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The Encapsulation of Bleomycin Within Chitosan Based Polymeric Vesicles Does Not Alter its Biodistribution

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Abstract

Polymeric vesicles have recently been developed from an amphiphilic chitosan derivative — palmitoyl glycol chitosan. Their potential as a drug delivery system was evaluated using the anti-cancer compound bleomycin as a model drug.

Palmitoyl glycol chitosan (GCP41) was synthesised by conjugation of palmitoyl groups to glycol chitosan. Bleomycin-containing vesicles (669 nm diameter) were prepared from a mixture of GCP41 and cholesterol by remote loading. The vesicles were imaged by freeze-fracture electron microscopy and their in-vitro stability tested. Incubation of the larger vesicles with plasma in-vitro led to a reduction of mean size by 49%, a reaction not seen with control sorbitan monostearate niosomes (215 nm in size). They also showed a higher initial drug release (1 h), but GCP41 and sorbitan monostearate vesicles retained 62% and 63% of the encapsulated drug after 24 h, respectively. The biodistribution of smaller vesicles (290 nm) prepared by extrusion through a 200-nm filter was also studied in male Balb/c mice. Encapsulation of bleomycin into polymeric vesicles did not significantly alter the pharmacokinetics of biodistribution of bleomycin in male Balb/c mice although plasma and kidney levels were slightly increased.

It is concluded that the extruded GCP41 vesicles break down in plasma in-vivo and hence are unlikely to offer any therapeutic advantage over the free drug.

Bleomycin, a mixture of at least seven water-soluble glycopeptides produced by *Streptomyces verticillis* (Mir 1996), is frequently used for the treatment of head and neck squamous cell carcinoma, testicular cancer and Hodgkin and non-Hodgkin lymphomas (Figure 1). The dose-limiting toxicity is a mucocutaneous interstitial pneumonia leading to potentially fatal pulmonary fibrosis (Jules-Elysee & White 1990).

Bleomycin exerts its cytotoxic effect by cleaving DNA, acting mainly on cells in G2 phase that are undergoing mitosis. Optimum in-vivo activity would therefore require prolonged exposure to the drug. The terminal elimination half-life of bleomycin in man is only 2–4 h (Oken et al 1981). Consequently, a prolonged infusion would be expected to exert a greater antitumour effect. Evidence exists that prolonged low-dose administration is actually more effective and less toxic than intermittent high-dose bolus injections in both mice (Sikic et al 1978; Peng et al 1980) and man (Krakoff et al 1977; Cooper & Hang 1981).

At present, the only means of maintaining a prolonged low-dose bleomycin regimen is administering the drug by continuous intravenous infusion. There have been a few attempts to design new drug delivery systems for bleomycin, including an emulsion preparation (Slevin et al 1984), incorporation of the drug into non-ionic surfactant vesicles (niosomes) (Naresh & Udupa 1996), microcapsules (Lin et al 1986), poly-*d*,*l*-lactic acid implants (Kumanohoso et al 1997) and liposomes (Roy & Kim 1991).

Vesicles based on phospholipids (liposomes) or non-ionic surfactants (niosomes) have been extensively studied as carriers for anti-cancer drugs. More recently, we have developed polymeric vesicles from palmitoyl glycol chitosan (GCP41),

