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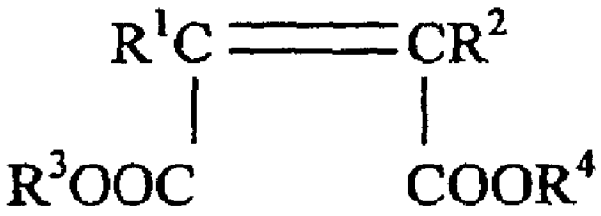
(54) Title: LIPID-CONTAINING NANOPARTICLE



(57) Abstract: A lipid-containing nanoparticle, the nanoparticle comprising: a membrane-forming polar lipid; and a lipid- solubilising agent including a synthetic amphipathic polymer which is a copolymer of a first monomer of formula: where R^1 and R^2 are each independently hydrogen or C_1 - C_9 alkyl, and at least one of R^3 and R^4 is C_1 - C_9 alkyl and the other is hydrogen or C_1 - C_9 alkyl; and a second monomer of formula R^5-CH=CH_2 where R^5 is selected from hydrogen, C_1-C_8 alkyl, C_1-C_6 alkoxy, phenyl, benzyl and phenyl or benzyl substituted with an alkyl or other hydrophobic group and uses of this in contact lens applications and membrane extraction

Lipid-containing Nanoparticle

Field

[0001] The invention relates to a lipid-containing nanoparticle, methods of making such particles, use of nanoparticles in contact lens applications, use of nanoparticles in membrane protein extraction and a process for extracting membrane proteins from cell membranes.

Background

[0002] A variety of interfaces exist in biological systems, many of which make use of some form of membrane or film to isolate one region from the other. These interfaces are found across all scales from the nanoscale interfaces of organelles and cellular membranes to macroscale structures such as the films of surfactant solution lining the air sacs of the lungs. These biological structures have been studied in varying degrees of detail and have been found to have a range of interesting properties. This has promoted attempts to mimic these biological structure to create synthetic analogues which are expected to have a wide range of potential applications.

[0003] By way of example, EP1007002 describes a polymer/lipid complex comprising an alternating copolymer of an unsaturated dicarboxylic acid and a second, typically hydrophobic, monomer. These mixtures form non-micellular disc-like assemblies when exposed to solutions of the correct pH. The assemblies are polymer/lipid complexes in which the lipid is trapped within the polymer network. This structure has been found to liberate the lipid component in response to blinking and so can be used in the treatment of dry eye syndrome. However, these compounds are not stable at a physiologically acceptable pH making them inappropriate for use in some biological situations.

[0004] Also, EP 1890675 describes compositions comprising macromolecular assemblies of lipids and copolymer of styrene and maleic acid, wherein the ratio of styrene to maleic acid monomer units is greater than 1:1. The pH of the composition must be

carefully controlled if the formation of the assembly is to occur. These compositions may be used in cosmetic and biomedical applications.

[0005] Biological membranes are complex structures. They include a variety of structures both embedded within them or on their surfaces and are typically formed of one or more surfactant based layers to encase biological contents. Structures associated with these membranes may be proteins that actively move components across membranes, compounds positioned within the membrane adapted maintain the membrane integrity or structures which scavenge and release excess quantities of compounds which contribute to the stability of the membrane.

[0006] The structures of particular interest are those which exist on the macroscale, such as surfactant rich films found in parts of the body, such as the eyes or lungs. The liquids which make up these films comprise a range of compounds including a blend of phospholipids which ensure good lubrication and permeability to air.

[0007] It has been discovered that the surfactants found in areas of the body such as the lungs, bear close similarities with those surfactants found in the tear film. These films contain a large proportion of membrane forming lipid compounds such as phospholipids. However, emulating these naturally occurring lubricants with synthetic alternatives has proven difficult and only recently have techniques and compositions been developed suitable for the task.

[0008] It is thought that synthetic mimics of these naturally occurring lubricants could be useful in, for instance, creating artificial tear films such lubricants may be useful in reducing the disruption of the natural tear film associated with the wearing of contact lenses. This could improve the comfort of contact lenses and reduce the incidence of "dry eye syndrome".

[0009] There are many contact lens commonly produced from polymeric materials having self lubricating surfaces, the self lubrication being intended to address the problems arising from the disruption of the tear film. However, discomfort still arises when the lens is worn over the course of a day (or longer). An alternative method for overcoming this

problem is the use eye drops comprising a synthetic lachrymal fluid; however, this method is undesirable as it requires the user to apply the eye drops regularly, a requirement which is often viewed as an inconvenience by the user.

[0010] As mentioned above, many membrane-like structures found in biology, including cell membranes, include within them a wide variety of different compounds. One common category of compound found in membranes are proteins. Membrane proteins are of particular interest as targets for drug discovery as these structures frequently govern trans-membrane movements. Known processes for extracting proteins from films and/or membranes are not ideal as these systems conventionally make use of surfactants which disrupt the chemistry and complex environment around the membrane proteins of interest.

[0011] In order to accurately study the function and structure of these proteins, it is essential that the modelling and analysis of membrane protein systems studied ex-vivo substantially correspond to the behaviour of the proteins in their native environment.

[0012] Various methods have been proposed to isolate membrane proteins without the requirement to utilise an aggressive surfactant type approach. These methods include using bicelles made from combinations of phospholipids and surfactants which form disc like particles. Whilst this approach works well for proteins having a reasonable tolerance to surfactant, many proteins are not suitable for isolation in this manner. Another method has been to use so called "amphipols" (amphipathic polymers) having hydrophobic side groups and a hydrophilic backbone. These compounds can "hypercoil" around transmembrane regions of proteins which help to maintain the proteins in their folded configurations.

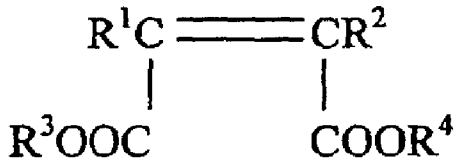
[0013] A technique still in its relative infancy is the use of polymers to form lipid containing nanoparticles which are capable, in some cases, of undergoing self-assembly with membranes to encapsulate membrane proteins. However, as this technique is relatively new, only a limited number of polymers have been investigated for their ability to function as membrane extraction tools.

[0014] Accordingly, there is a need for improved surfactant technology. In particular, there is a need for surfactant technology which can be applied in biological situations

including, for instance, where the formation or supplementation of the tear film can improve the comfort of contact lenses or in the provision of surfactant which can be used in membrane protein extraction processes. The invention seeks to overcome or ameliorate at least some of these problems.

Summary of Invention

[0015] Accordingly, in a first aspect of the invention there is provided a lipid-containing nanoparticle, the nanoparticle comprising a membrane-forming polar lipid and a lipid-solubilising agent including a synthetic amphipathic polymer which is a copolymer of one or more first monomers according to the formula:



Where R^1 and R^2 are each independently hydrogen or C_1 - C_9 alkyl, and at least one of R^3 and R^4 is C_1 - C_9 alkyl and the other is hydrogen or C_1 - C_9 alkyl; and a second monomer of formula R^5 -CH=CH_2 where R^5 is selected from hydrogen, C_1-C_8 alkyl, C_1-C_6 alkoxy, phenyl, benzyl and phenyl or benzyl substituted with an alkyl or other hydrophobic group. Desirably, the copolymer is a substantially alternating copolymer as would be understood by the skilled reader. The polymer may comprise regions of alternation separated by regions of block polymers. Typically, the copolymer is formed from a 1:1 ratio of first and second monomers. However, there is no particular limitation of the range of ratios of monomers which may be used. The ratios of monomers may range from 1:10 to 10:1, 1:5 to 5:1, 1:3 to 3:1 or 1:2 to 2:1. Typically the ratios are selected from; 5:1, 4:1, 5:2, 2:1, 3:2, 1:1, 2:3, 1:2, 2:5, 1:3, 1:4 and 1:5.

[0016] Typically, at least one of R^3 and R^4 will be hydrogen, such that a half-ester is formed. Often at least one of R^3 and R^4 is selected from methyl, ethyl and n-propyl, in some cases both R^3 and R^4 will be independently selected from methyl, ethyl and n-propyl, in some cases one of R^3 or R^4 is hydrogen and the other selected from methyl, ethyl and n-propyl. It will most often be the case that methyl is used in the formation of the half-ester or di-ester, in many cases one of R^3 or R^4 is hydrogen and the other is methyl.

[0017] The inventors have found that by replacing at least one of the two acidic protons on the carboxylic acid moieties with an alkyl group the stability of the nanoparticle can be improved. Without being bound by theory, this is considered to be attributable to the increased hydrophobicity arising from ester formation. Surprisingly, however, having at least one of R^3 and R^4 as a methyl group, with the other as hydrogen, was found to offer greatly improved stability over both the dicarboxylic acid, other half esters using ethyl groups or longer alkyl chains, and diesters. In particular, the methyl substituted polymer provided polymer/lipid assemblies with good stability at a pH of between 6 and 8, facilitating easy use of the polymers in biological applications.

