



(51) International Patent Classification:

A61K 9/50 (2006.01) A61K 9/00 (2006.01)

(21) International Application Number:

PCT/EP2020/053825

(22) International Filing Date:

14 February 2020 (14.02.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

19305186.9 15 February 2019 (15.02.2019) EP

(71) Applicants: INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)

[FR/FR]; 101, rue de Tolbiac, 75013 PARIS (FR). UNIVERSITE DE MONTPELLIER [FR/FR]; 163 rue Auguste Broussonnet, 34090 MONTPELLIER (FR). CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE - CNRS - [FR/FR]; 3, rue Michel Ange, 75016 PARIS (FR). UNIVERSITE D'ANGERS [FR/FR]; 40 rue de Rennes, 49000 ANGERS (FR).

(72) Inventors: VENIER-JULIENNE, Marie-Claire; MINT/INSERM1066/CNRS6021, IBS-CHU ANGERS, 4 rue Larrey, 49933 ANGERS Cedex 9 (FR). LE, Minh Quan; Equipe de Technologie Pharmaceutique, 41 rue Dinh Tien Hoang, Quartier Ben Nghe, Arrondissement 1, HCMV (VN). GARRIC, Xavier; Institut des Biomolécules Max Mousseron (UMR 5247,UM/CNRS/ENSCM), Département Biopolymères Artificiels, Faculté de Pharmacie, 15 avenue Charles Flahault, 34093 MONTPELLIER CEDEX 5 (FR).

(74) Agent: PLASSERAUD IP; 66 rue de la Chaussée d'Antin, 75440 PARIS CEDEX 09 (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,

OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH,

GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: PLGA-BASED MICROSPHERES, PREPARATION METHODS AND USES THEREOF

(57) Abstract: The present invention relates to the sustained delivery of protein and peptide therapeutics. In particular, the present invention relates to protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 k Da, and wherein the protein-loaded microspheres are obtained using a S/O/W solvent extraction/evaporation method, or by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer. The inventors showed that microspheres thus obtained exhibit a biphasic protein release profile with an insignificant burst after the first 24 hours, followed by a continuous and complete protein release over a period of 8 to 10 weeks. The present invention also relates to the use of such protein-loaded microspheres for the treatment of diseases of the human or animal body. Pharmaceutical compositions comprising such protein-loaded microspheres and kits are also described.



PLGA-based Microspheres, Preparation Methods and Uses Thereof

Related Application

The present application claims priority to European Patent Application No. EP 19 305 186.9 filed on February 15, 2019, which is incorporated herein by
5 reference in its entirety.

Background of the Invention

Since the late 20th century, numerous therapeutic proteins and peptides have emerged in the market. The market for protein and peptide drugs is estimated to be around 10% of the entire pharmaceutical market and will make up an even larger
10 proportion of the market in the future (Craig *et al.*, Chem. Biol. Drug Des., 2013, 81: 136-147; Bruno *et al.*, Ther. Deliv., 2013, 4: 1443-1467). Since the early 1980s, a total of 239 therapeutic proteins and peptides have been approved for clinical use by the US-FDA (Fosgerau *et al.*, Drugs Discov. Today, 2015, 20: 122-128). Due to their high affinity and specificity, proteins and peptides have a significant and rapidly
15 expanding role as pharmaceuticals useful in the treatment of a variety of clinical conditions and syndromes, such as cancer, diabetes, infectious diseases and many other disorders that have long been studied for a solution (Leader *et al.*, Nat. Rev. Drug Discov., 2008, 7: 21-39; Boohaker *et al.*, Curr. Med. Chem., 2012, 19: 3794-37804; Vlieghe *et al.*, Drug Discov. Today, 2010, 15: 40-56).

20 Despite the several advantages, there are a few drawbacks associated with protein and peptide-based therapeutics. Limitations including proteolytic degradation, relatively short circulating half-life, physiochemical instability and immunogenicity restrict their ease of administration and compliance at the user end (Antosova *et al.*, Trends Biotechnol., 2009, 27: 628-635; Bruno *et al.*, Ther. Deliv., 2013, 4: 1443-
25 1467; Otvos *et al.*, Front Chem., 2014, 2:62). Proteins generally have high molecular weights, low lipophilicity and contain some charged functional groups. Several strategies have been evaluated in an effort to improve the current limitations of therapeutic peptides and proteins. Most efforts center around one of two approaches – either a modification of the agent itself (*e.g.*, mutations in protein structure or covalent
30 attachment of moieties) or by a change in formulation (*e.g.*, liposomes, and polymeric microspheres or nanoparticles).

Among the approaches that have been developed for the delivery of protein and peptide therapeutics, protein/peptide encapsulation in polymeric microspheres using biodegradable and biocompatible polymers is of interest. In particular, there is a well-established track record in the use of poly(lactic-co-glycolic acid) (PLGA) microspheres for the controlled delivery of protein/peptide therapeutics (Pagels and Prud'homme, J. Control. Release, 2015, 219: 519-535), PLGA having been approved for use in humans by the US-FDA and the European Medicine Agency. PLGA is a polymer of lactic acid and glycolic acid joined by ester bonds. The ratio of lactic acid and glycolic acid controls numerous aspects of the polymer properties. The PLGA composition of 50:50 has demonstrated the fastest biodegradation rate of the polymers with near complete degradation in 50-60 days (Esmaeili *et al.*, Int. J. Pharm., 2008, 349: 249-255). Once administered to the body, the ester bond is hydrolyzed, releasing the drug from the polymer and allowing the polymer acids to become metabolized and cleared from the body.

Encapsulation of proteins/peptides into PLGA particles can improve the pharmacokinetic profiles of the drugs, providing release rates on the magnitude of days, weeks or months, and can subsequently reduce the required frequency of administration. However, the favorable characteristics of the vehicle itself must be balanced against the negative effects on protein stability during preparation and storage. Degradation of PLGA leads to accumulation of lactic and glycolic acids within the drug delivery device, thereby causing a significant reduction in pH of the surrounding microenvironment which can, in turn, facilitate denaturation of the encapsulated protein, aggregation and loss of activity (Carrasquillo *et al.*, J. Cont. Release, 2001, 76: 199-208; Wu and Jin, AAPS PharmSciTech, 2008, 9: 1218-1229; Cossé *et al.*, AAPS PharmSciTech, 2016, 18: 15-26). Furthermore, true zero-order release from PLGA microspheres can also be difficult to achieve as release is proportional to surface area, a property that will fluctuate with erosion of the vehicle (Ravi Kumar *et al.*, Biomaterials, 2004, 25: 1771-1777).

Therefore, there is still a need in the art for new, improved PLGA-based microsphere systems for the delivery of protein/peptide therapeutics.

Summary of the Invention

Using lysozyme as a model protein, different PLGA-based penta-block copolymers, and a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation method to prepare microspheres, the present Applicants have discovered that a PLGA with a molecular weight of 20 kDa yields highly porous microspheres which exhibit a biphasic protein release profile with an insignificant burst after the first 24 hours, followed by a continuous and complete protein release over a period of 8 to 10 weeks, making these microspheres interesting candidates for protein sustained release delivery. The present Applicants have also found that it is possible to modulate the protein release of these microspheres by mixing them with other protein-loaded microspheres. In particular, the Applicants have shown that it is possible to improve the controlled protein release profile by mixing the above-mentioned lysozyme-loaded PLGA(20 kDa)-based penta-block copolymer microspheres with lysozyme-loaded microspheres prepared in the same manner but using a PLGA-based penta-block copolymer, wherein the PLGA has a molecular weight of 40 kDa. Indeed, the mixture of microspheres thus obtained was observed to exhibit a biphasic protein release, with an insignificant initial burst during the first 24 hours, followed by a second phase exhibiting zero-order kinetics (which allows the delivery of a constant dose of protein over a long period of time). The present Applicants have further shown that by mixing the above-mentioned lysozyme-loaded PLGA(20 kDa)-based penta-block copolymer microspheres with lysozyme-loaded PLGA(20 kDa)-based penta-block copolymer microspheres obtained by encapsulating lysozyme after formation of the PLGA-based pentablock copolymer microspheres, the resulting microspheres mixture exhibits an increased initial protein burst, which is useful under particular circumstances, such as pharmacologically active microcarriers (PAMs).

Consequently, the present invention relates to protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein the protein-loaded microspheres are obtained:

- using a S/O/W solvent extraction/evaporation method, or
- by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer.

The term “a protein”, as used herein, means one protein or a mixture of two (or more than two) proteins.

The present invention also relates to a protein-loaded microsphere mixture comprising:

- 5 - a first population of protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a first protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa; and
- 10 - a second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer and comprising a second protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 40 kDa,

wherein each of the first and second populations of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method, and wherein the first
15 protein and the second protein are the same protein or are different proteins.

The present invention also relates to a protein-loaded microsphere mixture comprising:

- 20 - a first population of protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a first protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein said first population of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method; and
- 25 - a second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer and comprising a second protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein said second population of protein-loaded microspheres is obtained by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer,

wherein the first protein and the second protein are the same protein or are different
30 proteins.

The present invention also relates to a composition of cell-carrying, protein-loaded microspheres comprising

- protein-loaded microspheres, wherein at least part of the outer surface of the microspheres is coated with a cell adhesion surface coating; and
- whole cells and/or cell fragments bound to the cell adhesion surface coating,

wherein the protein-loaded microspheres are composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprise a protein embedded in the microspheres, the PLGA has a mean molecular weight of 20 kDa, and the protein-loaded microspheres are obtained by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer.

The present invention also relates to a composition of cell-carrying, protein-loaded microspheres comprising:

- a first population of protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a first protein embedded in the microspheres, wherein at least part of the outer surface of the protein-loaded microspheres is coated with a cell adhesion surface coating, and whole cells and/or cell fragments are bound to the cell adhesion surface coating, wherein the PLGA has a mean molecular weight of 20 kDa and wherein the first population of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method; and
- a second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer and comprising a second protein embedded in the microspheres, wherein at least part of the outer surface of the protein-loaded microspheres is coated with a cell adhesion surface coating, and whole cells and/or cell fragments are bound to the cell adhesion surface coating, wherein the PLGA has a mean molecular weight of 20 kDa and wherein the second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer is obtained by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer,

wherein the first protein and the second protein are the same protein or are different proteins.

In certain embodiments, in a composition of cell-carrying, protein-loaded microspheres according to the invention, the cell adhesion surface coating comprises fibronectine and poly-D-lysine.

5 In certain embodiments, in the protein-loaded microspheres according to the invention, or in the protein-loaded microsphere mixture according to the invention, or in the composition of cell-carrying, protein-loaded microspheres according to the invention, the poloxamer is poloxamer 188 (P188).

10 In certain embodiments, in the protein-loaded microspheres according to the invention, or in the protein-loaded microsphere mixture according to the invention, or in the composition of cell-carrying, protein-loaded according to the invention, the PLGA has a molar ratio of lactic acid/glycolic acid (LA/GA) of 50/50.

15 The present invention also relates to the protein-loaded microspheres according to the invention, or the protein-loaded microsphere mixture according to the invention, or the composition of cell-carrying, protein-loaded microspheres according to the invention for use in a method of treatment of the human or animal body.

In certain embodiments, the method of treatment is a method of treatment of a degenerative disease of the human or animal body.

20 The present invention also relates to a pharmaceutical composition comprising an effective amount of the protein-loaded microspheres according to the invention, or the protein-loaded microsphere mixture according to the invention, or the composition of cell-carrying, protein-loaded microspheres according to the invention, at least one pharmaceutically acceptable carrier or excipient, and optionally at least one therapeutic and/or prophylactic agent.

The present invention also relates to a kit comprising:

- 25 - a PLGA-poloxamer-PLGA penta-block copolymer, wherein the PLGA has a mean molecular weight of 20 kDa, and a first protein, and instructions to prepare protein-loaded microspheres according to the invention or a protein-loaded microsphere mixture according to the invention;
- or
- 30 - a first PLGA-poloxamer-PLGA penta-block copolymer, wherein the PLGA has a mean molecular weight of 20 kDa, a second PLGA-poloxamer-PLGA penta-

block copolymer, wherein the PLGA has a mean molecular weight of 40 kDa and a second protein, and instructions to prepare protein-loaded microsphere mixture according to the invention,

wherein the first protein and the second protein are the same protein or are different proteins.

The present invention also relates to a kit comprising:

- a PLGA-poloxamer-PLGA penta-block copolymer, wherein the PLGA has a mean molecular weight of 20 kDa, a first protein and a second protein, at least one adhesion molecule, and instructions to prepare a composition of cell-carrying, protein-loaded microspheres according to the invention,

wherein the first protein and the second protein are the same protein or are different proteins.

Such a kit may further comprise whole cells and/or cell fragments.

These and other objects, advantages and features of the present invention will become apparent to those of ordinary skill in the art having read the following detailed description of the preferred embodiments.

Detailed Description of Certain Preferred Embodiments

I - Protein-Loaded Microspheres and Protein-Loaded Microsphere Mixtures

Protein-Loaded Microspheres. A protein-loaded microsphere according to the present invention comprises a PLGA-based penta-block copolymer composing the microsphere and a biologically active protein that is encapsulated or embedded within the microsphere.

As used herein, the term “*microspheres*” refers to small spherical particles, with mean diameters in the micrometer range (typically 1 μm to 1000 μm (1 mm)). The term “*mean diameter*”, as used herein, generally refers to the statistically mean particle diameter of the microspheres. Two populations of microspheres can be said to have a “substantially equivalent mean particle size” when the statistical mean diameter of the first population of microspheres is within 20% of the statistical mean diameter of the second population of microspheres, more preferably within 15%, more preferably within 10%.

The protein-loaded microspheres according to the present invention have mean diameters in the micrometer range, typically between 1 μm and 1000 μm , preferably between 5 μm and 500 μm , in particular between 10 μm to 250 μm or between 25 μm and 150 μm , quite particularly between 30 μm to 80 μm .

5 Depending on the PLGA used and the method of preparation employed, the protein-loaded microspheres of the present invention exhibit different porosity patterns, from being highly porous and exhibiting interconnected pores on the outer surface as well as inside the microspheres, to being porous and exhibiting closed pores (*i.e.*, non-interconnected pores) both on the outer surface and inside the microspheres,
10 and to being dense on the outer surface and porous and exhibiting closed pores inside the microspheres.

As used herein, the term “**protein-loaded microspheres**” refers to microspheres that contain a protein (or a mixture of different proteins) embedded or encapsulated inside de microspheres. By contrast, “**blank or empty microspheres**” refers to therein,
15 microspheres that do not comprise any protein or any other molecule, agent or compound, embedded or encapsulated inside de microspheres.

A. PGLA-Based Penta-Block Copolymer

The PLGA-based penta-block copolymer composing the microspheres described herein is a penta-block copolymer with a A_2-B-A_2 (or $A-A-B-A-A$) structure, wherein
20 A is a PLGA and B is a poloxamer or a poloxamine, and wherein two molecules of PLGA are bound on each side of one molecule of poloxamer or poloxamine. The terms “ **A_2-B-A_2 copolymer**” and “ **$A-B-A$ penta-block copolymer**” are used herein interchangeably.

PLGA or poly(D,L-lactide-co-glycolide) (also known as poly(D,L-lactic-co-glycolic acid) is a copolymer synthesized by means of ring-opening copolymerization of
25 two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. During polymerization, successive monomeric units (of glycolic acid or lactic acid) are linked together in PLGA by ester linkages, thus yielding a linear, aliphatic polyester as a product. In the context of the present invention, the PLGA to
30 be used in the preparation of a PLGA-based penta-block copolymer has a mean molecular weight (M_w) of 20 kDa. The molar ratio of lactic acid/glycolic acid

(LA/GA) within the PLGA may be of any value, such as for example: 80/20, 70/30, 75/25, 60/40, 50/50, 40/60, 25/75, 30/70, or 20/80. The crystallinity of PLGAs varies from amorphous to crystalline depending on block structure and molar ratio. In certain preferred embodiments, the molar ratio LA/GA is 50/50.

5 As described below, when mixtures of microspheres are used, protein-loaded microspheres may be prepared using a PLGA-based penta-block copolymer with a mean molecular weight (M_w) of 40 kDa.

