UNIVERSITÀ DEGLI STUDI DI MILANO

DEPARTMENT OF PHARMACEUTICAL SCIENCES

Ph.D. PROGRAM IN PHARMACEUTICAL SCIENCES

XXXI CYCLE

DEVELOPMENT OF NOVEL BIODEGRADABLE MATERIALS STABLE TO STERILIZATION FOR THE PREPARATION OF DRUG DELIVERY SYSTEMS

CHIM/09 - APPLIED PHARMACEUTICAL TECHNOLOGY

Ph.D. thesis of:

Giulia MAGRI

R11302

Supervisor: Prof. Francesco CILURZO

Program Coordinator: Prof. Giancarlo ALDINI

ACADEMIC YEAR

2017/2018

UNIVERSITÀ DEGLI STUDI DI MILANO

DIPARTIMENTO DI SCIENZE FARMACEUTICHE

SCUOLA DI DOTTORATO IN SCIENZE FARMACEUTICHE

XXXI CICLO

DEVELOPMENT OF NOVEL BIODEGRADABLE MATERIALS STABLE TO STERILIZATION FOR THE PREPARATION OF DRUG DELIVERY SYSTEMS

SETTORE CHIM/09 FARMACEUTICO TECNOLOGICO APPLICATIVO

Tesi di dottorato di:

Giulia MAGRI

Matricola: R11302

Supervisore: Prof. Francesco CILURZO

Coordinatore del dottorato: Prof. Giancarlo ALDINI

ANNO ACCADEMICO

2017/2018

To my family

and my beloved grandmother Rosa

for their incessant love and unlimited support.

Abstract

Poly(lactide-co-glycolide) [PLGA] is the most exploited biodegradable and biocompatible material in the pharmaceutical field for the preparation of long-acting parenteral formulations, despite there are limitations related to the PLGA itself or to the final product to face with. These mainly include the limited ability in encapsulating hydrophilic compounds, the physical and chemical instabilities in aqueous media, the detrimental effect of the sterilization methods and the drop off in the micro-environmental pH upon degradation. Hence, there is the need to find new strategies for their overcoming.

This doctoral thesis aimed to exploit the functionalization of PLGA backbone with anti-oxidants (g-AA-PLGA) and a novel biodegradable material, containing polyesters segments in a multi-block poly(urethane) organization, to address the main limitations related to PLGA, with emphasis on nano-particulate drug delivery systems.

PLGA grafted to caffeic acid (g-CA-PLGA) nanoparticles (NP) showed an improved uptake in endothelial cells (EC) and smooth muscle cells (SMC), the representative cell populations in the artery wall. Thus, they were worth of interest for the loading of fluvastatin in restenosis prevention. The proliferation inhibition of human SMC was not significantly affected after the encapsulation of fluvastatin within g-CA-PLGA NP, with the effective concentration being 4 μM compared to the 1 μM of free fluvastatin, suggesting a control of the polymer on the drug

release. A higher dose was necessary in the case of EC, indicating the possibility to inhibit SMC proliferation while the healing of the endothelium is on-going. All these aspects highlight the suitability of this system in the prevention of restenosis, after the local delivery with the angioplasty balloon (*Chapter 1*). However, during the development of the formulation, the selection and optimization of the drying process is required, also with the aim to coat the angioplasty balloon with eluting-NP. In this context, a preliminary study was performed and the obtained results revealed that maltodextrins (MDX), an excipient widely used in the pharmaceutical industry, can be also advantageously used as drying auxiliary agent. Indeed, they permit an easily reconstitution of NP dispersion in aqueous media, independently of the selected drying technique, namely spray-drying or freeze-drying. The performances of such excipient were demonstrated in the case of both PLGA and g-CA-PLGA NP (*Chapter 2*).

The multi-block poly(ester-urethane) DegraPol® displays biocompatibility and biodegradability and consequently it is already used for the preparation of medical devices by electrospinning. Conversely, the possibility to design long-acting parenteral formulations is unknown as well as the possibility to conferee a spherical shape with the desired particle size and a narrow distribution. The performed work demonstrated that the emulsion/solvent evaporation method was the optimal process, with the possibility to cover size range from nano- to micro-meters. Nevertheless, the dispersant medium should be carefully studied, given the tendency of the particles to form aggregates due to the almost neutral Z-potential of the material *(Chapter 3)*.

All together the collected results, along with the known stability to sterilization process (ionizing radiations for g-AA-PLGA and ethylene oxide for DegraPol®), demonstrated that both PLGA grafted to anti-oxidant and DegraPol® are suitable materials for preparing particulate drug delivery systems that can overcome some of the limitations associated to PLGA.

As a general consideration, it should be underlined that formulation development cannot be disconnected from the regulatory framework. Particularly for long-

acting parenteral formulations, the elaboration of an appropriate *in vitro* test to study the release of the drug is critical, given the complexity in the set-up of methods able to efficiently discriminate products that can have different *in vivo* behaviour (*Chapter 4*). In the case of PLGA microspheres intended to sustain the release of a drug after the intra-articular administration, a bio-relevant approach was followed in the attempt to evaluate their performance under healthy and disease states simulated conditions. Formulation parameters such as PLGA lactide/glycolide ratio and the amount of drug encapsulated should be carefully considered to properly optimize the formulation. Furthermore, proteins contained in the release medium simulating the disease condition affected the release behaviour of microspheres. This suggests that simple buffers (*i.e.,* PBS at physiological pH) cannot correctly figure out the conditions occurring *in vivo* after the administration, much less the pathological situation (*Chapter 5*).

Riassunto

Il poli(lattide-co-glicolide) [PLGA] è un polimero biocompatibile e biodegradabile ampiamente utilizzato in campo farmaceutico, per l'allestimento di formulazioni parenterali a rilascio prolungato. Tuttavia, esistono delle limitazioni legate alle caratteristiche del materiale e/o del medicinale finito che devono essere tenute in considerazione. A titolo di esempio, il PLGA presenta una limitata capacità di incapsulare molecole idrofile e una elevata instabilità fisica e chimica in mezzi acquosi; subisce inoltre gli effetti degradativi dei metodi di sterilizzazione e causa la diminuzione del pH ambientale durante la fase di degradazione.

Al fine di superare questi limitazioni, nella presente tesi di dottorato sono state studiate le prestazioni di due famiglie di materiali, quali quella dei PLGA coniugati con anti-ossidanti (g-AA-PLGA) e quella dei polimeri a blocchi a struttura poli-uretanica con segmenti di poli-esteri (DegraPol®).

g-AA-PLGA – Uno studio effettuato *in vitro* utilizzando cellule endoteliali (EC) e cellule muscolari lisce (SMC) ha dimostrato che le nanoparticelle (NP) di g-AA-PLGA, in particolare di PLGA graffato con acido caffeico (g-CA-PLGA), sono captate in maggiore quantità rispetto a quelle costituite da PLGA, indipendentemente dal rapporto tra i due monomeri. Al fine di valutare il possibile impiego di g-CA-PLGA nella progettazione di drug eluting balloon (DEB) da angioplastica, NP contenenti fluvastatina sono state preparate e caratterizzate. Le NP hanno dimostrato un'attività anti-proliferativa ed anti-migratoria nei confronti di SMC umane per un

periodo di tempo di almeno 1 settimana, senza variare in modo significativo la concentrazione efficace del principio attivo (NP/fluvastatina: 4 μM; soluzione fluvastatina: 1 μM). Concentrazioni maggiori sono risultate necessarie per ottenere un effetto simile nelle EC, indicando la possibilità di inibire la proliferazione delle SMC senza compromettere i processi di riparazione dell'endotelio vasale. Questi risultati indicano chiaramente le potenzialità di questo sistema nella prevenzione della restenosi conseguente ad angioplastica (*Capitolo 1*). Poiché le NP devono essere essiccate per poter essere caricate in un DEB, è stato effettuato uno studio volto a selezionare ed ottimizzare il processo di essiccamento più idoneo e gli eccipienti che meglio consentano la ricostituzione delle NP. I risultati hanno dimostrato che le maltodestrine (MDX) possono essere vantaggiosamente usate come agente ausiliario durante spray-drying e freeze-drying, in quanto hanno permesso la facile e rapida ricostituzione delle NP sia di PLGA che di g-CA-PLGA, indipendentemente dalla tecnica di essiccamento considerata [*Capitolo 2*].

DegraPol® - Si tratta di una famiglia di polimeri multi-blocco biocompatibili e biodegradabili a struttura poli-uretanica progettata per generare fibre mediante la tecnica dell'elettrospinning. La possibile applicazione nella progettazione di formulazioni nano- e micro-particellari idonee per la chemo-embolizzazione ed il rilascio prolungato di principi attivi non è mai stata studiata. È stato quindi dimostrato che è possibile ottenere particelle sferiche con dimensioni nano- e micrometriche mediante il metodo di emulsione/evaporazione del solvente. È stato inoltre evidenziato che la composizione del mezzo disperdente deve essere attentamente studiata, data la tendenza delle particelle di DegraPol® di formare aggregati a causa del potenziale zeta neutro del materiale (*Capitolo 3*).

Considerata anche la nota stabilità al processo di sterilizzazione (radiazioni ionizzazioni per g-AA-PLGA ed etilene ossido per DegraPol®), è possibile concludere che sia il PLGA coniugato con anti-ossidanti sia il DegraPol® sono materiali appropriati per la preparazione di sistemi particolati per la veicolazione di farmaci, che possano superare alcune delle limitazioni associate al PLGA.

In generale, lo sviluppo di questi prodotti non può essere distaccato dal corrispondente quadro regolatorio. Particolarmente per sistemi parenterali a rilascio prolungato, l'elaborazione di un appropriato test *in vitro* per valutare il rilascio del farmaco è critica, data la complessità nella definizione di metodi in grado di discriminare *in vitro* le prestazioni biofarmaceutiche di prodotti che hanno un comportamento *in vivo* diverso (*Capitolo 4*). Nel caso di microsfere di PLGA da somministrare per via intra-articolare, è stato proposto un approccio bio-rilevante al fine di valutare la prestazione di microsfere costituite da PLGA con diverso rapporto lattide/glicolide e con diversa quantità di farmaco incapsulata, in condizioni simulate di salute e patologia. Inoltre, è stato dimostrato che le proteine contenute del mezzo di rilascio simulante la condizione di osteoartrite hanno un'influenza sul rilascio del principio attivo dal sistema micro-particellare. Questo indica che semplici sistemi tampone non possono correttamente interpretare le condizioni che si verificano *in vivo* dopo la somministrazione intra-articolare, in particolar modo nello stato patologico (*Capitolo 5*).

Table of contents

Introduction

Parenteral preparations are defined as "*sterile preparations intended for administration by injection, infusion or implantation into the human or animal body*" [1]. Several advantages characterize this route of administration, such as a rapid availability of the drug with a consequently fast onset of action, particularly useful in case of emergency. However, in some circumstances, the maintenance of systemic drug concentrations within the therapeutically effective range, for a prolonged period of time, is fundamental for assuring an effective treatment.

To purse this aim, over the last decades, sustained release formulations intended for parenteral administration have gained an exponential interest due to unquestionable advantages compared to the conventional ones [2,3]. The predictable and reproducible drug release profile over a defined period of time and the reduced incidence of side effects, generally result in enhanced therapeutic outcomes [2,4–6]. Indeed, the drug concentrations are maintained constant over time with respect to immediate dosage forms and the lower number of administration significantly improve the adherence of patients, reducing the problems associated to the skipped doses.

Among all the technologies proposed for sustaining the release of a drug after a parenteral administration there are also polymer-based systems which consist in complex platforms, where the polymer controls the drug release according to different mechanisms as discussed below. The introduction of biodegradable polymers, particularly those with a remarkable biocompatibility, has completely renewed the concept of *depot* system, with the development of different sized and shaped products – from rods, films and wafers to microspheres and nanoparticles – depending on the intended parenteral route [7–12]. The biocompatibility concerns with "*the ability of the material to perform with an appropriate host response in a specific application*" [13]. This is a general definition, given the wide range of today applications of biomaterials. However, a key factor at the basis of the classification of a material as "*biocompatible*" is its acceptance by a living body [14]. The biodegradability is related to the degradation of the material in contact with tissue's fluids [7]. This process leads to the

formation of degradation byproducts that, as the original material, have to be accepted by the biological environment, without causing toxic reactions [14,15]. Again, on the basis of the intended use, biomaterials should display the optimal thermal/mechanical/degradation properties and, thus, polymers with the desired residence time in a physiological environment, hardness, toughness and with the possibility of chemical/biological functionalization are needed [13]. The loading of an active principle ingredient (API) is, in some cases, a fundamental step to be considered. In addition, the requirement of the sterility must be met, since these systems are intended to be parenterally administered or implanted.

In literature, there are many examples of synthetic polymers proposed or already applied in the formulation of products available on market, for applications in the biomedical or pharmaceutical fields [7,16]. Among these, the lactide/glycolide polymers, namely poly(lactic acid) [PLA], poly(glycolic acid) [PGA] and their copolymer poly(lactic-co-glycolic acid) [PLGA] have been extensively exploited in the last 35 years. PLGA has always dominated the scenes regarding pharmaceutical applications. It is approved by the main Regulatory Agencies (*i.e.,* European Medicine Agency – EMA – and Food and Drug Administration – FDA) for parenteral use, since it is recognized as safe and given the good biocompatibility with both hard and soft tissues. It is also possible to tailor the mechanical, thermal and drug release properties of the final product other than PLGA biodegradation rate, by varying the molecular weight, the monomer ratio and the end-group functionalization of the polymer itself [7,17]. However, the main drawbacks of this type of polymer are related to the limited ability in encapsulating hydrophilic compounds [18–20], the physical and chemical instabilities in aqueous media, which in turn prevent the possibility to have an aqueous-based product [21,22], and the detrimental effect of the sterilization methods [23–25]. Moreover, the drop off in the micro-environmental pH upon degradation can enhance the polymer degradation rate, following the so-called "*auto-catalysis phenomenon*", or the inflammation events at the implantation site [26–28], or even create a harsh environment for highly vulnerable macromolecules being encapsulated [29].

Several chemical modifications of PLGA backbone are reported in literature in the attempt to address these issues [30,31]. Among these, the grafting of antioxidants and the synthesis of novel biodegradable materials, containing polyesters segments in a multi-block organization, appear attractive. [32,33].

The covalent bonding of small anti-oxidant molecule to PLGA backbone, such as pyrogallic acid or caffeic acid, led to some improvements in the design of microparticles for sustain the release of protein(s). The grafting modified the degradation mechanism of the polymer, favouring the random chain scission rather than the chain-end scission, and determined a significant lower decrease in medium pH, during the degradation, compared to the native PLGA [32]. And again, by a double emulsions process, caffeic acid grafted to PLGA improved the encapsulation efficiency, as exemplified by the case of ovalbumin, suggesting that the anti-oxidant residues improved the compatibility between the components. Besides, this structural modification gave to PLGA and to encapsulated ovalbumin an amelioration of the chemical stability upon sterilization by ionizing radiations. These observations suggested that the functionalization with anti-oxidants represents a suitable approach to overcome three of the major drawbacks associated to PLGA systems, with promising exploitations also in the design of nano-metric particles [32,33].

Considering the multi-block polymeric materials, poly(urethane)s are used in the preparation of medical devices for tissue engineering. The modulation of their composition, as an example with polyesters domains, allows to tailor the biodegradation profile of the final material [34,35]. In particular, DegraPol®, a class of biocompatible poly(ester-urethane) materials [35,36], is currently used in the design of electrospun fibers for scaffolds in the tissue engineering [37–40], while its possible application in the preparation of spherically shaped particles for drug delivery is still lacking. Furthermore, DegraPol® can overcome issues related to the drop off in the micro-environmental pH during degradation and the stability upon the sterilization process [41,42].

Aim of the thesis

The aim of this doctoral thesis was to explore the possible application of antioxidant grafted to PLGA (g-AA-PLGA) and DegraPol® in the design of drug delivery systems, with particular emphasis on nanoparticulate drug delivery systems.

In the case of g-AA-PLGA, aiming to demonstrate their potentialities in the field of nanomedicine, the work was focused on the design of nanoparticles containing fluvastatin sodium for the prevention of restenosis after angioplasty (*Chapter 1*). Since the drying of such nanoparticulate drug delivery systems presents some critical aspects, related to the reconstitution procedure of the drug product and the cost of the process, the possibility to use a low-cost excipient (maltodextrins) in the two main techniques (*i.e.,* spray-drying and freeze-drying) was also investigated (*Chapter 2*).

In the case of DegraPol®, the work dealt with the set-up of the operative conditions suitable for the preparation of particles with tailored dimensions (*Chapter 3*).

Lastly, after looking at the regulatory issues associated to the development of long-acting parenteral drug delivery systems (*Chapter 4*), the experimental work was dedicated to the study of the release behaviours of controlled release formulations of methylprednisolone, namely PLGA microspheres and drug suspension, in media simulating the *in vivo* micro-environment at the joint level, under healthy and disease conditions (*Chapter 5*).

Parenteral sustained release: rationale and advantages

Parenteral administration is characterized by a rapid availability of the drug with a consequently fast onset of action, particularly useful in case of emergency. However, in some circumstances, the maintenance of systemic drug concentrations within the therapeutically effective range, over a prolonged period of time, is fundamental for assuring an effective treatment. Suitable approaches include repeated oral, transdermal, pulmonary or parenteral administrations, or the use of long-acting injections, or implants [3]. The design and development of the latter two dosage forms remain one of the most important research area in the pharmaceutical technology [2]. Indeed, long-acting injections and implants allow a predictable and reproducible drug release profile over a defined period of time and a reduced incidence of side effects, resulting in enhanced therapeutic outcomes [4–6]. Moreover, the lower number of administrations can significantly improve the adherence of patients to the therapeutic regime and reduce the problems associated to the skipped doses. An overall reduction of the cost associated to medical care must be taken into account. The formulation of repositioned active principle ingredients (API) into a long-acting parenteral product can also permit to extend its life via new indications or new formulations. This is applicable only when significant changes are introduced as the addition of new indications, dosage form or strength, delivery method, target patient population or conditions of use. For instance, leuprolide acetate has different medical indications depending on the dosage form. Leuprolide acetate is a synthetic nona-peptide, superactive (the biological activity is 10 times higher than the endogenous hormone) luteinizing hormone-releasing hormone (LH-RH) analogue. The instability in body fluids, the rapid excretion and the low bioavailability after oral, rectal, nasal and vaginal administrations has made leuprolide acetate a good candidate for a parenteral formulation [43]. At acute doses, it stimulates gonadotropin secretion by the pituitary gland and, therefore, the steroidogenesis in the genital organs. Its chronical administration at higher dosing, through a long-acting parenteral formulation, produces an opposite

pharmacological effect, resulting in the final inhibition of the steroidogenesis from testes or ovary and finds clinical applications in the treatment of hormone-dependent tumours, endometriosis, uterine fibroids, central precocious puberty and adenomyosis [44]. An example mainly related to the improvement in patients' compliance is Vivitrol® (naltrexone, Alkermes). It was firstly approved in 1984 as tablets for the oral treatment of opioid addition and alcohol dependence; while in 2006 the extended release injectable suspension (*i.e.,* PLGA microspheres) was approved for preventing opioid and alcohol dependence relapse, increasing the patient compliance thanks to the decreased number of administrations compared to the oral treatment (once per month injection).

Long-acting parenteral formulations: an overview

Among the main long-acting parenteral formulations, drug suspensions, oil-based injectable solutions, liposomes and polymer-based implantable systems are generally subcutaneously or intra-muscularly administered. In some cases, approved procedures may be defined, establishing as an example the needle size for microspheres injection, or the small surgery with a special injector needed in the case of large implants. Other special routes of administration can be also considered in the attempt to realize a local sustained release of the drug, such as the intra-articular or intra-ocular ones.

Injectable drug suspensions consist in lipophilic drug finely dispersed in an aqueous solvent, while in oil-based solutions the lipophilic drug is dissolved in vegetable oils, suitable for parenteral application. These formulations assure the prolonged release of the drug for few weeks. However, different mechanisms govern the release. Considering the drug suspensions, the rate-limiting step for the absorption is the drug dissolution, due to the low solubility of the drug in the formulation solvent and in the interstitial fluids surrounding drug particles upon injection [45]. The suspension of water-soluble drug in oils has also been proposed, even if controversial results indicate the ability to properly control the

drug release [46,47]. In this context, the design of water insoluble prodrugs is often exploited to tailor the physicochemical properties of the drug suspension. As an example, the aqueous suspension of olanzapine pamoate monohydrate (Zypadhera® in Europe and Zyprexa Relprevv® in US, Eli Lilly) is administered every 2 or 4 weeks in the chronic treatment of schizophrenia [48]. The approach of esterification is also applied to formulate oil-based solutions, in which the drug release is ruled by its partition between the oily vehicle of the formulation and the tissue fluids, beyond the bioconversion of the drug ester into the parent drug [49]. As an example, the esterification of haloperidol with decanoic acid allows to obtain a highly lipophilic prodrug which is dissolved in sesame oil [50–53]. This formulation (Haldol® Injections, Janssen) is administered by intra-muscular route for the maintenance therapy of psychoses, forming an oily *depot* from which the prodrug diffuses out towards the blood circulation, where esterase acts to release the active moiety. Compared to the conventional tablet formulation, it permits to reduce the administration to every 3-4 weeks [54].

Liposomes represent a lipid-based carrier both for hydrophilic and hydrophobic drugs. They consist in a self-closed spherical structure composed at least by one curved lipid layer called "*lamella*" that shows a liquid core. In this configuration, a hydrophilic compartment is created inside the vesicle where hydrophilic drug can be loaded. Conversely, within the interior of the lamella, a hydrophobic pocket allows to host lipid-soluble drugs [55]. The most famous example of liposome formulation available on the market is Doxil® product, PEGylated liposomes loaded by doxorubicin, approved in 1995 for the treatment of ovarian cancer, Kaposi's sarcoma and multiple myeloma. After intravenous administration, it shows enhanced circulation time and accumulation in the target tumour tissue thanks to the PEGylation.

Polymer-based systems consist in complex platforms, in which the polymer controls the drug delivery according to different mechanisms. The first commercialized systems were based on non-biodegradable materials and mainly consisted in a drug reservoir associated to a membrane controlling the drug

release. Examples of this technology are the contraceptive implants, such as the Norplant® system (Wyeth), a cylindrical implant composed of a poly-dimethylsiloxane membrane that governs the release of the steroid levonorgesterol, and Implanon® (Organon) where a poly(ethylene-co-vinyl acetate) membrane regulates the release of etonogestrel. Beyond to this class, systems in which the release of the drug is controlled by osmosis were proposed, such as Duros® implants, which assures the release of leuprolide acetate over 1 year according to a zero-order kinetic [56]. Additionally, hydrogel is used as controlling-release system, as in Supprelin LA® where the Hydron® technology permits to control the release of a peptide (histrelin) over 1 year, for the treatment of central precocious puberty in children [57].

However, in case of technologies prolonging the treatment from months to years, the response of the host must be considered. Indeed, the first inflammatory response due to the presence of a foreign body leads to the activation of more complex phenomena that results in the formation of a thin fibrous capsule of collagen around the implant. This capsule can become thicker at the later stages, as occurs in the case of some non-degradable polymers [*e.g.,* polyethylene or poly(ethylene-vinyl acetate)] [15,58]. The extent of tissue's response also depends on the injury created at the moment of implantation [59,60]. It should be considered that this fibrous capsule can modify the drug release from the implant, particularly in the late phase, determining variations in the expected pharmacological response [61]. Moreover, at the end of the treatment, the non-biodegradable implant must be removed, requiring the intervention of healthcare professionals and the use of local anesthesia. This can be seen in a positive manner if an early termination of the treatment is needed.

Biodegradable polymers

The introduction of biodegradable polymers, particularly those with a remarkable biocompatibility, has completely renewed the concept of *depot* systems. As reported above, the biocompatibility concerns with "*the ability of the material to perform with an appropriate host response in a specific application*" [13]. Despite being a general definition, it is implied that the classification of a material as "*biocompatible*" is its acceptance by a living body [14]. The biodegradability is related to the degradation of the material upon contact with tissue's fluids [7]. The degradation byproducts formed have to be accepted by the biological environment, without causing toxic reactions.

Among materials with these properties applied in the pharmaceutical field, the lactide/glycolide polymers, namely poly(lactic acid) [PLA], poly(glycolic acid) [PGA] and their copolymer poly(lactic-co-glycolic acid) [PLGA] have been extensively exploited in the last 35 years. These materials are synthetized by the ring-opening polymerization of lactone monomers, with an easily tailoring of the final product properties by varying the extent of the reaction, crystallinity, ratio between the monomers in case of copolymer and esterification of the end-residues [7]. The degradation occurs by hydrolysis of the ester bonds and leads to the formation of lactic and/or glycolic acids that are then metabolize by Krebs' cycle [17]. Several studies demonstrated that polyester-based objects implanted both in soft tissues and bones caused low inflammation responses, which is generally dependent on the tissue type, the size and topography of the object [62,63].

PLGA-based implants available on market include rods, microspheres and *in-situ* forming gels. As an example, Zoladex® (AstraZeneca) is a rod-shape implant that releases gosereline acetate over a period of 1-3 months to treat prostate cancer, endometriosis and uterine fibroids. On the other hand, microspheres are the most successful PLGA-based long-acting injections, covering a wide range of applications, from prophylactic treatment of prostate cancer to growth hormone deficiency to antipsychotic therapy (**Table 1**) [64]. Microspheres are spherical

shaped micro-matrices that release the encapsulated drug by diffusion and/or polymer degradation. The release can be tunable based on PLGA properties, such as monomers ratio, molecular weight, crystallinity, balance between hydrophilicity and hydrophobicity [65].

In-situ forming systems are the most recent among *depot* technologies. They usually consist in a solution of a biodegradable material and a drug(s), that undergoes to the transformation into a semi-solid or, generally, a more viscous fluid upon injection in a specific body site [66]. The solidification of the solution can be obtained by different mechanisms: temperature reduction or increment towards the body one for thermoplastic pastes and thermally induced gelling systems, solubility variation for *in-situ* polymer precipitation systems, thermal or photo-induced cross-linking process for *in-situ* cross-linking systems and the formation of organogels in the case of lyotropic liquid crystals [8,45]. In 2002, an *in-situ* gel forming formulation based on PLGA has been placed on market with the trade name of Eligard®. It is loaded by leuprolide acetate and it is indicated for the palliative treatment of advanced prostate cancer.

Both microspheres, and above all *in-situ* gelling systems, show an improvement in injectability compared to larger parenteral implants, improving the patient's acceptability and simplifying the procedure necessary for their administration.

Nowadays, PLGA are also studied to formulate nanoparticles as drug delivery carrier. They consist in spherically shaped matrices, with a narrow size distribution in the nanometric scale (*i.e.,* below 1000 nm), able to load a variety of drugs - from small molecules to macromolecules, such as vaccines and proteins. Different strategies have been also proposed to target specific organs, tissues or cells [9,10,67]. However, no PLGA nanoparticles-based product, or more in general polymeric nanoparticles, is currently approved by the main Regulatory Agencies as drug delivery systems.

Table 1 – PLGA microspheres-based drug products available on market.

Drug product (Date of approval)	Active ingredient	Indication(s)	Strength, dosing frequency	Route of administration
Lupron Depot (1989)	Leuprolide acetate	Palliative treatment of prostate cancer	7.5 mg every month; 22.5 mg, every 3 months; 30 mg, every 4 months; 45 mg, every 6 months;	Intramuscular
Lupron Depot-PED (1993)	Leuprolide acetate	Treatment of children with central precocious puberty	7.5 mg, 11.25 mg or 15 mg, every month; 11.25 mg or 30 mg, every 3 months;	Intramuscular
Lupron (1995)	Leuprolide acetate	Endometriosis managing	3.75 mg, every month	Intramuscular
Sandostatin LAR (1998)	Octreotide	Acromegaly, severe diarrhea/flushing episodes associated to metastatic carcinoid tumors, profuse watery diarrhea associated to VIP-secreting tumors	10 mg, 20 mg, or 30 mg, every 4 weeks	Subcutaneous
Somatuline LA (1998-2004)	Lanreotide acetate	Treatment of acromegaly, thyrotropic adenomas and neuroendocrine tumors	30 mg, every 14 days	Intramuscular
Trelstar (2000, 2001, 2010)	Triptorelin pamoate	Palliative treatment of prostate cancer	3.75 mg, every 4 weeks; 11.25 mg, every 12 weeks; 22.5 mg, every 24 weeks;	Intramuscular
Arestin (2001)	Minocycline HCl	Adjunct to scaling and root planning procedure in patient with adult periodontitis	1 mg, variable dosing frequency	Periodontal
Risperdal Consta (2003)	Risperidone	Treatment of schizophrenia and bipolar disorder	12.5 mg, 25 mg, 37.5 mg or 50 mg, every 2 weeks	Intramuscular
Vivitrol (2006)	Naltrexone	Treatment of alcohol dependence	380 mg, every 4 weeks	Intramuscular
Decapeptyl SR (2006)	Triptorelin pamoate	Treatment of prostatic cancer	3mg, every month; 11.25 mg, every 3 months; 22.5 mg, every 6 months;	Intramuscular
Ozurdex (2009)	Dexamethasone	Treatment of macular edema, non-infectious uveitis, diabetic macular edema	0.7mg, variable dosing frequency	Subcutaneous
Bydureon (2012)	Exenatide	Adjunct to diet and exercise to control glycemic levels in adults with diabetes type II	2 mg, every 7-days	Subcutaneous
Lupaneta Pack	Leuprolide acetate (microspheres); Norethindrone acetate (tablets)	Managing of initial painful symptoms of endometriosis and managing of recurrent symptoms	11.25 mg microspheres, every 3 months	Intramuscular (microspheres), oral (tablets)
Signifor LAR	Pasireotide pamoate	Treatment of acromegaly	20 mg, 40 mg or 60 mg, every 28 days	Intramuscular

As an alternative to PLGA, some Authors also proposed the use of poly(ε-caprolactone) [PCL], a semi-crystalline homopolymer prepared by the ring-opening polymerization of ε-caprolactone. Being more hydrophobic, it results in longer degradation time of at least 2 years [68]. In the attempt to tailor the degradability, copolymers with PLA, PLGA, poly(ethylene glycol) or poly(ethylene oxide) have been prepared [69,70].

Poly(anhydrides) are copolymers prepared by the condensation reaction of two fatty acids, to form a hydrophobic construct linked by anhydride bonds. Again, the scission of the chains is through a hydrolytic process. It is possible to synthetize polymers with different features by varying the properties of the fatty acids, *e.g.* hydrophobic *vs* more hydrophilic ones, and their ratio [71,72]. An example of a successfully commercialized poly(anhydrides) implant is Gliadel® Wafer (Guilford Pharmaceuticals) that is composed of 1,3-bis(p-carboxypenoxy) propane and sebacic acid copolymer microspheres loaded with carmustin, compressed to form a wafer, used for the treatment of recurrent glioblastoma multiforme. The wafer releases the drug over 5 days and the polymer completely degrades between 6-8 weeks [11,73].

Poly(ortho esters) [POE] are prepared by the condensation reaction between diols and diketene acetal. The degradation time can be further optimized copolymerized acids such as glycolic acid, leading to a new class of POE called Biochronomer® (AP Pharma) [74]. This novel material has shown good stability upon sterilization by ionizing radiations and they are under studies for the development of *depot* formulations [75,76]. To date, their main uses fall in the biomedical applications.

Poly(phosphoesters) [PPE] are a quite novel class of polymers in which monomers are linked with repeating phosphoester bonds, prepared by the ring opening reaction of the corresponding ring monomers. These materials have a great potential as alternative to carboester polymers (*i.e.,* PLA, PGA and PLGA) since, in principle, a great variety of structures can be designed based on the different oxidation status of phosphorous and on the alkyl or alkoxy side groups covalently

linked to the backbone [12]. Recently, a copolymer between PPE and PLA, that combined the fast degradation of phosphate groups and the lower of lactide bonds (Paclimer®, Guilford Pharmaceuticals), has been used for the preparation of paclitaxel loaded microspheres currently in Phase I clinical trial for the ovarian cancer treatment with promising results [77].

The last class discussed is the block copolymers of poly(butylene terephthalate). They are polyether ester multiblock copolymers between poly(ethylene glycol) [PEG] and polybutylene terephthalate (PBT), developed and commercialized with the brand name of PolyActive® by OctoPlus. The variations of both amount and length of PEG and PBT allow to modify the rate of degradation that occurs by hydrolysis of the ester bonds and oxidation of the ethers [78]. Since their biocompatibility and biodegradability, the Food and Drug Administration (FDA) approved them for the use in bone replacement applications.

Drawbacks of PLGA and PLGA-based particulate systems

Despite the variety of biodegradable polymers presented, aliphatic poly(esters) and poly(anhydrides) are the only two classes approved by the FDA and the European Medicine Agency (EMA) for application in medicinal products. In particular, PLGA with tailored physicochemical properties permits to easy prepare particles spherical in shape, covering a wide range of dimensions, following different production methods [79]. However, the development of a micro- or a nano-particulate drug loaded system is flanked by some critical issues that must be dealt with. Most of them are PLGA-dependent and thus they are common in both micro- and nano-particles, while others are related to the size of the system.

Considering the main steps in the workflow of particles preparation, the first aspect is the encapsulation efficiency of the drug. Independently of particle size, several drugs can be loaded in PLGA particles, but significant difficulties have been highlighted in loading hydrophilic drugs, inversely proportional to the size of the particles: the smaller the system – and hence the higher the surface area - the bigger the troubles [18]. Particles' preparation methods have a great influence on the encapsulation efficiency of the drug. Regarding nanoparticles, the simplest process, the nanoprecipitation, relies on the rapid diffusion of organic solvent (*e.g.,* acetone) into a non-solvent phase (*e.g.,* water) and is more efficacious for lipophilic drugs due to the hydrophobic nature of PLGA [19,80,81]. On the contrary, other methods that involves the preparation of single or multiple emulsions are successfully applied for encapsulating hydrophilic drugs or even hydrophilic macromolecules, despite nanoparticles with higher diameter are generally produce by these techniques compared to nanoprecipitation [20,82]. Nevertheless, residues of stabilizers used for emulsion preparation (*e.g.,* polyvinyl alcohol) can remain onto nanoparticles' surface and exert, in some cases, cellular toxicity [83]. The entrapment of hydrophilic compound in PLGA microspheres is challenging as well. Emulsion-based, spray-drying and microfluidics processes are the major methods reported in literature [84].

Another material-related aspect to take into account during the development stages is the poor long-term stability of PLGA micro- and nanoparticles. Both these drug delivery platforms can be produced as aqueous suspension, but the degradation of the polymer in water-based media, the physical instability of the suspension and the risk of microorganisms' growth make a drying step mandatory [21]. For this purpose, spray- and freeze-drying are the methods of choice, although stresses, such as variations in temperature, physical state or atomization, can induce the irreversible aggregation of the particles, invalidating the quality of the final product. Indeed, different classes of compounds (*i.e.,* sugars, polyols, cellulose derivatives or surfactants) are used as drying auxiliary agents in the attempt to obtain an easy and fast reconstitution of the dried product [22,85].

The first requirement that must be complied for parenteral preparations is the sterility. Generally speaking, the optimal sterilization process should not chemically or physically alter the properties of the polymer and the loaded drug, and it must produce a safety level, namely Sterility Assurance Level (SAL), of 10^{-6}. Based on the European Guideline 3AQ4a, the method of choice for sterilizing these medicinal products is the ionising radiations (γ-rays and electron beam) due to PLGA heat sensitivity [86]. However, both radiations generally lead to PLGA degradation due to the formation of relative stable radical species that propagate the chain scission by oxidation reactions [23,25,87]. Additionally, ionising radiations can cause cross-links between the PLGA monomers, determining changes in polymer's properties or in drug release, as well as drug degradation [88]. Unfortunately, the effects of irradiation on drug loaded delivery systems have to be evaluated case-by-case because the experimental set-up and the loaded active ingredient deeply influence the polymer degradation process [24,25,89,90]. Nowadays, to control the bioburden in the raw materials, PLGA is often irradiated and held in quarantine until the radical decay takes place. Then, the production process is carried out in aseptic conditions or in aseptic units, with the risk of product contaminations and having to comply with different and complicated procedures [91].

Regarding nanoparticles, one of the aspects that can affect their *in vivo* performance, is the cellular uptake, especially if the nanosystem has to release its payload inside the cells. Hence, it could be necessary to study the nanoparticles uptake efficiency as well as the intracellular localization in tissues or cells population target, in the attempt to optimize the formulation and the drug therapy [92,93]. Different particles features, such as size and surface charge, can influence their interaction with cells and, consequently their fate. Generally, PLGA nanoparticles poorly interacts with cells, since both the particles and phospholipid membrane are negatively charged, and due to the rapid exocytosis. This could determine a scarce payload delivery in the target cell and, thus, an unsatisfying pharmacological effect [94]. Strategies concerning the functionalization of particle surface can be applied, promoting the recognition with a specific extracellular target or the internalization through specific pathways [95].

The drop off in pH medium upon PLGA degradation is an issue related mostly to the material. As a matter of fact, when the hydrolysis of the polymer takes place, monomeric acids, *i.e.* lactic and glycolic acids, are formed. These degradation by-products determine the acidification of the surrounding microenvironment and within the core of PLGA micro- and nanoparticles, causing the "*auto-catalysis*" phenomenon. In other words, the decreased pH catalyses the scission of other polymer's ester bonds, accelerating the degradation of the system [26,27]. However, the presence of a drug dispersed throughout the polymer matrix could alter this process. If the drug is a base, two scenarios are possible: the base can catalyse the ester bonds cleavage as well, acting as a nucleophile agent [28], or neutralize the chains terminal carboxylic acid and minimize (or even eliminate) the auto-catalysis [96]. Thus, the degradation rate can be accelerated or slowed down by a basic drug, depending on the relative importance of the two effects [97]. Acidic drug can boost the hydrolysis of the polymer as per the auto-catalytic mechanism already described [98]. This phenomenon has implications not only in the release of the drug, but also in the inflammatory response of the surrounding

tissues where the system is implanted or in the drug stability, as in the case of Lupron Depot® where the acylation of leuprolide increased [99].

In vitro testing of parenteral long-acting formulations

The *in vitro* testing for parenteral long-acting drug products is still an open issue since no compendial assays and protocols are reported both in the European Pharmacopoeia (Ph.Eur.) and United States Pharmacopoeia (USP) for all the technologies available. This has led to the spreading of non-compendial set-ups available in literature that, in some cases, are not reproducible or applicable at an industrial scale. Despite the earlier *in vitro* dissolution tests were developed for immediate release oral dosage forms, the same principles can be extended to controlled and/or modified release formulations administered also by parenteral routes, such as suspensions, liposomes, microparticles or implants [100]. However, given the complexity of such formulations, it is not possible to set-up a single test able to address the requirements and the needs of all products. Hence, apparatus and methods should be evaluated case-by-case [101]. And again, the extended release of such formulations - from weeks to months or even years - makes impossible to match these times with those from industry. Thus, real-time release testing should be replaced or at least joined with accelerated tests for rapidly assess the impact of formulation and process variables on drug release profile. A correlation with real-time release is desirable and will allow using a short-term assay as predictive tool. However, accelerated test should not alter the mechanism of release and should be bio-relevant as well [102,103].

Another aspect that has to be considered is the bio-relevance of the *in vitro* release testing. Despite the several objectives pursed, the final goal behind a release test should be the ensure of the clinical performance of the medicinal product [103,104]. In the attempt to achieve *in vivo* relevance and then to establish, if possible, an *in vitro-in vivo* correlation (IVIVC), physiological variables at the site of interest such as body temperature, blood flow, vascularity, pH,

osmolarity and buffer capacity must be considered [104]. Furthermore, tissue responses have to be taken into account, since they can affect *in vivo* drug release [105]. As an example, regarding injectable formulations, sink conditions should be applied when the purpose of the release test is the quality control, while the bio-relevance of such conditions must be evaluated case-by-case, since in particular sites sink conditions may not exist [106]. Regulatory considerations on the role played by the *in vitro* release testing is extensively discussed in *Chapter 4*.

References

[1] Parenteral preparations, Eur. Pharmacopoeia. (2018) 871–873.

[2] A.S. Hoffman, The origins and evolution of “controlled” drug delivery systems, J. Control. Release. 132 (2008) 153–163. doi:DOI 10.1016/j.jconrel.2008.08.012.

[3] R. Langer, New methods of drug delivery, Science (80-.). 249 (1990) 1527–1533. doi:10.1126/science.2218494.

[4] C. Larsen, S.W. Larsen, H. Jensen, A. Yaghmur, J. Ostergaard, Role of in vitro release models in formulation development and quality control of parenteral depots, Expert Opin. Drug Deliv. 6 (2009) 1283–1295. doi:10.1517/17425240903307431 PM - 19941410 M4 - Citavi.

[5] L.E. Hollister, Site-specific drug delivery to CNS: Old and new, Neurobiol. Aging. 10 (1989) 631. doi:https://doi.org/10.1016/0197-4580(89)90156-5.

[6] R.J. Levy, V. Labhasetwar, S.A. Strickberger, T. Underwood, J. Davis, Controlled release implant dosage forms for cardiac arrhythmias: Review and perspectives, in: Drug Deliv. J. Deliv. Target. Ther. Agents, 1996: pp. 137–142. doi:10.3109/10717549609029442.

[7] K.E. Uhrich, S.M. Cannizzaro, R.S. Langer, K.M. Shakesheff, Polymeric Systems for Controlled Drug Release, Chem. Rev. 99 (1999) 3181–3198. doi:10.1021/cr940351u.

[8] C.B. Packhaeuser, J. Schnieders, C.G. Oster, T. Kissel, In situ forming parenteral drug delivery systems: An overview, Eur. J. Pharm. Biopharm. 58 (2004) 445–455. doi:10.1016/j.ejpb.2004.03.003.

[9] F. Danhier, E. Ansorena, J.M. Silva, R. Coco, A. Le Breton, V. Préat, PLGA-based nanoparticles: An overview of biomedical applications, J. Control. Release. 161 (2012) 505–522. doi:10.1016/j.jconrel.2012.01.043.

[10] M.. Hans, A.. Lowman, Biodegradable nanoparticles for drug delivery and targeting, Curr. Opin. Solid State Mater. Sci. 6 (2002) 319–327. doi:10.1016/S1359-0286(02)00117-1.

[11] A.J. Domb, Z.H. Israel, O. Elmalak, D. Teomim, A. Bentolila, Preparation and characterization of carmustine loaded polyanhydride wafers for treating brain tumors, Pharm. Res. 16 (1999) 762–765. doi:10.1023/A:1011995728760.

[12] Z.E. Yilmaz, C. Jérôme, Polyphosphoesters: New Trends in Synthesis and Drug Delivery Applications, Macromol. Biosci. 16 (2016) 1745–1761. doi:10.1002/mabi.201600269.

[13] B.D. Ratner, A.S. Hoffman, F.J. Schoen, J. Lemons, Biomaterials Science : A Multidisciplinary Endeavor, 2004. doi:10.1016/B978-0-08-087780-8.00153-4.

[14] H. Chen, L. Yuan, W. Song, Z. Wu, D. Li, Biocompatible polymer materials: Role of protein-surface interactions, Prog. Polym. Sci. 33 (2008) 1059–1087. doi:10.1016/j.progpolymsci.2008.07.006.

[15] J.M. Anderson, In vivo biocompatibility of implantable delivery systems and biomaterials, Eur. J. Pharm. Biopharm. 40 (1994) 1–8.

[16] S. Kim, J.H. Kim, O. Jeon, I.C. Kwon, K. Park, Engineered polymers for advanced drug delivery, Eur. J. Pharm. Biopharm. 71 (2009) 420–430. doi:10.1016/j.ejpb.2008.09.021.

[17] T.G. Park, Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition, Biomaterials. 16 (1995) 1123–1130. doi:10.1016/0142-9612(95)93575-X.

[18] T. Govender, S. Stolnik, M. Garnett, L. Illum, S. Davis, PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug., J. Control. Release. 57 (1999) 171–85. http://www.ncbi.nlm.nih.gov/pubmed/9971898.

[19] J.M. Barichello, M. Morishita, K. Takayama, T. Nagai, Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nanoprecipitation method, Drug Dev. Ind. Pharm. 25 (1999) 471–476. doi:10.1081/DDC-100102197.

[20] M. Iqbal, N. Zafar, H. Fessi, A. Elaissari, Double emulsion solvent evaporation techniques used for drug encapsulation, Int. J. Pharm. 496 (2015) 173–190. doi:10.1016/j.ijpharm.2015.10.057.

[21] R.F. Ribeiro, M.H. Motta, A.P.G. Härter, F.C. Flores, R.C.R. Beck, S.R. Schaffazick, C. De Bona Da Silva, Spray-dried powders improve the controlled release of antifungal tioconazole-loaded polymeric nanocapsules compared to with lyophilized products, Mater. Sci. Eng. C. 59 (2016) 875–884. doi:10.1016/j.msec.2015.10.035.

[22] W. Abdelwahed, G. Degobert, S. Stainmesse, H. Fessi, Freeze-drying of nanoparticles: Formulation, process and storage considerations, Adv. Drug Deliv. Rev. 58 (2006) 1688–1713. doi:10.1016/j.addr.2006.09.017.

[23] L. Montanari, M. Costantini, E.C. Signoretti, L. Valvo, M. Santucci, M. Bartolomei, P. Fattibene, S. Onori, A. Faucitano, B. Conti, I. Genta, Gamma irradiation effects on poly(DL-lactictide-co-glycolide) microspheres, J. Control. Release. 56 (1998) 219–229. doi:10.1016/S0168-3659(98)00082-0.

