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Polymeric Chitosan-based Vesicles for Drug Delivery

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Abstract

A simple carbohydrate polymer glycol chitosan (degree of polymerization 800 approx.) has been investigated for its ability to form polymeric vesicle drug carriers. The attachment of hydrophobic groups to glycol chitosan should yield an amphiphilic polymer capable of self-assembly into vesicles. Chitosan is used because the membrane-penetration enhancement of chitosan polymers offers the possibility of fabricating a drug delivery system suitable for the oral and intranasal administration of gut-labile molecules.

Glycol chitosan modified by attachment of a strategic number of fatty acid pendant groups (11–16 mol%) assembles into unilamellar polymeric vesicles in the presence of cholesterol. These polymeric vesicles are found to be biocompatible and haemocompatible and capable of entrapping water-soluble drugs. By use of an ammonium sulphate gradient bleomycin (MW 1400), for example, can be efficiently loaded on to these polymeric vesicles to yield a bleomycin-to-polymer ratio of 0·5 units mg^{-1}.

Previously polymers were thought to assemble into vesicles only if the polymer backbone was separated from the membrane-forming amphiphile by a hydrophilic side-arm spacer. The hydrophilic spacer was thought to be necessary to decouple the random motion of the polymer backbone from the ordered amphiphiles that make up the vesicle membrane. However, stable polymeric vesicles for use in drug delivery have been prepared from a modified carbohydrate polymer, palmitoyl glycol chitosan, without this specific architecture. These polymeric vesicles efficiently entrap water-soluble drugs.

It had been assumed that polymeric vesicles could be produced only from polymers of a specific molecular shape. A normal requirement was thought to be a hydrophilic side-chain spacer, for example oxyethylene groups, between the polymer backbone and the membrane-forming amphiphilic pendant group (Kunitake et al 1981; Elbert et al 1985; Ringsdorf et al 1988; Furhop & Köning 1994), as illustrated in Figure 1, to enable decoupling of the motion of the polymer backbone from the amphiphile's tendency to order into a bilayer membrane (Elbert et al 1985; Ringsdorf et al 1988; Furhop & Köning 1994). Alternatively vesicles might be polymerized after being formed from polymer-forming amphiphilic monomers (Bader et al 1981; Fendler & Tundo 1984; Sackmann et al 1985; Samuel et al 1985). Vesicles can, however, also be fabricated from cholesterol and a simple amphiphilic polymer (Figure 1) in a manner similar to the small molecular-weight single-chain amphiphiles that are known to form vesicles (Uchegbu & Florence 1995). Chitosan-based vesicles will be particularly useful for the oral and intranasal delivery of peptides because this macromolecule is said to enhance the epithelial permeation of peptides (Artusson et al 1994; Aspden et al 1996). Here we present preliminary results on the design and characterization of chitosan-based vesicles as drug carriers. These results pave the way for use of a number of carbohydrate polymers such as starch, dextran and some oligosaccharides to form the basis of drug-carrier vesicles.

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Materials and Methods

Synthesis of palmitoyl glycol chitosan

Glycol chitosan (Sigma UK; 500 mg), sodium bicarbonate (Fluka, UK; 376 mg) in a mixture of absolute ethanol (Rathburn Chemicals, UK; 24 mL) and water (76 mL) was reacted with palmitic acid *N*-hydroxysuccinimide (Sigma; 198 mg) in absolute