The B component of the PLGA-based penta-block copolymer is a amphiphilic polymer. Indeed, it has been documented that the combination of amphiphilic
10 polymer and PLGA is useful for improving protein release profiles from microspheres (Tran *et al.*, Eur. J. Pharm. Sci., 2012, 45: 128-137; Feng et al., J. Appl. Polym. Sci., 2015, 132: 41431-41439, LE et al., Int. J. Pharm., 2018, 535: 428-437). More specifically, the B component of the PLGA-based penta-block copolymer to be used in the preparation of microspheres according to the present invention is a poloxamer
15 or a poloxamine. Preferably, the B component is a poloxamer.

Poloxamers are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophobic chains of polyoxyethylene (poly(ethylene oxide)). Because of their amphiphilic structures, poloxamers have surfactant properties. Any poloxamer may
20 be used in the practice of the present invention, and one skilled in the art knows how to select poloxamers to obtain a PLGA-based penta-block copolymer with desired properties. In certain preferred embodiments, the poloxamer is such that the polyoxypropylene has a mean molecular weight of between 1,000 and 3,500 Da, preferably between 1,000 and 2,000 Da, and the polyoxyethylene content is between
25 40% and 90%, preferably between 80% and 85% by weight. Preferably, the poloxamer is poloxamer 188 (or P188), which has a mean molecular weight of 8,400 Da and which comprises about 80% of polyoxyethylene by weight.

The proportion of poloxamer in the PLGA-based penta-block copolymer is between 0.4% and 50%, preferably between 8% and 30%, more preferably between
30 9% and 18%, by weight.

A PLGA-based penta-block copolymer to be used in the practice of the present invention may be prepared according to any suitable method known in the art.

B. Biologically Active Proteins

The second component of the microspheres according to the present invention is a protein, which is encapsulated or embedded within the microsphere for subsequent delivery or release in the human or animal body.

5 The terms “*protein*”, “*peptide*” and “*polypeptide*” are used herein interchangeably. They refer to a polymer of amino acid residues linked together by peptide (amide) bonds. The terms refer to a protein, peptide or polypeptide of any size, structure, or function. Typically, a protein, peptide, or polypeptide will be at least three amino acids long, generally at least 20, at least 30, at least 50, at least 100,
10 at least 200, at least 300 amino acids, or at least 500 amino acids long. One or more the amino acids in a protein, peptide or polypeptide may be modified, for example, by the addition of a chemical entity, such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. A protein suitable for
15 use in the practice of the present invention may have a molecular weight in the range of about 5 kDa to about 150 kDa. A protein, peptide, or polypeptide may also be a single molecule or may be a multi-molecular complex. A protein, peptide, or polypeptide may be just a fragment of a naturally occurring protein or peptide. A protein, peptide, or polypeptide may be naturally occurring, recombinant, or synthetic,
20 or any combination thereof. A protein useful in the practice of the present invention may comprise two or more different domains (for example a binding domain, an effector domain, a nucleic acid cleavage domain, a transcriptional activator, a repressor domain, etc...). A microsphere according to the present invention may encapsulate one type of protein or a mixture of two or more proteins.

25 Proteins that can be encapsulated in microspheres described herein include any protein, peptide or polypeptide that is “*biological active*” (*i.e.*, that has an action or effect in the target cell or target tissue to which it is delivered and consequently is beneficial to the human or animal body). Thus, suitable biologically active proteins include enzymes (*e.g.*, oxidoreductases, transferases, hydrolases, lyases, isomerases,
30 or ligases); transcriptional activators, transcriptional repressors, genome editing proteins, Cas9 proteins, TALEs (Transcription Activator-Like Effectors), TALENs (Transcription Activator-Like Effectors), nucleases, binding proteins (*e.g.*, ligands,

receptors, antibodies, antibody fragments, nucleic acid binding proteins, etc...); structural proteins (*e.g.*, albumins, globulins, histones, collagens, elastins and keratins), therapeutic proteins (*e.g.*, tumor suppressor proteins, therapeutic enzymes, growth factors, growth factor receptors, transcription factors, proteases, etc...); as
5 well as variants and fusions thereof.

Examples of therapeutic proteins include cytokines, such as transforming growth factor-beta (TGF- β), interferons (*e.g.*, interferon-alpha, interferon-beta, interferon-gamma), colony stimulating factors (*e.g.*, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF)), thymic stromal
10 lymphopoietin (TSLP), and the interleukins (*e.g.*, interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6; IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-22, IL-23, and IL-35).

Other examples of biologically active proteins include hormones or peptide hormones such as estrogens (diethylstilbestrol, estradiol), androgens (testosterone, fluoxymesterone), progestins (megestrol acetate), medroxyprogesterone acetate); and
15 corticosteroids (prednisone, dexamethasone, hydrocortisone); amylin, anti-Mullerian hormone, calcitonin, cholecystokinin, corticotropin, endothelin, enkephalin, erythropoietin (EPO), follicle-stimulating hormone, gallanin, gastrin, ghrelin, glucagon, gonadotropin-releasing hormone, growth hormone-releasing hormone,
20 hepcidin, human chorionic gonadotropin, human growth hormone (hGH), inhibin, insulin, insulin-like growth factor, leptin, luteinizing hormone, luteinizing hormone-releasing hormone, melanocyte stimulating hormone, motilin, orexin, oxytocin, pancreatic polypeptide, parathyroid hormone, prolactin, secretin, somatostatin, thrombopoietin, thyroid-stimulating hormone, vasoactive intestinal peptide, and
25 vasopressin.

Other examples of therapeutic proteins include therapeutic antibodies, *e.g.*, anticancer antibodies (*e.g.*, abagovomab, adecatumumab, afutuzumab, alacizumab pegol, altumomab pentetate, amatuximab, anatumomab mafenatox, apolizumab, arcitumomab, bavituximab, bectumomab, belimumab, bevacizumab, bivatuzumab
30 mertansine, blinatumomab, brentuximab vedotin, cantuzumab mertansine, cantuzumab ravtansine, capromab pendetide, cetuximab, citatuzumab bogatox, cixutumumab, clivatuzumab tetraxetan, dacetuzumab, demcizumab, detumomab,

drozitumab, ecromeximab, eculizumab, elotuzumab, ensituximab, epratuzumab, etaracizumab, farletuzumab, figitumumab, flanvotumab, galiximab, gemtuzumab, ozogamicin, girentuximab, ibritumomab tiuxetan, imgatuzumab, ipilimumab, labetuzumab, lexatumumab, lorvotuzumab mertansine, nimotuzumab, ofatumumab, 5 oregovomab, panitumumab, pemtumomab, pertuzumab, tacatuzumab tetraxetan, tositumomab, trastuzumab, totumumab, zalutumumab, and the like), and anti-inflammatory antibodies (*e.g.*, adalimumab, alemtuzumab, atlizumab, canakinumab, certolizumab, certolizumab pegol, daclizumab, efalizumab, fontolizumab, golimumab, infliximab, mepolizumab, natalizumab, omalizumab, ruplizumab, ustekinumab, 10 visilizumab, zanolimumab, vedolizumab, belimumab, orelizumab, teplizumab, rituximab, ofatumumab, ocrelizumab, epratuzumab, eculizumab, briakinumab, and the like).

Other examples of therapeutic proteins include antioxidant enzymes which are capable of reducing oxidative damage by decomposition or degrading reactive oxygen 15 species. Antioxidant enzymes particularly useful include, without limitation, catalase, glutathione peroxidase, superoxide dismutase, hemeoxygenase, glutathione-S-transferase, or synthetic or mimetic enzymes thereof.

Other useful enzymes are enzymes that detoxify a xenobiotic such as insecticides, drugs, pharmaceutical agents, organic chemicals, chemical warfare 20 agents, toxic (including endotoxins), and the like which can have an adverse effect on a subject. Xenobiotic detoxifying enzymes include cytochrome P450 enzymes such as Cyp3A4 and Cyp3A5, Cyp1A1, Cyp1A2, Cyp2D6, Cyp2E1, Cyp2C, Cyp2C9, Cyp2B6, Cyp2C19 and the like which are responsible for the metabolism of a variety of drugs including cyclosporin, nifedipine, warfarin, phenacetin, caffeine, aflatoxin 25 B1, ethanol, carbon tetrachloride, coumarin, sparteine, cyclophosphamide. Other xenobiotic detoxifying suitable enzymes further include alcohol dehydrogenase; epoxide hydrolase; glucuronyl transferases (detoxifying phenols, thiols, amines, and carboxylic acids); sulfotransferase (detoxifying phenols, thiols, and amines); N- and O-methyl transferases (detoxifying phenols and amines); N-acetyl transferase 30 (detoxifying amines); and other peroxisomal enzymes including peroxidases, catalase, phytanoyl-CoA hydroxylase, and α -methylacyl-CoA racemase.

Other suitable proteins that can be encapsulated in microspheres of the present invention will be apparent to the skilled artisan based on the present disclosure, and the disclosure is not limited in this respect.

5 A microsphere formulation according to the present invention can comprise protein in an amount of 0.5% to about 50% by weight of the formulation, based on the total weight of the microsphere formulation. According to some embodiments, the protein can be present in a microsphere formulation in an amount of about 0.5% to about 5% by weight, about 5% to about 30% by weight, about 5% to about 40% by weight, about 10% to about 25% by weight, or about 1%, about 5%, about 10%, about 10
20%, or about 30% by weight protein, based on the total weight of the microsphere formulation. According to some embodiments, the microsphere formulation comprises about 0.5%, about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 10%, about 15%, about 20%, about 25%, about 30%, or about 35% by weight protein based on the total weight of the microsphere
15 formulation.

C. Other Agents

Additional agents may also be incorporated into the microspheres, such additional agents include additives such as poloxamer, poloxamine, BSA, surfactant, alkaline substance (e.g., magnesium hydroxide ($\text{Mg}(\text{OH})_2$)).

20 Other suitable agents include chromophores, dyes, colorants and combinations thereof.

In certain embodiments, an affinity moiety may be used to modify the outer surface of the protein-loaded microspheres of the present invention. As used herein, the term “*affinity moiety*” refers to any material or substance which can promote
25 targeting of protein-loaded microspheres or protein-loaded microsphere compositions to particular cells, tissues and/or receptors *in vivo* or *in vitro*. The affinity moiety can be synthetic, semi-synthetic, or naturally-occurring. Particularly suitable affinity moieties include molecules which specifically bind to receptors or antigens found on cells. Other suitable affinity moieties target endothelial receptors, tissues or other
30 targets accessible through a body fluid or receptors or other targets upregulated in a tissue or cell adjacent to or in a bodily fluid.

In other embodiments, the microspheres and/or microsphere compositions according to the present invention are used for carrying cells of interest (see PAM application below). In such embodiments, the microspheric core composed of the PLGA-based penta-block copolymer, is coated with a cell adhesion surface coating and whole cells and/or cell fragments are bound to the cell adhesion coating.

The term “*cell adhesion surface coating*”, as used herein, refers to a surface comprising or consisting of extracellular matrix molecules or cell adhesion molecules, or fragments thereof, enhancing cell adhesion. The cell adhesion surface coating may also modify cell function, particularly cell proliferation and/or cell survival and/or cell differentiation. For this purpose, a microsphere may be completely or partially coated with a cell adhesion surface coating or may present a cell adhesion surface coating obtained by another manner. Examples of compounds that may composed a cell adhesion coating include, but are not limited to, poly-D-lysine (PDL), poly-L-lysine, arginine-glycine-aspartic acid tripeptide (RGD), polyornithine, polyethyleneamine, other synthetic molecules such as fibronectin-like compounds, or extracellular matrix molecules (fibronectin (FN), laminin, collagen) or cell adhesion molecules (N-CAM, selectines) or fragments of extracellular matrix molecules or cell adhesion molecules. A cell adhesion surface coating may be composed of a single compound or of a mixture of such compounds. One skilled in the art knows how to select a cell adhesion surface coating depending on the nature of the cells or cell fragments to be carried by the microspheres and/or depending on the tissue or cell type to which the cells or cell fragments are intended to be delivered. An example of a cell adhesion surface coating is a mixture comprising fibronectin and poly-D-lysine, preferably at a 50/40 molar ratio.

As used herein, the term “*cell*” has its art understood meaning and refers to the basic structural, functional, and biological unit of all known living organisms. The term “*whole cell*”, as used herein, refers to a functioning cell. The term “*cell fragment*”, as used herein, refers to a pharmacologically active part of a whole cell. Pharmacologically active cell fragments may be obtained from whole cells, for example by sonication, which disrupts cell membranes and releases cellular contents, or by enzyme-based disruption.

The binding mechanism of whole cells, or cell fragments to microspheres may be of different nature. Cell attachment may be obtained by intermolecular forces, for example by ionic interactions, van der Waals interactions or hydrogen bonds. Cell attachment may particularly be obtained *via* a receptor present on the cell surface, according to conventional ligand-receptor binding (integrins on the cell surface and extracellular matrix molecules such as fibronectin or laminin present in the cell adhesion coating for example, or cell adhesion molecules (N-CAM) on the cell surface and N-CAM present on the cell adhesion coating).

Cells useful in the practice of the present invention may be any cell, cell population or cell mixture whose delivery to the human or animal body is beneficial to the host. Suitable cells, preferably human cells, may be for example adult autologous cells, foetal cells, transformed or non-transformed cell lines, stem cells, multipotent or pluripotent cells, reprogrammed cells, genetically modified cells and the like, and more generally cells that have proved useful in the treatment of various diseases, such as for example in regenerative medicine. This is for example the case of transplantation of hepatocytes and islets of Langerhans (*i.e.*, pancreatic islets) for the treatment of diabetes. In neurotransplantation, a cell carried by microspheres according to the invention may be a cell from the PC12 cell line capable of secreting dopamine and differentiating into sympathetic neurons-like under the effect of NGF. Other examples of suitable cells include cells used in cell transplantation for repair of the liver, the myocardium or the central nervous system, bone marrow cells, nerve cells, muscle cells, hematopoietic cells, bone cells and the like, and cells producing recombinant virus defective for replication that infects host cells nearby. Other suitable cells are cells for gene transfer *in vivo*, such as cells containing a transgene.

As will be recognized by one skilled in the art, the protein encapsulated within cell-carrying microspheres may be chosen to modulate the cells behavior (*e.g.*, favoring cell proliferation and/or differentiation), the cells environment (avoiding immunological rejection processes, promoting angiogenesis, and the like) or the cells effects. Thus, for example, the protein may be selected from growth factors, cytokines, or immunomodulatory factors affecting cell differentiation, including those selected from the group consisting of neurotrophins such as NGF, BDNF, NT-3, etc... the TGFs, GDNF family, the FGFs, EGFs, PDGFs, interleukins, chemokines, retinoic

acid, erythropoietin etc..., and combinations thereof. Often, in such embodiments, preferred proteins will promote cell survival or function, or direct the differentiation of stem cells to a determined phenotype, or yet control the expression of a gene present in a genetically modified cell.

5 ***D. Specific Examples of Protein-Loaded Microspheres and Protein-Loaded Microsphere Mixtures***

In certain embodiments, the present invention provides protein-loaded microspheres composed of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprising a protein embedded in the microspheres, wherein the
10 PLGA has a mean molecular weight of 20 kDa. Preferably, the poloxamer is poloxamer 188 (or P188). Preferably, the protein-loaded microspheres are obtained using a S/O/W solvent extraction/evaporation method. Alternatively, the protein-loaded microspheres are obtained by loading (*i.e.*, embedding or encapsulating) the protein after preparation of blank/empty microspheres composed of the PLGA-
15 poloxamer (or poloxamine)-PLGA penta-block copolymer (see below). Preferably, the blank/empty microspheres composed of the PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer are obtained using an S/O/W solvent extraction/evaporation method.

In other embodiments, the present invention provides a protein-loaded
20 microspheres mixture comprising:

- a first population of protein-loaded microspheres composed of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprising a protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa; and
- 25 - a second population of protein-loaded microspheres composed of the PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprising the protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 40 kDa.

Preferably, the poloxamer is poloxamer 188 (or P188). Preferably, the first population
30 of protein-loaded microspheres and the second population of protein-loaded microspheres are each obtained using a S/O/W solvent extraction/evaporation method.

