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(54) Title: METHOD FOR PREPARING SOLID LIPID NANOPARTICLES CONTAINING ANTIBODIES IN ION PAIR FORM USING THE FATTY ACID COACERVATION TECHNIQUE

(57) Abstract: A method for preparing solid lipid nanoparticles containing antibodies, comprising the steps of: (a) preparing a micellar solution comprising: from 0.02 mg/ml to 10 mg/ml of at least one anionic surfactant selected from the group consisting of alkylphosphates, alkylsulfates and alkylsulfonates; from 0.1% to 30% by weight on the total weight of the micellar solution of at least one alkaline soap; from 0.1% to 30% by weight on the total weight of the micellar solution of at least one non-ionic amphiphilic polymer selected from the group consisting of partially hydrolyzed polyvinyl acetate, polyoxyethylene/polyoxypropylene copolymers, polyacrylamides, polyvinylpyrrolidone and derivatives thereof, dextrane derivatives and agarose derivatives with various molecular weights, cellulose derivatives, non-ionic rubbers, cyclodextrins and derivatives thereof; said micellar solution being prepared by means of the following steps: (i) solubilizing in water the at least one anionic surfactant; (ii) adding the at least one alkaline soap, heating to a temperature comprised between 35°C and 90°C; and (iii) adding the at least one non-ionic amphiphilic polymer; (b) adding to the micellar solution prepared in step (a) a solution that contains at least one antibody of interest at a concentration comprised between 0.1 mg/ml and 10 mg/ml with respect to the final solution resulting from step (b); (c) adding to the solution obtained in step (b) at least one acid at a concentration comprised between 0.01M and 5M with respect to the final solution resulting from step (c); (d) cooling in an ice bath the suspension obtained in step (c) down to ambient temperature.

METHOD FOR PREPARING SOLID LIPID NANOPARTICLES CONTAINING ANTIBODIES IN ION PAIR FORM USING THE FATTY ACID COACERVATION TECHNIQUE

The present invention relates to a method for preparing solid lipid
5 nanoparticles containing antibodies.

Solid lipid nanoparticles (SLN) are used widely in the pharmaceutical field to transport active ingredients thanks to their low toxicity and simple preparation.

In the field of pharmaceutical technology, several methods are known
10 for preparing solid lipid nanoparticles, such as for example the cold homogenization method, the hot homogenization method (Müller et al., EP0605497), the microemulsion dilution method (Munper et al., US2006/0292183), the method of evaporation of the solvent from the emulsion (Siekmann et al., European Journal of Pharmaceutics and
15 Biopharmaceutics 1996; 43: 104-109), the method of dilution of the solvent from the emulsion (Trotta et al., International Journal of Pharmaceutics 2003; 257(1-2): 153-160) and the solvent injection method (Schubert et al., European Journal of Pharmaceutics and Biopharmaceutics 2003; 55(1): 125-131).

20 All of the methods cited above allow to produce nanoparticles with dimensions comprised between 10 and 1000 nm that have a reduced size distribution. The only exception is the cold homogenization method, which consists in a milling method and results in larger particles (microparticles with dimensions between 1 and 100 μm).

25 However, all the methods cited above have disadvantages. For example, the hot homogenization method requires the use of complex and expensive instruments and of high temperatures; the dilution method and the microemulsion cooling method require the use of large quantities of surfactants and cosurfactants and of high temperatures, which therefore do
30 not allow the embedding of heat-sensitive drugs; moreover, the methods

that entail the use of solvents are unable to ensure complete elimination of the solvent used, which sometimes can be toxic.

A new method for preparing solid lipid nanoparticles has been developed recently which is based on the coacervation of fatty acids (Battaglia et al., Journal of Microencapsulation 2010; 27(1): 78-85, WO 2008/149215). This method has allowed to overcome, at least partially, the disadvantages of the other methods described in the literature.

The encapsulation of peptides and proteins within solid lipid nanoparticles is one of the emerging challenges in the field of the transport of drugs and active ingredients, many of which are indeed proteins or peptides. The therapeutic application of peptides and proteins is in fact limited by some characteristics, such as high molecular weight, hydrophilic nature and limited chemical stability. These characteristics are the basis for low bioavailability, limited ability to cross biological membranes and short half-life in the circulatory stream of peptides and proteins administered for therapeutic use.