[0018] In the situation that R^1 and R^2 are both hydrogen and R^5 is alkyl, the alkyl chain typically contains less than eight carbon atoms. Usually, the synthetic amphipathic polymer is an alternating copolymer wherein the first monomer is a maleic acid ester and the second monomer is selected from styrene, indene, a C_1 - C_4 alkyl substituted styrene or indene, and an alkyl vinyl ether selected from propyl, isopropyl and butyl vinyl ether, or combinations thereof.

[0019] The second monomer or monomers will generally be selected from indene or naphthalene and compounds of formula R-CH=CH_2 where R is hydrogen, C_1-C_8 alkyl, C_3-C_6 alkoxy, or is phenyl or benzyl which may be optionally substituted with an alkyl or other hydrophobic group. Typically, the second monomer is a compound according to formula R-CH=CH_2. Even more typically, R is phenyl or benzyl which may be optionally substituted with an alkyl or other hydrophobic group.

[0020] The number of carbon atoms in the hydrophobic side groups of the polymer or copolymer may be equal to or greater than the number of carbon atoms in the backbone of the polymer. Typically the second monomer is styrene.

[0021] Historically, preparation of the copolymer has required the presence of a catalyst, typically a pyridine catalyst such as 4-dimethylaminopyridine (DMAP). It has been found that the use of a catalyst is not essential when forming the esters and half esters (R^3 and/or R^4 are C_1 - C_9 alkyl) to form the polymers used in the nanoparticles of the invention. In

other words, the catalyst can be absent. This can be advantageous as many catalysts, and in particular DMAP (the favoured catalyst of the prior art) are toxic, and must be removed from the polymer mixture if the polymers are to be accepted for biological use. The removal of the need to include a catalyst therefore ensures that the polymers are suitable for use in biological applications as no purification is needed and so any risk of trace amounts of catalyst remaining is removed. The absence of any requirement for a purification step also simplifies the copolymer preparation process.

[0022] The lipid used in the lipid-containing nanoparticle is typically a phospholipid, often a phosphatidic acid derivative in which the non-polar acyl ester groups contain eight to twenty five carbon atoms, and even more often selected from a phosphatidylcholine selected from dipalmitoylphosphatidylcholine (DPPC), dilauroylphosphatidylcholine (DLPC) and dimyristoylphosphatidylcholine (DMPC). These phospholipids are found naturally in lachrymal fluid and therefore show excellent biocompatibility.

[0023] In some embodiments, the lipid-containing nanoparticle has physiologically and pharmaceutically acceptable non-toxic properties for administration to mammals. These properties may be desirable if the nanoparticles are to be used in the eye. Typically, the synthetic polymer used in the lipid-containing nanoparticle has a molecular weight (number average) or relative mass of the synthetic polymer has a range of 1,500 to 500,000 daltons, often in the range 5,000 - 50,000 daltons.

[0024] Examples of typical number average molecular weights of the polymers used in carrying out this invention are as follows:

Poly(maleic anhydride-styrene)	7,000
Poly(maleic anhdyride-propyl vinyl ether)	5,500
Poly(maleic anhydride-butyl vinyl ether)	43,300

[0025] It is typically the case that the synthetic polymer used in the lipid-containing nanoparticle is a poly(maleic acid ester-styrene) copolymer (known as PMAS or PSMA). The steric and hydrophobic properties of this alternating arrangement of monomers in a polymer chain has been found to provide optimal stability for the lipid-containing

nanoparticle. Additionally, the composition may further comprise a pH stabilising agent. Although this is not particularly limited, the stabilising agent will often be sodium hydroxide.

[0026] The size of the lipid-containing nanoparticle is usually in the range of 1 - 50 nm in diameter under physiological conditions of temperature and pH. More typically the lipid-containing nanoparticles have a size in the range of 10-40 nm in diameter, more typically 20 nm in diameter or 5-7 nm in diameter.

[0027] In a second aspect of the invention there is provided a suspension comprising a lipid containing nanoparticle according to the first aspect of the invention and a fluid continuous phase, wherein the suspension is of pH in the range 6 - 9, often 6 - 8 and more often 6.5 - 7.5. In general, the suspension will be of biological pH, by which it is intended to mean at a pH compatible with biological systems, in particular human biological systems. Often the suspension will comprise water as the fluid continuous phase, forming an aqueous suspension of nanoparticles. Water is generally selected due to its ready availability and excellent compatibility with biological systems. The nanoparticles of the invention are particularly advantageous as depending on the esters or half esters used, the nanoparticles can be made to resist changes to their structure when exposed to a change in pH or be made to disrupt on exposure to a change in pH. This allows the nanoparticles to act as pH sensitive delivery systems.

[0028] In the lipid containing nanoparticles a membrane-forming polar lipid is generally dispersed in the aqueous medium together with a synthetic amphipathic polymer that interacts with the polar lipid to produce a nanoparticle whereby said polymer interacts with and solubilises the polar lipid in the aqueous medium successful nanoparticle formation being indicated by:

(a) the composition having the visual appearance of a substantially clear solution;

(b) the nanoparticle produced is in the form of non-liposomal micellar particles or assemblies of discoidal form in which the polar lipid forms a bilayer core;

(c) the synthetic amphipathic polymer has a linear backbone along which hydrophobic groups and anionic hydrophilic groups may be evenly arranged and is typically a copolymer, other than a block copolymer, of a first monomer which is a derivatised unsaturated dicarboxylic acid, or an anhydride thereof, and a second monomer which is a monoenoic compound, said first and second monomers being arranged in alternating relationship along said backbone; and

(d) the number of carbon atoms in hydrophobic side groups of the amphipathic polymer may be equal or greater than the number of carbon atoms in the polymer backbone.

[0029] An example of one lipid-solubilising synthetic amphipathic polymer including both hydrophobic groups and anionic hydrophilic groups which can be use in carrying out the invention is the homopolymer poly(2-ethyl acrylic acid) (PEAA) that has previously been reported as interacting in aqueous solutions at pH>7 with phosphatidylcholines such as dilauroylphosphatidylcholine (DLPC) and dipalmitoylphosphatidylcholine (DPPC) to yield suspensions of multilamellar vesicles which clear when the pH is lowered below a critical value of approximately 6.5. See for example K. Seki *et al.* (1984) "pH-Depéndent Complexion of Poly(acrylic acid) Derivatives with Phospholipid Vesicle Membranes", *Macromolecules,* 17, 1692-1698, D.A. Tirrell *et al.* (1985) "pH Sensitisation of Phospholipid Vesicles via Complexion with Synthetic Poly(carboxylic acid)s", *Ann. N.Y. Acad. Sci* 446, 237-248, and K.A. Borden *et al.* (1987) "Polyelectrolyte adsorption induces a vesicle-to-micelle transition in aqueous dispersions of dipalmitoylphosphatidyl-choline, *Polymer Preprints*, 28, 284-285).

[0030] The solubilisation effect described in the literature above was attributed to a break-up and reorganisation of the vesicle structures accompanying conformational changes occurring in the polymer upon lowering of the pH, leading to the formation of lipid/polymer complexes producing small micellar discoidal particles or assemblies.

[0031] In carrying out the invention, instead of PEAA other similar vinyl homopolymers of an acrylic acid derivative having a hydrophobic side chain, e.g. 2-propyl acrylic acid, or other poly(carboxylic acid) polymers having pendant hydrophobic side groups in addition to anionic hydrophilic groups, may be used. In some instances, however, the selected

synthetic lipid-solubilising amphipathic polymer will be a copolymer, especially a linear alternating vinyl copolymer formed by free radical addition polymerisation of an unsaturated dicarboxylic acid, or an anhydride or monoester of said dicarboxylic acid, with a monoenoic vinyl monomer or monomers in alternating relationship.

[0032] Especially suitable polymers may be formed as alternative copolymers of maleic acid (or the anhydride thereof) with styrene, indene or a C_{1-4} alkyl, e.g. methyl, substituted styrene or indene, or with propyl (or isopropyl) or butyl vinyl ether. It is also possible to use a mixture of the styrene; or indene, or alkylated styrene or indene, and alkyl vinyl ether components. A number of suitable copolymers that may be used are commercially available from Aldrich Chemical Co., e.g. those marketed under the Aldrich Chemical Co. catalogue number 23,529-5 (CAS Registry No. 25736-61-2). Pharmaceutical grade polymers or copolymers that can be used are available from Kuraray Co. Ltd. of Japan.

[0033] The polymer may have physiologically or pharmaceutically acceptable non-toxic properties, and the molecular weight (number average) or relative mass of the polymer will generally be within the range of 1,500 to 20,000 Daltons. In some cases, however, the molecular weight may be higher, e.g. up to 500,000 Daltons as for example with poly (maleic anhydride-butyl vinyl ether) that has a number average molecular weight equal to 43 kDa approximately.

[0034] The particular synthetic method used in synthesising the maleic anhydride, styrene copolymers described herein involves a step of quenching the reaction mixture after a certain interval and favours the formation of alternating copolymers which is an essential feature in the formation of a coil with an amphipathic character such that one facet is hydrophobic and one is hydrophilic. This cannot generally be achieved in copolymers which are “blocky” or produced by other means, e.g. in the poly(maleic anhydride-styrene) copolymers supplied by Sigma Chemical Co. St. Louis, Missouri and sold as 50% styrene (number average molecular weight 350,000), or those sold by Scientific Polymer Products Inc. Ontario, New York as 50/50 maleic anhydride-styrene copolymers with a molecular weight of 50,000.