In yet embodiments, the present invention provides a protein-loaded microsphere mixture comprising:

- a first population of protein-loaded microspheres composed of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprising a protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein said first population of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method; and

- a second population of protein-loaded microspheres composed of the PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprising the protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein said second population of protein-loaded microspheres is obtained by loading (*i.e.*, embedding or encapsulating) the protein after preparation of blank/empty microspheres composed of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer. Preferably, the blank/empty microspheres composed of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer are obtained using an S/O/W solvent extraction/evaporation method.

Preferably, the poloxamer is poloxamer 188 (or P188).

In yet other embodiments, the present invention provides some of the above-described protein-loaded microspheres and protein-loaded microsphere mixtures as cell-carrying microsphere compositions.

More specifically, the present invention provides a composition of cell carrying microspheres composition comprising:

- protein-loaded microspheres, wherein at least part of the outer surface of the microspheres is coated with a cell adhesion surface coating; and

- whole cells and/or cell fragments bound to the cell adhesion surface coating, wherein the protein-loaded microspheres are composed of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprise a protein embedded in the microspheres, and wherein the PLGA has a mean molecular weight of 20 kDa. Preferably, the poloxamer is poloxamer 188 (or P188). In certain embodiments, the protein-loaded microspheres are preferably obtained by loading (*i.e.*, embedding or encapsulating) the protein after preparation of blank/empty microspheres composed of the PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer. Preferably, the

blank/empty microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer are obtained using an S/O/W solvent extraction/evaporation method.

In other embodiments, the present invention provides a composition of cell carrying microspheres comprising:

- 5 - a first population of protein-loaded microspheres composed of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprising a protein embedded in the microspheres, wherein at least part of the outer surface of the protein-loaded microspheres is coated with a cell adhesion surface coating, and whole cells and/or cell fragments are bound to the cell adhesion surface coating, wherein the
10 PLGA has a mean molecular weight of 20 kDa and wherein the first population of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method; and
- a second population of protein-loaded microspheres composed of the PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer and comprising the protein
15 embedded in the microspheres, wherein at least part of the outer surface of the protein-loaded microspheres is coated with a cell adhesion surface coating, and whole cells and/or cell fragments are bound to the cell adhesion surface coating, wherein the PLGA has a mean molecular weight of 20 kDa and wherein the second population of protein-loaded microspheres composed of the PLGA-poloxamer (or poloxamine)-
20 PLGA penta-block copolymer is obtained by loading (*i.e.*, embedding or encapsulating) the protein after preparation of blank/empty microspheres composed of the PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer. Preferably, the blank/empty microspheres are obtained using an S/O/W solvent extraction/evaporation method. Preferably, the poloxamer is poloxamer 188 (or
25 P188).

II – Methods of Preparation of Protein-Loaded Microspheres and Protein-Loaded Microsphere Compositions

In another aspect, the present invention provides methods for the preparation of protein-loaded microspheres and protein-loaded microsphere compositions, as
30 described above, *i.e.*, microspheres made of a PLGA-based penta-block copolymer, and more specifically of a PLGA-poloxamer (or poloxamine)-PLGA penta-block copolymer. The preparation of such microspheres includes the encapsulation

(i.e., incorporation or embedment) of protein (or a mixture thereof) in microspheres. The term “**encapsulation**”, as used herein, has its art understood meaning and refers to the containment, immobilization and/or entrapment of a protein within a three-dimensional structural (here a microsphere) delineated by a physical barrier (i.e., a barrier that reduces or controls the permeability of said structure).

A. Solid-in-Oil-in-Water Solvent Extraction/Evaporation Methods

In certain embodiments, the microspheres according to the present invention are obtained using a standard solid-in-oil-in-water (S/O/W) solvent extraction/evaporation method. Such S/O/W solvent extraction/evaporation methods are well known in the art. Indeed, such S/O/W methods have been developed to protect protein from denaturation during formation of a W/O (water-in-oil) emulsion. Protein adsorption and denaturation at the aqueous/organic solvent interface is responsible for the decreased protein bioactivity occurring during the encapsulation process. In S/O/W methods, the (co)polymer is dissolved in an organic solvent, in which solid protein particles are dispersed to generate the primary solid-in-oil (S/O) suspension. This suspension is then added to an aqueous phase containing an emulsifier such as polyvinyl alcohol (PVA) to form the S/O/W emulsion. The resultant emulsion is then maintained under stirring to allow both the extraction and the evaporation of the organic solvent and subsequent recovery of the protein-loaded microspheres.

Thus, in certain embodiments, protein-loaded microspheres according to the present invention are obtained using a S/O/W solvent extraction/evaporation method comprising steps of:

- suspending the protein in a solution of the PLGA-based pentablock copolymer (as described above) dissolved in an organic solvent or organic solvent mixture, wherein the protein is in the form of solid particles;
- emulsifying the suspension thus obtained by adding it into an aqueous solution containing a surfactant agent;
- stirring the emulsion thus obtained;
- adding the emulsion to water and stirring the mixture to extract/evaporate the organic solvent(s); and
- recovering the protein-loaded microspheres formed.

Preferably, the protein to be used in the S/O/W solvent extraction/evaporation method is formulated as solid particles by nanoprecipitation. Nanoprecipitation allows for reduced interactions between the protein and the PLGA-based pentablock copolymer. Nanoprecipitation of the protein may be performed using any suitable method known in the art. In certain preferred embodiments, the nanoprecipitated protein is obtained using a method comprising steps of:

- dissolving the protein and a surfactant (for example a poloxamer, preferably poloxamer 188) in water to obtain a solution;
- inducing protein nanoprecipitation by adding glycofurol to the solution, thereby obtaining a suspension;
- incubating the suspension; and
- recovering the nanoprecipitated protein, for example by centrifugation.

In the S/O/W solvent extraction/evaporation method, the organic solvent or organic solvent mixture used to dissolve the PLGA-based pentablock copolymer may be any suitable organic solvent or organic solvent mixture. In certain embodiments, the PLGA-based pentablock copolymer is dissolved in a mixture of dichloromethane and acetone, for example dichloromethane/acetone (3/1, v/v).

The surfactant agent used to emulsify the solid protein/PLGA-based pentablock copolymer suspension may be any suitable surfactant, such as for example polyvinyl alcohol (PVA).

Removal of the organic solvent or organic solvent mixture is achieved by addition of water, which acts as an extraction solvent, and stirring of the mixture which favors organic solvent evaporation.

The recovery of the protein-loaded microspheres formed using the S/O/W solvent extraction/evaporation method, may be performed using any suitable technique known in the art, for example by physical separation such as filtration.

The protein-loaded microspheres thus recovered can be freeze-dried and then preserved, for example at -20°C, until use.

The Examples section below provides a description of a S/O/W solvent extraction/evaporation method.

B. Methods of Protein Loading After Preparation of Blank Microspheres

In certain embodiments, the protein-loaded microspheres according to the present invention are obtained by loading, with the desired protein or protein mixture, already formed microspheres. Such a method comprises steps of:

- 5 - providing blank microspheres composed of a PLGA-based penta-block copolymer (as described herein);
- suspending the blank microspheres in an aqueous buffer containing the protein to be loaded;
- shaking the suspension obtained; and
- 10 - recovering the protein-loaded microspheres formed.

Preferably, the blank (or empty) microspheres used in the method are obtained using an S/O/W (solid-in-oil-in-water) solvent extraction/evaporation method, *i.e.* a S/O/W solvent extraction/evaporation method, as described above, except that no nanoprecipitated protein is encapsulated in the microspheres.

- 15 The shaking step may be performed using any suitable method, for example using a vortex. One skilled in the art will know how to select the time, speed, and temperature of shaking to optimize the formation of protein-loaded microspheres.

- The step of recovery of the protein-loaded microspheres thus formed may be performed using any appropriate technique(s) and conditions. For example, the
- 20 reaction mixture may be centrifuged and the supernatants removed. The obtained protein-loaded microspheres may be washed with water and then freeze-dried to be preserved (for example at -20°C) prior to being used.

The Examples section below provides a description of method for preparing protein-loading microspheres by loading the protein in already formed microspheres.

25 C. Methods of Preparation of Protein-Loaded Microsphere Mixtures

The protein-loaded microsphere mixtures according to the present invention are prepared by simply mixing two different populations of protein-loaded microspheres. More specifically, such a method comprises steps of:

- providing a first population of protein-loaded microspheres (as described herein)
- 30 and a second population of protein-loaded microspheres (as described herein),

wherein the first and second populations of microspheres are loaded with the same protein or with two (or more than two) different proteins; and

- mixing the first and second populations of protein-loaded microspheres to obtain the protein-loaded microsphere mixture.

5 Preferably, before the mixing step, each of the first and second populations of protein-loaded microspheres has been freeze-dried and preserved at -20°C for 24 hours for stabilization.

10 The first and second populations of protein-loaded microspheres may be mixed in any appropriate proportions depending on the desired protein release profile modulation. Appropriate proportions may be equal amounts (*i.e.*, 50/50, w/w) of each of the two microsphere populations. Alternatively, proportions of the two population of protein-loaded microspheres may be 10/90; 20/80; 30/70; 40/60; 60/40; 70/30; 80/20, or 90/10 (w/w), or anything in between these ratios.

15 The mixing step may be performed using any suitable mixing method, for example using a vortex.

 The obtained protein-loaded microsphere mixture may be freeze-dried and preserved (for example at -20°C) until use.

D. Preparation of Cell-Carrying, Protein-Loaded Microsphere Compositions

20 Cell-carrying microsphere compositions may be obtained from protein-loaded microspheres (prepared using a method such as those described above).

 In particular, the present invention relates to a method for preparing a cell-carrying protein-loaded microsphere composition, comprising steps of:

- providing a population of protein-loaded microspheres (as described herein);
- fully or partly coating the protein-loaded microspheres with a cell adhesion compound or a mixture of cell adhesion compounds to obtain protein-loaded microspheres coated with a cell adhesion surface coating; and
- contacting the protein-loaded microspheres coated with a cell adhesion surface coating with whole cells and/or cell fragments to obtain a cell-carrying protein-loaded microsphere composition.

30 In certain embodiments, the cell adhesion surface is obtained by chemical surface modification of the polymeric matrix for example by grafting synthetic

adhesion peptides such as polylysine or RGD-like peptide such as RGD or peptides of extracellular matrix molecules such as IKVAV or of cell adhesion molecules such as KHIFSDDSSSE onto the surface of the microspheres.

In other embodiments, the coating step is performed by mixing the cell adhesion compounds of the coating with microspheres in suspension in appropriate proportions. Coating is preferably obtained by adsorption of the adhesion compound(s) onto the microspheres. As already mentioned above, the coating of the microsphere outer surface may be complete (*i.e.*, coating of the entire outer surface) or partial (*i.e.*, coating of less than the entire outer surface – *e.g.*, about 75%, about 60%, about 50%, about 40%, about 30%, or less than about 30% of the outer surface). Furthermore, coated microspheres of a given population may be coated to different extents.

In certain embodiments, the contacting step is performed by mixing the cell (or cell fragment) in suspension with a suspension of the coated protein-loaded microspheres in appropriate proportions.

III – Uses of Protein-Loaded Microspheres, Protein-Loaded Microsphere Mixtures, Cell-Carrying, Protein-Loaded Microsphere Compositions, and Pharmaceutical Compositions thereof

A. Indications of Protein-Loaded Microspheres and Microsphere Mixtures

Protein-loaded microspheres and microsphere compositions according to the present invention may be used in the treatment of any clinical disease or condition for which administration of the protein (encapsulated in microspheres) is beneficial to a subject. Thus, the present invention provides protein-loaded microspheres and microsphere compositions for use in the treatment of a disease or clinical condition in a subject. As used herein, the term “*subject*” refers to a human or another mammal (*e.g.*, primate, dog, cat, goat, horse, pig, mouse, rat, rabbit, and the like) that is suffering from a disease or clinical condition or is at risk of developing such a disease or clinical condition. Non-human subjects may be transgenic or otherwise modified animals. In many embodiments of the present invention, the subject is a human being. In such embodiments, the subject is often referred to as an “*individual*” or a “*patient*”. The terms “subject”, “individual” and “patient” do not denote a particular age, and

thus encompass newborns, children, teenagers, and adults. The term “patient” more specifically refers to an individual suffering from a disease or clinical condition.

The term “**treatment**” is used herein to characterize a method or process that is aimed at (1) delaying or preventing the onset of a disease or clinical condition; (2) slowing down or stopping the progression, aggravation, or deterioration of the symptoms of the disease or clinical condition; (3) bringing about amelioration of the symptoms of the disease or clinical condition; or (4) curing the disease or clinical condition. A treatment may be administered after initiation of the disease or clinical condition, for a therapeutic action. Alternatively, a treatment may be administered prior to the onset of the disease or clinical condition, for a prophylactic or preventive action. In this case, the term “**prevention**” is used.

Examples of diseases and clinical conditions that can be treated with protein therapeutics include, but are not limited to, cancer metabolic disorders, hematological disorders, immunological disorders, genetic disorders, hormonal disorders, bone disorders, cardiac disorders, infectious diseases, respiratory disorders, neurological disorders, adjunct therapy, eye disorders, and malabsorption disorders.

Protein-loaded microspheres and protein-loaded microsphere compositions according to the present invention (optionally after formulation with one or more appropriate pharmaceutically acceptable carriers or excipients), in a desired dosage, can be administered to a subject in need thereof by any suitable route. Methods of administration include, but are not limited to, dermal, intradermal, intramuscular, intraperitoneal, intralesional, intravenous, subcutaneous, intranasal, pulmonary, epidural, ocular, and oral routes. Microspheres and microsphere compositions of the present invention, or a pharmaceutical composition thereof, may be administered by any convenient or other appropriate route, for example, by infusion or bolus injection, by adsorption through epithelial or mucocutaneous linings (*e.g.*, oral, mucosa, rectal and intestinal mucosa, etc). Administration can be systemic or local. Parenteral administration may be directed to a given tissue of the patient, such as by catheterization.

Administration of protein-loaded microspheres and protein-loaded microsphere compositions (or a pharmaceutical composition thereof) according to the present invention will be in a dosage such that the amount delivered is effective for the

intended purpose. The route of administration, formulation dosage administered will depend upon the therapeutic effect desired, the severity of the disorder being treated, the presence of any infection, the age, sex, weight and general health condition of the patient as well as upon the potency, bioavailability and *in vivo* half-life of the protein-loaded microspheres or protein-loaded microsphere composition used, the use (or not) of concomitant therapies, and other clinical factors. These factors are readily determinable by the attending physician in the course of the therapy. Alternatively or additionally, the dosage to be administered can be determined from studies using animal models. Adjusting the dose to achieve maximal efficacy based on these and other methods are well known in the art and are within the capabilities of trained physicians.

A treatment according to the present invention may consist of a single dose or multiple doses.

B. Indications of Cell-Carrying, Protein-Loaded Microspheres

Cell-carrying, protein-loaded microspheres and microsphere compositions according to the present invention may be useful as pharmacologically active microcarriers (PAMs). PAMs are biocompatible and biodegradable protein-loaded microparticles conveying cells (or cell fragments) on their surface and exhibiting a controlled release of the encapsulated protein, wherein the protein is selected for its ability to promote cell survival and/or differentiation, favor cell integration in the host tissue, and/or to enhance cell (cell fragment) activity.

Cell-carrying, protein-loaded microspheres and microsphere compositions according to the present invention may be used in the treatment of any disease or clinical condition for which administration of the cells or cell fragments is beneficial to a subject. Examples of such diseases or clinical conditions include, but are not limited to, degenerative diseases, such as neurodegenerative diseases (*e.g.*, Parkinson's disease, Huntington's disease, Alzheimer's disease, and the like), spinal cord injury, osteo-articular diseases (*e.g.*, osteoarthritis, post-traumatic osteoarthritis), ischemic diseases (*e.g.*, cerebral ischemia, erection malfunctions, urinary incontinence, peripheral limb ischemia), kidney malfunction, and the like.

C. *Pharmaceutical Compositions*

Microspheres and microsphere compositions according to the present invention may be administered *per se* or as a pharmaceutical composition. Accordingly, the present invention provides pharmaceutical compositions comprising an effective
5 amount of microspheres or of a microsphere composition and at least one pharmaceutically acceptable carrier or excipient. In some embodiments, the composition further comprises one or more additional biologically active agents.