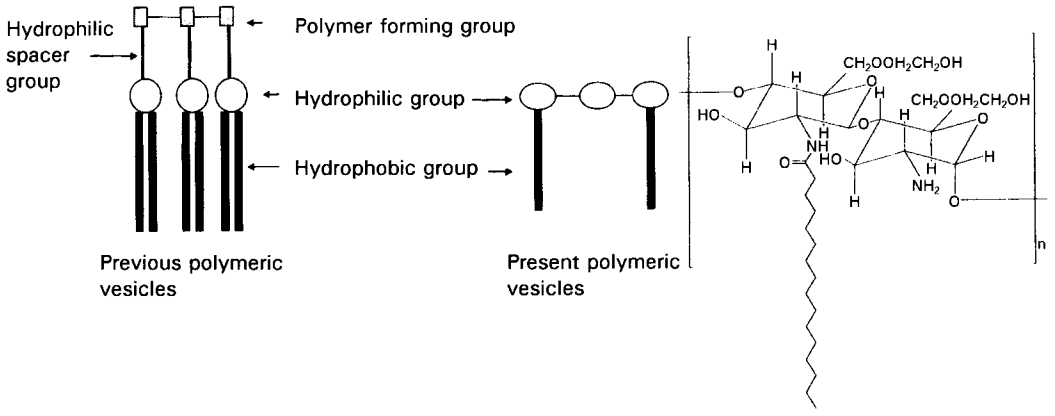

Figure 1. Schematic representation of the monomeric unit previously considered to be ideal for a vesicle-forming polymer (from data presented by Kunitake et al (1981), Elbert et al (1985), Ringsdorf et al (1988) and Furhop Köning (1994)) and a schematic representation (and the chemical structure) of the amphiphilic polymer described in this report which forms vesicles in the presence of cholesterol.

ethanol (150 mL). Palmitic acid *N*-hydroxysuccinimide solution was added dropwise. The product was isolated after stirring for 72 h by evaporating most of the ethanol, extraction of the remaining liquid with diethyl ether (3 × 100 mL) and exhaustive dialysis against water.

^1H NMR

Glycol chitosan is moderately soluble in water (2 mg mL^{-1}) and ^1H NMR (with integration) and ^1H correlation spectroscopy experiments (Bruker AMX 400 MHz spectrometer) were performed on a solution of glycol chitosan in D_2O, palmitoyl glycol chitosan in D_2O, and D_2O/CD_3OD mixture (Sigma, UK) to assign non-exchangeable coupled protons.

Fourier transform infrared spectroscopy (FTIR)

FTIR was performed with a Mattson Galaxy FTIR instrument on potassium bromide discs.

Preparation of vesicles

Palmitoyl glycol chitosan vesicles were prepared by sonication of palmitoyl glycol chitosan (8 mg) and cholesterol (Sigma; 4 mg) in water for 2 × 2 min with the instrument set at 20% of maximum power. Bleomycin palmitoyl glycol chitosan vesicles were prepared by sonicating palmitoyl glycol chitosan (8 mg) and cholesterol (4 mg) in ammonium sulphate (Sigma; 0·12 M, 2 mL). Unentrapped ammonium sulphate was removed by ultracentrifugation (150 000 *g* for 1 h (MSE 75 superspeed instrument). Vesicles were then incubated for 1 h at 60°C with bleomycin (Lundbeck, UK) solution (6 units mL^{-1}, 2 mL) and left to stand overnight at room temperature. Unentrapped bleomycin was also removed by ultracentrifugation (150 000 *g* for 1 h) and entrapment was measured by disrupting the vesicles with a tenfold volume of isopropanol (Rathburn) then ultraviolet absorption spectrophotometry at 254 nm (Unicam UV-1).

Release of 5(6)-carboxyfluorescein from vesicles

Vesicles were prepared from palmitoyl glycol chitosan (16 mg) and cholesterol (8 mg) as described above except that the hydrating solution was 5(6)-carboxyfluorescein (Sigma; 5·03 mM, 4 mL). Sorbitan monostearate vesicles were prepared by hydrating sorbitan monostearate (Sigma; 24 mg), cholesterol and poly-24-oxyethylene cholesteryl ether (D. F. Anstead, UK; 16 mg) in the presence of 4 mL 5(6)-carboxyfluorescein (5·03 mM). Unentrapped material was again removed by ultracentrifugation as described above. To monitor the release of 5(6)-carboxyfluorescein from palmitoyl glycol chitosan and sorbitan monostearate vesicles a 1 : 2 mixture of the vesicles and 2% w/w bile salts (sodium cholate and sodium deoxycholate; Sigma) was placed in 5 cm Visking tubing (MW cut-off 12 000–14 000) sealed at both ends. This mixture was dialysed against a 13-fold volume of a solution of the bile salts. 5(6)-Carboxyfluorescein external to the dialysis tubing was monitored fluorimetrically (λ_{exc} 486, λ_{em} = 514 nm; Perkin-Elmer LS-5) at regular intervals. A 1 : 2 mixture of 5(6)-carboxyfluorescein in phosphate buffered saline (PBS, pH 7·4; 0·5 mL) and 2% w/w bile salts (1 mL) was included as a control.