[24] L. Montanari, F. Cilurzo, L. Valvo, A. Faucitano, A. Buttafava, A. Groppo, I. Genta, B. Conti, Gamma irradiation effects on stability of poly(lactide-co-glycolide) microspheres containing clonazepam, J. Control. Release. 75 (2001) 317–330. doi:10.1016/S0168-3659(01)00401-1.

[25] L. Montanari, F. Cilurzo, F. Selmin, B. Conti, I. Genta, G. Poletti, F. Orsini, L. Valvo, Poly(lactide-co-glycolide) microspheres containing bupivacaine: Comparison between gamma and beta irradiation effects, J. Control. Release. 90 (2003) 281–290. doi:10.1016/S0168-3659(03)00153-6.

[26] S. Li, S. McCarthy, Further investigations on the hydrolytic degradation of poly (DL-lactide), Biomaterials. 20 (1999) 35–44. doi:10.1016/S0142-9612(97)00226-3.

[27] A. Shenderova, T.G. Burke, S.P. Schwendeman, The Acidic Microclimate in Poly(lactide-co-glycolide) Microspheres Stabilizes Camptothecins, Pharm. Res. 16 (1999) 241–248. doi:Doi 10.1023/A:1018876308346.

[28] H. V. Maulding, T.R. Tice, D.R. Cowsar, J.W. Fong, J.E. Pearson, J.P. Nazareno, Biodegradable microcapsules: Acceleration of polymeric excipient hydrolytic rate by incorporation of a basic medicament, J. Control. Release. 3 (1986) 103–117. doi:10.1016/0168-3659(86)90071-4.

[29] Y.Y. Huang, T.W. Chung, T.W. Tzeng, A method using biodegradable polylactides/polyethylene glycol for drug release with reduced initial burst, Int. J. Pharm. 182 (1999) 93–100. doi:10.1016/S0378-5173(99)00060-5.

[30] F.Y. Han, K.J. Thurecht, A.K. Whittaker, M.T. Smith, Bioerodable PLGA-based microparticles for producing sustained-release drug formulations and strategies for improving drug loading, Front. Pharmacol. 7 (2016). doi:10.3389/fphar.2016.00185.

[31] S.-L. Bee, Z.A.A. Hamid, M. Mariatti, B.H. Yahaya, K. Lim, S.-T. Bee, L.T. Sin, Approaches to Improve Therapeutic Efficacy of Biodegradable PLA/PLGA Microspheres: A Review, Polym. Rev. 58 (2018) 495–536. doi:10.1080/15583724.2018.1437547.

[32] F. Cilurzo, F. Puoci, F. Selmin, F. Iemma, P. Minghetti, Pyrogallic acid-PLGA conjugate as new biodegradable material suitable for final sterilization by irradiation, Polym. Adv. Technol. 22 (2011) 2201–2205. doi:10.1002/pat.1746.

[33] F. Selmin, F. Puoci, O. Parisi, S. Franzé, U. Musazzi, F. Cilurzo, Caffeic Acid-PLGA Conjugate to Design Protein Drug Delivery Systems Stable to Irradiation, J. Funct. Biomater. 6 (2015) 1–13. doi:10.3390/jfb6010001.

[34] A. Lendlein, P. Neuenschwander, U.W. Suter, Hydroxy-telechelic copolyesters with well defined sequence structure through ring-opening polymerization, Macromol. Chem. Phys. 201 (2000) 1067–1076. doi:10.1002/1521-3935(20000701)201:11<1067::AID-MACP1067>3.0.CO;2-Y.

[35] A. Lendlein, M. Colussi, P. Neuenschwander, U.W. Suter, Hydrolytic degradation of phase-segregated multiblock copoly(ester urethane)s containing weak links, Macromol. Chem. Phys. 202 (2001) 2702–2711. doi:10.1002/1521-3935(20010901)202:13<2702::AID-MACP2702>3.0.CO;2-I.

[36] T.D. Hirt, P. Neuenschwander, U.W. Suter, Synthesis of degradable, biocompatible, and tough block-copolyesterurethanes, Macromol. Chem. Phys. 197 (1996) 4253–4268. doi:10.1002/macp.1996.021971221.

[37] M. Borkenhagen, R.C. Stoll, P. Neuenschwander, U.W. Suter, P. Aebischer, In vivo performance of a new biodegradable polyester urethane system used as a nerve guidance channel, Biomaterials. 19 (1998) 2155–2165. doi:10.1016/S0142-9612(98)00122-7.

[38] B. Saad, T.D. Hirt, M. Welti, G.K. Uhlschmid, P. Neuenschwander, U.W. Suter, Development of degradable polyesterurethanes for medical applications: In vitro and in vivo evaluations, J. Biomed. Mater. Res. 36 (1998) 65–74. doi:10.1002/(SICI)1097-4636(199707)36:1<65::AID-JBM8>3.0.CO;2-J.

[39] B. Saad, Y. Kuboki, M. Welti, G.K. Uhlschmid, P. Neuenschwander, U.W. Suter, DegraPol-Foam: A degradable and highly porous polyesterurethane foam as a new substrate for bone formation, Artif. Organs. 24 (2000) 939–945. doi:10.1046/j.1525-1594.2000.06664.x.

[40] O. Evrova, J. Houska, M. Welti, E. Bonavoglia, M. Calcagni, P. Giovanoli, V. Vogel, J. Buschmann, Bioactive, Elastic, and Biodegradable Emulsion Electrospun DegraPol Tube Delivering PDGF-BB for Tendon Rupture Repair, Macromol. Biosci. 16 (2016) 1048–1063. doi:10.1002/mabi.201500455.

[41] H. Krynauw, L. Bruchmüller, D. Bezuidenhout, P. Zilla, T. Franz, Degradation-induced changes of mechanical properties of an electro-spun polyester-urethane scaffold for soft tissue regeneration, J. Biomed. Mater. Res. Part B Appl. Biomater. 99B (2011) 359–368. doi:10.1002/jbm.b.31907.

[42] G. Meier Bürgisser, M. Calcagni, A. Müller, E. Bonavoglia, G. Fessel, J.G. Snedeker, P. Giovanoli, J. Buschmann, Prevention of peritendinous adhesions using an electrospun DegraPol polymer tube: A histological, ultrasonographic, and biomechanical study in rabbits, Biomed Res. Int. 2014 (2014). doi:10.1155/2014/656240.

[43] H. Okada, One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate, Adv. Drug Deliv. Rev. 28 (1997) 43–70. doi:10.1016/S0169-409X(97)00050-1.

[44] P. Periti, T. Mazzei, E. Mini, Clinical pharmacokinetics of depot leuprorelin, Clin. Pharmacokinet. 41 (2002) 485–504. doi:10.2165/00003088-200241070-00003.

[45] Y.-S. Rhee, C.-W. Park, P.P. DeLuca, H.M. Mansour, Sustained-Release Injectable Drug Delivery, Pharm. Technol. (2010) 6–13. http://www.pharmtech.com/pharmtech/Id=43497%5Cnhttp://www.pharmtech.com/pharmtech/Drug+Delivery/Sustained-Release-Injectable-Drug-Delivery/ArticleStandard/Article/detail/694429?contextCategoryId=43497.

[46] M.L. Slevin, E.M. Piall, A. Johnston, D.A. Levison, G.W. Aherne, S.B. Tree, T.A. Lister, The pharmacokinetics of subcutaneous bolus cytosine arabinoside in an arachis oil plus aluminium distearate suspension, Invest. New Drugs. 2 (1984) 271–276. doi:10.1007/BF00175376.

[47] H.A.C. Titulaer, J. Zuidema, P.A. Kager, J.C.F.M. Wetsteyn, C.B. Lugt, F.W.H.M. Merkus, The pharmacokinetics of artemisinin after oral, intramuscular and rectal administration to volunteers, J. Pharm. Pharmacol. 42 (1990) 810–813. doi:10.1111/j.2042-7158.1990.tb07030.x.

[48] J. Kane, H. Detke, D. Naber, G. Sethuraman, D. Lin, R. Bergstrom, D. McDonnell, Olanzapine long-acting injection: a 24-week, randomized, double-blind trial of maintenance treatment in patients with schizophrenia, Am. J. Psychiatry. 167 (2010) 181–189. doi:10.1176/appi.ajp.2009.07081221.

[49] S. Weng Larsen, C. Larsen, Critical Factors Influencing the In Vivo Performance of Long-acting Lipophilic Solutions—Impact on In Vitro Release Method Design, AAPS J. 11 (2009) 762–770. doi:10.1208/s12248-009-9153-9.

[50] W.H. Chang, S.K. Lin, D.J. Juang, L.C. Chen, C.H. Yang, W.H. Hu, C.P. Chien, Y.W.F. Lam, M.W. Jann, Prolonged haloperidol and reduced haloperidol plasma concentrations after decanoate withdrawal, Schizophr. Res. 9 (1993) 35–40. doi:10.1016/0920-9964(93)90007-6.

[51] D.H. Wiles, R.G. McCreadie, A. Whitehead, Pharmacokinetics of haloperidol and fluphenazine decanoates in chronic schizophrenia, Psychopharmacology (Berl). 101 (1990) 274–281. doi:10.1007/BF02244140.

[52] J.P. Luo, J.W. Hubbard, K.K. Midha, Studies on the mechanism of absorption of depot neuroleptics: Fluphenazine decanoate in sesame oil, Pharm. Res. 14 (1997) 1079–1084. doi:10.1023/A:1012165731390.

[53] J. Dreyfuss, J.M. Shaw, J.J. Ross, Fluphenazine enanthate and fluphenazine decanoate: Intramuscular injection and esterification as requirements for slow-release characteristics in dogs, J. Pharm. Sci. 65 (1976) 1310–1315. doi:10.1002/jps.2600650912.

[54] Janssen, Haldol® Injection - Product information, 1991. (2017). https://www.janssen.com/australia/sites/www_janssen_com_australia/files/prod_files/live/haldol_pi.pdf.

[55] A. Sharma, U.S. Sharma, Liposomes in drug delivery: Progress and limitations, Int. J. Pharm. 154 (1997) 123–140. doi:10.1016/S0378-5173(97)00135-X.

[56] J. C Wright, A. E Chester, R. Skowronski, C. Lucas, Long-Term Controlled Delivery of Therapeutic Agents via an Implantable Osmotically Driven System: The DUROS Implant, (2003) 657–669.

[57] SUPPRELIN® LA (histrelin acetate), Endo Pharm. Inc. (n.d.). https://www.supprelinla.com (accessed September 13, 2018).

[58] J.M. Anderson, A. Rodriguez, D.T. Chang, Foreign body reaction to biomaterials, Semin. Immunol. 20 (2008) 86–100. doi:10.1016/j.smim.2007.11.004.

[59] J.M. Anderson, H. Niven, J. Pelagalli, L.S. Olanoff, R.D. Jones, The role of the fibrous capsule in the function of implanted drug-polymer sustained release systems, J. Biomed. Mater. Res. 15 (1981) 889–902. doi:10.1002/jbm.820150613.

[60] J. Anderson, A. Rodriguez, D. Chang, Foreign Body Reaction to Biomaterials, Semin. Immunol. 20 (2008) 86–100. doi:10.1097/MPG.0b013e3181ec1d2e.Somatic.

[61] J.M. Anderson, W.G. Brodbeck, Local tissue responses to polymer implants affecting pharmacokinetics, pharmacodynamics and metabolism of proteins and peptides, in: J.M. Randall, A. Daugherty (Eds.), Proteins Pept. Pharmacokinet. Pharmacodyn. Metab. Outcomes, Informa Healthcare Publishers, New York, USA, 2009.

[62] G.E. Visscher, R.L. Robison, H. V. Maulding, J.W. Fong, J.E. Pearson, G.J. Argentieri, Biodegradation of and tissue reaction to 50:50 poly(DL-lactide-co-glycolide) microcapsules, J. Biomed. Mater. Res. 19 (1985) 349–365. doi:10.1002/jbm.820190315.

[63] J. Tiainen, Y. Soini, P. Törmälä, T. Waris, N. Ashammakhi, Self-reinforced polylactide/polyglycolide 80/20 screws take more than 1½ years to resorb in rabbit cranial bone, J. Biomed. Mater. Res. Part B Appl. Biomater. 70B (2004) 49–55. doi:10.1002/jbm.b.30013.

[64] Y. Wang, W. Qu, S.H. Choi, FDA's Regulatory Science Program for Generic PLA / PLGA - Based Drug Products, Am. Pharm. Rev. 19 (2016) 5–9. http://www.americanpharmaceuticalreview.com/Featured-Articles/188841-FDA-s-Regulatory-Science-Program-for-Generic-PLA-PLGA-Based-Drug-Products/.

[65] R. Jain, N.H. Shah, A.W. Malick, C.T. Rhodes, Controlled drug delivery by biodegradable poly(ester) devices: Different preparative approaches, Drug Dev. Ind. Pharm. 24 (1998) 703–727. doi:10.3109/03639049809082719.

[66] A. Hatefi, B. Amsden, Biodegradable injectable in situ forming drug delivery systems, J. Control. Release. 80 (2002) 9–28. doi:10.1016/S0168-3659(02)00008-1.

[67] A. Prokop, J.M. Davidson, Nanovehicular intracellular delivery systems, J. Pharm. Sci. 97 (2008) 3518–3590. doi:10.1002/jps.21270.

[68] B. Saad, U.W. Suter, Biodegradable Polymeric Materials, in: K.H.J. Buschow, R.W. Cahn, M.C. Flemings, B. Ilschner, E.J. Kramer, S. Mahajan, P. Veyssière (Eds.), Encycl. Mater. Sci. Technol., Elsevier, Oxford, 2001: pp. 551–555. doi:https://doi.org/10.1016/B0-08-043152-6/00105-4.

[69] S. Zhou, X. Deng, H. Yang, Biodegradable poly(ε-caprolactone)-poly(ethylene glycol) block copolymers: characterization and their use as drug carriers for a controlled delivery system, Biomaterials. 24 (2003) 3563–3570. doi:https://doi.org/10.1016/S0142-9612(03)00207-2.

[70] C.M. Dong, Y.Z. Guo, K.Y. Qiu, Z.W. Gu, X. De Feng, In vitro degradation and controlled release behavior of D,L-PLGA50 and PCL-b-D,L-PLGA50 copolymer microspheres, J. Control. Release. 107 (2005) 53–64. doi:10.1016/j.jconrel.2005.05.024.

[71] N. Kumar, R.S. Langer, A.J. Domb, Polyanhydrides: an overview, Adv. Drug Deliv. Rev. 54 (2002) 889–910. doi:https://doi.org/10.1016/S0169-409X(02)00050-9.

[72] J.P. Jain, S. Modi, A.J. Domb, N. Kumar, Role of polyanhydrides as localized drug carriers, J. Control. Release. 103 (2005) 541–563. doi:https://doi.org/10.1016/j.jconrel.2004.12.021.

[73] A.B. Fleming, W.M. Saltzman, Pharmacokinetics of the carmustine implant, Clin. Pharmacokinet. 41 (2002) 403–419. doi:10.2165/00003088-200241060-00002.

[74] J. Heller, J. Barr, Biochronomer™ technology, Expert Opin. Drug Deliv. 2 (2005) 169–183. doi:10.1517/17425247.2.1.169.

[75] A. Rothen-Weinhold, K. Schwach-Abdellaoui, J. Barr, S.Y. Ng, H.R. Shen, R. Gurny, J. Heller, Release of BSA from poly(ortho ester) extruded thin strands, J. Control. Release. 71 (2001) 31–37. doi:10.1016/S0168-3659(00)00348-5.

[76] X.L. Bai, Y.Y. Yang, T.S. Chung, S. Ng, J. Heller, Effect of polymer compositions on the fabrication of Poly(ortho-ester) microspheres for controlled release of protein, J. Appl. Polym. Sci. 80 (2001) 1630–1642. doi:10.1002/app.1257.

[77] K.W. Li, W. Dang, B.M. Tyler, G. Troiano, T. Tihan, H. Brem, K.A. Walter, Polilactofate microspheres for paclitaxel delivery to central nervous system malignancies, Clin. Cancer Res. 9 (2003) 3441–3447.

[78] A.A. Deschamps, D.W. Grijpma, J. Feijen, Poly(ethylene oxide)/poly(butylene terephthalate) segmented block copolymers: the effect of copolymer composition on physical properties and degradation behavior, Polymer (Guildf). 42 (2001) 9335–9345. doi:https://doi.org/10.1016/S0032-3861(01)00453-0.

[79] K. Avgoustakis, Polylactic-Co-Glycolic Acid (PLGA), Encycl. Biomater. Biomed. Eng. (2005) 1–11. doi:10.1081/E-EBBE-120013950.

[80] T. Betancourt, B. Brown, L. Brannon-Peppas, Doxorubicin-loaded PLGA nanoparticles by nanoprecipitation: preparation, characterization and in vitro evaluation, Nanomedicine (Lond). 2 (2007) 219–232. doi:10.2217/17435889.2.2.219.

[81] K.S. Yadav, S. Jacob, G. Sachdeva, K. Chuttani, A.K. Mishra, K.K. Sawant, Long circulating PEGylated PLGA nanoparticles of cytarabine for targeting leukemia, J. Microencapsul. 28 (2011) 729–742. doi:10.3109/02652048.2011.615949.

[82] M.. Zambaux, F. Bonneaux, R. Gref, P. Maincent, E. Dellacherie, M.. Alonso, P. Labrude, C. Vigneron, Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method, J. Control. Release. 50 (1998) 31–40. doi:10.1016/S0168-3659(97)00106-5.

[83] N. Grabowski, H. Hillaireau, J. Vergnaud, N. Tsapis, M. Pallardy, S. Kerdine-Römer, E. Fattal, Surface coating mediates the toxicity of polymeric nanoparticles towards human-like macrophages, Int. J. Pharm. 482 (2015) 75–83. doi:10.1016/j.ijpharm.2014.11.042.

[84] F. Ramazani, W. Chen, C.F. Van Nostrum, G. Storm, F. Kiessling, T. Lammers, W.E. Hennink, R.J. Kok, Strategies for encapsulation of small hydrophilic and amphiphilic drugs in PLGA microspheres: State-of-the-art and challenges, Int. J. Pharm. 499 (2016) 358–367. doi:10.1016/j.ijpharm.2016.01.020.

[85] J. Broadhead, S.K. Edmond Rouan, C.T. Rhodes, The spray drying of pharmaceuticals, Drug Dev. Ind. Pharm. 18 (1992) 1169–1206. doi:10.3109/03639049209046327.

[86] ICH, 3AQ4a: THE USE OF IONISING RADIATION IN THE MANUFACTURE OF MEDICINAL PRODUCTS, Ich. (1992).

[87] A.G. Hausberger, R.A. Kenley, P.P. DeLuca, Gamma irradiation effects on molecular weight and in vitro degradation of poly(D,L-lactide-CO-glycolide) microparticles., Pharm. Res. 12 (1995) 851–6. http://www.ncbi.nlm.nih.gov/pubmed/7667189.

[88] J.A. Bushell, M. Claybourn, H.E. Williams, D.M. Murphy, An EPR and ENDOR study of γ- and β-radiation sterilization in poly (lactide-co-glycolide) polymers and microspheres, J. Control. Release. 110 (2005) 49–57. doi:10.1016/j.jconrel.2005.09.009.

[89] B. Bittner, K. Mader, C. Kroll, H.H. Borchert, T. Kissel, Tetracycline-HCl-loaded poly(DL-lactide-co-glycolide) microspheres prepared by a spray drying technique: influence of gamma-irradiation on radical formation and polymer degradation., J. Control. Release. 59 (1999) 23–32. doi:10.1016/S0168-3659(98)00170-9.

[90] C. Volland, M. Wolff, T. Kissel, The influence of terminal gamma-sterilization on captopril containing poly(d,l-lactide-co-glycolide) microspheres, J. Control. Release. 31 (1994) 293–305. doi:10.1016/0168-3659(94)90012-4.

[91] Y.W. Jo, B.H. Woo, A.M. Hazrati, P.P. DeLuca, Use of PharmASep unit for processing microspheres, AAPS PharmSciTech. 2 (2001) E—TN2. doi:10.1208/pt0201_tn2.

[92] A. Lamprecht, U. Schäfer, C.M. Lehr, Size-dependent bioadhesion of micro- and nanoparticulate carriers to the inflamed colonic mucosa, Pharm. Res. 18 (2001) 788–793. doi:10.1023/A:1011032328064.

[93] J. Panyam, S.K. Sahoo, S. Prabha, T. Bargar, V. Labhasetwar, Fluorescence and electron microscopy probes for cellular and tissue uptake of poly(D,L-lactide-co-glycolide) nanoparticles, Int. J. Pharm. 262 (2003) 1–11. doi:10.1016/S0378-5173(03)00295-3.

[94] C. Cella, F. Martello, S. Ghisletti, C. Lenardi, P. Milani, S. Argentiere, Amine-modified poly(vinyl alcohol) as a novel surfactant to modulate size and surface charge of poly(lactide-co-glycolide) nanoparticles, Polym. Int. 65 (2016) 792–797. doi:10.1002/pi.5122.

[95] S.M. Moghimi, A.C. Hunter, J.C. Murray, Long-circulating and target-specific nanoparticles: theory to practice., Pharmacol. Rev. 53 (2001) 283–318. doi:VL - 53.

[96] J. Mauduit, N. Bukh, M. Vert, Gentamycin/poly (lactic acid) blends aimed at sustained release local antibiotic therapy administered per-operatively. III. The case of gentamycin sulfate in films prepared from high and low molecular weight poly (DL-lactic acids), J. Control. Release. 25 (1993) 43–49. doi:https://doi.org/10.1016/0168-3659(93)90093-K.

[97] S. Li, M. Vert, Lactic Acid-based Degradable Polymers, in: Biodegrad. Aliphatic Polyesters, 1996: pp. 43–87. https://www.smithersrapra.com/SmithersRapra/media/Sample-Chapters/Handbook-of-Biodegradable-Polymers-2nd-Edition.pdf.

[98] T. Nakamura, S. Hitomi, S. Watanabe, Y. Shimizu, K. Jamshidi, S. -H Hyon, Y. Ikada, Bioabsorption of polylactides with different molecular properties, J. Biomed. Mater. Res. 23 (1989) 1115–1130. doi:10.1002/jbm.820231003.

[99] A. Lucke, J. Kiermaier, A. Göpferich, Peptide acylation by poly(alpha-hydroxy esters)., Pharm. Res. 19 (2002) 175–81. doi:10.1023/A:1014272816454.

[100] M. Siewert, J. Dressman, C.K. Brown, V.P. Shah, J.-M. Aiache, N. Aoyagi, D. Bashaw, C. Brown, W. Brown, D. Burgess, J. Crison, P. DeLuca, R. Djerki, J. Dressman, T. Foster, K. Gjellan, V. Gray, A. Hussain, T. Ingallinera, J. Klancke, J. Kraemer, H. Kristensen, K. Kumi, C. Leuner, J. Limberg, P. Loos, L. Margulis, P. Marroum, H. Moeller, B. Mueller, M. Mueller-Zsigmondy, N. Okafo, L. Ouderkirk, S. Parsi, S. Qureshi, J. Robinson, V. Shah, M. Siewert, R. Uppoor, R. Williams, FIP/AAPS guidelines to dissolution/in vitro release testing of novel/special dosage forms, AAPS PharmSciTech. 4 (2003) 43–52. doi:10.1208/pt040107.

[101] V.P. Shah, M. Siewert, J. Dressman, H. Moeller, C.K. Brown, J.M. Aiache, C.K. Brown, J.. Dressman, R. Djerki, K. Gjellan, Dissolution/in vitro release testing of special dosage forms, Dissolution Technol. 9 (2002) 1–5.

[102] C.K. Brown, H.D. Friedel, A.R. Barker, L.F. Buhse, S. Keitel, T.L. Cecil, J. Kraemer, J.M. Morris, C. Reppas, M.P. Stickelmeyer, C. Yomota, V.P. Shah, FIP/AAPS Joint Workshop Report: Dissolution/In Vitro Release Testing of Novel/Special Dosage Forms, AAPS PharmSciTech. 12 (2011) 782–794. doi:10.1208/s12249-011-9634-x.

[103] S.S. D'Souza, P.P. DeLuca, Methods to assess in Vitro drug release from injectable polymeric particulate systems, Pharm. Res. 23 (2006) 460–474. doi:10.1007/s11095-005-9397-8.

[104] D.J. Burgess, D.J.A. Crommelin, A.S. Hussain, M.-L. Chen, Assuring quality and performance of sustained and controlled release parenterals: EUFEPS workshop report, AAPS PharmSci. 6 (2004) 100–111. doi:10.1208/ps060111.

[105] S.S. Iyer, W.H. Barr, H.T. Karnes, Profiling in vitro drug release from subcutaneous implants: a review of current status and potential implications on drug product development., Biopharm. Drug Dispos. 27 (2006) 157–170. doi:10.1002/bdd.493.

[106] D.J. Burgess, A.S. Hussain, T.S. Ingallinera, M.L. Chen, Assuring quality and performance of sustained and controlled release parenterals: AAPS workshop report, co-sponsored by FDA and USP, in: Pharm. Res., 2002: pp. 1761–1768. doi:10.1023/A:1020730102176.

Chapter 1

Fluvastatin eluting nanomaterials for the prevention of neo-intimal hyperplasia: an *in vitro* study

Abstract

Restenosis is one of the major long-term concern after percutaneous transluminal angioplasty (PTA), determining the uncontrolled proliferation of smooth muscle cells (SMC). Among the strategies developed to overcome this issue, drug eluting balloon are gaining interest. Nevertheless, the rapid wash out of the drug by the blood flow remains the main limitation. Thus, the aim of this work was to evaluate the feasibility to design biodegradable eluting nanoparticles (NP) containing fluvastatin. The work was designed as follow. The biodegradable materials, namely PLGA grafted to caffeic acid (g-CA-PLGA) and resveratrol (g-RV-PLGA), were synthetized and checked for physicochemical properties and cytotoxicity. Based on the ability of g-CA-PLGA and g-RV-PLGA NP to get access into the cells, the most suitable material was selected. The *in vitro* efficacy of fluvastatin-loaded NP was assessed by proliferation and migration of SMC and endothelial cells. The obtained data clearly demonstrated that by using g-CA-PLGA NP, it was possible to effectively deliver fluvastatin to cells over at least one week, inhibiting the proliferation of human SMC by 50% at 4 μM dose. This concentration was close to the one determined for free fluvastatin (1 μM), suggesting that this nanosystem, properly coupled with a suitable device such as an angioplasty balloon, could be used to reduce or even prevent the restenosis following PTA.

1.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death for both men and women accounting approximately for 45% of all deaths in Europe and 37% of all deaths in the European Union [1]. Percutaneous transluminal angioplasty (PTA) is known to effectively improve the prognosis of patients with vascular diseases, particularly those with acute coronary syndrome and critic limb ischemia [2]. However, in-stent restenosis (ISR) is a major concern that can compromise the long-term outcome of PTA [3], with rates of angiographic restenosis of as much as 30% after bare metal stents (BMS) positioning and about 12-15% with drug eluting stents (DES) [4]. The cellular processes responsible for restenosis secondary to PTA are very complex and include local re-endothelialization and vascular remodeling mediated by a variety of inflammatory cells, cytokines and growth factors. The pathophysiology of post-PTA restenosis involves neointimal formation that consists of three phases: thrombosis (within 24 h), recruitment (3-8 days) and proliferation, which starts on day 8 of PTA [5]. Poor re-endothelialization and excessive migration and proliferation of vascular smooth muscle cells (SMC) in the tunica media peak at 6 months after stenting [6]. This can result in obstructive neointimal hyperplasia and is the major mechanism involved in restenosis following PTA [7].

There are no "*zero restenosis*" devices and simple, safe and durable solutions to restenosis are needed. The pharmaceutical agents could have different targets, such as the inhibitions of cell cycle, SMC proliferation and migration, synthesis of extra cellular matrix and inflammatory mediators.

Statins are widely used for the treatment of hypercholesterolemia and to reduce the risk of cardiovascular morbidity and mortality in patients with or at risk of coronary heart disease [8]. In addition, statins have effects that go beyond their mechanism of action, following the so called "*pleiotropic effects*" [9–12]. These include anti-inflammatory, antithrombotic, antioxidant, antimitotic, inhibition of SMC proliferation [12–14] and matrix metalloproteinase secretion activities [15].

Preprocedural statin therapy may reduce peri- and post-PTA myonecrosis and the need for repeat revascularization, while statin-eluting stents inhibit ISR in animal models [5]. Indeed, animal studies indicate that the local delivery of intimal hyperplasia-inhibiting drugs with the positioning of DES is able to promote vascular re-endothelialization and prevent restenosis [16], although this remains controversial. This may be due to inadequate drug concentrations or to the short time that effective concentrations of the drug are locally available at the blood vessel.

As mentioned above, DES exhibit positive early- and mid-term results, but tend to cause similar restenosis rates of bare stents or plain old balloon angioplasty at long term [4]. In addition, DES positioning may induce endothelial cell dysfunction, retarding endothelium healing [17]. Changes in DES structure and composition have tried to circumvent such problems [17], but clinical results with DES are the consequence of the combined effects of both DES backbone and drug-coating. Furthermore, BMS or DES cannot be used in all the patients who need a PTA. Predictors related to the patient, the vessel or to the procedure are used to evaluate the risk of restenosis [3] and, thus, patient at high risk are preferentially treated with plain balloon or with drug eluting balloon (DEB) [18]. The latter treatment can suffer of a limited residence time of the administered drug due to the rapid wash out effect of the blood flow. Thus, the development of drug eluting nanoparticles (NP) intended to be delivered intramurally by a balloon appears of interest to overcome the limitations of DES and conventional DEB. The most attractive material to design such NP is probably the biodegradable poly(lactide-co-glycolide) [PLGA], since it has been successfully and widely used in the development of implantable medicinal products and medical devices [19]. Nevertheless, during the degradation, acidic byproducts are formed, decreasing the microenvironment pH and, thus, evoking an acute inflammatory response at the administration site [20,21]. Given the intended parenteral use, the loaded nanocarrier must also meet the requirement of sterility. This is known to be critical

regarding PLGA-based products, since the instability of ester bonds upon different sterilization processes [22,23].

In the attempt to provide the polymer with better functional characteristics and improve its properties, worth of interest is the application of a radical grafting, that represents one of the most effective and easy methods to confer novel properties to a polymer [24,25]. The grafting process of bioactive molecules such as anti-oxidants to the polymeric backbone includes two main steps: polymer surface activation, producing macroradical species, and the subsequent insertion of the bioactive molecule onto the polymeric chain with the formation of covalent bonds. Radicals can be conveniently generated along polymer backbone in the presence of chemical initiators, such as diazo compounds, peroxides and hydroperoxides redox pairs. Furthermore, this strategy allows to overcome the main limitations intrinsic to some polymeric materials, such as PLGA, due to the low density of reactive groups on their surface [26]. The results available in literature on PLGA grafted to anti-oxidants (g-AA-PLGA) evidenced that these novel materials also reduced the pH drop off during the polymer degradation [26] and improved the drug loading with respect to the naïve PLGA [27]. Moreover, the grafting of anti-oxidants could add some beneficial effects on the restenosis process, as previously described [28].

In this work, the feasibility of using NP based on g-AA-PLGA, namely caffeic acid (g-CA-PLGA) and resveratrol (g-RV-PLGA) conjugates, to get access into cells at the arterial wall to deliver fluvastatin, was evaluated. Fluvastatin was selected since statins are gaining growing interest in managing the restenosis process as demonstrate in the case of pitavastatin [29–31]. To evaluate the potentialities of the proposed approach, the work was designed as follow. Firstly, NP made of PLGA grafted with caffeic acid or resveratrol were characterized in terms of their main physico-chemical properties. Secondly, *in vitro* biocompatibility of nanomaterials with cultured cells was examined. Thirdly, the cellular uptake and exocytosis of the prepared NP formulations were assessed using three cellular types present in the artery wall, namely smooth muscle cells, macrophages and endothelial cells.

Finally, NP were loaded with fluvastatin, and their effects on cell behavior monitored *in vitro*. The performances of g-AA-PLGA were also compared with those of raw PLGA.

1.2 Materials

For the purposes of this study, capped poly (D,L-lactide-co-glycolide) [PLGA] with a co-monomer ratio of 50:50 (lactic/glycolic acids) [Purasorb® PDLG 5002, inherent viscosity in $CHCl_3$ = 0.16 - 0.24 dl/g] was obtained from Corbion PURAC (NL). Caffeic acid (CA), resveratrol (RV), hydrogen peroxide (H_2O_2), ascorbic acid (AA), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), alpha-smooth muscle actin (ACTA-2) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [MTT] were obtained from Sigma-Aldrich (I). Isoflurane was bought by Forane (Abbot Laboratories Ltd, USA). Dulbecco's modified Eagle's medium (DMEM), modified Eagle's medium (MEM), fetal calf serum (FCS), glutamine, penicillin, streptomycin, hypoxanthineaminopterin-thymidine medium (HAT) and essentially fatty bovine serum albumin (EFAF) were purchased by Euroclone (I). Epon-Araldite resin was obtained by Polysciences, Inc. (G), while fluvastatin sodium salt (FLUVA) by Novartis.

All solvents were of analytical grade, unless specified.

1.3 Methods

1.3.1 g-AA-PLGA synthesis

In a 100-mL round-bottom flask, 0.5 g of PLGA were dissolved in 5 mL of THF and the obtained solution was evaporated leading to the formation of a thin polymeric film [27]. After the addition of 50 mL of a 1.0 M H_2O_2 solution and 1.2 g of AA, the reaction was allowed to stand for 30 min. Then, the H_2O_2/AA solution was removed and replaced with 50 mL of a mixture consisting of H_2O_2 (2.0 M) and ethanol (1:1 v/v) containing 1.2 g of AA and 0.8 mmol of the antioxidant agent (CA

or RV). The reaction mixture was maintained at 25±1 °C under atmospheric air and, after 24 h, the obtained functionalized film was purified by washing with distilled water and ethanol and dried under vacuum for 24 h at room temperature.

1.3.2 Characterization of g-AA-PLGA

1.3.2.1 Molecular weight

Polymer molecular weight was determined by using a HP1100 Chemstation (Agilent, Santa Clara, CA, USA), equipped with a G1379A degasser, a G1310A isocratic pump, a G1313A auto-sampler, a G1316A thermostated column compartment and a double detector: refractive index detector G1362A and UV/visible detector (G1314A) set at λ=210 nm. Two columns were connected in series: μStyragel™ Toluene 10^4 Å 7.8 × 300 mm and μStyragel™ Toluene 10^3 Å 7.8 × 300 mm (Waters, Milan, I). Samples of about 5-6 mg were dissolved in THF over 24 h. The mobile phase was THF at the flow rate of 1 mL/min and at the temperature of 25.0±0.1 °C. An injection volume of 20 μL was used. The weight-average molecular weight (M_w) and the number-weight molecular weight (M_n) of each sample were calculated using monodisperse polystyrene standards with M_w ranging from 1,000 to 45,000 Da and a GPC-Addon HP ChemStation software (Hewlett-Packard Co., USA) to compute molecular weight distribution (Agilent, USA). Dispersity index (DI) was calculated by the ratio between M_w and M_n.

1.3.2.2 Thermal properties

Thermal properties, namely glass transition temperature (T_g) of PLGA and g-AA-PLGA, were evaluated by differential scanning calorimetry (DSC). For this purpose, a differential scanning calorimeter DSC 1 Star^e System (METTLER TOLEDO, CH), equipped with a refrigerated cooling system, was used. Thermal behavior was studied by heating the sample placed into a crimped aluminum pan under nitrogen gas flow (80 mL/min). The reference was an empty pan. The sample was heated at 20 K/min until 50 °C over the presumed T_g to erase polymer's thermal history,

then cooled down until 0 °C or -10 °C at 20 K/min and finally re-hated at a scan heat rate of 20 K/min. The T_g was measured on the second heat scan.

1.3.2.3 Anti-oxidant activity

To investigate the antioxidant properties of the synthesized PLGA-based conjugates, the reactivity towards 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was evaluated [27]. For this purpose, 25 mg of each polymeric materials, namely PLGA grafted to caffeic acid (g-CA-PLGA) or to resveratrol (g-RV-PLGA), were mixed with 25 mL of an acetonitrile solution of DPPH (100 µM). The samples were incubated at 25 °C in dark conditions and, after 1, 2, 3 and 24 h, the absorbance was measured at 517 nm using a Jasco V-530 (UK) UV/Vis spectrometer. The same reaction conditions were adopted for the control polymer (blank PLGA) to evaluate the interference of the polymeric material on DPPH assay. The scavenging activity was expressed as percentage of inhibition of DPPH radicals and calculated according **Eq. (1)**.

$$Inhibition\ \% = \frac{A_0 - A_1}{A_0} \times 100 \qquad \textbf{Eq. (1)}$$

where A_0 is the absorbance of a standard prepared in the same conditions, but without any polymers, and A_1 is the absorbance of the polymeric samples. Each measurement was performed in triplicate and data expressed as mean ± SEM.

1.3.3 Placebo and drug loaded NP preparation

Placebo surfactant-free PLGA and g-AA-PLGA NP were prepared following the solvent displacement method [32]. Briefly, 1 mL of 1% w/v organic solution of the polymer was added dropwise to 10 mL of ultrapure water (prepared by the MilliQ® system) filtered with a nylon syringe filter of 0.2 µm nominal porosity (VWR, I), using an electronic pipette (PIPETMAN M® Gilson, USA). The polymer organic solutions were composed of a mixture of acetone:absolute ethanol (7:3 v/v) or of only acetone for PLGA and g-AA-PLGA, respectively. After the addition of the polymer solution, the aqueous phase was maintained at 4±1 °C and under magnetic stirring of 500 rpm for 15 min. The temperature was then gradually increased up to 30±1 °C and the nanosuspension was maintained under stirring over 3h, to allow the solvent to evaporate. Fluorescent NP were prepared following the same protocol, replacing 10% w/w of the polymer with a PLGA conjugated with fluorescein isothiocyanate (FITC-PLGA) [33].

For the preparation of FLUVA loaded NP, g-AA-PLGA solution in acetone (1.4% w/v) was mixed with FLUVA solution in THF in the ratio of 8:2 v/v, respectively, in order to get a theoretical drug loading of 20% w/w. 2 mL of ultrapure water maintained at 24±1 °C was rapidly added to the polymer/drug organic mixture under a vigorous magnetic stirring, using the same electronic pipette reported above at the maximum dispensing velocity. The system was cooled at 4±1 °C and diluted with 8 mL of ultrapure water. The organic solvents were evaporated as previously described. Loaded NP was collected by centrifugation at 11,000 rpm for 30 min at 4±1 °C (Universal 30 RF, Hettich GmbH & Co., G.) and suspended in ultrapure fresh water. For the evaluation of drug content, NP were freeze-dried (Martin Christ Alpha 1-4 LSC Plus, G) and stored under vacuum at 5±3 °C until use.

1.3.4 NP characterization

1.3.4.1 Particle size distribution

Particle size measurement was conducted using a Zetasizer Nano ZS DLS instrument (Malvern Instruments, Worcestershire, UK). The instrument uses a 4 mW He-Ne laser (λ=632.8 nm) with backscatter detection at 173° and a thermostated sample chamber set at 25.0±0.1 °C. The Zetasizer Nano ZS automatically adjusted the attenuator setting to optimize the amount of light scattered by a sample. The results, calculated using the Dispersion Technology Software (DTS, Malvern Instruments Ltd., Worcestershire, UK), were reported as mean ± SD of the hydrodynamic diameter (D_H) in the intensity distribution. All the samples were subjected of three measurements.

1.3.4.2 Zeta potential (ζ)

The zeta potential of NP was assessed by M3-PALS (Phase Analysis Light Scattering) technique, using the same equipment reported for the size measurement. The analyses were carried out into a capillary cuvette at 25.0±0.1 °C. All the samples were subjected of three measurements and the results are reported as mean ± SD.

1.3.4.3 Fluvastatin content in NP formulation

Fluvastatin was extracted from the PLGA matrix by incubating 2 mg exactly weighed of dried NP in 10 mL of a mixture of acetonitrile/phosphate buffer pH=3.0, in the volume ratio of 60:40, over 24 h at room temperature. The amount of drug was quantified by HPLC, using an Agilent HP1100 series (Agilent, UK), equipped with a G1311A quaternary pump, a G1313A auto-sampler, a G1316A thermostated column compartment and G1315B DAD-UV detector. A Phenomenex Luna® 5 µm (C18 150 x 46 mm) LC column was used for the reversed phase chromatography. The mobile phase was the same mixture used for drug

extraction at the flow rate of 1.2 mL/min. The temperature was set at 25.0±0.1 °C and the injection volume was 10 µL. The detection of FLUVA was performed at 230 nm. Calibration curve in the range of 1-20 µg/mL was freshly prepared by dissolving the drug in the mobile phase (R^2>0.99). All measurements were performed in triplicate.

The experimental fluvastatin loading % and the encapsulation efficiency (EE) % were calculated based on **Eq. (2)** and **Eq. (3)**, respectively.

$$Experimental\ FLUVA\ loading\ \% = \frac{amount\ of\ FLUVA\ entrapped\ in\ NP}{mass\ of\ NP} \times 100 \quad \textbf{Eq. (2)}$$

$$EE\ \% = \frac{amount\ of\ FLUVA\ entrapped\ in\ NP}{theoretical\ amount\ of\ FLUVA} \times 100 \quad \textbf{Eq. (3)}$$

1.3.5 Cell cultures

This study was performed conform to the declaration of Helsinki. Smooth muscle cells (SMC) were isolated from the intimal-medial layer of aortae of littermate C57/BL6 mice of both sexes (The Jackson Lab, Bar Harbor, ME, USA) [34,35]. All mice were housed in accordance with guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Mice were kept under standard laboratory conditions with temperature at 22±1 °C, dark/light cycles of 12/12 h, relative humidity of 55±5 % and 20 air changes per hour. Food and tap water were given *ad libitum*. Mice were anesthetized with 2% isoflurane and killed by cervical dislocation. Aorta was rapidly dissected from the aortic root to the iliac bifurcation, periadventitial fat was removed and SMC prepared according to the procedure described by Ross [36]. Cells were grown in monolayers at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 20% FCS, glutamine, penicillin and streptomycin. SMC lineage was confirmed by the presence of immunoreactivity for ACTA-2 in more than 99

% of the cells. Human SMC (A 617 from human femoral artery) were grown in the same culture conditions and used for the proliferation and migration experiments.

The human endothelial cell line EAhy.926 [37,38] (EC) was cultured in monolayer in DMEM supplemented with 10% FCS, 1% HAT, glutamine, streptomycin and penicillin [39] and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

The mouse macrophages cell line J774 was cultured in monolayer in DMEM supplemented with 10% FCS, glutamine, streptomycin and penicillin and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

The media of all the cell lines were changed every three days.

1.3.6 Cell viability assay

Cellular toxic effects of PLGA, g-CA-PLGA and g-RV-PLGA NP were assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [MTT] colorimetric assay. This assay relied on the ability of mitochondrial dehydrogenases of viable cells to reduce MTT to a blue formazan product [40], which was measured by using a scanning multi-well spectrophotometer (Multimode plate reader Enspire, PerkinElmer, I). Briefly, after incubation with NP (100 µg/mL) for 24 h, cells were washed with PBS and MTT was added at a concentration of 10 µg/mL in the culture medium. Following 90 min of incubation, the supernatants were decanted, the formazan precipitates were solubilized by the addition of 100% DMSO and plates placed on a plate shaker (Orbital shaker, Major Science, US) for 10 min. Absorbance was evaluated at 620 nm [41]. Results are reported as mean ± SD of cell viability % (n=3). Incubation medium was used as negative control, while a 4% Triton solution as the positive one.

1.3.7 NP cellular uptake

Cellular uptake of fluorescent PLGA, g-CA-PLGA and g-RV-PLGA NP was evaluated in SMC, macrophages and endothelial cells. Briefly, cells were seeded at the

cellular density of 30,000 cells/well in a 96-well plate. After the confluence was reached, cells were washed with PBS and culture medium with 0.2% EFAF containing the fluorescent nanosuspension at a final concentration of 100 µg/mL was added. Incubation times were 0.5, 2 and 4 h for macrophages and 4, 16 and 24 h for SMC and endothelial cells. After the incubation period, plates were cooled with ice, the media were removed, the cells were washed for three times with cold PBS and fixed with methanol for 20 min. After methanol removal, cells were washed again for three times with PBS. Culture medium without nanosuspensions was used as negative control. A microplate reader (Wallac 1420 Victor^2 Microplate Reader, PerkinElmer, I) was used to measure the fluorescence intensity from up-taken NP in each well at 25.0±0.1 °C, with lamp filter at 485 nm and an emission filter at 535 nm. The cellular uptake efficiency % was calculated normalizing the observed fluorescence intensity in each well (I_{OBS}) for the mean fluorescence intensity of the negative control (I_{NC}), as reported in **Eq. (4)** [42].

$$Uptake\ efficiency\ \% = \frac{I_{OBS} - I_{NC}}{I_{NC}} \times 100 \qquad \textbf{Eq. (4)}$$

Only the samples with a fluorescence higher than the highest fluorescence registered for the negative control were used for the analysis. Results are reported as mean ± SEM (n=6).

Cellular uptake was also monitored by fluorescence microscopy (Axiovert M220 Zeiss, G). Cells were seeded on sterile microscope slides positioned in 24-well plate and grew until confluence. The same protocol previously reported was followed. Slides microscopy were treated with DAPI at room temperature for 5 h and stored at dark until use. Pictures of the up-taken fluorescent NP were captured at the time points previously reported.