The pharmaceutical compositions of the present invention may be formulated in dosage unit form for ease of administration and uniformity of dosage. The expression
10 “unit dosage form”, as used herein, refers to a physically discrete unit for the patient to be treated. It will be understood, however, that the total dosage of the compositions will be decided by the attending physician within the scope of sound medical judgement.

Injectable preparations, for example, sterile injectable aqueous or oleaginous
15 suspensions may be formulated according to the known art using suitable dispersing or wetting agents, and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension or emulsion in a non-toxic parenterally acceptable diluent or solvent, for example, as an aqueous solution. Among the acceptable vehicles and solvents that may be employed are: water, Ringer’s solution,
20 U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solution or suspending medium. Fatty acids such as oleic acid may also be used in the preparation of injectable formulations. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration.

25 Oui mais ne s’applique pas dans le cas des microsphères, soit stérilisation terminale par gamma irradiation ou préparation dans conditions aseptiques

Injectable formulations can be sterilized, for example, by preparation under aseptic conditions or by gamma irradiation following preparation. Liquid
30 pharmaceutical compositions which are sterile solutions or suspensions can be administered by, for example, intravenous, intramuscular, intraperitoneal or subcutaneous injection. Injection may be *via* single push or by gradual infusion.

Where necessary or desired, the composition may include a local anesthetic to ease pain at the site of injection.

For parenteral or local administration (*e.g.*, by injection, preferably bolus injection or stereotaxic injection), the pharmaceutical compositions of the present invention may be formulated and may be presented in unit dose form in ampoules, bottles, pre-filled
5 syringes, small volume infusion or multi-dose containers with or without formulatory or additive agents.

Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions,
10 syrups, elixirs, and pressurized compositions. In addition to the microspheres or microsphere composition, the liquid dosage form may contain inert diluents commonly used in the art such as, for example, water or other solvent, solubilising agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol,
15 dimethylformamide, oils (in particular, cotton seed, ground nut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols, and fatty acid esters of sorbitan and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, suspending agents, preservatives, sweetening, flavouring, and perfuming agents, thickening agents,
20 colors, viscosity regulators, stabilizes or osmo-regulators. Examples of suitable liquid carriers for oral administration include water (potentially containing additives as above, *e.g.*, cellulose derivatives, such as sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols such as glycols) and derivatives thereof, and oils (*e.g.*, fractionated coconut oil and arachis oil). For
25 pressurized compositions, the liquid carrier can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Solid dosage forms for oral administration include, for example, capsules, tablets, pills, powders, and granules. In such solid dosage forms, the microsphere or microsphere composition may be mixed with at least one inert, pharmaceutically
30 acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and one or more of: (a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid; (b) binders such as, for example, carboxymethylcellulose,

alginates, gelatine, polyvinylpyrrolidone, sucrose, and acacia; (c) humectants such as glycerol; (d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (e) solution retarding agents such as paraffin; absorption accelerators such as quaternary ammonium compounds; (g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate; (h) absorbents such as kaolin and bentonite clay; and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. Other excipients suitable for solid formulations include surface modifying agents such as non-ionic and anionic surface modifying agents. Representative examples of surface modifying agents include, but are not limited to, poloxamer 188, benzalkonium chloride, calcium stearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatine capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition such that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delaying manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

In certain embodiments, the microspheres or the microsphere composition are/is the only active ingredient(s) in a pharmaceutical composition of the present invention. In other embodiments, the pharmaceutical composition further comprises one or more additional biologically active agents. Examples of suitable biologically active agents include, but are not limited to, anti-cancer agents, anti-inflammatory agents, immunomodulatory agents, analgesics, antimicrobial agents, antibacterial agents, antibiotics, antioxidants, antiseptic agents, and combinations thereof.

Materials and methods for producing various formulations, including formulations of cell-carrying microspheres, are known in the art and may be adapted for practicing the subject invention. Suitable formulations for the delivery of antibodies can be found, for example, in “*Remington’s Pharmaceutical Sciences*”,
5 E.W. Martin, 18th Ed., 1990, Mack Publishing Co.: Easton, PA.

IV – Kits

In another aspect, the present invention provides kits comprising materials useful for carrying out a method according to the invention. Materials and reagents for performing a method of the present invention may be assembled together in a kit.

10 In certain embodiments, a kit comprises a PLGA-based penta-block copolymer (as described above) and a protein (or more than one protein) to prepare protein-loaded microspheres or protein-loaded microsphere mixtures according to the invention. In other embodiments, a kit comprises a PLGA-based penta-block copolymer (as described above), a protein (or more than one protein), and whole cells or cell
15 fragments to prepare a cell-carrying, protein loaded microsphere composition.

Depending on the procedure, the kit may further comprise one or more of: chemical reagents (such as poloxamer 188, polyvinyl alcohol, glycofurol and/or cell adhesion compounds), washing buffer and/or reagents, dissolution buffer and/or reagents, suspending buffer and/or reagents, and the like. Protocols/instructions for
20 using these buffers and reagents to perform different steps of the preparation may be included in the kit.

The reagents may be supplied in a solid (*e.g.*, lyophilized) or liquid form. The kits of the present invention may optionally comprise different containers (*e.g.*, vial, ampoule, test tube, flask or bottle) for each individual buffer and/or reagent. Each
25 component will generally be suitable as aliquoted in its respective container or provided in a concentrated form. Other containers suitable for conducting certain steps of the disclosed methods may also be provided. The individual containers of the kit are preferably maintained in close confinement for commercial sale.

A kit according to the present invention may further comprise instructions for
30 using the kit according to a therapeutic method of the invention, such as for example method of administration, dosage of administration, indications, and the like. In

certain embodiments, a kit according to the present invention may comprise a device (e.g., a syringe and needle system) for administration to a patient.

Optionally associated with the container(s) can be a notice or package insert in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

An identifier, e.g., a bar code, radio frequency, ID tags, etc., may be present in or on the kit. The identifier can be used, for example, to uniquely identify the kit for purposes of quality control, inventory control, tracking movement between workstations, etc.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

Brief Description of the Drawing

Figure 1. Physical characteristics and encapsulation efficiency of microspheres produced using the PLGA-P188-PLGA penta-block copolymer 50P40 and using the PLGA-P188-PLGA copolymer 50P20. (A) External/internal morphology of 50P40-microspheres (white scale bar represents 10 μm). (B) External/internal morphology of 50P20-microspheres (white scale bar represents 10 μm). (C) Particle mean size and encapsulation efficiency of the microspheres.

Figure 2. (A) Lysozyme release profiles from microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 ($n = 10$) and from microspheres prepared using the PLGA-P188-PLGA copolymer 50P20 ($n = 24$). (B) Fitting of the release data obtained in (A) using the Higuchi's model by plotting the release rate versus time^{1/2}.

Figure 3. Size variation of microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 and of microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P20 during the release test.

Figure 4. Morphology changes of microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 (left column) and of microspheres

prepared using the PLGA-P188-PLGA penta-block copolymer 50P20 (right column) during the release test (white scale bar represent 10 μm) at different time points over a period of 28 days.

Figure 5. Internal structure of microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P20 incubated in release medium for two days at different temperatures: (A) 3°C, (B) 22°C and (C) 37°C.

Figure 6. Morphology and protein release profiles of (A) blend-microspheres (blend-MSs) and (B) mix-microspheres (mix-MSs). The porous microspheres correspond to the 50P20-microspheres while the microspheres exhibiting a non-porous surface correspond to the 50P40-microspheres. The white scale bars represent 10 μm .

Figure 7. Accumulated release rate plotted *versus* release time from day 1 to day 49 (release data at day 56 was excluded as a plateau was reached at day 49).

Figure 8. (A) Cross-sectional photograph of post-loading-microspheres (PL-MSs) after lysozyme loading process. (B) Physical mixture of PL-MS/50P20-MS (1/1) (PM-MSs) (white scale bar represents 10 μm). The more porous microspheres are the 50P20-MS while less porous microspheres are the PL-MSs. (C) Encapsulation rate of PL-MSs and PM-MSs compared with protein theoretical encapsulation.

Figure 9. Lysozyme release profile from post-loading microspheres (PL-MSs) (n = 9) and physical mixture of post-loading-microspheres/50P20-microspheres (1/1, w/w) (PM-MS) (n = 9).

Figure 10. Schematic diagram illustrating the different strategies used, in the present study, to modulate the protein release profile of PLGA-based microspheres.

Examples

The following examples describe some of the preferred modes of making and practicing the present invention. However, it should be understood that the examples are for illustrative purposes only and are not meant to limit the scope of the invention.

Materials and Methods

Materials. Lysozyme (chicken egg white), its substrate *Micrococcus lysodeikticus*, glycofurol, dimethylformamide (DMF), methylene chloride (DCM), acetone, and trizma base (Tris) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Pluronic[®] F68 (poloxamer 188 ou P188) was purchased from BASF (Levallois-Perret, France). Polyvinyl alcohol (PVA) (Mowiol[®] 4-88) was supplied by Kuraray Specialities Europe (Frankfurt, Germany). Two different PLGA-P188-PLGA penta-block copolymers were kindly supplied by CNRS Unit UMR 5247 (Montpellier, France). These copolymers are characterized by different PLGAs. The first PLGA-P188-PLGA penta-block copolymer, called 50P40, is made of a PLGA with a molecular weight (M_w) of 40 kDa and a molar ratio of lactic acid/glycolic acid (LA/GA) of 50/50 (or 1/1); and the second PLGA-P188-PLGA penta-block copolymer, called 50P20, is made of a PLGA with a molecular weight (M_w) of 20 kDa and a molar ratio LA/GA of 50/50 (or 1/1).

Preparation of Lysozyme-loaded Microspheres. Different methods have been used to prepare lysozyme-loaded microspheres.

Standard Procedure. Lysozyme-loaded penta-block PLGA-based microspheres were prepared as previously described (Tran *et al.*, Eur. J. Pharm., 2012, 45: 128-137). The theoretical protein loading was 0.6% (w/w). Briefly, lysozyme and P188 (with a ratio lysozyme/P188 of 1/10 (w/w) were dissolved in water, then glycofurol (3.12 g) was added to the obtained solution to form a suspension. After incubation at 4°C for 30 minutes, the precipitated lysozyme (in the form of precipitated lysozyme/P188 product) was recovered by centrifugation (10,000 g, 4°C, 30 minutes), and then re-dispersed in 2 mL of a mixture of dichloromethane and acetone (3/1, v/v) containing 150 mg of PLGA-P188-PLGA penta-block copolymer. The suspension was emulsified in 90 mL of polyvinyl alcohol (PVA) at 4% (o 6%) (w/v) at 1°C and mechanically stirred at 550 rpm (or 1000 rpm) for 1 minutes. After addition of 100 mL of deionized water, the emulsion was stirred for 10 additional minutes, and added to 500 mL of deionized water and stirred continuously during 20 minutes in order to extract the organic solvent. The microspheres were then filtered on a 5 µm filter (HVLP type, Millipore SA, Guyancourt, France), washed, freeze-dried and stored at -20°C.

Preparation of Microspheres using a Polymer Blend. This formulation process was quite similar to the “standard procedure” described above. The mixture of lysozyme and P188 was nano-precipitated in glycofurol using the same protocol. The precipitated lysozyme/P188 product was re-dispersed in 2 mL of a mixture of dichloromethane and acetone (3/1, v/v) containing 150 mg of copolymer blend – the copolymer blend being obtained by completely dissolving a physical blend of the 50P40 copolymer and of the 50P20 copolymer (ratio 1/1 by weight) in a mixture of dichloromethane and acetone (3/1, v/v). The emulsification and solvent extraction/evaporation steps were performed using the procedure as that described above.

Preparation of Microspheres using a Mixing Strategy. Lysozyme-loaded microspheres of 50P40 and lysozyme-loaded microspheres of 50P20 were separately prepared following standard procedure. After the freeze-drying steps, the microspheres obtained were preserved at -20°C for 24 hours for stabilization. The mixture was then formed by carefully mixing equal amounts of the 50P40-microspheres (50P40-MS) and 50P20-microspheres (50P20-MS) using a vortex. The mixture (50P40-MS/50P20-MS; 1/1 w/w) were preserved at -20°C prior to being used for further study.

Preparation of Lysozyme-loaded Microspheres by Post-Loading already formed Microspheres. Blank (or empty) microspheres were produced using the standard procedure described above, but without incorporating lysozymes. Then, following freeze-drying, they were loaded with the protein to obtain lysozymes post-loaded microspheres (PL-MSs). Briefly, 50 mg of blank microspheres were dispersed in 1.6 mL of TRIS buffer (Tris-HCl 0.01 M buffer and 0.09% w/v NaCl, pH 7.4) containing lysozyme. The concentration of lysozyme in TRIS buffer was 750 µg/mL. The suspension obtained was then shaken horizontally using a vortex and tube foam inserts (Scientific Industries, USA) during 45 minutes at room temperature. Then, the samples were centrifuged (20°C, 3000 g) for 5 minutes and the supernatants were removed. The obtained samples were washed three times with water, and then freeze-dried to be preserved prior to further studies.

Preparation of a Physical Mixture of Lysozyme Post-Loaded Microspheres (PL-MS) and 50P20 Microspheres. Lysozyme PL-MSs and blank (or empty) 50P20-

microspheres were prepared separately, as described above. After the freeze-drying step, the microspheres were all preserved at -20°C for 24 hours for stabilization. The mixture was then formed by carefully mixing equivalent amounts of PL-MSs and 50P20-MSs using a vortex. The mixture (PL-MSs/50P20-MSs, 1/1, w/w) was preserved at -20°C for further studies.

Microsphere Characterization Techniques.

Microsphere Morphology and Mean Size. Microsphere mean size was measured using a Coulter[®] Multisizer (Coultronics, Margency, France). The microspheres were dispersed in isotonic saline solution prior to be analyzed. Microsphere surface morphology was observed by scanning electron microscopy (SEM) (JSM 6310F, JEOL, Paris, France). Freeze-dried microspheres were mounted onto metal stubs using double-sided adhesive tape, vacuum-coated with a film of carbon using MED 020 (Bal-Tec, Balzers, Lichtenstein) before being investigated. The microspheres internal morphology was examined using the following process: an appropriate amount of microspheres was dispersed in 1 mL of Tissue-Tel[®] (Sakura Finetek, USA), then frozen (-20°C, 1 hour). The block obtained was then cut into (20 µm-thick) slices at -15°C maximum with a micro-cutting device (Leica, USA). The slices were rinsed three times with cold water (1°C) before being freeze-dried. The samples obtained were then analyzed by SEM, as described above.

Glass Transition Temperature of Polymers and Polymer Microspheres. To determine the glass transition temperature of the raw copolymer and of the copolymer under the form of microspheres, differential scanning calorimetry (DSC) analyses were carried out. DSC measurements were performed under nitrogen on a Perkin-Elmer DSC 6000 thermal analyzer. Samples (of raw copolymer or freeze-dried microspheres) were subjected to a first heating scan from -50°C to 200°C (+10°C/minute), followed by a cooling step (-10°C/minute), and then to a second heating scan from -50°C to 200°C (+10°C/minute). The glass transition temperature (T_g) was measured during a second heating scan.

Protein Encapsulation Efficiency. The amount of entrapped lysozyme (within microspheres) was determined by dissolving 5 mg of microspheres in 0.9 mL of DMF in a silanized glass tube at room temperature under agitation (1 hour). Then, 3 mL of TRIS solution (Tris-HCl 0.01 M buffer and 0.09% w/v NaCl, pH 7.4) were added to

the glass tube, and the resulting mixture was agitated for 1 additional hour. The obtained solution was then submitted to a *Micrococcus lysodeikticus* test for quantification of active lysozyme, as previously described (Morille *et al.*, J. Control. Release, 2013, 170: 99-110). Protein encapsulation efficiency (EE) was determined in triplicate.

