin very efficiently (≥ 80%)

but they always displayed, *in vitro*, a rapid and initial burst release of insulin (up to 40%) followed by a slow release.

This is the reason why we have proposed composite microparticles made with hydrophobic polymers. However, the encapsulation of a lipophilic polymer (as nanoparticles) in another lipophilic polymer (as microparticles) is really challenging with classical microparticles techniques.

So, the use of insulin in our composite microparticles would certainly have the potential to eliminate or at least decrease the burst effect. Furthermore, it could be interesting to develop a new injectable and possibly once a day dosage form of insulin (drug with a narrow therapeutic index so with serious risk of side effects from high serum levels), a short elimination half-life drug [33].

However, with a view to injecting the composite microparticles subcutaneously, it was mandatory to use two biodegradable polymers. This is why PLGA was selected as the polymer of microparticles.

In our already mentioned previous work [11], the original idea was to use a solvent (ethyl acetate) in which one of the polymers (PCL) would not be soluble during the double emulsion process. Replacing the previous non biodegradable polymers (ethylcellulose, Eudragit® RS) by PLGA did not change anything since the latter is also soluble in ethyl acetate. Indeed, microparticles were prepared by dissolving PLGA in ethyl acetate which is a poor solvent for PCL. Therefore, it was possible to use the PCL nanoparticles suspension as the internal aqueous phase in the preparation of the composite microparticles since this polymer is mostly not dissolved in ethyl acetate.

PCL nanoparticles were found in the classical particles size range i.e. under 500 nm [34]. It has to be noticed that loaded and unloaded nanoparticles bear almost no charge.

Insulin was encapsulated with a high efficiency both in nanoparticles and microparticles (simple microparticles or composite microparticles). This high encapsulation efficiency of insulin especially with microparticles may be explained by i) the use of a pre-emulsification step into a smaller volume of external aqueous phase before the final organic phase extraction (necessary in order to avoid premature polymer precipitation and to obtain microparticles) and ii) the overall fast PLGA precipitation when ethyl acetate was extracted in water [4] [20]. However, the encapsulation efficiency of insulin in PLGA composite microparticles (99 %) was higher than in both PLGA simple microparticles and PCL nanoparticles (90 and 80 % respectively). This might be due to the use of the insulin solid suspension as internal phase

(S/W/O/W).Thus, it would consequently be more difficult for insulin to diffuse towards the outer water phase than in the case of the two others particles types.

There are two ways to verify that the burst is controlled i.e. either by an *in vitro* dissolution test or by an *in vivo* approach for instance after subcutaneous or intramuscular administration. We have used both approaches to verify the potential burst reduction with composite microparticles.

*In vitro* (fig. 1 and table 3), it is obvious that the lowest burst was achieved with the composite microparticles but the same burst effect was observed with PCL nanoparticles and PLGA simple microparticles. Indeed, the burst at 15 minutes was almost 2 times more with the simple microparticles or with the PCL nanoparticles.

Several hypotheses can be taken into account to explain the insulin burst. Among them, the heterogeneous distribution of insulin including surface distribution, physico-chemical nature of the polymeric matrix, morphology of particles and insulin/matrix interactions are likely to happen. In the case of PCL nanoparticles, the main factor driving insulin release is the large exchange surface area developed with the outer medium due to the very small diameter of nanoparticles. Although PLGA simple microparticles have larger size (300 times more) than PCL nanoparticles, insulin burst effect is almost the same for two formulations. The size effect can be balanced by relative hydrophobicities of polymers. Indeed, PCL is more hydrophobic than PLGA and water uptake is faster for PLGA leading to faster insulin diffusion to the PBS external buffer in the case of PLGA simple microparticles.

Since simple and composite microparticles have been prepared according to the same conditions, differences in process and formulation parameters affecting insulin burst release can be ruled out. However, for composite microparticles it is not the drug but the PCL nanosuspension which is distributed in the PLGA polymer matrix; thus drug distribution could be different between simple and composite microparticles. Therefore, composite microparticles present mixed characteristics of both PCL nanoparticles and PLGA simple microparticles.

As for the polymer matrix, it is also obvious that, in composite microparticles, there are two barriers for the drug to diffuse through before reaching the outside release medium. The first barrier is due to the PCL polymer of nanoparticles and the second barrier is the outer PLGA matrix: so we can assume that there is no insulin on the particles surface in the case of PLGA composite microparticles. It is reasonable to make the assumption that the double layer of polymers is the main parameter explaining the marked *in vitro* burst reduction.

As observed in figure 1, composite microparticles release insulin very slowly since only around 39 % of insulin is found in the outer phase after 24 h. This is another interesting feature of the composite microparticles whose very slow release pattern could also be of interest in the development of a long lasting injectable dosage form.

Although the entire encapsulated insulin was not released within 24 h, the dissolution test was limited to this time since the goal of this research work was to demonstrate the influence of the encapsulation of nanoparticles into microparticles on the initial burst release.

However, it has to be noted that prolonging the time of the release study does not always lead to a 100 % insulin release. Indeed, Furtado *et al.*, [17] carried out an *in vitro* release study with insulin microparticles made of poly (fumaric-co-sebacic anhydride) and achieved only 40 % of insulin release after 12 days. In addition, Jiang et al., [30] prepared insulin composite microparticles made of an inner hydrogel [poly (acryloyl hydroxyethyl starch)] in a PLGA matrix and after 8 days of *in vitro* test, they only found 5 % of insulin released in a glycine buffer (pH 2.8).

Due to their small average diameter, the suspension of nanoparticles was easily injected subcutaneously. As for the simple microparticles and composite microparticles, it was first necessary to disperse them in a relatively viscous aqueous solution prior to injection. The carboxymethylcellulose aqueous suspension of simple microparticles and composite microparticles also allowed an easy subcutaneous injection and was characterized by i) a good physical stability (no sedimentation observed during preparation and injection), ii, good syringability (21 G) and iii) a physiological pH of 7.4.

It has also to be mentioned that different experimental models of diabetes and different dosages of insulin reported in the literature make results comparison very difficult. For instance, Furtado *et al.*, [17] used rats which are predisposed to spontaneously develop type I diabetes via cell-mediated autoimmune destruction of the pancreatic β cells, whereas Takenaga et al., [12] [30] used rats with streptozotocin-induced diabetes. Administrated doses of insulin ranged from 45 IU/kg [17], 250 IU/kg [12] to 345 IU/kg [30]. In our case, we have chosen to administer the lowest dose possible which was 20 IU/kg. Other differences included sampling fluids (plasma [12] [17] or serum [30]), insulin dosage method (ELISA [17] or RIA [30]) and type of insulin (human [12] or bovine [29]).

As already observed, the insulin nanoparticles display a fast absorption (Tmax 1 h) whereas absorption is much slower for simple microparticles (Tmax 2 h) and even more delayed for composite microparticles (Tmax 4 h). As well known for matrix microparticles, diffusion of the drug through the microparticles is generally the rate limiting step of absorption. The

diffusion pathway is even longer for the composite microparticles due to the double layer of polymers.

Cmax and Tmax are parameters which reflect the *in vivo* absorption rate of a drug. Therefore the initial burst is correlated to the values of the two latter parameters. In terms of burst effect, the three dosage forms can be ranked as: PCL nanoparticles > simple microparticles > composite microparticles. There is a significant difference (six fold times) in Cmax between the PCL nanoparticles and the PLGA composite microparticles. In addition, the Tmax are also very different between the two dosage forms (1 and 4 h for PCL nanoparticles and PLGA composite microparticles). The difference in Cmax between the composite microparticles and simple microparticles is less spectacular but is still of about 2.2 times whereas Tmax are 2 and 4 h for simple microparticles and composite microparticles, respectively. However, despite such difference in Cmax between composite and simple microparticles, it was not statistically different. Larger groups of animals should probably be used if one wants to demonstrate a significant difference.

Thus it is confirmed that the microparticle systems, and more particularly the composite microparticles, have a dramatic influence on the initial burst release *in vivo*. The same hypotheses, as already discussed for the *in vitro* dissolution results, may explain the observed *in vivo* results. It has been definitely confirmed that the composite microparticles present a strong potential in reducing the burst effect not only *in vitro* but also *in vivo*.

The *in vivo* study was performed for 96 h in order to evaluate the potential of the 3 multiparticular dosage forms as prolonged release compositions. The insulin serum concentrations reach the quantification limit of insulin in diabetic rats after 10 h and 24 h for PCL nanoparicles and PLGA simple microparticles, respectively. It probably means that insulin release (based on diffusion mechanism) from particles stopped and the second potential insulin release mechanism (based on polymers degradation) did not begin at least up to 96 h. For the PLGA composite microparticles, the insulin initial serum level for diabetic rats (22 µU/ml) was not reached (insulin serum concentration at 96 h is 101 µU/ml) after the $4^{th}$ day of the *in vivo* test which means that insulin was still released from particles till 96 h. Nevertheless, this definitely demonstrates that insulin is released for the longest period of time with composite microparticles and insulin release from particles is diffusion controlled till the next mechanism of release (based on degradation polymers) may eventually start. However, due to a sampling period limited to 96 h, we were not able to observe this later release phase.

However, taking into account the relatively low number of animals and the normal *in vivo* variability, the figures of absolute bioavailability for simple and composite microparticles are relatively close and it could be considered that the quantitative bioavailability are the same at 24 h (around 35%); indeed, this is confirmed by the lack of statistically difference between all the dosage forms. In the case of composite microparticles, the bioavailability increased with time because the insulin quantification limit was not reached till 96 h. This shows the great potential of such dosage forms to act as long release microparticles. Our bioavailability results are lower than the ones found by Furtado et al., [17] after subcutaneous administration of insulin poly(fumaric-co-sebacic anhydride) microparticles, who obtained an absolute bioavailability of 61%. However, our composite microparticles still have a potential to release insulin after 96 h and bioavailability could still be increased.

Our composite microparticles are characterised by delayed insulin Tmax and a decrease in Cmax. In control experiments, a bolus of insulin was injected; it enters into the circulation and bombards the tissues at a rate faster than these tissues can process the insulin. Consequently, tissue receptors quickly become saturated and the remaining unbound insulin is rapidly degraded by virtue of its short half-life [35]. The pharmacokinetic data of our work and other groups [12] [17] [30] imply that unencapsulated (or fast insulin release formulation as simple nanoparticles) insulin has an extremely high rate of absorption for a very brief period of time at the start of the experiment after which, the insulin has been taken up or degraded and is no longer present in the body for the remainder of the experiment. The inherent disadvantage with this type of un encapsulated insulin or fast insulin release formulation is that the short insulin duration fails to provide insulin for controlled glucose regulation throughout the entire experiment and is therefore unable to maintain basal plasma glucose levels past the initial hours of the experiment. In this respect, subcutaneous delivery of fast insulin release formulation fails to mimic the normal physiological insulin secretion. Additionally, excessive exposure of tissues and muscle to injected insulin increases insulin resistance and diabetes complications.

In contrast, the insulin composite microparticles (and to a lower degree the simple microparticles) degrade slowly over a period of several days or weeks, thereby providing closely controlled insulin diffusion throughout most of the experiment. The implications of such a system (average Tmax around 4h, average insulin Cmax of only 435µU/ml and minimum insulin concentration of 100µU/ml at 96 h) show that insulin is absorbed at a slower rate for a longer duration. The major benefit of this system is that insulin is presented to tissue receptors at a constant rate, as opposed to pure insulin injections where an overload of insulin

results in a high degree of insulin degradation. This prevents excessive tissue and muscle exposure to insulin and makes insulin available to regulate glucose levels in the body for a substantially longer time than subcutaneous unencapsulated insulin administrations. Therefore, this type of release more closely resembles the body's normal physiological processes whereby insulin is constantly secreted to keep plasma glucose at a basal level.