[0035] In many cases, especially for pharmaceutical applications, poly(maleic anhydride-styrene) (PMAS) will be a preferred polymer. This polymer, of molecular weight 4,000 Daltons, is disclosed in U.S. Patent No. 4,732,933 (Yamanouchi). JP 01061424A discloses a pharmaceutical formulation comprising a conjugate of a styrene/maleic acid copolymer bound to molecules of the drug neocarcinostatin (SMANCS), prepared by mixing a solution of SMANCS in ammonium carbonate buffer (pH 7.5 to 9.5) with a solution of a phospholipid such as egg yolk also in ammonium carbonate buffer (pH 7.5 to 9.5) to form a mixture which after being freeze dried to remove water is dispersed in a non-aqueous oily contrast medium so as to then provide a clear and transparent dispersion therein of the SMANCS conjugate.

[0036] It is believed that in aqueous media, at least over a particular pH range, the solubilising synthetic amphipathic polymers specified in the present invention will generally adopt a helical coil configuration with the hydrophobic side groups presented along one facet and the anionic hydrophilic groups presented along the opposite facet, and that they interact with the lipid in the aqueous medium to form discoidal micellar particles or assemblies of sub-liposomal dimensions in which the lipid forms a bilayer core. In any event, it has been found that these micellar particles or assemblies usually appear to be in the range 10-40 nm in diameter, typically 20 nm, and 5-7 nm thick. This compares favourably with the dimensions of lipoprotein micellar assemblies found in nature, such as the well characterised system between apolipophorin III and dimyristoylphosphatidylcholine (DMPC) that has been identified in insects, where the micelles are reported to have a diameter of 18.5 +/- 2.0 mn and a thickness of 4.8 +/- 0.8 nm (see Wientzek, M., Kay, C.M., Oikawa, K. and Ryan, R.O. (1994), "Binding of Insect Apolipophorin III to Dimyristoylphosphatidylcholine Vesicles." *J. Biological Chem.* 269 (6). 4605-4612). In comparison, typical phospholipid-containing liposomes currently used in drug delivery systems have a diameter of 50-1000 nm for unilamellar vesicles and 400 - 3500 nm for multilamellar vesicles.

[0037] The lipid will usually comprise a phospholipid and the synthetic amphipathic polymer with which it is combined will have a balance of hydrophobic and anionic hydrophilic groups evenly arranged along at least part a linear backbone.

[0038] The term "membrane-forming polar lipid" is used herein to denote lipids having a highly polar head portion attached to a non-polar hydrophobic tail, generally composed of a pair of relatively long hydrocarbon chains, such that in aqueous media the lipid molecules tend to associate and form membrane structures and interfaces, possibly as lipid monolayers or bilayers.

[0039] The polar lipids used in connection with the invention will usually be phospholipids based on glycerol in the form of phosphatidic acid derivatives in which the non-polar acyl ester groups contain between 8 and 25 carbon atoms. These acyl ester groups, however, are preferably selected from lauryl, palmitoyl and myristoyl, and the polar head of the molecule may be a phosphate group which may further comprise a choline substituent. Typically, the lipid is a phosphatidylcholine. It is possible to use other polar lipids, especially phospholipids, based on different structures, for example sphingosine or a ceramide from which may be derived the phospholipid sphingomyelin.

[0040] It should be pointed out that many of these polar lipids, especially phospholipids such as phosphatidylcholines, undergo phase transitional changes in aqueous media at predetermined temperatures at which they may change from a relatively ordered to a relatively disordered state. Dipalmitoylphosphatidylcholine (DPPC), for example, has a main thermal phase transition temperature (T_m) of around 42°C, although for dilauroylphosphatidylcholine (DLPC) the main thermal phase transition temperature is about -2°C so that it is in a disordered bilayer or liquid crystalline phase at room temperature.

[0041] The compositions in accordance with the invention will generally be prepared by mixing the polymer and the polar lipid in an aqueous medium. As described above with reference to the suspension, the fluid composition will generally be at biological pH, often in the ranges described above.

[0042] In a third aspect of the invention there is provided a method of making the lipid-containing nanoparticle of the first aspect of the invention, the method comprising the step of adding the membrane-forming polar lipid and the lipid-solubilising agent to water to form an aqueous solution having a pH in the range of 6 to 8. It has been found that when

using dicarboxylic acid monomers, in order for the lipid-containing nanoparticle to form, it is necessary to acidify the solution or nanoparticles will not be produced. However, where the first monomer in the synthetic amphipathic polymer is a half ester or diester, the pH at which stable nanoparticles form is much higher and therefore the need for this acidification step is removed. This simplifies the preparation procedure as the need to neutralise the nanoparticle solutions is removed, and provides nanoparticles which, having been formed at moderate pH, can be used in biological applications, there being no risk of damage to the biological system through the addition of an acidic solution, as would have been the case with known systems.

[0043] It is often the case that the nanoparticles will form more quickly with heating or stirring. As used herein the term 'stirring' is intended to include any form of physical agitation, and hence stirring, sonication, and shaking are all within the scope of this term. Heating of the mixture of nanoparticle forming components will generally be mild, and to a temperature in the range 50 - 75°C, often in the range 55 - 70°C. The duration of heating or stirring will generally be until the solution becomes clear, as this indicates the formation of the nanoparticles, such durations may be in the range 1 minute - 2 hours, often in the range 5 minutes - 60 minutes, often in the range 10 - 20 minutes and is typically about 15 minutes.

[0044] In a fourth aspect of the invention, there is provided a contact lens comprising a lipid-containing nanoparticle for inclusion in a contact lens, the nanoparticle comprising a membrane-forming polar lipid, a lipid-solubilising agent including a synthetic amphipathic polymer including both hydrophobic groups and anionic hydrophilic groups, a lens-forming material and water.

[0045] The lipid containing nano-particle is typically a nanoparticle according to the first aspect of the invention. The nanoparticle may consist essentially of or consist of a membrane-forming polar lipid, a lipid-solubilising agent including a synthetic amphipathic polymer including both hydrophobic groups and anionic hydrophilic groups and water. The material from which the lens is constructed is not particularly restricted but is typically selected from polymethylmethacrylate (PMMA), polyacrylamide, silicone, hydrogels or combinations thereof.

[0046] In a fifth aspect of the invention there is provided a packing solution for a contact lens comprising a lipid containing nanoparticle, often the lipid containing nanoparticle is a nanoparticle according to the first aspect of the invention, although those described below may also be used. Typically, the solution will be an aqueous solution and usually an aqueous salt or saline solution. Such solutions are used to suspend a contact lens in when the lens are not being used. An advantage of storing a contact lens in the packing solution of the invention is that it forms a thin layer over the surface of the lens when the lens is removed from the solution, reducing disruption of the tear film when the lens is in the eye, lubricating the lens and preventing the lens from drying out. Without being bound by theory, it is believed that when the lens is inserted into the eye, the packing solution combines with the tear film and over time, the lipid content of the lipid-containing nanoparticles is liberated to ensure that the tear film remains sufficiently supplied with lipids. A further advantage of the packing solution of the invention is that it can be used with existing contact lens technology.

[0047] As described above with reference to the suspension, the packing solution will generally be at biological pH, often in the ranges described above.

[0048] In a sixth aspect of the invention there is provided a fluid composition for making a contact lens comprising a lipid-containing nanoparticle for inclusion in a contact lens, the nanoparticle as described above.

[0049] The lipid containing nanoparticles which are used in the contact lens of the invention may be as described in EP1007002, which describes polymer/lipid complexes which could fall within the scope of the lipid-containing nanoparticles of these aspects of the invention. The disclosure of polymer/lipid complexes in EP1007002 is therefore incorporated herein in its entirety.

[0050] It is often the case that the lipid-containing nanoparticles are dispersed throughout the lens-forming material. The liquid nanoparticles are usually uniformly dispersed throughout the fluid composition of the invention. Accordingly, when contact lenses are created from the composition, the lipid-containing nanoparticle will form part of

the contact lens, being substantially uniformly distributed throughout. Alternatively, the lipid-containing nanoparticles may be adhered to one or both surfaces of the contact lens, for instance through immersion in a packing solution as described above, or through immersion in a coating solution, to form a coating (partial or total) which is physically or chemically bonded to the surface. Adherence to the surface, or coating, may also be achieved using spray application of the nanoparticles, whether in solution or dry form.

[0051] Usually, the lens forming material is selected from PMMA, Polyacrylamide, silicone, hydrogels or combinations thereof. These materials are capable of storing large quantities of water relative to their weight and the structures created are usually soft and flexible.