In vitro Release of Active Lysozyme from Microspheres. Lysozyme-loaded microspheres (5 mg) were dispersed in 375 μ L of a buffer solution (Tris-HCl 0.01 M buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl) in tubes. The mixture was incubated in a shaken thermostatic bath (37°C, 125 rpm). At defined time intervals, the tubes were centrifuged for 5 minutes at 3000 g. The supernatant was collected, tested for active lysozyme quantification, and replaced with fresh buffer. The release profiles were established using at least three different batches of microspheres and for each one, at least three independent experiments were carried out. In order to assess the effect of temperature on the release of active lysozyme from microspheres, the release test was carried out using the same protocol as previously described, except that the temperature of the thermostatic bath was set of 22°C or the samples were placed in a 4°C chamber.

Variations of Microspheres Morphology During Release Test. Lysozyme-loaded microspheres (15 mg) were dispersed in 1 050 μ L of a buffer solution (Tris-HCl 0.01 M buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl) in tubes. The mixture was incubated in a shaken thermostatic bath (37°C, 125 rpm). After a certain time, the tubes were centrifuged for 5 minutes at 3000 g. The supernatant was removed and the remaining particles were rinsed three times with water (1°C) before being freeze-dried. Before microsphere cross-sectional observation, the microspheres were cut and submitted to a SEM analysis, as described above.

Results and Discussion

Physical Characteristics and Protein Encapsulation Efficiency of the Prepared Microspheres

Morphology, Mean Size and Encapsulation Efficiency. The microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 were found to exhibit a smooth surface with visible, distinct pores. The internal structure was observed to be porous, having a number of closed-pores which distribute uniformly

throughout the microspheres (see Figure 1(A)). On the other hand, the microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P20 were found to be highly porous with interconnected pores on the surface as well as inside the microspheres (see Figure 1B).

5 The differences in morphology/porosity of the microspheres prepared using PLGA-based copolymers with different PLGA molecular weights can be attributed to different polymer solidification rates during solvent removal (Hiraoka *et al.*, Chem. Pharm. Bull. (Tokyo), 2014, 62: 654-660; Lee and Sah, J. Mater. Sci., 2016, 1-18). Since lower molecular weight polymers are more soluble in organic solvents (Ito *et al.*, Colloids Surfaces B Biointerfaces, 2007, 54: 173-178; Gaignaux *et al.*, Int. J. Pharm., 2012, 437: 20-28), the PLGA-P188-PLGA penta-block copolymer 50P20 was able to remain in a semi-solid state for a longer time than the PLGA-P188-PLGA penta-block copolymer 50P40, before turning to a solid state upon solvent extraction. The slower precipitation allows the water to penetrate more significantly, yielding
10 internally porous microspheres (Yeo *et al.*, Arch. Pharm. Res., 2004, 27: 1-12; Mao *et al.*, Eur. J. Pharm. Biopharm., 2008, 68: 214-223).

 In contrast to morphological aspects, the mean sizes of the microspheres prepared using the 50P40 PLGA-P188-PLGA penta-block copolymer and of the microspheres prepared using the 50P20 PLGA-P188-PLGA penta-block copolymer
20 were quite similar (39.6 ± 1.1 mm and 40.5 ± 2.9 μ m, respectively).

 The encapsulation efficiency (EE) values obtained ranged from 40% to 60% regardless of the PLGA-P188-PLGA penta-block copolymer used (see Figure 1D). The loss of lysozyme during the formulation process can be attributed to the porous structure of the microspheres, which resulted in protein leaking into the external
25 aqueous phase (Fu *et al.*, J. Microencapsul., 2005, 22: 705-714; Luan *et al.*, Int. J. Pharm., 2006, 324: 168-175).

Glass Transition Temperature. The data obtained in the DSC analyses performed, which are presented in Table 1 below, highlight the interaction between the poloxamer/lysozyme and the PLGA-P188-PLGA penta-block copolymer.

Table 1. Glass transition temperature of copolymer in studied samples

Samples	T _g (°C)
Copolymer 50P40	24.3
50P40-MS with no additives and no lysozyme	24.2
50P40-MS with additives and no lysozyme	23.7
50P40-MS with additives and with lysozyme	15.9
Copolymer 50P20	9.6
50P20-MS with no additives and no lysozyme	9.1
50P40-MS with additives and no lysozyme	5.5
50P40-MS with additives and with lysozyme	3.5

As can be seen in Table 1, the difference in PLGA molecular weight resulted in different T_g values. The PLGA-P188-PLGA penta-block copolymer prepared with the PLGA of higher molecular weight (40 kDa) was found to exhibit a higher T_g value compared to the PLGA-P188-PLGA penta-block copolymer prepared with the PLGA of lower molecular weight (20 kDa). The same was true for the blank (or empty) microspheres prepared from these copolymers. These results suggest that the microspheres formation process does not modify the glass transition temperature of the starting PLGA-P188-PLGA penta-block copolymer.

The presence of additives in the microspheres was found to have no influence on the T_g values of the 50P40-microspheres, and a small effect on the T_g values of the 50P20-microspheres (T_g decreased from 9.14°C to 5.49°C). Importantly, the encapsulation of lysozyme (and of additives) in a polymeric matrix contributed to a remarkable decreases in the T_g values of the copolymer (a decrease from 24.23°C to 15.89°C was observed in the case of the 50P40-microspheres and from 9.14°C to 3.49°C in the case of the 50P20-microspheres). Hydrophobic interactions between lysozyme and polymer has previously been identified (Hamishehkar *et al.*, Colloids Surfaces B Biointerfaces, 2009, 74: 340-349), and these interactions are probably responsible for the decrease in T_g values observed in the present study. Since protein-copolymer interactions have been observed, the effect of the nature of the copolymer on protein release profile is worth investigating.

Protein Release Profiles. Copolymer characteristics, microsphere porosity and mean size are recognized as critical factors to the release rate of encapsulated protein

from PLGA-based microspheres (Siepmann *et al.*, J. Control. Release, 2004, 96: 123-134; Klose *et al.*, Int. J. Pharm., 2006, 314: 198-206). In the present study, the microspheres mean size was kept the same to eliminate the impact of size on microspheres release behavior. The active protein release from 50P40-microspheres (dense surface/porous interior) and from 50P20-microspheres (porous surface/porous interior) was studied for 10 weeks.

Lysozyme release from the 50P40-microspheres was found to have a bi-phasic profile with no significant burst ($11.2\% \pm 1.3\%$ released after 24 hours). The lack of significant burst is due to the presence of a dense surface on the microspheres. The release was then sustained for 56 days, at which time accumulated release was $53.8\% \pm 6.2\%$. In contrast, highly porous 50P20-microspheres exhibited a successful bi-phasic protein profile. An insignificant burst was observed after the first 24 hours ($15.2\% \pm 1.1\%$), followed by a continuous and complete release over 8 weeks (ending up with $93.4\% \pm 3.1\%$ of active lysozyme released) (see Figure 2(A)). Interestingly, both release profiles fitted well to Higuchi's model (R-squared values were 0.990 and 0.992 for the lysozyme-loaded 50P40- and 50P20-microspheres, respectively) (see Figure 2B).

Drug release from PLGA-based microspheres can follow mono-, bi- or tri-phasic profiles depending on a variety of factors including their own hydrophilicity/hydrophobicity and the morphology of the microspheres (Fredenberg *et al.*, Int. J. Pharm., 2011, 415: 34-52). As far as protein-encapsulated microspheres are concerned, the bi-phasic profile is commonly achieved with porous microspheres (Ahmed and Bodmeier, Eur. J. Pharm. Biopharm., 2009, 71: 264-277; Boukari *et al.*, J. Biomater. Sci. Polym., 2015, 26: 796-811) or with microspheres having a non-porous surface but a porous interior (D'Aurizio *et al.*, Int. J. Pharm., 2011, 409: 289-296). However, a profile fitted to the Higuchi's model is rare with PLGA-based microspheres. This is because the Higuchi's model describes drug release principally due to a diffusion process following Fick's law (Costa and Lobo, Eur. J. Pharm. Sci., 2001, 13: 123-133). Meanwhile, the mechanism of drug release from PLGA depot is impacted not only by drug diffusion but also by polymer degradation and erosion (Siepmann *et al.*, Biomacromolecules, 2005, 6: 2312-2319; Berklund *et al.*, J. Pharm. Sci., 2007, 96: 1176-1191; Sackett *et al.*, Int. J. Pharm., 2011, 418: 104-114; Ford

Versypt *et al.*, J. Control. Release, 2013, 165: 29-37). In the present study, the release of lysozyme from 50P20-microspheres and from 50P40-microspheres exhibited a bi-phasic profile and fitted to the Higuchi's model, suggesting that they are matrix systems, where the main mechanism of protein release is dominated by diffusion (Costa and Lobo, Eur. J. Pharm. Sci., 2001, 13: 123-133). On the other hand, a burst effect was commonly observed when porous PLGA-based microspheres were used in previous studies (Patel *et al.*, J. Control. Release, 2012, 162: 310-320; Wei *et al.*, J. Colloid Interface Sci., 2016, 478: 46-53). However, this was not the case when 50P40-microspheres were used in the present study. All of these results raised the question of the nature of the release mechanism of lysozyme from the studied microspheres. With the goal of acquiring more understanding about this process, the variations in microspheres morphology and size during the release test were evaluated.

Microspheres Morphology and Size Change During the Release Test. To clarify the mechanism of protein release from the prepared microspheres, the variation of their particle size and morphology was evaluated during the release test. The results obtained are presented in Figure 3 and Figure 4.

As far as the microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 are concerned, after 2 days, many pores were found to appear on the surface of the microspheres. Over time, the internal structure did not change much with the exception of a slight increase of peripheral porosity and the formation of a thin outer layer (see the second picture of Figure 4(A), left column). Importantly, the mean size of the microspheres was found to increase noticeably during the first two days (about 110% compared to the initial mean size) (see Figure 3). The size increase probably results from the swelling of the microspheres due to the presence of P188 in the copolymer, as reported elsewhere (Witt and Kissel, Eur. J. Pharm. Biopharm., 2001, 51: 171-181). It has recently been demonstrated that the swelling of PLGA-based microspheres was responsible for the huge release observed during the third stage of the tri-phasic release profile (Gasmi *et al.*, J. Control. Release, 2015, 213: 120-127; Gasmi *et al.*, J. Drug Deliv. Sci. Technol., 2015, 30: 123-132; Gu *et al.*, J. Control. Release, 2016, 228: 170-178; Messaritaki *et al.*, J. Control. Release, 2005, 108: 271-281). In addition, microsphere swelling is suggested as the rate-limiting step

of drug release in the initial burst release (not the diffusion of water or the presence of drug in pores of the microspheres) (Messaritaki *et al.*, J. Control. Release, 2005, 108: 271-281). Therefore, it is believed that in addition to drug located on the surface, microsphere swelling contributes to the mechanism of lysozyme release from microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 during the first two days.

At day 10, the porosity of the microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 was observed to increase remarkably, especially in the peripheral area (see the third picture of Figure 4A, left column). Moreover, the pores were connected together and supported the release of protein due to a diffusion mechanism. From day 10 to day 28, no significant differences were observed in terms of mean size and of morphology, besides the erosion that was taking place inside the 50P40-microspheres. Referring to Higuchi's model fitting of the protein release profile of the 50P40-microspheres, it is probable that the lack of polymer erosion might be maintained until day 56, which explains the diffusion-dominated protein release mechanism from 50P40-microspheres over this period. The retardant effects of polymer degradation and erosion have previously been observed with small/porous PLGA-based microspheres. During the release test, the exchanges between the environment inside the microspheres and the external buffer medium were well facilitated. Consequently, the acidification of the internal micro-environment due to the accumulation of PLGA degraded products was limited, and hence the autocatalysis phenomenon was also limited (Klose *et al.*, Int. J. Pharm., 2008, 354: 95-103; Versypt *et al.*, PLoS one, 2015, 10: 1-14). Therefore, polymer erosion can be retarded, hence increasing drug release duration (Berkland *et al.*, J. Pharm. Sci., 2007, 96: 1176-1191).

Thus, in the mechanism of protein release from microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P40 during 10 weeks, it was identified that microsphere swelling contributes to the release for the first two days, while diffusion following Fick's law dominates the release mechanism afterwards. As no clear sign of polymer erosion was observed, it is believed that the release profile has not yet reached completion. However, a plateau drawn from day 56 to day 70 (data

not shown) indicates that this type of microspheres is of no interest considering the purpose of the current study.

The microspheres prepared using the PLGA-P188-PLGA penta-block copolymer 50P20 were found to behave differently. The mean size of the 50P20-microspheres decreased continuously from the beginning of the test until day 28, at which time the microspheres shrunk to less than 25 μm mean size. Furthermore, the morphology changes undergone by the 50P20-microspheres were also different. It was clear from the SEM images obtained that the pore-closing process occurred over the first two days. The 50P20-microspheres became denser with no visible pores on the surface as well as in the core. This phenomenon explained the reduced burst seen in Figure 2(A). A pore-closing process upon incubation of microspheres in a release buffer has been demonstrated by Wang *et al.* (J. Control. Release, 2002, 82: 289-307). In the case of PLGA-based microspheres, the pore-closing process was attributed to the temperature of the release test (generally 37°C), which is close to the PLGA glass transition temperature (Wang *et al.*, J. Control. Release, 2002, 82: 289-307; Lee and Sah, J. Mater. Sci., 2016, 1-18). It is probably also the reason for the pore-closing process observed in the present study since the 50P20 copolymer has a low T_g value of 9.6°C and the 50P20-microspheres have a T_g value of 3.5°C. In order to examine this hypothesis, the morphology of the 50P20-microspheres after two days of incubation in the release buffer medium was studied at different temperatures. The results obtained are presented in Figure 5. Microspheres with a highly porous internal structure were obtained after two days of incubation at 3°C. When the incubation was carried out at 22°C, the internal porosity was found to decrease remarkably, while totally dense microspheres were obtained under physiological temperature (37°C). Thus, the temperature of the buffer medium influenced the pore-closing process of porous 50P20-microspheres during the release test. The extent of this effect was related to the T_g value of the polymer. Indeed, as the temperature of the release medium gets close to the T_g value of the polymer, the polymeric chains become more mobile and closure of the pores occurs under shaking conditions (Kang *et al.*, Mol. Pharm., 2007, 4: 104-118; Lee and Sah, J. Mater. Sci., 2016, 1-18).

Following the pore-closing process, which was observed during the first two days of incubation in the release buffer medium, the internal structure of the 50P20-

microspheres was found to become more porous and the shape of the 50P20-microspheres was no more spherical at day 10. The porosity increase and associated non-spherical shape could be inferred to the onset of polymer degradation/erosion inside the microspheres. The shrinkage of the microspheres and concurrent decrease of their mean size were important at day 28.

Since the 50P20-microspheres achieve a complete protein release after 10 weeks, said release exhibiting no significant burst and following Higuchi's model release kinetics, they are highly interesting candidates for protein sustained release delivery.

Protein Release Profile Modulation. With the study purposes including protein release modulation, different strategies were carried out to achieve various types of protein release profile.

Biphasic Release with Second Phase Exhibiting Zero-order Kinetics. Although drug release well-fitted to the Higuchi model is relevant for a sustained delivery system, the release rate is still not constant over time. Instead, zero-order kinetics release represents the ideal system for pharmacological prolonged action – since the same amount of drug is released by unit of time (Costa and Lobo, Eur. J. Pharm. Sci., 2001, 13: 123-133). Therefore, the present Inventors have tried to prepare microspheres exhibiting a biphasic release profile with a second phase (from day 2 on) with zero-order kinetics. Two different strategies have been used: a polymer blending strategy and a physical mixing strategy.

The mean size and encapsulation efficiency of microspheres obtained from polymer blending and from physical mixture (hereafter called blend-MSs and mix-MSs, respectively) were found to be rather similar to those of the 50P40-microspheres and the 50P20-microspheres (Figure 6).

The blend-microspheres were found to exhibit a porous surface and an internal structure with numerous closed-pores distributed uniformly throughout the spheres. Moreover, the porosity of the blend-microspheres appeared to be between the porosity of the 50P40-microspheres and the porosity of the 50P20-microspheres. This observation is consistent with previous studies. An initial burst was observed with

20.4% $\pm$ 1.0% lysozyme released after the first 24 hours of incubation. The release was then continuous and reached 69.1% $\pm$ 4.9% at day 56.

