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(54) Title: PARTICULATE DRUG CARRIERS

(57) Abstract

The present invention is directed to particulate drug carriers, such as vesicles, formed from polysaccharide derivatives. A polysaccharide bearing at least one non–ionic hydrophilic group attached to an individual monosaccharide unit is hydrophobised to form a derivative bearing at least one long chain alkyl residue. Particle formation is then induced in the presence of cholesterol. The particles are suited for entrapment or conjugation of pharmaceutically active ingredients.

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PARTICULATE DRUG CARRIERS

Field of the invention

This invention relates to particulate drug carriers formed from polysaccharide derivatives. A polysaccharide bearing at least one non-ionic hydrophilic group attached to the individual monosaccharide units is hydrophobised to form a derivative bearing at least one long chain alkyl residue. Particle formation is then induced in the presence of cholesterol. The particles are suited for entrapment or conjugation of pharmaceutically active ingredients.

Backgroud to the invention

Chitosan (N-deacetylated chitin) has been investigated as a drug delivery agent when fabricated into cross-linked microspheres (Thanoo et al, J. Pharma. Pharmacol., 1992, 44, 283-286) and as a coating for liposomes (Henriksen et al, Int. J. Pharm., 1994, 101, 227-236). Chitosan solutions have also been used as penetration enhancers (Aspden et al, Eur. J. Pharm. Sci., 1996, 4, 23-32).

Yoshioka et al (Biosci. Biotech. Biochem., 1993, 57, 1053-1057) have shown that the precipitation (on standing) of a liposome suspension prepared from hydrogenated egg yolk lecithin could be prevented by treating the suspension with an aqueous solution of sulphated N-myristoyl chitosan (S-M-Chitosan). This result is explained by the surface of the liposomes being coated with S-M-chitosan, the ionisation of the sulphate group leading to a negative charge on the liposomes. This in turn causes repulsion between the liposome particles and prevents precipitation. The coating process was not found to destroy the lipid bilayer.

More recently, the same group (in Biosci. Biotech. Biochem., 1995, 59, 1901-1904) have examined the properties of aqueous solutions of chitosan derivatives, namely sulphated N-acyl chitosan (S-C_n-chitosan) having varying lengths of alkyl chain. S-C_n-chitosans from C_2 to C_{14}

dissolved completely in water to form transparent solutions. S-C_{16}-chitosan was also prepared but its properties were not examined since the aqueous mixture formed was not transparent and it was believed that an aggregate such as a liquid crystal had been formed. The solubilising capacity of the aqueous S-C_n-chitosan solutions towards a hydrophobic substance (azobenzene) was examined and it was found that solubility increased sharply with increasing carbon number above C_{10}. It was concluded by the authors that the long alkyl chains were aggregated to form micelles able to dissolve the azobenzene molecules. The micelles formed are described as "polymer micelles", although the type of micelle formed was not ascertained. The "polymer micelles" are suggested for use as drug carriers.

Sunamoto et al (Chem. Lett., 1991, 1263-1266) have reported that palmitoyl- or cholesterol-substituted derivatives of polysaccharides such as pullulan, amylopectin and dextran form self-aggregates in aqueous solution. A palmitoyl pullulan derivative substituted to 5.4 palmitoyl groups per 100 glucose units is reported, together with cholesterol-substituted pullulans having varying degrees of substitution. Investigation of the interaction between the pullulan derivatives and a fluorescent probe showed that the pullulan derivatives formed polymer self-aggregates above a critical concentration. The driving force for the aggregation is ascribed to a hydrophobic interaction between hydrophobic moieties and it is noted that the palmitoyl group was less effective for forming the self-aggregates. The aggregates are described as having the capacity to encapsulate various substances by hydrophobic interaction, for example drugs, proteins and nucleic acids.

The same group also reports (in Macromolecules, 1993, 26, 3062-3068) on the synthesis and solution properties of a non-ionic cholesterol-modified pullulan derivative (CHP) in water. In this work, pullulan was substituted by 1.6

cholesterol groups per 100 anhydroglucoside units. The authors state that the CHP self-aggregates formed relatively monodispersive particles and it is suggested that one CHP self-aggregate consists of approximately 13 CHP molecules. Experimental data suggests that the hydrophobic core of the CHP aggregates is completely and stably covered by the hydrophilic shell of the polysaccharide skeleton, forming colloidally stable nanoparticles above the critical concentration. The binding of various fluorescent probes was investigated and shown to increase with an increase in hydrophobicity of the probe. It is therefore concluded that the main driving force for complexation is a hydrophobic interaction.

These researches have further described (in Chem. Lett., 1995, 707-708) the complexation of the hydrogel nanoparticle formed by self-assembly of CHP with 5-10 insulin monomers in water. The number of complexed insulin molecules increased with an increase in the substitution degree of the cholesterol group of CHP. The insulin is stated as being complexed deeply inside the amphiphilic hydrogel matrix of the nanoparticle in which the hydrophobic microdomain of the associating cholesterol forms non-covalent crosslinks of gel structure. The number of crosslinks of one nanoparticle increases with an increase in the number of substitution degree of cholesterol, leading to an increase in the binding site for insulin.

Finally, the same group reports (in J. Am. Chem. Soc., 1996, 118, 6110-6115) a study of the complexation between CHP self-aggregate and bovine serum albumin (BSA). In all cases, approximately one BSA molecule was complexed by one nanoparticle of CHP self-aggregate, irrespective of the structure of the CHP self-aggregate. Unfolding of BSA by thermal means or by a denaturant such as urea was largely suppressed on complexation. This stabilisation of BSA upon complexation is ascribed to the formation of multiple non-covalent interactions between BSA and the hydrogel of CHP

self-aggregate.

Summary of the invention

According to the present invention, there is provided a compound which is a polysaccharide derivative bearing at least one non-ionic hydrophilic group and at least one hydrophobic group per molecule wherein said hydrophobic group is attached to the individual monosaccharide units and said hydrophobic group contains a C_{12-24} alkyl, alkenyl, alkynyl or acyl residue.