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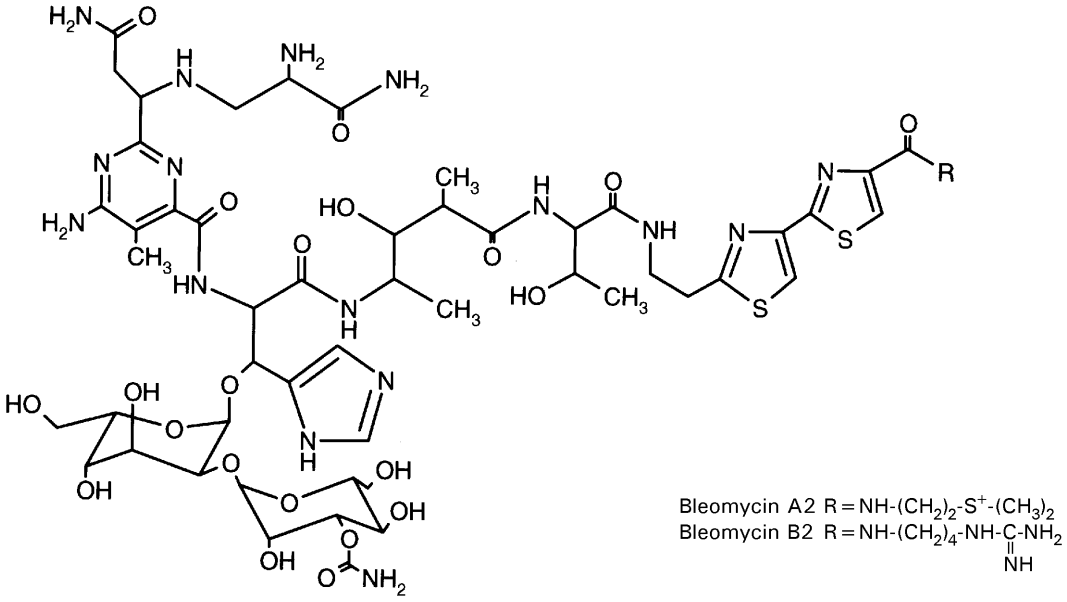

Figure 1. Structure of bleomycin.

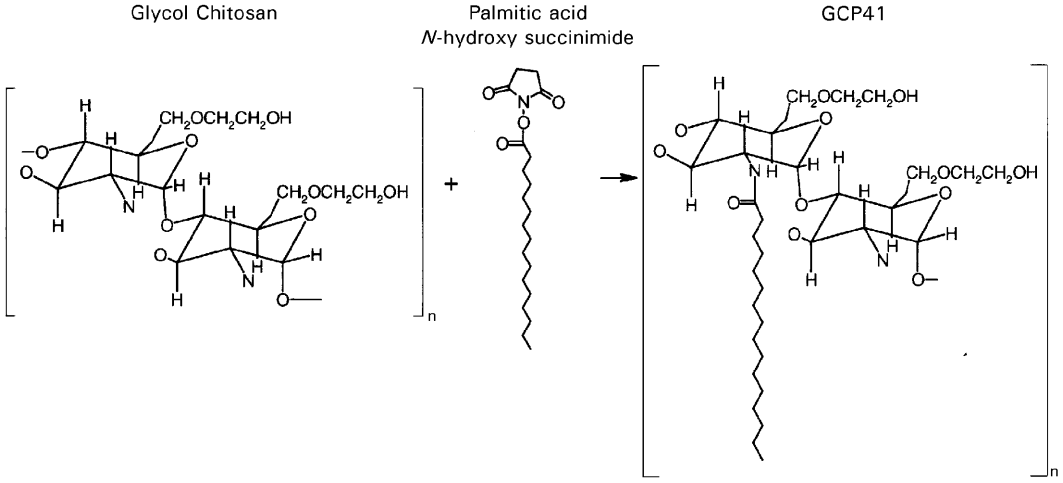

Figure 2. Synthesis and structure of palmitoyl glycol chitosan (GCP41).

an amphiphilic polymer in which hydrophobic pendant groups are attached directly to the sugar monomer units (Figure 2) (Uchegbu et al 1998). The object of this study was to assess the potential of these new polymeric vesicles as drug carriers using the model anti-cancer drug bleomycin.

Materials and Methods

Materials

Bleomycin sulphate was supplied by Lundbeck (Milton Keynes, UK). Cholesterol, sorbitan monostearate, palmitic acid *N*-hydroxysuccinimide ester and glycol chitosan were obtained from Sigma (Dorset, UK), ammonium sulphate and diethyl ether from Fisons (Loughborough, UK) and cholesteryl poly-24-oxyethylene ether (Solulan C24) from D. F. Anstead (Essex, UK). Sepharose 2B was purchased from Pharmacia Biotech (Upsalla, Sweden). All other chemicals were obtained from Merck Chemical Co. (Poole, Dorset, UK) and were of the highest purity available. Human plasma was kindly donated by the Glasgow and West of Scotland Blood Transfusion Service. Male Balb/c mice were obtained from Harlan (Olac, UK).