1.3.8 NP exocytosis

Macrophages and SMC were seeded at cellular density of 30,000 cells/well in a 96-well plate (n=6 for each treatment), grown until confluence and incubated with 100 µg/mL of fluorescence g-CA-PLGA NP for 0.5 and 16 h, respectively. Then, the cells were washed and fresh culture medium was added. At predetermined time points (4, 6, 8, 24, 48, 56 and 160 h), the back scattering of the culture medium was measured using DLS, as described in the paragraph 1.3.4.1. The derived count rate (DCR), in kilo counts per second (kcps), was also recorded during particle size measurements. In the absence of NP, the light scattered by ultrapure water according to DLS measurements ranged from 45-50 kcps. In contrast, samples of incubation media scatter an amount of light which can be quantified by DLS. Because light scattered is directly proportional to the size and number of NP present in the sample, the absolute light scattering (DCR) can be used to indicate the presence of NP in the sample. The comparison with the media incubated in negative control wells (cells treated with medium not containing NP) allowed to discriminate between NP and particulate matter present in the culture medium. Furthermore, to establish the presence of NP that underwent to exocytosis, the following criteria had to be satisfied: D_H comparable to those of NP after preparation and sigmoidal shape of correlation function. The detection of NP after exocytosis was expressed as DCR over time.

1.3.9 Transmission electron microscopy (TEM)

Exocytosis of NP was visualized also by TEM. Macrophages and SMC were seeded at cellular density of 300,000 cells in 35-mm diameter Petri dish and incubated for 96 h. Cells were then washed with PBS and treated for 4 h with placebo g-CA-PLGA NP diluted with MEM + 0,2% EFAF (MEM + 0,2% EFAF without NP was also used as negative control). After washing with PBS, cells were detached with trypsin and centrifuged at 1,500 rpm for 15 min. Pellets were washed with 0.1 M sodium cacodylate buffer at pH 7.3 and centrifuged as previously reported for two times.

The pellets were fixed overnight in a solution containing 2% of freshly prepared paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Samples were rinsed twice in the same cacodylate buffer for 30 min and post-fixed in 1% osmium tetroxide in cacodylate buffer 0.1 M at 0 °C for 90 min. After a washing with distilled water, pellets were stained with 2% aqueous uranyl acetate, dehydrated in a graded acetone series and embedded in Epon-Araldite resin. Ultrathin sections were cut by a Leica Supernova ultramicrotome (Reichert Ultracut E, SE) and counterstained with lead citrate. Transmission electron microscopy was performed with a Zeiss EM10 electron microscope.

1.3.10 Cell proliferation and migration

In order to evaluate the effect of free and NP encapsulated fluvastatin on cell proliferation, human SMC and endothelial cells were seeded at 5,000 cells/well in a 12-well plate until confluence. Human SMC were treated with fluvastatin dissolved in ethanol and FLUVA-NP at the drug concentration of 1, 2, 4, 6, 10 µM, using ethanol and placebo NP as negative controls. In the case of endothelial cells, the fluvastatin concentrations were fixed at 1, 2, 4, 6 and 10 µM. After the removal of the treatment and the wash with PBS, cells were detached with trypsin and diluted with fresh medium and physiological solution. Cells in 500 µL of each diluted sample were counted by Coulter Counter (Z1 Beckman Coulter, I), calibrated at the threshold of 6.8 µm. Results are reported as mean ± SEM (n=6).

For the evaluation of cell migration, the *in vitro* scratched wound method was used. Human SMC and endothelial cells were seeded at the density of 5,000 cells/well in a 12-well plate until confluence was reached. After a washing step with PBS, fresh medium was added on the day before treatment. A rectangular wound was made according to the diameter of the well by using a sterile disposable P200 cell TIP (Eppendorf, I) and detached cells were removed by washing with PBS. After injury of cell monolayer, the cells were incubated for 24 h with increasing concentrations (2, 4 and 10 µM) of FLUVA-loaded NP and the

matching concentrations of either placebo NP or fluvastatin solution in ethanol. Ethanol and plain culture medium were used as negative controls. Cell directional migration was evaluated by their ability to move and migrate across the wound by using phase-contrast microscope and photographed using a digital camera (Axiovert 200, Zeiss, G). Images were acquired after 24, 48, 72 and 144h. For quantitative representation of the results, the percentage of total distance migrated from the edge of the monolayer was determined by using Axiovision software (Zeiss, US) at five different positions (every 5 mm). Results are reported as mean ± SEM (n=6).

1.3.11 Statistical analysis

One-way or two-way analysis of variance (ANOVA) followed by Tukey's test as post ANOVA means comparison were performed using OriginPro® 2015 (OriginLab Corporation, USA) for analysing results from NP cellular uptake. The type of polymer (*e.g.* PLGA, g-CA-PLGA and g-RV-PLGA) and the time of incubation with cells (*e.g.* 0.5, 2 and 4h for macrophages and 4, 16 and 24 h for SMC and endothelial cells) were considered as factors. Differences were considered statistically significant at a level of α=0.05. Outliers were discarded according to Dixon's T-test.

Comparison of two experimental means from cytotoxicity, exocytosis, cell proliferation and migration experiments were performed using unpaired student t-test to determine two-tailed p values at 95% confidence level.

1.4 Results

1.4.1 g-AA-PLGA synthesis

In the present work, PLGA-based conjugates were synthesized by performing a two-step grafting procedure at room temperature, which involved the use of a biocompatible redox pair consisting of hydrogen peroxide and ascorbic acid. In the first step of the adopted synthetic strategy, the oxidation of ascorbic acid by H_2O_2 led to the formation of hydroxyl radicals able to activate the PLGA backbone; in the next step, the reactive sites present on the preformed PLGA macroradicals reacted with the antioxidant molecules resulting in CA and RV insertion. This synthetic strategy allowed avoiding the temperature induced degradation of the antioxidant agents and the generation of toxic by-products. Hence, most of the polymeric chains were preserved from side reactions and the M_w and the glass transition values of g-AA-PLGA were not significantly affected (**Table 1.1**).

DPPH radical has been widely used to test the ability of compounds as free radical scavengers or hydrogen donors in order to evaluate the antioxidant activity. DPPH tests were conducted to evaluate whether resveratrol and caffeic acid retained their antioxidant capacity after grafting to PLGA backbone. The radical scavenging activity of PLGA with and without incorporated the anti-oxidants was determined and is presented in **Table 1.1**. As expected, the control polymer (PLGA) did not show any antioxidant activity. g-CA-PLGA exhibited a higher level of radical scavenging activity with respect to g-RV-PLGA. Moreover, the ability to inhibit DPPH radicals was almost constant for g-CA-PLGA and doubled for g-RV-PLGA in a 24h-period. The activity values achieved for a g-CA-PLGA in this study were of the same order of magnitude as those reported for PLGA grafted with pyrogallic acid [26], confirming the effectiveness of this approach to confer scavenger properties to a well-known polymer.

Table 1.1 – Physico-chemical features of g-CA-PLGA and g-RV-PLGA and their anti-oxidant properties expressed as DPPH radical inhibition (%).

Polymer	*DPPH radical inhibition (%)*				M_w (KDa)	DI	T_g (°C)
	1 h	2 h	3 h	24 h			
PLGA	0±0.4	0±0.4	0±0.5	0±0.3	20.4	1.52	36.2±0.5
g-CA-PLGA	90±0.4	92±0.7	93±0.6	97±0.6	18.4	1.65	33.6±1.2
g-RV-PLGA	27±0.8	32±0.4	37±0.3	56±0.5	18.5	1.38	33.2±2.3

M_w: polymer weight-average molecular weight;
DI: dispersity index of the molecular weight distribution;
T_g: polymer glass transition temperature.

1.4.2 Placebo and drug-loaded NP characterization

Placebo PLGA, g-CA-PLGA and g-RV-PLGA NP prepared by the solvent displacement method without adding surfactants were monodispersed with a mean hydrodynamic diameter (D_H) lower than 200 nm (**Table 1.2**). NP were negatively charged with zeta potentials (ζ) ranging from -23 to -33 mV. Furthermore, their features were not significantly modified by replacing 10% w/w of the polymer with FITC-PLGA, used as fluorescent marker in the *in vitro* cellular studies (data not reported).

Table 1.2 – Hydrodynamic diameter (D_H), polydispersity index (PDI) and zeta potential (ζ) of placebo PLGA, g-CA-PLGA and g-RV-PLGA NP prepared by the solvent displacement method.

Polymer	D_H (nm)	PDI	ζ (mV)
PLGA	186±1	0.061±0.016	-22.4±0.6
g-CA-PLGA	171±2	0.082±0.020	-30.4±1.0
g-RV-PLGA	193±3	0.064±0.016	-32.5±0.6

To encapsulate fluvastatin into g-CA-PLGA NP (FLUVA-NP), minor modifications were introduced in NP preparation due to the water solubility of fluvastatin

sodium, avoiding the use of surfactants as for the placebo formulation. FLUVA-NP had a D_H of 175±3 nm (n=3) with a monodispersed size distribution, given the low polydispersity index (PDI) of 0.125±0.045 (n=3). These results were not statistically different compared to the placebo NP ($p>0.05$). Also regarding the ζ, no statistical differences were found after NP purification compared to unloaded formulation (-28.4±2.1 mV, $p>0.05$). The experimental fluvastatin loading was 4.3±0.3% with an encapsulation efficiency of 22.6±1.3%.

1.4.3 Cell viability assay

The effect of NP made of PLGA, g-CA-PLGA and g-RV-PLGA on the cell viability was assessed in macrophages, endothelial cells and SMC, considered representative of the main cell populations in the artery vessel. The exposure concentration of each type of NP was fixed at 100 µg/mL, while a 4% solution of Triton was used as positive cytotoxic control. As evidenced in **Fig. 1.1**, none of the type of nanoparticles considered exerted a toxic effect on cells, since no statistical differences in cell viability were found, comparing the results with the culture medium alone (negative control). Hence, the selected grafting procedure did not alter the intrinsic biocompatibility of PLGA.

Considering these results, both the NP prepared by the grafted PLGA, namely g-CA-PLGA and g-RV-PLGA, were subjected to subsequent experiments.

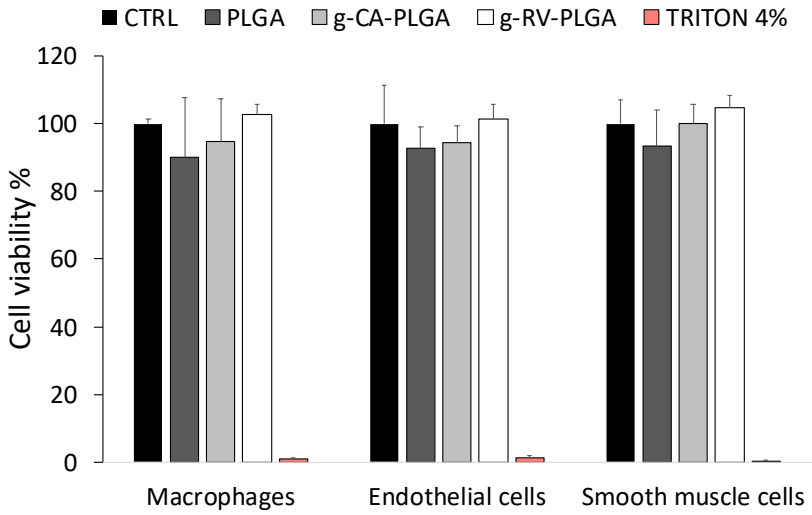

Fig. 1.1 – Cell viability after 24 h of incubation with placebo PLGA, g-CA-PLGA and g-RV-PLGA NP. A 4% solution of Triton was used to obtain a 100% toxicity (positive control), while culture medium alone was selected as the negative control (CTRL). Results are reported as mean ± SD (n=3)

1.4.4 NP uptake and exocytosis

The uptake of fluorescently labelled PLGA, g-CA-PLGA and g-RV-PLGA NP was evaluated over a time period of 4 or 24 h in the case of macrophages or endothelial cells and SMC, respectively. NP were up-taken by all the investigated cell types and it was observed that independently of the polymer with which they are made, NP seemed to be localized in the cytoplasm. Representative fluorescence microscopy pictures of g-CA-PLGA NP uptake, after 24 h of exposure in endothelial cells and SMC and 4 h in macrophages, are shown in **Fig. 1.2**. Nuclei are visualized by the blue colour, while the fluorescence emitted by NP is represented by the green dots.

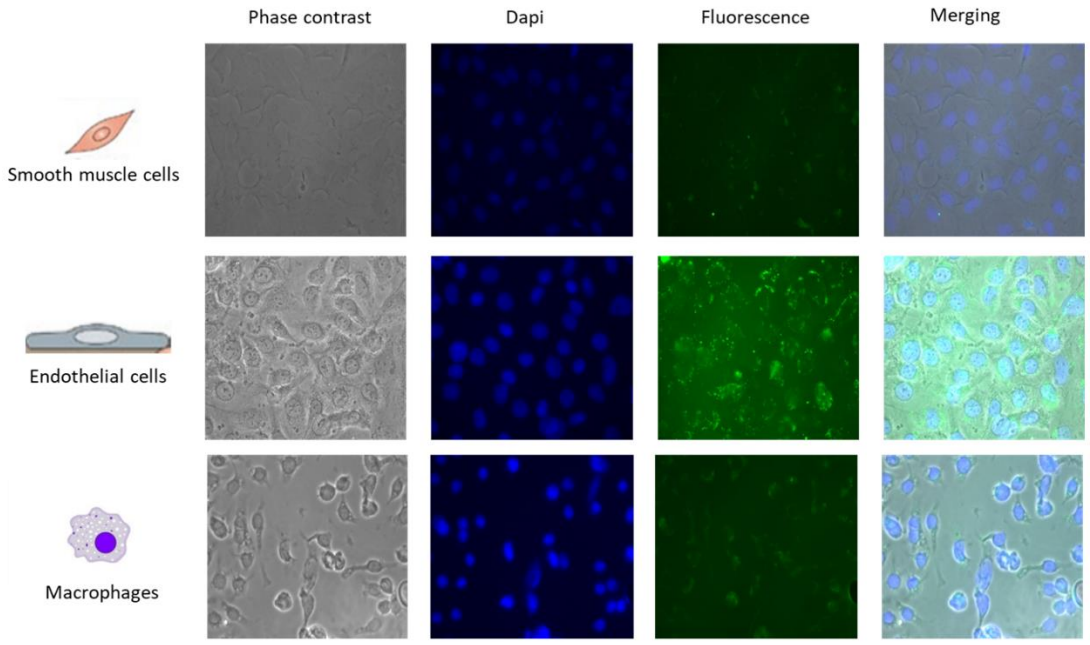

Fig. 1.2 – Representative pictures of cellular uptake of fluorescently labelled g-CA-PLGA NP after 24 h (SMC and endothelial cells) or 4 h (macrophages) of incubation. In the phase contrast pictures, the morphology of the cells is shown; in DAPI pictures, the blue colour is attributed to the staining of the nuclei; in fluorescence pictures, the fluorescence given by the up-taken NP is represented by the green dots; in the merging picture, DAPI and fluorescence pictures are overlapped, qualitative indicating the intra-cellular localization.

As expected, NP were faster up-taken by macrophages compared to the other cell lines, as fluorescence was detectable after 0.5 h of exposure. Moreover, the grafting of caffeic acid or resveratrol on the PLGA backbone did not influence the uptake process, independently of the incubation time (Two-way ANOVA $p>0.05$) [**Fig. 1.3**].

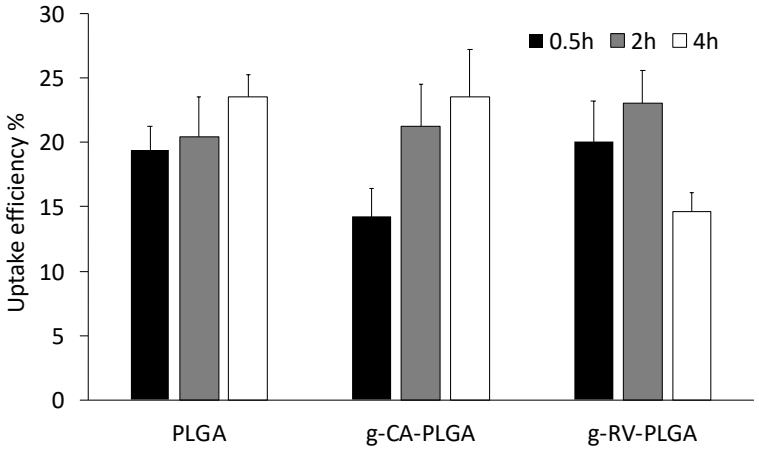

Fig. 1.3 – PLGA, g-CA-PLGA and g-RV-PLGA NP uptake in macrophages at different time points. The results are reported as mean uptake efficiency % ± SEM (n=6).

In the case of endothelial cells, after 16 h of exposure to the nanosuspensions, the fluorescence in the samples treated with g-CA-PLGA NP resulted significantly higher than in those treated with PLGA or g-RV-PLGA (Two-way ANOVA, g-CA-PLGA *vs* PLGA p=0.028 and g-CA-PLGA *vs* g-RV-PLGA p=0.032, **Fig. 1.4a**). On the contrary, as shown in **Fig. 1.4b**, SMC took up g-CA-PLGA NP more rapidly than the other formulations, as a more intense fluorescence was measured after 4 h of incubation (One-way ANOVA followed by Tukey's test, g-CA-PLGA *vs* PLGA p=0.002, g-CA-PLGA *vs* g-RV-PLGA p=0.004 and g-RV-PLGA *vs* PLGA p=0.984). However, only in SMC, a significant decrease in the uptake efficiency % of g-CA-PLGA NP was observed at later time points of incubation (*e.g.,* 16 and 24 h). Considering that any cytotoxicity effects were detected over 24h-period, the reduced ability of this cells to sustain the uptake of NP can be ascribed to the beginning of exocytotic mechanism(s), already reported in literature [43].

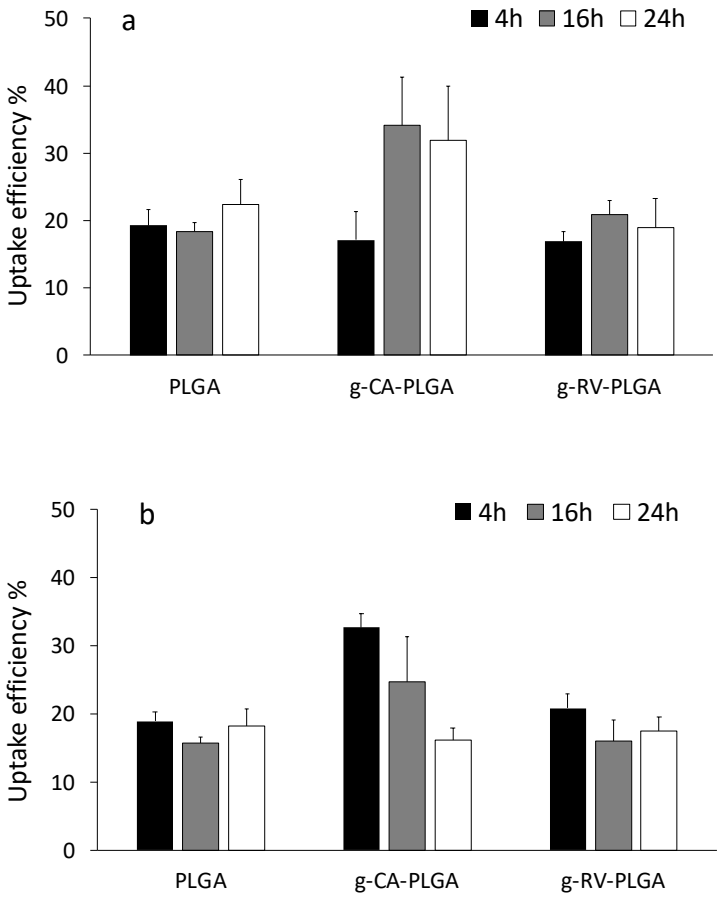

Fig. 1.4 – PLGA, g-CA-PLGA and g-RV-PLGA NP uptake in (a) endothelial cells and (b) SMC at different time points. The results are reported as mean uptake efficiency % ± SEM (n=6).

The exocytosis was qualitatively evaluated by TEM and semi-quantitively by DLS in macrophages and SMC. As exemplified in **Fig. 1.5**, the NP was evidenced inside and outside SMC and appeared surrounded by a membrane likely derived from the release of extracellular vesicles-containing NP. This suggested that exocytosis occurred.

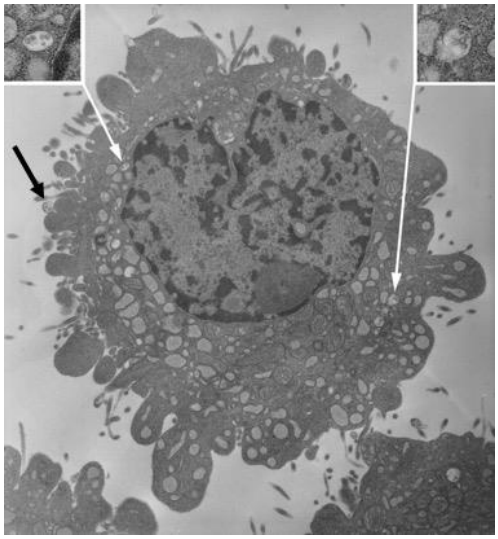

Fig. 1.5 – Representative transmission electron micrograph of g-CA-PLGA NP in human SMC. The white arrows indicate the NP visualized inside the cell, while the black one the NP outside, likely after the exocytosis process.

The DLS results, expressed as the derived count rate (DCR) over time, are reported in **Fig. 1.6**. The media incubated with both cell types after the exposition with the NP scattered light at about 30,000 and 25,000 kcps up to the 4 h time point. The correlation function of the DLS analysis maintained the sigmoidal shape and the measured D_H of the NP was around 200 nm up to 56 and 8 h in case of SMC and macrophages, respectively. The slightly higher value of the D_H resulted for NP after exocytosis can be attributed to the presence of residues of the cellular membrane that, as evidence in **Fig. 1.5**, surrounded the exocytotic material [43].

At longer time points, DLS analysis was associated with a high cumulant fit error probably because the amount of NP in the medium was drastically decreased and the data were discarded.

The DCR values were generally higher in SMC compared to macrophages, indicating that the presence of NP in the medium was detectable for a longer time and suggesting the suitability of the use of DLS to semi-quantitative evaluate the exocytosis of NP. Hence, these results were in agreement with the different phagocytic activities of the two cell lines, known to be much higher in the case of macrophages.

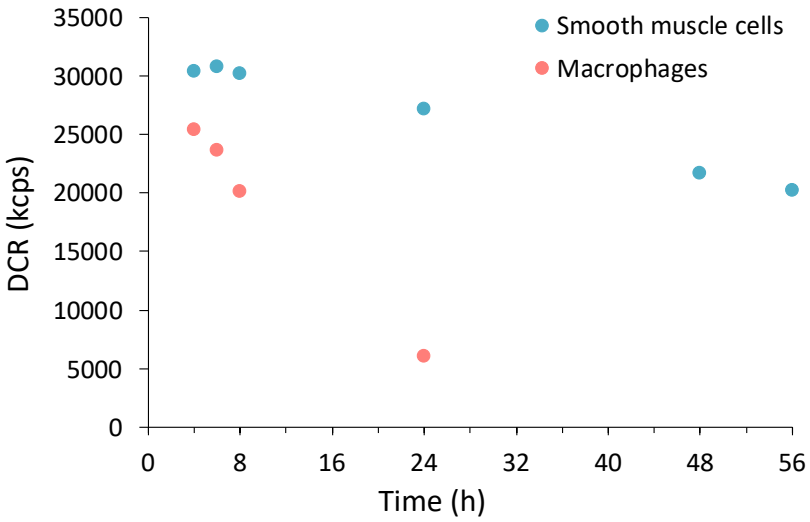

Fig. 1.6 – Variation of DCR over time in macrophages and SMC after incubation with g-CA-PLGA NP for 0.5 h and 16 h, respectively.

1.4.5 Effect of fluvastatin-loaded NP on human SMC and endothelial cells proliferation

To test the efficacy of fluvastatin loaded g-CA-PLGA NP (FLUVA-NP) in inhibiting SMC proliferation, human SMC were incubated for 24 h with increasing concentrations of FLUVA-NP and the matching concentrations of either placebo g-CA-PLGA NP or fluvastatin solution in ethanol. As expected [44], fluvastatin alone inhibited SMC proliferation in a statistically significant manner starting at concentrations higher than 1 µM (at 2 µM cell proliferation was inhibited by 100%) [**Fig. 1.7a**]. FLUVA-NP were also effective in inhibiting human SMC proliferation: at 4 µM cell proliferation was reduced by 50% and by 100% at 6 µM (**Fig. 1.7b**). This demonstrated that fluvastatin-loaded NP efficiently delivered to cells the encapsulated drug and that the drug was still pharmacologically active and able to affect cell behaviour.

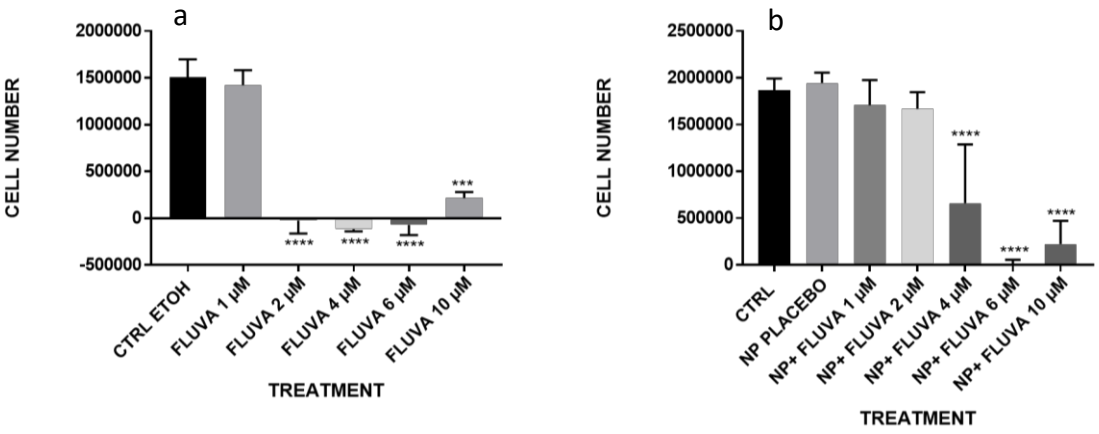

Fig. 1.7 – Effect of (a) fluvastatin and (b) fluvastatin loaded g-CA-PLGA NP (NP+FLUVA) on human SMC proliferation. Results are reported as mean ± SEM (n=6).

In the case of endothelial cells, the addition of fluvastatin resulted in a concentration-dependent and statistically significant reduction of cell proliferation, with the maximal inhibitory effect achieved at a concentration of 1.5 µM (**Fig. 1.8a**). Surprisingly, the addition of the placebo g-CA-PLGA NP resulted in a stimulation of endothelial cells proliferation of about 50% (**Fig. 1.8b**). On the contrary, FLUVA-NP did not affect endothelial cells proliferation, with the exception of the particles containing the highest fluvastatin concentration tested (10 µM, **Fig. 1.8b**).

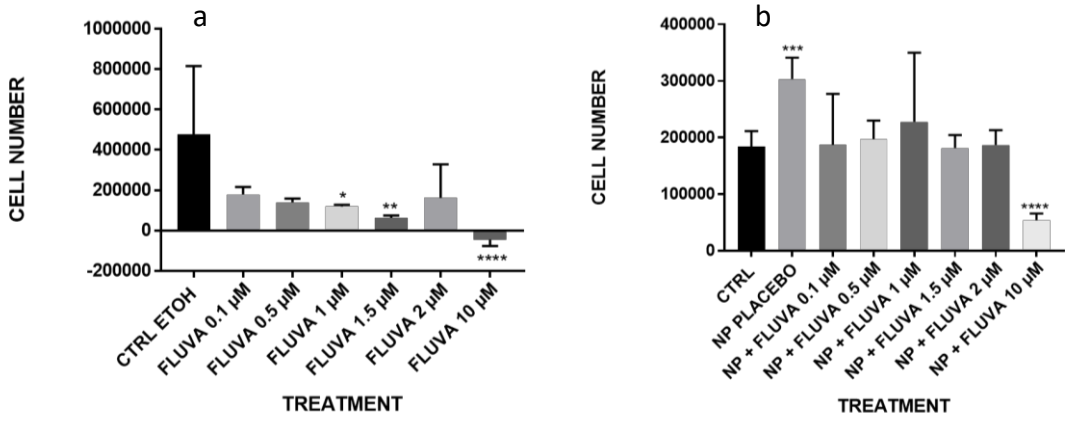

Fig. 1.8 – Effect of (a) fluvastatin and (b) fluvastatin loaded g-CA-PLGA NP (NP+FLUVA) on endothelial cells proliferation. Results are reported as mean ± SEM (n=6).

1.4.6 Effect of fluvastatin-loaded NP on human SMC and endothelial cells migration

Finally, considering that the occurrence of restenosis is also related to the activity of SMC to migrate in the tunica media of blood vessel, the effects of fluvastatin-loaded g-CA-PLGA NP (FLUVA-NP) on cell migration was assessed by using the directional migration assay [45]. As shown in **Fig. 1.9**, fluvastatin and FLUVA-NP reduced human SMC directional migration in a concentration and time-dependent manner, up to an 80% of inhibition at the longest time-point tested. These results were in agreement with the phase-contrast pictures and, in **Fig. 1.10**, representative pictures of the effects of the treatments at the 4 µM concentration at two different time-points (72 and 144 h) are shown. Enhanced migration of human SMC with an almost completed wound closure was evident after 144 h when cells were treated with ethanol as negative control, indicating that they preserved the motility. Similar results were obtained after the treatment with placebo g-CA-PLGA NP. When they were treated with fluvastatin as such or with FLUVA-NP, a significant area of the wound remained uncovered.

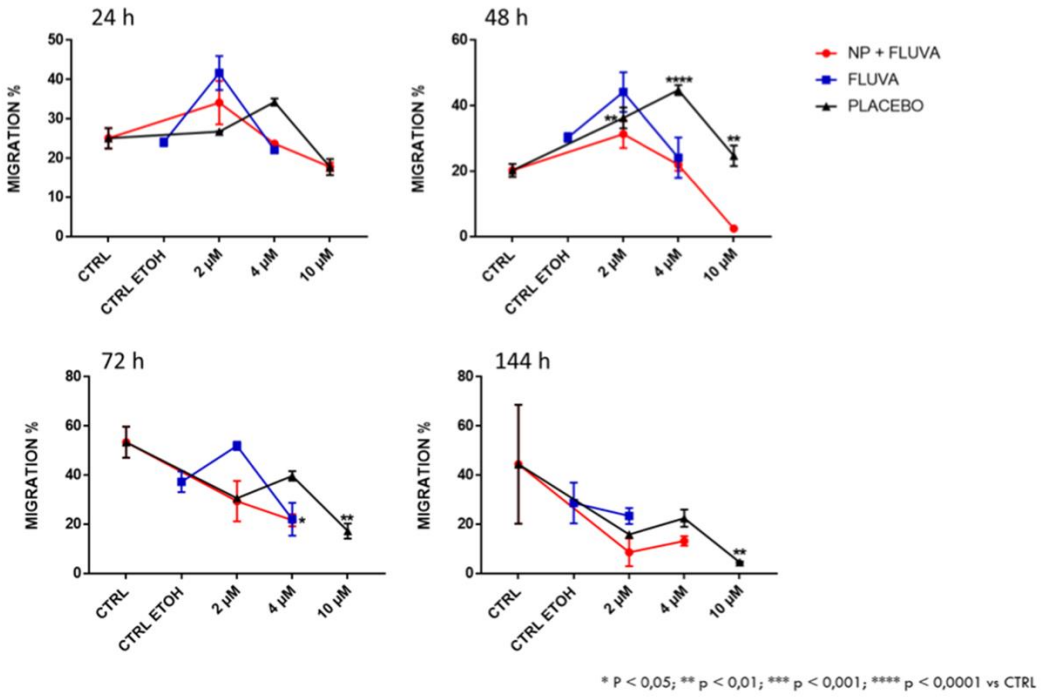

Fig. 1.9 – Effect of fluvastatin dissolved in ethanol, FLUVA-NP (NP+FLUVA in the plots) and placebo g-CA-PLGA NP on human SMC directional migration at different time points. Results are reported as mean ± SEM (n=6). Culture media and ethanol were used as double negative control.

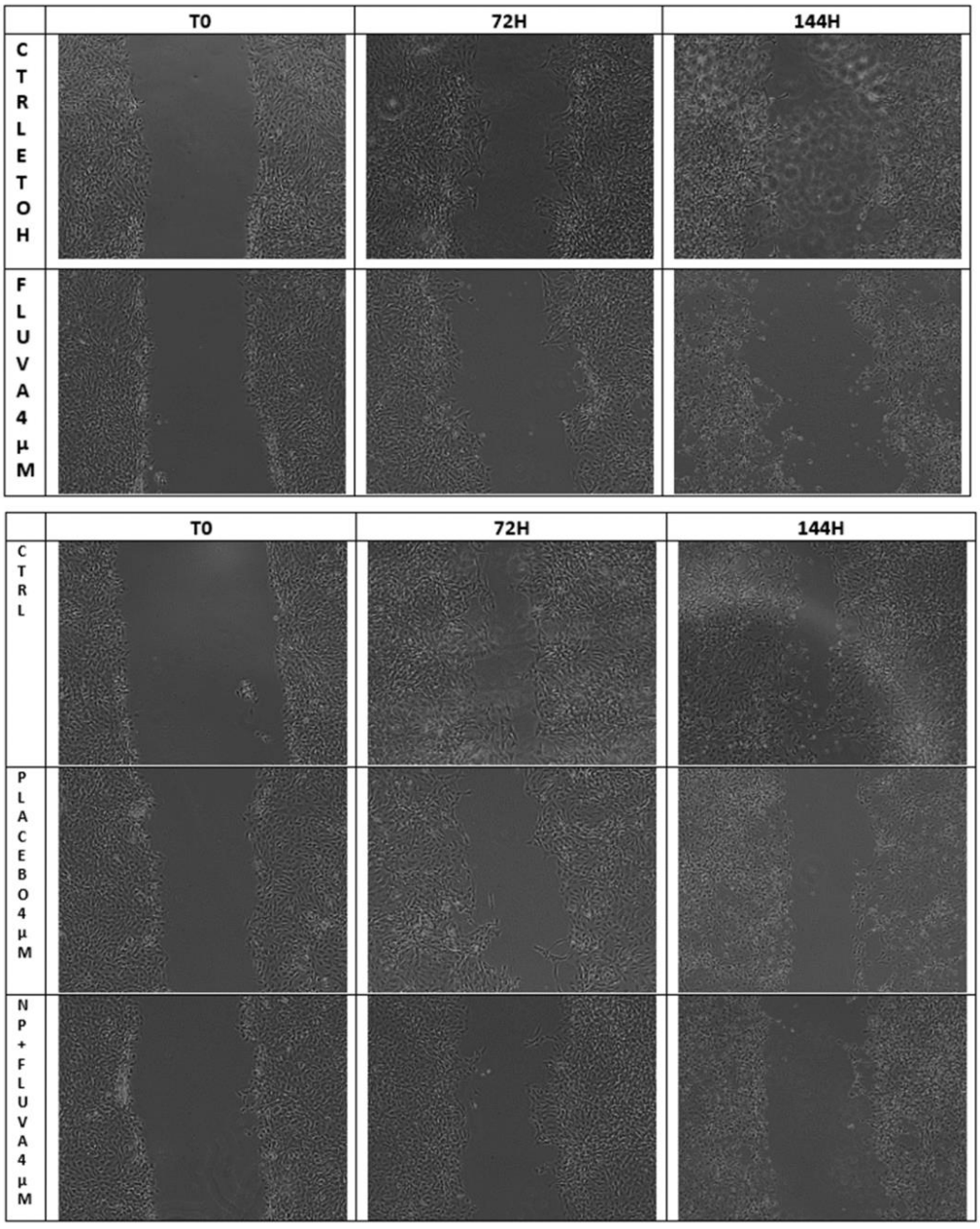

Fig. 1.10 – Representative pictures of the effects of the treatments at the concentration of 4 µM on human SMC directional migration at two time points (72 and 144 h of incubation). Treatments included culture medium (Ctrl in the plot) and ethanol (Ctrl EtOH in the plot) as negative controls, placebo g-CA-PLGA NP (Placebo in the plot), fluvastatin dissolved in ethanol (Fluva in the plot) and FLUVA-NP (NP+FLUVA in the plot).

The effect of the different treatments on directional migration was also evaluated in endothelial cells. As shown in **Fig. 1.11**, the cell migration was reduced both by fluvastatin as such or FLUVA-NP, following a concentration and a time-dependent manner. The placebo g-CA-PLGA NP did not affect this process.

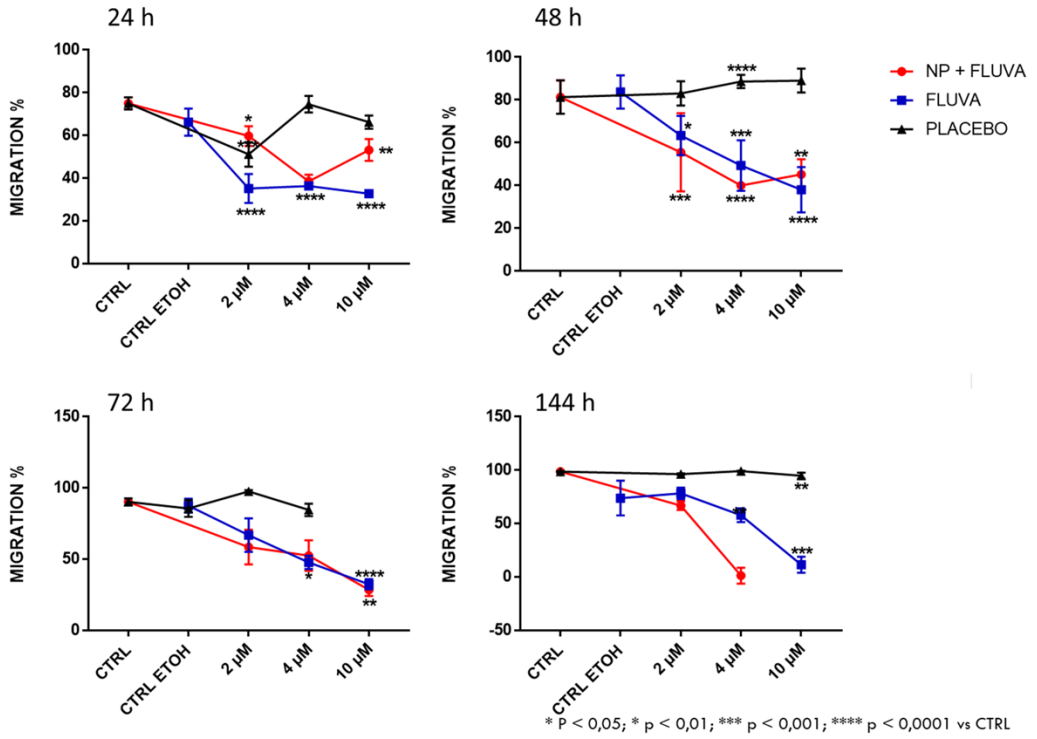

Fig. 1.11 – Effect of fluvastatin dissolved in ethanol, FLUVA-NP (NP+FLUVA in the plots) and placebo g-CA-PLGA NP on endothelial cells directional migration at different time points. Results are reported as mean ± SEM (n=6). Culture media and ethanol were used as double negative control.

Again, representative pictures of the effects of the treatments at the 4 µM concentration at two different time points (72 and 144 h) in endothelial cells are shown in **Fig. 1.12**. After the treatments with ethanol or culture medium (negative controls) or placebo g-CA-PLGA NP, the wound areas were completely covered by the migrated cells, while in the presence of fluvastatin as such or FLUVA-NP the wound areas were still uncovered at the later time point, particularly evident in the case of FLUVA-NP.

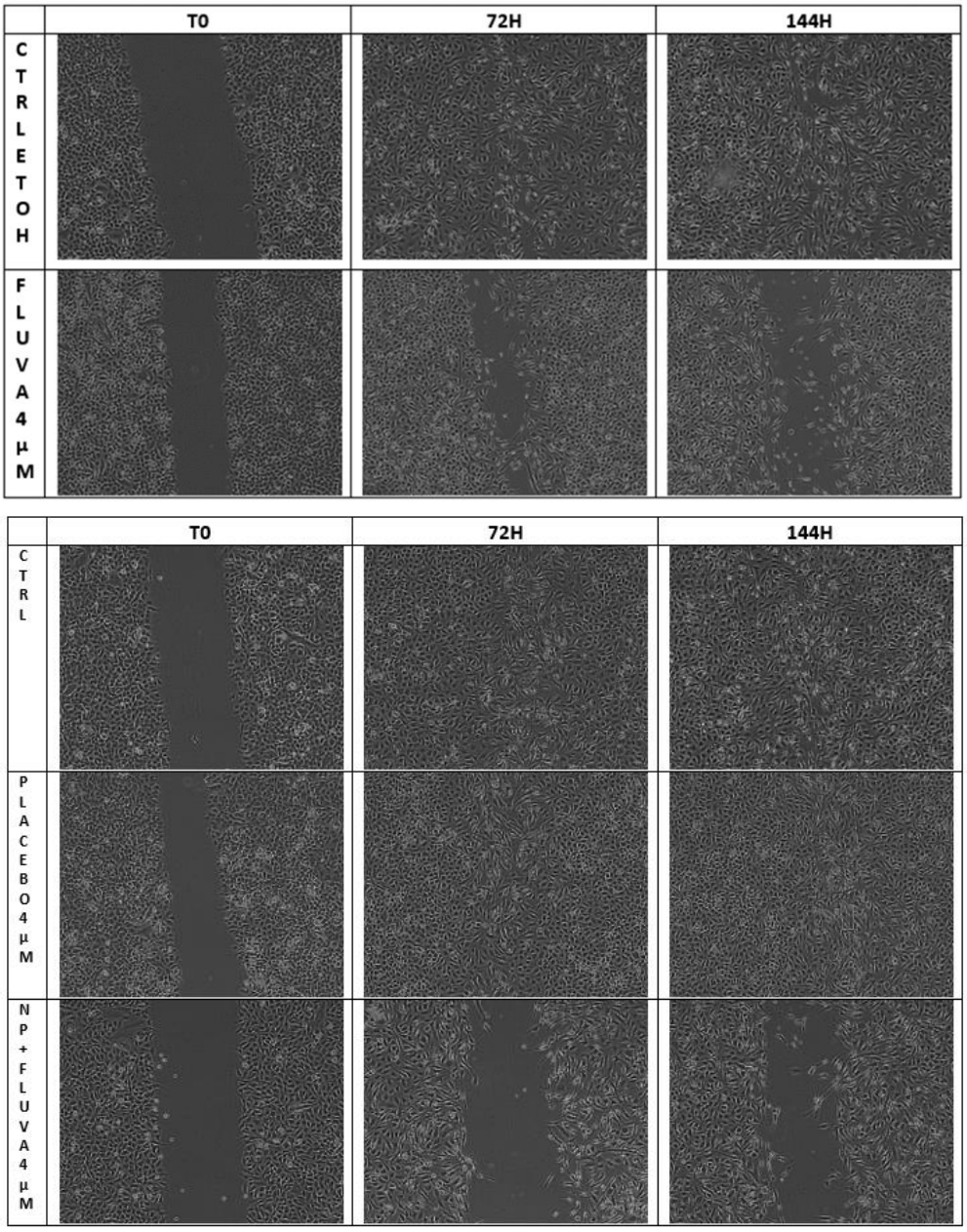

Fig. 1.12 – Representative pictures of the effects of the treatments at the concentration of 4 µM on endothelial cells directional migration at two time points (72 and 144 h of incubation). Treatments included culture medium (Ctrl in the plot) and ethanol (Ctrl EtOH in the plot) as negative controls, placebo g-CA-PLGA NP (Placebo in the plot), fluvastatin dissolved in ethanol (Fluva in the plot) and FLUVA-NP (NP+FLUVA in the plot).

1.5 Discussion

The risk of late in-stent thrombosis and restenosis following the use of DES is a clinical concern [45]. Therefore, the use of devices and treatments that assure a protection against thrombosis and reduce the risk of restenosis is needed.

The encapsulation of drug(s) in polymeric nanoparticles to be locally delivered in the artery wall with a suitable device can be a strategy to sustain its release over the needed time-period for inhibiting those cellular mechanisms at the basis of restenosis.

Data showed that the fluvastatin-containing g-CA-PLGA NP were able to effectively enter into smooth muscle cells and endothelial cells. In addition, they delivered intracellularly fluvastatin which still resulted pharmacologically active after the encapsulation into the polymer matrix. Indeed, after treatment with FLUVA-NP a concentration and time-dependent inhibition of human SMC proliferation was observed, with a 50% of inhibition at 4 μM and the maximal effect at a concentration of 6 μM. This effect was achieved at a concentration of fluvastatin, delivered by the platform of polymeric nanoparticles, that is slightly higher than what was obtained with fluvastatin as such, that completely abolished SMC proliferation at a concentration of 2 μM. This slight discrepancy in the effective concentrations may be due to the fact that, once encapsulated, not all of the fluvastatin is readily available for the release: the surface-associated drug particles will be quickly release upon contact with the medium, while drug properly entrapped within the matrix will be released in a longer time, following diffusion and/or polymer degradation mechanisms. This suggest an effective control of the g-CA-PLGA matrix on the fluvastatin release.

Interestingly, fluvastatin ethanol solution blocked endothelial cells proliferation in a concentration-dependent manner. But when the statin was delivered encapsulated in NP, any inhibitory effect on the endothelial cells was observed, except at the highest concentration tested (10 μM). This most probably highlights a toxic effect on the cell monolayer.