SLN can be useful for the transport of peptides and proteins within the body thanks to the potential of the lipid matrix to protect the peptide molecules from degradation and to promote their absorption. However, the embedding of peptides and proteins into SLN is still a difficult goal to reach, since every peptide molecule is characterized by its own particular characteristics (for example, molecular weight, hydrophilicity and stability), which vary from one peptide molecule to the next.

This situation often hinders the formulation of vehicles for the administration of peptides and proteins into a body, since each peptide and protein would require an individual study to determine the most suitable strategy for transporting it into the body. The choice of the correct formulation strategy is dictated mainly by considerations regarding the solubility and stability of the individual molecule (A. J. Almeida and E. Souto, Advanced Drug Delivery Reviews 2007 59 (6), 478–490).

The use of lipid matrices as materials for slow-release formulations of peptides and proteins has been reported only by a few authors (H. Reithmeier et al., Journal of Controlled Release 2001, 73, (2-3), 339–350; M. Garcia-Fuentes et al. Colloids and Surfaces B 2002, 27, 3, 159–168). This is due to the hydrophobic nature of the lipid matrix, in which it is possible to encapsulate lipophilic molecules more easily than hydrophilic ones such as peptides and proteins.

A valid strategy for promoting the encapsulation of peptide molecules in a solid lipid colloidal system is to increase the lipophilicity of the peptide molecules themselves, forming a complex between the protein or peptide having a pharmacological action and a surfactant by means of what is called the hydrophobic ion pairing technique (J. D. Meyer and M. C. Manning, Pharmaceutical Research 1998, 15, (2), 188–193). This technique is based on forming an interaction between a molecule that has an electrical charge and a surfactant having an opposite electrical charge, which leads to the formation of an insoluble lipophilic complex. In the specific case of proteins and peptides, the amino groups that are present on them can be protonated, thus acquiring a positive electrical charge that can easily form complexes (ion pairs) with surfactants having a negative charge.

Currently, in the biomedical field there is particular interest in research on antibodies, which are protein complexes that have a modular structure and are capable of binding in a highly specific manner to structurally complementary molecules known as antigens.

The application of the hydrophobic ion pairing technique to proteins has been described in the literature and has been patented (WO 1994/008599). Also the transport of ion pairs within solid lipid nanoparticles has been documented in the literature (Gallarate M. et al. Int J Chem Eng 2011). However, the transport within lipid nanoparticles of antibodies by means of the hydrophobic ion pairing technique has not been described yet. This is due to the high molecular weight of antibodies, which

can hinder encapsulation into lipid nanoparticles. Moreover, the particular tertiary structure of antibodies, which is fundamental for their biological activity, differentiates them from other proteins having a lower molecular weight. The particularity of forming an ion pair between an antibody and a 5 counterion with negative charge lies in optimizing the quantity of the counterion so as to increase the lipophilicity of the antibody without however saturating all the amino groups that are present on the antibody, so as to safeguard its biological activity.

The aim of the present invention is therefore to provide a method for 10 preparing, by means of the coacervation technique, solid lipid nanoparticles containing antibodies, embedded in said nanoparticles by means of the hydrophobic ion pairing technique.

Moreover, an object of the present invention is to provide a method for preparing solid lipid nanoparticles containing antibodies encapsulated in 15 the nanoparticles themselves in ion pair form.

Another object of the invention is to provide a method for preparing solid lipid nanoparticles containing antibodies that is highly reliable, relatively easy to provide and that has competitive costs.

This aim and these and other objects that will become more apparent 20 hereinafter are achieved by the method according to the present invention for preparing, by means of the coacervation technique, solid lipid nanoparticles containing antibodies encapsulated by means of the hydrophobic ion pairing technique.

Further objects, characteristics and advantages of the invention will 25 become more apparent from the detailed description that follows.

