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Pharmaceutical and biological characterisation of a doxorubicin-polymer conjugate (PK1) entrapped in sorbitan monostearate Span 60 niosomes

Elisabetta Gianasi [a], Fausto Cociancich [b], Ijeoma F. Uchegbu [a], Alexander T. Florence [b], Ruth Duncan [a,*]

[a] *Centre for Polymer Therapeutics, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK*
[b] *Centre for Drug Delivery Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK*

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Abstract

A doxorubicin *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate (PK1) designed for intracellular lysosomal cleavage following fluid phase pinocytic uptake is currently in early clinical development. This macromolecular prodrug has been encapsulated in niosomes prepared from Span 60/cholesterol/Solulan C24 (a cholesteryl poly-24-oxyethylene ether) (45:45:10). Lipid/surfactant films hydrated with a 3 mg ml^{-1} solution of PK1 using a modification of the dehydration rehydration vesicle (DRV) method gave an encapsulation efficiency of $49.0 \pm 1.54\%$. Vesicle size was 583 ± 191 nm. Span 60 PK1 niosomes were photographed by optical and transmission electron microscopy and found to have a bright fluorescence on the outside of the vesicles and diminished fluorescence in the vesicle core. This is thought to be due to fluorescence quenching of the higher concentration of PK1 inside the niosomes. Span 60 PK1 niosomes stored freeze dried at -40°C, 4°C and 25°C were completely stable and when stored as a liquid suspension at 4°C and 25°C retain 75% of their encapsulated material even after 28 days. Addition of excipients, e.g. polyethylene glycol 8000 and polyvinylpyrrolidone at the rehydration step increased the PK1 encapsulation efficiency to 62% and 65%, respectively. Incubation of PK1 niosomes with plasma resulted in less than 0.02% doxorubicin release after 72 h. When PK1 niosomes were incubated with a lysosomal enzyme preparation doxorubicin release increased to 7% after 72 h. PK1 niosomes have potential for use in targeted cancer chemotherapy.

Keywords: HPMA copolymer-doxorubicin; PK1; Niosomes

* Corresponding author. Tel.: +44 171 7535932; fax: +44 171 7535931.

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1. Introduction

The use of doxorubicin, a broad spectrum antineoplastic agent is hampered by a dose limiting cardiomyopathy and myelosuppression (Chabner et al., 1996). An *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate containing doxorubicin (PK1) (Fig. 1) has been described in which the drug is linked to a non-biodegradable backbone by a tetrapeptide (glycine-phenylalanine-leucine-glycine) biodegradable spacer (Duncan, 1992; Duncan et al., 1996). Following fluid phase pinocytic uptake of PK1, intracellular cleavage by lysosomal thiol-dependant proteases facilitates active doxorubicin release at the tumour site (Duncan, 1992). It has been shown that this macromolecular prodrug shows tumour tropism in animals (Seymour et al., 1994) due to the enhanced permeation and retention effect (EPR) (Matsumura and Maeda, 1986) which is mediated by a combination of a leaky tumour vasculature and the absence of lymphatic drainage leading to increased tumour accumulation of the macromolecular drugs. Decreased toxicity and profound antitumour activity in animal models was used as a basis to progress PK1 into early clinical development (Vasey et al., 1996). Already a 4-fold increase in the maximum tolerated dose of doxorubicin has been clinically established (Vasey et al., 1996).

However, on intravenous injection a significant proportion of the PK1 dose undergoes glomerular filtration (Seymour et al., 1990; Pimm et al., 1996). While this renal clearance prevents accumulation of PK1 in sites of toxicity it has been shown that increasing the molecular weight of the HPMA main chain, over and above the glomerular threshold, substantially increases polymer blood residence time with a consequent increase in solid tumour levels (Seymour et al., 1995). It was therefore hoped that the encapsulation of PK1 within non-ionic surfactant vesicles (niosomes) (Uchegbu et al., 1996a), would increase the blood residence time and promote PK1 tumour accumulation without the need to increase polymer molecular weight. The latter is not sensible clinically as the main chain is not biodegradable and thus could cause storage disease syndrome.

Niosomes are analogous to liposomes (Ballie et al., 1985) and are formed as the result of the self assembly of non-ionic surfactants (Uchegbu and Florence, 1995). Alkyl glycerol (Kerr et al., 1988; Rogerson et al., 1988) and sorbitan monostearate Span 60 niosomes (Uchegbu et al., 1995) have been used experimentally as drug carriers of doxorubicin. They alter the pharmacokinetics and metabolism of doxorubicin and can increase its therapeutic index in mouse tumour models.