The effects of the treatment with FLUVA-NP on cell migration was also assessed. The fluvastatin-loaded NP inhibited based on concentration-dependent manner the directional migration of human SMC, achieving its maximal inhibitory effect already after 48 h of incubation at the highest concentration tested. At later time points (72 and 144 h), the highest concentration (10 μM) became cytotoxic. Fluvastatin dissolved in ethanol inhibited SMC migration similarly. When these treatments were provided to the endothelial cells, at the later time point (144 h) fluvastatin and FLUVA-NP completely blocked endothelial cell migration at the highest concentrations tested of 10 and 4 μM, respectively. Again, the discrepancy in the concentrations can be ascribed to the polymeric matrix-controlled drug release.

Our data confirms that statins could be suitable drugs to inhibit smooth muscle cell-induced restenosis, since their ability in inhibiting not only SMC proliferation but also the migration, as confirmed by other authors in literature [12–14,44,46]. The data also demonstrated that the NP prepared by PLGA grafted to caffeic acid are suitable for delivering in an effective and rapid way fluvastatin to cells, maintaining its pharmacological properties and therapeutic effects. Some other examples of nanoparticle-mediated delivery of statins have been described in the literature. Pitavastatin-containing nanoparticles have been incorporated into eluting stent and reduced ISR in an animal model [30] or have been intravenously infused inhibiting left ventricular remodelling [31] or atherosclerotic plaque destabilization [29]. However, the intravenous injection of nanoparticles selectively delivers the drug to inflammatory cells, mainly monocytes [31], missing other cellular targets such as SMC and endothelial cells.

The coating of an angioplasty balloon with drug eluting nanoparticles can overcome the above-mentioned issues related to the injection, allowing the localization of the treatment in the restenosis-involved cells and, possibly, reducing the restenosis risk.

In particular, the generated data suggested that the use of PLGA grafted to caffeic acid could permit not only to overcome some limitations of PLGA as already discussed in literature [26,27], but also to enhance the performances of drug eluting nanoparticles, maintaining the PLGA biocompatibility properties. Interestingly, the presence of caffeic acid resulted advantageous in the design of this eluting NP, since the encapsulation of fluvastatin inhibited the proliferation of SMC at a lower dose compared to endothelial cells. Indeed, the stimulation of endothelial cells proliferation of about 30%, counterweighted the effect of fluvastatin with the unexpected result of slight but significant increase of the NP dose required to inhibit the proliferation of this cell line with respect to SMC. This would permit to inhibit the abnormal proliferation of SMC in the time laps necessary for the endothelium, that is known to be damaged by the PTA procedure, to heal and normally proliferate.

1.6 Conclusions

It is believed that restenosis is a form of atherosclerosis, which reflects complex interactions among mediators of tissue injury and inflammation. These aspects lead to the uncontrolled proliferation particularly of SMC, with the result of luminal re-narrowing of the artery and hampering the artery's patency.

The obtained data clearly demonstrated that by using NP prepared with g-CA-PLGA is possible to effectively deliver fluvastatin to the cells involved in those mechanisms at the basis of the restenosis process. Indeed, the proliferation of human SMC was selectively inhibited with the respect to endothelial cells. This system, properly coupled with a suitable device such as an angioplasty balloon, could be used to reduce or even prevent the restenosis which is one of the major concerns compromising the long-term outcome of a PTA.

References

[1] European Cardiovascular Disease Statistics 2017, (n.d.). http://www.ehnheart.org/cvd-statistics/cvd-statistics-2017.html. (accessed September 18, 2018).

[2] M. Singh, C.S. Rihal, P.B. Berger, M.R. Bell, D.E. Grill, K.N. Garratt, G.W. Barseness, D.R. Holmes, Improving outcome over time of percutaneous coronary interventions in unstable angina, J. Am. Coll. Cardiol. 36 (2000) 674—678. doi:10.1016/s0735-1097(00)00768-3.

[3] M.S. Kim, L.S. Dean, In-stent restenosis., Cardiovasc. Ther. 29 (2011) 190–198. doi:10.1111/j.1755-5922.2010.00155.x.

[4] S. Cassese, R.A. Byrne, T. Tada, S. Pinieck, M. Joner, T. Ibrahim, L.A. King, M. Fusaro, K.L. Laugwitz, A. Kastrati, Incidence and predictors of restenosis after coronary stenting in 10 004 patients with surveillance angiography, Heart. 100 (2014) 153–159. doi:10.1136/heartjnl-2013-304933.

[5] K. Prasad, Do statins have a role in reduction/prevention of post-PCI restenosis?, Cardiovasc. Ther. 31 (2013) 12–26. doi:10.1111/j.1755-5922.2011.00302.x.

[6] A. Kastrati, A. Schomig, R. Dietz, F.J. Neumann, G. Richardt, Time course of restenosis during the first year after emergency coronary stenting, Circulation. 87 (1993) 1498–1505. doi:10.1161/01.CIR.87.5.1498.

[7] J.W. Jukema, J.J.W. Verschuren, T.A.N. Ahmed, P.H.A. Quax, Restenosis after PCI. Part 1: pathophysiology and risk factors., Nat. Rev. Cardiol. 9 (2011) 53–62. doi:10.1038/nrcardio.2011.132.

[8] A.L. Catapano, I. Graham, G. De Backer, O. Wiklund, M.J. Chapman, H. Drexel, A.W. Hoes, C.S. Jennings, U. Landmesser, T.R. Pedersen, Z. Reiner, G. Riccardi, M.-R. Taskinen, L. Tokgozoglu, W.M.M. Verschuren, C. Vlachopoulos, D.A. Wood, J.L. Zamorano, M.-T. Cooney, 2016 ESC/EAS Guidelines for the Management of Dyslipidaemias., Eur. Heart J. 37 (2016) 2999–3058. doi:10.1093/eurheartj/ehw272.

[9] S. Bellosta, F. Bernini, N. Ferri, P. Quarato, M. Canavesi, L. Arnaboldi, R. Fumagalli, R. Paoletti, A. Corsini, Direct vascular effects of HMG-CoA reductase inhibitors., Atherosclerosis. 137 Suppl (1998) S101-9.

[10] S. Bellosta, N. Ferri, L. Arnaboldi, F. Bernini, R. Paoletti, A. Corsini, Pleiotropic effects of statins in atherosclerosis and diabetes., Diabetes Care. 23 Suppl 2 (2000) B72-8.

[11] C. Comparato, C. Altana, S. Bellosta, R. Baetta, R. Paoletti, A. Corsini, Clinically relevant pleiotropic effects of statins: drug properties or effects of profound cholesterol reduction?, Nutr. Metab. Cardiovasc. Dis. 11 (2001) 328–343.

[12] M. Takemoto, J.K. Liao, Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors., Arterioscler. Thromb. Vasc. Biol. 21 (2001) 1712–1719.

[13] A. Corsini, S. Bellosta, R. Baetta, R. Fumagalli, R. Paoletti, F. Bernini, New insights into the pharmacodynamic and pharmacokinetic properties of statins., Pharmacol. Ther. 84 (1999) 413–428.

[14] S. Bellosta, L. Arnaboldi, L. Gerosa, M. Canavesi, R. Parente, R. Baetta, R. Paoletti, A. Corsini, Statins Effect on Smooth Muscle Cell Proliferation, Semin. Vasc. Med. 4 (2004) 347–356. doi:10.1055/s-2004-869591.

[15] S. Bellosta, D. Via, M. Canavesi, P. Pfister, R. Fumagalli, R. Paoletti, F. Bernini, HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 1671–1678. doi:10.1161/01.ATV.18.11.1671.

[16] V. Tolva, S. Mazzola, P. Zerbi, R. Casana, M. Albertini, L. Calvillo, F. Selmin, F. Cilurzo, A successful experimental model for intimal hyperplasia prevention using a resveratrol-delivering balloon, J. Vasc. Surg. 63 (2016) 788–794. doi:10.1016/j.jvs.2014.09.035.

[17] R.A. Byrne, M. Joner, A. Kastrati, Stent thrombosis and restenosis: what have we learned and where are we going? The Andreas Gruntzig Lecture ESC 2014., Eur. Heart J. 36 (2015) 3320–3331. doi:10.1093/eurheartj/ehv511.

[18] R. Waksman, R. Pakala, Drug-eluting balloon: the comeback kid?, Circ. Cardiovasc. Interv. 2 (2009) 352–358. doi:10.1161/CIRCINTERVENTIONS.109.873703.

[19] F. Danhier, E. Ansorena, J.M. Silva, R. Coco, A. Le Breton, V. Préat, PLGA-based nanoparticles: An overview of biomedical applications, J. Control. Release. 161 (2012) 505–522. doi:10.1016/j.jconrel.2012.01.043.

[20] A.P. De Prado, C. Perez-Martinez, C. Cuellas, J.M. Gonzalo-Orden, A. Diego, M. Regueiro, B. Martinez-Fernandez, J.R. Altonaga, J.G. Marin Francisco, F. Fernandez-Vazquez, Preclinical evaluation of coronary stents: focus on safety issues., Curr. Vasc. Pharmacol. 11 (2013) 74–99.

[21] P. Erne, M. Schier, T.J. Resink, The road to bioabsorbable stents: reaching clinical reality?, Cardiovasc. Intervent. Radiol. 29 (2006) 11–16. doi:10.1007/s00270-004-0341-9.

[22] L. Montanari, M. Costantini, E.C. Signoretti, L. Valvo, M. Santucci, M. Bartolomei, P. Fattibene, S. Onori, A. Faucitano, B. Conti, I. Genta, Gamma irradiation effects on poly(DL-lactictide-co-glycolide) microspheres, J. Control. Release. 56 (1998) 219–229. doi:10.1016/S0168-3659(98)00082-0.

[23] L. Montanari, F. Cilurzo, L. Valvo, A. Faucitano, A. Buttafava, A. Groppo, I. Genta, B. Conti, Gamma irradiation effects on stability of poly(lactide-co-glycolide) microspheres containing clonazepam, J. Control. Release. 75 (2001) 317–330. doi:10.1016/S0168-3659(01)00401-1.

[24] F. Puoci, F. Iemma, M. Curcio, O.I. Parisi, G. Cirillo, U.G. Spizzirri, N. Picci, Synthesis of methacrylic-ferulic acid copolymer with antioxidant properties by single-step free radical polymerization, J. Agric. Food Chem. 56 (2008) 10646–10650. doi:10.1021/jf802302k.

[25] M. Curcio, F. Puoci, F. Iemma, O.I. Parisi, G. Cirillo, U.G. Spizzirri, N. Picci, Covalent insertion of antioxidant molecules on chitosan by a free radical grafting procedure, J. Agric. Food Chem. 57 (2009) 5933–5938. doi:10.1021/jf900778u.

[26] F. Cilurzo, F. Puoci, F. Selmin, F. Iemma, P. Minghetti, Pyrogallic acid-PLGA conjugate as new biodegradable material suitable for final sterilization by irradiation, Polym. Adv. Technol. 22 (2011) 2201–2205. doi:10.1002/pat.1746.

[27] F. Selmin, F. Puoci, O. Parisi, S. Franzé, U. Musazzi, F. Cilurzo, Caffeic Acid-PLGA Conjugate to Design Protein Drug Delivery Systems Stable to Irradiation, J. Funct. Biomater. 6 (2015) 1–13. doi:10.3390/jfb6010001.

[28] J.C. Tardif, J. Grégoire, P.L. L'Allier, Prevention of restenosis with antioxidants: Mechanisms and implications, Am. J. Cardiovasc. Drugs. 2 (2002) 323–334. doi:10.2165/00129784-200202050-00005.

[29] S. Katsuki, T. Matoba, S. Nakashiro, K. Sato, J. Koga, K. Nakano, Y. Nakano, S. Egusa, K. Sunagawa, K. Egashira, Nanoparticle-Mediated Delivery of Pitavastatin Inhibits Atherosclerotic Plaque Destabilization/Rupture in Mice by Regulating the Recruitment of Inflammatory Monocytes, Circulation. 129 (2013) 896–906. doi:10.1161/CIRCULATIONAHA.113.002870.

[30] N. Tsukie, K. Nakano, T. Matoba, S. Masuda, E. Iwata, M. Miyagawa, G. Zhao, W. Meng, J. Kishimoto, K. Sunagawa, K. Egashira, Pitavastatin-Incorporated Nanoparticle-Eluting Stents Attenuate In-Stent Stenosis without Delayed Endothelial Healing Effects in a Porcine Coronary Artery Model, J. Atheroscler. Thromb. 20 (2013) 32–45. doi:10.5551/jat.13862.

[31] Y. Mao, J. Koga, M. Tokutome, T. Matoba, G. Ikeda, K. Nakano, K. Egashira, Nanoparticle-Mediated Delivery of Pitavastatin to Monocytes/Macrophages Inhibits Left Ventricular Remodeling After Acute Myocardial Infarction by Inhibiting Monocyte-Mediated Inflammation, Int. Heart J. 58 (2017) 615–623. doi:10.1536/ihj.16-457.

[32] H. Fessi, F. Puisieux, J.P. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 55 (1989) R1–R4. doi:10.1016/0378-5173(89)90281-0.

[33] V. Laquintana, N. Denora, T. Musacchio, M. Lasorsa, A. Latrofa, G. Trapani, Peripheral Benzodiazepine Receptor ligand-PLGA polymer conjugates potentially useful as delivery systems of apoptotic agents, J. Control. Release. 137 (2009) 185–195. doi:10.1016/j.jconrel.2009.04.007.

[34] S. Castiglioni, M. Monti, G.A. Buscherini, L. Arnaboldi, M. Canavesi, A. Corsini, S. Bellosta, The dataset describes: Phenotypic changes induced by cholesterol loading in smooth muscle cells isolated from the aortae of C57BL/6 mice, Data Br. 16 (2018) 334–340. doi:https://doi.org/10.1016/j.dib.2017.11.050.

[35] S. Castiglioni, M. Monti, L. Arnaboldi, M. Canavesi, G.A. Buscherini, L. Calabresi, A. Corsini, S. Bellosta, ABCA1 and HDL3 are required to modulate smooth muscle cells phenotypic switch after cholesterol loading, Atherosclerosis. 266 (2017) 8–15. doi:https://doi.org/10.1016/j.atherosclerosis.2017.09.012.

[36] R. Ross, The smooth muscle cells. II. Growth of smooth muscle in culture and formation of elastic fibers., J. Cell Biol. 50 (1971) 172–186. doi:10.1083/jcb.50.1.172.

[37] G. Franceschini, F. Bernini, S. Michelagnoli, S. Bellosta, V. Vaccarino, C. Torre, F. Pazzucconi, R. Fumagalli, C.R. Sirtori, Increased affinity of LDL for their receptors after acipimox treatment in hypertriglyceridemia, Eur. J. Clin. Pharmacol. 40 (1991) S45--S48. doi:10.1007/BF03216289.

[38] C.J. Edgell, C.C. McDonald, J.B. Graham, Permanent cell line expressing human factor VIII-related antigen established by hybridization., Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 3734–3737. doi:10.1073/pnas.80.12.3734.

[39] I. Giunzioni, A. Bonomo, E. Bishop, S. Castiglioni, A. Corsini, S. Bellosta, Cigarette smoke condensate affects monocyte interaction with endothelium, Atherosclerosis. 234 (2014) 383–390. doi:https://doi.org/10.1016/j.atherosclerosis.2014.03.029.

[40] F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, J. Immunol. Methods. 89 (1986) 271–277. doi:https://doi.org/10.1016/0022-1759(86)90368-6.

[41] F. Bernini, M. Canavesi, E. Bernardini, N. Scurati, S. Bellosta, R. Fumagalli, Effect of lacidipine on cholesterol esterification: In vivo and in vitro studies, Br. J. Pharmacol. 122 (1997) 1209–1215. doi:10.1038/sj.bjp.0701469.

[42] S.A. Kulkarni, S.S. Feng, Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery, Pharm. Res. 30 (2013) 2512–2522. doi:10.1007/s11095-012-0958-3.

[43] J. Panyam, V. Labhasetwar, Dynamics of endocytosis and exocytosis of poly(D,L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells, Pharm. Res. 20 (2003) 212–220. doi:10.1023/A:1022219003551.

[44] S. Bellosta, N. Fed, F. Bernini, R. Paoletti, A. Corsini, Non-lipid-related effects of statins, Ann. Med. 32 (2000) 164–176. doi:10.3109/07853890008998823.

[45] C.-C. Liang, A.Y. Park, J.-L. Guan, In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro, Nat. Protoc. 2 (2007) 329. http://dx.doi.org/10.1038/nprot.2007.30.

[46] L. Arnaboldi, R. Baetta, N. Ferri, A. Granata, S. Bellosta, R. Paoletti, A. Corsini, Inhibition of smooth muscle cell migration and proliferation by statins, Immunol. Endocr. Metab. Agents Med. Chem. 8 (2008). doi:10.2174/187152208784587944.

Chapter 2

Maltrodextrins as drying auxiliary agent for the preparation of easily resuspendable nanoparticles

Abstract

The drying of biodegradable polymeric nanoparticles (NP) is mandatory to improve their physical and chemical stability over time. Spray- or freeze-drying can induce irreversible aggregation of NP and therefore the use of drying auxiliary agents is required. The ability of four grades of maltodextrins (DE2, DE6, DE12 and DE38) to protect PLGA NP from stresses was studied. High M_w maltodextrin (DE2) was not functional for obtaining an easily resuspendable dried product, since it needs a prolonged time to fully hydrate. Maltodextrin at intermediate DE showed a poor ability to protect NP from irreversible aggregation probably because too sensitive to environmental variation. DE38, which did not alter ζ-potential of NP, allowed to obtain an easily resuspendable nanosuspension independently of the drying process. The effectiveness of such material was attributed to the easiness of spray-drying a low viscous solution and to the ability of substitute the water molecules' hydrogen bonds with NP during freeze-drying.

2.1 Introduction

One of the main obstacles limiting the availability of products based on hydrophobic colloidal suspensions is their physical and chemical instability upon long-term storage. Thus, it is mandatory to convert these systems into solids of sufficient stability which can be easily and rapidly reconstituted preserving the initial physicochemical characteristics of the product. Among the possible drying processes, spray-drying and freeze-drying are the methods of choice [1–4]. Spray drying involves feeding a solution or a suspension into an atomizer, producing small droplets, which are exposed to air or nitrogen maintained to temperatures higher than the solvent boiling point favoring the rapid evaporation of the liquid. This thermal stress could impede the reconstitution of the hydrophobic colloidal suspension. During the lyophilization process, the colloidal system, mainly in the freezing step, is subjected to different stresses which can lead to the aggregation or coalescence of the dispersed phase.

Drawbacks due to both processes are particularly critical in the case of polymeric nanoparticles [5,6] which are generally constituted by materials characterized by low melting points and/or glass transition temperatures and, therefore, sensitive to the temperatures required to remove water in both spray-drying or freeze-drying. As an example, in the case of poly(ε–caprolactone) nanoparticles, the exposure to high temperatures can be prohibitive since it can jeopardize their structural integrity, leading to degradations or coalescences, despite the short residence time in the drying chamber [6]. Regarding the lyophilization, freezing is considered the most aggressive and critical step since it can induce the nanoparticle instability. As an example in freeze-dried poly(D,L-lactide acid-co-ethylene oxide) nanoparticles, the formation of intra- and inter-particle bridges of PEO crystallized on the surface upon freezing caused their irreversible aggregation [7]. The same problems are reported by the formation of ice-ice and ice-container interfaces caused spatial confinement of nanoparticles [8]. Moreover, difficulties in drying amorphous polymers are also dependent on the chemical affinity with the water molecules which are responsible for plasticizing

the polymer. The consequent decrease of the glass transition temperature (T_g) of the system causes processing problems, namely particle agglomeration, caking and stickiness. As an example, the moisture content can reduce the T_g up to 15°C of poly(lactide-co-glycolide) [9,10]. Hence, both in spray- and freeze-drying, two major challenges are to set-up the process conditions and to identify the drying auxiliary agents able to preserve the original physicochemical properties of nanoparticles.

Maltodextrins (MDX) appears especially useful to this purpose due to their good aqueous solubility, low viscosity and high T_g which provided a stable glassy matrix at room temperatures [11].

Based on these considerations, this work aims to evaluate the ability of four types of MDX differing in molecular weight on protecting poly(D,L lactide-co-glycolide) nanoparticles from thermal and mechanical stresses induced by spray-drying or freeze-drying. In this regard, an uncapped poly(D,L lactide-co-glycolide) with a low T_g was selected as critical material and nanoparticles were prepared by a solvent displacement method avoiding the use of any stabilizer, which can interfere with the effect of MDX.

2.2 Materials

Uncapped poly(lactide-co-glycolide) (PLGA) at lactide/glycolide ratio of 50:50 and having a T_g at about 36.5 °C was purchased by Evonik Industries (G). MDX with a dextrose equivalent (DE) of 2, 6, 12 and glucose syrup with a DE38 (Glucidex®) were kindly gifted by Roquette (F). All solvents were of analytical grade, unless specified.

2.3 Methods

2.3.1 MDX characterization

Size exclusion chromatography (SEC) was performed using an HP1100 Chemstation (Agilent, USA) equipped with refractive index signal as a detector. A combination of two columns in series, Superchrome Biobasic SEC300 (300x7.8 mm, 5µm, 300 Å) and 120 (300x7.8 mm, 5µm, 120 Å), were operated at the flow rate of 0.4 mL/min and temperature of 35 °C. Samples at the concentration of 5 mg/mL were eluted using a mobile phase constituted of pH 6.8 0.05 M PB and 0.25 M KCl and the injection volume was 50 µL. The weight- average molecular weight (Mw) and the number-weight molecular weight (M_n) was calculated using dextrans as standards, in the range from 1-410 KDa. Dispersity index (DI) is reported as the ratio between M_w and M_n.

Modulated differential scanning calorimetry (mDSC) experiments were performed using a DSC1 Star^e System (Mettler Toledo, CH) equipped with a refrigerated cooling system (RCS) to determine the T_g of MDX. Samples of about 10-15 mg exactly weighted were transferred to pin-holed aluminium pans, sealed and subjected to heating cycles from 10 to 100 °C, 150 °C or 200 at °C at the 5 K min–1 (period = 90s; amplitude = 0.5 °C). The temperature range was fixed based on preliminary DSC analysis. The mDSC cell and RCS were purged with dry nitrogen at 80 and 120 mL/min, respectively. The system was calibrated using an indium standard. All data were treated with Stare System software (Mettler Toledo, CH) and the T_g is expressed as an inflection point in the reversible curve.

Dynamic light scattering analysis (see paragraph 2.3.6) were performed on MDX solutions at 4 and 8% following the sample preparation as per the compatibility study (paragraph 2.3.3). Size distribution plots were further analyzed using the high-resolution mode to resolve multimodal or broad peaks.

All samples were subjected of three measurements and the results are reported as mean ± standard deviation.

2.3.2 Nanoparticles preparation

PLGA NP were prepared using a solvent displacement method [12]. Briefly, PLGA was dissolved in a mixture of acetone/absolute ethanol (7:3 v/v) at the concentration 1% w/v and 1 mL was added dropwise to 10 mL of MilliQ® water, using an electronic pipette (PIPETMAN M®- Gilson, USA). The system was maintained at 4 °C under magnetic stirring at 500 rpm for 15 min before increasing the temperature to 25 °C over a 3 h period.

2.3.3 Compatibility

An aliquot of 1 mL of MDX solution in MilliQ® water or 0.9% NaCl solution was added to 1 mL of nanosuspension to get the final concentration of 2, 4 or 8 % w/v. After stirring for 3 h at room temperature, the samples were visually observed, and the particle size was determined as reported in paragraph 2.3.6. The ζ–potential values were measured only for samples at 2% MDX concentration to limit interferences on electrophoretic mobility.

2.3.4 Spray-drying

Nanosuspension in presence of MDX was sprayed through a two-fluid nozzle, operating in a co-current manner, of a Format 4 M8 (ProCepT, Belgium). The process parameters, optimized according to the results of the Design of Experiments (*Annex*, **Table A1**), were set as follows: inlet temperature= 130 °C; feed flow rate = 6.5 mL/min; nozzle pressure = 1.7 atm; nozzle diameter = 0.4 mm; ΔP = 70 mbar. The dried powders were separated from the drying air in the cyclone (37–39 °C outlet temperature) and deposited in the collector.

2.3.5 Freeze-drying

Freeze-drying was performed using an Epsilon 2-6 LSC plus freeze dryer (Martin Christ, Germany).

To tailor the experimental set-up [13], the glass transition temperature of maximally cryo-concentrated solution (T_g') of a 20% w/v MDX solution was determined by differential scanning calorimetry (DSC). Briefly, an aliquot was cooled until -40 °C at 5 K·min^{-1}, kept at -40 °C for 20 min and then heated to 25 °C at 5 K·min^{-1}. T_g' were taken as the inflection point of the specific heat increment at the glass–rubber transition on the heat scan.

The influence of NP on ice melting ΔH of MDX solutions was evaluated by modulated DSC analysis, heating the frozen samples with a cycle from -40 °C to +25 °C (period = 90 s; amplitude = 0.5 °C).

All the samples were analyzed in triplicate and the results are reported as mean ± standard deviation.

Two different cycles were designed based on T_g'. In particular, samples containing DE2, DE6 and DE12 were frozen at -30 °C for 2 h; then, the main drying was carried out at 10 °C and 0.22 mbar for 6 h and the secondary drying at 35 °C and 0.22 mbar for 4 h. In case of DE38, the experimental conditions were: freezing: -40 °C for 2 h; main drying at 0 °C and 0.10 mbar for 6 h; secondary drying: 35 °C and 0.10 mbar for 6 h. Afterwards, the vacuum was broken by air injection and samples were stored at room temperature until reconstitution.

2.3.6 Dynamic light scattering

The Z-average diameter (D_H) and the size distribution (PDI) of samples were evaluated by photon correlation spectroscopy using a dynamic light scatter (DLS, Zetasizer Nano ZS, Malvern Instrument, UK), equipped with a backscattered light detector, operating at 173° and 25 °C. The results calculated using the Dispersion Technology Software (Malvern Instruments, UK) are reported as intensity distribution. Samples were subjected of three analysis and the results are reported as mean ± standard deviation.

2.3.7 Zeta-potential

Zeta potential (ζ) of nanosuspension was assessed by M3-PALS (Phase Analysis Light Scattering) technique, using Zetasizer Nano ZS (Malvern Instrument, UK) at 25 °C. Samples were subjected of three measurements and the results are reported as mean ± standard deviation.

2.3.8 Resuspendability

5 mg of the dried powders were reconstituted in 1 mL of MilliQ® water or 0.9% w/v NaCl and the size distribution was evaluated by DLS after 5, 30 and 60 min of shaking at 100 rpm, 25 °C in a benchtop incubation shaker (Sartorius Certomat IS, G). This system was selected since it allowed to obtain a gentle and reproducible shaking.

MDX were considered suitable drying agent if both the quality of DLS measurement resulted good and D_H (expressed as peak size) of the reconstituted NP was not significantly different with regards to the values from the compatibility study (α=0.05, ANOVA One-way). For this purpose, the size of the main peak was considered, accounting also its percentage on the whole size distribution.

The ζ of NP reconstituted in water without a sign of aggregation was also evaluated after 60 min of shaking.

2.4 Results and discussion

2.4.1 Characterization of MDX and evaluation of their physical compatibility with PLGA NP

Molecular weight distribution and thermal properties are two of the main parameters that can potentially impact the ability of MDX to act as a drying agent. The physicochemical features of the selected MDX are summarized in **Table 2.1**. According to literature data, M_n and M_w of MDX decreased increasing the DE value [14], following an exponential relationship (R^2>0.99). A similar dependence was found in the case of T_g: in a homologous polysaccharide series with different M_n, the decreased length of polymeric chains, namely the increased concentration of reducing sugars in MDX, determines the transition from glassy to rubbery state at lower temperature, compared to high M_w products [15]. Moreover, the change in heat capacity associated with T_g (ΔC_p, **Table 2.1**) was related to DE [16]: the higher the M_n, the smaller the ΔC_p. No statistical differences between ΔC_p of DE6 and DE12 was found, in agreement with the similarity in terms of T_g and M_n (**Table 2.1**).

Table 2.1 – Molecular weight distribution of raw MDX, glass transition temperature (T_g) and change in heat capacity (ΔC_p) associated with the transition of 20% MDX solution.

MDX	M_w (KDa)	M_n (KDa)	DI	T_g (°C)	ΔC_p (J/g·K)
DE2	172.0±7.1	67.9±0.8	2.57	158.5±1.7	0.01±0.00
DE6	71.3±1.5	35.5±0.9	2.00	112.8±0.4	0.08±0.01
DE12	47.4±0.6	27.9±0.2	1.70	111.9±0.3	0.06±0.03
DE38	1.7±0.0	1.5±0.0	1.13	55.5±2.0	0.13±0.08

PLGA NP were prepared avoiding the use of surfactants or steric stabilizers which can remain adsorbed onto NP surface [17] and cooperate to preserve NP size during drying [18]. The selected process conditions allowed to obtain batches of monodispersed PLGA NP (PDI = 0.059±0.012) with a mean hydrodynamic diameter lower than 170 nm (D_H = 157±7 nm) and a ζ-potential of about -31 mV.

PLGA NP were compatible with MDX solution as no aggregates or phase separations were evident after 3h of stirring. However, for MDX concentration higher than 2%, DLS analyses evidenced a slight increment of NP DH as a function of the MDX grade and concentration. This was concomitant to a shift of ζ–potentials towards higher values and was due to the adsorption of MDX on NP surface (**Table 2.2**).

Table 2.2 – Compatibility of PLGA NP, expressed as D_H (nm), PDI and ζ-potential (mV), with MDX solution at different percentages (%, w/v).

MDX	D_H (PDI)				ζ-potential (mV)	
	0 %	2 %	4 %	8 %	0 %	2 %
DE2	143±1 (0.06±0.02)	144±1 (0.21±0.01)	196±1 (0.28±0.00)	315±2 (0.32±0.01)	-31.1±1.0	-25.8±0.6
DE6		146±1 (0.13±0.02)	184±2 (0.17±0.01)	279±2 (0.270.01)		-23.4±0.4
DE12		143±1 (0.09±0.01)	175±1 (0.12±0.01)	171±1 (0.10±0.01)		-24.7±0.5
DE38		143±1 (0.06±0.01)	155±1 (0.07±0.03)	186±1 (0.04±0.02)		-26.8±0.8

The change of D_H became more noticeable increasing the ionic strength. PLGA NP as such were physically stable in 0.9% NaCl solution, but adding MDX, only DE2 and DE38 at all the concentrations tested, were able to maintain an acceptable monodisperse distribution of NP, even if D_H increased (**Table 2.3**). At intermediate DE values, the formation of aggregates was dependent on MDX concentration. In other words, DE6 was compatible with NP only at 2%, despite the significant increase of DH and the same behaviour was evident in case of 4% DE12 solution. Furthermore, increasing the DE12 concentration to 8%, D_H of PLGA NP shifted to the submicron range (**Table 2.3**).

Table 2.3 – Compatibility of PLGA NP, expressed as D_H (nm), PDI and ζ-potential (mV) with solutions in 0.9% NaCl of MDX at different percentages (%, w/v).

MDX	**D_H (PDI)**			
%	**0**	**2**	**4**	**8**
DE2	141±1 (0.05±0.01)	143±1 (0.22±0.02)	204±2 (0.25±0.00)	292±4 (0.14±0.06)
DE6		234±7 (0.27±0.01)	1163±87 (0.65±0.04)	-*
DE12		146±2 (0.09±0.01)	277±4 (0.19±0.02)	496±15 (0.15±0.01)
DE38		164±1 (0.07±0.01)	200±3 (0.02±0.01)	237±2 (0.09±0.02)

*Not performed

The compatibility data can be explained considering that the apparent persistence length relative to hydration linearly increases as a function of DE, until a maximum value is reached around DE10 and, then, it decreases by increasing the de-polymerization degree of MDX [19]. This would explain the relative lower ζ–potential values of DE6 and DE12 with respect to those measured after adding

DE2 and DE38. Moreover, it is also recognized that the apparent persistence length of high-M_w polysaccharides is influenced by the increase of ionic strength, which modified stiffness and the conformation shape of MDX [20]. In other words, a different organization of DE6 and DE12 chains adsorbed onto NP surface with respect to those of DE2 and the oligomers of DE38 can be supposed and this can reflect the different impact on ζ–potentials shielding (**Table 2.2**). This effect is amplified by increasing the dispersant ionic strength after adding sodium chloride, which it is known to compress the electrical double layer at the NP surface in a concentration dependent way and to reduce the ζ–potential [21,22]. Since PLGA NP without polysaccharides were stable in 0.9% NaCl, their aggregation might be related to the chain mobility of the MDX adsorbed onto NP surface. This hypothesis is supported by the DLS analysis on MDX dispersion in water or NaCl 0.9%. DE2 segregated in clusters stable to environmental variation such as ionic strength (**Fig. 2.1a**) probably because of their ability to form "*helical coils*" [23]. Conversely, DE6 and DE12, due to their limited capacity to organize ordered structures [24], generate clusters of different dimensions which resulted sensible to the increase of the ionic strength (**Fig. 2.1b-c**). Finally, DE38 is too small ($M_n \sim$ 1500 Da) to form clusters in agreement with the lack of any populations detectable by DLS.

Considering that the irreversible aggregation of PLGA NP depends on the DE6 and DE12 concentration, their potentiality of as drying auxiliary agent became less relevant for the purposes of this study.

In general, PLGA NP/MDX samples, which underwent an increase of D_H higher than 300 nm in water or sodium chloride solution, were not worthy of further investigation.

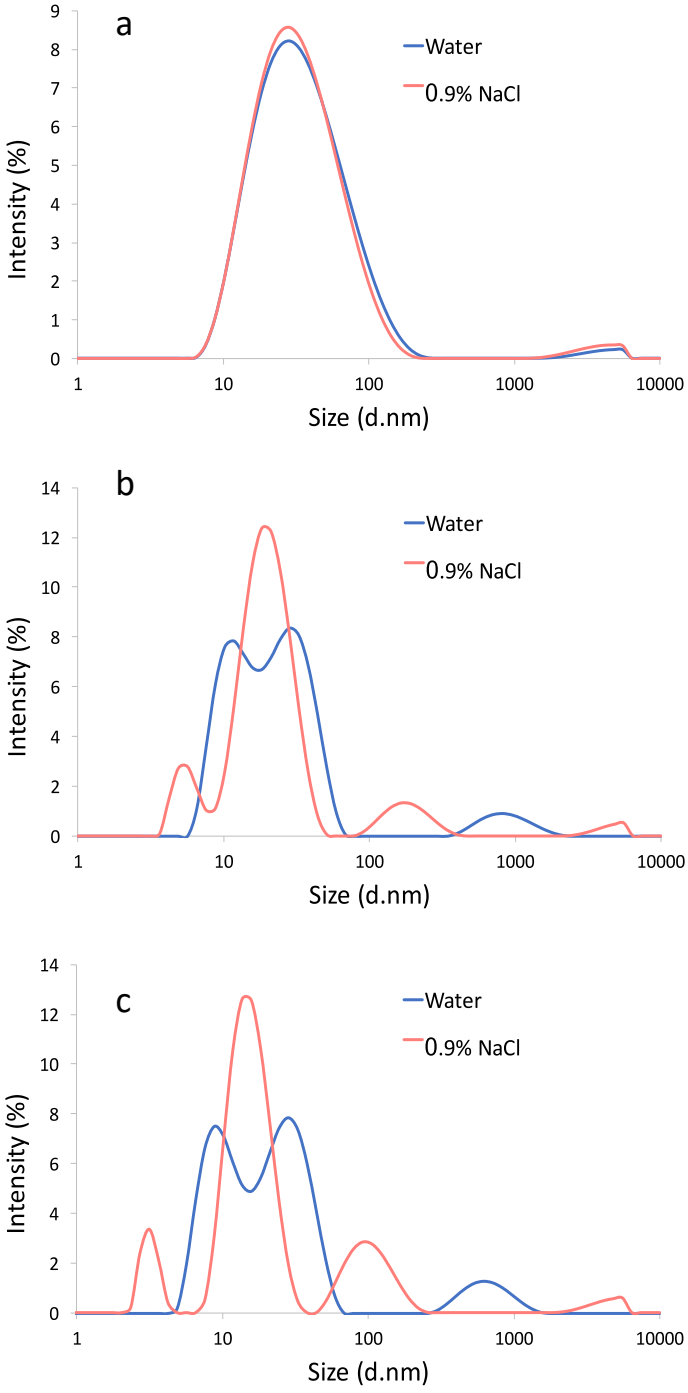

Fig. 2.1 – High resolution size distribution plots of (a) 4% DE2 and (b) DE6, and (c) 8% DE12 in water and 0.9% NaCl.

2.4.2 Drying of nanoparticles

2.4.2.1 Spray-drying

The performances of 2% MDX solution was tested using the optimized conditions (see *Annex*). In all cases, the yield was satisfactorily ranging from about 50 to 75 % and the outlet temperature did not exceed 40 °C, assuring that the T_g of raw PLGA was not significantly exceeded ($T_g \sim 36.5$ °C).

The data of reconstitution in water evidenced that the higher the DE value, the faster the recovery of the original NP D_H (**Fig. 2.2**): only DE38 guaranteed the reconstitution after 5 min; DE12 and DE6 based solids required 30 min- and 60 min-to obtain a monodispersed distribution, respectively; whilst DE2 was unable to guarantee the formation of a stable colloidal system.

This different ability can be related to MDX viscosity which controls the NP motion to the surface of the droplets during drying. Hence, it can be assumed that DE2 slowed the drying and diffusion rate of PLGA NP which were slowly transported by means of convection within the droplets. Thus, the probability of constrained NP to interact each other increased, causing the formation of irreversible aggregates. On the other hand, the rearrangement of NP can be considered limited in case of DE38 since the drying occurs almost instantaneously, maintaining NP secluded as in the feed. This hypothesis is in line with MDX Mw and the time needed for a complete NP reconstitution: DE2 promoted NP aggregation, while DE6 and DE12 favored the dispersion of NP over relatively prolonged time, according to their M_w, and DE38 allowed the fastest recovery of NP size.

Regarding the ζ–potential, in the case of DE12 and DE6 the values were superimposable to those obtained during the compatibility study (**Table 2.2**); meanwhile in presence of DE38 (ζ = -28.9±3.9 mV) the value was not statistically different compared to NP as such (t-test $p > 0.05$). DE38 permitted to re-suspend the NP in 0.9% NaCl in few minutes (**Fig. 2.2**).

These results confirmed that the formation of MDX clusters had a detrimental effect on the reconstitution of NP. Therefore, MDX at higher concentrations were not tested.

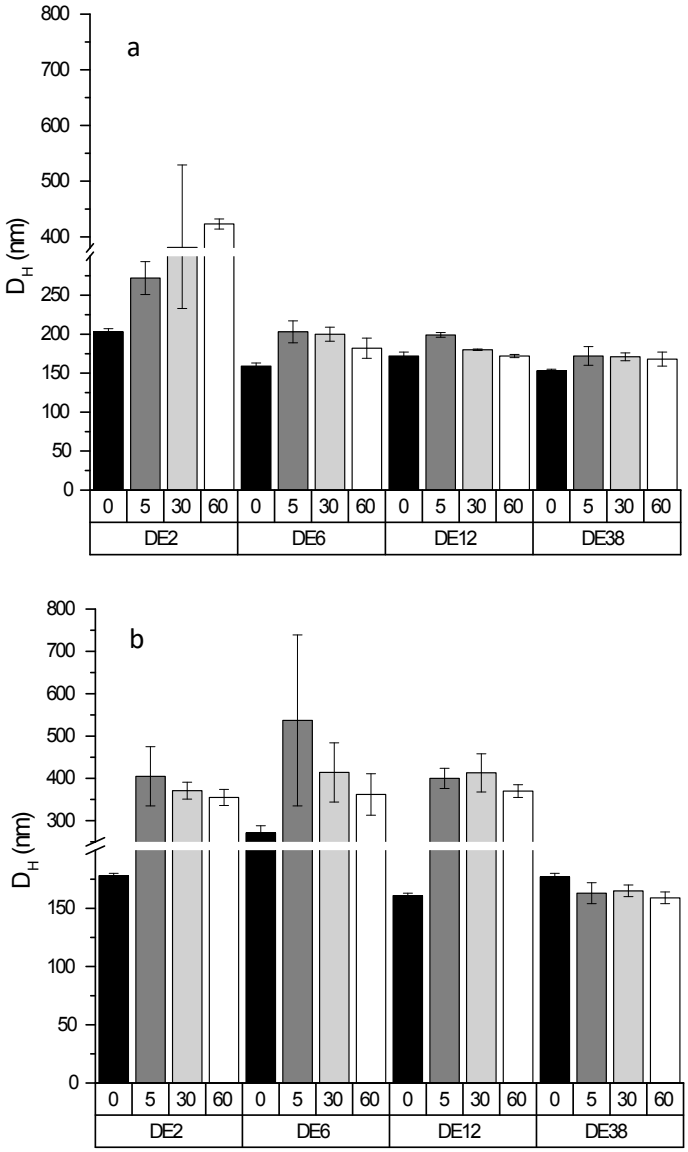

Fig. 2.2 – Hydrodynamic diameter (D_H) of PLGA NP in presence of 2% MDX before spray-drying (t = 0) and after 5, 30 and 60 min of gentle reconstitution in (a) water and (b) 0.9% NaCl.

2.4.2.2 Freeze-drying

A DSC investigation was preliminary carried out to determine the thermal properties of MDX solutions and, consequently, to establish the optimal set-up of the operative conditions.

For all MDX solutions at 20%, two thermal events were observed scanning the sample from -40 °C to 25 °C: an inflection point on heat flow signal attributed to the T_g' of maximally cryo-concentrated solution and an endotherm related to the melting of frozen water (T_m'). As expected, the higher the MDX molecular weight, the higher the T_g' [25,26] and the higher T_m' [27] (**Table 2.4**).

Table 2.4 – Glass transition temperature of maximally cryo-concentrated solutions (T_g'), associated change in heat capacity ($\Delta C_p'$) and onset temperature of ice melting (T_m') of 20% w/v MDX solutions. $\Delta(\Delta H_m)$ represents the variation of ice melting enthalpy values.

Sample	T_g' (°C)	$\Delta C_p'$ (J/g·K)	T_m' (°C)	$\Delta(\Delta H_m)$ * (mJ/mg)
Pure water	--	--	0.7±0.0	--
MDX DE2	-5.3±0.0	1.10±0.03	0.2±0.0	43
MDX DE6	-7.5±0.2	1.03±0.15	-0.1±0.2	42
MDX DE12	-11.0±0.0	0.75±0.03	-0.5±0.0	15
MDX DE38	-23.6±0.1	0.44±0.01	-2.0±0.0	3

*values referred to MDX solution with PLGA NP

Typically, T_g' value is used as a reference for designing both freezing and primary drying steps of lyophilization process, since the system undergoes to drastic changes in viscosity, heat capacity and molecular mobility as the glassy matrix is formed. Changes in heat capacity ($\Delta C_p'$) at T_g' also permitted to evaluate the temperature dependence of MDX molecular mobility. Among all tested materials, the lowest $\Delta C_p'$ of DE38 indicated the formation of the strongest glass due to the limited chain mobility during the transition, which also occurred at the lowest temperature (**Table 2.4**) [28].

The influence of PLGA NP on the thermal properties of MDX solutions cannot be considered negligible, despite they accounted for the minority of the formulation. Indeed, the dispersed solid caused the broadening of ice melting peaks, due to the possible structural heterogeneity of nucleation, with a concomitant variation of ice melting enthalpy values [$\Delta(\Delta H_m)$]. This feature can be attributed to the formation of hydrogen bonds between PLGA and water molecules which increased the amount of un-freezable water [9]. Interestingly, $\Delta(\Delta H_m)$ decreased as the DE value, suggesting that the colloidal system caused a great deal on instability in fragile glasses which are probably more sensitive to the variation of viscous flow generated by the increase of unfrozen water. This observation agreed with the appearance of cakes obtained after freeze-drying 2% MDX solution with or without NP added. As exemplified in **Fig. 2.3**, DE2 based cakes collapsed only in presence of NP, while DE38 did not show visible defects.

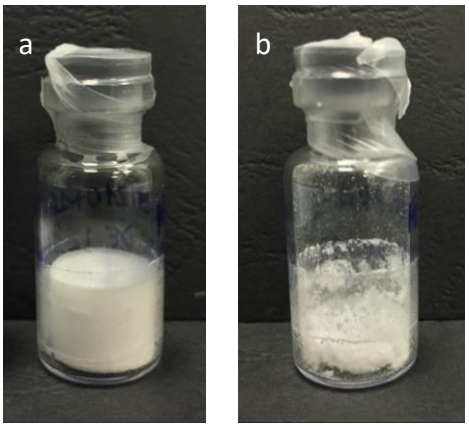

Fig. 2.3 – 2% MDX DE2-based freeze-dried product (a) without and (b) with PLGA NP added.

However, it is noteworthy that the loss of structure of the DE2, DE6 and DE12 dried products was not just a cosmetic issue, as NP massively aggregated after reconstitution in water and NaCl solution. Conversely, at the 2% concentration, DE38 permitted the formation of an elegant cake, but the reconstituted samples in water and 0.9% NaCl presented a too high polydispersity.

Regarding DE2 based cakes, only the increase of concentration to 8% permitted to obtain an elegant solid, but after 5 min of reconstitution the NP size increased up to about 350 nm and 500 nm in water or 0.9% NaCl, respectively (**Table 2.5**). DE12 matrix obtained from a 4% solution showed a good aesthetic but, again, the reconstituted sample was too polydispersed, independently of the media and the time points (**Table 2.5**).

Eventually, DE38 at 4% concentration was able to avoid size variation (at all the time points considered ($p>0.05$) with the respect of reference sample (**Table 2.5**). Moreover, the decrease of ζ (ζ = -47.4±1.10 mV) reflected the good stability of this reconstituted sample also in 0.9% NaCl ($D_H \sim 200$ nm at 5 min, $p>0.05$ at all the time points considered).