The non-ionic hydrophilic group is preferably a group of the formula R^1, wherein R^1 is selected from mono- and oligo-hydroxy C_{1-6} alkyl, mono- and oligo-hydroxy substituted C_{2-6} acyl, C_{1-2} alkoxy alkyl optionally having one or more hydroxy groups substituted on the alkoxy or alkylene groups, oligo- or poly-(oxa C_{1-3} alkylene) preferably polyoxyethylene comprising up to about 120 ethylene oxide units (i.e. up to a molecular weight of 5000), and C_{1-4} alkyl (oligo- or poly-oxa C_{1-3} alkylene) optionally hydroxy substituted preferably oligo- or polyglycerol ethers such as those described in GB-A-1,529,625, for example containing up to 10 glycerol units; and wherein R^1 is joined via an ether linkage to a saccharide unit of the polysaccharide. It is to be understood herein that the term acyl includes alkenoyl and alkynoyl groups as well as alkanoyl groups.

The requirement that the hydrophilic group is non-ionic is an important feature since a charged ionic group such as sulphate would repel anionic DNA which, in one embodiment, is associated with the particles as a means for gene delivery or vaccination.

The polysaccharide derivative is preferably a derivative of chitosan, pullulan or dextran and most preferably comprises 1,4-linked saccharide units. Normally, substitution by the non-ionic hydrophilic moiety occurs at the C6 position of a saccharide unit.

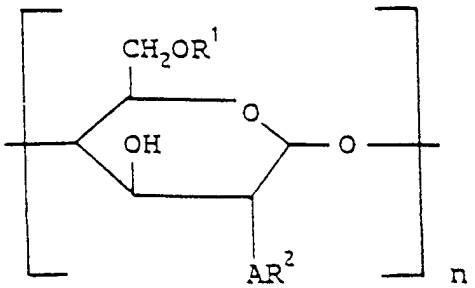
The hydrophobic group is preferably joined to a saccharide unit by an amide, ester, ether or amine linkage, most preferably by an amide linkage. In a further preferred embodiment, this group is substituted at the C2 position in a 1,4-linked saccharide unit.

The compound has a degree of substitution by non-ionic hydrophilic groups in the range 0.1-1.5, preferably greater than 0.9 and most preferably 1 per saccharide unit.

The ratio of hydrophilic:hydrophobic groups in the compounds of this invention is in the range 100:1 to 1:2, preferably between 10:1 and 2:1 more preferably 5:1 and 2:1. Compounds having a degree of hydrophobic substitution of 0.5 or above per hydrophilic group are found to be difficult to disperse due to the high hydrophobic burden. Consequently, compounds having a degree of substitution of 0.25 or less are preferred.

A preferred range of compounds according to the present invention are the N-substituted derivatives of poly-amino glycans most preferably N-acyl glycol chitosans, especially N-palmitoyl glycol chitosan (poly[β(1→4)-2-deoxy-2-hexadecanamido-6-0-(2-hydroxyethyl)-D-glucopyranose]. In this case, the presence of free amino groups is advantageous from a point of view of permitting complexing with anionic DNA. In addition, such groups could be used for the conjugation of drug molecules.

In a preferred embodiment, the compound has the formula:



wherein each R^1 is selected from hydrogen, mono- and oligo-hydroxy C_{1-6} alkyl, mono- and oligo-hydroxy substituted C_{2-6} acyl, C_{1-2} alkoxy alkyl optionally having one or more hydroxy groups substituted on the alkoxy or alkylene groups, oligo-

or poly-(oxa C_{1-3} alkylene) such as polyoxyethylene comprising up to about 120 ethylene oxide units and C_{1-4} alkyl (oligo- or poly-oxa C_{1-3} alkylene) optionally hydroxy substituted such as polyglycerol ethers, for example containing up to 10 glycerol units, provided that at least one of the groups R^1 is other than hydrogen;

A is -NH-, or -O-;

each R^2 is selected from hydrogen, C_{12-24} alkyl, alkanoyl, -alkenyl, alkenoyl, -alkynyl or alkynoyl, provided that at least one of the groups R^2 is other than hydrogen; and

n is 5-2000.

Preferably, the group R^1 has the formula $-CH_2CH_2OH$ or $-CH_2CH(OH)CH_2OH$, R^2 is C_{16-18} acyl and A is -NH-.

The compounds may be formed according to any of the standard techniques described in the prior art for the derivatisation of polysaccharides (see for example, the references by Yoshioka et al -op cit). The technique may involve derivatisation of a polysaccharide starting material by a hydrophilic group in a first step, followed by a second step comprising attachment of a hydrophobic group or vice-versa. Alternatively, commercially-available polysaccharide derivatives already possessing a hydrophilic group may be hydrophobised using standard techniques to form a compound according to this invention.

The compounds described are used in combination with cholesterol or a derivative thereof to form particles. In the absence of cholesterol, particle formation does not occur and the material precipitates. Consequently, the presence of cholesterol is required to promote self-assembly of the polysaccharide derivatives to form particles.

The particles are made by techniques similar to those used to form liposomes and niosomes, for instance by blending the compounds in an organic solvent and then contacting the dried mixture with an aqueous solution, optionally followed by a particle size reduction step.

The particles formed may be suspended in an aqueous vehicle or alternatively may be isolated in a dry state. The particles may optionally incorporate a steric stabilizer, for instance a non-ionic amphiphilic compound, preferably a poly-24-oxyethylene cholesteryl ether. The particles may be micro or nano-particulate, nano-particles being formed preferably in the presence of the steric stabilizer. In this case, the steric stabilizer is incorporated into the structure of the particle.

The particles preferably also comprise an associated pharmaceutically active ingredient. The active ingredient may be water soluble, in which case it will be associated with the hydrophilic regions of the particle, or water insoluble and consequently associated with the hydrophobic regions of the particle.

Such an ingredient is preferably physically entrapped within the particle but may also be held by covalent conjugation. The pharmaceutically active ingredient may be a peptide or protein therapeutic compound. A further preferred alternative for the pharmaceutically active compound is nucleic acid (eg. DNA), preferably in the form of a gene for gene therapy or gene vaccination.

These drug carriers may be used for the treatment of a human or animal by therapy, in particular for oral drug delivery of peptides or proteins or as gene delivery vectors. It is envisaged that this drug delivery system will also be useful when used via the intravenous, intramuscular, intraperitional or topical (inhalation, intranasal, application to the skin) routes.

The novel compounds according to this invention may also be used for the coating of pre-formed liposomes or niosomes for instance which are drug carriers suspended in an aqueous carrier.