Here we have studied the encapsulation of PK1 in sorbitan monostearate niosomes, the pharmaceutical stability of the formulation and measured the release of doxorubicin in vitro from PK1 entrapped in Span 60 niosomes during incubation in plasma or a lysosomal enzyme preparation (tritosomes). Also the ability of Span 60 niosomes to induce red blood cell lysis was assessed in vitro.

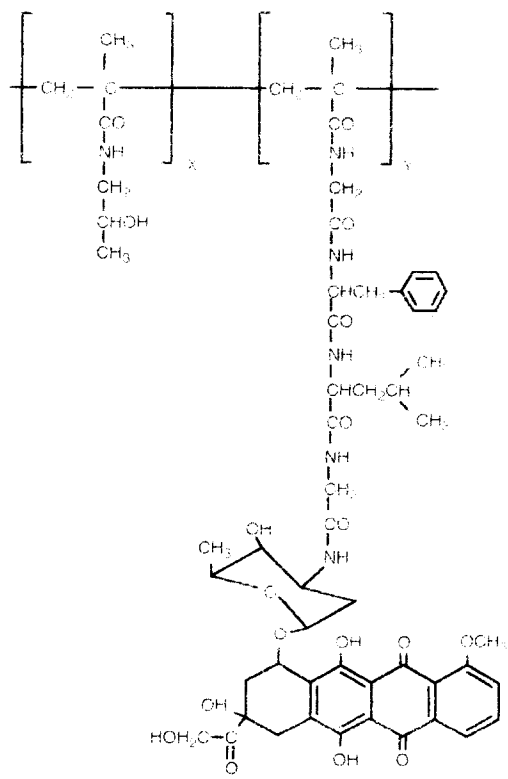

Fig. 1. Structure of doxorubicin *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate (PK1).

Table 1
Size and loading of PK1 into Span 60 niosomes[a]

Initial PK1: surfactant (g/g)	Final PK1 concentration (mg $ml^{-1} \pm$ S.D.)	PK1 entrapment (% ± S.D.)	Mean size (nm ± S.D.)
0.2	1.51 ± 0.19	49.0 ± 1.54	583 ± 191
1.3	4.1 ± 0.61	18.53 ± 0.54	417 ± 91

[a] Mean of three batches of niosomes.

2. Materials and method

2.1. Chemicals

PK1 was synthesised as previously described elsewhere (Ulbrich et al., 1996) and had the following characteristics: $M_w = 30\,000$; $M_w/M_n = 1.3$; 8% w/w of doxorubicin content. Keltone LV® (alginate, $M_w = 500\,000$) was from Kelco, UK. Solulan C24 (cholesteryl poly-24-oxyethylene ether) was a gift from Ellis and Everard, UK. Span 60, cholesterol, daunomycin HCl, polyethylene glycol (PEG) 8000, polyvinylpyrrolidone ($M_w = 40\,000$) were all from Sigma Chemical, UK. Citric acid, sodium hydrogen orthophosphate dodecahydrate, orthophosphoric acid, hydrochloric acid, methanol, chloroform and propan-2-ol were all from Merck, UK and phosphate buffered saline was prepared from preformulated tablets supplied by Oxoid, UK.

2.2. Preparation of Span 60 niosomes

Niosomes were prepared in a modification of the dehydration rehydration method used to prepare liposomes (Kirby and Gregoriadis, 1984) by hydrating lipid/surfactant films prepared from Span 60, cholesterol, Solulan C24 in the molar proportions (45:45:10) with a solution of PK1 (3 mg ml^{-1}) in water (nominally 5 ml) at 60°C. The resulting niosome dispersion was then probe sonicated (Soniprep 150 MSE) for 5 × 1 min with 30-s intervals for cooling. The dispersion was left to cool to room temperature, flash frozen in liquid nitrogen and subsequently freeze dried overnight (Edwards Modulyo freeze drier) in 1-ml aliquots. Freeze dried aliquots were then rehydrated with 1 ml water added in 0.2 ml fractions, with vigorous vortexing (1–2 min) in between. To separate the entrapped PK1 from the unentrapped material, rehydrated niosome dispersions were diluted to 6 × the original volume and ultracentrifuged for 2 × 1.5h at 146 000 × g (Beckman L8-55 Ultracentrifuge). The supernatant was removed in between centrifugations and at the end of the procedure the niosome pellet was resuspended in water or other suitable solvent.