[0052] A seventh aspect of the invention provides a method of making a contact lens, comprising the steps of exposing a contact lens to lipid containing nanoparticles, the nanoparticles typically being nanoparticles according to the first aspect of the invention. Typically the contact lens will not have been previously coated, although layered structures are possible, and it could be the case that the contact lens is first coated with an adhesive substance, to ensure bonding of the nanoparticles to the lens. Often, the nanoparticles will be in solution and the uncoated contact lens may be exposed to the nanoparticle solution for a period of time in the range of about 1 minute to 24 hours, often in the range 10 - 60 minutes, perhaps 30 - 45 minutes. This exposure often involves dipping the contact lens into the nanoparticle solution, although spray coating may also be used. Where spray coating is applied, the contact time will generally be reduced to 10 seconds - 2 minutes, often 30 seconds - 1 minute, such reductions in production time can be advantageous, although spray coatings can sometimes be less uniform resulting in lenses of lower quality.

[0053] In an eighth aspect of the invention there is provided a method of making a contact lens comprising curing the fluid composition according to the sixth aspect of the invention. This curing procedure may be photocuring, thermal curing, chemical curing or a combination thereof.

[0054] In another aspect of the invention there is provided a composition for use in the prevention and/or treatment of dry eye syndrome, the method comprising incorporating a lipid-containing nanoparticle, which will typically be a nanoparticle according to the first aspect of the invention, into a fluid composition for making contact lenses, into a contact lens, or into a packing solution for contact lenses and placing said fluid composition, contact lens or contact lens coated in said packing solution into the eye. Often a pharmaceutically acceptable excipient will be present in the packing solution. The lipid-containing nanoparticles are typically administered in combination with a contact lens but may also be administered as eye drops.

[0055] In another aspect of the invention, there is provided a use of a lipid containing nanoparticle, which is typically a nanoparticle according to a first aspect of the invention, in a fluid composition for making contact lens.

[0056] In another aspect of the invention, there is provided a use of a lipid containing nanoparticle, which is typically a nanoparticle according to a first aspect of the invention, in a contact lens.

[0057] In another aspect of the invention, there is provided a use of a lipid containing nanoparticle, which is typically a nanoparticle according to a first aspect of the invention, in the delivery of lipids to the tear film of the eye.

[0058] As mentioned above, one useful application that has been discovered of the nanoparticles of the invention is that they are able to remove compounds from their native membrane environment in a manner which minimises the structural, chemical and conformational changes to the extracted compounds.

[0059] Accordingly, in a still further aspect of the invention, there is provided a use of a lipid-containing nanoparticle according to a first aspect of in the invention in the extraction of membrane proteins from cell membranes.

[0060] In yet another aspect of the invention, there is provided, a process for extracting membrane proteins from cell membranes comprising the steps of: (a) exposing a cell to a

composition comprising a nanoparticle according to a first aspect of the invention (b) incubating the mixture from step (a) until at least a portion of one or more of the proteins present in the membrane is solubilised by the composition to form a polymer-membrane protein complex; and (c) separating the polymer-membrane protein complex from the mixture resulting from steps (a) and (b).

[0061] The term "membrane" as used herein is intended to refer to structures defining the interface between different regions within the body, in particular, boundaries between organelles and cytoplasm, between the contents of a cell and the exterior of a cell including cell walls or other layers surrounding the periphery of a cell. The "cell" could be a plant cell, mammalian cell, bacterial cell, fungal cell or other biological cell . Typically, the cell is a yeast cell.

[0062] The inventors have found that lipid-containing nanoparticles having acid, ester or half-ester functionality as defined herein are particularly suited for solubilising membrane proteins and are readily extractable from reaction mixtures.

[0063] Unless otherwise stated each of the integers described in the invention may be used in combination with any other integer as would be understood by the person skilled in the art. Further, although all aspects of the invention preferably "comprise" the features described in relation to that aspect, it is specifically envisaged that they may "consist" or "consist essentially" of those features outlined in the claims. In addition, all terms, unless specifically defined herein, are intended to be given their commonly understood meaning in the art.

[0064] Further, in the discussion of the invention, unless stated to the contrary, the disclosure of alternative values for the upper or lower limit of the permitted range of a parameter, is to be construed as an implied statement that each intermediate value of said parameter, lying between the smaller and greater of the alternatives, is itself also disclosed as a possible value for the parameter.

[0065] In addition, unless otherwise stated, all numerical values appearing in this application are to be understood as being modified by the term "about".

Brief Description of the Drawings

[0066] In order that the present invention may be more readily understood, it will be described further with reference to the figures and to the specific examples hereinafter.

[0067] Figure 1 schematically shows a nanoparticle of the invention;

[0068] Figure 2 shows the effect of pH on the optical density of a nanoparticle solution formed with unmodified PMAS 1600;

[0069] Figure 3 shows the effect of pH on the optical density of a nanoparticle solution formed with PMAS 1600 modified with methanol;

[0070] Figure 4 shows the effect of time on the optical density of a nanoparticle solution formed with unmodified PMAS 1600;

[0071] Figure 5 shows the effect of time on the optical density of a nanoparticle solution formed with methanol-modified PMAS 1600;

[0072] Figure 6 shows the effect of time on the optical density of a nanoparticle solution formed with ethanol-modified PMAS 1600; -

[0073] Figure 7 shows the effect of time on the optical density of a nanoparticle solution formed with propan-1-ol-modified-PMAS 1600;

[0074] Figure 8 shows a gel electrophoresis blot for the extraction of CD81; and

[0075] Figure 9 shows a gel electrophoresis blot for the extraction of Claudin-1.

[0076] It should be appreciated that the nanoparticles, fluids, contact lenses, solutions, methods and uses of the invention are capable of being incorporated in the form of a variety of embodiments, only a few of which have been illustrated and described above.

Examples

Example 1 - Polymer Formation

[0077] The copolymer is made by the polymerisation of maleic anhydride and styrene under conventional conditions. The resulting intermediate copolymer (PMAS) then under goes a ring opening and esterification process.

Ring-opening of Styrene Maleic Anhydride Copolymer (PMAS)

[0078] 3g of PMAS was added to 50ml of deionised water. The solution was heated at 50°C - 60°C with stirring for 15 minutes. 1M sodium hydroxide solution was added dropwise to increase and maintain the pH at 11 until the solution clarified. The resulting solution was made up to 100ml using de-ionised water and the resulting polymer was extracted using standard extraction procedures.

Esterification of Polystyrene Maleic Acid

[0079] 3g of the polymer was dissolved in 6ml of methyl ethyl ketone (MEK) and heated to 60°C - 70°C whilst stirring. 1.5ml of methanol was added. The solution was refluxed at 70°C - 80°C with stirring for 14 hours. An additional 3ml of MEK was added followed by a further 2.25ml aliquot of methanol 15 minutes later. The solution was allowed to reflux for a further 6 hours before being separated out using petroleum ether (60-80°C). The polymer was precipitated out, filtered and dried in a vacuum oven.

Example 2 - Nanoparticle Formation

[0080] The nanoparticles were formed by mixing 0.05g of DMPC with 1 ml of a 3% solution of PMAS at pH 7 using a vortex shaker for 30 seconds. The mixture was then placed in an ultrasound bath for 15 minutes, before removal from the bath and the addition of a further 4 ml of the 3% solution of PMAS at pH 7. The resulting mixture was again shaken in a vortex shaker for 30 seconds.

[0081] For esterified PMAS this resulted in the formation of a clear solution, indicating formation of the nanoparticles.

[0082] For unmodified PMAS examples it was necessary to add 1M hydrochloric acid dropwise until the solution clarified, the point of clarification was around pH 4.

Example 3 - Stability of Nanoparticles

[0083] An example of a nanoparticle in accordance with the invention is shown schematically in Figure 1, as can be seen the nanoparticles are substantially discoid.

[0084] The nanoparticle solutions are regarded as stable when the solution is clear, as such the clarity of the solution can be used to monitor the stability of the solution. The method used is UV-visible spectroscopy in the 400 - 700 nm range. Values close to zero in this range are indicative of a stable nanoparticle in solution.

[0085] The effect of pH on the nanoparticles is shown in Figures 2 and 3. Specifically, it can be seen that nanoparticles which have not been modified to form a half ester are stable in the pH range 3 - 4, but that outside this pH range the mixtures become opaque, indicating breakdown of the nanoparticles (Figure 2). The pH stability of nanoparticles formed using the half esters described in the invention is very different, the window of stability of these nanoparticles being around 7, with acidic pH's producing highly absorbing solutions, and basic pH's producing mildly cloudy solutions (Figure 3). This indicates that whilst stability is reduced at basic pH's, it is at acidic pH where the nanoparticles of the invention break down most rapidly.

[0086] The stability of nanoparticles over time was also studied. As can be seen from Figures 4 - 7, the unmodified (dicarboxylic acid) nanoparticles show significant loss of clarity, and hence nanoparticle breakdown after just 28 days whereas the methyl, ethyl and n-propyl functionalised nanoparticles remain stable.