Preparation of palmitoyl glycol chitosan (GCP41)

GCP41 was prepared as previously described (Figure 2, Uchegbu et al 1998). Briefly, glycol

chitosan (200 mg) and sodium bicarbonate (150 mg) were dissolved in water (35 mL). Absolute ethanol (10 mL) was added to this solution, followed by drop-wise addition, over 30 min, of a solution of palmitic acid *N*-hydroxysuccinimide ester (79 mg) in absolute ethanol (60 mL). The reaction mixture was stirred for 72 h. The ethanol was evaporated with a rotary evaporator at 40°C, until a residual volume of only 50 mL remained, which was extracted with 2 volumes of diethyl ether. Following extraction the solution was dialysed within Visking dialysis tubing (Molecular weight cut off = 12–14 kD) against water (5 L) with 6 changes over 24 h. The product was freeze-dried to give a white, cotton wool-like substance.

Vesicle preparation

Bleomycin palmitoyl glycol chitosan vesicles were prepared by the remote loading method (Haran et al 1993). Briefly, palmitoyl glycol chitosan (8 mg) and cholesterol (4 mg) were hydrated with ammonium sulphate solution (2 mL, 0·12 M) by probe sonication (2×2 min, Soniprobe Instruments). Unentrapped ammonium sulphate was separated by ultracentrifugation (150 000 *g* for 1 h) and the pellet resuspended in bleomycin (2 mL, 6 U mL^{-1}) dissolved in phosphate buffered saline (PBS; pH 7·4). This suspension was incubated overnight at room temperature. Unentrapped bleomycin was separated from the entrapped material by ultracentrifugation (150 000 *g* for 1 h). The supernatant was retained for quantification. The pellet was resuspended in PBS (pH 7·4, 2 mL). Control niosomes were prepared by hydrating sorbitan monostearate (12 mg), cholesterol (10 mg) and cholesteryl poly-24-oxyethylene ether (Solulan C24; 8 mg) with ammonium sulphate (2 mL, 0·12 M) by probe sonication as described above.

Bleomycin quantification

Vesicles and supernatant were separated by ultracentrifugation. The vesicles were disrupted by dilution with 10 volumes of isopropanol. Both the disrupted vesicles and the supernatant were then diluted appropriately and the level of bleomycin quantified by ultraviolet spectroscopy ($\lambda = 254$ nm). A bleomycin standard curve (0·01–0·10 U mL^{-1}) was prepared and the assay was found to be linear over this concentration range.

In-vitro plasma stability

Human plasma (400 μL) was mixed with 100 μL of bleomycin palmitoyl glycol chitosan vesicles or, alternatively, 100 μL of sorbitan monostearate niosomes, both prepared as described above. Samples were incubated at 37°C and at 0, 1, 5 and 24 h, a 200-μL sample of each incubation mixture was removed and fractionated over a Sepharose 2B column (0·5×10 cm). The first 0·5 mL was discarded. The subsequent 1·5-mL and 3-mL fractions were retained and contained the vesicles and unentrapped bleomycin, respectively (Uchegbu et al 1995). The bleomycin content of both the 1·5-mL and 3-mL fractions was measured using a modified HPLC method with UV detection (Roy & Kim 1991).

Biodistribution of bleomycin polymeric vesicles

Palmitoyl glycol chitosan vesicles for the biodistribution study. Palmitoyl glycol chitosan vesicles were prepared by hydrating palmitoyl glycol chitosan (32 mg) and cholesterol (16 mg) with ammonium sulphate (8 mL, 0·12 M) in a manner similar to that described above. Unentrapped ammonium sulphate was separated from the entrapped material by ultracentrifugation as described above and the pellet resuspended in bleomycin (8 mL, 7·5 U mL^{-1}) dissolved in PBS (pH 7·4). The remote loading procedure was then continued as described above. The resulting vesicle solution was then extruded through a 200-nm filter (Avanti Polar Extruder) and sized (Malvern Zetasizer 4). The amount of bleomycin entrapped was quantified after extrusion as described above and the bleomycin polymeric vesicles and solution diluted to a concentration of 2·5 U mL^{-1}.