2.3. The use of lactose and polymer excipient to improve niosome stability

Two methods of rehydrating freeze dried niosomes were used: (a) freeze dried niosomes were rehydrated with 1 ml solutions of lactose (50 μM), PEG 8000 (50 μM), polyvinylpyrrolidone (50 μM) or alginate (11 μM); (b) freeze dried niosomes were rehydrated with 0.5 ml water as described above. To this dispersion was then added 0.5 ml solutions of lactose (100 μM), PEG 8000 (100 μM), polyvinylpyrrolidone (100 μM) or alginate (22 μM). Concentrations of polymer excipient were chosen so as to be equimolar to PK1 with the notable exception of the alginate solutions where the low alginate solubility precluded this. Ultracentrifugation was carried out as described above using polymer or lactose solutions in place of water.

2.4. Characteristics of PK1 niosomes

Niosome dispersions were diluted 1:3 with propan-2-ol to disrupt the membranes and centrifuged at 4000 × g (Haraeus Varifuge 3.0RS). The supernatant was assayed for entrapped PK1 by ultraviolet (UV) absorption spectroscopy (Shi-

madzu UV-1601) at 478 nm. Preparations diluted (1:100) in filtered water (0.22 μm) were sized by dynamic laser light scattering (Malvern Autosizer 2c). To visualise the preparations, a thin film of the niosome dispersion was photographed either using a Nikon Microphot FXA 100 equipped with a fluorescent light source or a Philips 201 T.E.M. In the latter case the samples were negatively stained (1% phosphostungstic acid).

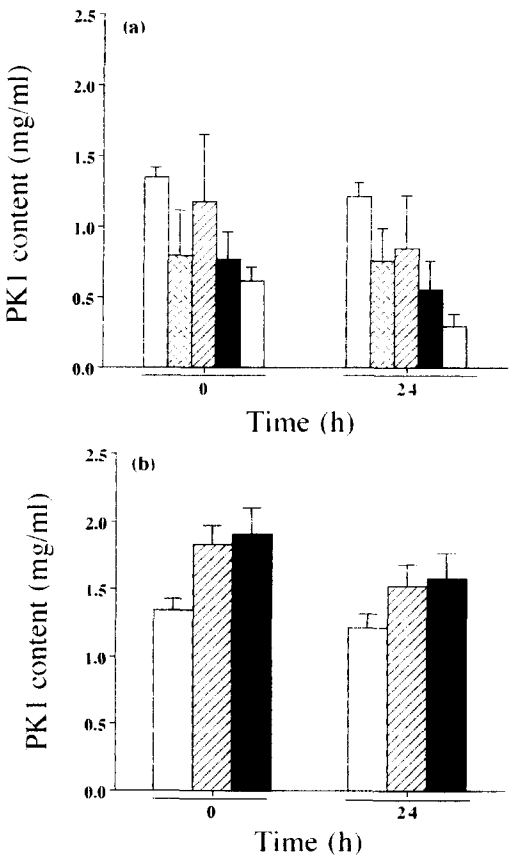

Fig. 2. PK1 content in niosomes prepared by: (a) rehydrating dehydrated niosomes with excipient solution; (b) rehydrating dehydrated niosomes with water and excipient solution added to resulting dispersion. Data as shown water (open square); lactose (dotted square); PEG 8000 (lined square); polyvinylpyrrolidone (filled square); alginate (shaded square). (Mean ± S.D. of three batches of niosomes).

Table 2
The effect of polymer excipient on niosome size

Excipient	Niosome size (nm ± S.D.)[a]	
	Method 1[b]	Method 2[c]
Water	483 ± 63	483 ± 63
Alginate	701 ± 151	–
Polyvinypyrrolidone	342 ± 44	538 ± 100
Polyethylene glycol	365 ± 47	514 ± 145
Lactose	387 ± 68	–

[a] Mean of three batches of niosomes.
[b] Method 1: niosomes prepared by rehydrating dehydrated niosomes with excipient solution.
[c] Method 2: niosomes prepared by rehydrating dehydrated niosomes with water and excipient solution added to resulting dispersion.

2.5. Niosome stability

Niosomes were isolated at four different stages of preparation cycle and their stability assessed. The conditions examined were as follow: 4°C or 25°C for the initial crude suspension and purified suspension respectively; −40°C, 4°C or 25°C for the freeze dried cake prepared from purified niosomes and the cake prepared without the rehydration and purification steps. At various time intervals niosomes were rehydrated (where necessary) and separated from any released PK1 by ultracentrifugation. PK1 content and size were assessed as above.