In the context of the present invention, the expression "aqueous solution" is understood to refer to a solution in which the solvent is water. The expression "micellar solution" is understood to refer to an aqueous solution comprising at least one anionic surfactant, at least one alkaline soap 30 and at least one non-ionic amphiphilic polymer. The term "nanoparticles" is

understood to refer to particles with a size comprised between 10 and 1000 nm. The expression "biocompatible substance" is understood to refer to a substance that is biologically compatible with tissues, organs and functions of the body and does not cause toxic or immune responses by said body. The expression "acid solution" is understood to refer to a solution of a biocompatible acid with a pH comprised between 0 and 7. The expression "alkaline soap" is understood to refer to an alkaline salt of a water-soluble fatty acid, for example a sodium or potassium salt. The expression "peptide molecule" is understood to refer to a natural or synthetic peptide or protein.

The present invention consists in a method for preparing solid lipid nanoparticles containing antibodies, comprising the steps of:

(a) preparing a micellar solution comprising:

from 0.02 mg/ml to 10 mg/ml of at least one anionic surfactant selected from the group consisting of alkylphosphates, alkylsulfates and alkylsulfonates;

from 0.1% to 30% by weight on the total weight of the micellar solution of at least one alkaline soap;

from 0.1% to 30% by weight on the total weight of the micellar solution of at least one non-ionic amphiphilic polymer selected from the group consisting of partially hydrolyzed polyvinyl acetate, polyoxyethylene/polyoxypropylene copolymers, polyacrylamides, polyvinylpyrrolidone and derivatives thereof, dextrane derivatives and agarose derivatives with various molecular weights, cellulose derivatives, non-ionic rubbers, cyclodextrins and derivatives thereof;

said micellar solution being prepared by means of the following steps:

(i) solubilizing in water the at least one anionic surfactant;

(ii) adding the at least one alkaline soap, heating to a temperature comprised between 35°C and 90°C; and

(iii) adding the at least one non-ionic amphiphilic polymer;

(b) adding to the micellar solution prepared in step (a) a solution that

contains at least one antibody of interest at a concentration comprised between 0.1 mg/ml and 10 mg/ml with respect to the final solution resulting from step (b);

(c) adding to the solution obtained in step (b) at least one acid at a concentration comprised between 0.01M and 5M with respect to the final solution resulting from step (c);

(d) cooling in an ice bath the suspension obtained in step (c) down to ambient temperature.

The anionic surfactant that is present in the micellar solution acts as a counterion for the antibody to be encapsulated in the solid lipid nanoparticle, which, as said above, being a protein molecule can be protonated on its amino groups, acquiring a positive electrical charge. The anionic surfactant and the antibody thus form a lipophilic ion pair.

Preferably, the at least one anionic surfactant is selected from the group consisting of sodium dioctylsulfosuccinate, sodium dodecyl sulfate, sodium deoxy taurocolate, sodium taurocolate, potassium cetyl phosphate, potassium decyl phosphate, potassium dodecyl phosphate and potassium tetradecyl phosphate.

It was said above that the expression "alkaline soap" refers to a water-soluble salt of a fatty acid. In a preferred embodiment of the invention, the alkaline soap is a salt selected from the group consisting of alkaline salts, such as for example sodium salts and potassium salts. In another preferred embodiment, the alkaline soap is a salt of a fatty acid that is solid at ambient temperature. Preferably, the fatty acid has an aliphatic chain with a length comprised between 10 and 24 carbon atoms. More preferably, the fatty acid is selected from the group consisting of stearic acid, palmitic acid, myristic acid, lauric acid, arachidic acid, behenic acid. Preferably, the at least one alkaline soap that is present in the micellar solution is selected from the group consisting of sodium stearate, sodium palmitate, sodium myristate, sodium laurate, sodium arachidate and sodium behenate. Moreover, the

concentration of the at least one alkaline soap is comprised preferably between 1% and 5% by weight on the total weight of the solution.

The non-ionic amphiphilic polymer that is present in the micellar solution acts as a stabilizer for the suspension, avoiding the aggregation and sedimentation of the particles.

Preferably, the concentration of the at least one non-ionic amphiphilic polymer is comprised between 1% and 5% by weight on the total weight of the solution.

Preferably, the at least one acid added to the solution in step (c) is an organic or inorganic acid. In a preferred embodiment, the at least one acid is an organic acid selected from the group consisting of acetic acid, carbonic acid, acrylic acid, lactic acid, glycolic acid, tartaric acid, maleic acid, pyruvic acid, malic acid, succinic acid, citric acid, amino acids, poly amino acids and polymers containing acid groups. In another preferred embodiment, the at least one acid is an inorganic acid selected from the group consisting of hydrochloric acid, phosphoric acid, nitric acid, sulfuric acid, acid polyphosphates and acid ammonium salts and derivatives thereof.