Release of bleomycin from vesicles

Vesicles were prepared as described above and stored at different temperatures. Released bleomycin was determined by ultracentrifugation then analysis of the supernatant and entrapped bleomycin was determined by analysis of the vesicle pellet as described above.

Biocompatibility studies

Cytotoxicity was evaluated by measurement of the IC50 value in a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide; Sigma)-based assay (Freshney et al 1994). Depending on the growth rate, 0·5–2·0 × 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. MTT (50 mg mL^{-1}, 50 μL) was added to each well. After incubation for 4 h in the dark, the medium and MTT solution were removed and the cells were lysed in dimethylsulphoxide (Sigma; 200 μL). After addition of Sorensen's glycine buffer (25 μL) the absorption was measured at 570 nm.

Haemocompatibility studies

Freshly drawn blood from man was centrifuged (3000 *g*) to separate the red blood cells. These were washed with PBS (pH 7·4) and weighed. The erythrocyte pellet (3 g) was dispersed in PBS (pH 7·4; 100 mL) and incubated for 5 h with different concentrations of palmitoyl glycol chitosan, cholesterol vesicles prepared as described above, or DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate; Sigma) vesicles. Haemolysis was assessed by centrifugation (3000 *g*) to isolate the released haemoglobin, addition of a twofold volume of isopropanol to the supernatant and measurement of the absorbance at 570 nm.

Results and Discussion

Synthesis of palmitoyl glycol chitosan

Palmitoyl glycol chitosan was prepared by the reaction of glycol chitosan with palmitic acid *N*-hydroxysuccinimide in a 4 : 1 molar ratio. The amount of hydrophobic modification in palmitoyl glycol chitosan and the original amount of acetylation in glycol chitosan were assessed by ^{1}H NMR (Vårum et al 1991; Yoshioka et al 1993). The batch of glycol chitosan used was found to be one third acetylated. Proton assignments: δ 0·86 ppm = CH_3 (palmitoyl), δ 1·25 ppm = CH_2 (palmitoyl), δ 1·89 ppm = CH_2 (palmitoyl shielded by carbonyl), δ 2·13 ppm = CH_3 (acetyl, palmitoyl glycol chitosan), δ 2·14 ppm = CH_2 (adjacent to carbonyl protons), δ 1·99 ppm = CH_3 (acetyl, glycol chitosan), δ 2·71 ppm = CH (C2 sugar proton, palmitoyl glycol chitosan), δ 2·64 ppm = CH (C2 sugar proton, glycol chitosan), δ 3·31 ppm = methanol protons, δ 3·3–4·0 ppm = non-exchangeable sugar protons, δ 4·4 ppm = water protons. The extent of hydrophobic modification in palmitoyl glycol chitosan was assessed by using the ratio of non-exchangeable C2 protons to methyl protons and was found to be 14·48 ± 2·88% (mean ± s.d., n = 3) with values lying between 11 and 16 mol%. The ratio *N*-acetyl protons/C2 sugar protons/nine additional sugar or glycol non-exchangeable protons was approximately 1 : 1 : 10.

Palmitoyl glycol chitosan was insoluble yet dispersible in D_2O to give a cloudy liquid which remained without sediment for at least four weeks. The ^{1}H NMR spectrum of a fresh sample of this dispersion was devoid of signals from fatty acid side-chain protons, suggesting 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 seems to be an integral part of the hydrophilic portion of the molecule in the modified polymer, because signals for the acetyl groups are clearly seen in the palmitoyl glycol chitosan-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.