SEM photography of mix-microspheres revealed two different types of morphology, which illustrated the mixture of the 50P40-microspheres the 50P20-microspheres. Protein release was found to be complete (82.3% $\pm$ 2.6%), and complied with a biphasic profile having a second phase of zero-order kinetics. The initial burst was limited with 15.2% $\pm$ 4.0% protein released after the first 24 hours of incubation. The release profile of the mix-microspheres was found to be intermediate between the release profile of the 50P40-microspheres and the 50P20-microspheres. Furthermore, when considering the protein release from day 1 (after the initial significant release) to day 49 (where the release was almost discontinued reaching a plateau), the accumulated release rate versus time was found to be linear ($R^2 = 0.9915$). This is also an improvement in released control compared to the non-linear plot of the corresponding release from the 50P20-microspheres (see Figure 7).

Complete Protein Release Profile with Burst. Theoretically, a burst effect is not desired in a drug delivery system because the massive release of drug in the human body can result in adverse effects. However, under particular circumstances, such as pharmacologically active microcarriers (PAMs), the significant burst release of protein during the first days is essential to induce stem cell commitment. In addition, the continuous protein release during the second phase contributes to the maintenance of a differentiated phenotype (Morille *et al.*, J. Control. Release, 2013, 170: 99-110).

Under protein post-loading conditions, the porosity of the resultant microspheres (PL-microspheres or PL-MSs) was found to decrease (see Figure 8(A)). Porosity reduction was attributed to the pore-closing process, which has already been observed above. The occurrence of the pore-closing process might be useful for improving protein loading efficiency of the post-loading process. Protein loading rate was 0.87% $\pm$ 0.32% in post-loading microspheres and 0.69% $\pm$ 0.10% in physical mixture microspheres (PM-MSs) (see Figure 8(C)). Interestingly, the loading rate of PL-MSs was approximately the theoretical protein encapsulated rate of microspheres prepared according to the standard procedure (0.6%).

A burst release was found to occur for both types of microspheres: post-loading-microspheres (PL-MSs) and physical mixture microspheres (PM-MSs) (see Figure 9). The PL-MSs exhibited a larger burst (with a $61.5\% \pm 8.1\%$ lysozyme release during the first 24 hours) than the PM-MSs ($42.6\% \pm 8.9\%$). Subsequently, the release from PL-MSs was continuous, following zero-order kinetics and was complete after 42 days with approximately 100% active protein released. Again, physical mixture microspheres showed a release profile that was intermediate between that of both microsphere components. The physical mixture microspheres, which release more than half of the encapsulated protein during the first 2 days, and release the rest continuously over several weeks, meet the biological requirements for pharmacologically active microcarriers (PAMs).

Overall, in the present study, the protein release profile of microspheres prepared with the PLGA-P188-PLGA penta-block copolymer 50P40 and with the PLGA-P188-PLGA penta-block copolymer 50P20 was modulated using different strategies (see Figure 10). Using a S/O/W (solid/oil/water) extraction/evaporation method for the preparation, the porosity of the microspheres thus obtained is explained by the presence of amphiphilic segments at the center of the copolymer structure. The extent of porosity of the microspheres was dependent on the molecular weight (M_w) of the PLGA. The differences in copolymer characteristics also resulted in distinct behaviors of the microspheres during the release test and the protein release profile. Complete release following a biphasic sustained profile was achieved with the microspheres prepared with the PLGA-P188-PLGA penta-block copolymer 50P20. On the other hand, the microspheres prepared with the PLGA-P188-PLGA penta-block copolymer 50P40 did not each complete protein release. The release profiles from both the 50P20-microspheres and the 50P40-microspheres fitted well to the Higuchi model, indicating that these microspheres are interesting for protein sustained delivery in which protein-protein is principally liberated *via* Fick's law diffusion. Although the Higuchi model fitted systems do not continuously deliver protein over time, the protein release from a physical mixture of these 50P20-microspheres and 50P40-microspheres was found to comply with zero-order kinetics. Practically, by mixing equivalent amounts of the 50P20-microspheres and 50P40-microspheres, the resultant samples were found to exhibit a biphasic protein release

with an insignificant burst and a release following zero-order kinetics in the second phase. Similarly, intermediate properties were observed by mixing equivalent amounts of 50P20-microspheres and post-loading microspheres. These results highlight the potential of microspheres physical mixing as a promising strategy for modulating protein release.

Microspheres resulting from physical mixture exhibit two types of morphology (*i.e.*, porous microspheres and less porous or non-porous microspheres), present the advantage of being not only useful for drug delivery applications but also for scaffold for tissue engineering. In tissue regeneration applications, PLGA-based scaffolds composed of non-porous microspheres exhibit relatively high compressive strength, which is one of their important properties (Bose *et al.*, Trends Biotechnol., 2012, 30: 546-554; Rahman *et al.*, J. Biomed. Mater. Res. – Part B Appl. Biomater., 2013, 101: 648-655). The porosity of a scaffold for tissue engineering is also known to play an important role for cell adhesion, proliferation and differentiation (Cheng *et al.*, J. Biomed. Mater. Res. – Part B Appl. Biomater., 2016, 104: 1056-1063; Qutachi *et al.*, Acta Biomater, 2014, 10: 5090-5098). The benefits of an open porous structure with interconnecting pores include: sufficient cell seeding density, and increased cell viability, proliferation and differentiation. Furthermore, the transport of nutrients and oxygen can be maintained for subsequent cell proliferation and differentiation (Kang *et al.*, J. Biomater. Sci. Polym. Ed 20, 2009, 20: 399-409; Chou *et al.*, J. Biomed. Mater. Res. A, 2013, 101: 2862-2869). For this reason, scaffolds have been fabricated by combining porous and non-porous microspheres in order to adjust the compressive strength as a function of the area where the scaffold is to be applied (Qutachi *et al.*, Acta Biomater, 2014, 10: 5090-5098; Boukari *et al.*, J. Biomater. Sci. Polym. Ed., 2015, 26: 796-811).

Other Embodiments

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of the specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope of the invention being indicated by the following claims.

Claims

What is claimed is:

1. Protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein the protein-loaded microspheres are obtained:
 - using a S/O/W solvent extraction/evaporation method, or
 - by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer.
2. A protein-loaded microsphere mixture comprising:
 - a first population of protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a first protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa; and
 - a second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer and comprising a second protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 40 kDa,wherein each of the first and second populations of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method and wherein the first protein and the second protein are the same protein or are different proteins.
3. A protein-loaded microsphere mixture comprising:
 - a first population of protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a first protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein said first population of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method; and
 - a second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer and comprising a

second protein embedded in the microspheres, wherein the PLGA has a mean molecular weight of 20 kDa, and wherein said second population of protein-loaded microspheres is obtained by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer,

wherein the first protein and the second protein are the same protein or are different proteins.

4. A composition of cell-carrying, protein-loaded microspheres comprising:
- protein-loaded microspheres, wherein at least part of the outer surface of the microspheres is coated with a cell adhesion surface coating; and
 - whole cells and/or cell fragments bound to the cell adhesion surface coating,

wherein the protein-loaded microspheres are composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprise a protein embedded in the microspheres, the PLGA has a mean molecular weight of 20 kDa, and the protein-loaded microspheres are obtained by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer.

5. A composition of cell-carrying, protein-loaded microspheres comprising:
- a first population of protein-loaded microspheres composed of a PLGA-poloxamer-PLGA penta-block copolymer and comprising a first protein embedded in the microspheres, wherein at least part of the outer surface of the protein-loaded microspheres is coated with a cell adhesion surface coating, and whole cells and/or cell fragments are bound to the cell adhesion surface coating, wherein the PLGA has a mean molecular weight of 20 kDa and wherein the first population of protein-loaded microspheres is obtained using a S/O/W solvent extraction/evaporation method; and
 - a second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer and comprising a second protein embedded in the microspheres, wherein at least part of the outer surface of the protein-loaded microspheres is coated with a

cell adhesion surface coating, and whole cells and/or cell fragments are bound to the cell adhesion surface coating, wherein the PLGA has a mean molecular weight of 20 kDa and wherein the second population of protein-loaded microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer is obtained by loading the protein in blank microspheres composed of the PLGA-poloxamer-PLGA penta-block copolymer,

wherein the first protein and the second protein are the same protein or are different proteins.

6. The composition of cell-carrying, protein-loaded microspheres according to claim 4 or claim 5, wherein the cell adhesion surface coating comprises fibronectine and poly-D-lysine.
7. The protein-loaded microspheres according to claim 1, or the protein-loaded microsphere mixture according to claim 2 or claim 3, or the composition of cell-carrying, protein-loaded microspheres according to any one of claims 4 to 6, wherein the poloxamer is poloxamer 188 (P188).
8. The protein-loaded microspheres according to claim 1 or claim 7, or the protein-loaded microsphere mixture according to any one of claims 2-3 and 7, or the composition of cell-carrying, protein-loaded microspheres according to any one of claims 4 to 7, wherein the PLGA has a molar ratio of lactic acid/glycolic acid (LA/GA) of 50/50.
9. The protein-loaded microspheres according to any one of claim 1 and 7-8, or the protein-loaded microsphere mixture according to any one of claims 2-3 and 7-8, or the composition of cell-carrying, protein-loaded microspheres according to any one of claims 4 to 8 for use in a method of treatment of the human or animal body.
10. The composition of cell-carrying, protein-loaded microspheres according to any one of claims 4 to 8 for use in a method of treatment of a degenerative disease of the human or animal body.

11. A pharmaceutical composition comprising an efficient amount of the protein-loaded microspheres according to any one of claim 1 and 7-8, or the protein-loaded microsphere mixture according to any one of claims 2-3 and 7-8, or the composition of cell-carrying, protein-loaded microspheres according to any one of claims 4 to 8, at least one pharmaceutically acceptable carrier or excipient, and optionally at least one therapeutic and/or prophylactic agent.
12. A kit comprising:
- a PLGA-poloxamer-PLGA penta-block copolymer, wherein the PLGA has a mean molecular weight of 20 kDa, and a first protein, and instructions to prepare protein-loaded microspheres according to claim 1 or a protein-loaded microsphere mixture according to claim 3;
- or
- a first PLGA-poloxamer-PLGA penta-block copolymer, wherein the PLGA has a mean molecular weight of 20 kDa, a second PLGA-poloxamer-PLGA penta-block copolymer, wherein the PLGA has a mean molecular weight of 40 kDa and a second protein, and instructions to prepare protein-loaded microsphere mixture according to claim 2,
- wherein the first protein and the second protein are the same protein or are different proteins.
13. A kit comprising:
- a PLGA-poloxamer-PLGA penta-block copolymer, wherein the PLGA has a mean molecular weight of 20 kDa, a first protein and a second protein, at least one adhesion molecule, and instructions to prepare a composition of cell-carrying, protein-loaded microspheres according to claim 4 or claim 5,
- wherein the first protein and the second protein are the same protein or are different proteins.
14. The kit according to claim 13 further comprising whole cells and/or cell fragments.