## 6. Conclusion:

Our results confirm the efficacy of composite microparticles to obtain high insulin encapsulation efficiency and limited burst effect with a good sustained release. Such insulin composite microparticles present an interesting behaviour both *in vitro* and *in vivo*. One way to increase or decrease the release rate of drugs from such composite microparticles would be to play on the type of biodegradable polymers used in their manufacturing. Indeed, higher or smaller molecular weights of the 2 types of polymers could lead to taylor made release. Such an approach will be used in the continuation of this work.

It can be concluded that composite microparticles gave better *in vivo* profile than simple PLGA microparticles. Longer *in vivo* studies (> 96 h) should be undertaken in order to verify whether insulin can still be recovered in serum after polymer degradation.

## References

[1] Y. Shi, L.C. Li, Current advances in sustained-release systems for parenteral drug delivery, Expert Opin Drug Deliv. 2 (2005)1039-58.

[2] H. Reithmeier, J. Herrmann, A. Gopferich, Lipid microparticles as a parenteral controlled release device for peptides, J. Control. Release 73 (2001) 339–350.

[3] X. Huang, C.S. Brazel, On the importance and mechanisms of burst release in matrix-controlled drug delivery systems, J. Control. Release 73 (2001)121-136.

[4] Y. Yeo, K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems, Arch. Pharm. Res. 27 (2004)1-12.

[5] R. C. Mehta, B. C. Thanoo, P.P. DeLuca, Peptide containing microspheres from low molecular weight and hydrophilic poly(DL-lactide-co-glycolide), J. Control. Release 41(1996) 249–257.

[6] Y.Y. Yang, T.S. Chung, N. Ping Ng, Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method, Biomaterials 22 (2001) 231-241.

[7] S. Nicoli, P. Santi, P. Couvreur, G. Couarraze, P. Colombo, E. Fattal, Design of triptorelin loaded nanospheres for transdermal iontophoretic administration, Int. J. Pharm. 214 (2001) 31-35.

[8] J. Wang, K.M. Chua KM, C.H. Wang, Stabilization and encapsulation of human immunoglobulin G into biodegradable microspheres, J Colloid Interface Sci 271 (2004) 92-101.

[9] A. Aubert-Pouëssel, M.C. Venier-Julienne, A. Clavreul, M. Sergent, C. Jollivet, C.N. Montero-Menei, E. Garcion , D.C. Bibby, P. Menei, J.P. Benoit, In vitro study of GDNF release from biodegradable PLGA microspheres, J. Control Release 95 (2004) 463-475.

[10] A. Sánchez , M. Tobío, L. González, A. Fabra , M.J. Alonso, Biodegradable micro- and nanoparticles as long-term delivery vehicles for interferon-alpha, Eur. J. Pharm. Sci. 18 (2003) 221-229.

[11] A. Sheikh Hasan, M. Socha, A. Lamprecht, F. Ghazouani, A. Sapin, M. Hoffman, P. Maincent, N. Ubrich, Effect of the microencapsulation of nanoparticles on the reduction of burst release, Int. J. Pharm., 344 (2007) 53-61.

[12] M. Takenaga, Y. Yamaguchi, A. Kitagawa, Y. Ogawa, S. Kawai, Y. Mizushima, R. Igarashi, Optimum formulation for sustained-release insulin, Int. J. Pharm. 271 (2004) 85-94.

[13] Y. Yamaguchi, M. Takenaga, A. Kitagawa, Y. Ogawa, Y. Mizushima, R. Igarashi, Insulin-loaded biodegradable PLGA microcapsules: initial burst release controlled by hydrophilic additives, J. Control. Release 81 (2002) 235-249.

[14] T. Haak, New developments in the treatment of type 1 diabetes mellitus, Exp. Clin. Endocrinol. Diabetes 107 (1999) S108–S113.

[15] K.D. Hinds, K.M. Campbell, K.M. Holland, D.H. Lewis, C.A. Piche, P.G. Schmidt, PEGylated insulin in PLGA microparticles. In vivo and in vitro analysis, J. Control. Release, 104 (2005) 447-460.

[16] M. Takenaga, Y. Yamaguchi, A. Kitagawa, Y. Ogawa, Y. Mizushima, R. Igarashi,
A novel sustained-release formulation of insulin with dramatic reduction in initial rapid release, J. Control. Release 79 (2002) 81-91.

[17] S. Furtado, D. Abramson, L. Simhkay, D. Wobbekind, E. Mathiowitz, Subcutaneous delivery of insulin loaded poly(fumaric-co-sebacic anhydride) microspheres to type 1 diabetic rats, Eur. J. Pharm. Biopharm., 63 (2006) 229-236.

[18] S. Choi, S. W. Kim, Controlled release of insulin from injectable biodegradable triblock copolymer depot in ZDF rats, Pharm. Res. 20 (2003) 2008-2010.

[19] V. Hoffart, N. Ubrich, C. Simonin, V. Babak, C. Vigneron, M. Hoffman, T. Lecompte, P. Maincent, Low molecular weight heparin-loaded polymeric nanoparticles: formulation, characterization, and release characteristics, Drug Dev. Ind. Pharm. 28 (2002) 1091–1099.

[20] T. Freytag, A. Dashevsky, L. Tillman, G.E. Hardee, R. Bodmeier, Improvement of the encapsulation efficiency of oligonucleotide-containing biodegradable microspheres, J. Control. Rel. 69 (2000) 197-207.

[21] P. Moslemi, A.R. Najafabadi, H. Tajerzadeh, A rapid and sensitive method for simultaneous determination of insulin and A21-desamido insulin by high performance liquid chromatography, J. Pharm. Biomed. Anal. 33 (2003) 45–51.

[22] C. Damgé, C. Michel, M. Aprahamian P. Couvreur, New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier, Diabetes 37 (1988) 246–251.

[23] J. Gerich, Novel insulins: expanding options in diabetes management, Am. J. Med. 113 (2002) 308–316.

[24] J. Mayfield, R. White, Insulin therapy for type 2 diabetes: rescue, augmentation and replacement of beta-cell function, Am. Fam. Physician 70 (2004) 489–500.

[25] C.P. Reis, A.J. Ribeiro, S. Houng, F. Veiga, R.J. Neufeld, Nanoparticulate delivery system for insulin: design, characterization and in vitro/in vivo bioactivity, Eur. J. Pharm. Sci. 30 (2007) 392-397.

[26] Y.F. Fan, Y.N. Wang, Y.G. Fan, J.B. Ma, Preparation of insulin nanoparticles and their encapsulation with biodegradable polyelectrolytes via the layer-by-layer adsorption, Int. J. Pharm. 324 (2006) 158-167.

[27] A. Grenha, B. Seijo, C. Remuñán-López, Microencapsulated chitosan nanoparticles for lung protein delivery, Eur. J. Pharm. Sci. 25 (2005) 427-437.

[28] R.L. Graves, M.C. Makoid, S. Jonnalagadda, The effect of coencapsulation of bovine insulin with cyclodextrins in ethylcellulose microcapsules, J. Microencapsul. 22 (2005) 661-670.

[29] G. De Rosa, D. Larobina, M. Immacolata La Rotonda, P. Musto, F. Quaglia, F. Ungaro, How cyclodextrin incorporation affects the properties of protein-loaded PLGA-based microspheres: the case of insulin/hydroxypropyl-beta-cyclodextrin system, J. Control. Release. 102 (2005) 71-83.

[30] G. Jiang, W. Qiu, P.P. DeLuca, Preparation and in vitro/in vivo evaluation of insulin-loaded poly(acryloyl-hydroxyethyl starch)-PLGA composite microspheres, Pharm. Res. 20 (2003) 452-459.

[31] J.M. Barichello, M. Morishita, K. Takayama, T. Nagai, Absorption of insulin from pluronic F-127 gels following subcutaneous administration in rats, Int. J. Pharm. 184 (1999) 189-198.

[32] A.L. Weiner, S.S. Carpenter-Green, E.C. Soehngen, R.P. Lenk, M.C. Popescu, Liposome-collagen gel matrix: a novel sustained drug delivery system, J Pharm Sci.74(1985) 922-5.

[33] www.theriaque.org, Update 08/04/2008, view 8 April.

[34] D. Attivi, P. Wehrlé, N. Ubrich, C. Damgé, M. Hoffman, P. Maincent, Formulation of insulin-loaded polymeric nanoparticles using response surface methodology, Drug Dev. Ind. Pharm. 31 (2005)179-189.

[35] D.J. Chetty and Y.W. Chen, Novel methods of insulin delivery: an update, Critical Reviews in Therapeutic Drug Carrier Systems 15 (1998) 629–670.

# DISCUSSION GENERALE

Ces dernières années, différentes formes particulaires ont été élaborées pour permettre de délivrer dans l'organisme, par voie parentérale, des molécules thérapeutiques (Sinha *et al*., 2005). Certaines de ces formes développées sont des particules synthétisées à base de polymères qui délivrent progressivement le principe actif, par diffusion du principe actif dans la matrice jusqu'au milieu extérieur, mais aussi tout au long de la dégradation du polymère avec une forme biodégradable (Shi *et al.,* 2005). La plupart des études s'intéressant à ce type de particules évoque une libération initiale rapide du principe actif, difficile à contrôler, appelée « effet burst » (« burst effect » en anglais) (Shi *et al.,* 2005, Sinha *et al*., 2005). Cette libération initiale pose plusieurs problèmes. Elle peut avoir des conséquences cliniques importantes pouvant expliquer une toxicité initiale liée à une concentration trop élevée du principe actif dans l'organisme. De plus, en dehors des problèmes toxiques éventuels, la libération initiale d'une quantité importante de principe actif a pour conséquence une diminution de la quantité encapsulée. Ainsi, cela peut aussi entraîner des modifications de libération dans les dernières semaines de la vie des systèmes multiparticulaires soit en ralentissant la cinétique de libération du principe actif soit en abaissant sa concentration sanguine (Heya et al., 1994 ; Okada et al., 1995 ; Shi *et al.,* 2005). Les causes de cette libération sont multiples et les moyens employés par différentes équipes sont variés. Le développement et l'évaluation d'une nouvelle forme particulaire permettant de diminuer l'effet « burst » ont donc constitué l'objectif du travail présenté ici.

## I. Développement de microparticules composites :

Pour réduire l'effet « burst », les microparticules composites présentent plusieurs atouts. Plusieurs types de microparticules composites ont été décrits ces dernières années : dans ces systèmes le principe actif est complexé avec des cyclodextrines par exemple (De Rosa *et al*., 2005; Zheng *et al*., 2006 ) ou encapsulé dans des nanoparticules (nanoparticules de polymère (Li et al., 1997), liposomes (Dhoot *et al*., 2003) ou hydrogel (Mandal *et al*., 2002 ; Jiang *et al*., 2003). Ces différents vecteurs sont eux-mêmes encapsulés dans des microparticules généralement de nature polymérique. Plusieurs études *in vitro* et *in vivo* ont montré l'efficacité de ces systèmes à réduire l'effet « burst », notamment en permettant une meilleure homogénéité du principe actif dans la matrice et donc le contrôle de sa diffusion dans cette double paroi. Les systèmes décrits jusqu'alors présentent quelques limites résidant notamment dans l'utilisation d'excipient ou de polymère de nature hydrophile. En effet, ces constituants favorisent l'entrée d'eau dans la matrice et ainsi la fuite du principe actif vers le milieu extérieur (Zheng *et al*., 2006 ; Chung *et al*., 2006 ; Wang *et al*., 2004). Cette entrée

d'eau peut également entraîner le gonflement des particules, l'augmentation de la taille des pores voire même l'éclatement des particules. C'est pourquoi nous nous sommes proposés de développer des particules composites uniquement à base de polymères hydrophobes, polymères limitant l'entrée d'eau et la diffusion du principe actif vers le milieu extérieur. L'élaboration de tels systèmes constitue un véritable challenge et constitue toute l'originalité de ce travail.