2.6. Degradation of PK1 niosomes at different pH and in the presence of plasma and tritosomes

Niosomes were prepared as described above except that rehydration of the freeze dried material was carried out with phosphate buffered saline (PBS, pH 7.4). A portion of the dispersion was adjusted to pH 5.5 with 1 M HCl. Niosomes were incubated at 37°C and the amount of PK1 released at pH 7.4 and pH 5.5 was estimated spectrophotometrically as described above.

Niosomes prepared as described above, rehydrated with PBS (pH 7.4) were incubated with freshly prepared rat plasma at 37°C in a 1:1 ratio. Aliquots of the incubation mixture were taken at various time intervals over 72 h and analysed for released doxorubicin. Extraction and analysis of

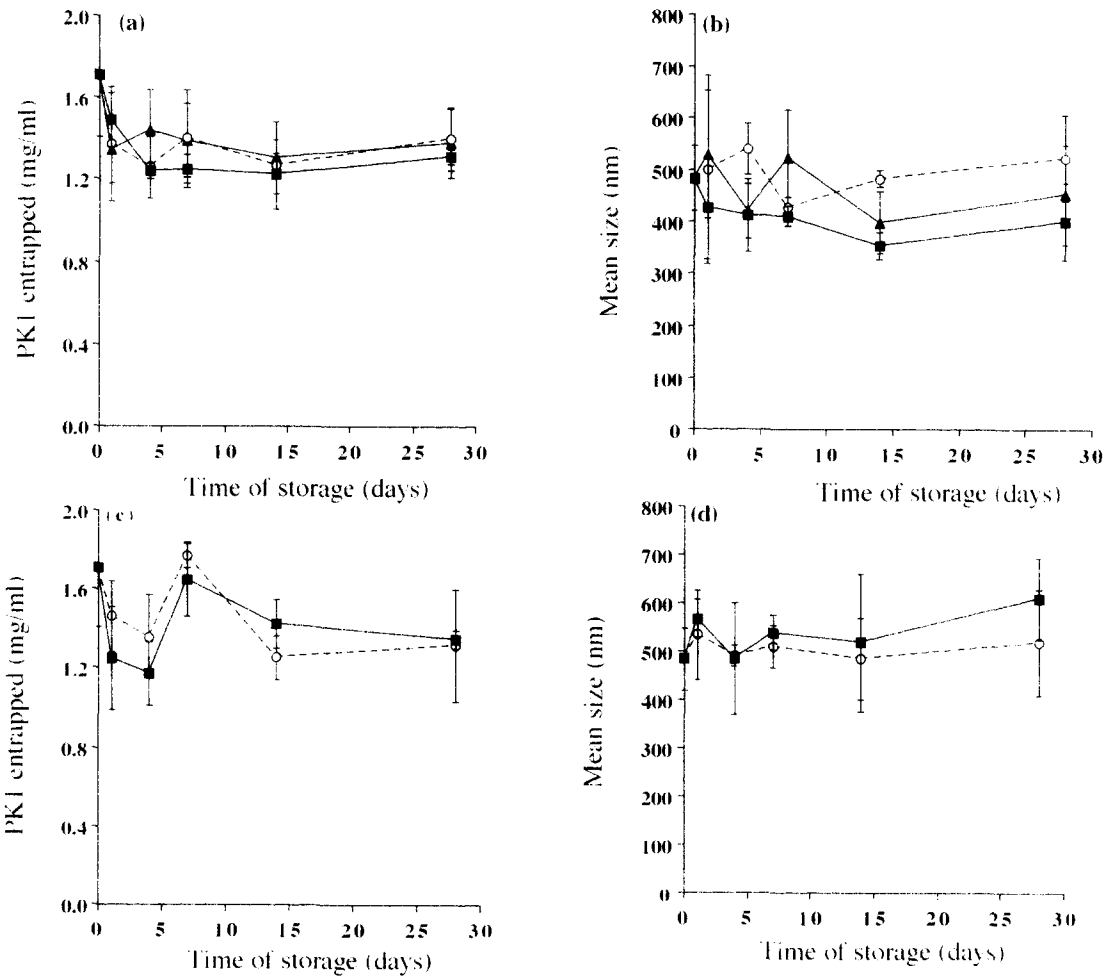

Fig. 3. Pharmaceutical stability of unpurified PK1 niosomes over 28 days. (a) PK1 entrapped in the freeze dried cake prepared without the rehydration and purification steps; (b) mean size of the freeze dried cake prepared without the rehydration and purification steps; (c) PK1 entrapped in the initial crude; (d) mean size of the initial crude suspension. Temperature of storage: −40°C (▲); 4°C (○); 25°C (■). (Mean ± S.D. of three batches of niosomes).