The invention will now be further illustrated with reference to the following non-limiting examples, wherein the fluorescent aqueous marker 5(6)-carboxy fluoroscein (CF) is used as a model drug and with reference to the

figures in which:

Figure 1 shows the stability of bleomycin GCP41 based vesicles after storage at 4°C (●, ■) and room temperature 16 - 25°C (○, □). ■, □ = % encapsulation, ●, ○ = mean size. Data points = mean ± s.d., n = 3;

Figure 2 shows the release of 5(6)-carboxyfluorescein from GCP41, cholesterol vesicles. Data points = mean of 3 determinations. Δ = palmitoyl glycol chitosan based vesicles (mean ± s.d., n = 6), ▲ = Span 60 vesicles (mean, n = 3), ♦ = 5(6)-carboxyfluorescein solution;

Figure 3 shows the biocompatibility of GCP41 based vesicles against 3 cell lines, ■ = A549, ● = A431, ▲ = A2780. Data points = mean ± s.d., n = 3; and

Detailed description of the invention

Example 1

SYNTHESIS OF N-PALMITOYL GLYCOL CHITOSAN (poly[β(1-4)-2-deoxy-2-hexadecanamide-6-0-(2-hydroxyethyl)-D-glucopyranose]

a) GCP41 (4:1 initial ratio of glycol chitosan: palmitoyl units)

200mg glycol chitosan (GC) and 150mg sodium bicarbonate was dissolved in 35mL water. 10mL absolute ethanol was added, followed by a drop-wise addition of a solution of 79mg palmitoyl N-hydroxysuccinimide ester dissolved in 60mL absolute ethanol. Addition of the palymitoyl N-hydroxysuccinimide ester was carried out with stirring over 30min. The reaction mixture was initially cloudy but turned clear after about 1h. The reaction mixture was left to stir for 72h. After this time, 100mL acetone was added with formation of a slight precipitate. This mixture was then evaporated to reduced volume under reduced pressure at 60°C. The resuting liquid was extracted with 3 volumes of

ether and exhaustively dialysed against water for 24h. The dialyzed mixture was freeze dried to give a white fluffy cotton wool like substance.

b) GCP21 (initial 2:1 ratio of glycol chitosan: palmitoyl units)

200mg glycol chitosan (GC) and 150mg sodium bicarbonate was dissolved in 35mL water. 10mL absolute ethanol was added, followed by a drop-wise addition of a solution of 150mg palmitoyl N-hydroxysuccinimide ester dissolved in 120mL absolute ethanol. Addition of the palmitoyl N-hydroxysuccinimide ester was carried out with stirring over 30min. The reaction mixture was initially cloudy but turned clear after about 6h. The reaction mixture was left to stir for 72h. After this time, 100mL acetone was added with formation of a slight precipitate. This mixture was then evaporated to reduced volume under reduced pressure at 60°C. The resulting liquid was extracted with 3 volumes of diethyl ether and exhaustively dialyzed against water for 24h. The dialyzed mixture was freeze dried to a white fluffy cotton wool like substance. This was washed with water and the sticky mass freeze dried to give a fluffy cotton wool like substance.

c) Characterisation of GCP41

^1H NMR.

Glycol chitosan is moderately soluble in water (2mg mL^{-1}) and ^1H NMR (with integration) and H ^{1}- H ^{1}COSY experiments were carried out on glycol chitosan in (D_2O, Sigma Chemical Co., UK) and GCP41 in a CD_3OD/D_2O mixture **using** a Bruker AMZ 400MHz in order to assign the non-exchangeable coupled protons.

FT-IT.

FT-IR was performed in potassium bromide discs on a Mattson Galaxy FT-IR.

The level of hydrophobic modification in GCP41 and the original level of acetylation in glycol chitosan were assessed by ^1H NMR (Vårum et al 1991, Yoshioka et al 1993). In this way the batch of glycol chitosan (Sigma Chemical Co, UK - 105H0111) that was used was found to be one third acetylated. Proton assignments, δ0.86p.p.m = CH_3 (palmitoyl) δ1.25p.p.m = CH_2 (palmitoyl), δ1.89p.p.m = CH_2 (palmitoyl - shielded by carbonyl), δ2.13p.p.m = CH_3 (acetyl - GCP41), δ2.14p.p.m. = CH (adjacent to carbonyl protons), δ1.99p.p.m = CH_3 (acetyl - glycol chitosan), δ2.71p.p.m = CH (C2 sugar proton - GCP41), δ2.64p.p.m = CH (C2 sugar proton - GCP41), δ3.31p.p.m = methanol protons, δ3.3 - 4.0p.p.m = non-exchangeable sugar protons, δ4.4p.p.m = water protons. The level of hydrophobic modification in GCP41 was assessed by using the ratio of non-exchangeable C2 protons to methyl protons (spectrum b) and was found to be 14.48 ± 2.88% (mean ± s.d., n = 3) with values lying between 11 and 16 mole %. The ratio of N-acetyl protons, C2 sugar protons, 9 additional sugar/glycol non-exchangeable protons remains at (~1:1:10) in all three spectra.

GCP41 was insoluble yet dispersible in D_2O to give a cloudy liquid which remained without a sediment for at least 4 weeks. The ^1H NMR spectra of a fresh sample of this dispersion is devoid of signals for the fatty acid side chain protons. This suggests that palmitoyl glycol chitosan in water adopts an orientation in which the fatty acid side chains exist in hydrophobic domains separated from the hydrophilic part of the polymer. The acetyl group appears to be an integral part of the hydrophilic portion of the molecule in the modified polymer as signals for the acetyl groups are clearly seen in the GCP41 - D_2O spectra. Hence there was no co-operative association between the acetyl group and the hydrophobic side chains when palmitoyl glycol chitosan was dispersed in water. Freeze fracture electron microscopy did not reveal the existence of any discernible particulate matter in this cloudy liquid.

The GCP41 FT - IR spectrum revealed a sharpening of the amide peak at 1648 cm^{-1}. The starting material glycol chitosan contains a relatively smaller amide peak at 1653 cm^{-1}.

Example 2

PREPARATION AND CHARACTERIZATION OF GCP41 AND GCP21 MICRO- AND NANO-PARTICLES

a) GCP21 - Cholesterol Particles

7.2mg cholesterol was dissolved in 10mL chloroform. To this solution was added 12.2mg GCP21. The organic solvent was removed under vacuum and the solid deposit dried under a stream of nitrogen. 2mL of aqueous CF (5mM) was added to this solid deposit and the mixture shaken for 1h at 70°C to form a homogenous dispersion of micro-particles.