Animal studies. Male Balb/c mice were housed under a 12-h light–dark cycle and allowed free access to food and water (Harlan Olac, UK). Mice were administered either bleomycin solution or bleomycin palmitoyl glycol chitosan vesicles (16 U kg^{-1}) via the tail vein. At various times 4 mice from each treatment group were killed and blood taken by cardiac puncture. Plasma was then isolated by centrifugation (2500 *g* for 30 s) and stored at −70°C. Lung, heart, liver, spleen and kidneys were also removed and immediately frozen.

Tissue analysis. Tissue samples were weighed and a small piece of each tissue was removed and weighed before being homogenised in 500 μL of distilled water on ice using a micropestle. The resulting homogenate was centrifuged (21 000 *g* for 10 min) at 4°C and the supernatant transferred to a clean microcentrifugation tube containing 2 volumes of ice-cold acetonitrile. This mixture was then centrifuged (21 000 *g* for 30 s) and 100 μL of

the supernatant was injected onto a C-18 reversed phase Inertsil ODS-2·5-μm column (250×4·6 mm; Chrompack, London, UK) eluted with a mobile phase of methanol : acetonitrile : water : acetic acid (225 : 68 : 756 : 2 by volume) and containing 0·2 mM heptane sulphonic acid and 29 mM triethylamine (final pH 4·3) (Roy & Kim 1991). Bleomycin was detected at 254 nm using a Waters photodiode array detector. Retention times of bleomycin A2 and B2 were 4 and 10 min, respectively and the standard curve (obtained by adding the peak areas of bleomycin A2 and B2) was linear between 0·006 U mL^{-1} and 0·6 U mL^{-1}.

Results

Palmitoyl glycol chitosan (GCP41) vesicle size and encapsulation efficiency

GCP41, in the presence of cholesterol, formed stable unilamellar vesicles (Figure 3) with a mean size distribution of 669 nm (n = 3). Smaller vesicles with a mean diameter of 290 nm could be produced by extrusion through 200-nm membranes. The larger vesicles encapsulated bleomycin with a high efficiency of 30·5 ± 9·2% (n = 3) and 0·5 U of bleomycin per gram polymer when a 6 U mL^{-1} bleomycin solution was used for loading. The smaller vesicles had an encapsulation efficiency of 33% and 0·6 U g^{-1} bleomycin per gram polymer using a 7·5 U mL^{-1} bleomycin loading solution.

Plasma stability

GCP41 bleomycin vesicles decreased in size by 49% over the 24-h period, from 684 nm (n = 3) to 340 nm (n = 3). Niosomes showed a small increase in size from 215 nm (n = 3) to 270 nm (n = 3) in the same experiment. The amount of bleomycin encapsulated decreased by 38% in the case of the GCP41 bleomycin vesicles and 37% in the case of the sorbitan monostearate niosomes, respectively (Figure 4). In these in-vitro experiments the GCP41 vesicles were less stable than the sorbitan monostearate niosomes.

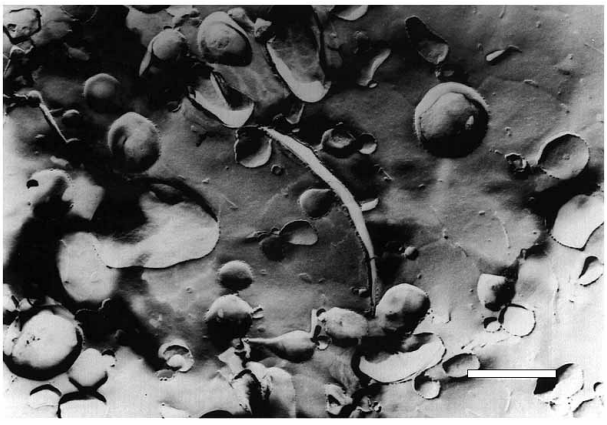

Figure 3. Freeze-fracture electron micrograph of palmitoyl glycol chitosan-based bleomycin vesicles. Bleomycin : polymer ratio = 0·5 U mg^{-1}, scale bar = 0·6 μm.