Example 4 - Extraction of $hA_{2a}R$

Membrane preparation

[0087] All work was carried out at 0–4 °C. Typically 20 g cells were suspended in 40 ml ice-cold breaking buffer (50 mM Tris-HCl, 5% glycerol, 2 mM EDTA, pH 7.4, 0.2% protease inhibitor cocktail set IV (Calbiochem)) and broken by 3 passages through a

chilled Avestin C3 cell disrupter. Breaking efficiency was typically > 90% as determined by light microscopy. Unbroken cells and cell debris were removed from the membrane suspension by low-speed centrifugation in (10,000xg, 30 min). Membranes were then collected by ultracentrifugation (150,000xg, 45 min), suspended in 50 mM Tris-HCl, 10% glycerol, 500 mM NaCl, flash frozen in liquid N_2 and stored at –80 °C.

Production of $hA_{2a}R$ in yeast

[0088] A glycosylation variant of $hA_{2a}R$ has previously been produced in *P. pastoris* membranes from cultures grown in shake flasks. When purified in DDM/CHS, a fully-functional protein was obtained. By optimising the protocol in a 1 l bioreactor (Applikon Biotechnology) and inducing with 50% methanol, we obtained a factor of 10 increase in cell density compared with shake-flask cultures (average OD_{600} was 160 and 16 respectively), whilst maintaining the receptor abundance (B_{max} = 9.5 $pmolmg^{-1}$). All subsequent preparations were therefore derived from bioreactor cultures.

Solubilisation of $hA_{2a}R$ with PMAS.

[0089] $hA_{2a}R$ was solubilised from *P. pastoris* membranes using either (i) SMA in the presence of DMPC or (ii) the detergent, n-Dodecyl *β*-D-Maltopyranoside (DDM), in the presence of cholesteryl hemisuccinate (CHS).

Analysis of solubilised $hA_{2a}R$

[0090] Radioligand binding with [^{3}H]ZM241385 established that the binding capability (B_{max}) of the purified $hA_{2a}R$ preparation was consistent with the theoretical value of 21.3 nmol mg^{-1} for fully active GPCR. It is noteworthy that there was no decrease in the $hA_{2a}R$ B_{max} value upon polymer solubilisation from the yeast membranes. The affinity (K_d) of ZM241385 was comparable to previous reports. To confirm that the binding profile of the solubilised product was not significantly different from that observed in the native membranes, the product was pharmacologically characterised using a range of ligands, which varied in affinity and efficacy, in competition radioligand binding assays. These data show that the receptor is still able to bind to a range of ligands including theophylline, XAC an NECA with near native affinities.

[0091] Yeast membrane with $hA_{2a}R$ was combined with a mixture of 1% DMPC and 2.3% PMAS and re-suspended in 20mM HEPES, 50mM NaCl, 10% glycerol and water at pH 7. The suspension was placed in a rocker over night at ambient temperature and the sample was spun at 100,000g for 1 hr. The resulting supernatant was analysed. The results show the specific activity via a single-point radioligand ($[^3H]$ ZM241385) binding assay. The experiment was repeated at pH 11 and using a range of different polymers. The results are shown in Table 1 below:

Table 1

Polymer	**pH**	**B_{max}Estimate (pmol mg^{-1})**	**Kd (nM)**
SMA 2000P§ - Powdered	7	4.9	5.6
SMA 2000P§ - Powdered	11	6.4	1.4
SMA 1000F‡ - Flaked	7	0 or negligible	not measured
SMA 1000F‡ - Flaked	11	0.0004 or negligible	not measured
PMAS 1600#	7	0.0001 or negligible	not measured
PMAS 1600#	11	0.0004 or negligible	not measured
PMAS 1600ME	7	5.4	4.6
PMAS 1600ME	11	5.2	3.6
DDM plus CHS	7	5.6	5.3

§ - styrene maleic anhydride copolymer, approx. ratio 2:1, MW approx. 7500

‡ - styrene maleic anhydride copolymer, approx. ratio 1:1, MW approx. 5500

- styrene maleic anydride copolymer approx. ratio 1:1, MW approx. 1600

[0092] It can be seen from Table 1 that the compositions according to the present invention have comparable activity to PMAS 2000 and n-dodecyl-beta-D-maltoside (DDM) and cholesteryl hemisuccinate (CHS).

Example 5 - Extraction of CD81

[0093] Example 4 was repeated with CD81, the results are shown in Figure 8 and in Table 2 below:

Table 2

Sample	Polymer	pH
1	PMAS 1600ME - Pellet	7
2	PMAS 1600ME - Supernatant	7
3	PMAS 1600ME - Pellet	11
4	PMAS 1600ME - Supernatant	11
5	SMA 2000P§ - Pellet	11
6	SMA 2000P§ - Supernatant	11
7	ND Ladder	

§ - styrene maleic anhydride copolymer, approx. ratio 2:1, MW approx. 7500

[0094] As can be seen from Figure 8, the nanoparticles directly extract CD81 from the membrane, primarily into the supernatant.

Example 6 - Extraction of Claudin-1

[0095] Example 4 was repeated with Claudin 1, the results are shown in Figure 9 and in Table 3 below:

Table 3

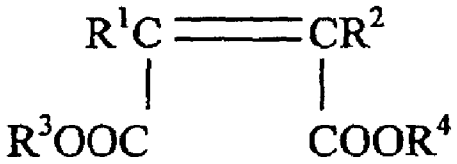
Sample	Polymer	pH
1	PMAS 1600ME - Pellet	11
2	PMAS 1600ME - Supernatant	11
3	PMAS 1600ME - Pellet	7
4	PMAS 1600ME - Supernatant	7
5	SMA 2000P§ - Pellet	11
6	SMA 2000P§ - Supernatant	11
7	ND Ladder	
8	ND Ladder	

§ - styrene maleic anhydride copolymer, approx. ratio 2:1, MW approx. 7500

[0096] As can be seen from Figure 9, the nanoparticles directly extract Claudin-1 from the membrane, the extraction of Claudin-1 being primarily into the pellet.

Claims

1. A lipid-containing nanoparticle, the nanoparticle comprising:
a membrane-forming polar lipid; and
a lipid-solubilising agent including a synthetic amphipathic polymer which is a copolymer of a first monomer of formula:



where R^1 and R^2 are each independently hydrogen or C_1 - C_9 alkyl, and at least one of R^3 and R^4 is C_1 - C_9 alkyl and the other is hydrogen or C_1 - C_9 alkyl; and
a second monomer of formula R^5-CH=CH_2 where R^5 is selected from hydrogen, C_1-C_8 alkyl, C_1-C_6 alkoxy, phenyl, benzyl and phenyl or benzyl substituted with an alkyl or other hydrophobic group.

2. A lipid-containing nanoparticle according to claim 1 wherein at least one of R^3 and R^4 is C_1 - C_9 alkyl and the other is hydrogen.

3. A lipid-containing nanoparticle according to claim 1 or claim 2 wherein at least one of R^3 and R^4 is methyl.

4. A lipid-containing nanoparticle according to any preceding claim, wherein the lipid comprises a phospholipid.

5. A lipid-containing nanoparticle according to claim 4, wherein the phospholipid is a phosphatidylcholine selected from dipalmitoylphosphatidylcholine (DPPC), dilauroylphosphatidylcholine (DLPC) and dimyristoylphosphatidylcholine (DMPC).

6. A lipid-containing nanoparticle according to any preceding claim, wherein when R^1 and R^2 are both hydrogen, R^5 if alkyl, contains less than eight carbon atoms.

7. A lipid-containing nanoparticle according to any preceding claim, wherein the synthetic amphipathic polymer is an alternating copolymer wherein the first monomer is a maleic acid ester and the second monomer is selected from styrene, indene, a C_1 - C_4 alkyl substituted styrene or indene, and an alkyl vinyl ether selected from propyl, isopropyl and butyl vinyl ether, or combinations thereof.

8. A lipid-containing nanoparticle according to any preceding claim, wherein said synthetic polymer has physiologically and pharmaceutically acceptable non-toxic properties for administration to mammals.

9. A lipid-containing nanoparticle according to any preceding claim, wherein the molecular weight (number average) or relative mass of the synthetic polymer has a range of 1,500 to 500,000 daltons.

10. A lipid-containing nanoparticle according to any preceding claim, wherein said synthetic polymer is a poly(maleic acid ester-styrene) copolymer (PMAS).

11. A lipid-containing nanoparticle according to any preceding claim, further comprising a pH stabilising agent.

12. A lipid-containing nanoparticle according to any preceding claim, wherein the pH stabilising agent is sodium hydroxide.

13. A lipid-containing nanoparticle according to any preceding claim, of size in the range of 1 - 50nm under physiological conditions of temperature and pH.

14. A suspension comprising, a lipid containing nanoparticle according to any preceding claim, and a fluid continuous phase, wherein the suspension is of pH in the range 6 - 8.

15. A suspension according to claim 14, wherein the fluid continuous phase comprises water.

16. A suspension according to claim 14 or claim 15, wherein the suspension is at biological pH.

17. A packing solution for a contact lens comprising, a lipid containing nanoparticle according to claims 1 to 13.

18. A solution according to claim 17, wherein the solution is an aqueous solution.

19. A solution according to claim 17 or claim 18, wherein the solution is an aqueous saline solution.

20. A fluid composition for making a contact lens comprising:
a lipid-containing nanoparticle for inclusion in a contact lens, the nanoparticle consisting of:
a membrane-forming polar lipid; and
a lipid-solubilising agent including a synthetic amphipathic polymer including both hydrophobic groups and anionic hydrophilic groups;
a lens-forming material; and
water.