FTIR

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

Palmitoyl glycol chitosan vesicles, preparation and drug encapsulation

Unlike previously described vesicle-forming polymers in which a hydrophilic side-arm spacer such as polyoxyethylene glycol (Kunitake et al 1981; Elbert et al 1985; Furhop & Köning 1994) was used to separate the amphiphilic pendant group from the polymer backbone (Figure 1), the hydrophilic portion of the current amphiphilic polymer is an integral portion of the polymer backbone (Figure 1). Despite this, stable vesicles are formed (Figure 2). The incomplete acylation of glycol chitosan enables the polymer to adopt the conformation shown in Figure 3. The inclusion of a hydrophilic co-monomer (main-chain spacer) in a polymer which bore double-chained amphiphilic pendant groups led to an increase in the fluidity of these alkyl chains in monolayers (Ringsdorf et al 1988). However, we were unable to find any reference to vesicles produced by these monolayer-forming systems.

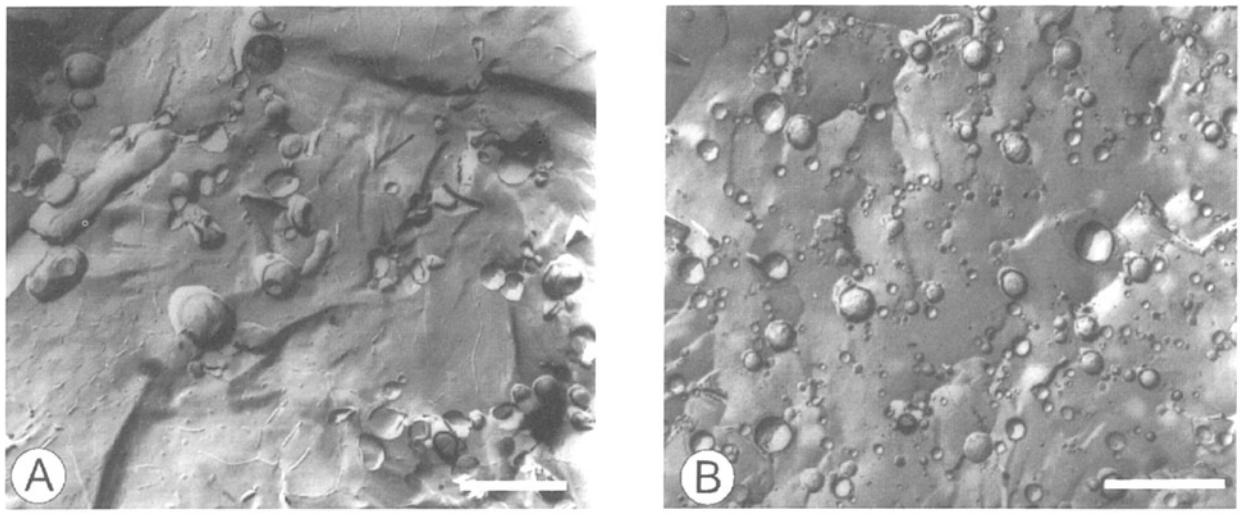

Figure 2. Freeze-fracture electron micrograph of A. palmitoyl glycol chitosan-based bleomycin vesicles, frozen in native conditions and, B. palmitoyl glycol chitosan-based vesicles after storage for one month (bar = 2·5 μm).

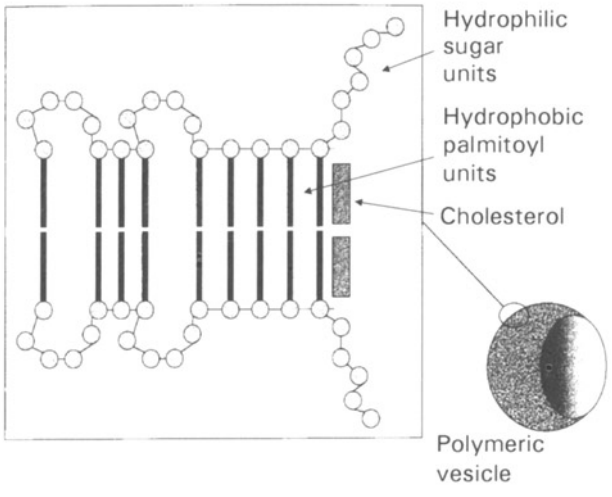

Figure 3. Proposed conformation of palmitoyl glycol chitosan in the vesicle membrane with the hydrophobic units present as evenly spaced, random or blockwise units.