0.1mL of this dispersion was then fractionated over a Sephadex G50 column (205 x 8mm) and the sample eluting in the void volume collected. This sample was sized in a Malvern Mastersizer. Assay for entrapped material was carried out by solubilizing the particles in isopropanol (0.1mL dispersion to 1mL isopropanol). CF was then assayed by fluorometry (exc.= 486nm, em.= 514nm).

b) GCP21 - Cholesterol - Solulan C24 Particles

6.2mg cholesterol and 5.4mg Solulan C24 were dissolved in 10mL chloroform. To this solution was added 11mg GCP21. The organic solvent was removed under vacuum and the solid deposit dried under a stream of nitrogen. 2mL of aqueous CF (5mM) was added to this solid deposit and the mixture shaken for 1h at 70°C to form a homogenous dispersion of micro-particles.

Nano-particles were prepared by filtration of this dispersion (0.22μm).

0.1mL of these dispersions was then fractionated over a Sephadex G50 column (205 x 8mm) and the sample eluting in the void volume collected. This sample was sized in a Malvern Mastersizer or Autosizer depending on the particle

size. Assay for entrapped material was carried out by solubilizing the particles in isopropanol (0.1mL dispersion to 1mL isopropanol). CF was then assayed by fluorometry (exc.= 486,em.+ 514nm).

c) GCP41 - Cholesterol Particles

7.3mg cholesterol was dissolved in 10mL chloroform. To this solution was added 19.8mg GCP41. The organic solvent was removed under vacuum and the sold deposit dried under a stream of nitrogen. 2mL of aqueous CF (5mM) was added to this solid deposit and the mixture shaken for 1h at 70°C to form a homogenous dispersion of micro-particles.

0.1mL of this dispersion was then fractionated over a Sephadex G50 column (205 x 8mm) and the sample eluting in the void volume collected. This sample was sized in a Malvern Mastersizer. Assay for entrapped material was carried out by solubilizing the particles in isopropanol (0.1mL dispersion to 1mL isopropanol). CF was then assayed by fluorometry (exc.= 486nm, em.= 514nm).

d) GCP41 - Cholesterol - Solulan C24 Particles

6.5mg cholesterol and 5.4mg Solulan C24 were dissolved in 10mL chloroform. To this solution was added 17.3mg GCP41. The organic solvent was removed under vacuum and the solid deposit dried under a stream of nitrogen. 2mL of aqueous CF (5mM) was added to this solid deposit and the mixture shaken for 1h at 70°C to form a homogenous dispersion of micro-particles.

Nano-particles were formed by filtration of this dispersion (0.22μm).

0.1mL of this dispersion was then fractionated over a Sephadex G50 column (205 x 8mm) and the sample eluting in the void volume collected. This sample was sized in a Malvern Mastersizer or Autosizer. Assay for entrapped material was carried out by solubilizing the particles in isopropanol (0.1mL dispersion to 1mL isopropanol). CF was then assayed by fluorometry (exc.= 486nm, em.= 514nm).

The sizes and encapsulation efficiencies are given in Table 1.

Example 3

PREPARATION OF BLEOMYCIN ENTRAPPED VESICLES

GCP41 vesicles were prepared by the sonication of GCP41 (8mg) and cholesterol (4mg, Sigma Chemical Co., UK) in water for 2 X 2 minutes with the instrument set at 20% of its maximum capacity. Bleomycin GCP41 vesicles were prepared by sonicating GCP41(8mg) and cholesterol (4mg) in 2mL ammonium sulphate (0.12M, Sigma Chemical Co., UK). Unentrapped ammonium sulphate was removed by ultracentrifugation (150,000g X 1h - MSE 75 superspeed). Vesicles were then incubated for 1h at 60°C with bleomycin (Lundbeck, UK) solution (2mL 6U mL^{-1}) and left to stand overnight at room temperature. Unentrapped bleomycin was also removed by ultracentrifugation (150,000g X 1h) and entrapment was measured by disrupting the vesicles in 10X volume isopropanol (Rathburn Chemical Co., UK) followed by ultraviolet absorption spectrophotometry at 254nm (Unicam UV-1).

On storage at room temperature there was an initial loss of bleomycin although over 60% of the drug is retained within the vesicles (see Figure 1). Particle size is also seen to change very little. The stability data suggests that there is a loosely bound and a tightly bound fraction of bleomycin associating with GCP41 vesicles. The tightly bound fraction is presumed to be that fraction of bleomycin that traverses the membrane of the polymeric vesicle and actually accumulates within it in response to the ammonium sulphate gradient.

Example 4

5(6)-CARBOXYFLUORESCEIN RELEASE FROM VESICLES

Vesicles were prepared as described in Example 3 from GCP41(16mg) and cholesterol (8mg) except that the hydrating solution was 4mL 5(6)-carboxyfluorescein (5.03mM, Sigma Chemical Co., UK). Sorbitan monostearate vesicles were prepared by hydrating sorbitan monostearate (24mg, Sigma Chemical Co., UK), cholesterol and poly-24-oxyethylene cholesteryl ether (16mg, D.F. Anstead, UK) in the presence of 4mL 5(6)-carboxyfluorescein (5.03mM). Unentrapped material was again removed by ultracentrifugation as described in Example 3. The release of 5(6)-carboxyfluorescein from GCP41 and sorbitan monostearate vesicles was monitored as follows. A 1:2 mixture of the vesicles and 2% w/w bile salts (sodium cholate and sodium deoxycholate, Sigma Chemical Co., UK) was placed in a 5cm piece of Visking tubing (Mw cut off 12,000 - 14,000) sealed at both ends. This mixture was dialysed against a 13-fold volume of the bile salts solution. 5(6)-carboxyfluorescein external to the dialysis tubing was monitored fluorimetrically (exc. 486, em. = 514nm, Perkin Elmer LS-5) at regular time intervals. A 1:2 mixture of 5(6)-carboxyfluorescein in phosphate buffered saline (PBS, pH = 7.4) (0.5mL) and 2%w/w bile salts (1mL) was included as a control.