In step (c), the addition of the acid induces the exchange of a proton between the acid and the alkaline soap molecules, which precipitate in the form of solid lipid nanoparticles. By precipitating, the solid lipid nanoparticles encapsulate the antibody within themselves, in the form of a lipophilic ion pair with the anionic surfactant.

The invention is now described further by means of examples the content of which is to be understood as non-limiting for the scope of the present invention.

Example 1: preparation of SLN containing rat immunoglobulins.

A solution of AOT (sodium dioctylsulfosuccinate) at a concentration of 0.8 mg/ml is prepared. 80 mg of PVA (partially hydrolyzed polyvinyl acetate) 9000 are dissolved in 1 ml of the AOT solution; then 40 mg of sodium stearate are added at a temperature of 50°C, thus obtaining a

micellar solution. Separately, the antibody is dissolved in 1 ml of water at a concentration of 2 mg/ml. Also, this solution is brought to 50°C before being added to the micellar solution. At this point the nanoparticles are precipitated by acidifying under agitation with 0.1 ml of 2M lactic acid. The
5 solution is then cooled in an ice bath.

The resulting SLN have an average diameter of 850 nm and a polydispersion of 0.250.

The encapsulation efficiency of the antibody is determined following centrifugation of the SLN at 26,000 rpm and washing of the resulting
10 precipitate with a 0.1M phosphate buffer at pH=7.4 and then with 30% ethanol: the antibody is then extracted by dissolving the precipitate with ethanol at 50°C and by reprecipitating the lipid with the addition of 0.1M phosphate buffer at pH=7.4. The resulting mixture is centrifuged and the supernatant is injected in SEC-HPLC. The measured encapsulation
15 efficiency is approximately 90%.

Example 2: preparation of SLN containing bevacizumab.

A solution of AOT (sodium dioctylsulfosuccinate) at a concentration of 0.33 mg/ml is prepared. 25 mg of HPMC (hydroxypropyl methylcellulose) 15cP are dissolved in 2.4 ml of the AOT solution; then 25
20 mg of sodium stearate are added at a temperature of 50°C, thus obtaining a micellar solution. 0.1 ml of an aqueous solution of bevacizumab at a concentration of 25 mg/ml (which corresponds to 2.5 mg) are then added to the micellar solution. At this point the nanoparticles are precipitated, by acidifying under agitation with 0.1 ml of 1 M lactic acid. The solution is
25 then cooled in an ice bath.

The resulting SLN have an average diameter of 690 nm and a polydispersion of 0.150.

The encapsulation efficiency is determined following centrifugation of the SLN at 26,000 rpm and washing of the precipitate with a 0.1M
30 carbonate buffer at pH=10; the precipitate is then dissolved in acetic acid

and analyzed in a spectrophotometer at the wavelength of 280 nm. The measured encapsulation efficiency is approximately 30%.

Actually it has been found that the method according to the invention fully achieves the intended aim since, through the fatty acid coacervation technique, it allows the preparation of solid lipid nanoparticles containing antibodies in the lipophilic ion pair form. In particular, it has been observed that the method according to the invention allows an effective forming of the solid lipid nanoparticles and an effective encapsulation of the antibody molecules inside them, as indicated by the encapsulation efficiency value.

The method thus conceived is susceptible of numerous modifications and variations, all of which are within the scope of the inventive concept; all the details may furthermore be replaced with other technically equivalent elements.

Actually, the materials used, as well as the quantities, may be any according to the requirements and the state of the art.

The disclosures in Italian Patent Application No. BO2013A000376 from which this application claims priority are incorporated herein by reference.