21. A fluid composition according to claim 20, wherein the pH of the composition is in the range 6 - 9.

22. A fluid composition according to claim 20 or claim 21, wherein the composition is at biological pH.

23. A contact lens comprising:
a lipid-containing nanoparticle for inclusion in a contact lens, the nanoparticle consisting of:
a membrane-forming polar lipid; and
a lipid-solubilising agent including a synthetic amphipathic polymer including both hydrophobic groups and anionic hydrophilic groups;
a lens-forming material; and

water.

24. A fluid composition or a contact lens according to any of claims claim 20 to 23, wherein the lens forming material is selected from PMMA, Polyacrylamide, silicone, hydrogels or combinations thereof.

25. A contact lens according to claim 23 or claim 24, wherein the lipid-containing nanoparticle is dispersed throughout the lens-forming material.

26. A contact lens according to claim 23 or claim 24, wherein the lipid-containing nanoparticle is adhered to one or both surfaces of the contact lens.

27. A contact lens according to claim 23 or claim 24, wherein the lipid-containing nanoparticle forms a coating on one or both surfaces of the contact lens.

28. A method of making a lipid-containing nanoparticle according to claim 1 comprising the step of adding the membrane-forming polar lipid and the lipid-solubilising agent to an aqueous solution having a pH in the range of 6 to 8.

29. A method according to claim 28, wherein the solution is heated to a temperature in the range 50 - 75°C and/or stirred for a time in the range 30 - 120 minutes.

30. A method of making a contact lens according to claim 26 or claim 27, comprising the step of exposing a contact lens to the nanoparticles according to any of claims 1 to 13.

31. A method according to claim 30, where the nanoparticles are in solution.

32. A method according to claim 30 or claim 31, wherein the uncoated contact lens is exposed to the nanoparticle solution for a period of time in the range of about 1 minute to 24 hours.

33. A method according to any of claims 30 to 32, wherein the step of exposing comprises dipping the contact lens into the nanoparticle solution.

34. A method of making a contact lens comprising, curing the fluid composition according to any of claims 20 to 22.

35. Use of a lipid-containing nanoparticle according to any of claims 1 to 13, in a fluid composition for making contact lenses.

36. Use of a lipid-containing nanoparticle according to any of claim 1 to 13 in a contact lens.

37. Use of a lipid-containing nanoparticle according to claims any of 1 to 13, in the delivery of lipids to a tear film of an eye.

38. Use of a lipid-containing nanoparticle according to claims any of 1 to 13, in the extraction of membrane proteins from cell membranes.

39. A composition for the prevention and/or treatment of dry eye syndrome, the composition comprising a lipid-containing nanoparticle according to claims 1 to 13.

40. A method of prevention and/or treatment of dry eye syndrome, the method comprising incorporating a lipid-containing nanoparticle according to claims 1 to 13 into a fluid composition for making a contact lens, or into a contact lens.

41. A nanoparticle, contact lens, use, composition, method or packing solution substantially as described herein with reference to the examples.

42. A process for extracting membrane proteins from cell membranes comprising the steps of:

(a) exposing a cell to a nanoparticle according to any one of claims 1 to 13;

(b) incubating the mixture from step (a) until at least a portion of one or more of the proteins present in the membrane is solubilised by the composition to form a polymer-membrane protein complex; and

(c) separating the polymer-membrane protein complex from the mixture resulting from steps (a) and (b).