Table 2.5 – Main populations detected by DLS after reconstitution of freeze-dried NP with compatible concentrations of MDX at different time points.

MDX		**5 min**		**30 min**		**60 min**	
Concentration	**Grade**	**water**	**NaCl**	**water**	**NaCl**	**water**	**NaCl**
2%	DE2	Collapsed cake					
	DE6	Collapsed cake					
	DE12	Collapsed cake					
	DE38	236±130[*]	188±9[*]	288±76[*]	240±15[*]	317±90[*]	245±15[*]
4%	DE2	Collapsed cake					
	DE12	285±34	438±158	350±28	560±173	289±40	--[**]
	DE38	188±6	188±9	209±19	233±15	205±12	235±16
8%	DE2	316±46[*§]	392±104[*§]	358±38[*§]	482±233[*§]	371±20[*§]	649±138[*§]

[*]Poor quality of DLS analysis.

[**]Sample too dispersed.

[§]The main population accounted for about 50% of the size distribution.

Generally speaking, the stabilization of NP during lyophilization can be driven by two main mechanisms occurring concomitantly [29]. First, the "*vitrification hypothesis*" suggests that glassy matrices formed by amorphous protectants (*i.e.,* saccharides) upon freezing allow the immobilization of NP preserving them from detrimental effects of ice crystals. Secondly, the "*water replacement theory*" proposes that the hydrogen bonds between water and NP are replaced by similar interactions occurring onto NP surface with the adsorbed excipient, thus avoiding particle aggregation or fusion.

Among the selected MDX, DE38 is a glass former material which was demonstrated to remain adsorbed to PLGA NP despite the environmental condition as both the size and the ζ–potential values of NP increased. Hence, its ability to protect NP during lyophilization can be ascribed to the concomitant occurrence of both mechanisms.

2.4.3 Drying of g-CA-PLGA nanoparticles

Considering that MDX DE38 was effective in preserving the main features of the model PLGA at a lower concentration in the spray-drying process (*i.e.,* 2% w/v) compared to the freeze-drying (*i.e.,* 4% w/v), this drying set-up was also applied for placebo and fluvastatin sodium (FLVNa) loaded g-CA-PLGA (see *Chapter 1* for the details). Again, both nanosuspensions tested were compatible with the aqueous solution of 2% MDX DE38, since no NP aggregation or unacceptable increment in NP size were detected (**Table 2.6**). The reconstitution in water of the spray-dried powders demonstrated the fast and complete resuspension of NP within 5 min of stirring ($p>0.05$, **Table 2.6**). Interestingly, the presence of FLVNa encapsulated in g-CA-PLGA matrix did not cause a variation in the ability of MDX DE38 in acting as drying auxiliary agent during the spray-drying, suggesting the suitability and versatility of this set-up in the drying of different PLGA-based NP.

Table 2.6 – Water compatibility and reconstitution of the spray-dried powder of placebo and fluvastatin sodium (FLUVA) loaded g-CA-PLGA NP with 2% w/v MDX DE38 solution. Results are expressed as Z-average diameter (D_H) and Z-potential (ζ).

g-CA-PLGA NP	Compatibility		Reconstitution			
			D_H (nm)			ζ (mV)
	D_H (nm)	ζ (mV)	5 min	30 min	60 min	
Placebo	196±1	-27.6±0.7	223±3	212±3	206±2	-27.7±0.4
FLUVA loaded	202±1	-35.0±1.1	208±2	208±2	208±2	-34.7±0.5

2.5 Conclusions

The overall data suggests that MDX DE38 can be proposed to preserve the stability of PLGA NP both during spray- or freeze-drying. It should be underlined that such material was effective at low concentrations for both drying processes. This advantage is of relevance in spray-drying since it was possible to reduce the amount of MDX DE38 required to obtain an easily resuspendable nanosuspension using a technique which permits to reduce the time and the operation costs with respect to lyophilization [30].

Furthermore, the possibility of preparing a dried powder of FLUVA loaded g-CA-PLGA nanoparticles pave the way for the processability of this formulation into a NP-rich coating of the angioplasty balloon.

References

[1] P. Blasi, A. Schoubben, G.V. Romano, S. Giovagnoli, A. Di Michele, M. Ricci, Lipid nanoparticles for brain targeting II. Technological characterization, Colloids Surfaces B Biointerfaces. 110 (2013) 130–137. doi:10.1016/j.colsurfb.2013.04.021.

[2] K.L. Christensen, G.P. Pedersen, H.G. Kristensen, Preparation of redispersible dry emulsions by spray drying, Int. J. Pharm. 212 (2001) 187–194. doi:10.1016/S0378-5173(00)00596-2.

[3] D.M.K. Jensen, D. Cun, M.J. Maltesen, S. Frokjaer, H.M. Nielsen, C. Foged, Spray drying of siRNA-containing PLGA nanoparticles intended for inhalation, J. Control. Release. 142 (2010) 138–145. doi:10.1016/j.jconrel.2009.10.010.

[4] R.F. Ribeiro, M.H. Motta, A.P.G. Härter, F.C. Flores, R.C.R. Beck, S.R. Schaffazick, C. De Bona Da Silva, Spray-dried powders improve the controlled release of antifungal tioconazole-loaded polymeric nanocapsules compared to with lyophilized products, Mater. Sci. Eng. C. 59 (2016) 875–884. doi:10.1016/j.msec.2015.10.035.

[5] P. Fonte, S. Reis, B. Sarmento, Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery, J. Control. Release. 225 (2016) 75–86. doi:10.1016/j.jconrel.2016.01.034.

[6] K. Kho, W.S. Cheow, R.H. Lie, K. Hadinoto, Aqueous re-dispersibility of spray-dried antibiotic-loaded polycaprolactone nanoparticle aggregates for inhaled anti-biofilm therapy, Powder Technol. 203 (2010) 432–439. doi:10.1016/j.powtec.2010.06.003.

[7] F. De Jaeghere, E. Allémann, J.C. Leroux, W. Stevels, J. Feijen, E. Doelker, R. Gurny, Formulation and lyoprotection of poly(Lactic acid-co-ethylene oxide) nanoparticles: Influence on physical stability and In vitro cell uptake, Pharm. Res. 16 (1999) 859–866. doi:10.1023/A:1018826103261.

[8] L. Niu, J. Panyam, Freeze concentration-induced PLGA and polystyrene nanoparticle aggregation: Imaging and rational design of lyoprotection, J. Control. Release. 248 (2017) 125–132. doi:10.1016/j.jconrel.2017.01.019.

[9] P. Blasi, S.S. D'Souza, F. Selmin, P.P. DeLuca, Plasticizing effect of water on poly(lactide-co-glycolide), J. Control. Release. 108 (2005) 1–9. doi:10.1016/j.jconrel.2005.07.009.

[10] N. Passerini, D.Q.. Craig, An investigation into the effects of residual water on the glass transition temperature of polylactide microspheres using modulated temperature DSC, J. Control. Release. 73 (2001) 111–115. doi:10.1016/S0168-3659(01)00245-0.

[11] M.M. Kenyon, Modified Starch, Maltodextrin, and Corn Syrup Solids as Wall Materials for Food Encapsulation, in: 1995: pp. 42–50. doi:10.1021/bk-1995-0590.ch004.

[12] H. Fessi, F. Puisieux, J.P. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 55 (1989) R1–R4. doi:10.1016/0378-5173(89)90281-0.

[13] X. Tang, M.J. Pikal, Design of Freeze-Drying Processes for Pharmaceuticals: Practical Advice, Pharm. Res. 21 (2004) 191–200. doi:10.1023/B:PHAM.0000016234.73023.75.

[14] K. Pycia, L. Juszczak, D. Gałkowska, R. Socha, G. Jaworska, Maltodextrins from chemically modified starches. Production and characteristics, Starch/Staerke. 69 (2017). doi:10.1002/star.201600199.

[15] Y.H. Roos, M. Karel, J.L. Kokini, Glass transitions in low moisture and frozen foods: Effects on shelf life and quality, Food Technol. 50 (1996) 95–108.

[16] K. Pycia, L. Juszczak, D. Gałkowska, M. Witczak, G. Jaworska, Maltodextrins from chemically modified starches. Selected physicochemical properties, Carbohydr. Polym. 146 (2016) 301–309. doi:10.1016/j.carbpol.2016.03.057.

[17] N. Grabowski, H. Hillaireau, J. Vergnaud, N. Tsapis, M. Pallardy, S. Kerdine-Römer, E. Fattal, Surface coating mediates the toxicity of polymeric nanoparticles towards human-like macrophages, Int. J. Pharm. 482 (2015) 75–83. doi:10.1016/j.ijpharm.2014.11.042.

[18] V.R. Garipova, C.G.M. Gennari, F. Selmin, F. Cilurzo, R.I. Moustafine, Mucoadhesive interpolyelectrolyte complexes for the buccal delivery of clobetasol, Polymers (Basel). 10 (2018). doi:10.3390/polym10010085.

[19] K. Aeberhardt, J.Y. De Saint Laumer, P.E. Bouquerand, V. Normand, Ultrasonic wave spectroscopy study of sugar oligomers and polysaccharides in aqueous solutions: The hydration length concept, Int. J. Biol. Macromol. 36 (2005) 275–282. doi:10.1016/j.ijbiomac.2005.04.006.

[20] L. Yang, L.M. Zhang, Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources, Carbohydr. Polym. 76 (2009) 349–361. doi:10.1016/j.carbpol.2008.12.015.

[21] E.J.W. Verwey, J.T.G. Overbeek, Theory of the stability of lyophobic colloids, J. Colloid Sci. 10 (1955) 224–225. doi:10.1016/0095-8522(55)90030-1.

[22] B. Derjaguin, L. Landau, Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes, Prog. Surf. Sci. 43 (1993) 30–59. doi:10.1016/0079-6816(93)90013-L.

[23] H. Soini, M. Stefansson, M.L. Riekkola, M. V. Novotny, M.L. Riekkola, Maltooligosaccharides as Chiral Selectors for the Separation of Pharmaceuticals by Capillary Electrophoresis, Anal. Chem. 66 (1994) 3477–3484. doi:10.1021/ac00092a028.

[24] N. Castro, V. Durrieu, C. Raynaud, A. Rouilly, Influence of DE-value on the physicochemical properties of maltodextrin for melt extrusion processes, Carbohydr. Polym. 144 (2016) 464–473. doi:10.1016/j.carbpol.2016.03.004.

[25] H. Levine, L. Slade, A polymer physico-chemical approach to the study of commercial starch hydrolysis products (SHPs), Carbohydr. Polym. 6 (1986) 213–244. doi:10.1016/0144-8617(86)90021-4.

[26] S.K. Pansare, S.M. Patel, Practical Considerations for Determination of Glass Transition Temperature of a Maximally Freeze Concentrated Solution, AAPS PharmSciTech. 17 (2016) 805–819. doi:10.1208/s12249-016-0551-x.

[27] N. Harnkarnsujarit, S. Charoenrein, Y.H. Roos, Microstructure formation of maltodextrin and sugar matrices in freeze-dried systems, Carbohydr. Polym. 88 (2012) 734–742. doi:10.1016/j.carbpol.2012.01.028.

[28] S.L. Nail, L.A. Gatlin, Pharmaceutical Dosage Forms, in: S. Nema, J.D. Ludwing (Eds.), Pharm. Dos. Forms Parenter. Medicat., Third, Informa Healthcare, New York, USA, 2010: pp. 353–381.

[29] W. Abdelwahed, G. Degobert, S. Stainmesse, H. Fessi, Freeze-drying of nanoparticles: Formulation, process and storage considerations, Adv. Drug Deliv. Rev. 58 (2006) 1688–1713. doi:10.1016/j.addr.2006.09.017.

[30] J. Broadhead, S.K. Edmond Rouan, C.T. Rhodes, The spray drying of pharmaceuticals, Drug Dev. Ind. Pharm. 18 (1992) 1169–1206. doi:10.3109/03639049209046327.

Annex:

Optimization of spray-drying process using a Central Composite Design

Preliminary, a Central Composite Design (2^{5-1}; $2^{*}5$; n_C=2) was used to optimize the spray-drying condition. Since the weight ratio of NP/excipient was 1/20, it was decided to find the appropriate process input parameter ranges without using NP. Thereby, assuming that such low NP weight ratio would not significantly influence the output parameter, a 5% solution of MDX DE6 was spray-dried using a spray-drier Format 4 M8 (ProCepT, B) equipped by a two-fluid nozzle operating in a co-current manner, namely the sprayed product and the drying air flow are in the same direction

The factors include inlet temperature (T_{in}), feed flow rate (FFR), nozzle diameter (D_N), nozzle pressure (P_N) and the difference of pressure between drying chamber and cyclone (ΔP). Before the application of the design, a number of preliminary trials were conducted to determine the conditions at which the process resulted in a dried powder. The levels of each factor determined by this procedure are reported in **Table A1**. In particular, three levels were taken into account: level -1 was the lowest, level +1 was the highest and level 0 was the central level identified as the mean between the level -1 and +1.

Table A1 - Levels and values of the process factors [feed flow rate (FFR); inlet temperature (T_{in}), nozzle pressure (P_N) and diameter (D_N) and difference of pressure between cyclone and chamber (ΔP)] considered in the Central Composite Design.

Level	FFR (mL/min)	P_N (atm)	D_N (mm)	T_{in} (°C)	ΔP (atm)
-1	5.0	1.0	0.4	130	30
0	7.5	1.5	0.8	155	50
+1	10.0	2.0	1.2	180	70

During the optimization of a multivariable process, the responses desirability is combined in order to produce a product of desired characteristics. In the case of this study, the product yield, the span and the outlet temperature were considered the main dependent variables. In particular, the desirability goals were defined as following: the process yield should be maximized to avoid waste of material; the span should be minimized to have a narrow monomodal particle distribution; the outlet temperature should be lowered below 40 °C as a product temperature lower than the glass transition of PLGA would avoid the formation of aggregates. The matrix of the experiments (28 runs) is listed in **Table A2.**

The statistical evaluation of the results was carried out by one-way analysis of variance (ANOVA) and lack of fit analysis using a commercially available statistical software package (JMP Pro VERSION 13, SAS Institute, USA). Pareto charts were used to report the factors and the interaction between them that exert a statistical influence on the responses.

Results on optimization of spray-drying process

To limit the NP aggregation during the spray-drying, the outlet temperature should be lower than the glass transition of PLGA. At the same time, inadequate process conditions due to too low drying air or temperature can cause particles to adhere to the inside wall of the spray-dryer decreasing the powder yield. This is disadvantageous since it limits the amount of material available for powder-consuming tests [i]. Moreover, the experimental set-up dictates the particle size and size distribution, which is responsible for the main technological and biopharmaceutical feature of the powder [ii]. Indeed, it is well-known that not-agglomerated spherical particles with a narrow size distribution (monodispersed) are preferred for applications and technologies. Hence, the impact of the factor interrelation on the properties of the spray-dried powder was preliminarily elucidated by using a Design of Experiments (DoE) approach.

The statistical analysis evidenced the significance of the contribution of the process parameters on the selected responses. The equation describing the impact of factors on the yield is reported in **Eq. 1** (p=0.014, R^2=0.94):

$$Yield = 35.17 - 6.90\left(\frac{FFR - 7.5}{2.5}\right) - 4.80\left(\frac{D_N - 0.8}{0.4}\right) + 4.03\left(\frac{T_{in} - 155}{25}\right) + 3.81\left(\frac{P_N - 1.5}{0.5} \cdot \frac{D_N - 0.8}{0.4}\right) + 3.22\left(\frac{FFR - 7.5}{2.5} \cdot \frac{T_{in} - 155}{25}\right) - 3.81\left(\frac{D_N - 0.8}{0.4} \cdot \frac{\Delta P - 50}{20}\right)$$

Eq. (1)

This equation points out that the yield resulted negatively influenced by the feed flow rate (p<0.001) and D_N (p=0.006); whereas T_{in} had a positive effect (p=0.015). Moreover, the interaction between P_N and D_N (p=0.024), feed flow rate and T_{in} (p=0.046), and D_N and ΔP (p=0.024) resulted also significant. In another word, the product yield progressively increases on increasing T_{in} and decreasing the amount or the size of droplets to be dried.

Similar analyses performed on span and outlet temperature evidenced that pump speed is the most critical parameter among those investigated (p<0.05). In

addition, D_N improved significantly the span (p=0.033), whereas, as expected, T_{in} influenced positively (p=0.001) on the outlet temperature.

The combination of these results with the highest desirability (0.6595) permitted to optimize the experimental conditions:

- feed flow rate = 6.5 mL/min;
- nozzle pressure = 1.7 atm;
- nozzle diameter = 0.4 mm;
- T_{in} = 130 °C;
- ΔP = 70 mbar.

Table A2 - Experimental design matrix. The design matrix shows the input parameters set for spray drying [feed flow rate (FFR); inlet temperature (T_{in}), nozzle pressure (P_N) and diameter (D_N) and difference of pressure between cyclone and chamber (ΔP)] and the output parameters that were experimentally determined [(powder yield, span, outlet temperature (T_{out})].

Exp. no.	Pattern	FFR (mL/min)	P_N (atm)	D_N (mm)	T_{in} (°C)	ΔP (atm)	Yield %	Span	T_{out}
1	−+−+−	5.0	2.0	0.4	180	30	31.50	2.9	56.5
2	+−−+−	10.0	1.0	0.4	180	30	30.75	3.1	49.6
3	−++++	5.0	2.0	1.2	180	70	33.25	3.1	43.8
4	−++−−	5.0	2.0	1.2	130	30	35.00	3.4	39.7
5	-0000	5.0	1.5	0.8	155	50	34.00	4.4	57.0
6	−−+−+	5.0	1.0	1.2	130	70	11.50	5.4	34.0
7	+−+−−	10.0	1.0	1.2	130	30	9.50	2.8	39.6
8	0	7.5	1.5	0.8	155	50	33.75	1.8	45.7
9	0000+	7.5	1.5	0.8	155	70	37.00	2.1	39.1
10	−−−++	5.0	1.0	0.4	180	70	40.25	1.9	51.2
11	++−−−	10.0	2.0	04	130	30	3.50	1.5	47.2
12	00+00	7.5	1.5	1.2	155	50	19.25	3.4	38.0
13	++−++	10.0	2.0	0.4	180	70	28.50	2.3	37.2
14	−−++−	5.0	1.0	1.2	180	30	26.75	6.0	57.9
15	0	7.5	1.5	0.8	155	50	36.50	3.4	51.9
16	0+000	7.5	2.0	0.8	155	50	35.50	4.2	52.7
17	+−+++	10.0	1.0	1.2	180	70	9.25	2.0	37.0
18	000-0	7.5	1.5	0.8	130	50	30.75	3.6	40.1
19	0000-	7.5	1.5	0.8	155	30	36.25	2.3	49.0
20	+−−−+	10.0	1.0	0.4	130	70	14.75	2.3	33.8
21	−−−−−	5.0	1.0	0.4	130	30	35.00	3.1	49.5
22	000+0	7.5	1.5	0.8	180	50	33.25	4.7	54.2
23	+++−+	10.0	2.0	1.2	130	70	6.75	1.9	35.5
24	0-000	7.5	1.0	0.8	155	50	30.00	3.8	46.8
25	00-00	7.5	1.5	0.4	155	50	39.25	3.5	51.0
26	++++−	10.0	2.0	1.2	180	30	26.75	3.6	56.5
27	+0000	10.0	1.5	0.8	155	50	34.25	3.6	47.0
28	−+−−+	5.0	2.0	0.4	130	70	41.00	1.6	37.8

Annex references

[i] Y.F. Maa, P.A. Nguyen, K. Sit, C. Hsu, Spray drying performance of a bench-top spray dryer for protein aerosol powder preparation, Biotech. Bioeng. 60 (1998) 301-309. doi: 10.1002/(SICI)1097-0290(19981105)60:3<301::AID-BIT5>3.0.CO;2-L.

[ii] J. Elversson, A. Millqvist-Fureby, Particle size and density in spray drying—effects of carbohydrate properties. J. Pharm. Sci. 94 (2005) 2049–2060. doi: 10.1002/jps.20418.

Chapter 3

Feasibility study of novel biodegradable DegraPol® microparticles for embolization application

Abstract

Spherically-shaped particles represent the most recent device proposed for the (chemo)embolization. However, there is need of novel biocompatible polymeric material with suitable properties. DegraPol® is a class of biocompatible and biodegradable poly(ester-urethane) polymers currently used for preparing scaffolds for tissue engineering, given the good ability to form fibers, the stability upon Et_2O sterilization and the ability to encapsulate small and complex molecules within the fibers' matrix. This work aimed to exploit the feasibility of prepare spherical-shaped DegraPol® particles with a size ranging from nano- (*i.e.* 100-300 nm) to micrometric scale (*i.e.* 1-10 µm and 30-40 µm). Among the methods considered, only the emulsion/solvent evaporation one was proven to be feasible. Poly(vinyl alcohol) and cellulose derivatives were suitable emulsifier/stabilizer for emulsifying DegraPol® solution, while the equipment used allowed to determine the size of the droplets of the dispersed phase and, hence, the dimension of the particles obtained. Together these results demonstrated the feasible application of DegraPol® as a novel material in the preparation of spherically-shaped particles for embolization but also as drug delivery carrier, particularly in the case of nanoparticles.

3.1 Introduction

Trans-arterial embolization (TAE) concerns the transient or permanent block of the blood provision to a specific and defined area of the body. Focusing on applications in tumors' treatment, chemoembolization (TACE) also combines the local delivery of a chemotherapeutic agent when needed [1,2].These techniques have been extensively studied as suitable loco-regional approaches mainly for treating confined tumors, *i.e.,* hepatocellular carcinoma, juvenile angiofibromas [3,4] or arteriovenous malformations [5] or acute hemorrhage of varying etiology [6]. Different embolic agents have been proposed over the past years, such as liquids, coils and particulate systems both spherical and non-spherical [6]. Among them, polymeric spherical-shaped microparticles, namely microspheres, are the most recent agent proposed, paving the way for better targeted embolization, inducing a temporary or a permanent occlusion. Even though the nature of the material plays an important role, size and shape of the particles represent critical factors as well. Indeed, spherical and smooth-surface particles with a precisely controlled sized are the optimal features to avoid particles aggregation, assuring an easy injectability through a micro-catheter, a targeted and predictable artery occlusion [7]. Both natural and synthetic polymers have been used, such as gelatine, starch and chitosan for the former and poly(vinyl alcohol) [PVA] or poly(lactide-co-glycolide) [PLGA] for the latter [6]. PLGA has seen widely applications in the pharmaceutical field, due to the possibility of tailoring its mechanical, thermal and degradation properties as well as the processability into different shaped-objects [8–10]. However, PLGA has some technological drawbacks, including the difficult encapsulation of hydrophilic compounds in spherical shaped micro- and nano-particles [11,12], the limited stability upon sterilization (*e.g.,* vapor stream) [13] and the drop off in pH medium upon degradation [14]. Hence, the need to overcome these issues or to find new alterative materials are still unmet. A class of poly(ester-urethanes), collectively traded with the name of DegraPol® (DP), has demonstrated appealing features for applications in the biomedical area. They are a phase segregated multiblock

copolymers prepared by the poly-addition of two macrodiols, using a diisocyanate (*e.g.,* lysinmethylester-diisocyanate or 2,2,4- trimethyl-hexamethylene-diisocyanate). The macrodiol present in all the grades of DegraPol® is the crystalline *hard segment*, composed of α,ω-dihydroxy-oligo[((R)-3-hydroxy-butyrate-co-(R)-3-hydroxyvalerate)-block-ethylene glycol]) (PHB/HV). It reacts with a second macrodiol, referred to as *soft segment*, that is amorphous and with a variable poly(ester diol) composition, including *e.g.* ethylene glycol, ε–caprolactone or di-glycolide [15,16]. The mechanical properties of such materials are dominated by the amount and characteristics of the *hard segment*, with an elastic modulus that can range from 30 to 1.2 GPa [17]. In other words, increasing the fraction of the *non-crystallizable segment*, the elongation at break decreases while the tensile strength increases. On contrary, the chemical nature of the esters in the *soft segmen*t regulates the degradation rate in an independent manner, ranging from weeks to years [18,19]. It is noteworthy that degradation of these polymers is not only independent from the mechanical properties but also from the pH of the medium in which the material is dispersed [19]. Unlike what happen in the case of PLGA, upon degradation of DegraPol® film the pH decreased only from 7.2±0.1 to 5.9±0.2 within the first 6 days of study, and then remained constant over more than a one month-period [20].

Data available in literature about DegraPol® family also demonstrated optimal interactions with different cell types and biological environments as 2D scaffolds prepared by electrospinning favored the adhesion, proliferation and differentiation of skeletal muscle cells [21], bladder endothelial cells [22] and also acted as good guidance channel for nerve regeneration [23]. And again, highly porous DegraPol® 3D foam scaffolds obtained by a congealing-dipping-precipitation process resulted suitable in supporting the formation and proliferation of smooth muscle and bone tissues [24,25]. All these applications, strongly confirmed the biocompatibility of DegraPol® polymers and their degradation byproducts [26,27]. The loading of model active ingredients from small molecules as fluorescein (FITC) to more complex one as fluorescein

isothiocyanate-conjugated bovine serum albumin (FITC-BSA) or a platelet-derived growth factor(PDGF-BB) has been demonstrated for electrospun nanofibers. FITC release was mainly characterized by burst-mediated release, reaching almost 95% cumulative release after 2 days, while for FITC-BSA less than 20% was released after 24h, followed by a gradual increment at the later time points [28]. The *in vitro* release of PDGF-BB was similar to the one of FITC-BSA, *i.e.* a more sustained, rather than a burst release exhibited for FITC [28].

Considering that these objects are implantable systems, they must meet the sterility requirement. DegraPol® raw material and scaffolds can be sterilized by treatment with ethylene oxide without causing any detrimental effects [29].

Despite these materials show appropriate properties, poly(ester urethanes) have never been used for the preparation of spherical shaped particles suitable for embolization applications or, in a future perspective, as a carrier for designing drug delivery systems. However, since particles are usually prepared in aqueous environment, the engineering of DegraPol® was expected to be complicated due to the tendency of the material to form fibers and its low z-potential value which in turn can promote the aggregation of the particles.

Hence, the feasibility of prepare spherical shaped DegraPol® particles with a size distribution ranging from nano- to micrometric scale was explored. In particular, three main target sizes were selected, *i.e.* 100-300 nm, 1-10 µm and 30-40 µm, with low dispersity indexes. The suitability of solvent displacement methods, emulsion/solvent evaporation methods and spray-drying was studied.

3.2 Materials

Three different grades of DegraPol® were kindly provided by ABmedica:

- DP4: DP4/PC1250/60/B1/20141118; batch B1.1 (10 g);
- DP15: DP15/PC1000/25/B1/20141111; batch B1.1 (15 g);
- DP30: DP30/PC1250/40/B1/20110329; batch B1.9 (15 g).

The following materials were purchased and used as such:

- Methocel® E50p LV (HPMC E50) (hydroxypropyl methylcellulose substitution: % methoxyl 28-30; % hydroxylpropyl 7-12; nominal viscosity 2% in water at 20°C: 50 cP, Colorcon, I);
- Methocel® K100 LV (HPMC K100) (hydroxypropyl methylcellulose substitution: % methoxyl 19-24; % hydroxylpropyl 7-12; nominal viscosity 2% in water at 20°C: 80–120 cP, Colorcon, I);
- poly(vinyl alcohol) 10-98 (PVA) with a molecular weight of about 61 KDa and a degree of hydrolysis of 98.0-98.8 mol % (Fluka Chemika, I);
- poly(vinyl alcohol) fully hydrolysed (Sigma Aldrich, I);
- poly(vinyl alcohol) 56-98 (PVA) with a molecular weight of about 195 KDa and a degree of hydrolysis of 98.0-98.8 mol % (Fluka Chemika, I);
- Tween® 20 (Sigma-Aldrich, I);
- Lutrol F127 (poloxamer 407, BASF, G);

All solvents used were of analytical grade, unless specified.

3.3 Methods

3.3.1 Preparation of nanoparticles (NP)

3.3.1.1 Method 1: solvent displacement method

Preliminary, the solubility of DP4, DP15 and DP30 in different water miscible organic solvents was evaluated. The concentration was fixed at 10% w/v. The solvents and the solvents mixtures tested were the follow:

- tetrahydrofuran (THF);
- acetone (ACE);
- acetonitrile;
- dimethyl sulfoxide (DMSO);
- absolute ethanol (EtOH);
- dimethylformamide (DMF);
- ACE/EtOH in the ratios of 7:3 and 6:4;
- THF/EtOH in the ratios of 7:3 and 8:2;
- THF/DMSO in the ratios of 5:5, 6:4, 7:3, 8:2 and 9:1.

DP15 dissolved in DMF (1% w/v) and DP30 solution in 8:2 THF/DMSO (1% w/v) were added dropwise to 10 mL of MilliQ® water filtered at 0.22 µm with a nylon syringe filter (VWR International, I), at 4 °C.

DP4 dissolved in 8:2 THF/DMSO (0.5% w/v) was added dropwise to 10 mL of different stabilizers aqueous solutions. The system was stirred at 500 rpm, at different temperature (**Table 3.1**).

The organic solvent was evaporated over 3 h of stirring at 25 °C.

Table 3.1 - Main process and formulation parameters to prepare nanoparticles by solvent displacement method from 0.5% w/v DP4 solution in THF/DMSO in the ratio of 8:2.

Formulation ID	Stabilizer		T aqueous phase (°C)
	Type	Concentration (% w/v)	
P1	PVA 56-98	0.05	4
P2	PVA fully hydrolysed	0.05	4
P3	Poloxamer 407	0.05	7
P4	Poloxamer 407	0.1	4
P5	Poloxamer 407	0.1	10

3.3.1.2 Method 2a: emulsion/solvent evaporation method

The emulsion/solvent evaporation method was used to prepare NP and the optimization of the process parameters was performed using DP4 as polymer. Briefly, DP4 was dissolved overnight in DCM at the concentration of 1 % w/v (phase O) and an aliquot of 1 mL was emulsified with PVA 10-98 aqueous solution (phase W) in different concentrations and ratios (**Table 3.2**) in an ice bath, using an ultrasound titanium probe with a diameter of 7 mm (UP200st, Hielscher, G). The O/W emulsion was then poured in 7 mL HPLC-grade water or PVA 10-98 solution under magnetic stirring of 600 rpm (**Table 3.2**). The particles hardening occurred after the evaporation of the organic solvent over 3 h at 25 °C.

The process parameters reported for formulation F3 were also applied for the preparation of DP15 and DP30 NP (F10 and F11, **Table 3.2**). Moreover, DP30 NP were washed by centrifugation at 5000 rpm for 15 min at 10 °C for three times and resuspended in MilliQ® water filtered at 0.22 µm with a nylon syringe filter (VWR International, I).

After the preparation, the obtained dispersions were visually observed to assess the lack of macroscopic aggregates or film. The suitable nanosuspensions were characterized by DLS analyses in terms of size and polydispersity index that were selected as quality parameters.

Table 3.2 - Experimental parameters and formulation compositions used to prepare DP4 nanoparticles by the emulsion/solvent evaporation method.

Formulation ID	Phase O (% w/v)			Phase W (% w/w)	O/W ratio (v/v)	Sonication		Dilution	
	DP4	DP15	DP30			A %	time (s)	PVA (% w/w)	rpm
F1	1	-	-	5	1:2	20	60	0.1	600
F2	1	-	-	5	1:2	30	60	0.1	600
F3	1	-	-	5	1:2	40	60	0.1	600
F4	1	-	-	5	1:2	50	60	0.1	600
F5	1	-	-	5	1:2	40	90	0.1	600
F6	1	-	-	5	1:2	40	120	0.1	600
F7	1	-	-	5	1:3	40	60	0.1	600
F8	1	-	-	3	1:2	40	60	0.1	600
F9	1	-	-	5	1:2	40	60	0	600
F10	-	1	-	5	1:2	40	60	0.1	600
F11	-	-	1	5	1:2	40	60	0.1	600

3.3.2 Preparation of submicron-particles (sMP)

3.3.2.1 Method 2b: emulsion/solvent evaporation method

A DP30 solution in DCM at the concentration of 1 % w/v was emulsified with an aqueous solution of cellulose derivatives (HPMC K100 or HPMC E50) at different concentrations (phase W, **Table 3.3**) using the ultrasound titanium probe (UP200st, Hielscher, G) at the amplitude of 40 % over 1 min. The volume ratio between the aqueous and organic phases was set at 1:2. Then, the emulsion was diluted with 7 mL MilliQ® water solution under magnetic stirring of 600 rpm (**Table 3.3**). The particles hardening occurred after the evaporation of the organic solvent over 3 h at 25 °C.

Table 3.3 - Experimental parameters and formulation compositions used to prepare submicron-particles made of DP30 by the emulsion/solvent evaporation method.

ID	Emulsion				Sonication	
	Phase O	Phase W (% w/v)		O/W	A	time
	(% w/v)	HPMC K100	HPMC E50	(v/v)	%	(s)
F12	1	0.5	-	20	40	60
F13	1	1.0	-	30	40	60
F14	1	2.0	-	40	40	60
F15	1	-	0.5	50	40	60
F16	1	-	1.0	40	40	60
F17	1	-	2.0	40	40	60

3.3.3 Preparation of microparticles (MP)

3.3.3.1 Method 2c: emulsion/solvent evaporation method

Three different approaches were investigated for the preparation of microparticles (MP) by the emulsion/solvent evaporation method, as follow.

Approach #1

An aliquot of 1 mL of DP15 solution in DCM at 5 or 10% w/v (phase O) was added dropwise with a glass syringe into 200 mL of HPMC K100 solution (phase W) at 4 °C under constant stirring of the propeller (**Table 3.4**). The hardening of the MP was achieved following the thermal cycle reported:

- 15 min at 4 °C;
- 15 min at 10 °C;
- 120 min at 25 °C;
- 60 min at 30 °C.

The particle size was evaluated visualizing a sample of the MP under a light microscope (Carl Zeiss Axiolab E optical transmission microscope, G).

Table 3.4 - Experimental parameters and formulations compositions used to prepare DP15 microparticles by emulsion/solvent evaporation method (approach #1).

ID	Phase O (% w/v)	Phase W (% w/v)	O/W ratio (v/v)	Stirring rate (rpm)
F18	10	0.2	1:200	600
F19	10	0.4	1:200	600
F20	5	0.2	1:200	400
F21	5	0.2	1:200	600
F22	5	0.2	1:200	800

Approach #2

An aliquot of 2 mL of a DP15 solution in DCM (phase O) was emulsified with 25 mL HPMC K100 solution (phase W) using a homogenizer (Ultra-Turrax® T25, IKA, G) at 4 °C, according to the compositions and the process parameters reported in **Table 3.5**. The resulting O/W emulsion was transferred into 250 mL of HPLC-grade water maintained at 4 °C. The organic solvent was evaporated following the thermal cycle reported in Approach #1. The microparticles suspension was finally cooled at 15 °C for 60 min and then recovered by filtration under vacuum with a 1.2 µm pores nitrocellulose filter (Millipore, Milan, I) and washed with MilliQ® water.
The recovered MP were suspended in 0.1 % Tween® 20 and stored at 5 ± 3 °C until the determination of particle size distribution.

Approach #3

An aliquot of 2 mL of DP15 at the concentration of 5% w/v (phase O) was emulsified with HPMC K100 solution (phase W) at 4 °C according to ratio and process parameters reported in **Table 3.6**. The O/W emulsion was transferred into 250 mL of HPLC-grade water or 0.1% HPMC K100 solution at 4 °C (**Table 3.6**). DCM was evaporated following the thermal cycle reported for approach #1 and MP recovered by filtration. MP were suspended in 0.1% Tween® 20 and stored at 5 ± 3 °C until the determination of particle size distribution.

Table 3.5 - Experimental parameters and formulations compositions used to prepare DP15 microparticles by the solvent/evaporation method (approach #2).

ID	Emulsion					Dilution	Final HPMC conc. (% w/v)
	Phase O (% w/v)	Phase W (% w/v)	O/W (v/v)	Speed (rpm)	Time (min)	Speed (rpm)	
F23	10	1.0	1:12.5	9500	5	500	0.09
F24	10	1.0	1:12.5	9500	2	500	0.09
F25	10	1.0	1:12.5	8000	2	500	0.09
F26	10	2.5	1:12.5	8000	2	500	0.23
F27	5	2.5	1:12.5	9500	2	250	0.23
F28	5	2.5	1:12.5	9500	2	500	0.23

Table 3.6 - Experimental parameters and formulation compositions used to prepare DP15 microparticles by emulsion/solvent evaporation method (approach #3).

ID	Emulsification					Dilution		Final HPMC conc. (% w/v)
	Phase O (% w/v)	Phase W (% w/v)	O/W ratio (v/v)	Stirring (rpm)	Time (min)	Stirring (rpm)	HPMC conc. (% w/v)	
F29	5	2.0	1:25	100	3	250	--	0.33
F30	5	2.5	1:25	100	3	250	--	0.42
F31	5	2.5	1:25	100	3	250	0.1	0.50
F32	5	2.5	1:20	150	3	250	0.1	0.43
F33a	5	2.5	1:15	100	3	250	0.1	0.36
F33b	5	2.5	1:15	100	3	250	0.1	0.36
F33c	5	2.5	1:15	100	3	250	0.1	0.36
F34	5	2.5	1:15	150	3	250	0.1	0.36
F35	5	2.5	1:15	250	3	250	0.1	0.36

3.3.3.2 Method 3: spray-drying

The feed was obtained by dissolving DP15 or DP30 in DCM or chloroform, according to the composition reported in **Table 3.7**.

Table 3.7 - Experimental parameters and feed compositions for the preparation of DP microparticles by spray-drying.

ID	Polymer concentration (%)		Solvent	T_{in} (°C)	Nozzle pressure (atm)
	DP15	DP30			
SD1	2	--	DCM	60	1.5
SD2	4	--	DCM	55	2
SD3	2	--	chloroform	50	2
SD4	--	2	DCM	50	2

Process parameters of the spray-drier (Format 4 M8, ProCepT, B):

- nozzle diameter: 4 mm;
- pump speed: 5 mL/min;
- difference of pressure between the drying chamber and the cyclone: 70 mbar;
- inlet air speed: 0.3 m^3/min;

3.3.4 Nanoparticles characterization

The size distribution of the nanosuspension was evaluated by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK) equipped with a backscattered light detector, operating at 173°. The instrument uses a 4 mW He-Ne laser (λ = 632.8 nm) with backscatter detection at 173° and a thermostated sample chamber set to 25 °C. The Zetasizer Nano ZS automatically adjusted the attenuator setting to optimize the amount of light scattered by a sample. The results, calculated using the Dispersion Technology Software (DTS, Malvern Instruments Ltd., Worcestershire, UK), were reported as intensity distribution.

The nanosuspension zeta potential was determined by M3-PALS (Phase Analysis Light Scattering) technique at 25 °C, using the same equipment reported for the size measurement. The nanosuspensions were characterized before and after the centrifugation step.

3.3.5 Submicron-particles and microparticles characterization

After the preparation, sMP and MP were characterized in terms of particle size by using an Accusizer 770 (PSS Inc. USA). This instrument employs a Single Particle Optical Sensing (SPOS), namely a light scattering and light extinction dual detection system that allows for single particle sizing in the range from 0.5 to 400 μm. In the SPOS technique, particles in liquid suspension flow through a photozone - a narrow and rectangular region of uniform light produced by light from a laser. As a particle passes through the photozone, light is either absorbed or refracted due to the physical presence of the particle or it can be scattered at some oblique angle. The magnitude of this pulse is dependent on the cross-sectional area of the particle and the physical principle of detection. As each successive particle passes through the sensor, a particle size distribution is created by comparing the detected pulse heights with a standard calibration curve, obtained from a set of uniform particles of known diameters.

Particle size was expressed as undersize cumulative percentage of the volume distribution and the population dispersity was referred as span and calculated as reported in the following equation:

$$span = \frac{d_{90} - d_{10}}{d_{50}}$$

where d_{10}, d_{50} and d_{90} represent the mean diameters at the 10%, 50% and 90% of the distribution, respectively.

3.4 Results and discussion

3.4.1 Preparation of nanoparticles

The application of the solvent displacement method required the solubilisation of the polymer in a water miscible or partly miscible solvent(s) [11]. Hence, preliminarily the solubility of DP4, DP15 and DP30 at the concentration of 1% w/v in different organic solvents or their mixtures were assessed. Among all the solvents tested, DP4 and DP30 resulted partial soluble in THF/DMSO 8:2, while DP15 was soluble in DMF at the prefixed concentration. Unfortunately, when the solution of DP15 in DMF was added dropwise to MilliQ® water under magnetic stirring, part of the polymer quickly aggregated around the stirrer. However, the DLS analysis revealed the formation of NP in the range of 112±1 nm with an acceptable monomodal distribution (PDI=0.083 ± 0.008) and a measured zeta potential of -24.9 ± 0.8 mV. This value falls in the instability range of colloidal systems and it may be related to the formation of macroscopic aggregates on the magnetic stirrer.

When the solution of DP30 in THF/DMSO 8:2 was added dropwise to MilliQ® at 4 °C and 20 °C under magnetic stirring, the system presented a great instability and visible aggregates were evident independently of the temperature. To avoid this issue, the concentration of DP4 solution in THF/DMSO 8:2 was reduced to 0.5% w/v and the aqueous phase was stabilized with different polymers according to the compositions reported in **Table 3.1**. However, despite the presence of a stabilizer, a significant amount of the polymer still aggregated and it was not possible to perform the DLS analysis.

Thanks to the great solubility of the different grades of DP in a water immiscible solvent, such as dichloromethane, the possibility of preparing nanoparticles by an emulsion/solvent evaporation method was also investigated. The formulation and process parameters considered to influence the final size of the particles are summarized in the *Ishikawa* diagram, reported in **Fig. 3.1**. However, the most critical have been considered the ones occurring in the emulsification step, namely the choice of the appropriate stabilizer, the aqueous and the organic phases ratio

and the ultrasound probe setting. Hence, the optimization of such parameters was performed starting from solutions of DP4 in DCM (1% w/v). The main features of the nanoparticles prepared are summarized in **Table 3.8**. The stabilizer selected for their preparation was the poly(vinyl alcohol), since it is the most popular used in the case of PLGA NP [30].

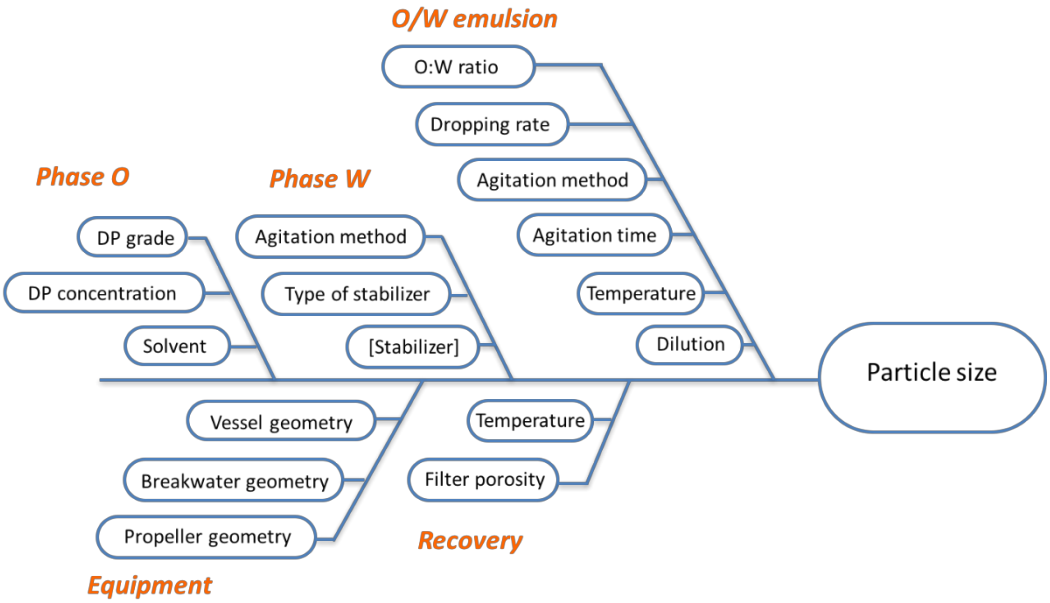

Fig. 3.1 - Ishikawa diagram on the main formulation and process parameters affecting the final size of the particles prepared by the emulsion/solvent evaporation method.

Table 3.8 - Effect of process parameters on size (D_H), size distribution (PDI) and zeta potential (ζ) of DP4 NP obtained by method 2a (refer to **Table 3.2** for experimental details).

ID	D_H (nm)	PDI	Visible aggregates
F1	549±16	0.152±0.011	+++
F2	411±10	0.072±0.036	++
F3	450±9	0.127±0.019	-
F4	496±17	0.165±0.008	+++
F5	482±8	0.136±0.040	-
F6	451±10	0.132±0.028	+
F7	581±13	0.086±0.023	-
F8	557±44*	0.255±0.011	++++
F9	403±9	0.081±0.013	++

*The peak corresponded to 96% of the population.

Regarding the emulsification step, ultrasounds were used, in the attempt to produce an emulsion with very small oily droplets and, hence, to obtain nanometric particles after the solvent evaporation. Increasing the sonication amplitudes from 20 to 40% for 60 s, the hydrodynamic diameter of the nanoparticles decreased of about 100 nm (F1 and F3), while the increase in amplitude (F4) did not allow a further reduction in size. Considering an amplitude of 40% as necessary to prepare a stable emulsion, the time of sonication was increased in order to understand if it was possible to prepare particles with a smaller size. Again, this variation did not significantly influence the particle size distribution (F5) and, the overcoming of 90 s as sonication time caused a partial aggregation of the polymer (F6). In the same way, a reduction in the PVA concentration (F8) or the use of only water as emulsion dilution phase (F9) caused a massive aggregation of DP. Meanwhile, increasing the volume ratio between the solution of DP (phase O) and 5% PVA (phase W) from 1:2 to 1:3, a significant enlargement of the D_H was detected, probably because a higher amount of PVA remained adsorbed on the nanoparticle surface (F7).