doxorubicin by HPLC with fluorometric detection was performed as previously described (Seymour et al., 1994). Briefly 100-μl samples to which had been added 100 μl daunomycin HCl (1 μg ml^{-1}) as internal standard, 100 μl ammonium formate (1 M, pH 8.5) and 800 μl water, were extracted into 5 ml chloroform, propan-2-ol (2:1), the organic layer evaporated and the residue reconstituted in 100 μl methanol. The sample were then injected onto a C_{18} μbondapak column (150 × 3.9 mm), running phase 29% propanol-2-ol (in water), adjusted to pH 3.2 with orthophosphoric acid and using a fluorescence detector (fixed wavelength excitation 490 nm, emission 550 nm).

Tritosomes (a lysosomal enzyme preparation) were prepared as previously described (Trouet, 1974) and enzymes released by freeze thaw disruption of their lysosomal membrane. The enzyme suspension was incubated with niosomes in citrate-phosphate buffer (pH 5.5) containing 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA) and 5 nM reduced glutathione at 37°C for 72 h. Released doxorubicin was extracted and estimated by HPLC as above.

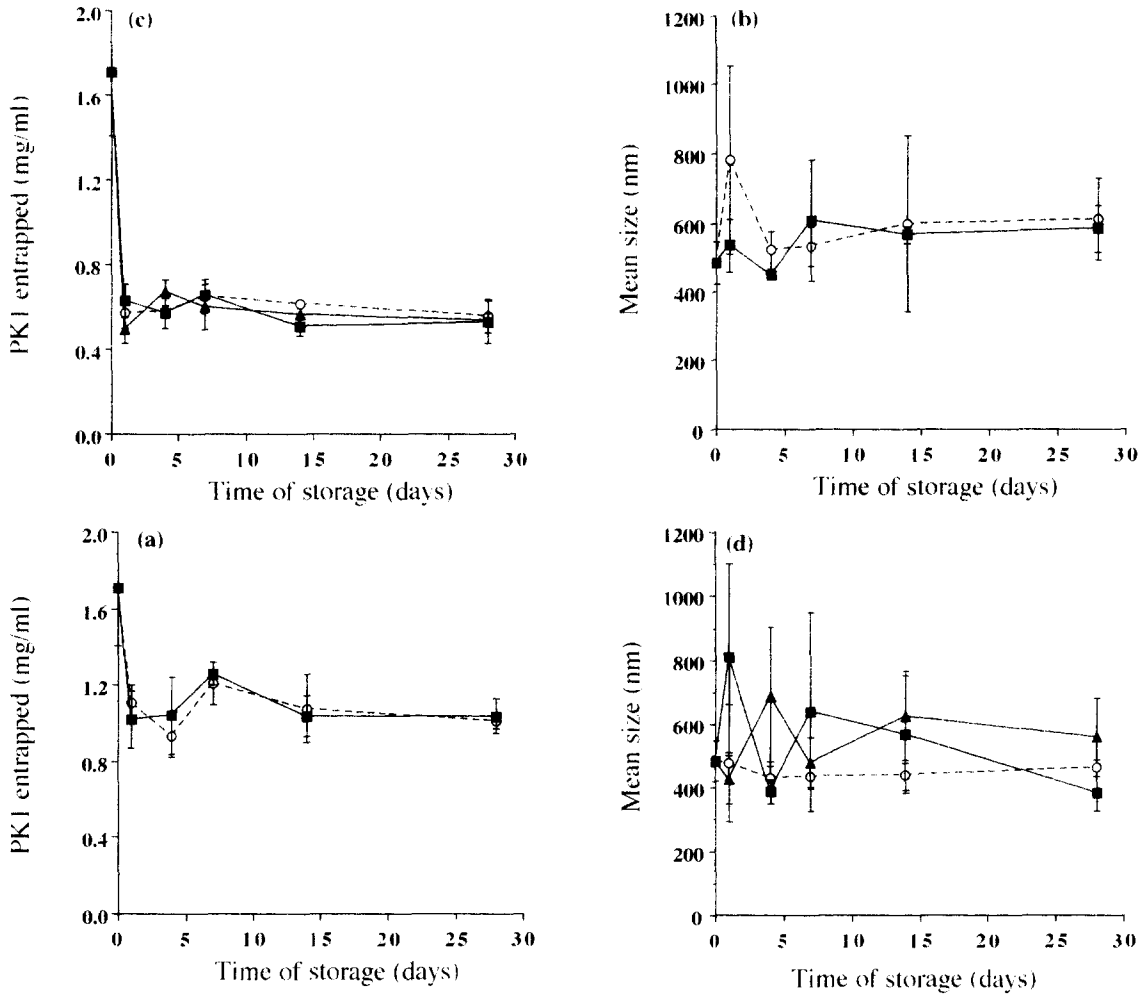

Fig. 4. Pharmaceutical stability of purified PK1 niosomes over 28 days of storage. (a) PK1 entrapped in the purified suspension; (b) mean size of the purified suspension; (c) PK1 entrapped in the freeze dried cake prepared from purified niosomes; (d) mean size of the freeze dried cake prepared from purified niosomes. Temperature of storage: −40°C (▲); 4°C (○); 25°C (■). (Mean ± S.D. of three batches of niosomes).

2.7. Effect of Span 60 niosomes on red blood cell stability

The unloaded niosomes prepared as described above were incubated with rat erythrocytes for 1 h or 24 h at 37°C. The percentage of haemoglobin released with time was determined by spectrophotometric assay (ELISA microtiter plate reader) (Duncan et al., 1991). The percentage haemoglobin released was calculated by reference to a control sample which contained Triton X-100 (100% haemoglobin release). Dextran was used as negative control and poly-L-lysine as positive control.

3. Results and discussion

Ultracentrifugation effectively separated free PK1 from the entrapped material, whereas other methods of separation such as gel filtration were less successful due to the high salt conditions