Ainsi, notre projet consistait à formuler des nanoparticules de polycaprolactone (PCL) - polymère hydrophobe - elles-mêmes encapsulées dans des microparticules préparées selon la technique de double émulsion et composées de polymères hydrophobes tels que l'éthylcellulose, un polyméthylmétacrylate (Eudragit® RS) ou un copolymère d'acide lactique et glycolique (PLGA). D'un point de vue technique cela nécessitait l'utilisation d'un polymère très hydrophobe pour la formulation des nanoparticules. Les microparticules étaient alors formulées dans un solvant organique, non-solvant des nanoparticules. Cette différence d'hydrophobicité des deux barrières polymériques entourant le principe actif devait permettre d'une part de ralentir la diffusion d'eau vers l'intérieur de la matrice et d'autre part de maintenir le principe actif le plus longtemps possible à l'intérieur des particules.

Le phénomène de « burst » dépend aussi du principe actif utilisé. Un « burst » important est particulièrement visible pour des formes particulaires libérant des principes actifs de faible masse moléculaire (Li *et al*., 2005) et dépend de la solubilité de ce dernier dans l'eau (Thote *et al*., 2005). C'est ainsi que dans nos premiers travaux, nous nous sommes intéressés à deux molécules de faible masse moléculaire et différant de par leur niveau de solubilité dans l'eau : l'ibuprofène, molécule de nature lipophile et l'acétate de triptoréline, décapeptide de nature hydrophile. Ces « molécules modèles » ont permis de mettre au point le protocole de formulation des microparticules composites, sur la base des travaux réalisés préalablement au laboratoire (Hoffart *et al*., 2003 ; Attivi *et al*., 2005). Dans cette première étude, des nanoparticules de PCL contenant l'un ou l'autre des principes actifs ont été formulées selon la technique de double émulsion. Puis elles ont été encapsulées par cette même méthode, dans des microparticules d'Eudragit RS ou d'éthylcellulose, polymères utilisés seuls ou en mélange (1 :1), et solubilisés dans l'acétate d'éthyle. La méthode de double émulsion s'est en effet imposée d'elle-même car il était nécessaire d'avoir une phase aqueuse continue et une phase aqueuse interne contenant la suspension de nanoparticules. La solution organique de polymère devait alors séparer les deux phases aqueuses. Les particules obtenues présentaient des caractéristiques intéressantes :

- un taux d'encapsulation important, atteignant même 85% pour les deux principes actifs en utilisant l'ethylcellulose seule. Ceci peut être attribué à la viscosité élevée de l'ethylcellulose dans l'acétate d'éthyle, empêchant ainsi la diffusion du principe actif, quel qu'il soit, vers le milieu extérieur lors de la préparation des microparticules composites.
- Une taille variable selon la nature du polymère et du principe actif utilisé, dépendant principalement d'interactions liées aux charges présentes sur le polymère (Eudragit RS) ou au principe actif (triptoréline). De plus, il est à noter que toutes les microparticules composites synthétisées présentaient des tailles supérieures aux microparticules simples (ne contenant pas de nanoparticules), confirmant ainsi l'encapsulation des nanoparticules dans les microparticules à la fin du procédé de fabrication.
- Des études *in vitro* de libération au cours du temps des principes actifs ont été réalisées en comparant les profils obtenus pour les nanoparticules, les microparticules simples et les microparticules composites. Les profils obtenus différaient selon le principe actif et la nature des polymères utilisés mais ont montré une réduction de l'effet « burst », plus ou moins marquée, dans le cas des microparticules composites.

En conclusion, nous avons développé des microparticules composites, capables de libérer *in vitro* de façon contrôlée des principes actifs de faible masse moléculaire et de nature lipophile et hydrophile. Afin de pouvoir évaluer l'efficacité de ces systèmes *in vivo*, administrés par voie parentérale, il nous fallait disposer de techniques de dosage suffisamment sensibles des principes actifs d'intérêt dans le plasma. La seconde partie de notre travail a donc consisté à mettre en place au laboratoire et à valider une technique de dosage de l'ibuprofène dans le plasma par chromatographie liquide à haute performance (CLHP).

## II.Mise au point et validation d'une technique de dosage de l'ibuprofène dans le plasma par méthode CLHP :

Dans la littérature, la limite de détection de l'ibuprofène dans le plasma par technique CLHP se situe à 1µg/mL, limite suffisante pour déceler l'effet pharmacologique de l'ibuprofène dont la concentration thérapeutique est de l'ordre de 10µg/mL (Doshi Abha and Deshpande SG 2007). Cette valeur relativement élevée de la concentration thérapeutique est liée à la dose administrée chez l'Homme. En effet, cette dose est elle-même élevée puisque pouvant atteindre 200 à 400 mg per os, en une seule prise, ce qui revient à environ une dose de 3 à 6 mg/kg chez l'Homme. Dans notre étude chez le rat, la dose administrable était limitée par la

quantité de microparticules qui pouvaient être injectées en une seule fois. Ceci nous a obligés à n'administrer qu'une dose de 1 mg/kg chez le rat. Cette plus faible dose administrée, liée à l'administration de formes à libération prolongée a nécessité la mise au point d'une méthode analytique plus sensible dans le décours de l'étude pharmacocinétique. Cette méthode couplait l'utilisation d'une méthode CLHP à pH = 6,5, couplée à un détecteur à fluorescence. A ce pH, l'ibuprofène est déprotoné, chargé négativement (Manzoori *et al.,* 2003 ; Wang *et al*., 2005). Travailler à ce pH a permis d'augmenter la sensibilité de la méthode (10 fois plus qu'au pH = 2,5 classiquement utilisé) (Manzoori *et al.,* 2003, Sheikh Hassan A *et al*., 2008).

Disposant ainsi d'une méthode de dosage de l'ibuprofène suffisamment sensible pour le détecter dans des échantillons de plasma de rats, nous avons engagé l'étude de la libération *in vivo* de l'ibuprofène à partir de microparticules composites.

## III.Evaluation *in vivo* de l'effet de microparticules composites libérant de l'ibuprofène.

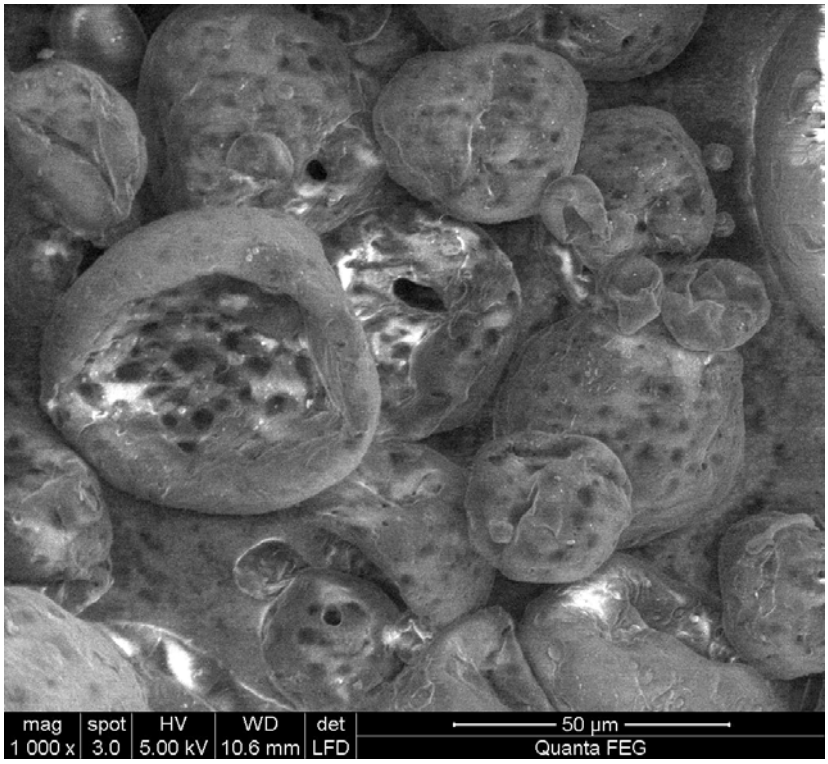
Pour élaborer une formulation plus adaptée à l'administration d'ibuprofène sous-cutanée chez le rat, certains paramètres de formulation ont été modifiés :

- Les polymères d'éthylcellulose et d'Eudragit RS ont été remplacés par le PLGA 504S (50/50 ; « endcapped », MW 48000), polymère biodégradable et soluble dans l'acétate d'éthyle. De plus, ce PLGA présente une Tg de 48°C, compatible avec une libération dans les conditions physiologiques à 37°C, ce qui n'altèrera pas la structure particulaire. Ce PLGA se dégrade plus lentement que d'autres PLGA, en raison de sa structure plus lipophile due à l'estérification de la fonction carboxylique terminale.
- Le Span 60 a été ajouté à la formulation pour limiter la formation de filaments de polymère liée à une précipitation trop rapide du polymère au contact de la phase aqueuse. En outre, ce surfactif ajouté dans la phase organique stabilise la première émulsion eau dans huile.
- L'étape de centrifugation pour récupérer les nanoparticules de PCL a été remplacée par une étape de concentration de la suspension de nanoparticules par une prolongation de l'évaporation jusqu'à l'obtention d'un faible volume (1 à 2 mL). Cette suspension est alors utilisée comme phase interne pour préparer la première émulsion eau dans huile lors de la préparation des microparticules composites.
- La phase externe utilisée correspondait uniquement à de l'eau et non du PVA 0,1% comme précédemment. En effet, l'existence d'un premier tensioactif (Span 60) rendait inutile l'utilisation de PVA dans la phase externe continue. Dans ces conditions, il n'était plus nécessaire d'éliminer l'excès de PVA par lavage.

- Les microparticules finales ont été lyophilisées pendant 24 heures ce qui a permis de limiter l'agrégation des particules entre elles.

Une caractérisation complète des microparticules ainsi obtenues a été réalisée. Les microparticules composites présentaient une taille de 30µm environ, taille compatible à une injection sous-cutanée [<125µm, taille maximum pour une administration parentérale (Tice and Tabibi 1991 ; Jain 2000)]. Des observations effectuées en microscopie électronique à balayage ont permis de confirmer la forme sphérique des particules et mettre en évidence une hétérogénéité de la taille des pores en surface (figure 1). La présence de ces pores peut jouer un rôle dans la libération du principe actif au travers de la matrice des microparticules composites et peut contribuer à la diffusion vers le milieu extérieur de l'ibuprofène.

**Figure 1 :** Observation en microscopie électronique à balayage de microparticule composite encapsulant de l'ibuprofène.