The formulation and process parameters applied for the preparation of formulation F3 were considered the best conditions, since no polymer aggregation occurred and the hydrodynamic diameter of the particles fell within the target range. For these reasons, they were also used for the preparation of NP made of DP15 and DP30. Interestingly, the different grades of DP did not particularly affect the feature of the nano-system, such as the polydispersivity or the formation of macroscopic aggregates. However, bigger particles were obtained when DP15 was used (F10, **Table 3.9**), with the respect of NP prepared with DP4 or DP30 (F3 and F11, respectively).

The zeta potential (ζ) values of formulations F3, F10 and F11 was near the neutrality (-0.92±0.01 mV for F3) because of the presence of PVA adsorbed on the nanoparticles surface that prevents their aggregation. In the attempt to better understand the influence of PVA on the hydrodynamic diameter of the nanoparticles, a series of washing was carried out to eliminate most of the PVA adsorbed. In particular, formulation F11 was centrifuged at 5000 rpm, 10 °C for 15 min, the supernatant was removed and NP were washed with MilliQ®. These operations were repeated three times. The decrease of the values of size and zeta potential was due the removal of PVA, highlighting the issue related to the removal of PVA adsorbed on nanoparticle surface (F11w, **Table 3.9**) [31].

Table 3.9 - Hydrodynamic diameter (D_H), polydispersity index (PDI) and zeta potential (ζ) of NP made of DP15 and DP30 (refer to experimental set-up of F3 in **Table 3.2**).

ID	Polymer	D_H (nm)	PDI	ζ (mV)
F10	DP15	535±5	0.160±0.035	-0.45±0.01
F11	DP30	484±16	0.098±0.026	-0.98±0.11
F11w*	DP30	279±2	0.067±0.024	-4.92±0.42

*Three washings were carried out to remove PVA adsorbed on NP surface.

3.4.2 Preparation of submicron-particles

For the preparation of particles with a size ranging from 1 to 10 µm, the emulsion/solvent evaporation method was considered the most suitable technique. Submicron-particles (sMP) made of DP30 were obtained by two different grades HPMC, successfully used as emulsifying agent in the preparation of PLGA microparticles by the emulsion/solvent evaporation method [32]. The results of size and size distribution are reported in **Table 3.10**.

Table 3.10 - Effect of process and formulation variables on size and size distribution of DP30 sMP, obtained by method 2b (refer to **Table 3.3** for experimental details).

ID	d_{10} (µm)	d_{50} (µm)	d_{90} (µm)	Span
F12	0.60	0.88	1.22	0.70
F13	0.57	0.79	0.98	0.52
F14	0.57	0.81	0.98	0.58
F15	0.60	0.88	1.29	0.78
F16	0.67	1.04	2.21	1.48
F17	0.61	0.89	1.20	0.76

No aggregates were visually detected in all formulations. When HPMC K100 was used to stabilize the water phase, the higher the concentration, the lower the particle size of the sMP, probably due to a better stabilization of the oily phase in the emulsion (**Table 3.10**). On contrary, the lower viscosity of HPMC E50 solution did not allow to have a satisfactory control on size and particle size distribution since there were no relationship between its concentration and the size of the particles (**Table 3.10**).

3.4.3 Preparation of microparticles

Considering the potential application of DegraPol® particles as embolic agent, the target mean diameter of microparticles made of DP15 was fixed in the range between 30 and 40 µm. To pursue this aim, three different approaches was investigated for emulsion preparation in the case of the emulsion/solvent evaporation method. Furthermore, the spray-drying was also investigated.

Preliminary, a 2% HPMC K100 solution and 1% DP30 solution in DCM at the volume ratio of 1:2 were emulsified by magnetic stirring at 1200 rpm. Indeed, the particle size distribution analysis revealed that the d_{10}, d_{50}, d_{90} and the mean diameter (volume distribution) were 3.74 µm, 9.99 µm, 19.10 µm and 10.77 µm, respectively.

Considering the well-known relation between the dimensions of the DCM globules dispersed in the emulsion and the hardened particles, it was necessary to prepare a coarser emulsion for obtaining bigger hardened particles [33].

Following the approach #1, MP were prepared by adding dropwise with a glass syringe the organic phase into 200 mL of HPMC K100 at different concentrations under constant stirring of the propeller (**Table 3.4**).

Despite the variation of DP15 and HPMC concentrations and rotation speed of the propeller, the MP obtained resulted too polydispersed in term of particles size since they presented a dimension that ranged between few microns and 250 µm, upon visualization with an optical transmission microscope (**Fig. 3.2**). This feature can be ascribed to an ineffective emulsification step that led to the formation of polydispersed globules and, consequently, solid particles.

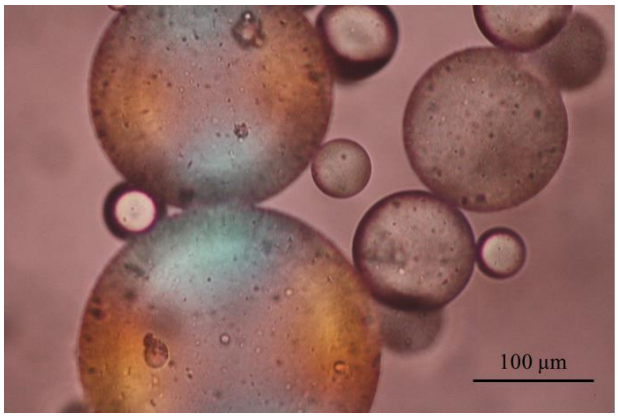

Fig. 3.2 - Light microscopy image of microparticles obtained by the emulsion/solvent evaporation method, approach #1.

On the other hand, the use of a homogenizer (approach #2, **Table 3.11**) led to the formation of the particles having a diameter smaller than the diameter target (30 – 40 µm), independently of the process parameters set. Formulation F23, F24 and F25 presented a bimodal size distribution, although the variation of parameters in the emulsification step. The reduction of DP concentration and the increase of HPMC concentration allowed dramatically reducing the bimodal shape of the distribution.

Table 3.11 - Effect of process and formulation variables on size and size distribution of DP15 MP obtained by approach #2 - method 2c (refer to **Table 3.5** for experimental details).

ID	d_{10} (µm)	d_{50} (µm)	d_{90} (µm)	Mean diameter (µm)	Span
F23	2.33	5.10	10.55	5.79	1.61
F24	2.49	5.52	13.09	6.98	1.92
F25	2.68	5.31	11.13	6.41	1.59
F26	1.65	4.69	7.23	4.59	1.19
F27	1.51	4.92	13.09	6.38	2.32
F28	0.98	2.16	3.99	2.36	1.39

To obtain a coarse emulsion and, subsequently, microparticles with the desired size, the organic and the aqueous phases (phase O and phase W) were emulsified by mechanical stirring (approach #3).

To collect MP, it is mandatory to pour the O/W emulsion into an aqueous phase stabilized by 0.1 % HPMC. The recovered MP were easily dispersible after a short period of sonication (F31, **Table 3.12**). Unfortunately, after the emulsification step, a film strictly adhering to the propeller was formed, leading to the formation polymer fibers visible under light microscopy (**Fig. 3.3**).

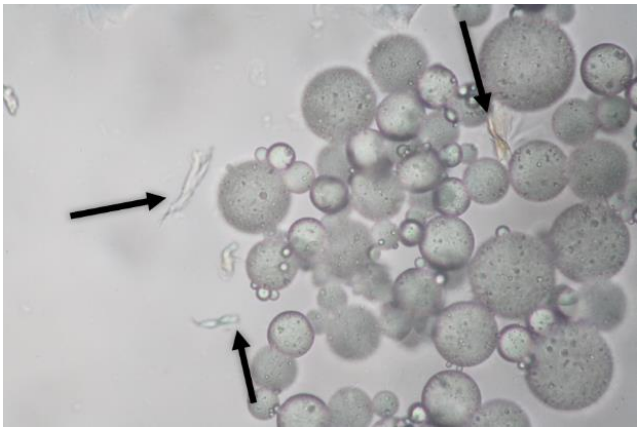

Fig. 3.3 - Light microscopy image of DP microparticles with polymer fibers (indicated by the arrows) formed after the emulsification step.

In order to avoid the polymer aggregation, in formulations F32-F35, the effect of the volume of phase W and the rotation speed of the propeller were investigated to improve the homogeneity of the emulsion. These experiments permitted also to individuate the temperature of emulsion formation as a critical point to avoid the formation of polymer aggregates, such as film or fibers. Indeed, the temperature should not exceed 4 °C. Moreover, the increment of the propeller rotation speed up to 250 rpm (F35) improved the homogeneity of the emulsion, without a significant variation of the particles size. The formulation F35 was prepared in duplicate (F35-1 and F35-2, **Table 3.13**), confirming the reproducibility of the result.

Table 3.12 - Effect of process and formulation variables on size and size distribution of DP15 MP obtained by approach #3 - method 2c (refer to **Table 3.6** for experimental details).

ID	d_{10} (µm)	d_{50} (µm)	d_{90} (µm)	Mean diameter (µm)	Span
F29	n.d.	n.d.	n.d.	n.d.	n.d.
F30	3.40	9.96	20.16	10.14	1.68
F31	21.28	39.74	59.32	40.28	0.96
F32	22.80	42.91	62.61	43.52	0.93
F33a	18.60	38.52	62.61	39.78	1.14
F33b	22.46	42.91	62.61	43.21	0.94
F33c	17.91	34.58	53.25	34.84	1.02
F34	22.46	42.01	56.20	41.13	0.80
F35	13.96	32.76	52.53	33.41	1.18

Table 3.13 - Particle size distribution of F35 prepared in duplicate. The diameters refer to the cumulative undersize volume distribution and the results are reported as mean ± standard deviation (n=3).

ID	d_{10} (µm)	d_{50} (µm)	d_{90} (µm)	Mean diameter (µm)	Span
F35-1	14.4±1.3	32.1±1.9	49.2±2.5	32.5±1.9	1.09±0.03
F35-2	13.7±2.5	34.3±1.5	57.1±5.1	35.4±2.3	1.26±0.16

A further investigation was carried out in the attempt to prepare DP microparticles by spray-drying. **Table 3.14** reports the outlet temperatures (T_{out}) recorded during the processes.

Table 3.14 - Outlet temperatures recorded during the time required to spray-dry 20 mL of the feed. The results are reported as mean ± standard deviation (refer to **Table 3.7** for experimental details).

ID	T_{out} (°C)	
	Chamber	**Cyclone**
SD1	33.2±0.3	28.7±0.2
SD2	31.7±0.5	27.6±0.2
SD3	28.3±0.5	28.9±0.1
SD4	29.5±0.2	26.1±0.5

Preliminary, it was verified that a 2% DP solution in DCM can be sprayed without particle deposition on the chamber wall, setting a nozzle pressure up to 1.5 bar In all cases, no nozzle clogging occurred or visible particles/aggregated were detected on the chamber wall. Meanwhile increasing the polymer concentration in the feed (SD2), needle shaped particles were clearly visible on the chamber.

In all cases, it was impossible to properly recover the sample and evaluate the process yield because particles were tightly adherent to the glass wall and the grinding procedure caused the formation of aggregates which appeared similar to the raw polymer. Only in the case of DP30, it was possible to collect some dried particles. In the attempt to collect the dried sample, a 0.05 % Tween® 80 solution was used to wash out particles. In the case of formulation SD2, a large mash net was obtained and, therefore, discarded. Under light microscopy (**Fig. 3.4**), all spray-dried powders appeared as irregular aggregates mainly constituted of needle shaped particles. For these reasons, we concluded that it is not possible to prepared DegraPol® MP by spray-drying and, therefore, the emulsification/solvent evaporation technique is the most promising method to prepare MP with the desired size distribution.

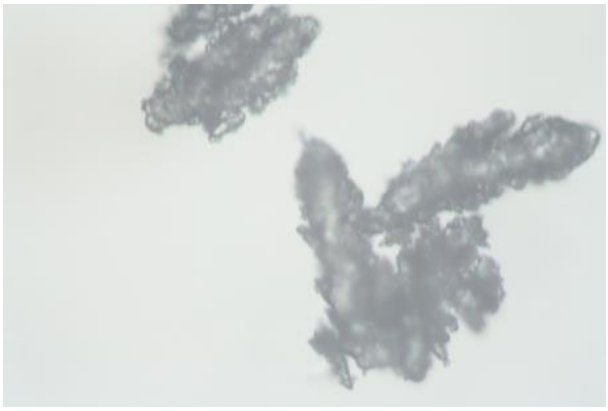

Fig. 3.4 - DP aggregates recovered after all the spray-drying processes.

3.5 Conclusions

In conclusion, the overall data suggest that it is possible to prepare DegraPol® spherical-shaped particles, with a size ranging from nano- to micro-meters by the emulsion/solvent evaporation method. Indeed, selecting the proper process parameters of the emulsification step, DegraPol® solution can be emulsified with PVA obtaining nanoparticles of about 280 nm; while using the ultrasound probe or the homogenizer with HPMC as emulsifier, particles of 1 and 5 µm were prepared. Finally, MP of 30 µm were obtained starting from a coarser emulsion prepared with a metallic propeller.

Together these results suggest the feasibility of preparing particles with different diameters by adjusting the features of the emulsion in terms of size of the oily droplets, type and concentration of the stabilizer. Despite further studies are still needed, DegraPol® can be proposed as a novel material for designing particles with a possible use in (chemo)embolization or drug delivery.

References

[1] P. Giunchedi, M. Maestri, E. Gavini, P. Dionigi, G. Rassu, Transarterial chemoembolization of hepatocellular carcinoma. Agents and drugs: An overview. Part 1, Expert Opin. Drug Deliv. 10 (2013) 679–690. doi:10.1517/17425247.2013.796359.

[2] P. Giunchedi, M. Maestri, E. Gavini, P. Dionigi, G. Rassu, Transarterial chemoembolization of hepatocellular carcinoma--agents and drugs: an overview. Part 2., Expert Opin. Drug Deliv. 10 (2013) 799–810. doi:10.1517/17425247.2013.796359.

[3] S.B. Paul, H. Sharma, Role of Transcatheter Intra-arterial Therapies for Hepatocellular Carcinoma, J. Clin. Exp. Hepatol. 4 (2014) S112–S121. doi:10.1016/j.jceh.2014.03.048.

[4] A. Laurent, M. Wassef, R. Chapot, Y. Wang, E. Houdart, L. Feng, P. Tran Ba Huy, J.J. Merland, Partition of calibrated tris-acryl gelatin microspheres in the arterial vasculature of embolized nasopharyngeal angiofibromas and paragangliomas, J. Vasc. Interv. Radiol. 16 (2005) 507–513. doi:10.1097/01.RVI.0000150038.99488.01.

[5] A. Laurent, M. Wassef, R. Chapot, E. Houdart, J.J. Merland, Location of Vessel Occlusion of Calibrated Tris-acryl Gelatin Microspheres for Tumor and Arteriovenous Malformation Embolization, J. Vasc. Interv. Radiol. 15 (2004) 491–496. doi:10.1097/01.RVI.0000124952.24134.8B.

[6] J.J. Leyon, T. Littlehales, B. Rangarajan, E.T. Hoey, A. Ganeshan, Endovascular Embolization: Review of Currently Available Embolization Agents, Curr. Probl. Diagn. Radiol. 43 (2014) 35–53. doi:10.1067/j.cpradiol.2013.10.003.

[7] E.P. Porcu, A. Salis, G. Rassu, M. Maestri, J. Galafassi, G. Bruni, P. Giunchedi, E. Gavini, Engineered polymeric microspheres obtained by multi-step method as potential systems for transarterial embolization and intraoperative imaging of HCC: Preliminary evaluation, Eur. J. Pharm. Biopharm. 117 (2017) 160–167. doi:10.1016/j.ejpb.2017.04.010.

[8] N. Harnkarnsujarit, S. Charoenrein, Y.H. Roos, Microstructure formation of maltodextrin and sugar matrices in freeze-dried systems, Carbohydr. Polym. 88 (2012) 734–742. doi:10.1016/j.carbpol.2012.01.028.

[9] R.C. Mundargi, V.R. Babu, V. Rangaswamy, P. Patel, T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(d,l-lactide-co-glycolide) and its derivatives, J. Control. Release. 125 (2008) 193–209. doi:https://doi.org/10.1016/j.jconrel.2007.09.013.

[10] X.S. Wu, N. Wang, Synthesis, characterization, biodegradation, and drug delivery application of biodegradable lactic/glycolic acid polymers. Part II: Biodegradation, J. Biomater. Sci. Polym. Ed. 12 (2001) 21–34. doi:10.1163/156856201744425.

[11] J.M. Barichello, M. Morishita, K. Takayama, T. Nagai, Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nanoprecipitation method, Drug Dev. Ind. Pharm. 25 (1999) 471–476. doi:10.1081/DDC-100102197.

[12] M. Iqbal, N. Zafar, H. Fessi, A. Elaissari, Double emulsion solvent evaporation techniques used for drug encapsulation, Int. J. Pharm. 496 (2015) 173–190. doi:10.1016/j.ijpharm.2015.10.057.

[13] B. Conti, R. Dorati, C. Colonna, I. Genta, Effects of ionizing radiation sterilization on microparticulate drug delivery systems based on poly-α-hydroxyacids: an overview, J. Drug Deliv. Sci. Technol. 19 (2009) 99–112. doi:https://doi.org/10.1016/S1773-2247(09)50017-2.

[14] A. Shenderova, T.G. Burke, S.P. Schwendeman, The Acidic Microclimate in Poly(lactide-co-glycolide) Microspheres Stabilizes Camptothecins, Pharm. Res. 16 (1999) 241–248. doi:Doi 10.1023/A:1018876308346.

[15] T.D. Hirt, P. Neuenschwander, U.W. Suter, Telechelic diols from poly[(R)-3-hydroxybutyric acid] and poly{[(R)-3-hydroxybutyric acid]-co-[(R)-3-hydroxyvaleric acid]}, Macromol. Chem. Phys. 197 (1996) 1609–1614. doi:10.1002/macp.1996.021970503.

[16] B. Saad, T.D. Hirt, M. Welti, G.K. Uhlschmid, P. Neuenschwander, U.W. Suter, Development of degradable polyesterurethanes for medical applications: In vitro and in vivo evaluations, J. Biomed. Mater. Res. 36 (1998) 65–74. doi:10.1002/(SICI)1097-4636(199707)36:1<65::AID-JBM8>3.0.CO;2-J.

[17] T.D. Hirt, P. Neuenschwander, U.W. Suter, Synthesis of degradable, biocompatible, and tough block-copolyesterurethanes, Macromol. Chem. Phys. 197 (1996) 4253–4268. doi:10.1002/macp.1996.021971221.

[18] A. Lendlein, P. Neuenschwander, U.W. Suter, Hydroxy-telechelic copolyesters with well defined sequence structure through ring-opening polymerization, Macromol. Chem. Phys. 201 (2000) 1067–1076. doi:10.1002/1521-3935(20000701)201:11<1067::AID-MACP1067>3.0.CO;2-Y.

[19] A. Lendlein, M. Colussi, P. Neuenschwander, U.W. Suter, Hydrolytic degradation of phase-segregated multiblock copoly(ester urethane)s containing weak links, Macromol. Chem. Phys. 202 (2001) 2702–2711. doi:10.1002/1521-3935(20010901)202:13<2702::AID-MACP2702>3.0.CO;2-I.

[20] H. Krynauw, L. Bruchmüller, D. Bezuidenhout, P. Zilla, T. Franz, Degradation-induced changes of mechanical properties of an electro-spun polyester-urethane scaffold for soft tissue regeneration, J. Biomed. Mater. Res. Part B Appl. Biomater. 99B (2011) 359–368. doi:10.1002/jbm.b.31907.

[21] S.A. Riboldi, M. Sampaolesi, P. Neuenschwander, G. Cossu, S. Mantero, Electrospun degradable polyesterurethane membranes: Potential scaffolds for skeletal muscle tissue engineering, Biomaterials. 26 (2005) 4606–4615. doi:10.1016/j.biomaterials.2004.11.035.

[22] M. Horst, V. Milleret, S. Noetzli, R. Gobet, T. Sulser, D. Eberli, Polyesterurethane and acellular matrix based hybrid biomaterial for bladder engineering, J. Biomed. Mater. Res. - Part B Appl. Biomater. 105 (2017) 658–667. doi:10.1002/jbm.b.33591.

[23] M. Borkenhagen, R.C. Stoll, P. Neuenschwander, U.W. Suter, P. Aebischer, In vivo performance of a new biodegradable polyester urethane system used as a nerve guidance channel, Biomaterials. 19 (1998) 2155–2165. doi:10.1016/S0142-9612(98)00122-7.

[24] C. Danielsson, S. Ruault, M. Simonet, P. Neuenschwander, P. Frey, Polyesterurethane foam scaffold for smooth muscle cell tissue engineering, Biomaterials. 27 (2006) 1410–1415. doi:S0142-9612(05)00784-2 [pii]\n10.1016/j.biomaterials.2005.08.026.

[25] B. Saad, Y. Kuboki, M. Welti, G.K. Uhlschmid, P. Neuenschwander, U.W. Suter, DegraPol-Foam: A degradable and highly porous polyesterurethane foam as a new substrate for bone formation, Artif. Organs. 24 (2000) 939–945. doi:10.1046/j.1525-1594.2000.06664.x.

[26] G. Ciardelli, B. Saad, T. Hirt, O. Keiser, P. Neuenschwander, U.W. Suter, G.K. Uhlschmid, Phagocytosis and biodegradation of short-chain poly [(R)-3-hydroxybutyric acid] particles in macrophage cell line, J. Mater. Sci. Mater. Med. 6 (1995) 725–730. doi:10.1007/BF00134308.

[27] B. Saad, G. Ciardelli, S. Matter, M. Welti, G.K. Uhlschmid, P. Neuenschwander, U.W. Suter, Cell response of cultured macrophages, fibroblasts, and co-cultures of Kupffer cells and hepatocytes to particles of short-chain poly[(R)-3-hydroxybutyric acid], J. Mater. Sci. Mater. Med. 7 (1996) 56–61. doi:10.1007/BF00121191.

[28] O. Evrova, J. Houska, M. Welti, E. Bonavoglia, M. Calcagni, P. Giovanoli, V. Vogel, J. Buschmann, Bioactive, Elastic, and Biodegradable Emulsion Electrospun DegraPol Tube Delivering PDGF-BB for Tendon Rupture Repair, Macromol. Biosci. 16 (2016) 1048–1063. doi:10.1002/mabi.201500455.

[29] G. Meier Bürgisser, M. Calcagni, A. Müller, E. Bonavoglia, G. Fessel, J.G. Snedeker, P. Giovanoli, J. Buschmann, Prevention of peritendinous adhesions using an electrospun DegraPol polymer tube: A histological, ultrasonographic, and biomechanical study in rabbits, Biomed Res. Int. 2014 (2014). doi:10.1155/2014/656240.

[30] J. Vandervoort, A. Ludwig, Biocompatible stabilizers in the preparation of PLGA nanoparticles: A factorial design study, Int. J. Pharm. 238 (2002) 77–92. doi:10.1016/S0378-5173(02)00058-3.

[31] S.K. Sahoo, J. Panyam, S. Prabha, V. Labhasetwar, Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake, J. Control. Release. 82 (2002) 105–114. doi:10.1016/S0168-3659(02)00127-X.

[32] F. Cilurzo, F. Selmin, P. Minghetti, L. Montanari, Design of methylprednisolone biodegradable microspheres intended for intra-articular administration., AAPS PharmSciTech. 9 (2008) 1136–1142. doi:10.1208/s12249-008-9158-1.

[33] E. Dickinson, Hydrocolloids as emulsifiers and emulsion stabilizers, Food Hydrocoll. 23 (2009) 1473–1482. doi:10.1016/j.foodhyd.2008.08.005.

Chapter 4

Regulatory aspects and quality controls of parenteral long-acting drug products

Abstract

The quality of a medicinal product, which is a key aspect to assure the safety and the efficacy, has to be demonstrated before the introduction in the market. The approaches followed in the European (Ph.Eur.) and US (USP) Pharmacopoeias are different, since the dosage forms are not classified in the same way and the quality tests are differently organized. Commonly, the assessment of drug release is fundamental, even though in the Ph.Eur. no indications are reported for any products, while specific tests are provided in the USP, according to the type of long-acting parenteral products. Product-dedicated monographies are also available in the USP, due to the necessity to implement a case-by-case quality evaluation. However, the development of suitable *in vitro* release models is critical for long-acting parenteral products, given the complexity in the set-up of methods able to efficiently discriminate products that can have different *in vivo* behaviour. All these aspects are reflected in lack of generics on the market.

In the present work, the actual regulatory frameworks in the EU and the US on the quality evaluation of long-acting parenteral are discussed, in the attempt to outline the direction followed by the Regulatory Agencies for the evaluation of the drug release.

4.1 Introduction

Therapeutic value of certain active principle ingredient(s) [API] can be improved by controlling and/or prolonging its release from the pharmaceutical dosage form over time. Possible approaches to reach this goal include repeated oral, transdermal, pulmonary, or parenteral administrations (*i.e.,* infusion pumps or injections). However, these routes of administration cannot be useful in all the circumstances in which prolonged drug concentrations are needed. As an example, drugs that induce gastric irritation or suffer from poor absorption or extensive first-pass metabolism cannot be efficiently delivered through a repeated oral therapy. In the same way, molecules unstable in a biological environment (*e.g.,* peptides or proteins) can be administered only by a parenteral route.

Long-acting parenteral formulations show several advantages, some of them are in common with other sustained release products, while others are specific. Indeed, only long-acting parenteral products permit to prolong the release of the drug over months or even years, whereas oral prolonged release and transdermal drug delivery systems extent the drug plasmatic concentrations for a day or a week, respectively. Consequently, the reduction of the number of administrations and the simple dosing regimen result in enhanced patient's compliance. Commonly to other prolonged release formulations, the sustained drug levels at the site of action, within the therapeutic range, permits to use a lower quantity of drug compared to conventional pharmaceutical dosage forms and hence, reduce the systemic exposure. Furthermore, the predictable and reproducible API release profile over a defined period of time, improved the API systemic availability and reduce the incidence of side effects, associated to an overall enhancement in the therapeutic outcomes [1, 2, 3].

Sustained parenteral formulations began to appear in the pharmaceutical research from middle-twentieth century. Nowadays, the technologies available for controlling the API release encompass a wide variety of platforms, from membrane-controlled to osmotic-controlled to polymers' diffusion/erosion-controlled systems. To do this, it is fundamental to know the physico-chemical

properties of the delivery platform (*e.g.,* kinetic of drug release, properties of polymer if present), its interactions with the biological environment, the physico-chemical properties of loaded molecule(s) as well as the pharmacokinetic and the pharmacodynamics of the drug being released. However, from a regulatory point of view, it is necessary to establish precise strategies to properly control the quality, the safety and, thus, the efficacy of the product.

Consequently, for a specific product or possibly for a class of product, the identification of the physico-chemical properties of the formulation that could alter its *in vitro* and *in vivo* performance is fundamental. This is challenging for parenteral long-acting formulations as the establishment of *in vitro*/*in vivo* correlations is not easy as happened for conventional dosage forms, probably due to the complexity of the delivery routes and/or of the site of implantation. Thus, *in vitro* tests might be missing to properly check the biopharmaceutical properties of the product being tested with the risk of not discriminating formulations that would behave differently *in vivo*. The raise of uncertainties in the development of a product claimed to be the generic of an innovator one is a possible scenario.

The present work aimed to discuss the regulatory requirements needed to develop generic products of long-acting parenteral formulations (*e.g.*, polymeric microspheres and implants) with attention to the critical issue of the quality assessment according to the current EU and US regulatory frameworks. Thus, the role that could be played by the *in vitro* testing in the evaluation and, possibly, in the prediction of product's *in vivo* performance is presented. The potential applications of *in vitro*-*in*-*vivo* correlations for quality control and waiver purposes are also matter of discussion in this context.

4.2 Parenteral long-acting drug products: definitions in European and United States Pharmacopoeias

Quality standards of medicinal products (*i.e.,* raw materials, preparations, dosage forms, containers) throughout the EU and the US are legally bound to the European (Ph.Eur.) and the United States (USP) Pharmacopoeias, respectively.

The technological progress offers different types of platforms to realize a parenteral sustained release of an API. However, the classification(s) raising from technological considerations can, in some cases, be different compare to the one(s) based on regulatory aspects. The latter available from Ph.Eur. and USP are different and not harmonized in all their parts. In particular, in Ph.Eur. implants are classified only as sterile and solid preparations that can be parenterally implanted and release the loaded drug(s) over an extended period of time [4]. No specific definitions and characteristics are provided in Ph.Eur. monograph for other technologies, such as microspheres or suspension-based *depot* dosage forms. On the other side, the USP gives more details about parenteral formulations for prolonged release. Indeed, USP definitions are not limited to the implantable solid dosage forms but includes all the preparations that are placed subcutaneously or in a specific region of the body (*e.g.,* sinus, artery, eye, brain) to provide a continuous release of the drug substance for a long period of time (*e.g.,* month or years). Consequently, both dosage forms that can be injected by needle or implanted by special injector fall in the definition accepted by USP. In particular, pellet implants, resorbable microparticles and drug suspensions are injectable *depot*, whereas polymeric implant and drug-eluting stents implantable ones [5]. It is noteworthy that from FDA point of view, extended-release injections and implants are considered as *complex formulations*, with critical issues related to the need of dedicated regulations and the development of suitable standards [6].

Interestingly, drug-eluting stents are differently classified in the EU and the US regulatory frameworks. Indeed, they are included in the USP monograph of pharmaceutical dosage forms [5], where they are not in the Ph.Eur. one [4]. Based

on the mechanical mechanism of action, stents are classified as medical devices in both regulatory frameworks. The loading of a drug substance in the stent does not modify the product classification if the pharmacological effect is considered ancillary to the primary mechanical action of the stent [7]. Although this definition is common in both the regulatory frameworks, there are significant differences in its application for placing a drug-eluting stent on the market. In Europe, they follow the same assessment process of other implantable medical devices [8]. The device dossier should be evaluated by a notified body. If the results of the assessment are positive, the device receives the CE-mark and can be placed on market. However, for drug-eluting stents, the quality, the safety and the usefulness of the API cannot be performed directly by the notified body but should be verified in cooperation with the EMA or a national authority competent for medicinal products, according to the Directive 2001/83/EU. In the US, drug-eluting stents are classified as combination product [9]. For reaching the market, the manufacturer should submit a premarket approval (PMA) application to the FDA, which is the only authority in charge to assess the device dossier [10]. The applicant should submit detailed data regarding both individual components (*e.g.,* drug, polymer, and stent) and finished drug-eluting stent to permit the FDA a comprehensive evaluation of the product benefit/risk balance. The assessment is performed by the Center for Devices and Radiological Health (CDRH) and the Center for Drug Evaluation and Research (CDER). The former office is competent for the device part of the application, the latter for the aspects related to the loaded drug substance.

4.3 Design of the main parenteral long-acting technologies

As per the USP definition, pellet implants are solid and sterile objects able to control the release of the drug for months. A specific device for the injection is usually prescribed otherwise a generic surgical procedure is followed. The release of the drug follows the first-order kinetic and it can be tailored by modifying the size of the implant. Testopel™ (Auxilium Pharms Inc.) is the only pellet implant available in the US market. It is a testosterone pellet to be implanted subcutaneously, for replacement testosterone therapy. The release of the drug is controlled from 3 to 6 months [11]. Pellet are generally produced by extrusion-based processes, where the mixture of drug(s) and matrix-forming polymer(s) are wetted with a binder solution and then extruded through a die. This is followed by spheronization or cutting into the desired shape.

Resorbable microparticles (or microspheres) are sphere-shaped implants with a diameter ranging from 20 to 100 μm. They are composed of a polymeric bioresorbable and biocompatible matrix, considered as an excipient, in which the drug is throughout dispersed [5]. They are designed to be injected by intra-muscular or sub-cutaneous routes, otherwise, USP states that they can also be deposited in a specific site of the body with the aim of realizing a site-specific release [5]. The most successful example of this type of implant are the ones based on the poly(lactide-*co*-glycolide) [PLGA]. It is a copolymer of lactic and glycolic acids, linked by ester bonds, that undergoes to a complete degradation upon contact with aqueous based fluids. Drugs formulated in PLGA microspheres are generally released by diffusion through the polymeric matrix or by the erosion of the polymer or by a combination of these processes. Additionally, the overall physical properties of the drug-polymer system as well as the release kinetic can be tuned from weeks to months by means of modifications in polymer molecular weight, ratio between monomers, esterification of the end-side chains or drug concentration [12, 13]. Microspheres preparation processes mainly include the emulsion/solvent evaporation methods, the phase separation method and the spray-drying [14]. Emulsion/solvents evaporation methods regard with the single

and double (or multiple) emulsions. In brief, the simplest procedure involves the dissolution of the polymer in an appropriate water immiscible and volatile solvent (*e.g.,* dichloromethane), the dissolution/dispersion of the drug and then the emulsification with an aqueous phase containing stabilizer(s) (*e.g.,* polyvinyl alcohol) to prepare typically an oil-in-water emulsion. The organic solvent is evaporated at reduced or atmospheric pressure or extracted with the auxiliary of a quench medium to obtain hardened particles [15, 16]. The double emulsion process is usually more suitable for hydrophilic drugs compared to the single emulsion, since the drug is dissolved in an aqueous medium and then properly emulsified with the volatile organic solution of the polymer, forming a water-in-oil emulsion. This system is in turn emulsified with a second aqueous phase and, in the last stage, the volatile solvent is evaporated or extracted from the water-in-oil-in-water emulsion [17].

Phase separation process, known also as “coacervation”, involves the preparation of polymer and drug solutions that are mixed together. The phase separation is achieved by the gradual addition of organic solvent to the system that extract the polymer solvent. Final microspheres are obtained by the rapid dipping of the soft drug-rich coarcevates into a medium in which they are not soluble [14].

The last technique applied for microspheres preparation is the spray-drying. It involves the atomization of a feed that is immediately hit by a desiccant fluid stream, leading to the fast evaporation of the solvent and the formation of solid particles. The composition of the feed depends on the solubility of the polymer and drug, so as solution in a volatile organic solvent or solid-in-oil dispersion or water-in-oil emulsion can be sprayed. The advantages of this method compared to those previously reported are mainly the relative easy scalability of the process and the ideal possibility to encapsulate within the particles a great variety of molecules (*i.e.,* hydrophobic or hydrophilic small drugs, proteins or peptides) by optimizing the feed configuration [18, 19].

Extended-release injectable suspensions are defined as liquid preparations composed of a suitable vehicle in which a solid drug is suspended, allowing the

drug to be available for an extended period of time. In most of the cases, the final product is composed of a lipophilic drug finely dispersed in an aqueous solvent. They can be supplied as a dried solid that must be dispersed in the provided vehicle or as a product ready to use; excipients such as surfactants are needed to assure the correct dispersion of the solid particles prior the injection. The suspension of water-soluble API in an oily solvent has also been proposed, even though controversial results in literature demonstrate the ability to sustain the release of the drug after administration [20, 21]. In these systems, the absorption of the drug is governed by its dissolution that, thereby, represent the rate-limiting step of the process.

Polymer implants are single masses with a specific shape (*e.g.,* cylinder) that are composed by a biocompatible biodegradable or non-biodegradable polymer. They are generally implanted by means of an injector and must be removed in the case of the non-biodegradability of the polymer. The release of the drug is controlled by diffusion in the polymer matrix, the erosion of the polymer if biodegradable or by the presence of a polymeric rate-controlling membrane. Depending on the design of the implant, the release can follow non-zero-order or zero-order kinetics. The main industrial preparation process is the extrusion. The polymer-drug mixture is heated, and shear stressed up to a semi-liquid state from an extrusion screw and then pushed through a die. The extrudate is cooled, solidified and cut in the desired shape [22].

Among the parenteral dosage forms that sustain the release of an API, drug substance-eluting stents are mentioned only in the USP [5]. The overall effect of this technology is double: the mechanical support of the stent allows to maintain the arterial patency while the prolonged release of the drug provides the pharmacological effect, for example to reduce restenosis or to inhibit the clot formation or to combat infections. The mechanism through which the release of the drug is controlled concerns the embedding of the active ingredient in a non-biodegradable or biodegradable polymeric coating. The fabrication of metallic stent is more complex compared to the dosage forms presented so far and the

process deeply depends on the raw material form. Today, the laser cutting from tubing is the most applied process. Then the drug can be loaded directly onto the metallic surface or spray-coated using a biodegradable or a non-biodegradable polymer. The latter is more convenient since the better drug retention during the development and the modulation of the drug-elution kinetic [23].

4.4 Considerations on the drug release evaluation in EU and US

The approaches followed in the Ph.Eur. and the USP to discuss the long-acting parenteral formulations and, hence, the quality tests reported in the monographs are different. In the case of USP, two general monographs are available. In the "*<1151> Pharmaceutical dosage forms*" monograph a detailed classification of the different types of long-acting parenteral products is reported [5], meanwhile in the "*<1> Injections and implanted drug products (parenterals) – product quality tests*" both general and class-specific quality tests are provided [24]. The general tests, which are common with other parenteral dosage forms, are the identification assay, impurities of the drug substance, assessment of the foreign and particulate matter, sterility, bacterial endotoxins, container content, packaging systems, container-closure integrity and labelling. The monograph dedicated to implanted drug products listed quality tests specific for the type of implant: the uniformity of dosage units is required for all implants, whereas the water content and the "*biological reactivity tests, in vivo*" only for microparticles and drug-eluting stents, respectively. A test to assess the drug release is not included in the quality test list for implants and microparticles. However, precise information are also reported in monographies dedicated to specific long-acting parenteral products, as in the case of goserelin implants [25], where a protocol to perform *in vitro* release studies from 3.6-mg and 10.8-mg doses is reported (**Table 4.1**). In particular, the study should be performed in 120-mL flat-bottomed glass jar filled with pH 7.4 buffer solution. The test should be performed in accelerated conditions at 39 °C up to 672 hours (28 days) for the 3.6-mg dose and to 2016 h (84 days) for the 10.8-mg one. Regarding other implants, information can be also

found in a database on dissolution methods established by the FDA to rationalize the comparison of *in vitro* performances of a specific drug product. In **Table 4.1** the specifications extrapolated for long-acting parenterals from the FDA database are listed. The availability of these information could be very important for the pharmaceutical development of new and generic products, other than for post-marketing variations. Indeed, harmonized dissolution protocols to be applied in the release test would make the comparison between products easier.

In the Ph.Eur., long-acting parenteral products fall within the general monograph on "*Parenteral preparation*" under the classification of *Implants*. However, no detailed sub-classification is available. In this monograph, it is stated that the sterility and the particulate contamination should be evaluated for implants, other than an appropriate test to properly demonstrate the release of the active substances [4]. However, no details are provided regarding the apparatus and protocols that should be applied for such drug release studies. Few information are available from the Pharmacopoeias of specific European Member States, as again in the case of goserelin implants. In the current British Pharmacopeia (BP) [26], a protocol to properly assess the release of the drug is reported, without any significant differences with respect to the USP monograph.

Unlike the US situation, in EU the lack of specific and harmonized information on protocols to be applied in release study leaves room for different interpretations. At the moment of the application submission to get a marketing authorization, EMA will give its opinion on the goodness of the chosen method.

The development of suitable *in vitro* release models is critical for long-acting parenteral formulations, both for formulation development and quality control purposes. Furthermore, the possible establishment an *in-vitro-in-vivo* correlation (IVIVC), namely an *in vitro* model that can predict the *in vivo* performance of the product, make the *in vitro* testing a more powerful tool. Thus, the consideration of variables accounting for the physiological environment is essential for reaching *in vivo* relevance, such as body temperature, vascularity, pH, buffer capacity, osmolarity, volumes or any tissue responses [2]. And again, the set-up of bio-

relevant *in vitro* protocols should not alter the mechanism(s) of *in vivo* drug release and is applicable only when the API release (dissolution) is the rate-limiting step for its absorption [27]. Ideally, meaningful IVIVC could find applications in bioequivalence studies or for biowaivers granting (more detailed aspects are discussed in the later sections). On the other hand, is not always easy to simulate the conditions occurring in the biological environment or to identify which variables are actually significant [2, 27]. However, all these evaluations have to be addressed on a case-by-case basis.

Table 4.1 – Protocols for release tests included in Pharmacopeia monographs or accepted by a Regulatory Agency.

Drug product	Dose	Dissol. apparatus	Sample	Medium	Volume	Temp.	Stirring	Sampling time
Dexamethasone IMT (FDA [28])	NA	USP VII apparatus (with reciprocating 50 mesh baskets	NA	Phosphate buttered saline + 0.05 g/L SDS	30 mL	45°C	30 cycles/min	12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 h
Goserelin implant (USP [25]/BP [26])	3.6 mg/ 10.8 mg	Flat-bottomed, borosilicate glass jar (120 mL) with a tight plastic cap	1/5 unit (3.6/10.8 mg)	pH 7.4 phosphate/ citrate buffer	50 mL	39°C	NA	7, 14, 17, 21 and 28 days (3.6mg) 3, 14, 35, 56, 84 days (10.8 mg)
Goserelin implant (FDA [28])	3.6 mg/ 10.8 mg	Wheaton jar (120 mL)	NA	pH 7.4 phosphate buttered saline	50 mL	39°C	Swirl orbit at 205 rpm for 6 s	7, 14, 17, 21 and 28 days (3.6mg) 3, 14, 35, 56, 84 days (10.8 mg)
Leuproline acetate ERS (FDA [28])	NA	USP II or IV apparatus	NA	NA	NA	NA	NA	NA
Naltrexone ERS (FDA [29])	380 mg	250 mL HDPE plastic bottle	600 mg	pH 7.4 phosphate buffered saline + 0.02% Tween 20 + 0.02% sodium azide (osmolarity: 270 mOsm	200 mL	37°C	NA	1, 7, 14, 28 days
Octreotide ERS (FDA [28])	NA	USP II or IV apparatus	NA	NA	NA	NA	NA	NA
Risperidone MPs (FDA [30])	25 mg	Cylinder bottle	NA	pH 7.4 HEPES buffer + sodium azide + NaCl + Tween 20	200 mL	37 and 45°C	NA	1, 21 Days (37°C) Multiple time points from 0 to 8 days (45°C)
Triamcinolone acetonide MPs (Zilretta® PQR [31])	40 mg	USP II apparatus	160 mg of MPs	pH 7.2 PBS (10 mM) + 0.3% SDS + 0.02% sodium azide	1000 mL	35°C	75 rpm	4, 24, 48, 120 h
Triptorelin Pamoate ERS (FDA [28])	NA	USP II apparatus	NA	50 mL of methanol to 950 of water	950 mL	NA	75rpm	1, 8, 24, 96, 168 h

ERS: extended release suspension; ID: Injectable Depot; IM: Intramuscular suspension/injection; IMT: Implant; MPs: microparticles; NA: not available; PBS: phosphate buffer solution; PQR: product quality review.

4.5 How to reach the market: the regulatory environments in EU and US

Parenteral route is in some cases the only logical approach for drugs suffer from poor absorption due to poor membrane transport or instability, *e.g.* in the gastro-intestinal tract. Additionally, if considering a chronic use, sustained release parenteral systems allow to maintain the drug concentration within the therapeutic range for the needed period of time. Rarely, this strategy is applied to new API; in the majority of the cases, it has been applied for reformulating "old drugs" into new formulations or formulations with new indications that permit to significantly improve their efficacy. In any case, as other medicinal products, for placing on market a novel long-acting parenteral drug product, the applicant must submit to a Regulatory Agency a Common Technical Document (CTD). The information that applicants must provide to obtain the marketing authorization varies according to the type of product and the application.

4.5.1 Marketing authorization of new active principle

The first parenteral long-acting drug products were authorized on the market in the early '80s. Nowadays, more than fifteen products are available in the US market [32] and more than five have been authorized by the EMA after a centralized authorization application [33]. However, within the European Member States, many other products are available, approved by a decentralized, mutual recognition or national procedures, since they got the marketing authorization prior the establishment of the centralized process.

In most of the cases, long-acting parenteral products have been developed to treat cancer (*e.g.,* prostatic cancer), acromegaly, alcohol dependence or psychiatric diseases (*e.g.,* schizophrenia, bipolar disorder).

To get a marketing authorization for a first-in-man medicinal product, a full dossier must be submitted to the FDA, EMA or a national Regulatory Agency. In US, the procedure to be followed is the New Drug Application (NDA) while in EU a

centralized, decentralized or mutual recognition application are possible ways (or a national procedure relative to a specific Member State). The CTD should include a full description of the quality, safety and efficacy profile of the proposed drug product and only if its benefit/risk balance results positive after the Agency assessment, the applicant can obtain the marketing authorization. Thus, the pharmaceutical development of the drug product should be described in detail, including all the information regarding the physico-chemical properties of both drug substance and excipients, the product design and its characterization in terms of biopharmaceutical performances *in vitro* and *in vivo* [34]. All the critical quality attributes of the product should be identified and studied on the basis of the intended use and route of administration. Moreover, comparative *in vitro* (*e.g.,* release studies) or *in vivo* (*e.g.,* bioequivalence) studies may be also included to link the results obtained with formulation(s) used in clinical studies to those of final commercial formulation(s). Any changes in the formulation during the late stage of pharmaceutical development should be justified with respect to their impact on the clinical performance. In this context, the establishment of an *in-vitro-in-vivo* correlation (IVIVC) can be also applied for selecting the appropriate quality acceptance criteria for batch release or for wavering further bioequivalence studies following changes to the product or its manufacturing process [35, 36]. However, only IVIVC based on point-to-point relationship between *in vitro* and *in vivo* data (Level A IVIVC) can be relevant from a regulatory point of view. And again, Level A IVIVC is considered a prerequisite to support the use of biowaiver, as it allows to predict the *in vivo* time course from the *in vitro* data [36]. Despite these great potentialities, to date this type of correlation has been demonstrated only for few long-acting systems [37, 38]. The lack of compendial methods for *in vitro* release testing and the complexity of the implantation site are probably the main limitations in the establishment of IVIVC. As a matter of facts, the drug release from implant/microspheres is a critical issue that should be taken into consideration during the pharmaceutical development of such products. The set-up of a proper release study for comparing the *in vitro*

performance of formulations is not always easy to perform. The classical compendial apparatus are not biorelevant for most of the marketed long-acting systems and, thus, other methods should be considered (*e.g.,* modified flow-through cell or even separate-and-sample method). Furthermore, the composition of the medium and the physiology of the implantation site should be carefully studied to mimic the *in vivo* conditions: osmolarity, pH, buffer capacity and volume are just some of the parameters that should be set-up similarly to the conditions at implantation site [39, 40].