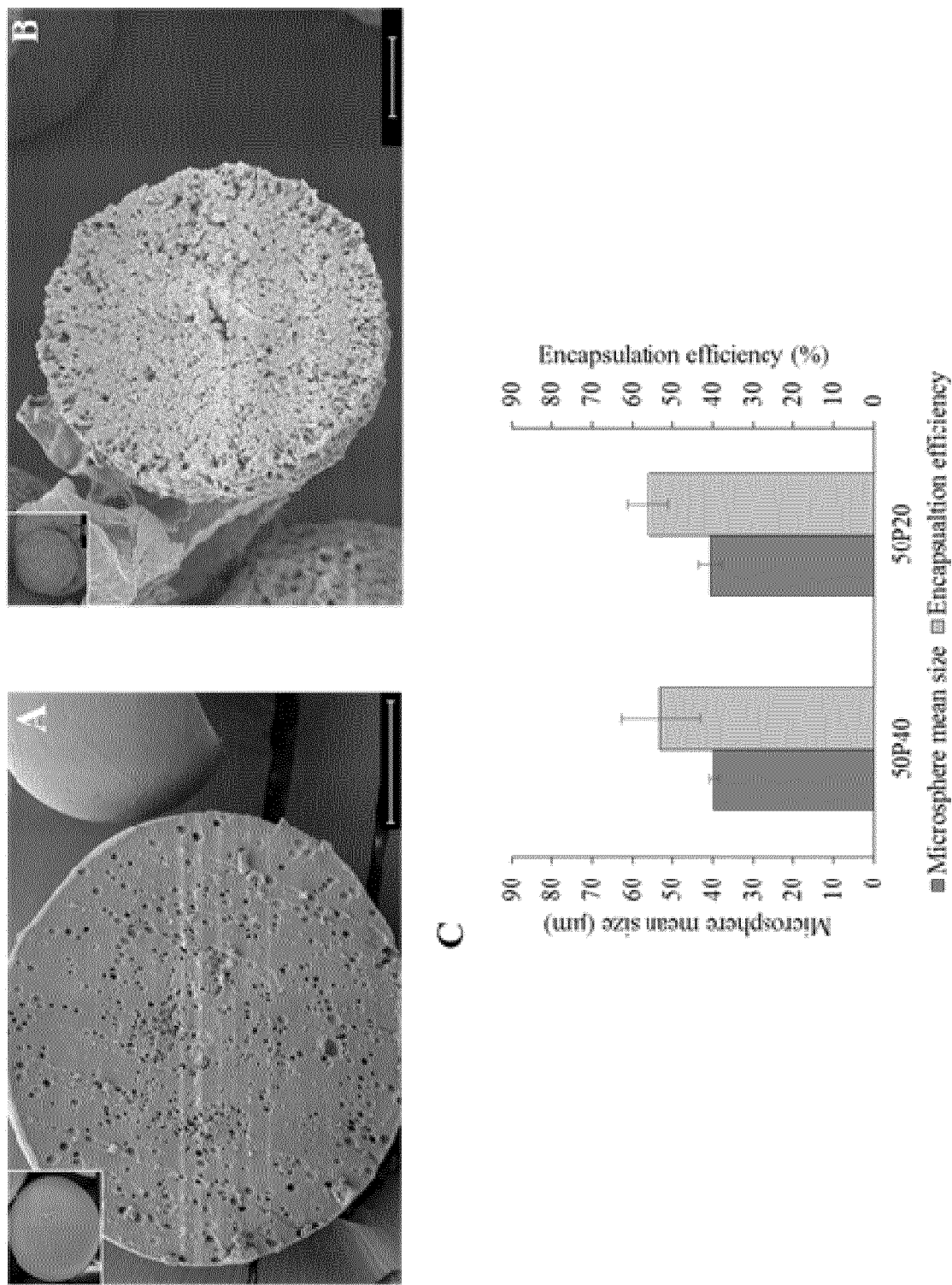


Figure 1

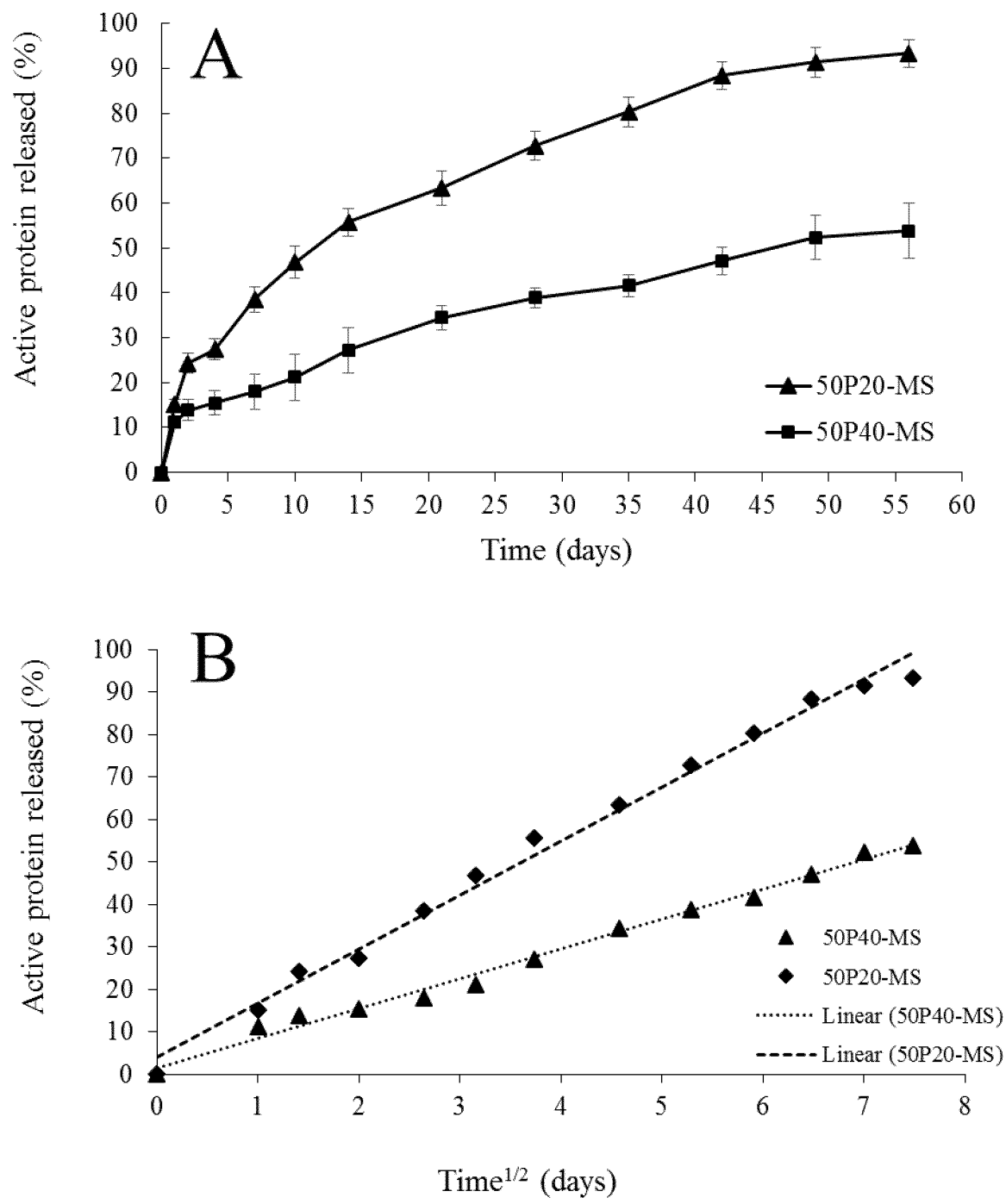
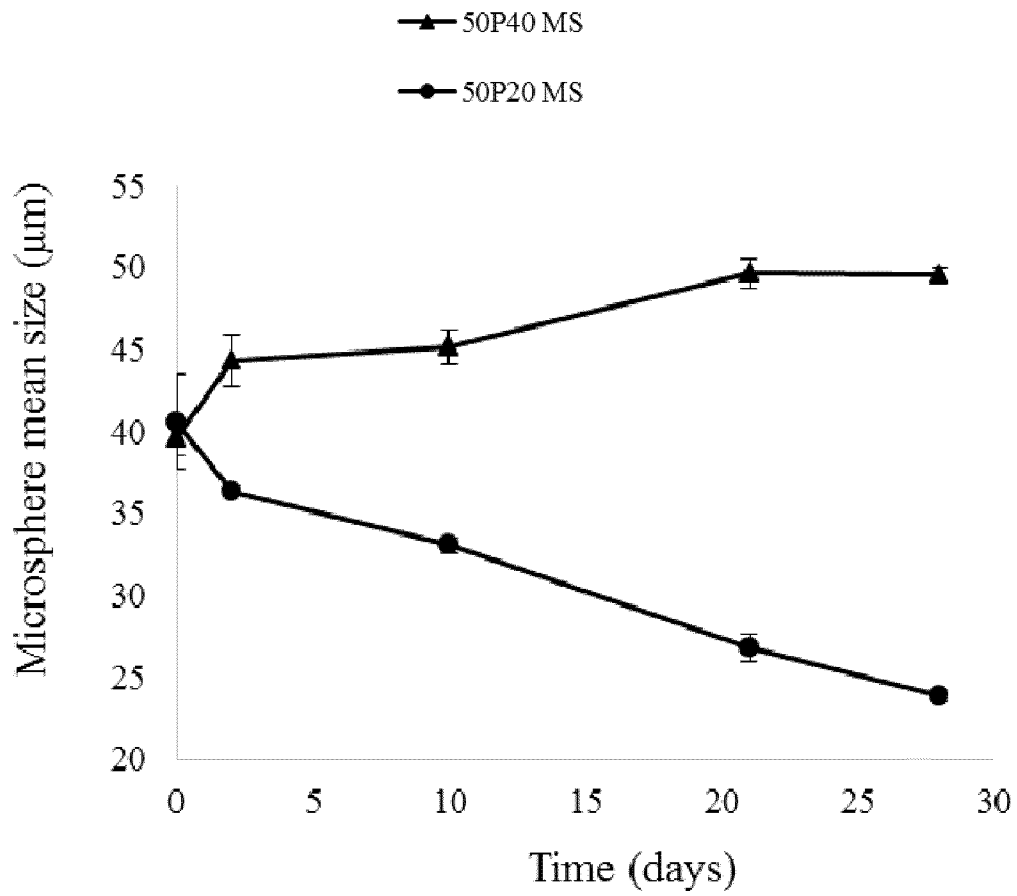
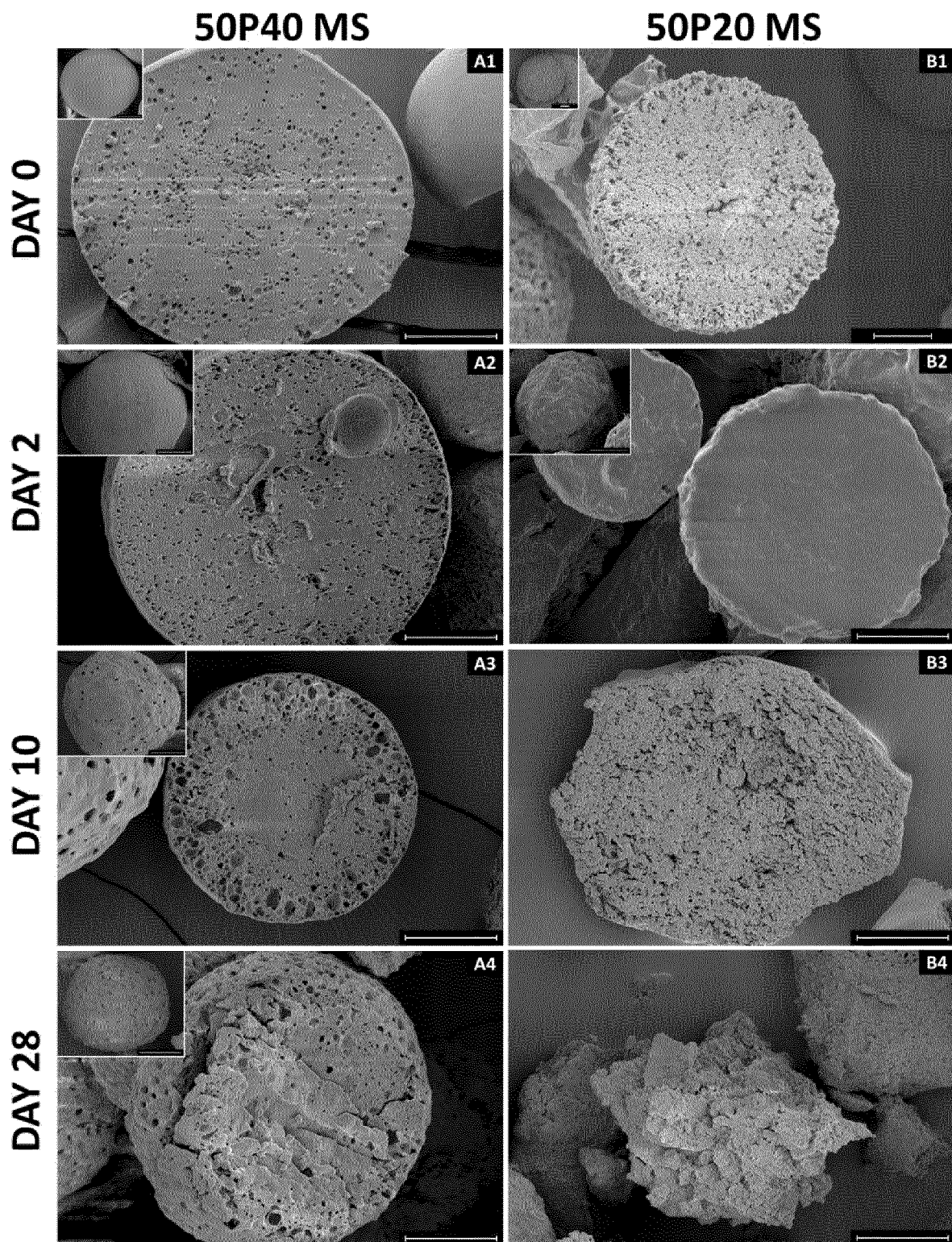