necessary which damaged the PK1 niosomes. The modified DRV method yielded niosomes with a high entrapment efficiency (Table 1) which, however, decreased with increasing PK1 concentrations, as PK1 precipitates at high temperature in a concentration-dependent manner (Uchegbu et al., 1996b) making it unavailable for entrapment at the elevated temperature required for the initial hydration step. The DRV method (Kirby and Gregoriadis, 1984) encourages an intimate association between the dehydrated niosome bilayers and PK1 thus maximising entrapment, values up to 50% being observed.

Optical microscopy revealed with a 'doughnut' appearance, there being a fluorescent, bright outer shell and decreased fluorescence in the niosome core (results not shown). This could either be due to the amphiphilic nature of PK1 (Fig. 1) which encourages bilayer localisation or due to the fluorescence quenching of PK1 at high concentration in the interior. Electron microscopy also revealed electron dense niosome core (results not shown). It is likely that the reduced fluorescence in the core is due to the dense packing of the doxorubicin residues which are known to self-quench at high concentration. Calculations using a niosome of diameter of 500 nm diameter and PK1 molecular diameter of 8 nm (by light scattering), would suggest that the final PK1 concentration in the niosome is some 10-fold in excess of the internal space available, consistent with the hypothesis of dense PK1 packing, polymer coil collapse and elimination of water of hydration.

The use of lactose solutions as the rehydrating liquid offered no apparent benefit to entrapment or stability and the use of aqueous polymer solutions to rehydrate dehydrated PK1 niosomes, decreases the amount of PK1 entrapped probably due to simple displacement of PK1 from areas around and within the niosome (Fig. 2a). However when aqueous polymer solutions are added to already hydrated niosomes, the percentage of PK1 entrapment increased (Fig. 2b). Thus the presence of a macromolecule external to the niosome increases PK1 niosomal association probably by osmotically protecting the niosomes during the ultracentrifugation steps. There was no protection with addition of polymer excipients over the first 24 h and the niosomes lost 14–20% of PK1 during this time. The formulations containing polymer excipients were more viscous and also contained a disproportionately high amount of excipient and thus were deemed not suitable for further development. Niosome mean size increased with the use of the high molecular weight polymer excipient alginate (Table 2) although there were no significant size changes with the use of the other excipients. The addition of polymer after the initial hydration of the dehydrated niosomes with water, also does not appear to increase the niosome size to significant degree.