Compared to immediate-release drug products, additional clinical studies are required by Regulatory Agencies for characterizing the *in vivo* performance of a novel long-acting system. For example, the EMA requires that applicant designs the pharmacokinetic studies to properly evaluate the drug diffusion from the implantation site, the rate-limiting steps that determine the systemic availability and the risks of dose-dumping [36]. In particular, different clinical-relevant aspects, such as the site-dependent absorption pattern, the fluctuation in drug concentration and the lag-times, should be studied in-depth during both single-dose or multiple-dose studies. In addition, when more than one strength is designed, the dose proportionality in absorption profile should also be investigated by the applicant.

4.5.2 Generic and abridged applications

If the drug product contains an API which has obtained a previous marketing authorization, even if in a different pharmaceutical form, the information regarding its efficacy or safety profile may be derived from those available in literature or provided by medicinal products already on the market.

Moreover, if the product on the market has the same pharmaceutical form, the strategies for obtaining the marketing authorization can follow the possibility to demonstrate its therapeutic equivalence with respect to the originator.

If it can be demonstrated by bioequivalence studies, a simplified dossier must be submitted to the FDA, EMA or a national Regulatory Agency. Since most of the

long-acting parenteral products available on the EU and US markets are out-of-patent, the development of their copies is a feasible strategy for reducing costs sustained by patients and healthcare systems.

In US, the procedure to be followed is the Abbreviated New Drug Application (ANDA) while in EU a generic application should be submitted through a centralized, decentralized or mutual recognition procedure (or a national procedure relative to a specific Member State).

The data that should be included in the CTD is simplified in the preclinical and clinical parts with respect to a new marketing application. The quality part of the CTD (Module 3) is the most critical part as the formulation of a modified release products plays a crucial role in the overall quality of the new medicinal product. In both the EU and the US, the generic product should have the similar qualitative and quantitative composition to be pharmaceutically equivalent to the reference product [41, 42]. In addition, for parenterals, the FDA states that the qualitative (Q1) and quantitative (Q2) composition of the test and reference products should be the same as demonstrated by a proper assessment [43, 44]. Even if parenteral long-acting products are classified as complex formulations [45], the product copies should fulfil the Q1/Q2 requirement for being FDA-authorized with an abbreviated new drug application (ANDA) [44]. On the other side, European regulation states that generic product should have same qualitative and quantitative composition in active substances and the same pharmaceutical form of the reference medicinal product [42]. Besides these differences, no generic products of long-acting parenteral products are available on EU and US market on the best of our knowledge. This is due to the difficulties in assuring the pharmaceutical equivalence of the copy. Criticisms of materials, product design and manufacturing process can strongly affect the biopharmaceutical properties of the final product. As an example, the physico-chemical properties of the matrix-former polymers (*e.g.,* PLA, PLGA) can influence significantly the *in vitro* performance (*e.g.,* drug release rate) and *in vivo* persistence of the drug product after implantation [46, 47]. And again, regarding PLGA, the copolymer

composition, molecular weight or end-side functionalization influence significantly drug's diffusion and system's degradation. Given the complexity of this context, the FDA requires a complete characterization of PLA/PLGA to fulfil Q1/Q2 requirements by the comparison of polymer composition (ratio between glycolic and lactic acids), molecular weight, weight distribution and polymer architecture (*e.g.,* linear or star-branched) [48]. Moreover, since both polymeric microparticles and implants are complex formulations, changes in the manufacturing process (*e.g.,* solvent, preparation method) can greatly impact the morphology of particles, the biopharmaceutical properties and the bioavailability of the drug and, thus, they are classified as major-changes in case of variations submission [37].

Besides such criticisms in the sameness assessment, the lack of compendial *in vitro* release testing and validated IVIVC limits the development of copies of parenteral long-acting systems. In the attempt to help applicants in the development of generics, the FDA has released eight guidelines specific for different type of systems (**Table 4.2**). The strategies included in these product-specific guidelines can be classified in three scenarios:

i. bioequivalence studies should be performed for all strengths available;
ii. bioequivalence studies should be performed for some strengths, but waivers can be accepted for other strengths available (a linear relationship between the strength and the pharmacokinetics of the drug is needed);
iii. both bioequivalence studies and *in vitro* release studies should be performed to support the equivalence between tested and reference products (*e.g.,* risperidone).

Moreover, the FDA establishes a database of dissolution methods that can be used to compare the *in vitro* performances of a specific drug product (**Table 4.1**).

On the other side, only one guideline on octreotide acetate *depot* powder was released by the EMA [49] (**Table 4.2**). Unlike the FDA guidelines, the EMA released guidance on the protocol to be used for demonstrating the bioequivalence of the highest strength (*i.e.*, 30 mg) of octreotide acetate *depot* powder, without provide

any details about the strategies applicable to the comparison of the lower strengths (*i.e.*, 10 mg, 20 mg).

When a long-acting parenteral product is not pharmaceutically equivalent to an authorized medicinal product or the bioequivalence cannot be demonstrated, an abridged application can be possible. In particular, this application can be used when: a) the new product has not the same qualitative and qualitative composition with respect to an originator (*e.g.*, changes in active substance, strength, pharmaceutical form); b) the bioequivalence cannot be considered as a surrogate of the therapeutic equivalence (*e.g.*, locally-applied and locally-acting drug products); c) therapeutic indications, pharmacokinetic profile or route of administration are changed with respect to the reference product.

In EU, these conditions fall in the "hybrid" procedure described by Article 10(3) of Directive 2001/83/EC [42]. In US, since a product with the above-mentioned conditions is not eligible for an ANDA, it can be for the 505(b)(2) NDA application, which is a similar approach to European hybrid procedure [50]. In both the cases, although the preclinical and clinical data are generally less than those required by the EMA and the FDA for a first-in-man drug product, the authorization dossier should appropriately support the safety and the efficacy of the new parenteral long-acting product. Specifically, the EMA requires at least that applicant performs single-dose and multi-dose studies to compare *in vivo* performances of intramuscular and subcutaneous *depot* with respect to an authorized reference [36]. The multi-dose study is needed unless the drug bioavailability after the single-dose (expressed as AUC_{0-t}) is higher than 90% of the global drug bioavailability (expressed as $AUC_{0-\infty}$) in both test and reference. The investigations should be performed using only one strength only if others are proportional in composition, exhibit a similar *in vitro* profile and there is a linear correlation between the strength and the pharmacokinetic profile of the drug.

4.6 Post-approval changes for approved long-acting parenteral drug products

After the marketing authorization, the applicant can introduce some changes in all the parts of the authorized version of CTD. However, the procedures required to make in place a change vary according to its impact on the quality, safety, efficacy profiles of the drug product. Both the EMA and FDA establish different kinds of procedures that marketing authorization holders (MAH) have to follow for implementing the changes. Based on the impact on the drug product benefit/risk balance, the FDA establishes a three-level classification (*i.e.,* major, moderate, minor), whereas the EMA a two-level one (*i.e.,* major, minor) [51, 52]. Although differences can be found in the change-impact classification, similarities in post-approval changes procedures are present. For major-impact changes, MAH must submit to the Regulatory Agency a formal application [EMA: Type II variation; FDA: prior approval supplemental (PAS) application]. The change must be authorized by the Regulatory Agency before that it can be applied. For moderate-impact changes in US and minor-impact changes in EU, the MAH should notify the dossier variation to the Regulatory Agency. In this context, according to the change type, the applicant can implement it immediately after the notification [EMA: Type IA variation that required an immediate notification; FDA: Supplement – Changes being Effected (CBE-0)] or after 30 days [EMA: Type IB variation; FDA: Supplement – Changes Being Effected in 30 Days (CBE-30)]. In EU, the notification can be done within 12 months following the change implementation if the variation should not be communicated immediately. On contrary, in the US, the MAH can implement minor-impact change directly and notify them in an annual report to the FDA. The information required to support the application can vary according to the change type, but MAH should submit additional studies to demonstrate the maintenance of the drug product quality profile. They can be *in vitro* or *in vivo* studies. As a general rule, if the drug dosage form is particularly critical, as in the case of a long-acting systems, or the proposed changes may influence the clinical pattern of the drug product, the changes are classified as major by both the EMA and FDA. The

EMA classified as Type II variation changes in the concentration of a single dose parenteral product where the strength remains the same or in the coating if it is critical for the release mechanism from the *depot* [53]. As well, changes in the manufacturing process of implant or microparticle for extended-release are considered major variations by the FDA, since they are complex processes and their modifications can have a significant impact on the quality, safety and efficacy of the medicinal product [51]. Emblematic cases are the risperidone-loaded or naltrexone-loaded microparticles, where changes in the manufacturing process can affect significantly the bioavailability of the drug substance [37, 38]. In this context, proper *in vitro* (*e.g.,* dissolution/release studies) or *in vivo* comparative studies (*e.g.,* bioequivalence studies) should be submitted to the Regulatory Agency for assuring that the change does not affect the benefit/risk balance of the drug product. To date, there are no specific EMA or FDA guidelines on the protocols that can be applied to determine the *in vitro* similarity of two long-acting formulations nor waivers of *in vivo* bioequivalence (if it is needed according to the specific type of change). Indeed, the EMA does not define specific bio-waivers for long-acting parenteral products and the demonstration of bioequivalence for modified release intramuscular or subcutaneous dosage forms still remains link to the rules applied to other extravascular modified release formulations (*e.g.,* transdermal dosage forms) [54]. On the other hand, the FDA states that a waiver for parenteral dosage forms can be accepted only when the bioequivalence is self-evident (*e.g.,* injections with the same qualitative and quantitative composition) or when the bioequivalence can be demonstrated by evidence obtained *in vitro* in lieu of *in vivo* data [55]. However, the latter option is not applicable to delayed or extended-release products [56]. Moreover, no specific scale-up and post-approval changes (SUPAC) guidelines for long-acting systems have been released by the FDA. The statistical approaches reported in the available SUPAC on oral (*e.g.,* SUPAC-IR, SUPAC-MR) and semisolid dosage forms (SUPAC-SS) cannot be applied to implants because the drug release is pre-programmed and, therefore, the

similarity factor (f2) cannot be used to determine the dissolution profile similarity due to the lack in predicting the *in vivo* performance [57].

According to the US regulatory framework, clinical data may be waived if a so-called comparability protocol (CP) has been submitted to the FDA. Indeed, based on 21 CFR Ch. I, 314.70 (e), the FDA admits the simplification of the change-implementation procedures in presence of a valid CP. It is a "*comprehensive, prospectively written plan for assessing the effect of a proposed chemistry, manufacturing and control post-approval changes on the identity, strength, quality, purity and potency of a drug product*" [58]. The CP should be submitted within an original marketing authorization application by applicant or as PAS by MAH. CP allows the FDA to review in advance the implementation plan that the MAH will submit for one or more proposed changes, including the type of supporting information will be provided, the analysis and the risk assessment activities that will be done to implement the change. If it is approved, CP facilitates the subsequent implementation and the reporting activity of the changes. It can also result in a reduction of the estimated change-impact level. Although not all the possible changes can be covered by a CP, it is possible to hypothesize that, in presence of a strong IVIVC, the CP can be a very useful tool to assure quality in long-acting system during change implementation. Indeed, the *in vitro* release method can be used as a surrogate of bioequivalence studies, especially when a point-to-point IVIVC is established [35, 36]. Such approach seems to be applied in the case of Zilretta® extended-released suspension (PLGA microparticles) as reported in the product quality review published on FDA portal [31]. Indeed, *in vitro* release data were accepted by the FDA to support a both a minor change in formulation (e.g. volume of diluent was increased to 5 mL) between the Phase II and Phase III, and a major one in the manufacturing site between that used in the production of Phase III batches and commercial ones. In particular, in the latter the FDA accepted *in vitro* release data instead of the results of a proper bioequivalence study extended-released suspension (PLGA microparticles).

Table 4.2 – Guidelines release by the EMA and FDA on long-acting parenteral products.

API	Dosage forms	Source	Recommended study to assess bioequivalence	Strengths	Waiver
Goserelin acetate [59]	IMT	FDA	2 single-dose, parallel in vivo studies	3.6 mg, 10.8 mg	NA
Leuprolide acetate [60]	ID	FDA	2 single-dose, randomized, parallel in vivo studies	30, 45 mg/vial	For 11.25 and 22.5 mg/vial (vs 30 mg/vial)
Leuprolide acetate [61]	IMT	FDA	1 single-dose, parallel, crossover in vivo study	Equivalent to 65 mg of base	NA
Leuprolide acetate, Norethindrone acetate [48]	ID + oral tablet	FDA	2 single-dose, randomized, parallel in vivo studies	11.25, 3.75mg/vial Leuprolide acetate ID	NA
			1 steady state, crossover in vivo study	5 mg Norethindrone acetate tablet	
Naltrexone [29]	ERS	FDA	1 parallel in vivo study	380 mg/vial	NA
Octreotide acetate [49]	MPs	EMA	1 single-dose, parallel design in vivo study	30 mg	NA
Octreotide acetate [62]	MPs	FDA	1 single-dose, parallel design in vivo study	30 mg	For 10, 20 mg (vs 30 mg)
Risperidone [30]	MPs	FDA	1 in vitro drug release	25 mg/vial	For 12.5, 37.5, 50 mg/vial (vs 25 mg/vial)
			1 in vivo, two period, crossover, steady-state study	12.5, 25, 37.5, 50 mg/vial	
Triptorelin pamoate [63]	IM	FDA	3 single-dose, parallel design in vivo with pharmacokinetics endpoints	3.75, 11.5, 22.5 mg base/vial	NA

ERS: extended release suspension; ID: Injectable *Depot*; IM: Intramuscular Injection/suspension; IMT: Implant; IS: Injectable suspension; MPs: microparticles; NA: not available.

4.7 Conclusions

The boost in the pharmaceutical technology development has led and is continuously leading towards the preparation of increasingly complex parenteral long-acting formulations to meet specific medical needs. The complexity and the variety of the available technologies to parenterally sustain the release of a drug as well as the few marketed products dictate an assessment of the *in vitro* testing in a case-by-case fashion, since different products necessitates different requirements. This, in turn, determines the impossibility to have a unique regulatory approach for all those products.

Additionally, the paucity of public technical information on innovator products creates other limitations to the generic development.

In line with the variety of the technologies, an equal increment in the complexity of the methods to control the *in vitro* quality of long-acting parenteral products is reasonable to be expected.

The establishment of a Level A *in-vitro-in-vivo* correlation is not always feasible, and it requires efforts also from an economic point of view.

All the aspects make the development of generic products very challenging and to date no generic of a long-acting parenteral is available. To increase the possibility, more guidelines product-dedicated, to support the formulations, the testing and thus the approval of generic parenteral long-acting products have to be elaborated. Furthermore, in EU the issues related to those products approved in the past before the introduction of the centralized procedure need to be addressed. Indeed, the approval through applications to single European Member States has determined the lack or, at least, the impossibility to find public information on the *in vitro* characterization of marketed products. This can represent another factor that, specifically in EU, is limiting the development of generics.

References

[1] L.E. Hollister, Site-specific drug delivery to CNS: Old and new. Neurobiol. Aging 10 (1989) 631-650. doi: 10.1016/0197-4580(89)90156-5.

[2] C. Larsen, S.W. Larsen, H. Jensen, A. Yaghmur, J. Ostergaard, Role of in vitro release models in formulation development and quality control of parenteral depots, Expert Opin. Drug Deliv. 6 (2009) 1283–1295. doi: 0.1517/17425240903307431.

[3] R.J. Levy, V. Labhasetwar, S.A. Strickberger, T. Underwood, J. Davis, Controlled release implant dosage forms for cardiac arrhythmias: Review and perspectives. In Drug Delivery: Journal of Delivery and Targeting of Therapeutic Agents 3 (1996) 137–142. doi: 0.3109/10717549609029442.

[4] Monograph on “parenteral preparations” (04/2015:0520). European Pharmacopoeia, ed. 9.0.

[5] Monograph on “pharmaceutical dosage forms <1151>”. US Pharmacopeia, ed. 41.

[6] D.J. Burgess, A.S. Hussain, T.S. Ingallinera, M.L. Chen, Assuring Quality and Performance of Sustained and Controlled Release Parenterals, AAPS Workshop Report, Co-Sponsored by FDA and USP. Workshop Report, Pharm. Res. 19 (2002) 1761-1768. doi: 10.1023/A:1020730102176.

[7] FDA. Guidance for Industry and FDA Staff. Classification of products as drugs and devices & additional product classification issues. September 2017; Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices.

[8] Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices.

[9] $3.2(e)(1), Subpart A, Part 3 Product Jurisdiction, Subchapter A, Chapter I, Section 21 ‘Food and Drugs’ of the Code of Federal Regulations. United States Government Publishing Office.

[10] FDA. Guidance for Industry – Coronary drug-eluting stents – non-clinical and clinical studies. March 2008.

[11] A. McCullough, A Review of testosterone pellets in the treatment of hypogonadism, Curr. Sex. Health Rep. 6 (2014), 265–269. doi: 10.1007/s11930-014-0033-7.

[12] M.L. Houchin, E.M. Topp, Physical properties of PLGA films during polymer degradation, J. Appl. Polym. Sci. 114 (2009) 2848-2854. doi: 10.1002/app.30813.

[13] K. E. Uhrich, S.M. Cannizzaro, R. S. Langer, K. M. Shakesheff, Polymeric systems for controlled drug release, Chem. Rev. 99 (1999) 3181–3198. doi: 10.1021/cr940351u.

[14] H.K. Makadia, S.J. Siegel, Poly Lactic-co-glycolc acid (PLGA) as biodegradable controlled drug delivery carrier, Polym. J. 3 (2011) 1377-1397. doi: 10.3390/polym3031377.

[15] R. Arshady, Preparation of biodegradable microspheres and microcapsules: 2. Polyactides and related polyesters, J. Contr. Rel. 17 (1991) 1-21. doi: 10.1016/0168-3659(91)90126-X.

[16] I.D. Rosca, F. Watari, M. Uo, Microparticles formation and its mechanism in single and double emulsion solvent evaporation, J. Contr. Rel. 99 (2004) 271-280. doi: 10.1016/j.jconrel.2004.07.007.

[17] S. Mao, J. Xu, C. Cai, O. Germerhaus, A. Schaper, T. Kissel, Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres, Int. J. Pharm. 334 (2007) 137-148. doi: 10.1016/j.ijpharm.2006.10.036.

[18] R. Vehring, Pharmaceutical particle engineering via spray drying, Pharm. Res. 25 (2008) 999-1022. doi: 10.1007/s11095-007-9475-1.

[19] E. Gavini, P. Chetoni, M. Cossu, M.G. Alvarez, M.F. Saettone, P. Giuchedi, PLGA microspheres for the ocular delivery of a peptide drug, vancomycin using emulsification/spray-drying as the preparation method: In vitro/In vivo studies, Eur. J. Pharm. Biopharm. 57 (2004) 207-212. doi: 10.1016/j.ejpb.2003.10.018.

[20] M. L. Slevin, E.M. Piall, A. Johnston, D.A. Levison, G.W. Aherne, S.B. Tree, T.A. Lister, The pharmacokinetics of subcutaneous bolus cytosine arabinoside in an arachis oil plus aluminium distearate suspension, Invest. New Drugs 2 (1984) 271–276. doi: 10.1007/BF00175376.

[21] H.A.C. Titulaer, J. Zuidema, P.A. Kager, J.C.F.M. Wetsteyn, C.B. Lugt, F.W.H.M. Merkus, The pharmacokinetics of artemisinin after oral, intramuscular and rectal administration to volunteers, J. Pharm. Pharmacol. 42 (1990) 810–813. doi: 10.1111/j.2042-7158.1990.tb07030.x.

[22] C. Wang, W. Wang, R.F. Meyer, Y. Liang, K.I. Winey, S.J. Siegel, A rapid method for creating drug implants: translating laboratory-based methods into a scalable manufacturing process, J. Biomed. Mater. Res. B 93 (2010) 562-572. doi: 10.1002/jbm.b.31617.

[23] M.J. Patel, S.S. Patel, N.S. Patel, N.M. Patel, Current status and future prospects of drug eluting stents for restenosis, Acta Pharm. 62 (2012) 473–496. doi: 10.2478/v10007-012-0036-8.

[24] Monograph on "Injections and implanted drug products (parenterals) – product quality tests <1>". U.S. Pharmacopeia, ed. 41.

[25] Monograph on "Goserelin implants". U.S. Pharmacopeia, ed. 41.

[26] Monograph on "Goserelin implants". British Pharmacopeia, ed. 2018.

[27] V.R.S. Uppoor, Regulatory perspectives on in vitro (dissolution)/in vivo (bioavailability) correlations, J. Contr. Rel. 72 (2001) 127-132. doi: 10.1016/S0168-3659(01)00268-1.

[28] FDA portal. Drug database. Dissolution Methods. Available at: *https://www.accessdata.fda.gov/scripts/cder/dissolution/* [web access: August 12th, 2018].

[29] FDA. Draft guidance on naltrexone. September 2015.

[30] FDA. Draft guidance on risperidone. August 2016.

[31] FDA. Zilretta™ Extended-release injectable suspension. Product quality review (application No. 208845Orig1s000). Available at: *https://www.accessdata.fda.gov/drugsatfda_docs/nda/2017/208845Orig1s000TOC.cfm* [web access: August 12th, 2018].

[32] Y. Wang, W. Qu, S.H. Choi, FDA's Regulatory Science Program for Generic PLA/ PLGA-Based Drug Products. Am. Pharmaceut. Rev. 20 (2017), Available at: *https://www.americanpharmaceuticalreview.com/Featured-Articles/188841-FDA-s-Regulatory-Science-Program-for-Generic-PLA-PLGA-Based-Drug-Products/*.

[33] EMA portal. European public assessment reports. Available at: *http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/epar_search.jsp&mid=WC0b01ac058001d124* [web access: August 12th, 2018].

[34] EMA. Guideline ICH Q8 (R2) on Pharmaceutical development. EMEA/CHMP/167068/2004. 22 June 2017.

[35] FDA. Guidance for Industry. Extended release oral dosage forms: development, evaluation, and application of in vitro/in vivo correlation. September 1997.

[36] EMA. Guideline on the pharmacokinetic and clinical evaluation of modified release dosage forms. EMA/CPMP/EWP/280/90 Corr 1. 20 November 2014.

[37] J. Shen, S. Choi, W. Qu, Y. Wang, D.J. Burgess, In vitro-in vivo correlation of parenteral risperidone polymeric microspheres, J. Contr. Rel. 218 (2015) 2–12. doi: 10.1016/j.jconrel.2015.09.051.

[38] J.V. Andhariya, J. Shen, S. Choi, Y. Wang, Y. Zou, D.J. Burgess, Development of in vitro-in vivo correlation of parenteral naltrexone loaded polymeric microspheres, J. Control. Rel. 255 (2017) 27-35. doi: 10.1016/j.jconrel.2017.03.396.

[39] M. Siewert, J. Dressman, C.K. Brown, V.P. Shah, et al. FIP/AAPS Guidelines to dissolution/in vitro release testing of novel/special dosage forms, AAPS PharmSciTech 4 (2003) E7. doi: 10.1208/pt040107.

[40] S.S. Iyer, W.H. Barr, H.T. Karnes, Profiling in vitro drug release from subcutaneous implants: a review of current status and potential implications on drug product development, Biopharm. Drug Dispos. 27 (2006) 157-170. doi: 10.1002/bdd.493.

[41] $314.92, Subpart C, Part 314 Application for FDA approval to market a new product, Subchapter D, Chapter I, Section 21 'Food and Drugs' of the Code of Federal Regulations. United States Government Publishing Office; Article 10, Dir. 2001/83/CE of 6 November 2001 as amended.

[42] Article 10, Dir. 2001/83/CE of 6 November 2001 as amended.

[43] $314.94(a)(9)(iii), Subpart C, Part 314 Application for FDA approval to market a new product, Subchapter D, Chapter I, Section 21 'Food and Drugs' of the Code of Federal Regulations. United States Government Publishing Office

[44] FDA. Guidance for Industry. Controlled correspondence related to generic drug development. Draft guidance. November 2017.

[45] FDA. Guidance for Industry. Formal Meetings between FDA and ANDA applicants for complex products under GDUFA. Draft guidance. October 2017.

[46] S. D'Souza, J.A. Fara, S. Giovagnoli, P.P. DeLuca In vitro-in vivo correlation from lactide-co-glycolide dosage forms, Progress Biomat. 3 (2014) 131-142. doi: 10.1007/s40204-014-0029-4.

[47] J. Garner, S. Skidmore, H. Park, K. Park, S. Choi, Y. Wang, Beyond Q1/Q2: the impact of manufacturing conditions and test methods on drug release from PLGA-based microparticle depot formulations, J. Pharm. Sci. 107 (2018) 353-361. doi: 10.1016/J.XPHS.2017.10.027.

[48] FDA. Draft guidance on Leuprolide Acetate/Norethindrone Acetate. February 2018.

[49] EMA. Octreotide acetate depot powder and solvent for suspension for injection 10 mg, 20 mg, 30 mg product – specific bioequivalence guidance, draft (EMA/CHMP/291571/2018). May 2018.

[50] Subpart B, Part 314, Subchapter D, Chapter I, Section 21 'Food and Drugs' of the Code of Federal Regulations. United States Government Publishing Office.

[51] FDA. Guidance for industry – Changes to an approved NDA or ANDA. April 2004.

[52] Regulation (EU) 712/2012 of the European commission of 3 August 2012 amending Regulation (EC) No. 1234/2008 concerning the examination of variations to the terms of marketing authorisations for medicinal products for human use and veterinary medicinal products.

[53] European Commission. Guidelines on the details of the various categories of variations, on the operation of the procedures laid down in Chapter II, IIa, III and IV of Commission Regulation (EC) No 1234/2008 of 24 November 2008 concerning the examination of variations to the terms of marketing authorizations for medicinal products for human use and veterinary medicinal products and on the documentation to be submitted pursuant to those procedures. August 2013.

[54] EMA. Guideline on the investigation of bioequivalence (CPMP/EWP/QWP/1401/98 Rev. 1/ Corr **). January 2010.

[55] $320.22(d), Subpart B, Part 320 Bioavailability and bioequivalence requirements, Subchapter D, Chapter I, Section 21 'Food and Drugs' of the Code of Federal Regulations. United States Government Publishing Office.

[56] 21 CFR, $ 320.22, letter (d)(1)(iv).

[57] Stevens R.E., Gray V., Dorantes A., Gold L., Pham L. (2015) Scientific and regulatory standards for assessing product performance using the similarity factor, f2. The AAPS Journal, 17, 301-306.

[58] FDA. Guidance for Industry – Comparability protocols for human drugs and biologics: chemistry, manufacturing, and controls information. Draft guidance, revision 1. April 2016.

[59] FDA. Draft guidance on Goserelin Acetate. October 2008.

[60] FDA. Draft guidance on Leuprolide Acetate. February 2014.

[61] FDA. Draft guidance on Leuprolide Acetate. July 2008.

[62] FDA. Draft guidance on octreotide acetate. February 2014.

[63] FDA. Draft guidance on triptorelin pamoate. February 2014.

Chapter 5

Biorelevant release testing of biodegradable microspheres intended for intra-articular administration

Abstract

Characterization of controlled release formulations used for intra-articular (IA) drug administration is challenging. Bio-relevant synovial fluids (BSF), containing physiologically relevant amounts of hyaluronic acid, phospholipids and proteins, were recently proposed to simulate healthy and osteoarthritic conditions. This work aims to evaluate the performance of different controlled release formulations of methylprednisolone (MP) for IA administration, under healthy and disease states simulated conditions. Microspheres differed in grade of poly(lactide-co-glycolide) and in the theoretical drug content (*i.e.* 23 or 30% w/w). Their performance was compared with the commercially available suspension of MP acetate (MPA). Under osteoarthritic state simulated condition, proteins increased the MPA release and reduced the MPA hydrolysis rate, over 48h. Regarding microspheres, the release patterns over 40 days were significantly influenced by the composition of BSF. The pattern of the release mechanism and the amount released was affected by the presence of proteins. Protein concentration affected the release and the concentration used is critical, particularly given the relevance of the concentrations to target patient populations, *i.e.* patients with osteoarthritis.

5.1 Introduction

Corticosteroids locally administered by intra-articular injections represent one of the major treatment for arthritis, osteoarthritis or musculoskeletal disorders to reduce pain and inflammation, facilitate motion and function [1]. Due to lymph drainage of synovial fluids, the drug residence into the joint is very short even when prodrugs are used [2,3] and, thus, systemic side effects have been frequently reported [2]. To overcome this limitation, the controlled release of drugs loaded in microspheres made of poly(lactide-co-glycolide) [PLGA] have been proposed [4,5] and the efficacy and bioavailability of methylprednisolone loaded in PLGA matrix has been demonstrated in an animal model [6]. The optimization of these drug delivery systems is challenging, as compendial *in vitro* drug release tests are not described in the main Pharmacopoeias. The sample-and-separate or modified USP 4 apparatus methods using a buffer as release medium and sink conditions have been proposed in the literature for the screening of different PLGA-based microspheres formulations and the evaluation of batch-to-batch variability [7]. However, an *in vitro* release experimental set-up reflecting the *in vivo* conditions, which would assist in formulation development and prediction of the *in vivo* performance, is missing. For example, sink conditions which are generally applied in quality control testing are not bio-relevant in some anatomic sites, such as in the sub-cutaneous or the intra-muscular environment [8,9]. Moreover, simple buffers do not reflect the composition of physiological fluids in healthy or disease states. In the case of joints synovial fluids of healthy subjects and osteoarthritic patients, it has been demonstrated that these fluids significantly differ qualitatively and quantitatively in their composition [10], and they present different physicochemical properties, such as viscosity, osmolarity, surface tension and pH [11]. The simulation of the synovial fluid in both healthy and disease states can play a crucial role in the development of *in vitro* release/dissolution testing for intra-articular formulations. Up to date, there are no synovial fluid-simulating media approved by Regulatory Agencies, and limited information on the impact of their composition on the drug release and

dissolution are reported. For instance, the addition of hyaluronic acid in a buffer system is the main focus of the published simulated synovial fluids used as release media [12–15]. Recently, bio-relevant synovial fluids containing physiologically relevant amounts of hyaluronic acid, phospholipids and proteins were proposed to evaluate the release profile of an approved triamcinolone suspension and predict performance of intra-articular formulations [16].

The main goal of the present study was to evaluate the *in vitro* release behaviour of PLGA microspheres loaded by methylprednisolone, in media simulating synovial fluids under healthy and disease state conditions. Microspheres were prepared by using two grades of PLGA, differing in the lactide/glycolide ratio, and encapsulating different amounts of drug. Preliminarily, the proposed bio-relevant synovial fluids were used to test the release of methylprednisolone acetate from the aqueous suspension of the drug available on market (Depo-Medrone®), approved for the treatment of joint disease such as osteoarthritis. For both the types of formulations (*i.e.* drug loaded PLGA microspheres and drug aqueous suspension) the influence of the main components of bio-relevant synovial fluids was evaluated.

5.2 Materials

Two different grades of poly(D,L-lactide-co-glycolide) (PLGA) were kindly donated by Corbion Purac Biomaterials (NL): Purasorb® PDLG 5002 (PLGA 5050) and Purasorb® PDLG 7502 (PLGA 7550; their characteristics are presented in **Table A1**). A grade of hydroxypropyl methylcellulose at low viscosity (Methocel® K100 LV, HPMC) was kindly provided by Colorcon (I). Methylprednisolone (MP) was obtained by Farmalabor (I), and Depo-Medrone® (methylprednisolone acetate [MPA] aqueous suspension, 40 mg/ml) was purchased from Pfizer Ltd (UK). Hyaluronidase from bovine testes type VIII (lyophilized powder, range of activity between 300 and 1000 Units/mg) and γ-globulin from bovine blood were purchased by Sigma-Aldrich (UK). Sodium hyaluronate 95% (HA) and bovine serum

albumin (BSA) were obtained from Fischer Scientific (UK). Egg phosphatidylcholine (PC) was purchased from Lipoid (G) and polysorbate 80 (Tween® 80) from Croda (I). Glass microfiber membrane and syringe filter (GF/D, pore size 2.7 μm) and regenerated cellulose syringe filter (RC, pore size 0.45 μm) were obtained from Whatman GE Healthcare Life Sciences (UK). Nitrocellulose membrane filters (NC, pore size 1.2 μm) were purchased by Millipore (I). Syringe filters of 0.2 and 0.45 μm pore size were purchased by VWR International (USA). All the other chemicals were bought by Fischer Scientific (UK) and all the solvents used were of analytical grade.

5.3 Methods

5.3.1 Bio-relevant synovial fluids preparation

Bio-relevant synovial fluids (BSF) were prepared according to the compositions and the protocols reported by Nikolettos I. [16]. BSF reflecting healthy (H-BSF) and osteoarthritic (OA-BSF) conditions contained physiologically relevant amounts of HA and PC with their ratio being 1:1.7 and 1:0.6 for OA and H conditions, respectively, γ-globulin and BSA. The pH of H-BSF was 7.4 and the pH of OA-BSF was 8. The OA-BSF was also prepared without BSA and γ-globulin [OAwp-BSF] in order to investigate the influence of the presence of proteins on the drug solubility and its release from the formulations under test.

5.3.2 Bio-relevant synovial fluid-sample treatment

The hyaluronidase type VIII solution was freshly prepared by dissolving the enzyme at the concentration of 1 mg/mL in sodium phosphate buffer at pH 7.0 with 77 mM NaCl and 0.01% w/v BSA. Biorelevant synovial fluid samples were treated with hyaluronidase solution to obtain a final concentration of 150 units/mL of the enzyme prior the HPLC analysis [17].

5.3.3 Solubility study

The MP solubility was studied by the shaking-flask method in healthy and disease states BSF and in PBS (pH 7.4) containing 0.02% w/v of SDS (PBS/SDS). Briefly, after the addition of an excess amount of the drug to each medium studied, the suspension was vortexed and incubated in a horizontal shaking water bath (Fisher Scientific, UK) at 37.0 ± 0.5°C for 48h. Samples were withdrawn at 24 and 48h, filtered through a 0.45 μm RC (samples from PBS/SDS) or 2.7 μm GF (samples from BSF) filters to remove the undissolved particles and the amount of dissolved drug was quantified by HPLC. Before injection, samples from BSF were treated with a hyaluronidase solution (section 5.3.2), filtered through 0.45 μm RC filter and diluted accordingly. Solubility studies were performed in triplicate.

5.3.4 Preparation of drug loaded microspheres

MP loaded microspheres were produced by the solid-in-oil-in-water (S/O/W) method, as described by Cilurzo et al., with minor modifications [5]. Briefly, MP was dispersed in 1 mL of 20 wt. % PLGA solution in dichloromethane by sonication with an ultrasound probe (UP200St, 7 mm diameter, Hielscher, G) at an amplitude of 20% for 5 s, and cooling the sample in an ice-bath. The amount of MP was fixed to obtain a theoretical drug loading of 23 or 30%. The S/O suspension was added dropwise into 25 mL of 2.5 % w/v solution of HPMC at 4.0 ± 0.5 °C, under mechanical stirring with a propeller (600 rpm, 5min). The S/O/W system was poured into 250 mL of ultrapure water cooled at 4.0 ± 0.5 °C and the temperature was gradually increased till 30 ± 1 °C. Hardened particles were recovered by filtration under vacuum using a 1.2 μm NC membrane filter, washed with ultrapure water, suspended in water and freeze-dried (Martin Christ Alpha 1-4 LSC Plus, G). Dried samples were stored under vacuum at 5 ± 3 °C until use. All formulations were prepared in duplicate.

5.3.5 Determination of polymer molecular weight

Molecular weight distribution of raw polymers and loaded microspheres before and after the 40-day release studies were measured by gel permeation chromatography (GPC). Samples of about 5-6 mg were dissolved in dichloromethane and filtered through a 0.45 μm PTFE syringe filter prior the injection, to remove the undissolved particles. The instrument was equipped with a G1379A degasser, a G1310A isocratic pump, a G1313A auto-sampler, a G1316A thermostated column compartment and double detector: refractive index detector G1362A and UV/visible detector (G1314A) set at λ=230 nm. Three columns (Phenogel™ 300x4.6 mm, Phenomenex, I) with gel pore size of 104 Å, 103 Å and 500 Å were connected in series. The mobile phase was dichloromethane at a flow rate of 0.35 mL/min at a temperature of 25.0 ± 0.1 °C. An injection volume of 70 μL was used. The weight-average molecular weight (M_w) and the number-weight molecular weight (M_n) of each sample were calculated using monodisperse polystyrene standards with M_w ranging from 486 to 188,000 Da and a GPC-Addon HP ChemStation software (Hewlett-Packard Co., USA) to compute molecular weight distribution (Agilent, USA). Dispersity index (DI) was calculated by the ratio between M_w and M_n.

5.3.6 Microspheres size distribution and morphology

The mean particle size and the size distribution of microspheres suspended in 0.05% polysorbate 80 solution were evaluated by the single particle optical sensing (SPOS) technique, using an Accusizer 770 (PSS Inc. USA). The results were expressed as undersize cumulative percentages and the dispersion of the size distribution as Span [**Eq. (1)**].

$$Span = \frac{d_{90} - d_{10}}{d_{50}}$$

Eq. (1)

where d_{10}, d_{50} and d_{90} represent the diameters at 10, 50 and 90% of the size volume distribution, respectively.

All the samples were analyzed in triplicate and the results reported as mean ± SD. Microspheres morphology before and after the 40-day release was investigated by scanning electron microscopy (SEM, JEOL 6480 LV, JEOL, USA), at an electron beam voltage of 10 kV. Dried samples were rigidly mounted on an aluminum stub using a carbon adhesive and placed under vacuum overnight in order to remove residual moisture. Before images collection and to improve their resolution, samples were coated with a thin layer of gold, using a sputter coater S150B (Edwards, UK) for 5 min.

5.3.7 MP content in the microsphere formulations

The amount of MP encapsulated in the microspheres was quantified by the HPLC method described in paragraph 5.3.10. MP was extracted from 10 mg of dried microspheres placed in 50 mL of a water/acetonitrile mixture (1:1) for 24h at room temperature. Each sample was filtered through a 0.2 μm nylon syringe filter before the HPLC analysis. All the measurements were performed in triplicate. The experimental MP loading % and the encapsulation efficiency (EE) % were calculated based on **Eq. (2)** and **Eq. (3)**, respectively.

$$Experimental\ MP\ loading\ \% = \frac{amount\ of\ MP\ entrapped\ in\ microspheres}{mass\ of\ microspheres} x\ 100$$

Eq. (2)

$$EE\ \% = \frac{amount\ of\ MP\ entrapped\ in\ microspheres}{theoretical\ amount\ of\ MP} x\ 100$$

Eq. (3)

5.3.8 ATR-FTIR spectroscopy

IR spectra were recorded using a Spectrum™ One spectrophotometer (PerkinElmer, USA) equipped with a diamond crystal mounted in a ATR cell (PerkinElmer, USA). Samples of MP and drug loaded microspheres were scanned with a resolution of 4 cm-1 over a wavenumber region between 4000 and 650 cm-1. 64 scans for each sample were collected. Baseline and ATR correction were performed on each spectrum.

5.3.9 *In vitro* release studies

All the release studies were carried out by the sample-and-separate method in a 50 mL-glass bottle closed by screwed cap at 37.0 ± 0.5 °C, in a horizontal shaking water bath set at the mild agitation of 250 strokes/min. An exact volume of MPA aqueous suspension from the marketed formulation (Depo-Medrone®), corresponding to 2 mg of MP, was placed in 20 mL of PBS/SDS or BSF (H-BSF, OA-BSF and OAwp-BSF). After 0.5, 1, 2, 3, 7, 24 and 48h a 4 mL-sample was withdrawn through a 2.7 μm GF filter and the sampled volume was replaced with fresh medium. The quantification of MPA and its hydrolysis product (MP) was performed by HPLC (paragraph 5.3.10). The formation rate constant of MP (k_{MP}) was calculated from the first order fitting of MP amount over time [**Eq. (4)**] using OriginPro® 2015 software (OriginLab Corporation, USA).

$$Y = Y_{\max}(1 - e^{k_{MP}t})$$

Eq. (4)

where Y is the % amount of MP formed at time t and Y_{max} is the maximum % of MP formed over time.

The goodness of the fit was evaluated by the adjusted R^2 and by the residual sum of squares.

The release profiles of MPA were also corrected for hydrolysis by transformation of the MP quantified amount to MPA amount.

Similarly, drug loaded microspheres were exactly weighed to obtain 2 mg of MP, properly dispersed in 1 mL of the buffer used for the preparation of each BSF and then transferred in the release medium, reaching a final volume of 20 mL. After 1, 3, 7, 24h, 3, 7 days and then once a week until 40 days, a 4 mL-sample was withdrawn as described above. Each sample from the studies in BSF was treated with a hyaluronidase solution prior to HPLC analysis (paragraph 5.3.2). A release study of formulation MS 50-23 in PBS with 0.02% w/v SDS was also performed. Sink conditions were achieved for all the release studies. All release studies were performed in triplicate. The release rate constant was calculated according to Higuchi's models [**Eq. (5)**].

$$\frac{M_t}{M_\infty} = kt^{0.5}$$

Eq. (5)

where M_t represents the amount of MP released at time t, M_∞ is the amount of MP loaded in the matrix and k is the constant rate of drug diffusion.

After 40 days and prior to the recovery of the microspheres, the pH of each medium was measured and reported as mean ± SD. Microspheres were then recovered by centrifugation (Hettich 1605-13 Universal 32 Centrifuge, G) at 8,000

rpm for 10 min at room temperature, washed three times with ultra-pure water, filtered (1.2 µm RA membrane) and dried under vacuum before GPC and SEM analyses.

5.3.10 HPLC analysis

MP and MPA were quantified by HPLC, using an Agilent HP1200 series (Agilent, UK) equipped with a G1312A binary pump, a G1329A auto-sampler, a G1316A thermostated column compartment and a G1315D UV detector. A Phenomenex® Inertsil ODS-2 (C18, 250 x 4.60 mm, 5 µm) column was used for the reversed phase chromatography [18]. The mobile phase was a mixture of water and methanol in the volume ratio of 70:30, at the flow rate of 1 mL/min. The injection volume was 50 µL and the temperature 35 °C. The detection of MP and MPA was performed at 247 nm. For the BSF samples, a gradient method was applied after the isocratic elution of MP and MPA, increasing the water content up to 90% over 12 min. MP and MPA calibration curves in the range of 1-10 µg/mL were freshly prepared in each medium prior each experiment (R^2 > 0.99). Standard solutions of MP and MPA in BSF were prepared by adding the relative amount of MP or MPA working solution at 20 µg/mL (in PBS) to the BSF and treating them as reported previously (paragraph 5.3.2).

5.3.11 Statistical analysis

Release data from marketed formulation were analyzed by one-way ANOVA followed by Tukey test for pair-wise means comparison (α=0.05), using OriginPro® 2015 software (OriginLab Corporation, USA).

Comparison of two experimental means from solubility data and release data from microsphere formulations were performed using unpaired student t-test to determine two-tailed p values at 95% confidence level.

5.4 Results and discussion

5.4.1 Solubility study

The equilibrium solubility of MP in healthy and disease states BSF (with and without proteins) was reached after 24h and the results are summarized in **Table 5.1**. Among the different BSF studied, the highest value of MP solubility was found in the osteoarthritic medium with proteins ($p<0.05$ in all the cases); while the lowest in the same medium without proteins (OAwp-BSF vs H-BSF $p=0.035$ and OAwp-BSF vs OA-BSF $p<0.01$). The MP solubility in OAwp-BSF was similar to the one in PBS/SDS ($p=0.190$), in accordance also with the value reported in literature [19]. The lower solubility values in the media without proteins compared to the ones where they were present can be attributed to the fact that particularly BSA can bind molecules through hydrophobic and electrostatic interactions [20] and act like a solubilizing agent, as in the case of ketoconazole, danazole, felodipine [21], itraconazole [22] and cholesterol [23]. Considering that MP has an albumin binding of approximately 78% [24], BSA can act similarly in the tested BSF influencing its solubility, as also revealed by the higher value resulted in H-BSF compared to PBS/SDS (H-BSF vs PBS/SDS $p=0.011$, **Table 5.1**). Being a surfactant [25], PC impacts MP solubility, even though in a lower extent, as demonstrated by the comparison of the values in OA-BSF and H-BSF (**Table 5.1**).

Table 5.1 – MP solubility in different media after 24h, expressed as mean ± SD (n=3).

Medium	Ratio % HA:PC	Ratio % BSA:γ-globulin	MP solubility (μg/mL)
PBS/SDS	-	-	$118.7 \pm 5.3^{*}$
OAwp- BSF	95:5	-	$112.8 \pm 0.9^{*}$
OA-BSF	95:5	87:13	161.3 ± 0.2
H-BSF	98:2	87:13	136.9 ± 3.2

*MP solubility in PBS/SDS and OAwp-SDS were not statistically different (p>0.05)

Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS].