Using the release of the small molecular weight compound 5(6)-carboxyfluorescein (Mw = 387) as a marker for vesicle integrity, these polymeric vesicles are found to be more resistant to attack by detergents than vesicles prepared from the non-ionic surfactant sorbitan monostearate (see Figure 2). This is believed to be due to the difficulty the soluble bile salt surfactants have in inserting into a polymeric bilayer as opposed to the ease of insertion into a bilayer resulting from the self-assembly of monomers.

Example 5

BIOCOMPATIBILTY AND HAEMOCOMPATIBILITY STUDIES

Biocompatibility studies

Cytotoxicity was evaluated by the IC50 value in a standard MTT based assay (Freshney et al Clulture of Animal Cells, 3rd edition, Wiley-Liss, New York, 1994). Depending on the growth rate, 0.5-2.0 X 10^3 cells per well were seeded into 96 well plates and incubated for 24 h. Serial dilutions of the suspensions were added and incubated with the cells for 12 h. The suspensions were replaced with fresh medium and the cells were incubated with repeated feeding for 72 h. 50mg mL^{-1} MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 50μL, Sigma Chemical Co., UK) was added to each well. After incubation for 4 h in the dark, the medium and MTT solution was removed and the cells were lysed in DMSO (200μL, Sigma Chemical Co., UK). Following the addition Sorensen's glycine buffer (25μL) the absorption was measured at 570nm.

Haemocompatibility studies

Freshly drawn human blood was centrifuged (3,000g) to separate the red blood cells. These were washed in PBS (pH = 7.4) and weighed. 3g of the erythrocyte pellet was dispersed in 100mL PBS (pH = 7.4) and incubated for 5h with various concentrations of GCP41, cholesterol vesicles prepared as described above or DOTAP vesicles (Sigma Chemical Co., UK). Haemolysis was assessed by centrifugation (3,000g) to isolate the released haemoglobin, addition of 2X volume of isopropanol to the supernatant and the measurement of the absorbance (570nm).

GCP41 vesicles were biocompatible with 3 human cell lines A2780 (ovarian cancer cell line), A549 (lung carcinoma) and A431 (epidermoid carcinoma) with no toxicity evident at concentrations of GCP41 below 150μg mL^{-1} and IC50 values of 0.2, 1.0 and 1.0mg mL^{-1} respectively (Figure 3). GPC41 vesicles showed good haemocompatibility with human erythrocytes and an ability to modulate the haemolytic

activity of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP) - the DNA transfection agent (Porteous et al, (1997) Gene Therapy 4, 210-218) (Table 2). These biocompatibility data are in good agreement with that reported for soluble glycol chitosan against the B16F10 cell line and rat erythrocytes (Carreno-Gomez and Duncan (1997) Int. J. Pharm. 148, 231-240.

Example 6

PREPARATION OF INSULIN AND LHRH ENTRAPPED VESICLES

GCP21 was prepared according to Example 1

a) Insulin. GCP21 vesicles were prepared by sonication of a mixture of GCP21 (8mg) and cholesterol (4mg) in 2mL water. GCP21 vesicles were loaded with insulin by either incubating 1ml of the vesicle dispersion with 1ml of insulin (160IU mL^{-1}) for 16h at room temperature or by the use of the dehydration-rehydration (DRV) method (Kirby C., Gregoriadis G (1984) Biotechnology 979-984) in which insulin vesicles mixtures, as described above, were lyophilised overnight and subsequently rehydrated to 2mL volume. The amount of insulin encapsulated was assessed by HPLC after separation of encapsulated insulin from the unencapsulated material by ultracentrifugation (150,000g) and disruption of the vesicles with isopropanol (1ml isopropanol to 1ml of the vesicle dispersion). Vesicles were also sized by photon correlation spectroscopy and the zeta potential of the dispersion measured.

b) Lutenizing hormone releasing hormone (LHRH) LHRH was loaded onto GCP21 vesicles by the use of ammonium sulphate gradients (Haran, G et al (1993) Biochym. Biophys. Acta 1151 , 201-205). Vesicles encapsulating ammonium sulphate were prepared by sonicating GP21 (8mg), cholesterol (4mg) mixtures in 2mL of a solution of ammonium sulphate (0.03M). Unentrapped ammonium sulphate was separated by ultracentrifugation (150,000g) and the pelleted ammonium sulphate vesicles were incubated with 2mL

LHRH (2.5mg mL^{-1}). Unentrapped LHRH was also removed by ultracentrifugation (150,000g for one hour). These vesicles were also sized by photon correlation spectroscopy.

a) Insulin. Insulin GCP21 vesicles could be prepared by incubating pre-formed GCP21 vesicles with insulin at room temperature for approximately 16h (Table 3). No improvement in the level of insulin associated with the vesicles was observed with the DRV method. The zeta potential of the GCP21 vesicles increased from -5mV to +10mV on loading with insulin, indicating that the insulin associates with the surface of the vesicles to a certain extent.

b) LHRH. LHRH vesicles could also be prepared by the use of ammonium sulphate gradients (Table 4).

PARTICLE	SIZE	% CF ENCAPSULATION
GCP21/Cholesterol micro-particles	34.6μm	7.4%
GCP21/Cholesterol/Solulan C24 micro-particles	30.7μm	4.6%
GCP41/Cholesterol micro-particles	nd	9.3%
GCP41/Cholesterol/Solulan C24 micro-particles	nd	4.2%
GCP21/Cholesterol/Solulan C24 nano-particles	325nm	6.88%
GCP41/Cholesterol/Solulan C24 nano-particles	333nm	4.6%

Table 1: Size and CF encapsulation efficient of GCP21 and GCP41 particles.

Table 2: The haemocompatibility of GCP41 vesicles

Formulation	Erythrocyte, polymer/DOTAP ratio	% Haemolysis (n=3)*
DOTAP	30 (erythrocyte, DOTAP ratio)	101.4 ± 20.4
DOTAP	300 (erythrocyte, DOTAP ratio)	71.0 ± 10.6
GCP41, cholesterol (8:4)	30 (eythrocyte, GCP41 ratio)	4.2 ± 1.6
GCP41, cholesterol, DOTAP (6:2:1)	60 (erythrocyte, DOTAP ratio)	10.7 ± 1.3
GCP41, cholesterol, DOTAP (6:2:1)	600 (erythrocyte, DOTAP ratio)	6.6 ± 1.8

*Haemocompatibility is expressed with respect to 100% haemolysis produced by an erythrocyte, triton X-100 weight ratio of 3:1 and 0% haemolysis produced by PBS (pH = 7.4).