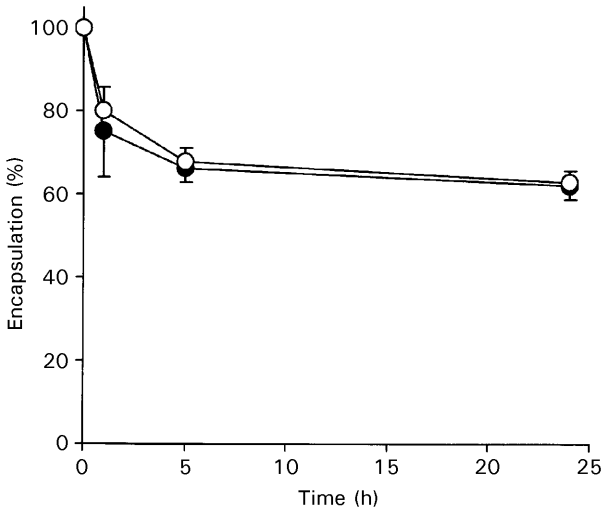

Figure 4. 24-h stability of palmitoyl glycol chitosan-based vesicles (●) and sorbitan monostearate vesicles (○) in plasma incubated at 377°C. Values are mean ± s.d.

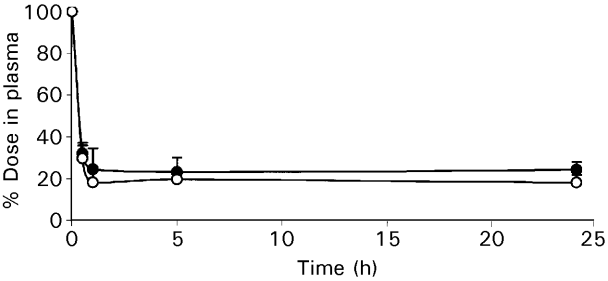

Figure 5. Distribution of palmitoyl glycol chitosan-based bleomycin vesicles (●) and bleomycin solution (○) in plasma following intravenous bolus administration to Balb/c mice. Values are mean ± s.d.

Biodistribution study

The vesicle suspension used for this study had a mean size of 290 nm and a bleomycin concentration of 2·5 U mL^{-1}. Mice showed no obvious toxic reaction to administration of any of the formulations. There were no statistically significant differences in the distribution of the drug to all of the tissues studied ($P > 0·5$) (Table 1). However in plasma there was a slightly decreased clearance of the drug when administered as the polymeric vesicle formulation (Figure 5). Distribution to the

Table 1. Biodistribution of bleomycin administered in GCP41 vesicles or as solution to Balb/c mice.

	Time (h)							
	0·5		1		5		24	
	Vesicles	Solution	Vesicles	Solution	Vesicles	Solution	Vesicles	Solution
Liver	2·52 ± 1·32	2·46 ± 0·59	2·09 ± 0·44	2·17 ± 0·30	2·32 ± 0·14	2·26 ± 0·70	2·49 ± 1·28	2·44 ± 0·27
Kidneys	1·42 ± 0·58	1·99 ± 0·94	2·64 ± 0·49	2·47 ± 0·48	2·63 ± 0·69	2·33 ± 1·27	1·78 ± 1·29	1·67 ± 0·49
Spleen	0·13 ± 0·05	0·24 ± 0·08	0·20 ± 0·02	0·17 ± 0·03	0·11 ± 0·03	0·19 ± 0·09	0·28 ± 0·18	0·21 ± 0·04
Heart	0·11 ± 0·04	0·05 ± 0·00	0·11 ± 0·05	0·05 ± 0·00	n.d.	0·08 ± 0·00	0·17 ± 0·05	0·12 ± 0·05
Lungs	0·37 ± 0·12	0·35 ± 0·23	0·39 ± 0·17	0·32 ± 0·02	0·34 ± 0·13	0·60 ± 0·00	0·40 ± 0·32	0·13 ± 0·02
Plasma	32·02 ± 3·88	29·65 ± 7·60	24·56 ± 10·10	18·34 ± 5·50	23·27 ± 7·02	24·50 ± 3·80	19·80 ± 4·20	18·40 ± 3·70

Data are given as mean ± s.d. of the percentage of the administered dose. n.d., not determined.

tissues was minimal and most of the drug was confined to the plasma compartment. On analysis of tissue distribution the liver and kidneys contained the greatest proportion of administered drug over the 24-h period. Levels in the liver were, however, consistently low over the 24-h period for both formulations. On the other hand, kidney levels of the drug when administered in the form of polymeric vesicles were slightly higher when compared with administration as a solution. Cumulative levels in the lung, spleen and heart represented less than 1% of the dose half an hour after administration.