Figure 2

3 / 10

**Figure 3**

**Figure 4**

5 / 10

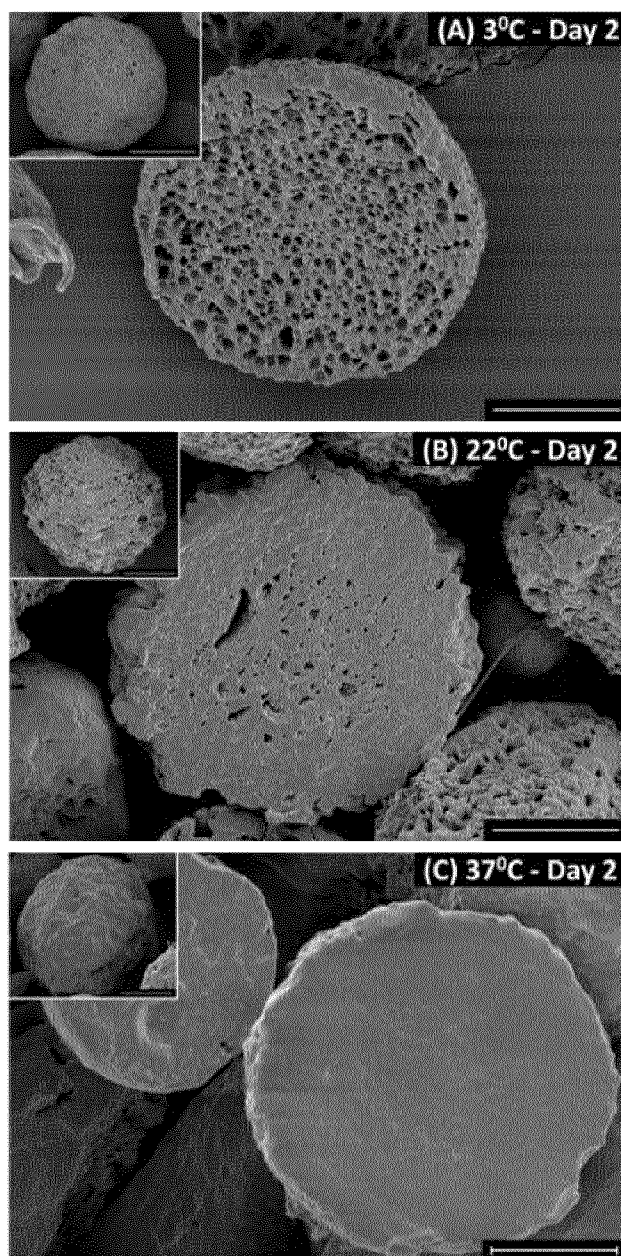


Figure 5

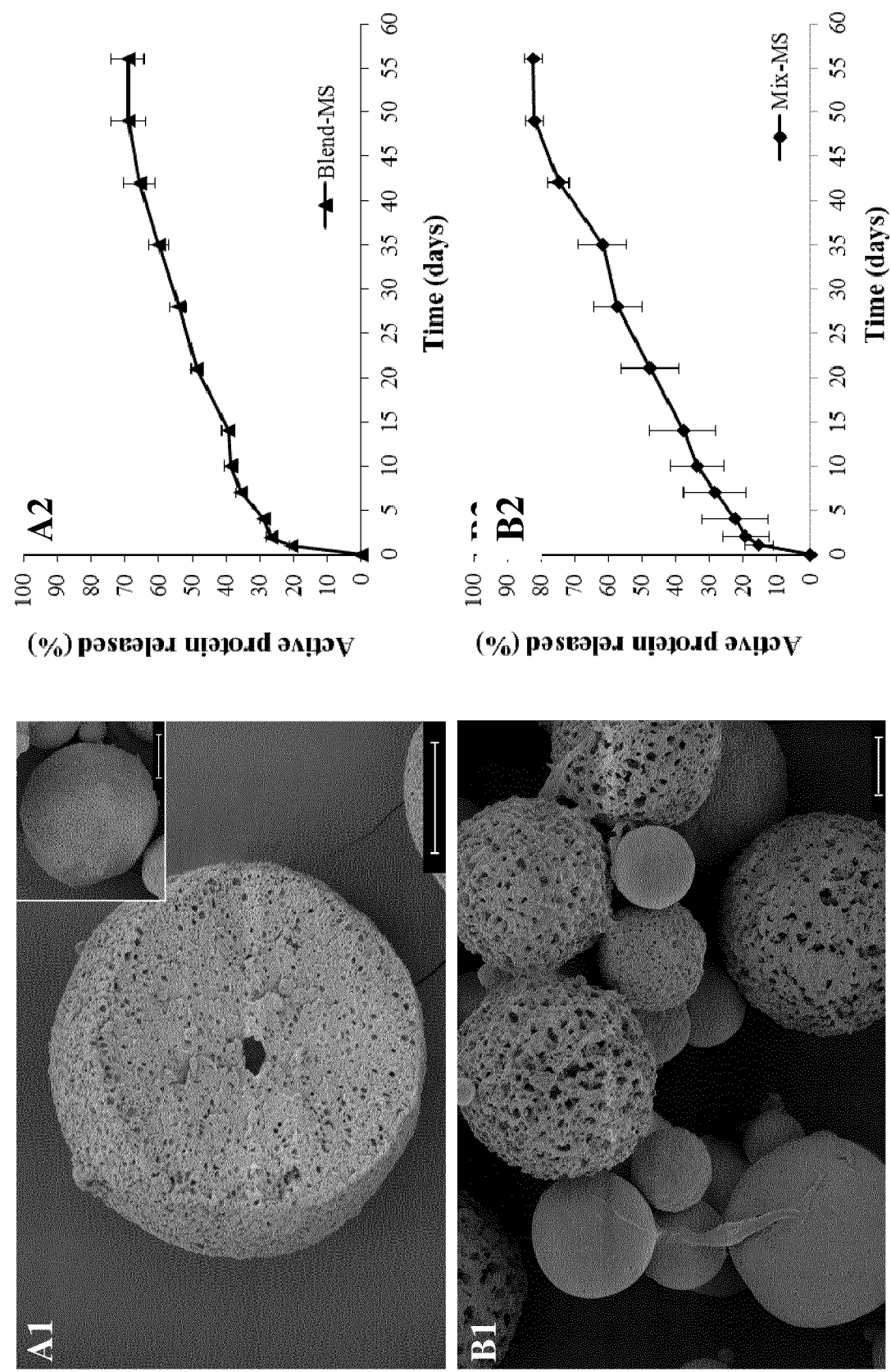


Figure 6

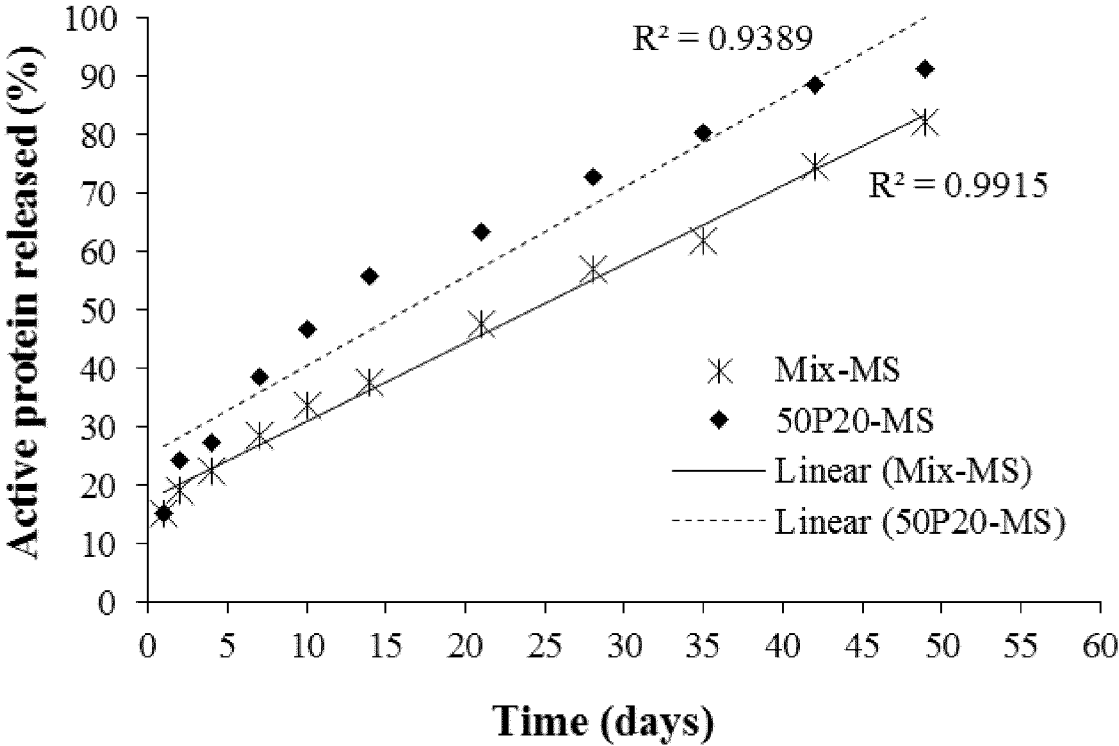


Figure 7

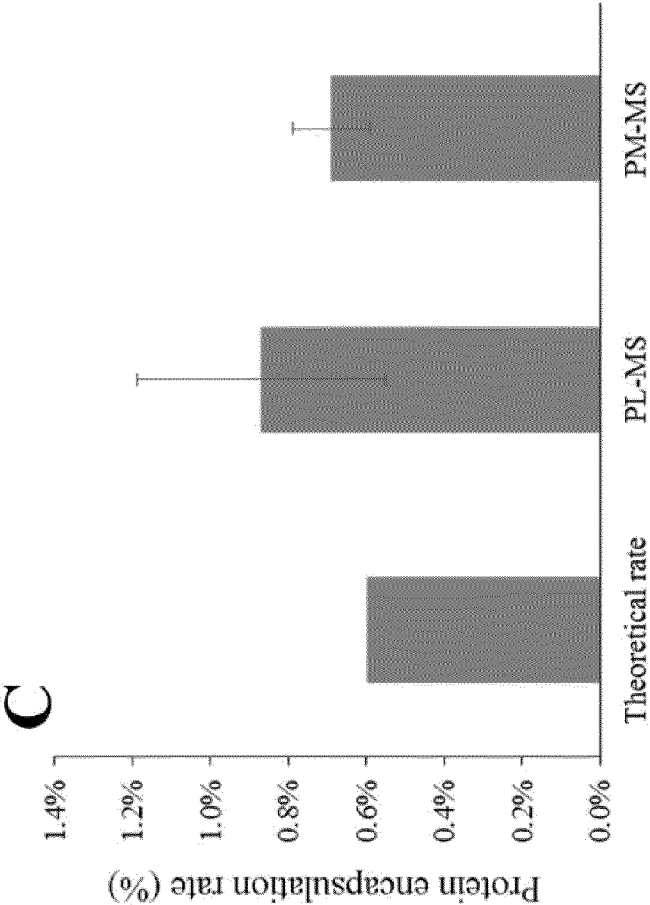
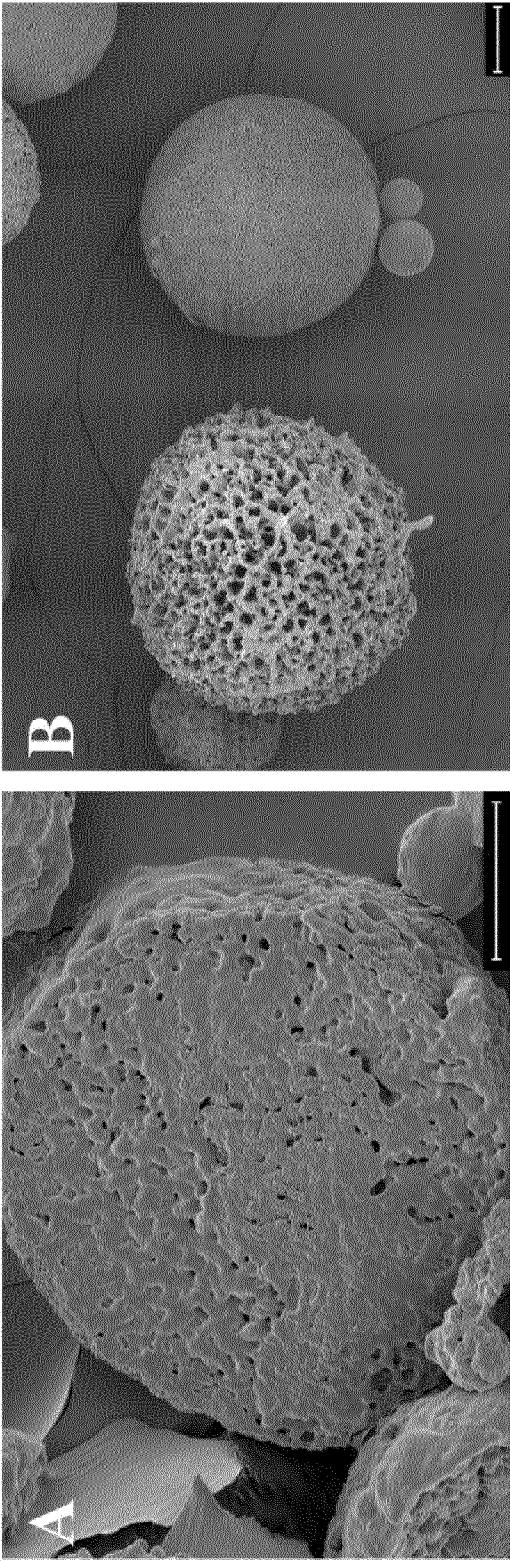


Figure 8

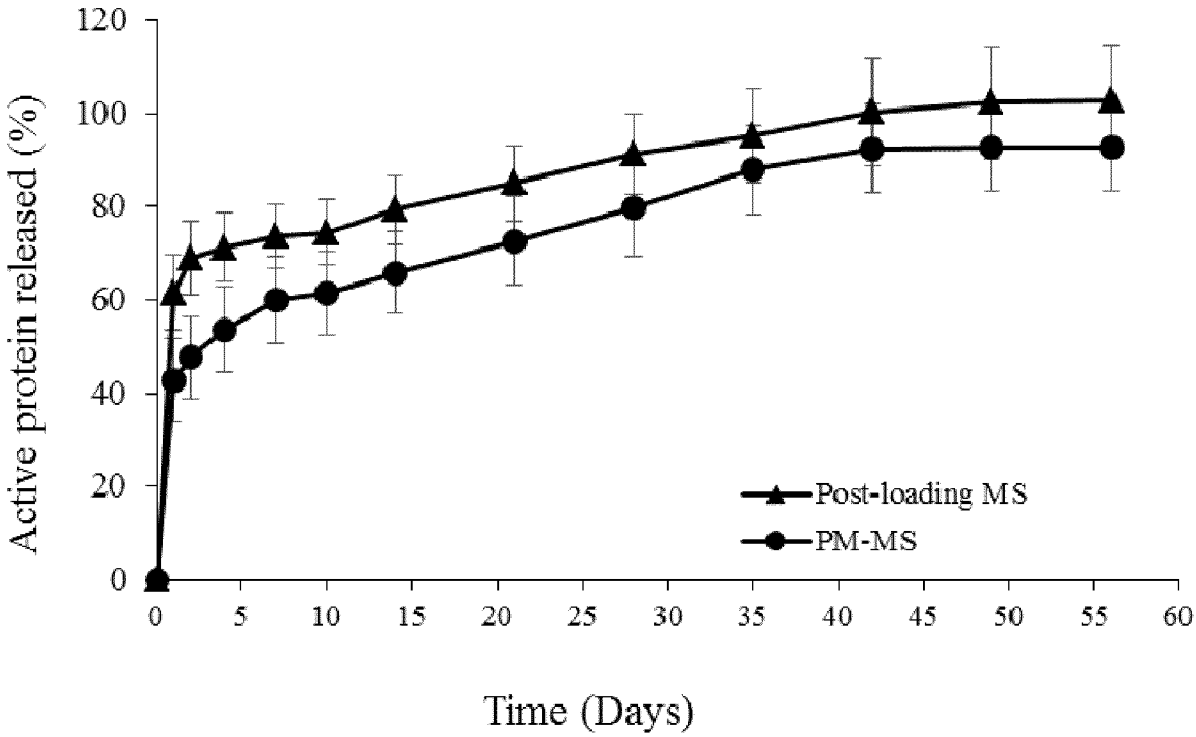


Figure 9

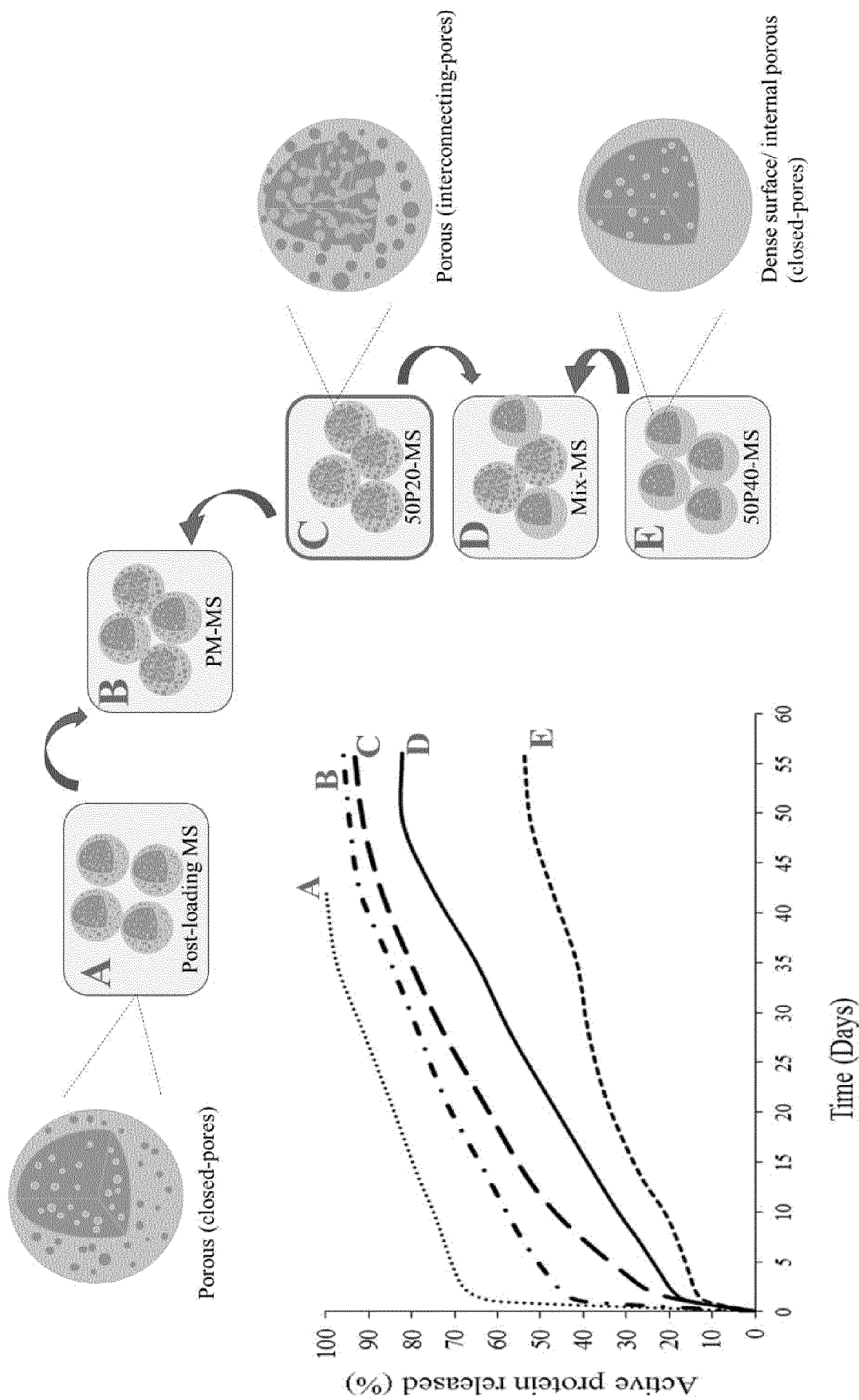


Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/053825

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/50 A61K9/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LE MINH-QUAN ET AL: "Penta-block copolymer microspheres: Impact of polymer characteristics and process parameters on protein release", INTERNATIONAL JOURNAL OF PHARMACEUTICS, ELSEVIER, NL, vol. 535, no. 1, 20 November 2017 (2017-11-20), pages 428-437, XP085328514, ISSN: 0378-5173, DOI: 10.1016/J.IJPHARM.2017.11.033 cited in the application	1-14
Y	abstract page 429, column 2, paragraph 2 page 430, column 2, paragraph 2 - page 431, column 1, paragraph 1 page 431, column 2, paragraph 2 - page 433, column 1, paragraph 2 page 436, column 1, paragraph 3 -/--	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 May 2020

Date of mailing of the international search report

29/05/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Estañol, Inma

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/053825

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	tables 1-3 figures 1-6 -----	
X	WO 2013/144341 A1 (INST NAT SANTE RECH MED [FR]) 3 October 2013 (2013-10-03)	1-14
Y	claims 1-3, 6-8, 10 Preparations 1, 11-13 Examples 1-2	1-14
Y	----- MORILLE MARIE ET AL: "PLGA-based microcarriers induce mesenchymal stem cell chondrogenesis and stimulate cartilage repair in osteoarthritis", BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 88, 18 February 2016 (2016-02-18), pages 60-69, XP029443941, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2016.02.022 abstract page 61, column 1, paragraph 3 - column 2, paragraph 1 page 62, column 1, paragraph 2 page 66, column 1, lines 4-8 -----	1-14
Y	ANGOTTI C ET AL: "Nanoprecipitated catestatin released from pharmacologically active microcarriers (PAMs) exerts pro-survival effects on MSC", INTERNATIONAL JOURNAL OF PHARMACEUTICS, ELSEVIER, NL, vol. 523, no. 2, 22 November 2016 (2016-11-22), pages 506-514, XP029987145, ISSN: 0378-5173, DOI: 10.1016/J.IJPHARM.2016.11.050 abstract page 507, column 2, paragraph 4 -----	1-14
Y	KANDALAM SAIKRISHNA ET AL: "Pharmacologically active microcarriers delivering BDNF within a hydrogel: Novel strategy for human bone marrow-derived stem cells neural/neuronal differentiation guidance and therapeutic secretome enhancement", ACTA BIOMATERIALIA, ELSEVIER, AMSTERDAM, NL, vol. 49, 16 November 2016 (2016-11-16), pages 167-180, XP029885872, ISSN: 1742-7061, DOI: 10.1016/J.ACTBIO.2016.11.030 abstract page 169, column 2, paragraph 3 - page 170, column 1, paragraph 1 -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2020/053825

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013144341 A1	03-10-2013	CA 2863109 A1	03-10-2013
		CN 104470505 A	25-03-2015
		EP 2830598 A1	04-02-2015
		ES 2612530 T3	17-05-2017
		JP 6177873 B2	09-08-2017
		JP 2015511622 A	20-04-2015
		KR 20140147880 A	30-12-2014
		US 2015056290 A1	26-02-2015
		WO 2013144341 A1	03-10-2013