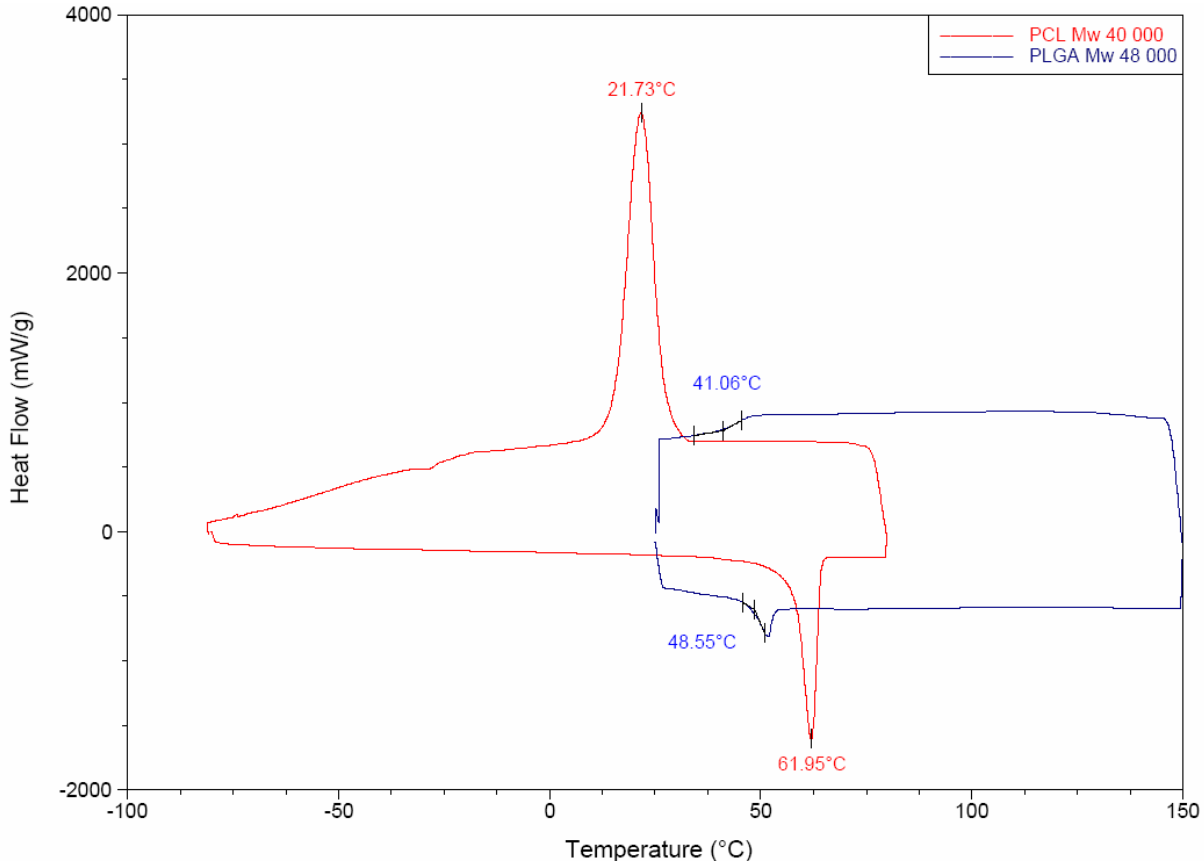



Le taux d'encapsulation de l'ibuprofène dans ces particules était élevé et atteignait 90%. D'autre part, la suspension particulaire était stable dans la solution d'injection de carboxyméthyle cellulose à 2%, et suffisamment dispersée pour permettre le passage à travers l'aiguille d'injection. L'étude de libération *in vitro* de ces particules a montré un profil comparable à celui obtenu lors de la formulation décrite dans la première partie, avec les

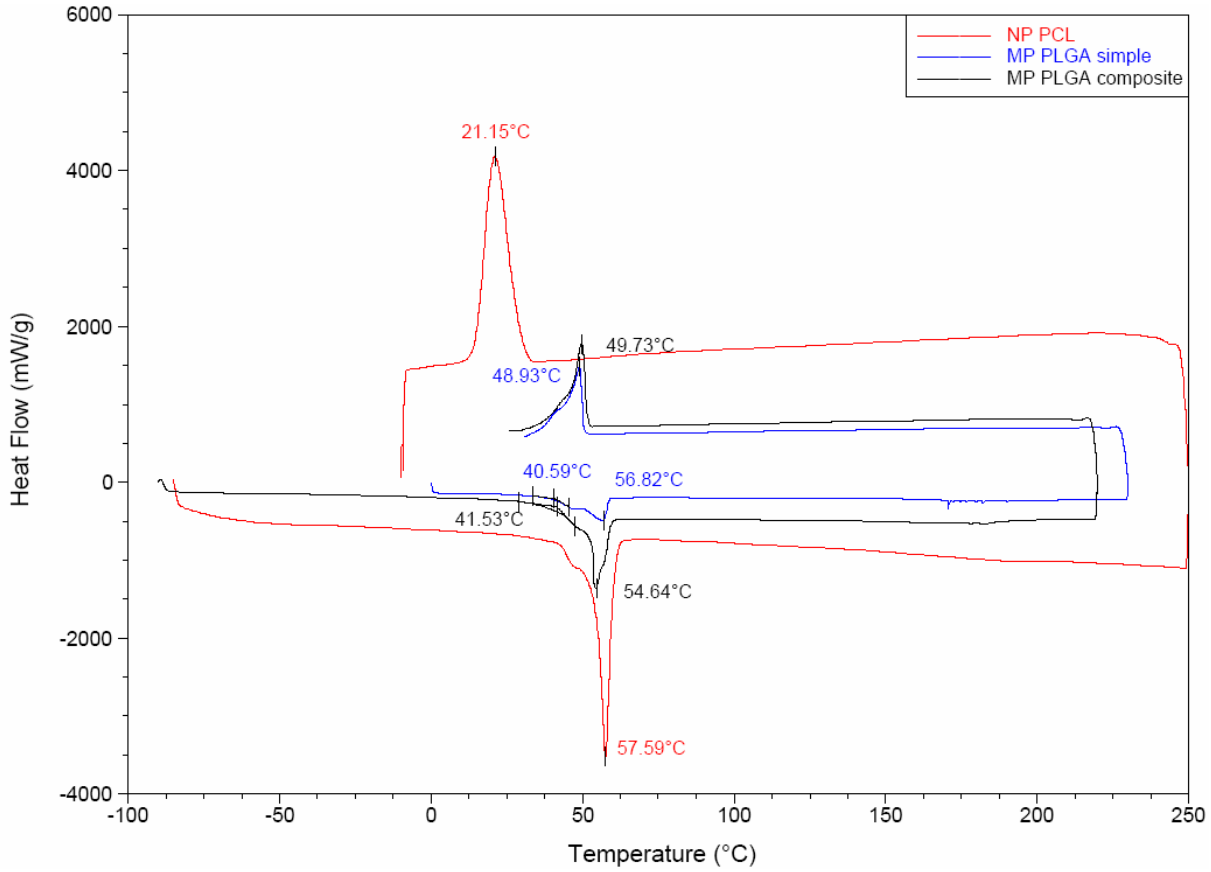
polymères non biodégradables. Enfin, pour étudier plus avant l'état du principe actif dans les microparticules composites, une étude de calorimétrie différentielle à balayage (Differential Scanning Calorimetry ou DSC) a été réalisée (DSC $Q_{10}$®, TA Instruments, Guyancourt, France) à l'aide d'un système de réfrigération, sous atmosphère d'azote, et d'un système d'acquisition de données Q Séries explorer®. Les différents évènements thermiques (température de fusion, température de cristallisation et température de transition vitreuse (*Tg*)) ont été mesurés par la méthode des tangentes. Les échantillons testés ont été placés dans des creusets d'aluminium, scellés, puis soumis à un refroidissement jusqu'à une température précise suivi de cycles de chauffage/refroidissement effectués avec une vitesse de 10°C/min pour le chauffage et de 20°C/min pour le refroidissement. Chaque échantillon a été soumis à deux cycles de chauffage/refroidissement et le deuxième cycle a été pris en compte pour l'analyse des évènements thermiques. Les courbes résultantes sont exprimées en mWatts par gramme de matière (wW/g), en fonction de la température. Sur les thermogrammes, il peut être noté que les évènements thermiques caractéristiques du PLGA et de la PCL sont toujours bien distincts (Figure 2 A) : la température de transition vitreuse du PLGA est de 48°C et le pic de fusion se situe à 62°C pour la PCL. Cette différentiation des pics est également retrouvée, bien que légèrement modifiée, sur les thermogrammes correspondant aux nanoparticules, microparticules simples et microparticules composites (Figure 2B). En effet, la température de transition vitreuse du PLGA est passée de 48°C à 41°C alors que le pic de fusion de la PCL se situe à environ 58°C au lieu de 62°C auparavant. Dans de telles structures, les deux polymères ne semblent pas présenter d'interactions et semblent se comporter du point de vue thermodynamique, comme un simple mélange physique. Cette modification peut être attribuée aux traces d'eau résiduelles dans les systèmes après séchage car il est connu que l'eau peut plastifier les polymères et en conséquence modifier les évènements thermiques caractéristiques de ces polymères comme la température de fusion ou la température de transition vitreuse. De plus, dans les microparticules composites, l'ibuprofène semble présenter un état moléculaire ou amorphe, constaté par la disparition du pic de fusion à 77°C, pic caractéristique de l'ibuprofène à l'état libre (cristallin) et retrouvé dans un simple mélange physique. Cette disparition du pic de fusion est également retrouvée sur les thermogrammes de microparticules simples et de nanoparticules véhiculant de l'ibuprofène (Figure 2C et 2D).

**Figure 2 :** Thermogrammes obtenus par calorimétrie différentielle à balayage des polymères seuls (PCL et PLGA) **A)** des particules sans ibuprofène **B)** des microparticules contenant l'ibuprofène **C)** mélange physique et les nanoparticules d'ibuprofène **D)**