Table 3: The size and encapsulation efficiency of insulin GCP21 vesicles

Method of Preparation	% Encapsulation (mean ± s.d.)	Mean Insulin, GP21 ratio (IU mg^{-1})	Size (nm, (mean ± s.d.)	Zeta Potential (mV, mean ± s.d.)
Empty GCP21 vesicles	-	-	559 ± 78	-
Incubation at room temperature	16.21 ± 0.70	3.24	-	-
DRV method	15.12 ± 0.62	3.02	945 ± 20	10.13 ± 4.82

Table 4: The encapsulation of LHRH in GCP21 vesicles

% Encapsulation	LHRH, GCP21 ratio (mg mg^{-1})
57.5	0.54

CLAIMS

1. A compound which is a polysaccharide derivative bearing at least one non-ionic hydrophilic group and at least one hydrophobic group per molecule wherein said hydrophilic group is attached to the individual monosaccharide units and said hydrophobic group contains a C_{12-24} alkyl, alkenyl, alkynyl or acyl residue.

2. A compound according to claim 1 wherein the non-ionic hydrophilic group is a group R^1, where R^1 is selected from mono- and oligo-hydroxy C_{1-6} alkyl, mono- and oligo-hydroxy substituted C_{2-6} acyl, C_{1-2} alkoxy alkyl optionally having one or more hydroxy groups substituted on the alkoxy or alkylene groups, oligo- or poly- (oxa C_{1-3} alkylene) such as polyoxyethylene comprising up to about 120 ethylene oxide units and C_{1-4} alkyl (oligo- or poly-oxa C_{1-3} alkylene) optionally hydroxy substituted such as oligo- or polyglycerol ethers; and wherein R^1 is joined via and ether linkage to a saccharide unit of the polysaccharide.

3. A compound according to claim 1 or claim 2 wherein the polysaccharide has 1,4-linked saccharide units.

4. A compound according to claim 3 wherein each non-ionic hydrophilic group is substituted at the C6 position of a saccharide unit.

5. A compound according to claim 3 or claim 4 in which the hydrophobic group is substituted at the C2 position.

6. A compound according to any preceding claim wherein the degree of substitution by non-ionic hydrophilic groups is 0.1-1.5, preferably at least 0.9 per saccharide unit.

7. A compound according to any preceding claim wherein the ratio of hydrophilic: hydrophobic groups is in the range 100:1 to 1:2, preferably between 10:1 and 2:1, more preferably between 5:1 and 2:1.

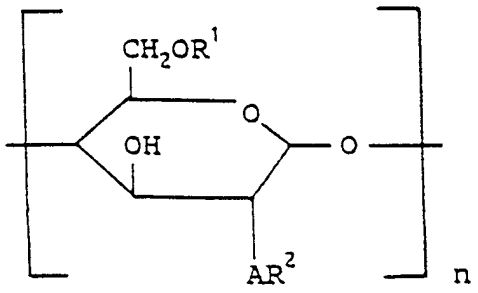
8. A compound according to any preceding claim wherein the hydrophobic group is joined to a saccharide unit by an amide, ester, ether or amine linkage.

9. A compound according to any preceding claim wherein the polysaccharide is a derivative of chitosan, pullulan or dextran.

10. A compound according to any preceding claim which is an N-substituted derivative of a poly-amino glycan.

11. A compound according to claim 10 which is an N-acyl glycol chitosan, preferably N-palmitoyl glycol chitosan.

12. A compound having the formula:



wherein each R^1 is selected from hydrogen, mono-and oligo-hydroxy C_{1-6} alkyl, mono- and oligo-hydroxy substituted C_{2-6} acyl, C_{1-2} alkoxy alkyl optionally having one or more hydroxy groups substituted on the alkoxy or alkylene groups, oligo- or poly-(oxa C_{1-3} alkylene) such as polyoxyethylene comprising up to about 120 ethylene oxide units and C_{1-4} alkyl (oligo- or poly-oxa C_{1-3} alkylene) optionally hydroxy substituted such as polyglycerol ethers, for example containing up to 10 glycerol units, provided that at least one of the groups R^1 is other than hydrogen;

A is -NH- or -O-;

R^2 is selected from hydrogen, C_{12-24} alkyl, -alkanoyl, -alkenyl -alkenoyl, -alkynyl or alkynoyl, provided that at least one of the groups R^2 is other than hydrogen; and
n is 5-2000.

13. A compound according to claim 12 wherein R^1 is $-CH_2CH_2OH$ or $-CH_2CH(OH)CH_2OH$.

14. A compound according to claim 12 or claim 13 wherein R^2 is C_{16-18} acyl.

15. A compound according to any of claims 12 to 14 wherein A is -NH-.

16. A composition comprising particles formed from a compound according to any preceding claim.

17. A composition according to claim 16 which comprises an aqueous vehicle in which the particles are suspended.

18. A composition according to claim 16 or claim 17 which additionally comprises cholesterol or a derivative thereof.

19. A composition according to claim 18 which further comprises a steric stabilizer, preferably a non-ionic amphiphilic compound, most preferably a poly-24-oxyethylene cholesteryl ether.

20. A pharmaceutical composition comprising particles of compound according to any of claims 1 to 15 and a pharmacologically acceptable carrier.

21. A composition according to any of claims 16 to 20 which comprises a pharmaceutically active ingredient associated with the particles.

22. A composition according to claim 21 which comprises an entrapped pharmaceutically active ingredient.

23. A composition according to any of claim 21 which comprises a covalently conjugated pharmaceutically active ingredient.

24. A composition according to any of claims 21 to 23 in which the pharmaceutically active ingredient is a peptide or protein therapeutic compound or DNA, preferably a gene for gene therapy or gene vaccination.

25. A compound according to any of claims 1 to 15 for use in a pharmaceutical composition.

26. Use of a compound according to any of claims 1 to 15 in the manufacture of a composition for use in a method of treatment of a human or animal by therapy.

27. Use of a particle comprising a compound according to any of claims 1 to 15 and a pharmaceutically active ingredient in a method of manufacturing a composition for use in a method of treatment of a human or animal.