Discussion

Bleomycin sorbitan monostearate niosomes (non-ionic surfactant vesicles) (Naresh & Udupa 1996) have been shown to increase the tumouricidal activity of bleomycin. Drug toxicity was also modulated with both liposome and niosome formulations (Roy & Kim 1991; Naresh & Udupa 1996). That the encapsulation of bleomycin within lecithin-based liposomes (Fichtner et al 1991) increased the area under the plasma level–time curve by about 250-fold and concomitantly the tumouricidal activity of the drug (Fichtner et al 1991; Roy & Kim 1991), led us to expect a similar result with the polymeric vesicle formulations.

The present chitosan-based polymeric vesicles (Figures 3 and 4), were stable on storage at refrigeration and room temperatures (Uchegbu et al 1998). The vesicles showed efficient drug encapsulation by remote loading with 0·5–0·6 U of drug encapsulated per gram of polymer. The drug release on incubation with plasma showed a very similar release from both the polymeric vesicles and the niosomes, with values of 75 ± 11% and 80 ± 6% after one hour, respectively. This, and our previous experience with doxorubicin-loaded niosomes (Uchegbu et al 1995), suggested that the GCP41 vesicles might also be useful carriers for in-vivo delivery of anti-cancer drugs.

The mean vesicle size of the GCP41 vesicles was reduced from 684 nm to 340 nm after 24 h incubation in plasma. The niosomal sorbitan monostearate formulation, however, showed a slight increase in vesicle mean size from 215 nm to 270 nm. This was an early indication that the two formulations differed in the degree, or mode, of interaction with plasma components.

The biodistribution of bleomycin administered as solution or vesicle suspension did not show any significant differences between the formulations at any of the time points studied. The kidney profiles of the drug indicate that there was a slight reduction in kidney clearance rates for the vesicle formulation when compared with the drug in solution. The peak kidney levels were obtained at 1 h for the drug in solution while the drug levels plateaued at 1 h and 5 h for the vesicular drug. This slight alteration in profile is mirrored by the plasma levels which were slightly higher for the vesicle formulation. The concentrations found in organs that typically show higher levels of drug after administration of vesicular formulations (e.g. liver, spleen, lung) were quite low throughout the time-course of the experiment. These data suggest that the drug concentrations detected in the plasma or organs after administration of the bleomycin vesicles predominantly reflect the level of free drug. It appears that the bleomycin initially encapsulated in the GCP41 vesicles was released almost immediately after administration to a much greater extent than expected from the results of the release studies carried out in plasma. Degradative processes operating at early time points in an in-vivo situation will be detrimental to overall vesicle performance; although vesicles do circulate on injection (Uchegbu et al 1995), the majority of the vesicle

dose is cleared within the first hour (Fichtner et al 1991; Uchegbu et al 1995; Uchegbu & Duncan 1997). The main difference between the formulations tested in-vitro and in-vivo was the size, the latter being much smaller with a mean size of 290 nm. Polymeric chitosan vesicles prepared by sonication, however, rarely achieve a mean size distribution of less than 350 nm (unpublished data). The extruded vesicles may have assumed a conformation and membrane curvature that could not be stably accommodated. The process could also have introduced persisting membrane defects, thus causing a slow leakage of the drug. The size changes observed on incubation of the GCP41 vesicles with plasma also suggested an intensive interaction with plasma proteins, although this was not reflected in the drug release.

It is concluded that the polymeric vesicles, on injection into the blood, suffer degradative influences that cause them to lose their drug payload and that this particular formulation of bleomycin is unlikely to offer any advantage over the free drug. It is unclear whether this will be the case for other encapsulated drugs.

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