Unilamellar vesicles, obtained on sonication of palmitoyl glycol chitosan in the presence of cholesterol in aqueous media, are 300–600 nm in size (Figure 2). These vesicles are large and yet unilamellar, because of the rigidity of the polymer backbone which resists too great a curvature. Glycol chitosan, which has been hydrophobically (palmitoyl) modified by as much as 29% also forms vesicles in the presence of cholesterol. In addition, sulphated *N*-myristoyl chitosan forms micelles when acylated to between 80 and 90 mol% (Yoshioka et al 1995). This micellar system does not require a hydrophilic spacer between the hydrophobic side chains and the hydrophilic head group, the hydrophilic head group being an integral part of the polymer backbone.

Storage of palmitoyl glycol chitosan-based vesicles for five weeks at room temperature in polystyrene containers unprotected from light does not affect their morphology (Figure 2B). Small molecular-weight compounds such as 5(6)-carboxyfluorescein (MW 387; Figure 4A) and larger molecules such as bleomycin (MW approx. 1400; Figure 4) have been encapsulated in palmitoyl glycol chitosan vesicles. Bleomycin can be loaded by the method of ammonium sulphate gradients (Haran et al 1993) to yield a bleomycin-to-polymer ratio of 0·5 units mg^{-1}. Ammonium sulphate entrapped in vesicles causes the amine drug doxorubicin (MW 580) to traverse vesicle membranes and accumulate within vesicles as a gel (Lasic et al 1995). Bleomycin has several amine groups and also accumulates within palmitoyl glycol chitosan-based vesicles in response to an ammonium sulphate gradient. On storage there was an initial loss of material although over 60% of the drug is retained within the vesicles (Figure 4B). Particle size is also seen to change very little. The stability data suggest that there are loosely bound and tightly bound fractions of bleomycin associating with palmitoyl glycol chitosan 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. No previous accounts have investigated the passage of large molecular-weight compounds across polymeric vesicle membranes although polymerized vesicles (produced by polymerization of vesicles from polymerizable monomers) are actually rather impermeable to the passage of entrapped small molecular-weight solutes (Bader et al 1981; Sackmann et al 1985; Samuel et al 1985; Fendler & Tundo 1984). The concept of a water-soluble macromolecule traversing a polymeric vesicle in