To determine stability, niosomes derived from the different stages of the preparation were stored at different temperatures. Niosomes subject to

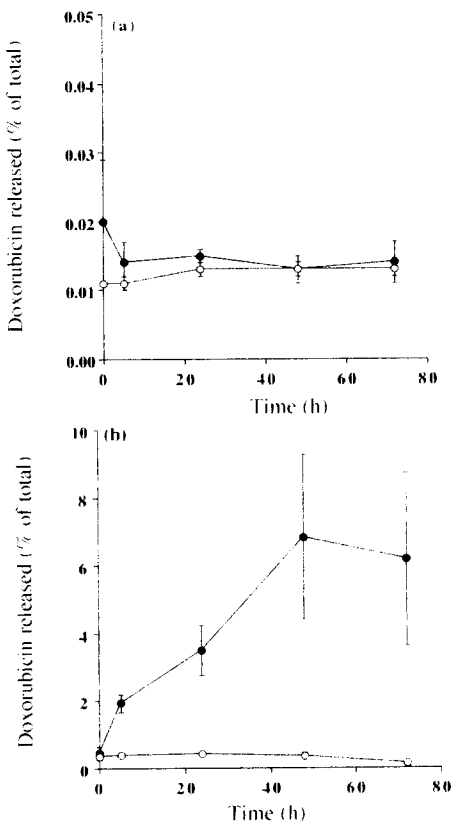

Fig. 5. Release of doxorubicin from PK1 niosomes. Data as shown incubation with (a) plasma (●) and control (PBS, pH 7.4) (○); and (b) lysosomal enzyme preparation (tritosomes) (●) and control (citrate-phosphate buffer, pH 5.5) (○). (Mean ± S.D. of three release studies).

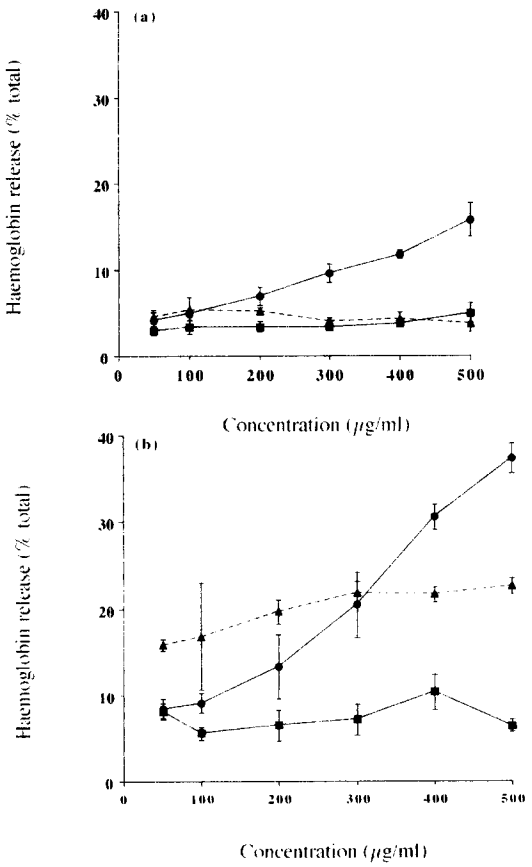

Fig. 6. Release of haemoglobin from rat erythrocytes by incubation with Span 60. Panel a, 1 h; panel b, 24 h. Data as shown for Span 60 niosomes (●); and the controls: dextran (■); poly-L-lysine (▲). (Mean ± S.D. of three studies).

freeze drying, and on rehydration purified by ultracentrifugation, were found to retain the same amount of PK1 over the entire 28 day period, irrespective of the storage temperature (Fig. 3a). Although the mean size varied, there was no trendancy towards increased aggregation and the storage temperature had little effect on particle size (Fig. 3b). Niosomes which had been rehydrated but the unentrapped material not separated by ultracentrifugation were also found to retain the same level of entrapped PK1 on purification by centrifugation, irrespective of the storage temperature (Fig. 3c). Size fluctuations were also minimal at both storage temperatures (Fig. 3d). This indicates a very stable system with no diffusion of material across niosome membrane.

If PK1 niosomes were stored as the purified suspension with the unentrapped material removed by ultracentrifugation, initially there was 25% drop in the level of PK1 entrapped within the first 24h, thereafter the level of PK1 entrapped changed very little (Fig. 4a). Purified niosomes retain a greater proportion of associated PK1 on storage and it is difficult to explain the initial losses seen as this is not simply an osmotic effect (evidenced by addition of polymeric excipient) and it is unlikely that sufficient drug is associated loosely with the niosome membrane. Although mean size was seen to fluctuate, no substantial aggregation was seen (Fig. 4b). Purified niosomes, prepared by the modified DRV method, on refreeze drying suffered a loss of 50% of the encapsulated material (Fig. 4c). This is because freeze drying effectively destroys the niosomes and on rehydration 50% of the PK1 available in solution (actually 25% of the initial amount present during the initial hydration of the lipid/surfactant film step) is entrapped. A similar 50% entrapment efficiency is seen with initial rehydration step (Table 1). This loss of material entrapped was observed regardless of the storage temperature. Size fluctuations were observed although no aggregation of niosomes could be seen on storage (Fig. 4d).