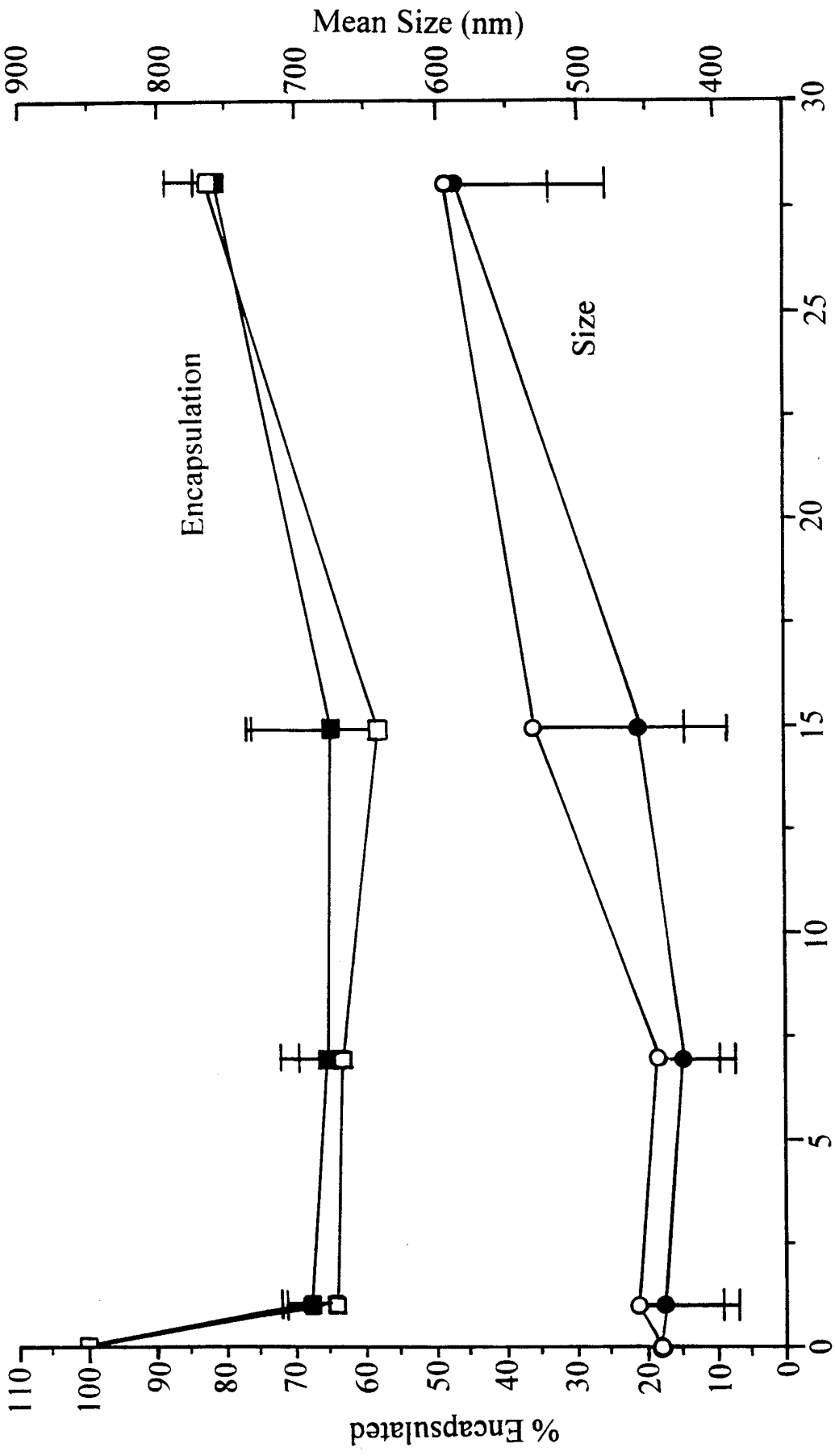

Mean Size (nm)
900
800
700
600
500
400
Encapsulation
Size
% Encapsulated
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (days)