Figure 1

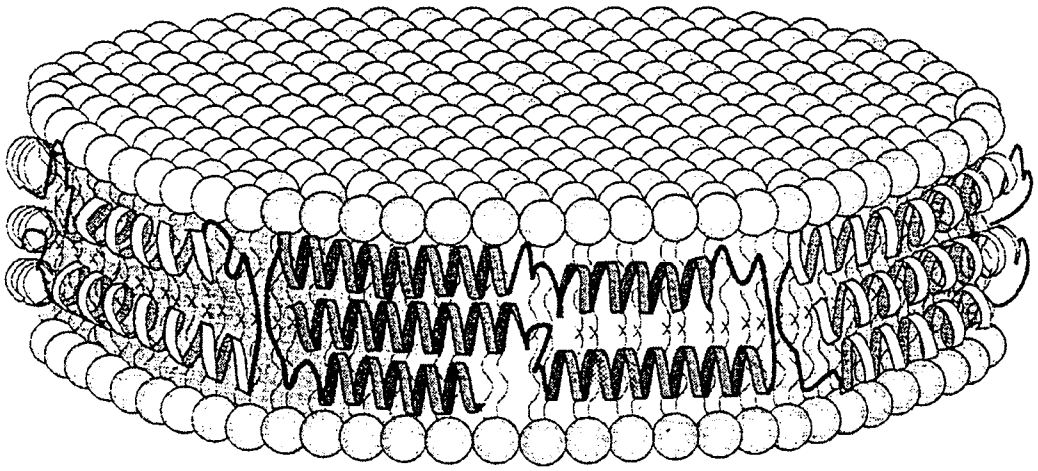


Figure 2

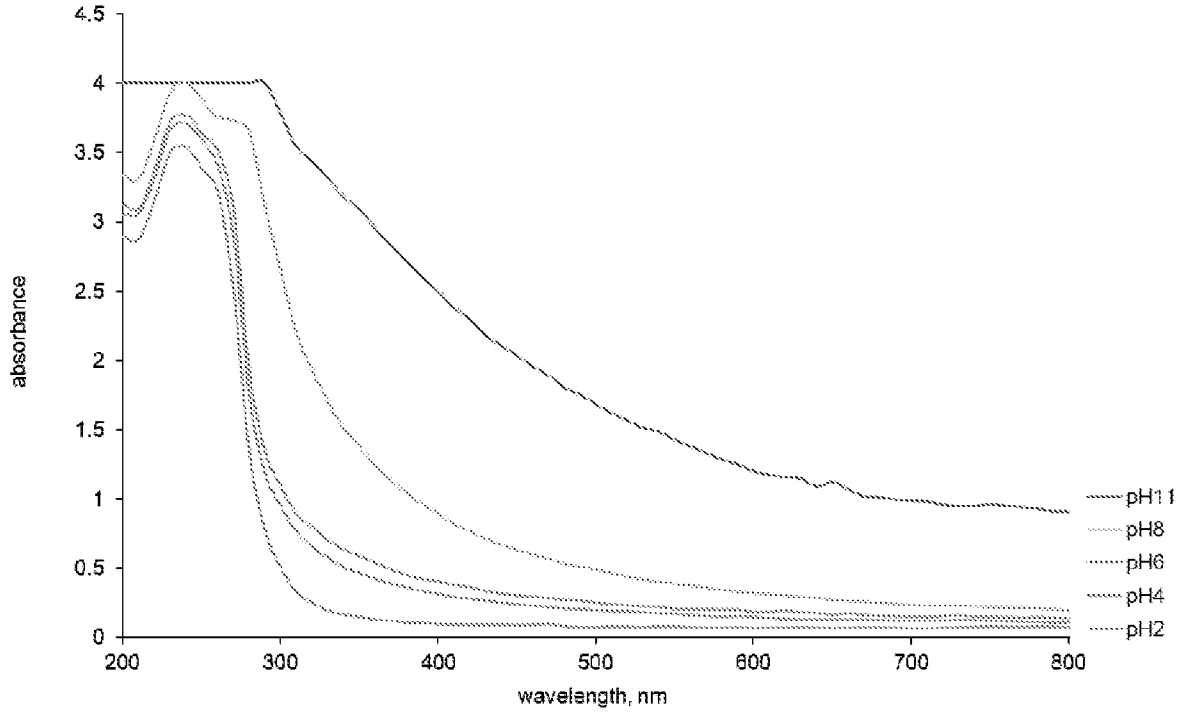


Figure 3

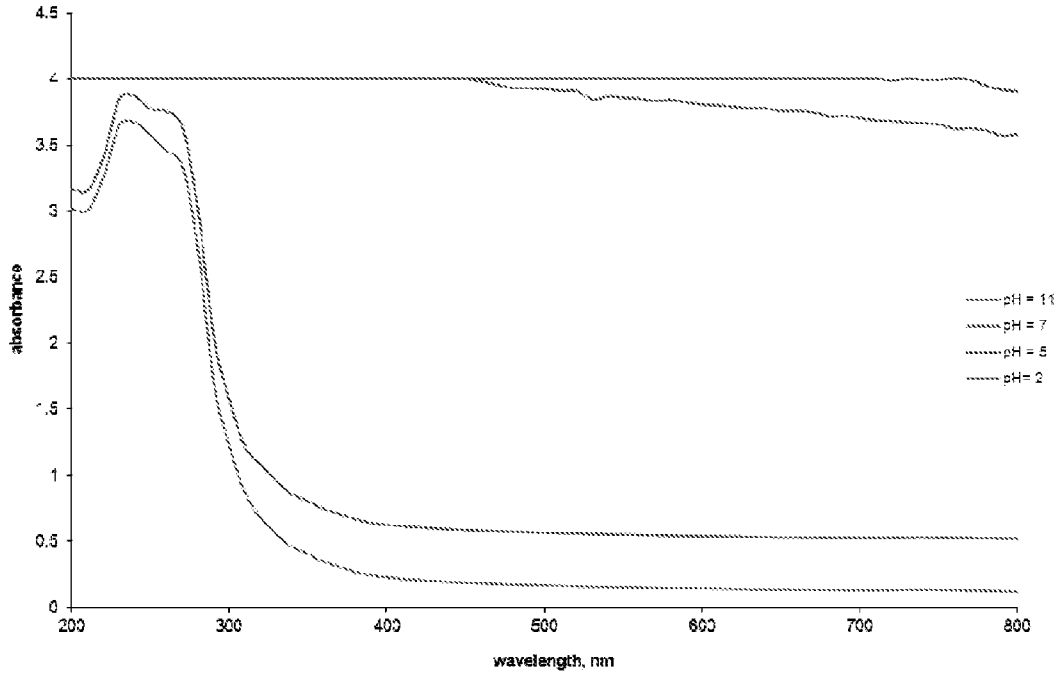


Figure 4

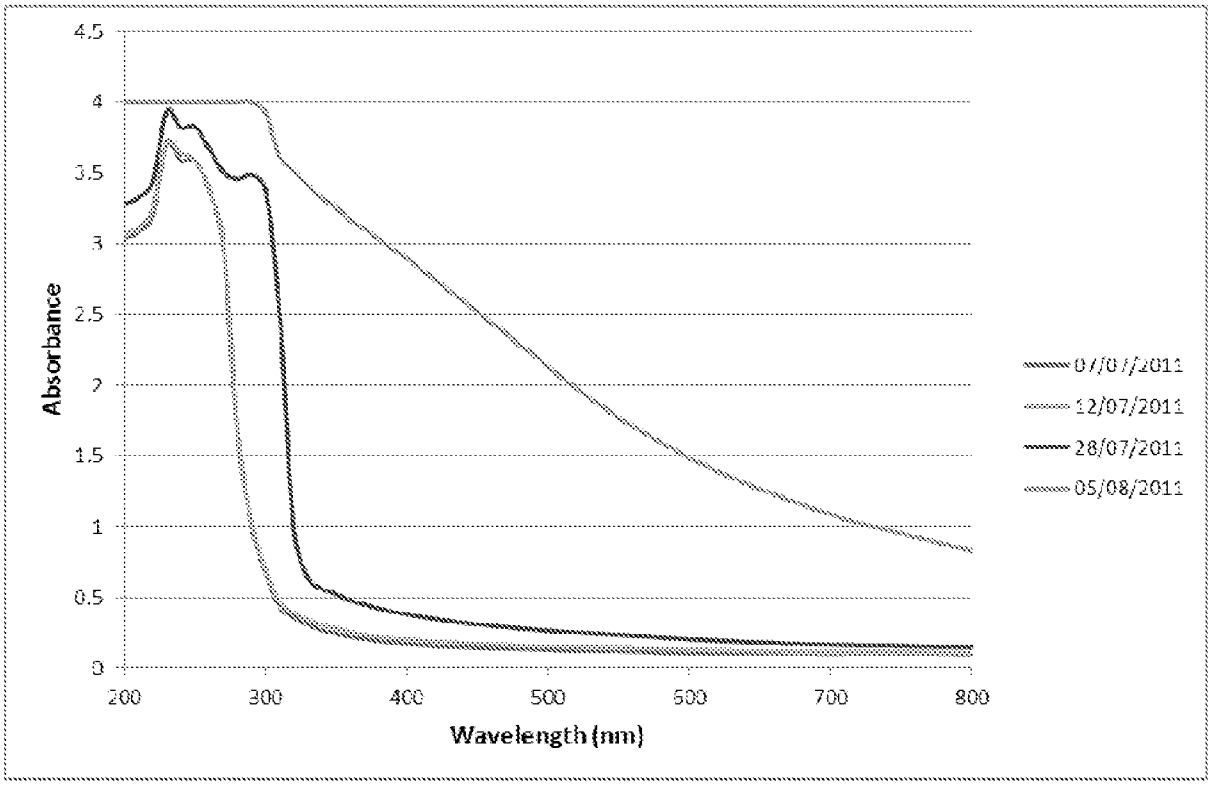


Figure 5

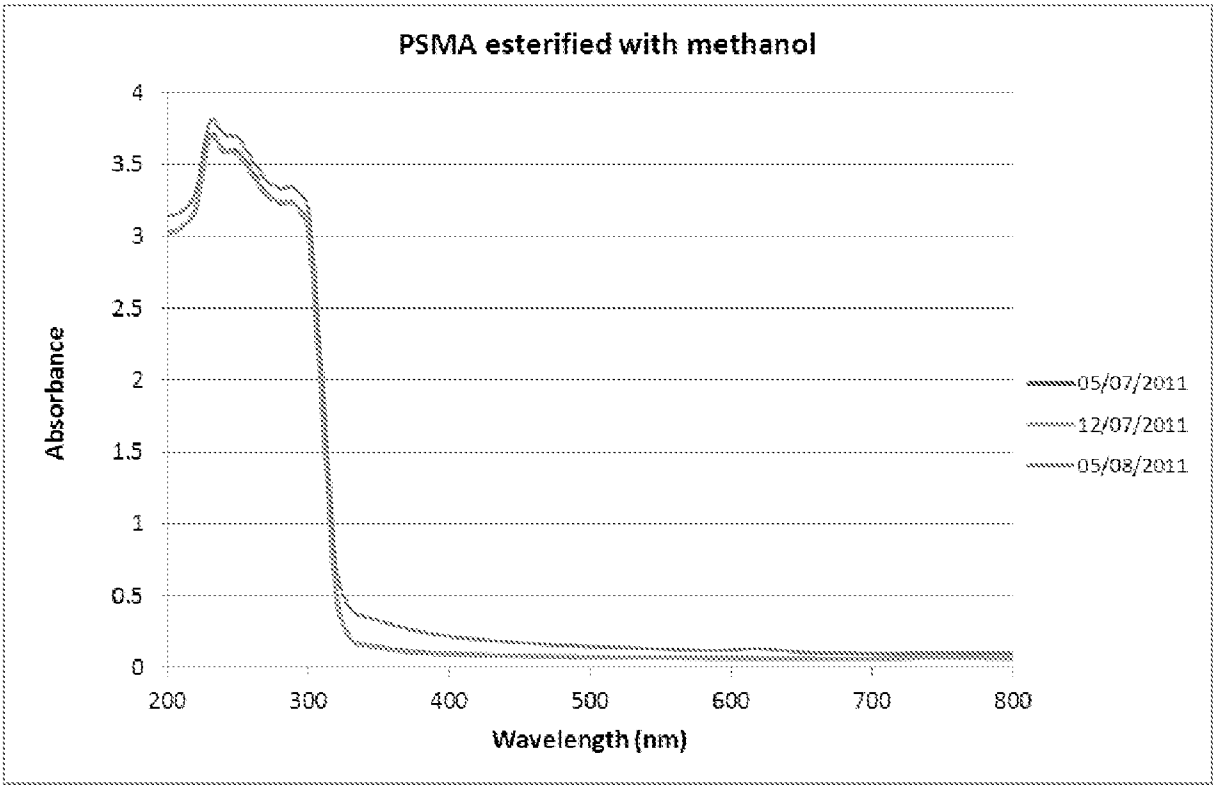


Figure 6

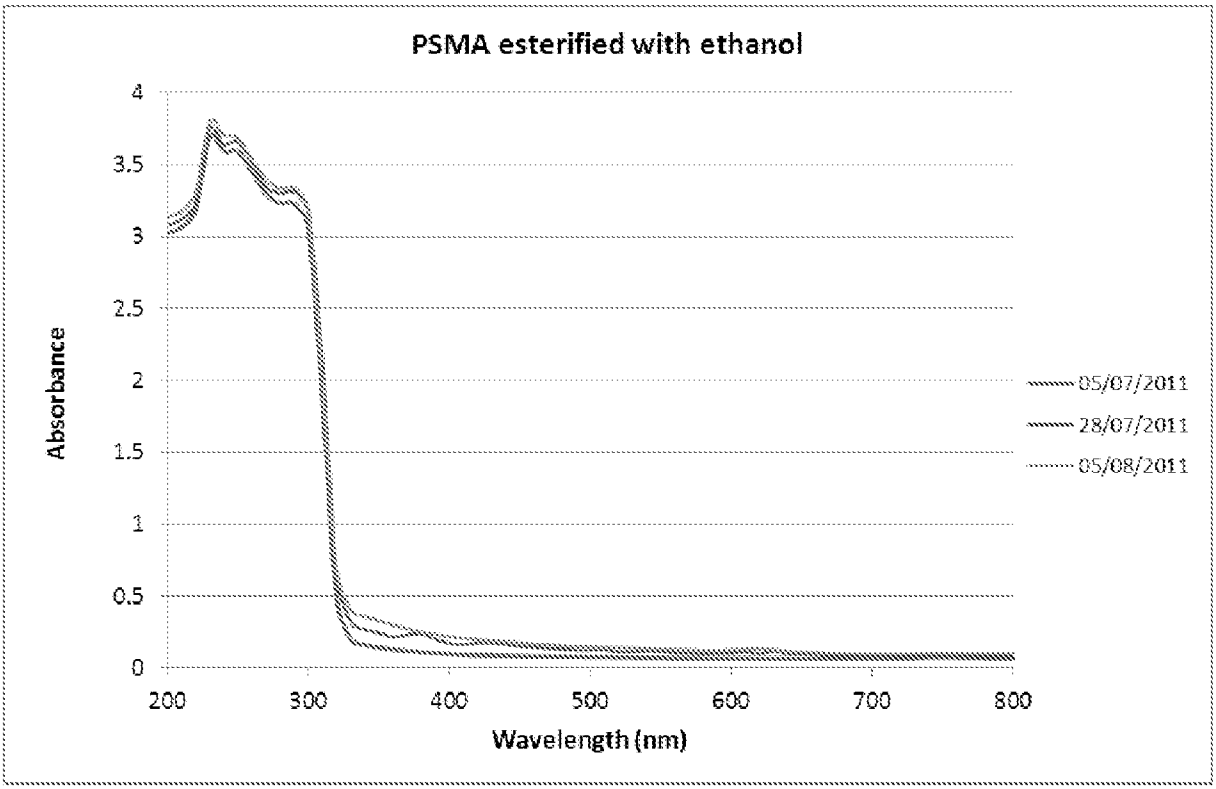


Figure 7

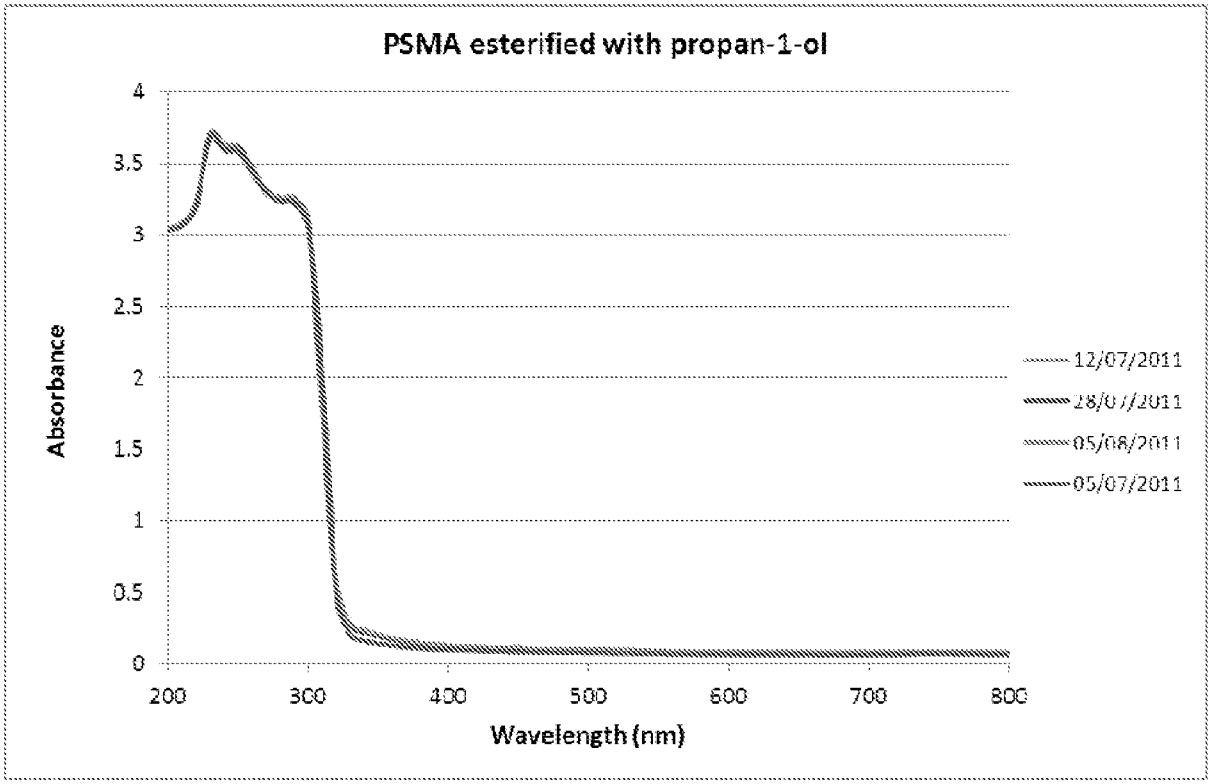


Figure 8

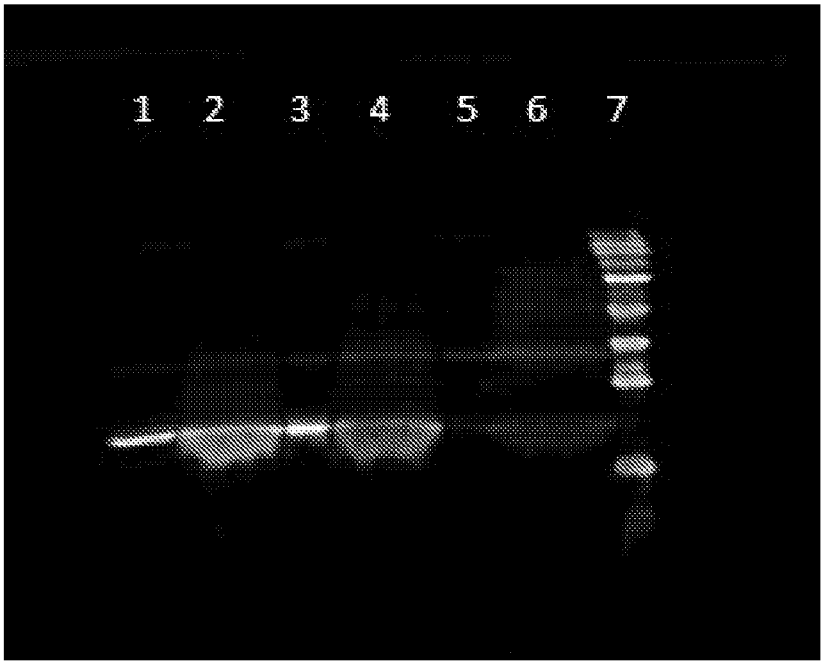
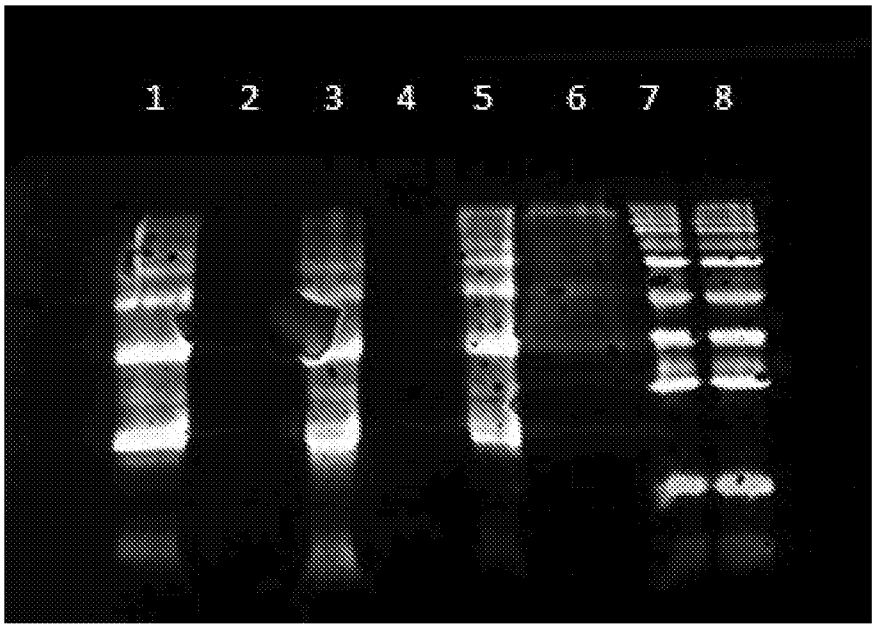


Figure 9



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/052103

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/00 A61K9/51 A61L12/08 C11D3/00
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 A61L C11D

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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/09955 A2 (UNIV ASTON [GB]; TONGE STEPHEN RONALD [GB]; TIGHE BRIAN JOHN [GB]) 4 March 1999 (1999-03-04) cited in the application page 12, lines 5-16; claims 1-35; examples 1-7 page 26, lines 3-10	1-42
X	GB 2 426 703 A (MALVERN COSMECEUTICS LTD [GB]) 6 December 2006 (2006-12-06) cited in the application page 16, lines 3-9; claims 1-89; examples 1-7 page 17, lines 19,28	1-42
A	GB 2 464 393 A (MALVERN COSMECEUTICS LTD [GB]) 21 April 2010 (2010-04-21) claims 1-154	1-42

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
16 September 2014	24/09/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Konter, Jörg

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2014/052103

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