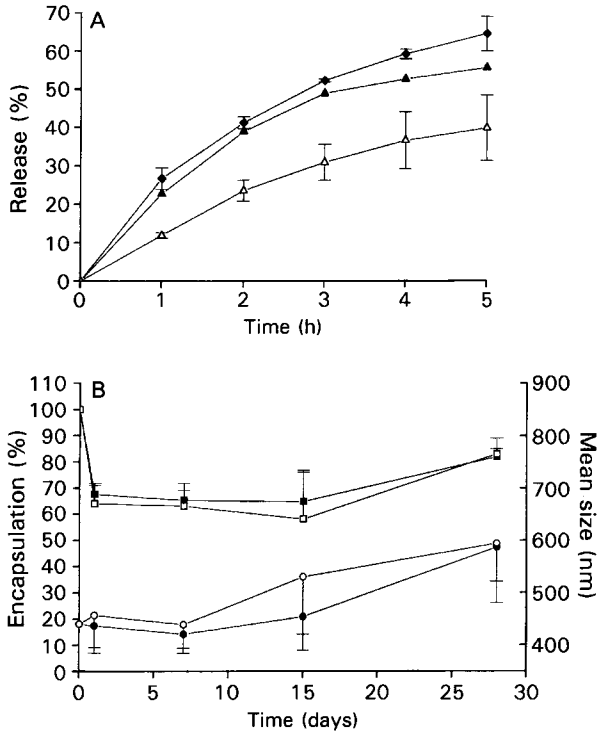

Figure 4. A. The release of 5(6)-carboxyfluorescein from palmitoyl glycol chitosan-cholesterol vesicles. Data points are means of results from three determinations. △ Palmitoyl glycol chitosan-based vesicles (mean ± s.d., n = 6); ▲ Span 60 vesicles (mean, n = 3); ◆ 5(6)-carboxyfluorescein solution. B. The stability of bleomycin palmitoyl glycol chitosan-based vesicles after storage at 4°C (●, ■) and at room temperature (16–25°C) (○, □). % Encapsulation (■, □); mean size (●, ○). Data points are means ± s.d., n = 3.

response to an ionic gradient is a difficult one, unless one assumes that the membrane is fluid because of the incorporation of cholesterol.

By using the release of the small molecular-weight compound 5(6)-carboxyfluorescein (MW 387) as a marker of vesicle integrity, these polymeric vesicles were found to be more resistant to attack by detergents than vesicles prepared from the non-ionic surfactant sorbitan monostearate (Figure 4A). It is believed that this is because of the difficulty soluble bile salt surfactants have in becoming inserted into a polymeric bilayer as opposed to the ease of insertion into a bilayer resulting from the self-assembly of monomers. The solubilization of vesicle drug carriers by detergents with the resultant production of mixed micelles has been described elsewhere (Uchegbu et al 1992, 1996).

Biocompatibility and haemocompatibility

Palmitoyl glycol chitosan vesicles were biocompatible with the three cell-lines from man A2780 (ovarian cancer cell-line), A549 (lung carcinoma) and A431 (epidermoid carcinoma), with no toxicity evident for concentrations of palmitoyl glycol chitosan below $150\,\mu g\,mL^{-1}$ and IC50 values of 0·2, 1·0 and $1·0\,mg\,mL^{-1}$, respectively (Figure 5). Palmitoyl glycol chitosan vesicles were highly haemocompatible with erythrocytes from man and could 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) (Table 1). These biocompatibility data are in good agreement with those reported for soluble glycol chitosan against the B16F10 cell line and against rat erythrocytes (Carreño-Gomez & Duncan 1997).

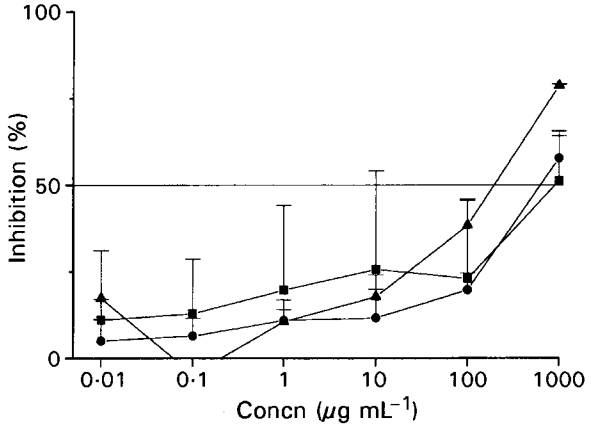

Figure 5. The biocompatibility of palmitoyl glycol chitosan-based vesicles with three cell-lines: ■ A549, ● A431, ▲ A2780. Data points are means ± s.d., n = 3.

Table 1. Haemocompatibility of palmitoyl glycol chitosan 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
Palmitoyl glycol chitosan/cholesterol (8 : 4)	30 (erythrocyte, palmitoyl glycol chitosan ratio)	4·2 ± 1·6
Palmitoyl glycol chitosan/cholesterol/DOTAP (6 : 2 : 1)	60 (erythrocyte, DOTAP ratio)	10·7 ± 1·3
Palmitoyl glycol chitosan/cholesterol/DOTAP (6 : 2 : 1)	600 (erythrocyte, DOTAP ratio)	6·6 ± 1·8

* Haemocompatibility is expressed relative to 100% haemolysis obtained with an erythrocyte to Triton X-100 weight ratio of 3 : 1 and 0% haemolysis obtained with PBS (pH = 7·4).

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