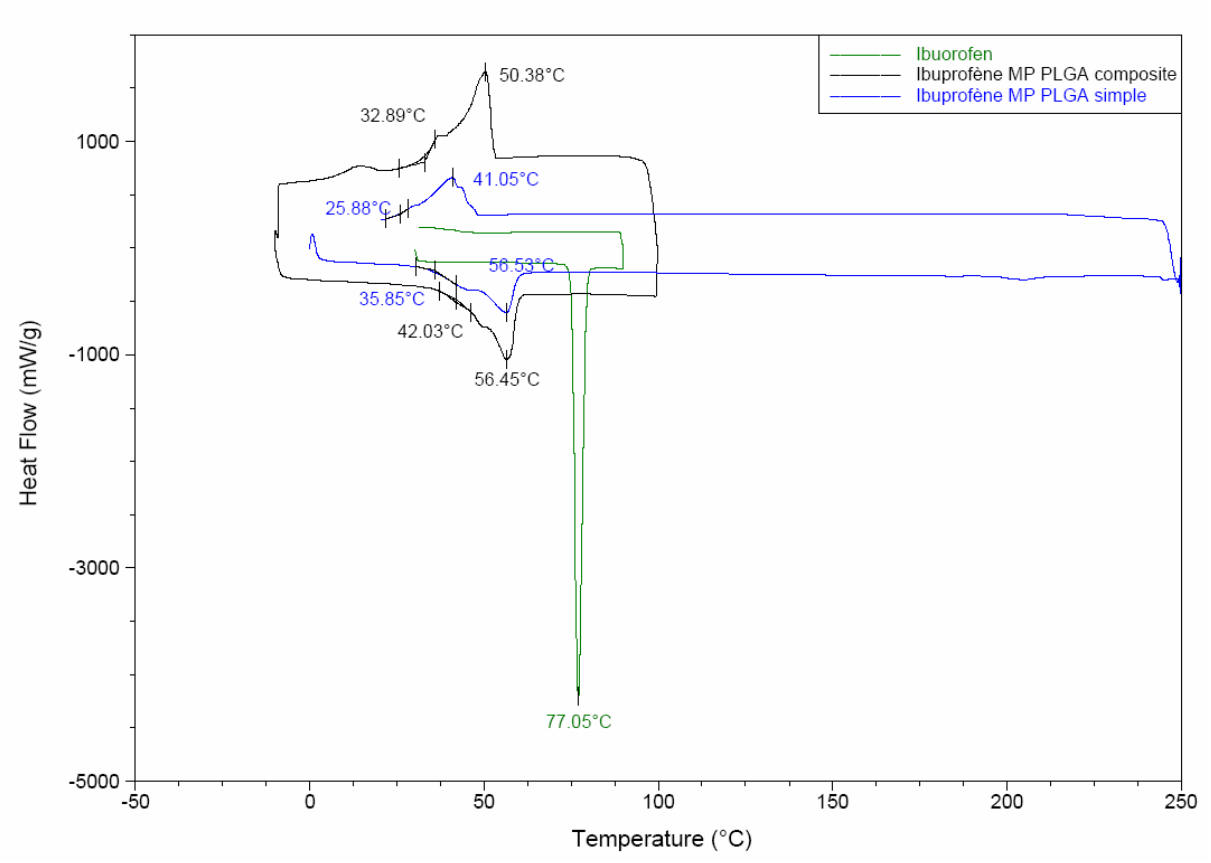
**A)**



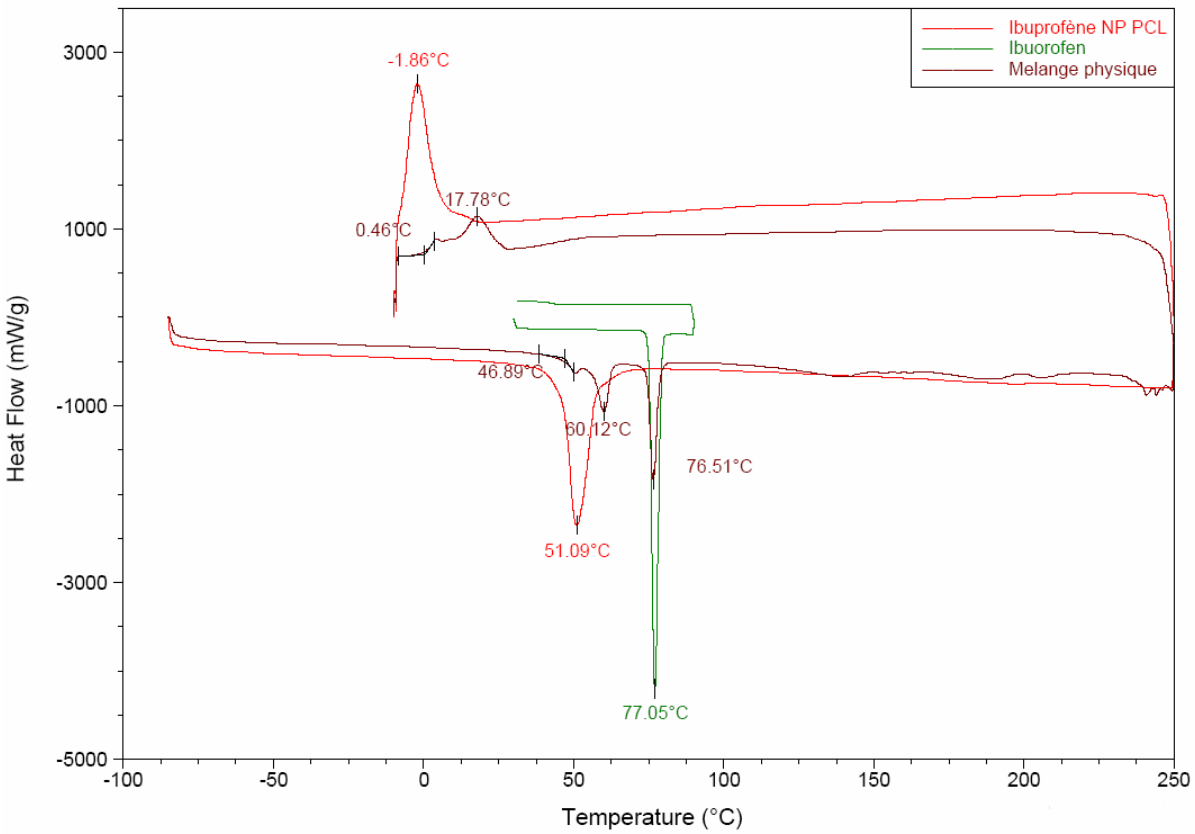
**B)**


NP PCL
MP PLGA simple
MP PLGA composite
21.15°C
49.73°C
48.93°C
40.59°C
56.82°C
41.53°C
54.64°C
57.59°C
Heat Flow (mW/g)
Temperature (°C)
6000
4000
2000
0
-2000
-4000
-100
-50
0
50
100
150
200
250

**C)**


Ibuorofen
Ibuprofène MP PLGA composite
Ibuprofène MP PLGA simple
50.38°C
32.89°C
41.05°C
25.88°C
56.53°C
35.85°C
42.03°C
56.45°C
77.05°C
Heat Flow (mW/g)
Temperature (°C)
1000
-1000
-3000
-5000
-50
0
50
100
150
200
250

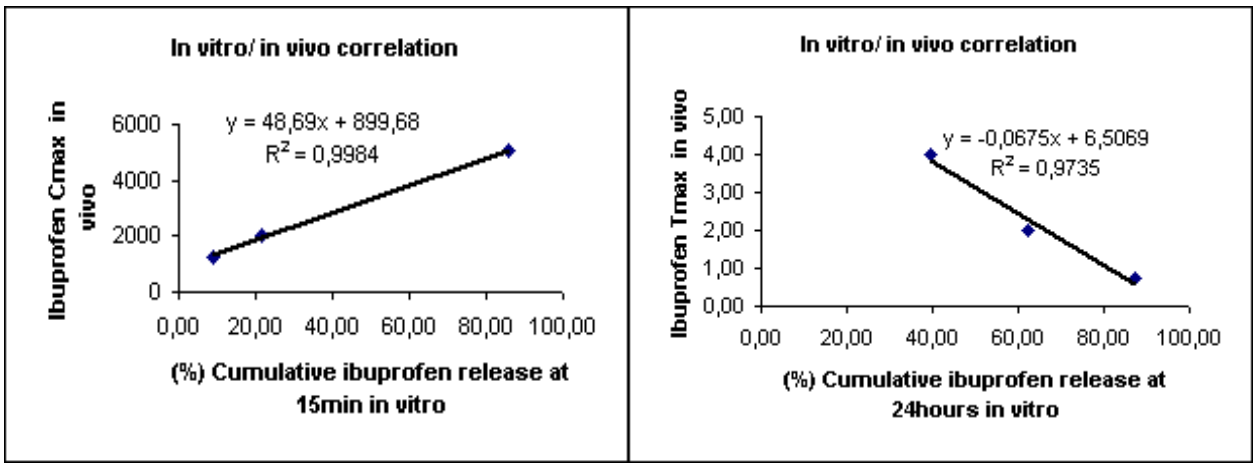
**D)**


Ibuprofène NP PCL
Ibuorofen
Melange physique
-1.86°C
17.78°C
0.46°C
46.89°C
60.12°C
76.51°C
51.09°C
77.05°C
Heat Flow (mW/g)
Temperature (°C)
3000
1000
-1000
-3000
-5000
-100
-50
0
50
100
150
200
250

Une fois les microparticules caractérisées *in vitro*, une étude *in vivo* a été effectuée chez le rat Wistar mâle adulte. Une injection sous-cutanée des différentes particules véhiculant l'ibuprofène (nanoparticules, microparticules simples et microparticules composites) ou d'une solution commerciale d'ibuprofène (Pedea®) a été réalisée dans différents groupes de rats. Le taux d'ibuprofène retrouvé dans le plasma à différents temps a alors été déterminé en utilisant la technique de dosage CLHP validée et décrite précédemment. Les résultats obtenus confirment la réduction de l'effet « burst » de l'ibuprofène par utilisation de microparticules composites. Les résultats observés ne sont pas systématiquement significatifs en termes statistiques. Toutefois, la tendance semble bien marquée et on peut sans doute attribuer la significativité partielle au relativement faible nombre d'animaux utilisés. Les résultats obtenus *in vitro* peuvent être corrélés à ceux obtenus *in vivo*. En effet, nos résultats *in vivo* montrent une bonne corrélation entre les paramètres pharmaceutiques et les valeurs issues des profils de libération de l'ibuprofène *in vitro*. Le niveau C de corrélation in vivo – in vitro défini selon les lignes directives européennes en vigueur (*Note for guidance on quality of modified release products. CPMP/QWP/604/96, EMEA, London*), correspond à une relation en un point entre la quantité dissoute à un temps donné et un paramètre pharmacocinétique moyen tel que l'AUC, le Cmax ou le Tmax. Ainsi, un effet « burst » important d'ibuprofène après 15 minutes avait été remarqué *in vitro* lors de l'utilisation de nanoparticules d'ibuprofène. Ceci s'est vérifié *in vivo* puisque les valeurs de concentrations maximales Cmax les plus élevées ont été obtenues avec ces nanoparticules et la solution immédiatement disponible. La figure 3A illustre cette première corrélation *in vitro - in vivo* concernant les deux formes à action plus rapide *(r = 0,9984)*. D'autre part, une diminution de l'effet « burst » avait été constatée lors de l'utilisation de microparticules simples et composites, *in vitro* comme *in vivo*. Ainsi, la libération progressive et continue observée *in vitro* a permis également d'établir une seconde corrélation, mettant en relation le Tmax d'ibuprofène et le pourcentage d'ibuprofène libéré à 24h *(r = 0,9735 figure 3 B).* Malgré ces données intéressantes, il est nécessaire d'approfondir les travaux pour confirmer cette approche de corrélation *in vivo – in vitro*, par exemple en préparant des microparticules ou nanoparticules présentant d'autres profils de libération *in vitro* et d'observer si les corrélations révélées par ces premiers travaux peuvent encore s'appliquer.

**Figure 3:** Corrélations *in vivo – in vitro* avec les différentes formulations d'ibuprofène.

(**A**) (**B**)




(A)

| Formulation | Cumulative ibuprofen release (%) after 15 minutes | Ibuprofen Cmax in plasma of rats (ng/ml) |
|---|---|---|
| PCL nanoparticules | 85.9 | 5 071 |
| PLGA simple microparticules | 21.4 | 2 033 |
| PLGA composite microparticules | 8.6 | 1 249 |

(B)

| Formulation | Cumulative ibuprofen release (%) after 24 heures | Ibuprofène Tmax in plasma of rats (hours) |
|---|---|---|
| PCL nanoparticules | 87.4 | 0.75 |
| PLGA simple microparticules | 62.2 | 2 |
| PLGA composite microparticules | 39.5 | 4 |

**IV. Etude des microparticules composites libérant de l'insuline.**

Compte tenu des résultats intéressants et encourageants obtenus avec la molécule modèle mais lipophile d'ibuprofène, nous avons souhaité poursuivre ce projet de recherche par l'exploration de la réduction de l'effet « burst » par l'utilisation de microparticules composites adaptées à un principe actif hydrophile. L'intérêt de cette partie du projet est triple : i) d'une part, la plupart des macromolécules thérapeutiques sont d'origine peptidique ou protéique. ii) D'autre part, les formes multiparticulaires injectables aujourd'hui sur le marché correspondent à ce type de principe actif (Reichert 2003 ; Degim and Celebi 2007). Outre une prolongation d'action remarquable de ces principes actifs incorporés dans des systèmes particulaires permettant une amélioration de la qualité de vie des patients (Sinha and Trehan 2005), certaines de ces formes pharmaceutiques peuvent éventuellement permettre de cibler des principes actifs ou au minimum de réduire leur toxicité systémique (Mohamed and van der Walle 2008 ; Sinha and Trehan 2005). iii) Enfin, un effet « burst » a souvent été décrit avec ce type de principe actif (Yeo and Park 2005).