These experiments reveal that the Span 60 niosome formulations described herein are generally very stable, with storage as an unpurified suspension yielding the most stable product. The purified suspension also offers a relatively stable formulation, retaining at least 75% of entrapped material after 28 days. Storage at expensive sub-ambient temperatures offers no advantage and as Span 60 has a high phase transition temperature (Yoshioka et al., 1994), room temperature would offer a suitable impermeable system for PK1 entrapment.

Before in vivo biological study it was necessary to investigate PK1 niosome behaviour under physiological conditions. Incubation of PK1 Span 60 niosomes with plasma revealed less than 0.02% doxorubicin release over 72 h (Fig. 5a), thus indicating likely stability during long circulation in the bloodstream. However incubation of PK1 with the lysosomal enzyme preparation in vitro (tritosomes) resulted in 7% of the doxorubicin

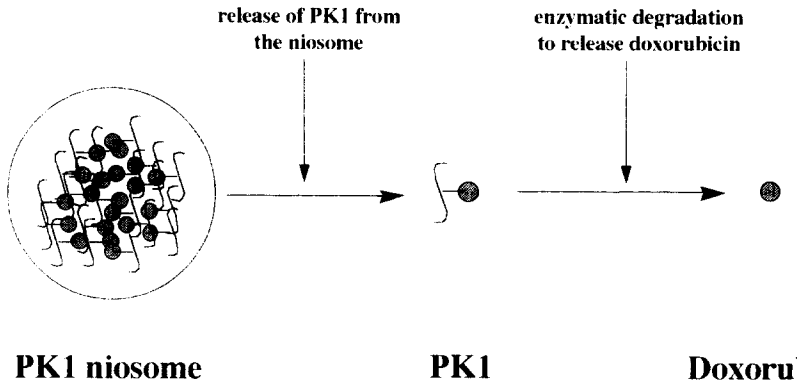

Fig. 7. Mechanism of release of doxorubicin from PK1 niosomes.

entrapped being released over 72 h (Fig. 5b). Release of the bioactive doxorubicin at the tumour target is essential for pharmacological activity. Incubation pH has no effect on the release of PK1 and thus did not contribute to PK1 or doxorubicin liberation. Span 60 niosomes displayed some ability to lyse rat erythrocytes in a concentration- and time-dependent manner (Fig. 6). After a 1-h incubation no lysis was observed but haemolysis reached 40% after a 24 h incubation at niosome concentration of 500 μg/ml. These results do not preclude the use of niosomes in vivo since the dose injected is not more than 200 μg/ml niosomes.

PK1 niosomes offer a novel approach which combines two technologies, the niosome and a new polymer drug in clinical development. To exert improved antitumour effect, PK1 must be liberated from the niosome at the tumour site and subsequently doxorubicin released by intracellular lysosomal enzyme cleavage (Fig. 7). PK1 has already been shown to accumulate selectively in solid tumour models (Seymour et al., 1994). Incorporation into niosomes should prolong circulation in the blood, as Span 60 niosomes have been shown to persist in the plasma for at least 24 h after administration (Uchegbu et al., 1995). The postulate is that the combined formulation will accumulate in solid tumour tissue due to the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986) as it known that polymers, particles and liposomes (up to 500 nm in diameter) (Yuan et al., 1995) can be selectively captured. Thus we believe that the intravenous administration of a PK1 niosomal formulation should improve the therapeutic index of doxorubicin. Work is currently under way to test this hypothesis in vivo.

4. Conclusions

A novel drug delivery system is described in which a doxorubicin HPMA copolymer conjugate is entrapped within niosomes prepared from a high phase transition temperature surfactant, Span 60. Niosomes showed a maximum 50% entrapment efficiency and were about 500 nm in size. This formulation may be stored as a suspension and retains 75% of entrapped material after 28 days of storage at room temperature. No advantages were seen on storage at refrigeration temperatures. On incubation with plasma, the niosomes release less than 0.02% doxorubicin but release 7% doxorubicin on incubation with a lysosomal enzyme preparation, an indication that this niosomal formulation will release less drug to potential sites of toxicity.

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