5.4.2 Microspheres formulations: preparation and characterization

The S/O/W method allowed to prepare particles suitable for the intra-articular administration [4], with a size ranging between 10 and 43 μm, a narrow size distribution and satisfactory drug encapsulation (**Table 5.2**).

SE micrographs of MP loaded microspheres (**Fig. 5.2**) showed that all the particles were spherical in shape. When the lowest amount of MP was loaded (formulation MS 50-23 and MS 75-23), small pores were evident on the surface of the particles, while when the highest amount of drug was encapsulated (formulation MS 50-30), particles had many holes since that the polymeric matrix seemed to be not completely formed.

Experimental MP loading and encapsulation efficiency were slightly higher when PLGA 5050 was used (formulation MS 50-23) instead of PLGA 7525 (formulation MS 75-23, **Table 5.2**), probably due to the lower rigidity of PLGA with a similar content between lactic and glycolic acids (**Table A1**). The increase of MP amount led to a higher loading of the drug in the microspheres with PLGA 5050 (MS 50-30 vs MS 50-23), with an encapsulation efficiency comparable to the one obtained for formulation MS 75-23. The GPC data confirmed that the use of an ultrasound probe to prepare microspheres did not have a detrimental effect on polymers,

since the M_w calculated for all the microsphere formulations were superimposable to those of the starting materials (**Table 5.4**). Additionally, the ultrasounds or the evaporation of the solvent during microspheres' formation did not change the solid state of MP that was encapsulated as Form I. This is evidenced by the three strong absorption bands between 1800 and 1580 cm^{-1} of the ATR-FITR spectrum that were attributed to the stretching of carboxylic acid and ketone groups (1650 and 1720 cm^{-1}, respectively) and to the aromatic bending (1592 cm^{-1}) [**Fig. 5.2**] [26].

Table 5.2 – Characterization of microspheres in terms of particle size distribution (undersize cumulative percentage of the volume distribution), and polydispersity of the size distribution (Span). All the results are expressed as mean ± SD (n=3).

Form. ID	L/G ratio	*Drug loading*			*Size distribution*			
		Theoretical (%, w/w)	Actual (% w/w)	EE%	d_{10}[a] (µm)	d_{50}[b] (µm)	d_{90}[c] (µm)	Span
MS 50-23	50:50	23	18.8±0.3	81.7±1.2	11.1±1.2	14.4±1.7	31.6±3.7	1.2±0.1
MS 75-23	75:25	23	17.0±0.4	73.5±1.9	10.7±1.0	23.1±2.8	42.5±3.7	1.4±0.1
MS 50-30	50:50	30	22.1±0.5	73.8±1.8	9.6±0.2	18.6±0.5	36.4±2.4	1.4±0.1

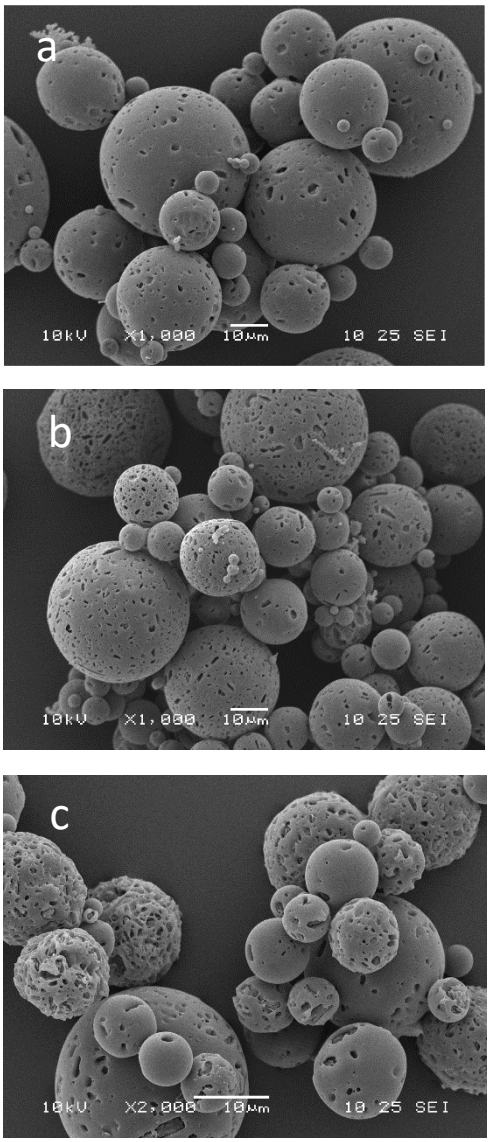

Fig. 5.1 – SE micrographs of MP loaded microsphere formulations. (a: formulation MS 50-23; b: formulation MS 75-23; c: formulation MS 50-30).

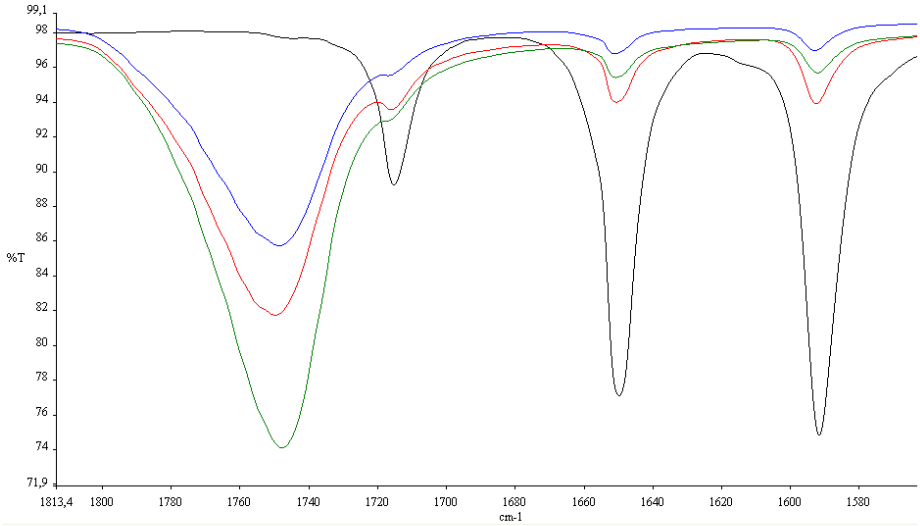

Fig. 5.2 – ATR-FTIR spectra of MP (black line), formulation MS 50-23 (blue line), formulation MS 50-30 (red line) and formulation MS 75-23 (green line).

5.4.3 *In vitro* release studies

5.4.3.1 Marketed formulation of methylprednisolone acetate

The release profiles of MPA aqueous suspension in all BSF and PBS/SDS showed that both the release and the hydrolysis of MPA were depended on the medium composition (**Fig. 5.3a**). After 24h the highest MPA release from the suspension was observed in the OA-BSF (29.2±2.2%, $p<0.05$ compared to the other media tested), whereas a lower and similar release was seen in the H-BSF and in PBS/SDS (22.6±1.6% and 21.6±1.9% respectively, $p=0.557$). This trend confirms the influence of proteins on drug release and this was even more evident from the significantly lower amount of MPA released in OAwp-BSF (8.2±1.2%) compared to OA-BSF. In all media tested, MPA underwent hydrolysis according to first order kinetics (**Fig. 5.3b**). The rate of MP formation was medium-depended (**Table 5.3**), with the MPA hydrolysis rate constant in H-BSF being significantly different than the hydrolysis rate constants in all the other media ($p<0.05$). These differences reveal the importance of the simulation of healthy and pathological status during the in vitro studies. After the correction of the

release profiles of MPA to account for the hydrolyzed drug (**Table 5.3**), at 48h the highest MPA release was observed in OA-BSF ($p<0.05$). MPA release from the suspension in the other media was lower, following the rank order: H-BSF, OAwp-BSF and PBS/SDS. The biorelevant simulation of the synovial fluid under healthy and osteoarthritic conditions is critical and the release in these conditions was significantly different than in a simple buffer with a surfactant (% corrected MPA release at 48h: PBS/SDS *vs* OA-BSF $p=0.008$ and PBS/SDS *vs* H-BSF $p=0.023$). The presence of proteins in the release medium led to an increased % amount of MPA released, suggesting that their presence should be carefully considered (OA-BSF *vs* OAwp-BSF $p=0.038$, **Table 5.3**).

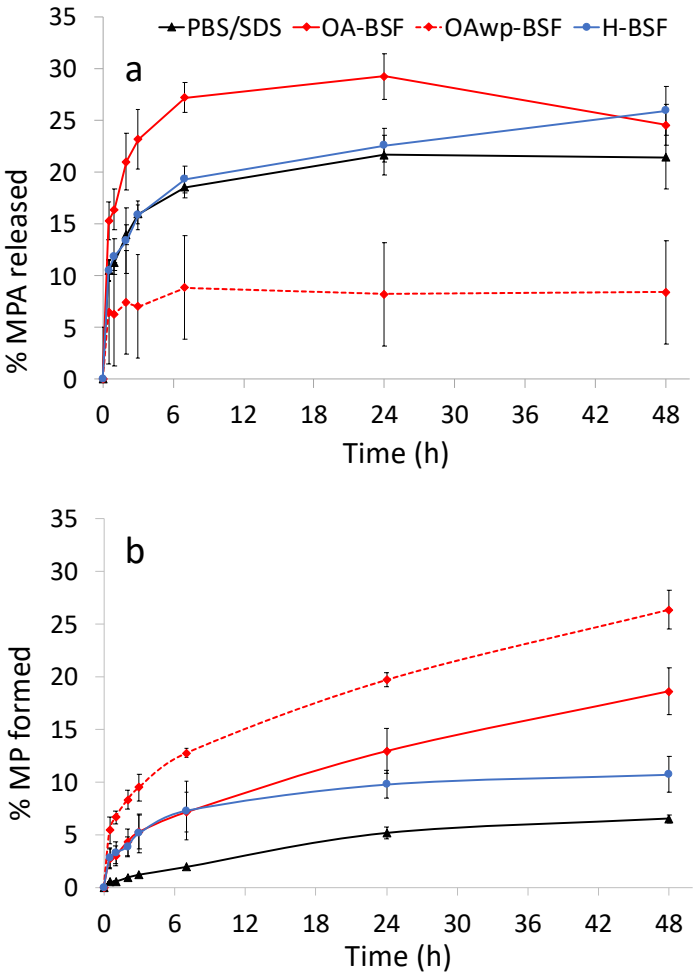

Fig. 5.3 – Cumulative percentage of MPA released (a) and MP formed (b) in the tested media from Depo-Medrone® formulation (osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS]).

Table 5.3 – Maximum % MPA released from Depo-Medrone® corrected for hydrolysis and goodness (adjusted R^2 (Adj R^2) and residual sum of square (RSQ)) of the first order fitting of the MP formation (k_{MP}). Results are reported as mean ± SD (n=3).

Release medium	MPA (%)	k_{MP} (h^{-1})	Adj R^2	RSQ
PBS/SDS	29.4±2.7	0.053±0.003	0.99±0.00	0.001±0.000
OAwp-BSF	34.7±2.2	0.166±0.015	0.87±0.04	0.163±0.060
OA-BSF	43.1±3.7	0.084±0.051	0.93±0.02	0.051±0.019
H-BSF	36.6±2.0	0.293±0.080	0.93±0.01	0.018±0.006

Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS].

5.4.3.1 Methylprednisolone loaded PLGA microspheres

In all tested media, a high burst release of MP from the formulation with the highest drug loading (MS 50-30) was observed in the first hour (**Fig. 5.4a**). This effect can be explained based on the IR spectrum of this formulation in which the high intensities of MP bands at 1592 and 1650 cm^{-1} was attributed to the high amount of surface-associated drug particles (**Fig. 5.2**). Moreover, the high discontinuity of PLGA matrix at microspheres' surface (**Fig. 5.1**) allowed the medium to enter quickly, fill the empty channels and dissolve the drug that then diffused out [27]. The burst effect of MS 50-30 was less pronounced in OAwp-BSF (MP released in 1h in OA-BSF=47.5±0.7% and in OAwp-BSF=29.4±1.1%, $p<0.05$), suggesting that the presence of proteins in the release medium influenced not only the drug solubility, but also the wettability of the matrix, as revealed by the SEM (**Fig. 5.5a-c**). After the burst effect, MP release reached a plateau after 3 days only in H-BSF and OA-BSF (49.6±4.3% and 46.3±2.5% in H-BSF and OA-BSF, respectively). On the contrary, a constant release occurred in OAwp-BSF ($k_{50\text{-}30\_OAwp}$: 0.032±0.002 $days^{-0.5}$; R^2=0.96±0.03). In this medium, a 59.4±2.6% MP was released after 40 days, that is significantly higher than the % MP released in the other media (OAwp-BSF *vs* H-BSF p=0.004, OAwp-BSF *vs* OA-

BSF p=0.048), revealing the effect of proteins on the amount of drug released from MS 50-30.

Concerning the formulation MS 75-23, only the burst MP release of around 20% after the first hour was seen in the H-BSF (**Fig. 5.4b**). The SE micrograph of the microspheres recovered after 40 days in this medium showed that the particles had a completely smooth surface, called the "*skin*" type structure (**Fig. 5.5d**), probably due to a remodelling/healing process occurred in the plasticized particles over time [28]. This phenomenon determined the occlusion of the pores and the inability of the drug to be continuously released out of the particles. MS 75-23 behaved differently in OA-BSF as a typical tri-phasic release was noticed, with a burst effect similar to the other two media (22.4±0.2 % at t=1h, p>0.05), a lag-phase of about 7 days and a second release phase which fitted the Higuchi model ($k_{75\text{-}23\_OA}$=0.051±0.024 $days^{-0.5}$; R^2=0.90±0.04) [**Fig 5.4b**]. After day 21, a plateau on MP release was reached (37.5±2.3 %). In absence of proteins (OAwp-BSF), a completely different shape of the MP release profile was obtained: after the burst effect, a continuous release of MP without lag phase was obtained and the release data was well characterized by the Higuchi model ($k_{75\text{-}23\_OAwp}$=0.025±0.002 $days^{-0.5}$; R^2=0.84±0.11, p>0.05 compared to $k_{75\text{-}23\_OA}$), indicating that the MP release was mostly governed by the diffusion, as also reported for the MS 50-30. However, at day 40, the % MP released was similar to the one in OA-BSF (about 37%). This can be explained by both morphological analysis (**Fig. 5.5e** and **f**) and GPC data of the recovered microspheres (**Table 5.4**) which demonstrated that the degradation of PLGA 7525 occurred similarly in OA-BSF and OAwp-BSF, with a reduction of the molecular weight of about 7 KDa.

Also in the case of MS 50-23, the MP release was dependent on the composition of the release medium (**Fig. 5.4c**), and a prolonged and controlled MP release was reached. The burst effect of about 6% at 1h was similar in H-BSF, OA-BSF and PBS/SDS (p>0.05). The % MP released from these microspheres in the first hour and after day 1 were lower than the corresponding ones from the MS 50-30 and MS 75-23. This difference can be attributed to the different distribution of the

drug within the polymer matrix during the microspheres preparation when PLGA 5050 and the lowest theoretical drug loading was used (MS 50-23) [29]. In H-BSF and PBS/SDS, MP release started after a lag phase of about 28 days, revealing that for releasing the drug it was necessary that polymer chains degradation reached a certain critical PLGA Mw. According to the SEM of the recovered microspheres after 40 days (**Fig. 5.5g** and **l**), a bulk-erosion controlled release was observed, that is typical of microspheres made of PLGA with relative low molecular weight (as PLGA 5050 used in this study) and encapsulating poorly soluble small molecules [30]. Despite the similarity in the release profiles, the microspheres did not behave in the same way in PBS/SDS and H-BSF, with microspheres recovered from H-BSF having a wrinkled surface compared to the ones from PBS/SDS (**Fig. 5.5g** and **l**). GPC data confirmed that MS 50-23 underwent a greater degradation in H-BSF than in the PBS/SDS, with a molecular weight loss of about 74% and 37%, respectively (**Table 5.4**). In the case of the MP release in BSF mimicking the disease state (OA-BSF), even though the burst effect was similar to the one observed in the simulated healthy conditions (H-BSF), MP release started after day 7, reaching a 27.8±1.6 % MP released at day 40, indicating that the simulation of disease state had an impact on the release. The absence of proteins resulted in a decrease of the burst effect, as a burst of 4.4% was measured in OAwp-BSF ($p=0.004$). Furthermore, in the same BSF medium, there was no lag phase and the MP release was characterised by the Higuchi model ($k_{50\text{-}23\_OAwp}$=0.050±0.004 $days^{-0.5}$; R^2=0.95±0.05, **Fig. 5.4c**), with about 37% of MP released after 40 days. Conversely, the MP released from MS 50-23 in OA-BSF seemed to follow the tri-phasic release: burst effect, lag phase of about 1 week and second pulse zero-order release from day 7 to day 21, fitting the Higuchi model with $k_{50\text{-}23\_OA}$=0.119±0.014 $days^{-0.5}$ (R^2=0.84±0.11). Afterwards, a plateau was reached (27.8±1.6 % after 40 days) probably due to the occurrence of healing processes, as evident in the SE micrograph (**Fig. 5.5h**). No significant differences in terms of M_w were detected in the microspheres recovered from OA-BSF and OAwp-BSF after 40 days (**Table 5.4**).

The overall release data from the performed studies clearly indicated that the presence of proteins influenced significantly the drug release, both in terms of the amount released and the release mechanism of MP from all types of microspheres. The presence of proteins in the bio-relevant synovial fluid has to be carefully considered. Their interactions with other components of the synovial fluid are not completely understood, but they affect properties of the synovial fluid, such as the surface tension, and consequently the performance of a drug delivery system inside the joint [31,32]. For these reasons, the disease state BSF was prepared with and without the proteins. Proteins interact with hydrophobic polymers, such as PLGA, and they can be adsorbed in a selective and a competitive manner onto the surface of nanoparticulate PLGA systems, forming the so called "*protein corona*" [33]. Among them, BSA, which has a good sequence identity with human serum albumin, is adsorbed onto PLGA nanoparticles better than other proteins, such as γ-globulin [34,35]. Based on these considerations, it can be assumed that similar interactions could also occur in the release studies carried out in BSF, determining the different mechanisms in MP release from the different microsphere formulations. A tri-phasic MP release in OA-BSF was observed from formulations MS 75-23 and MS 50-23, reaching a plateau at day 21 probably due to a polymer remodelling that closed the surface pores of microspheres, as previously discussed (**Fig. 5.5e** and **h**). These formulations after 40 days of incubation in OAwp-BSF presented a sponge-like structure which favours the release of MP according to a drug diffusion mechanism. For all the formulations, the protein content of the tested media also influenced the Higuchi constants, with the highest values obtained when proteins were added to the medium.

Both types of PLGA used in the microspheres (MS 50-23 and MS 50-23 prepared with PLGA 5050 and MS 75-23 prepared with PLGA 7525) underwent a more pronounced degradation after incubation in H-BSF compared to the other media. The hydrolysis of PLGA ester bonds starts immediately upon contact with the release medium and the acidic degradation by-products accumulate within

microsphere until a critical M_w is reached. As a result, the drop of micro-environmental pH catalyses the hydrolysis reaction, causing in some cases a heterogeneous degradation inside PLGA matrices [36,37]. The high viscosity of H-BSF could slow down the diffusion of PLGA oligomers allowing the establishment of the auto-catalysis phenomenon which accelerated PLGA degradation. Afterwards, the diffusion of PLGA degradation by-products outside microspheres determined the acidification of the H-BSF medium, which presents a low buffering capacity. On the other hand, in both the osteoarthritic media (OA-BSF and OAwp-BSF) the lower viscosity and the basic pH allowed a better oligomers' diffusion out of microspheres and their further neutralization. This hypothesis is supported by the pH of the BSF measured after the 40-day release (**Table 5.4**). The massive degradation occurred in H-BSF determined the greater drop in the pH value compared to the osteoarthritic media.

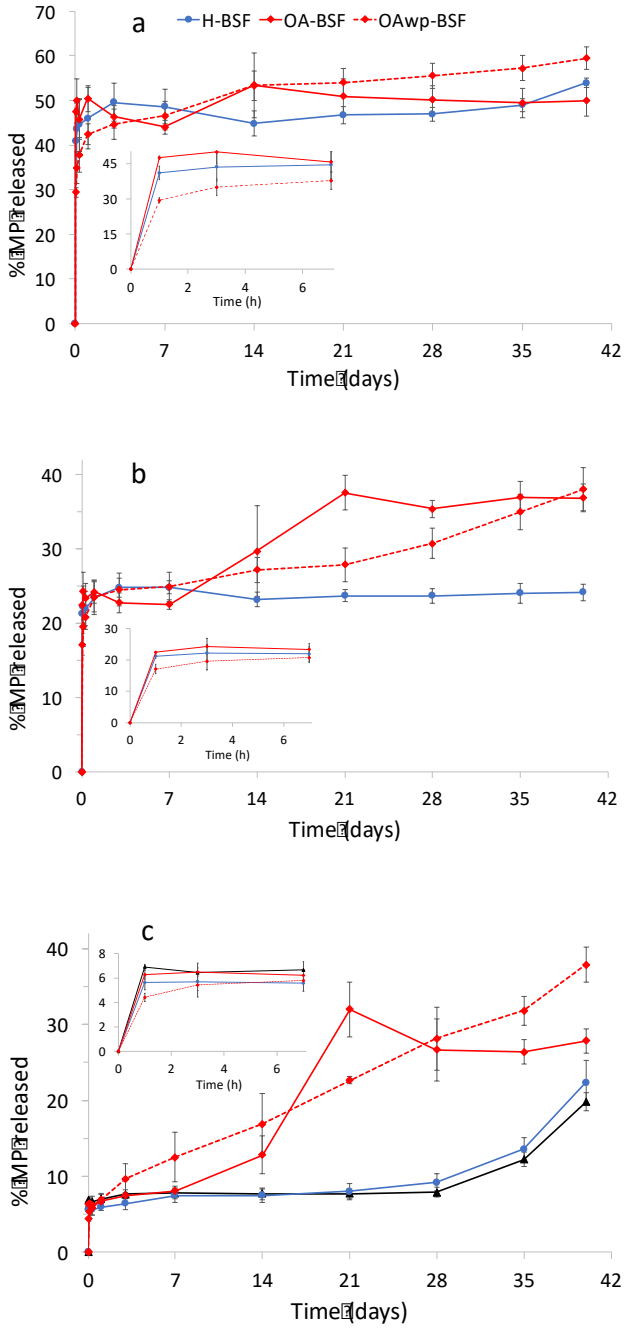

Fig. 5.4 – Cumulative percentage of MP released from formulation (a) MS 50-30, (b) MS 75-23 and (c) MS 50-23 in the tested media with the sample-and-separate method (osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS]); insert graphs: close up of first 7h.

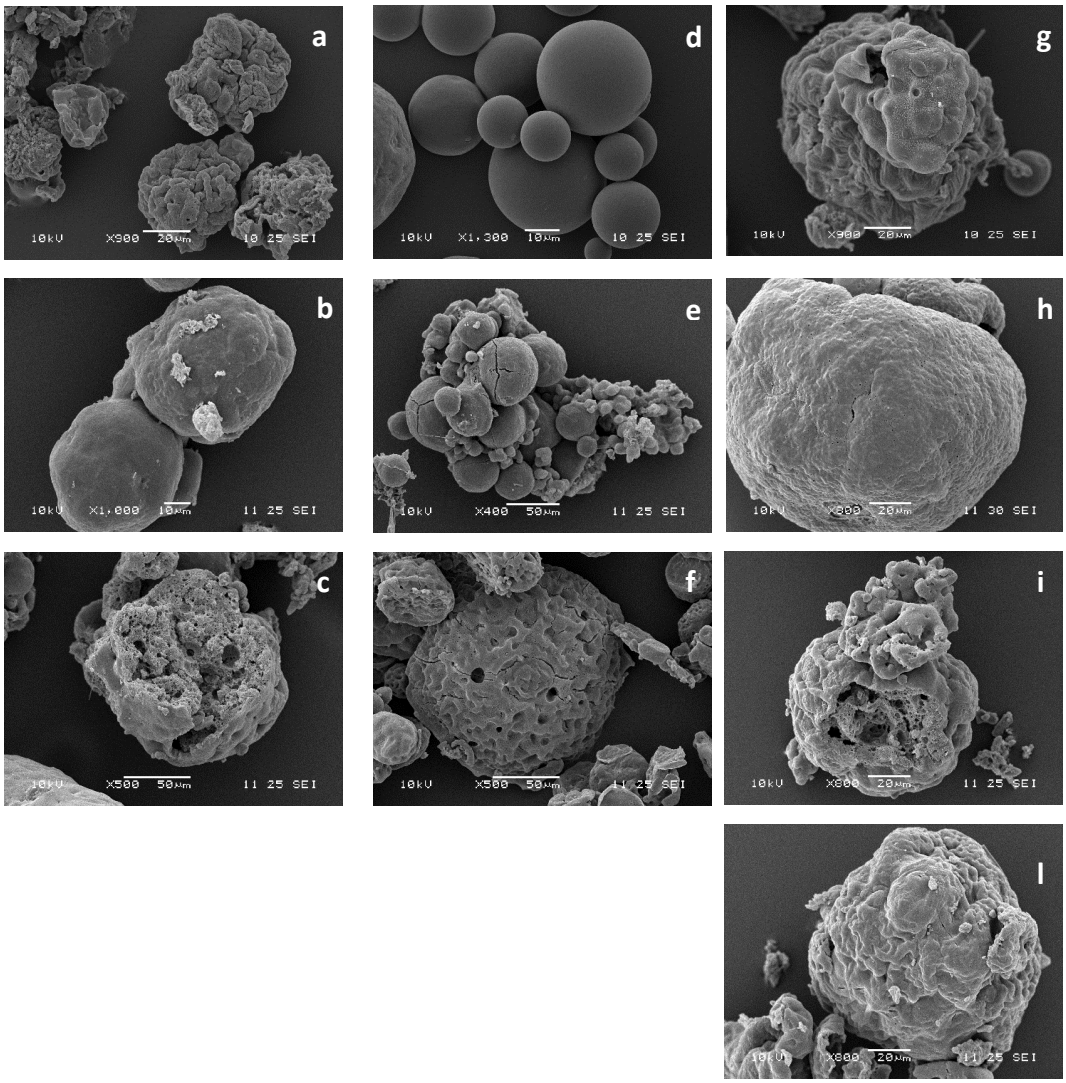

Fig. 5.5 – SE micrographs of MP loaded microsphere formulation recovered after day 40 of release; formulation MS 50-30 in (a) H-BSF, (b) OA-BSF and (c) OAwp-BSF; formulation MS 75-23 in (d) H-BSF, (e) OA-BSF and (f) OAwp-BSF; formulation MS 50-23 in (g) H-BSF, (h) OA-BSF, (i) OAwp-BSF and (l) PBS/SDS.

Table 5.4 – GPC data of PLGA microspheres after preparation (t=0 day) and after the 40-day release in different media. Molecular weight distribution is reported as weight average molecular weight (M_w) and polydispersity index (DI). The pH value of the release medium was measured after the 40 day-release and reported as mean ± SD (n=3).

Release medium	Time (days)	*MS 50-23*			*MS 75-23*			*MS 50-30*		
		M_w (KDa)	**DI**	**pH**	**M_w (KDa)**	**DI**	**pH**	**M_w (KDa)**	**DI**	**pH**
--	0	20.1	1.6	--	22.2	1.6	--	20.2	1.5	--
PBS/SDS	40	12.6	1.4	7.23±0.03	n.d.	--	n.d	n.d	--	n.d
OAwp-BSF	40	11.8	1.7	7.93±0.04	15.1	1.7	7.98±0.04	14.1	1.7	7.98±0.02
OA-BSF	40	10.5	1.5	7.39±0.05	13.7	1.7	7.38±0.02	12.6	1.6	7.43±0.12
H-BSF	40	5.2	1.3	5.85±0.09	9.7	1.6	5.76±0.24	4.7	1.3	6.03±0.03

Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS].

5.5 Conclusions

In the design and quality control of long-term release drug delivery systems, the availability of *in vitro* testing to characterize their biopharmaceutical performance is fundamental. This aspect is of great importance in the case of PLGA microspheres intended to locally administer a drug in a specific anatomic site. In the case of joints, the composition of the synovial fluid depends on the state of the subject (healthy *vs* pathological state) and such differences can impact the efficacy of an intra-articular medicinal product. In the present work, we proposed an advanced way to characterize MP loaded PLGA microspheres, simulating healthy and osteoarthritic status of the synovial fluid, that set the stage for the bio-relevant approach in an *in vitro* set up. The experimental results suggested that the release from both the marketed and microsphere formulations was affected by the medium composition, with a significant impact by the presence of proteins on the release mechanism and the hydrolysis rate. Furthermore, the proposed bio-relevant conditions permitted to discriminate among all formulations and individuate a possible candidate able to control MP prolonged release over a 30 day-period.

References

[1] W. Lavelle, E.D. Lavelle, L. Lavelle, Intra-articular injections., Med. Clin. North Am. 91 (2007) 241–50. doi:10.1016/j.mcna.2006.12.002.

[2] H. Derendorf, H. Möllmann, A. Grüner, D. Haack, G. Gyselby, Pharmacokinetics and pharmacodynamics of glucocorticoid suspensions after intra-articular administration, Clin. Pharmacol. Ther. 39 (1986) 313–317. doi:10.1038/clpt.1986.45.

[3] A. Panusa, L. Regazzoni, G. Aldini, M. Orioli, A. Giombini, P. Minghetti, C. Tranquilli, M. Carini, Urinary profile of methylprednisolone acetate metabolites in patients following intra-articular and intramuscular administration, Anal. Bioanal. Chem. 400 (2011) 255–267. doi:10.1007/s00216-011-4744-6.

[4] J.H. Ratcliffe, I.M. Hunneyball, A. Smith, C.G. Wilson, S.S. Davis, Preparation and evaluation of biodegradable polymeric systems for the intra-articular delivery of drugs, J. Pharm. Pharmacol. 36 (1984). doi:10.1111/j.2042-7158.1984.tb04419.x.

[5] F. Cilurzo, F. Selmin, P. Minghetti, L. Montanari, Design of methylprednisolone biodegradable microspheres intended for intra-articular administration., AAPS PharmSciTech. 9 (2008) 1136–1142. doi:10.1208/s12249-008-9158-1.

[6] A. Panusa, F. Selmin, G. Rossoni, M. Carini, F. Cilurzo, G. Aldini, Methylprednisolone-loaded PLGA microspheres: A new formulation for sustained release via intra-articular administration. A comparison study with methylprednisolone acetate in rats, J. Pharm. Sci. 100 (2011) 4580–4586. doi:10.1002/jps.22722.

[7] C. Larsen, S.W. Larsen, H. Jensen, A. Yaghmur, J. Ostergaard, Role of in vitro release models in formulation development and quality control of parenteral depots, Expert Opin. Drug Deliv. 6 (2009) 1283–1295. doi:10.1517/17425240903307431 PM - 19941410 M4 - Citavi.

[8] D. Burgess, A. Hussain, T. Ingallinera, M.L. Chen, Assuring quality and performance of sustained and controlled release parenterals: Workshop report, AAPS J. 4 (2002) 13–23. doi:10.1208/PS040205.

[9] C.K. Brown, H.D. Friedel, A.R. Barker, L.F. Buhse, S. Keitel, T.L. Cecil, J. Kraemer, J.M. Morris, C. Reppas, M.P. Stickelmeyer, C. Yomota, V.P. Shah, FIP/AAPS Joint Workshop Report: Dissolution/In Vitro Release Testing of Novel/Special Dosage Forms, AAPS PharmSciTech. 12 (2011) 782–794. doi:10.1208/s12249-011-9634-x.

[10] I. Kushner, J.A. Somerville, Permeability of human synovial membrane to plasma proteins. Relationship to molecular size and inflammation, Arthritis Rheum. 14 (1971) 560–570. doi:10.1002/art.1780140503.

[11] I. Nikolettos, O. Savvidou, P. Katsibri, N. Fotaki, Characterisation and drug solubility studies in Rheumatoid Arthritis and Osteoarthritis synovial fluid towards the development of in vitro disease state biorelevant media, in: AAPS Annu. Meet. 2016, Denver, Colorado (USA), 2016.

[12] M.R.C. Marques, R. Loebenberg, M. Almukainzi, Simulated biological fluids with possible application in dissolution testing, Dissolution Technol. 18 (2011) 15–28. doi:10.14227/DT180311P15.

[13] K.M.N. Oates, W.E. Krause, R.L. Jones, R.H. Colby, Rheopexy of synovial fluid and protein aggregation, J. R. Soc. Interface. 3 (2006) 167–74. doi:10.1098/rsif.2005.0086.

[14] A.M. Smith, L. Fleming, U. Wudebwe, J. Bowen, L.M. Grover, Development of a synovial fluid analogue with bio-relevant rheology for wear testing of orthopaedic implants, J. Mech. Behav. Biomed. Mater. 32 (2014) 177–184. doi:10.1016/j.jmbbm.2013.12.009.

[15] B. Sterner, M. Harms, M. Weigandt, M. Windbergs, C.M. Lehr, Crystal suspensions of poorly soluble peptides for intra-articular application: A novel approach for biorelevant assessment of their in vitro release, Int. J. Pharm. 461 (2014) 46–53. doi:10.1016/j.ijpharm.2013.11.031.

[16] I. Nikolettos, Predictive models for intra-articular drug delivery, Thesis (Ph.D.) - University of Bath, 2018, 2018.

[17] E. Sottofattori, M. Anzaldi, L. Ottonello, HPLC determination of adenosine in human synovial fluid, in: J. Pharm. Biomed. Anal., 2001: pp. 1143–1146. doi:10.1016/S0731-7085(00)00574-4.

[18] N.K. Hopkins, C.M. Wagner, J. Brisson, T.E. Addison, Validation of the simultaneous determination of methylprednisolone and methylprednisolone acetate in human plasma by high-performance liquid chromatography, J. Chromatogr. B Biomed. Sci. Appl. 577 (1992) 87–93. doi:10.1016/0378-4347(92)80601-L.

[19] R. Dannenfelser, S.H. Yalkowsky, Database for aqueous solubility of nonelectrolytes, Bioinformatics. 5 (1989) 235–236. doi:10.1093/bioinformatics/5.3.235.

[20] P. Macheras, C. Reppas, Dissolution and in vitro permeation behaviours of dicumarol nitrofurantoin and sulfamethizole in the presence of protein, Int. J. Pharm. 37 (1987) 103–112. doi:10.1016/0378-5173(87)90014-7.

[21] M. Vertzoni, A. Diakidou, M. Chatzilias, E. Söderlind, B. Abrahamsson, J.B. Dressman, C. Reppas, Biorelevant media to simulate fluids in the ascending colon of humans and their usefulness in predicting intracolonic drug solubility, Pharm. Res. 27 (2010) 2187–2196. doi:10.1007/s11095-010-0223-6.

[22] H.S. Ghazal, A.M. Dyas, J.L. Ford, G.A. Hutcheon, In vitro evaluation of the dissolution behaviour of itraconazole in bio-relevant media, Int. J. Pharm. 366 (2009) 117–123. doi:10.1016/j.ijpharm.2008.09.003.

[23] K. Fukudome, K. Chijiiwa, T. Furusawa, F. Nakayama, Effect of albumin on the solubility of cholesterol in bile, Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab. 922 (1987) 155–161. doi:10.1016/0005-2760(87)90149-4.

[24] S. Rohatagi, G. Hochhaus, H. Mollmann, J. Barth, H. Derendorf, Pharmacokinetic interaction between endogenous cortisol and exogenous corticosteroids, Pharmazie. 50 (1995) 610–613.

[25] R.G. Strickley, Solubilizing Excipients in Oral and Injectable Formulations, Pharm. Res. 21 (2004) 201–230. doi:10.1023/B:PHAM.0000016235.32639.23.

[26] W.I. Higuchi, W.E. Hamlin, S.C. Mehta, Infrared attenuated total reflectance (ATR) method for observing the water-mediated surface phase reversion of methylprednisolone II to I during dissolution, J. Pharm. Sci. 58 (1969) 1145–1146. doi:10.1002/jps.2600580926.

[27] S. Fredenberg, M. Wahlgren, M. Reslow, A. Axelsson, The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems - A review, Int. J. Pharm. 415 (2011) 34–52. doi:10.1016/j.ijpharm.2011.05.049.

[28] J. Wang, B.M. Wang, S.P. Schwendeman, 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. doi:10.1016/S0168-3659(02)00137-2.

[29] C. Berkland, K. Kim, D.W. Pack, PLG microsphere size controls drug release rate through several competing factors, Pharm. Res. 20 (2003) 1055–1062. doi:10.1023/A:1024466407849.

[30] C. Wischke, S. P. Schwendeman, Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles, Int. J. Pharm. 364 (2008) 298–327.

[31] H. Fam, J.T. Bryant, M. Kontopoulou, Rheological properties of synovial fluids, Biorheology. 44 (2007) 59–74.

[32] D. Tercic, B. Bozic, The basis of the synovial fluid analysis, Clin. Chem. Lab. Med. 39 (2001) 1221–6. doi:10.1515/CCLM.2001.196.

[33] S. Tenzer, D. Docter, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, F. Schlenk, D. Fischer, K. Kiouptsi, C. Reinhardt, K. Landfester, H. Schild, M. Maskos, S.K. Knauer, R.H. Stauber, Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology, Nat. Nanotechnol. 8 (2013) 772–781. doi:10.1038/nnano.2013.181.

[34] C. Fornaguera, G. Calderó, M. Mitjans, M.P. Vinardell, C. Solans, C. Vauthier, Interactions of PLGA nanoparticles with blood components: Protein adsorption, coagulation, activation of the complement system and hemolysis studies, Nanoscale. 7 (2015) 6045–6058. doi:10.1039/c5nr00733j.

[35] Y.M. Thasneem, S. Sajeesh, C.P. Sharma, Effect of thiol functionalization on the hemo-compatibility of PLGA nanoparticles, J. Biomed. Mater. Res. - Part A. 99 A (2011) 607–617. doi:10.1002/jbm.a.33220.

[36] A. Shenderova, T.G. Burke, S.P. Schwendeman, The Acidic Microclimate in Poly(lactide-co-glycolide) Microspheres Stabilizes Camptothecins, Pharm. Res. 16 (1999) 241–248. doi:Doi 10.1023/A:1018876308346.

[37] S. Li, S. McCarthy, Further investigations on the hydrolytic degradation of poly (DL-lactide), Biomaterials. 20 (1999) 35–44. doi:10.1016/S0142-9612(97)00226-3.

Annex:

Characterization of raw PLGAs used for microspheres preparation

For sack of clarity, the thermal properties and the molecular weights of the raw polymers used in the present study were characterized.

Thermal analysis

Thermal analyses of raw polymers were performed by differential scanning calorimetry (DSC), by using DSC 1 Star^{e} System (METTLER TOLEDO, CH). The instrument was calibrated with indium for melting point and heat of fusion. Samples of about 10 mg exactly weighted were crimped in 40 µL aluminium pan and scanned from room temperature to 90 °C at 20 K/min to erase polymers' thermal history. After a cooling step, samples were re-heated up to 60 °C at 10 K/min. Glass transition temperature were calculated on the second heat scan and reported as inflection point value. All the analyses were performed under inert atmospheres of nitrogen (80 mL/min) and using an empty aluminium pan as reference material.

Polymers molecular weight

Molecular weight distribution of raw polymers were measured by gel permeation chromatography (GPC). Samples of about 5-6 mg were dissolved in dichloromethane and filtered through a 0.45 µm PTFE syringe filter prior the injection, to remove undissolved particles. Three columns (Phenogel™ 300x4.6 mm, Phenomenex, I) with gel pore size of 104 Å, 103 Å and 500 Å were connected in series. The mobile phase was dichloromethane at a flow rate of 0.35 mL/min at a temperature of 25.0±0.1 °C. An injection volume of 50 µL was used. The instrument was equipped with a double detector: refractive index detector and UV/visible detector set at λ=230 nm. The weight-average molecular weight (M_w) and the number-weight molecular weight (M_n) of each sample were calculated

using monodisperse polystyrene standards with M_w ranging from 486 to 188,000 Da and a software to compute molecular weight distribution (Agilent, USA). Dispersity index (DI) resulted from the ratio between M_w and M_n

Results

Table A1 enlists the main properties of the selected PLGAs. As expected, PLGA 75:25 had a higher glass transition temperature with a lower heat capacity associated with the glass-rubber transition, indicating a higher rigidity of the polymer chains compared to PLGA 50:50 [i]. Both polymers were quite homogenous in terms of molecular weight distribution, with a M_w of about 20 KDa.

Table A1- Main properties of the two types of PLGA selected to prepare the microspheres for this study.

PLGA	DL-lactide content*	T_g (°C)	ΔC_p (J/g·K)	M_w (KDa)	DI
50:50	47-53 %mol	36.5±0.1	0.602±0.027	20.4±0.4	1.5±0.0
75:25	72-78 %mol	37.7±0.1	0.458±0.021	23.6±0.3	1.7±0.2

*as reported by the supplier.

Annex reference

[i] J.H. Gibbs, E.A. DiMarzio, Nature of the glass transition and the glassy state, J. Chem. Phys. 28 (1958) 373–383. doi:10.1063/1.1744141.

Final remarks

All together the collected results demonstrated that both PLGA grafted to anti-oxidant (g-AA-PLGA) and DegraPol® are suitable materials for preparing particulate drug delivery systems that can, in turn, overcome some of the limitations associated to PLGA as such. In particular, g-AA-PLGA can be considered suitable materials also in the field of nanomedicine. Indeed, nanoparticles prepared by PLGA grafted to caffeic acid (g-CA-PLGA) was biocompatible and showed an improved cellular uptake in the selected cell lines, in terms of amount and rate, compared to the ones made of the native PLGA. This positive aspect, particularly critical when considering PLGA-based nanoparticulate systems that should interact with cells, was associated to the maintenance of the biodegradability, a key feature of PLGA. Also, considering the reduced pH drop off while g-CA-PLGA degrades and the stability upon ionizing sterilization, this material appears worth of interest for delivering drug(s) as demonstrated by the loading of fluvastatin for the prevention of restenosis (*Chapter 1*). However, during the development of the formulation, the optimization of the drying process, in order to handle a dried powder that permits the fast and easy reconstitution of the biodegradable nanosystem, is necessary. Maltodextrins was demonstrated to be a good cost-saving drying auxiliary agent with the most promising application in the spray-drying, where they can be used for different PLGA-based nanoparticles (*Chapter 2*).

The multi-block poly(ester-urethane) DegraPol® represent a feasible non-traditional material in the preparation of particles for applications in the pharmaceutical field. Although it displays the necessary biodegradability and biocompatibility properties, other features need to be figure out compared to the well-established PLGA. For instance, the almost neutral Z-potential or the propensity to form fibers, determined the rapid loss of structure and, thus, the tendency of the material to form aggregates. These aspects could create some limitations but, on the other side, identifies very precise requirements that must be met in the production of spherically-shaped particles, such as the presence of

a proper stabilizer(s) [*i.e.* cellulose derivatives and poly(vinyl alcohol)] to avoid the irreversible aggregation of particles (*Chapter 3)*.

As a general consideration, formulation development cannot be disconnected from the regulatory framework. Particularly regarding long-acting parenteral formulations, the development should also address the need to have a suitable *in vitro* test for properly studying the drug release and, hence, assuring the quality, the safety and the efficacy of the final medicinal product. However, the elaboration of appropriate *in vitro* release models is critical, given the complexity in the set-up of methods able to efficiently discriminate products that can have different *in vivo* behaviours (*Chapter 4*). In the case of PLGA microspheres intended to sustain the release of a drug after the intra-articular administration, the followed bio-relevant approach revealed that formulation parameters such as PLGA lactide/glycolide ratio and the amount of drug encapsulated should be carefully considered to properly optimize the formulation. Furthermore, proteins contained in the release medium simulating the disease condition of the synovial fluid affected the release behaviour of microspheres, in terms of release pattern and amount of drug released. This suggests that, also regarding the intra-articular environment, simple buffers (*i.e.,* PBS at physiological pH) cannot correctly figure out the conditions occurring *in vivo* after the administration, much less the pathological situation (*Chapter 5*).

Acknowledgments

Come to an end, it is duty to thank all the people who helped and worked with me.

Firstly, I want to express my gratitude to my supervisor Prof. Francesco Cilurzo, for being an example of open-mind scientist. Besides, I would like to thank Prof. Paola Minghetti, for her insightful comments and encouragement, but also for incenting me to widen my research from other perspectives, such as the regulatory ones. I have to mention and thanks Prof. Francesca Selmin for the constant presence and with whom I shared most of my experimental discussions and Dr. Antonella Casiraghi for the kindness always shown.

A thanks must be given to Dr. Nikoletta Fotaki, for giving me the possibility to spend a period at the University of Bath.

I want to thank Prof. Stefano Bellosta and Dr. Silvia Castiglioni from the Dept. of Pharmacological and Biomolecular Sciences - University of Milan, Prof. Francesco Puoci from the Dept. of Pharmacy - Università della Calabria - and all the R&D group of ab medica spa for the multi-disciplinary and wonderful work we had together.

A huge thanks also to my lab mates, Dr. Chiara Gennari, Dr. Silvia Franzè, Dr. Gaia Quaroni and Dr. Umberto Musazzi. They have been not only my colleagues, but also good friends and I hope I can continue to cultivate this friendship of ours.

I want to address my most important thought to my beloved family. Their unconditioned support and love throughout all my PhD always gave me the power to move forward, without losing sight of my goals. I want to include and acknowledge at this stage also my best friend Dr. Arianna Quadri.

This thesis is also in loving memory of my grandmother Rosa, the truly driving force of my everything.