L'insuline a retenu notre attention et représente un bon candidat à notre étude. Il s'agit d'une protéine thérapeutique de masse moléculaire moyenne (5808 Da.) ayant déjà fait l'objet de plusieurs travaux d'encapsulation dans différents systèmes particulaires (Yamaguchi et al., 2002 ; Jiang et al. 2003 ; Trotta et al., 2005 ; Kang and Singh 2005 ; Wang et al, 2006). En effet, l'insuline a une demi-vie très courte dans le plasma, de l'ordre de quelques minutes (Takenaga *et al*., 2004). Pour cette raison, chez le malade atteint de diabète de type I ou de type II l'injection d'insuline est nécessaire jusqu'à plusieurs fois par jour pour atteindre le seuil physiologique normal d'insuline et palier au manque dans l'organisme. Ces injections répétées quotidiennes sont inconfortables et nécessitent un contrôle du traitement permanent avec des intervalles entre les différentes injections à respecter. Dans la littérature, beaucoup de tentatives pour obtenir des formulations à libération prolongée d'insuline destinées à la voie parentérale ont été décrites (Yamaguchi *et al.*, 2002 ; Jiang *et al*. 2003 ; Takenaga *et al*., 2004 ; Hinds *et al*., 2005 ; Trotta *et al*., 2005 ). Elles permettraient de réduire le nombre d'injections quotidiennes, tout en protégeant la molécule d'une dégradation rapide. L'important effet « burst » constaté dans ces études constitue le principal obstacle pour leur administration par cette voie parentérale.

L'encapsulation des nanoparticules PCL chargées d'insuline dans des microparticules de PLGA (microparticules composites) est la stratégie que nous nous proposions donc d'étudier pour l'administration de l'insuline par voie parentérale, pour réduire l'effet « burst » d'insuline et obtenir une libération prolongée. A notre connaissance, il s'agissait de la

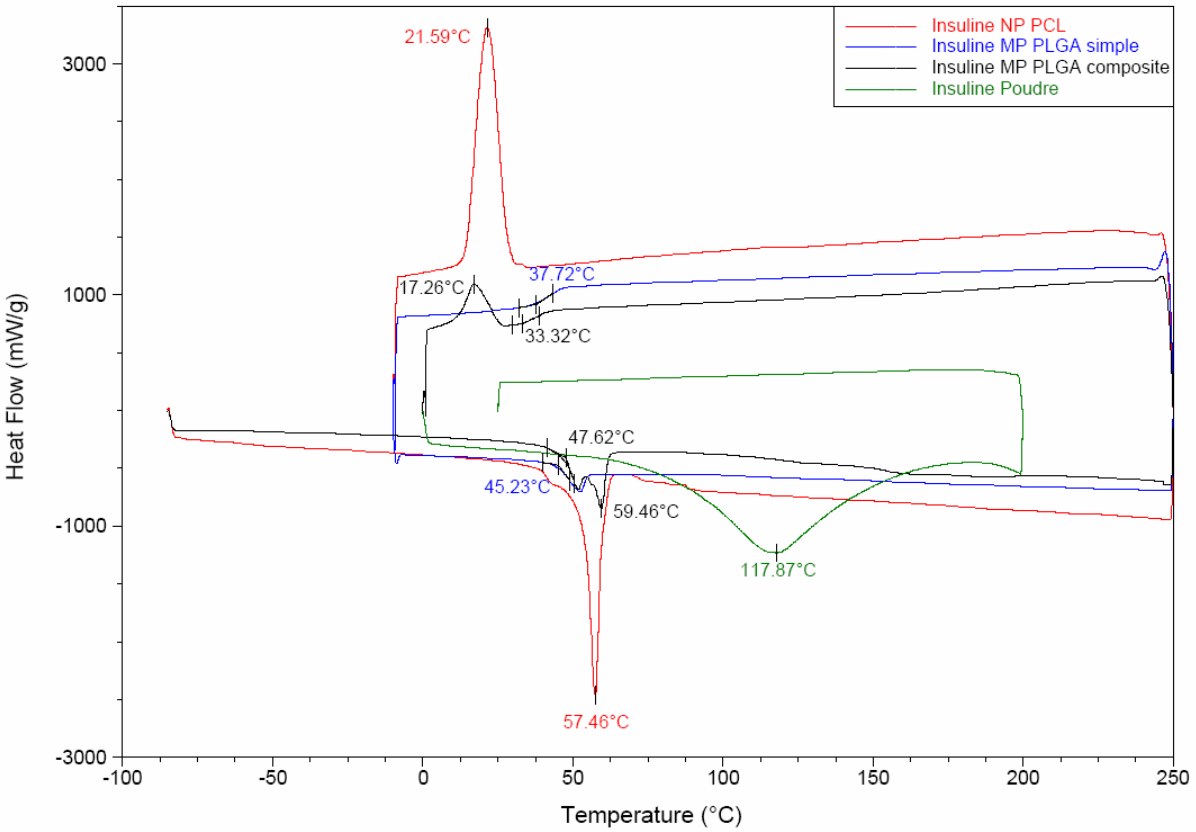
première formulation encapsulant l'insuline dans un polymère hydrophobe lui-même contenu dans un autre polymère hydrophobe.

Il nous a fallu dans un premier temps modifier quelques paramètres de formulation des microparticules composites pour l'adapter à cette molécule thérapeutique : i) il était inutile d'utiliser le Span 60 dans la phase organique car la suspension des nanoparticules d'insuline dans l'acétate d'éthyle était stable, ii) la quantité de PLGA a été réduite par rapport à l'étude des microparticules composites d'ibuprofène (de 400 mg à 150 mg). En effet, le ratio polymère / principe actif a été adapté à l'insuline. A cette même quantité de polymère de 400mg, une faible quantité d'insuline était libérée : ceci était sans doute lié à une diffusion plus difficile de l'insuline dans la matrice polymérique, compte tenu de sa masse moléculaire plus élevée que l'ibuprofène.

En utilisant ce protocole adapté, le taux d'encapsulation de l'insuline dans microparticules composites atteignait 99 %. La taille des particules était de 111µm ce qui est convenable pour une administration par voie sous-cutanée. Ces microparticules composites d'insuline présente une taille plus importante que les microparticules composites d'ibuprofène. Les deux modifications du protocole de formulation (absence de span 60 et quantité de polymère moins importante) en sont sans doute responsables.

Pour compléter la caractérisation physico-chimique des particules, une étude de calorimétrie différentielle à balayage a été entreprise selon les mêmes paramètres de manipulation que ceux décrits lors de l'étude des formes contenant de l'ibuprofène (Figure 4). L'état amorphe de l'insuline dans la matrice polymérique a été vérifié par disparition du pic de fusion (à 117°C) caractéristique de l'insuline.

**Figure 4**. Thermogrammes obtenus par calorimétrie différentielle à balayage des différentes particules contenant de l'insuline. Comparaison à l'insuline poudre.




L'étude de la libération *in vitro* effectuée dans le tampon phosphate a montré des résultats intéressants : l'insuline en solution (Actrapid®) est parfaitement miscible dans ce tampon mais devient instable après 8 heures de cinétique de libération (seulement 30 % de l'insuline est libérée après 24 heures). En revanche, les microparticules composites ont permis de libérer environ 20 % d'insuline après 15 minutes et 40 % après 24 heures. De plus, l'effet « burst » d'insuline a été remarquablement réduit par l'utilisation de microparticules composites par rapport aux nanoparticules ou aux microparticules simples chargées en insuline.

Compte tenu de ces résultats encourageants, une étude *in vivo* a été menée dans un modèle de rats diabétiques de type I. Ce modèle souvent utilisé pour l'étude de formes particulaires (Jiang *et al.* 2003 ; Takenaga *et al.*, 2004 ; Hinds *et al.*, 2005 ) est induit par une injection intraveineuse de streptozotocine à 10 mM dans un tampon citrate, cytotoxique pour les cellules β du pancréas. Dans ce cas, le niveau de sécrétion basal d'insuline est très faible. Ceci présente l'avantage d'une observation plus aisée de l'effet de l'insuline administrée lors

de l'étude. En utilisant des rats non diabétiques, la distinction entre l'insuline injectée pour l'étude ou l'insuline physiologiquement sécrétée est plus délicate et oblige à utiliser des doses importantes d'insuline. Ainsi, l'administration sous-cutanée des formulations d'insuline a été réalisée après vérification de l'état diabétique des animaux par mesure de la glycémie (> 300 mg/dL). *In vivo*, la libération d'insuline à partir des microparticules composites est plus lente que celle obtenue à partir des microparticules simples et des nanoparticules. Le niveau physiologique basal d'insuline (22 µUI/ml dans le sérum) n'était pas atteint 4 jours après l'injection sous-cutanée des microparticules composites. Les microparticules composites semblent donc constituer de véritables systèmes réservoirs permettant une libération contrôlée capable de palier au déficit de la sécrétion basale d'insuline qui fait défaut dans ce modèle de diabète.

En conclusion, les microparticules composites contenant de l'insuline ont montré leur capacité à contrôler la libération d'insuline. La recherche de formes à libération prolongée capables de délivrer l'insuline sur plusieurs semaines avait constitué notre objectif de base, sous réserve d'un intérêt clinique encore à démontrer. Il va de soit qu'une telle forme n'élimine pas la nécessité de l'injection d'une solution lors de la prise d'un repas.

## V. Perspectives et optimisations :

Notre travail a contribué à la mise au point d'un protocole de formulation aboutissant à l'obtention de microparticules composites capables de contenir un principe actif lipophile comme l'ibuprofène ou un principe actif hydrophile telle que la triptoréline ou l'insuline. Par cette encapsulation de nanoparticules dans des microparticules, à base de polymères hydrophobes, il a été possible de réduire l'effet « burst » de ces deux principes actifs, *in vitro* comme *in vivo*. Nous nous étions attachés à utiliser des polymères biodégradables. Pour poursuivre la caractérisation des formes galéniques ainsi développées, un suivi à plus long terme de la libération des principes actifs serait nécessaire. En effet, un deuxième effet « burst » est généralement constaté plus tardivement lors de l'utilisation de ces polymères, lié notamment à la dégradation de ces derniers (Sinha *et al*., 2005), ce qui représente donc un problème majeur à l'utilisation de ces polymères. Cependant, dans les microparticules composites que nous avons développées, nous avons utilisé deux polymères biodégradables qui présentent des temps de dégradation différents, ce qui pourrait permettre d'atténuer ce second effet « burst » et de libérer le principe actif de façon plus progressive. Ceci nécessiterait d'être vérifié.

D'autres modes de fabrication des nanoparticules seraient intéressants à explorer afin d'éviter l'utilisation du dichlorométhane (solvant toxique du groupe I). D'autres solvants organiques pourraient être testés (acétate d'éthyle, butanol, glycofurol…). D'autres techniques n'utilisant pas de solvants organiques ou une quantité très réduite telles que la méthode de CO2 supercritique (Thote and Gupta 2005) ou les méthodes de formulation de nanoparticules lipidiques (Almeida and Souto 2007) pourraient être envisagées. Ces modes de préparation permettraient de limiter la dénaturation des principes actifs comme les protéines, se produisant généralement à l'interface solvant/eau.

En effet, les protéines sont particulièrement sensibles aux contraintes imposées par le mode de fabrication de particules polymériques. Ainsi, les protéines utilisées dans notre étude (triptoréline et insuline) peuvent être sensibles à la sonication, à la présence de tensioactifs, à l'interface eau/huile, au pH, à la température et au degré d'humidité résiduelle dans les particules. Tous ces paramètres influencent la stabilité de ce type de principe actif et peuvent limiter l'utilisation à long terme des formes galéniques développées. Ainsi, une étude plus approfondie de la stabilité des principes actifs au cours du protocole de formulation mais aussi au cours de la conservation des particules et au cours des études de libération serait nécessaire. De plus, dans les formulations que nous proposons, l'utilisation de polymère comme le PLGA pourrait être un facteur supplémentaire d'instabilité des protéines : au cours de sa dégradation, des résidus acides sont relargués et peuvent entraîner une diminution de pH à l'intérieur des particules, diminution préjudiciable pour un principe actif particulièrement sensible aux pH acides (Determan et al., 2006).