FIG. 1

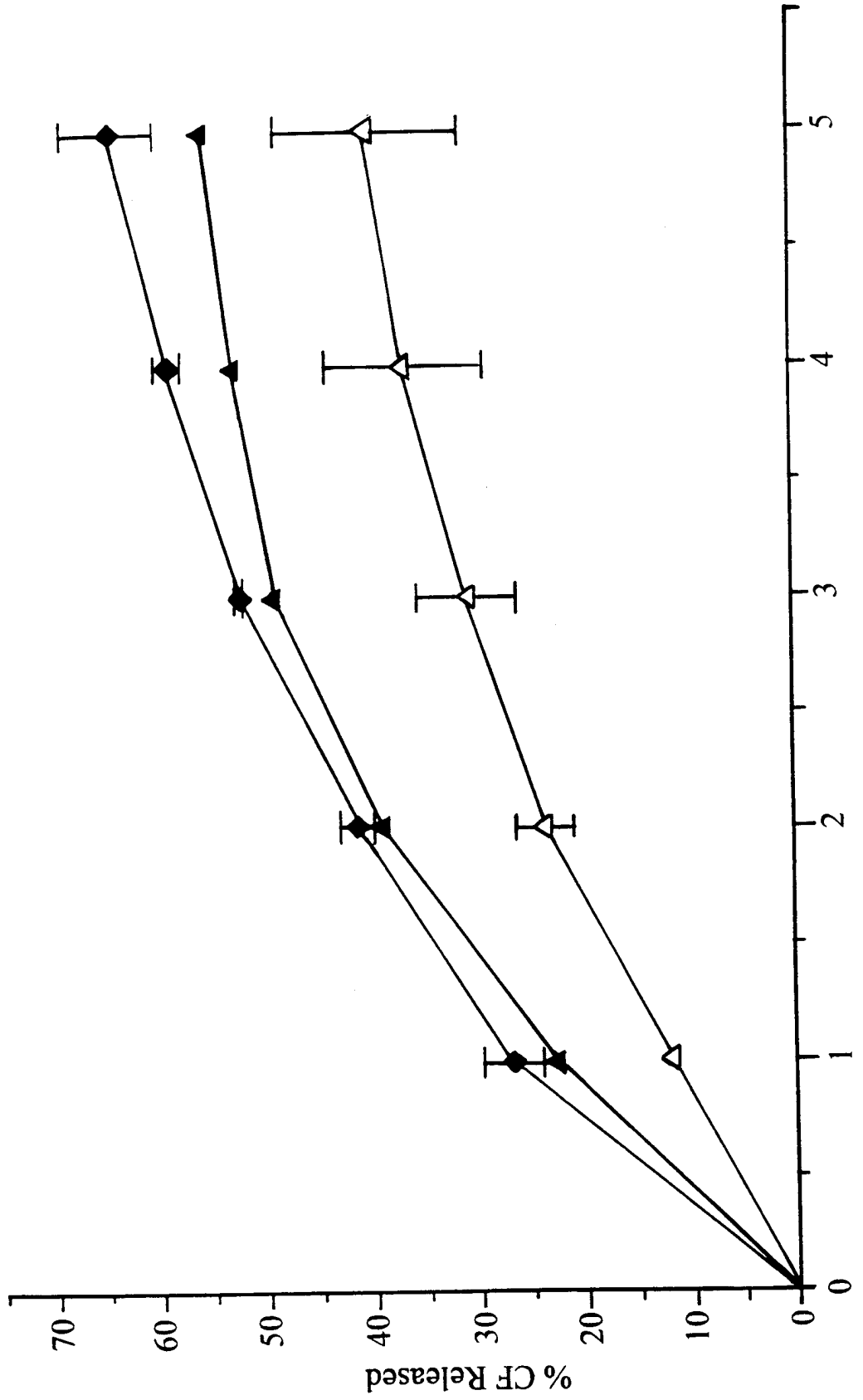


FIG. 2

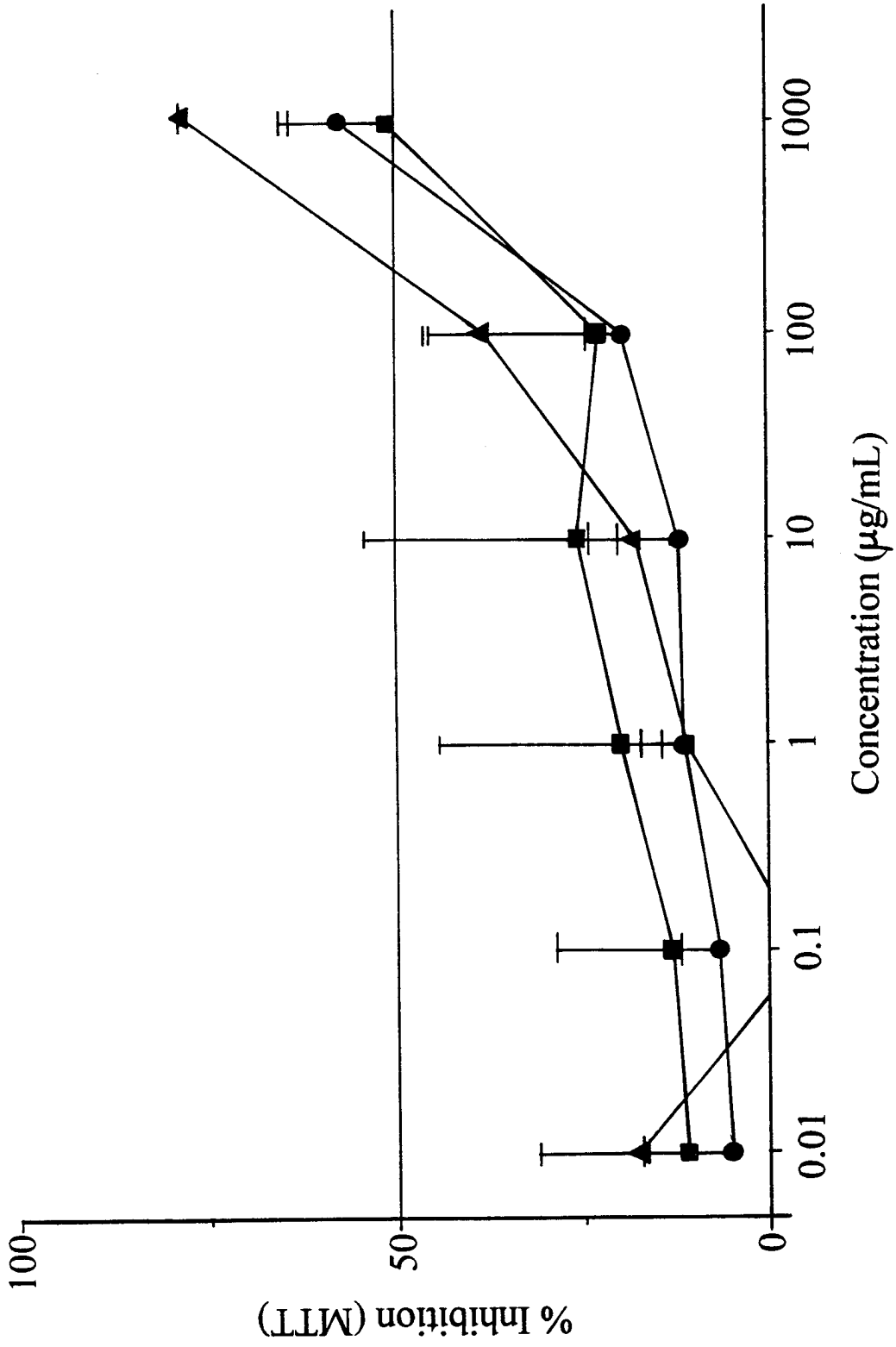

% Inhibition (MTT)
100
50
0
0.01
0.1
1
10
100
1000
Concentration (µg/mL)

FIG. 3

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/00903

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C08B37/08 A61K9/127

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)
IPC 6 C08B 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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 18, no. 601 (C-1274), 16 November 1994 & JP 06 227965 A (SHIN ETSU CHEM CO LTD), 16 August 1994, & DATABASE WPI Week 9437 Derwent Publications Ltd., London, GB; AN 299679 see abstract	1-8, 12-27
P,X	EP 0 773 229 A (L'OREAL) 14 May 1997 see page 3, line 46 - page 4, line 14	1-8, 12-27
X	EP 0 323 627 A (FRATELLI LAMBERTI) 12 July 1989 see page 3, line 35-49	1-8, 12-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 July 1998	16/07/1998

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lensen, H

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/00903

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 904 772 A (SAU ARJUN C.) 27 February 1990 see examples 1-3	1-8
A	PATENT ABSTRACTS OF JAPAN vol. 11, no. 259 (C-441), 21 August 1987 & JP 62 059215 A (IHARA CHEM IND CO), 14 March 1987, see abstract	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/GB 98/00903

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