CLAIMS

1. A method for preparing solid lipid nanoparticles containing antibodies, comprising the steps of:

(a) preparing a micellar solution comprising:

5 from 0.02 mg/ml to 10 mg/ml of at least one anionic surfactant selected from the group consisting of alkylphosphates, alkylsulfates and alkylsulfonates;

from 0.1% to 30% by weight on the total weight of the micellar solution of at least one alkaline soap;

10 from 0.1% to 30% by weight on the total weight of the micellar solution of at least one non-ionic amphiphilic polymer selected from the group consisting of partially hydrolyzed polyvinyl acetate, polyoxyethylene/polyoxypropylene copolymers, polyacrylamides, polyvinylpyrrolidone and derivatives thereof, dextrane derivatives and 15 agarose derivatives with various molecular weights, cellulose derivatives, non-ionic rubbers, cyclodextrins and derivatives thereof;

said micellar solution being prepared by means of the following steps:

(i) solubilizing in water the at least one anionic surfactant;

(ii) adding the at least one alkaline soap, heating to a temperature 20 comprised between 35°C and 90°C; and

(iii) adding the at least one non-ionic amphiphilic polymer;

(b) adding to the micellar solution prepared in step (a) a solution that contains at least one antibody of interest at a concentration comprised between 0.1 mg/ml and 10 mg/ml with respect to the final solution resulting 25 from step (b);

(c) adding to the solution obtained in step (b) at least one acid at a concentration comprised between 0.01M and 5M with respect to the final solution resulting from step (c);

(d) cooling in an ice bath the suspension obtained in step (c) down to 30 ambient temperature.

2. The method according to claim 1, wherein the at least one anionic surfactant that is present in the micellar solution is selected from the group consisting of sodium dioctylsulfosuccinate, sodium dodecyl sulfate, sodium deoxy taurocolate, sodium taurocolate, potassium cetyl phosphate, potassium decyl phosphate, potassium dodecyl phosphate and potassium tetradecyl phosphate.

3. The method according to claim 1 or 2, wherein the alkaline soap that is present in the micellar solution is a salt selected from the group consisting of sodium salts and potassium salts.

4. The method according to one or more of the preceding claims, wherein the alkaline soap is a salt of a fatty acid that is solid at ambient temperature.

5. The method according to claim 4, wherein the fatty acid has an aliphatic chain with a length comprised between 10 and 24 carbon atoms.

6. The method according to claim 4 or 5, wherein the fatty acid is selected from the group consisting of stearic acid, palmitic acid, myristic acid, lauric acid, arachidic acid, behenic acid.

7. The method according to one or more of the preceding claims, wherein the at least one alkaline soap is selected from the group consisting of sodium stearate, sodium palmitate, sodium myristate, sodium laurate, sodium arachidate and sodium behenate.

8. The method according to one or more of the preceding claims, wherein the concentration of the at least one alkaline soap is comprised between 1% and 5% by weight on the total weight of the micellar solution.

9. The method according to one or more of the preceding claims, wherein the concentration of the at least one non-ionic amphiphilic polymer is comprised between 1% and 5% by weight on the total weight of the micellar solution.

10. The method according to one or more of the preceding claims, wherein the at least one acid added to the solution in step (c) is an organic

or inorganic acid.

11. The method according to claim 10, wherein the at least one acid is an organic acid selected from the group consisting of acetic acid, carbonic acid, acrylic acid, lactic acid, glycolic acid, tartaric acid, maleic acid, 5 pyruvic acid, malic acid, succinic acid, citric acid, amino acids, poly amino acids and polymers containing acid groups.

12. The method according to claim 10, wherein the at least one acid is an inorganic acid selected from the group consisting of hydrochloric acid, phosphoric acid, nitric acid, sulfuric acid, acid polyphosphates and acid 10 ammonium salts and derivatives thereof.

INTERNATIONAL SEARCH REPORT

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PCT/EP2014/051812

A. CLASSIFICATION OF SUBJECT MATTER
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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	MARINA GALLARATE ET AL: "Peptide-Loaded Solid Lipid Nanoparticles Prepared through Coacervation Technique", INTERNATIONAL JOURNAL OF CHEMICAL ENGINEERING, vol. 132, no. 7, 1 January 2011 (2011-01-01), pages 1-6, XP55109205, US ISSN: 1687-806X, DOI: 10.1016/S0168-3659(97)00046-1 abstract page 2, left-hand column, lines 19-29 page 2, left-hand column, line 50 - right-hand column, line 9 page 2, right-hand column, line 51 - page 3, left-hand column, line 8; table 1 -----	1-12

☐ 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

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