Un point majeur de notre étude nécessitant d'être compléter serait les études de corrélation in *vitro – in vivo*. Nous n'avons pu dégager une tendance que lors de l'étude de l'ibuprofène. Les résultats étaient plus difficiles à interpréter dans le cadre de l'insuline. Lors de nos études, des conditions de libération *in vitro* simples ont été utilisées, nous permettant d'accéder à quelques informations sur la façon dont les principes actifs étaient libérés à partir des matrices polymériques, sur les interactions polymères / principes actifs, sur la stabilité du principe actif et sur la reproductibilité de la préparation des particules. Le but de ces expériences n'était pas, à priori, de « simuler » *in vitro* les conditions retrouvées *in vivo*. Pour tenter d'établir des corrélations *in vitro - in vivo* plus pertinentes, nous pourrions modifier les conditions des études de cinétiques de libération *in vitro*, en terme de volume de milieu, de mode et de vitesse d'agitation, et effectuer cette étude en présence d'enzymes par exemple… Le but serait de se rapprocher, autant que faire se peut, des conditions physiologiques et des

études *in vivo*. Nous pourrions alors obtenir de plus amples informations sur le mécanisme de l'effet « burst ».

Enfin, nous avons développé une forme galénique originale de microparticules composites, basées sur des polymères hydrophobes et permettant de libérer de façon plus progressive des molécules de faible à moyenne masse moléculaire, aussi bien lipophiles qu'hydrophiles. Même si pour chaque principe actif, le protocole doit être adapté et présente quelques modifications pour convenir aux propriétés physico-chimiques de chaque principe actif d'intérêt (taille, charge, masse moléculaire, cristallinité…), les étapes principales du protocole sont toujours respectées et identiques. Il pourrait être intéressant d'appliquer ce protocole pour des principes actif de masse moléculaire plus importante (comme des immunoglobulines-G ou l'interféron-α), afin d'observer l'impact de cette encapsulation particulière sur l'effet « burst », dont les mécanismes (mentionnés dans la revue bibliographique) sont connus pour être différents de celui des peptides et des molécules de plus petite masse (Li *et al*., 2005). Nous aurions ainsi montré la possibilité de généraliser l'utilisation des microparticules composites à bon nombre de principes actifs thérapeutiques.

## VI. Références bibliographiques :

Almeida AJ, Souto E. Solid lipid nanoparticles as a drug delivery system for peptides and proteins, Adv Drug Deliv Rev.10 (2007) 478-90.

Attivi D, Wehrle P, Ubrich N, Damge C, Hoffman M, Maincent P. Formulation of insulin-loaded polymeric nanoparticles using response surface methodology, Drug Dev Ind Pharm. 31 (2005) 179-89.

Chung TW, Tsai YL, Hsieh JH, Tsai WJ. Different ratios of lactide and glycolide in PLGA affect the surface property and protein delivery characteristics of the PLGA microspheres with hydrophobic additives, J Microencapsul. 23 (2006) 15-27.

Degim IT, Celebi N, Controlled delivery of peptides and proteins, Curr Pharm Des. 13 (2007) 99-117.

De Rosa G, Larobina D, Immacolata La Rotonda M, Musto P, Quaglia F, Ungaro F. How cyclodextrin incorporation affects the properties of protein-loaded PLGA-based microspheres: the case of insulin/hydroxypropyl-beta-cyclodextrin system, J Control Release. 102 (2005)71-83.

Determan AS, Wilson JH, Kipper MJ, Wannemuehler MJ, Narasimhan B. Protein stability in the presence of polymer degradation products: consequences for controlled release formulations, Biomaterials. 27 (2006) 3312-20.

Dhoot NO, Wheatley MA. Microencapsulated liposomes in controlled drug delivery: strategies to modulate drug release and eliminate the burst effect, J Pharm Sci. 92 (2003) 679-89.

Doshi Abha, Deshpande SG. In vivo pharmacokinetic studies of prodrugs of ibuprofen, Indian J. of pharmaceutical Sciences, 69 (2007) 824-827.

Heya T, Mikura Y, Nagai A, Miura Y, Futo T, Tomida Y, Shimizu H, Toguchi H. Controlled release of thyrotropin releasing hormone from microspheres: evaluation of release profiles and pharmacokinetics after subcutaneous administration, J Pharm Sci. 83 (1994) 798-801.

Hinds KD, Campbell KM, Holland KM, Lewis DH, Piché CA, Schmidt PG. PEGylated insulin in PLGA microparticles. In vivo and in vitro analysis, J Control Release 104 (2005) 447-60.

Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices, Biomaterials 21 (2000) 2475-90.

Jiang G, Qiu W, DeLuca PP. Preparation and in vitro/in vivo evaluation of insulin-loaded poly(acryloyl-hydroxyethyl starch)-PLGA composite microspheres, Pharm Res. 20 (2003) 452-9.

Kang F, Singh J. Preparation, in vitro release, in vivo absorption and biocompatibility studies of insulin-loaded microspheres in rabbits, AAPS PharmSciTech. 24 (2005) E487-94.

Li JK, Wang N, Wu XS. A novel biodegradable system based on gelatin nanoparticles and poly (lactic-co-glycolic acid) microspheres for protein and peptide drug delivery.J Pharm Sci. 86 (1997) 891-5

Li S, He Y, Li C, Liu X.In vitro release of protein from poly(butylcyanoacrylate) nanocapsules with an aqueous core. Colloid & Polymer Science 283 (2005) 480-485.

Mandal TK, Bostanian LA, Graves RA, Chapman SR. Poly(D,L-lactide-co-glycolide) encapsulated poly(vinyl alcohol) hydrogel as a drug delivery system, Pharm Res. 19 (2002) 1713-9.

Manzoori JL, Amjadi M. Spectrofluorimetric study of host-guest complexation of ibuprofen with beta-cyclodextrin and its analytical application. Spectrochim Acta A Mol Biomol Spectrosc. 59 (2003) 909-16.

Mohamed F, van der Walle CF. Engineering biodegradable polyester particles with specific drug targeting and drug release properties, J Pharm Sci. 97 (2008) 71-87.

Okada H, Toguchi H. Biodegradable microspheres in drug delivery, Crit Rev Ther Drug Carrier Syst. 12 (1995) 1-99.

Reichert JM. Trends in development and approval times for new therapeutics in the United States, Nat. Rev., Drug Discov. 2 (2003), pp. 695–702.

Sheikh Hassan A, Bolzan C, Sapin A, Ubrich N, Maincent P and Leroy P. Simple and sensitive HPLC method with fluorescence detection for the measurement of ibuprofen in rat plasma. Application to a long-lasting dosage form. Drug Dev Ind Pharm 2008 in press.

Sinha VR, Trehan A. Biodegradable microspheres for parenteral delivery, Crit Rev Ther Drug Carrier Syst. 22 (2005) 535-602.

Shi Y, Li LC. Current advances in sustained-release systems for parenteral drug delivery, Expert Opin Drug Deliv. 2 (2005) 1039-58.

Takenaga M, Yamaguchi Y, Kitagawa A, Ogawa Y, Kawai S, Mizushima Y, Igarashi R. Optimum formulation for sustained-release insulin, Int J Pharm. 271 (2004) 85-94.

Thote AJ, Chappell JT Jr, Gupta RB, Kumar R. Reduction in the initial-burst release by surface crosslinking of PLGA microparticles containing hydrophilic or hydrophobic drugs. Drug Dev Ind Pharm. 31 (2005) 43-57.

Thote AJ and Gupta RB. Formation of nanoparticles of a hydrophilic drug using supercritical carbon dioxide and microencapsulation for sustained release. Nanomedicine 1 (2005) 85-90.

Tice TR and Tabibi ES, Parenteral drug delivery: injectables. In: A. Kydonieus, Editor, Treatise on controlled drug delivery: fundamentals optimization, applications, Marcel Dekker, New York (1991), pp. 315–339.

Trotta M, Cavalli R, Carlotti ME, Battaglia L, Debernardi F. Solid lipid micro-particles carrying insulin formed by solvent-in-water emulsion-diffusion technique. Int J Pharm. 288 (2005) 281-8.

Hoffart V, Ubrich N, Lamprecht A, Bachelier K, Vigneron C, Lecompte T, Hoffman M, Maincent P. Microencapsulation of low molecular weight heparin into polymeric particles designed with biodegradable and nonbiodegradable polycationic polymers, Drug Deliv. 10 (2003) 1-7.

Wang J, Wang BM, Schwendeman SP. Mechanistic evaluation of the glucose-induced reduction in initial burst release of octreotide acetate from poly(D,L-lactide-co-glycolide) microspheres. Biomaterials, 25 (2004) 1919-27.

Wang LY, Gu YH, Su ZG, Ma GH. Preparation and improvement of release behavior of chitosan microspheres containing insulin. Int J Pharm. 27 (2006) 187-95.

Wang, P., Qi, M., Liu, L., & Fang, L. Determination of ibuprofen in dog plasma by liquid chromatography and application in pharmacokinetic studies of an ibuprofen prodrug in dogs. J. Pharm. Biomed. Anal. 38 (2005) 714-723.

Yamaguchi Y, Takenaga M, Kitagawa A, Ogawa Y, Mizushima Y, Igarashi R. Insulin-loaded biodegradable PLGA microcapsules: initial burst release controlled by hydrophilic additives. J Control Release 81 (2002) 235-49.

Yeo Y, Park K. Control of encapsulation efficiency and initial burst in polymeric microparticle systems, Arch Pharm Res. 27 (2004) 1-12.

Zheng CH, Gao JQ, Liang WQ, Yu HY, Zhang YL. Effects of additives and processing parameters on the initial burst release of protein from poly(lactic-co-glycolic acid) microspheres, PDA J Pharm Sci Technol. 60 (2006) 54-9.

# CONCLUSION

Les formes multiparticulaires injectables sont devenues des formes importantes de l'arsenal thérapeutique actuel notamment dans le traitement de certains types de cancer. Ces formes innovantes, la plupart du temps basées sur des polymères biodégradables, sont apparues dans les années 80 suite à des recherches intensives menées au cours des vingt années précédentes.

L'intérêt majeur de ces formes multiparticulaires, administrées soit par voie intramusculaire soit par voie sous-cutanée, réside dans la possibilité de libérer un principe actif pendant des semaines ou des mois à partir du site d'administration. Actuellement, les formes multiparticulaires à base de polymères biodégradables permettent des libérations pendant un maximum de 3 mois (Enantone®, Decapeptyl®). Le recours à des co-polymères d'acide polylactique et glycolique plus lentement biodégradables devrait permettre d'étendre la libération régulière et reproductible pendant des périodes pouvant couvrir jusqu'à une année.

Toutefois, une libération étendue sur une période plus prolongée exigera également des charges en principe actif plus importantes ce qui pourra avoir pour conséquence une libération initiale plus importante du principe actif incorporé. En effet, même avec les formes actuellement sur le marché, il est reconnu que la libération initiale suivant les premières heures après l'administration représente un inconvénient majeur de ce type de formulation. La conséquence principale peut être d'ordre toxicologique (quantité de principe actif trop importante dans la circulation systémique) entrainant, par ricochet, une diminution de la quantité encapsulée et donc une modification de la cinétique de libération.

L'objectif de notre travail a été de proposer une solution originale et innovante pouvant permettre de limiter cette libération initiale indésirable (également dénommée « burst effect ») survenant dans les premières heures après l'administration.

Sur la base des travaux antérieurs de la littérature, la stratégie utilisée a consisté à utiliser les propriétés synergiques de deux types de systèmes dispersés à savoir les nanoparticules et les microparticules. Pris séparément, ces deux systèmes permettent généralement de prolonger la libération des principes actifs qui y sont encapsulés. En ce qui concerne la libération initiale, les nanoparticules apparaissent moins avantageuses dans la mesure ou l'augmentation de la surface de contact (due à leur très faible diamètre) avec les fluides environnants conduit à des libérations souvent importantes. C'est pourquoi leur encapsulation dans des microparticules, en remplaçant le milieu extérieur directement à leur contact par une structure polymerique, nous a semblé être une approche originale à la modulation de la libération des principes actifs encapsulés. La diffusion du principe actif vers

le milieu extérieur devra d'abord se faire après avoir franchi les deux barrières de polymères (d'abord celle des nanoparticules puis ensuite celles des microparticules) ce qui devrait aboutir à un profond ralentissement. Le nouveau vecteur de médicament ainsi créé correspond à un système composite dans lequel des nanoparticules chargées en principe actif ont été directement encapsulées dans des microparticules.

Dans un premier temps, il fallait vérifier que ce concept original permettait effectivement de limiter la libération initiale des principes actifs encapsulés. Nous avons choisi deux principes actifs correspondant soit à une petite molécule (ibuprofène) soit à un peptide (acétate de triptoréline). En effet, ces 2 molécules permettent de tester le nouveau système sur les molécules dont la libération est la plus difficile à moduler en raison de leur faible masse moléculaire mais aussi de le démontrer sur le type de molécules thérapeutiques encapsulées dans les systèmes actuellement commercialisés. Pour mémoire, l'acétate de triptoréline est le principe actif du Decapeptyl®. Dans les méthodes d'encapsulation par évaporation/extraction de solvants, le polymère le plus souvent utilisé est le dichlorométhane. La difficulté de notre concept résidait dans la recherche d'un couple de polymères associé à un couple de solvants en sachant que les polymères devaient être uniquement solubles dans un solvant tout en étant insoluble dans l'autre. Tout couple répondant à cette définition pouvait permettre d'utiliser la méthode de double émulsion pour préparer le système composite nanoparticules dans microparticules. L'étude initiale in vitro a permis de sélectionner 1) la poly(ε-caprolactone) comme polymère des nanoparticules et le dichlorométhane comme solvant de ce polymère et 2) l'éthylcellulose ou un polymère polycationique comme constituant de la matrice des microparticules en utilisant l'acétate d'éthyle comme solvant de ces deux polymères. Les nanoparticules de poly(ε-caprolactone) étant insolubles dans l'acétate d'éthyle, il a été ensuite possible d'utiliser ce dernier solvant pour préparer les microparticules composites. Cette première étude a en effet permis de valider notre concept et de démontrer la diminution importante de la libération initiale in vitro par rapport aux nanoparticules seules ou à des microparticules simples (sans nanoparticules) mais incorporant les deux principes actifs d'intérêt.

Le concept ayant été démontré in vitro, il devenait important de le vérifier, dans un deuxième temps, in vivo afin de confirmer la mise au point de ce nouveau système original et innovant. Nous avons gardé l'ibuprofène comme molécule de faible masse moléculaire mais avons sélectionné une autre molécule, l'insuline, comme peptide modèle. Il convenait de résoudre alors un autre point qui était celui de la nature du matériau des microparticules. En effet dans les travaux ayant conduit à la démonstration du concept, nous avions utilisé deux

polymères non biodégradables. De tels polymères ne sont pas envisageables chez l'homme pour des administrations qui doivent être réitérées et des systèmes qui, de par leur nature multiparticulaire, ne peuvent être éliminées par intervention extérieure à la fin de leur période d'action. Nous avons alors porté notre choix sur un matériau biodégradable, largement utilisé pour les microparticules actuellement sur le marché, à savoir un copolymère d'acide lactique et glycolique qui présentait l'avantage d'être soluble dans l'acétate d'éthyle et donc de répondre au cahier des charges précédemment défini.

Dans le cadre des études in vivo sur les microparticules composites d'ibuprofène, nous avons d'abord été amenés à améliorer les techniques de dosage analytique aujourd'hui décrites dans la littérature. En effet, l'ibuprofène étant une molécule administrée à des quantités relativement importantes chez l'homme (200 à 400 mg au minimum), les techniques analytiques n'étaient pas assez sensibles pour suivre cette molécule administrée à faible dose chez un petit animal comme le rat. Suite à cette optimisation de la méthode analytique, nous avons pu démontrer que les microparticules composites étaient capables de réduire la libération initiale prématurée de cette petite molécule in vivo après administration sous-cutanée. Les mêmes observations ont ensuite été retrouvées pour l'administration de microparticules composites d'insuline chez des rats rendus diabétiques par administration intraveineuse de streptozotocyne.

Nous avons donc pu démontrer que les microparticules composites représentaient une forme pharmaceutique originale susceptible de ralentir fortement la libération initiale précoce des principes actifs encapsulés à la fois pour des molécules de faible masse moléculaire (cas le plus difficile) mais aussi pour des peptides (cas le plus fréquent en thérapeutique). De plus, ces microparticules composites ont montré une tendance très forte à une prolongation de la durée d'action des principes actifs encapsulés. Cette nouvelle propriété pourrait être mise à profit dans ce domaine de recherche puisque les laboratoires pharmaceutiques impliqués dans ce type de recherche souhaitent mettre sur le marché des formes multiparticulaires autorisant une libération pouvant s'étendre sur une année.

Les formes composites mises au point, reposant sur l'encapsulation de nanoparticules dans des microparticules, représentent une approche originale et innovante pour résoudre un problème majeur des formes multiparticulaires injectables. D'autres molécules thérapeutiques associées aux mêmes types de polymères mais avec des masses moléculaires plus importantes pourraient être testées dans l'avenir et offrir une solution originale aux deux problèmes que sont la réduction de la libération initiale et la nécessité d'augmenter la durée d'activité (et

donc la prolongation de la libération) des systèmes multiparticulaires actuellement sur le marché.

# Résumé

Les formes multiparticulaires injectables présentent l'inconvénient d'une libération initiale prématurée dont les conséquences sont une toxicité systémique si les concentrations sanguines du principe actif deviennent importantes ainsi qu'une modification de la libération. Pour résoudre ce problème, des microparticules composites ont été mises au point : il s'agit de microparticules encapsulant des nanoparticules.

Le concept a d'abord été démontré in vitro en encapsulant des nanoparticules de poly(epsilon-caprolactone) dans un polymère non biodégradable en choisissant comme modèles une molécule de faible masse moléculaire (ibuprofène) et un peptide (acétate de triptoréline). L'originalité du travail réside dans le choix des polymères et des solvants retenus pour la fabrication des microparticules. Le solvant utilisé pour fabriquer les microparticules doit être un non-solvant du polymère des nanoparticules. L'acétate d'éthyle répondait à ces conditions puisqu'il ne dissout pas la poly(epsilon-caprolactone) mais que c'est un excellent solvant de l'éthylcellulose ou du polymère polycationique utilisé dans la première partie du travail. Sur la base d'études de libération in vitro, il a ainsi été démontré que les microparticules composites permettaient effectivement de fortement réduire cette libération précoce tout en continuant d'assurer une libération prolongée. Dans un deuxième temps, la réduction de la libération initiale a été confirmée par une étude in vivo chez le rat avec 2 principes actifs modèles : ibuprofène et insuline. Toutefois, le polymère de la matrice des microparticules a été remplacé par un copolymère biodégradable constitué d'acides lactique et glycolique.

Il a été démontré que le nouveau concept de microparticules composites permettait de proposer une forme originale limitant la libération initiale des principes actifs suite à leur administration sous-cutanée ou intramusculaire tout en assurant une libération prolongée

***Mots clefs*** : Microparticules composites, Burst, Ibuprofène, Triptoréline, Insuline, Voie parentérale.

# Summary

Multiparticular injectable dosage forms present a burst effect known to lead to i) a systemic toxicoligal critical issue if blood concentrations of the drug are too high and ii) a change in the release profile due to a lower loading charge in microparticles. In order to solve this problem, composite microparticles have been developed: they consist in nanoparticles encapsulated in microparticles.

Such a concept has been demonstrated in vitro by encapsulating poly(epsilon-caprolactone) nanoparticles in a non-biodegradable polymeric matrix with two model drugs: a small molecular weight drug (ibuprofene) and a peptide (triptorelin acetate). The novelty of the research work lies on the adequate choice of polymers and solvents used for microparticles manufacturing. Indeed, the solvent used to manufacture microparticles has to be a non-solvent of the nanoparticles polymer. Ethyl acetate was a good candidate since it does not dissolve poly(epsilon-caprolactone) nanoparticles but is an excellent solvent for ethylcellulose and the polycationic polymer used in the first part of the work. Based on in vitro release studies, it was demonstrated that composite microparticles allowed the initial release to be strongly reduced together with a prolonged release. In a second part, the burst release reduction has been confirmed in vivo in rats with 2 drug models: ibuprofen and insulin. However, the microparticles polymer matrix was replaced by a biodegradable copolymer made of lactic and glycolic acids.

It has been demonstrated that the novel composite microparticles were an innovative dosage form able to control the initial burst release often associated to microparticles after sub-cutaneous or intramuscular administration while still maintaining the prolonged release of the encapsulated drugs. Such a result can be associated with the more difficult diffusion of the drug through the two consecutive polymeric barriers of nanoparticles and microparticles.

***Keywords*** : Composite microparticles, Burst release effect, Ibuprofen, Triptorelin, Insulin, Parenteral administration.

# Résumé

Les formes multiparticulaires injectables présentent l'inconvénient d'une libération initiale prématurée dont les conséquences sont une toxicité systémique si les concentrations sanguines du principe actif deviennent importantes ainsi qu'une modification de la libération. Pour résoudre ce problème, des microparticules composites ont été mises au point : il s'agit de microparticules encapsulant des nanoparticules.

Le concept a d'abord été démontré in vitro en encapsulant des nanoparticules de poly(epsilon-caprolactone) dans un polymère non biodégradable en choisissant comme modèles une molécule de faible masse moléculaire (ibuprofène) et un peptide (acétate de triptoréline). L'originalité du travail réside dans le choix des polymères et des solvants retenus pour la fabrication des microparticules. Le solvant utilisé pour fabriquer les microparticules doit être un non-solvant du polymère des nanoparticules. L'acétate d'éthyle répondait à ces conditions puisqu'il ne dissout pas la poly(epsilon-caprolactone) mais que c'est un excellent solvant de l'éthylcellulose ou du polymère polycationique utilisé dans la première partie du travail. Sur la base d'études de libération in vitro, il a ainsi été démontré que les microparticules composites permettaient effectivement de fortement réduire cette libération précoce tout en continuant d'assurer une libération prolongée. Dans un deuxième temps, la réduction de la libération initiale a été confirmée par une étude in vivo chez le rat avec 2 principes actifs modèles : ibuprofène et insuline. Toutefois, le polymère de la matrice des microparticules a été remplacé par un copolymère biodégradable constitué d'acides lactique et glycolique.

Il a été démontré que le nouveau concept de microparticules composites permettait de proposer une forme originale limitant la libération initiale des principes actifs suite à leur administration sous-cutanée ou intramusculaire tout en assurant une libération prolongée

***Mots clefs*** : Microparticules composites, Burst, Ibuprofène, Triptoréline, Insuline, Voie parentérale.

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Such a concept has been demonstrated in vitro by encapsulating poly(epsilon-caprolactone) nanoparticles in a non-biodegradable polymeric matrix with two model drugs: a small molecular weight drug (ibuprofene) and a peptide (triptorelin acetate). The novelty of the research work lies on the adequate choice of polymers and solvents used for microparticles manufacturing. Indeed, the solvent used to manufacture microparticles has to be a non-solvent of the nanoparticles polymer. Ethyl acetate was a good candidate since it does not dissolve poly(epsilon-caprolactone) nanoparticles but is an excellent solvent for ethylcellulose and the polycationic polymer used in the first part of the work. Based on in vitro release studies, it was demonstrated that composite microparticles allowed the initial release to be strongly reduced together with a prolonged release. In a second part, the burst release reduction has been confirmed in vivo in rats with 2 drug models: ibuprofen and insulin. However, the microparticles polymer matrix was replaced by a biodegradable copolymer made of lactic and glycolic acids.

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