Niosomes and Polymeric Chitosan Based Vesicles Bearing Transferrin and Glucose Ligands for Drug Targeting

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Purpose. To prepare polymeric vesicles and niosomes bearing glucose or transferrin ligands for drug targeting.

Methods. A glucose-palmitoyl glycol chitosan (PGC) conjugate was synthesised and glucose-PGC polymeric vesicles prepared by sonication of glucose-PGC/ cholesterol. N-palmitoylglucosamine (NPG) was synthesised and NPG niosomes also prepared by sonication of NPG/ sorbitan monostearate/ cholesterol/ cholesteryl poly-24-oxyethylene ether. These 2 glucose vesicles were incubated with colloidal concanavalin A gold (Con-A gold), washed and visualised by transmission electron microscopy (TEM). Transferrin was also conjugated to the surface of PGC vesicles and the uptake of these vesicles investigated in the A431 cell line (over expressing the transferrin receptor) by fluorescent activated cell sorter analysis.

Results. TEM imaging confirmed the presence of glucose units on the surface of PGC polymeric vesicles and NPG niosomes. Transferrin was coupled to PGC vesicles at a level of 0.60 ± 0.18 g of transferrin per g polymer. The proportion of FITC-dextran positive A431 cells was 42% (FITC-dextran solution), 74% (plain vesicles) and 90% (transferrin vesicles).

Conclusions. Glucose and transferrin bearing chitosan based vesicles and glucose niosomes have been prepared. Glucose bearing vesicles bind Con-A to their surface. Chitosan based vesicles are taken up by A431 cells and transferrin enhances this uptake.

KEY WORDS: polymeric vesicles; glucose vesicles; transferrin vesicles.

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ABBREVIATIONS: Con A-gold, concanavalin A gold; DMSI, dimethylsuberimidate; DMSO, dimethylsulphoxide; FACS, Fluorescent activated cell sorter; FITC-dextran, fluorescein isothiocyanate dextran; PGC, palmitoyl glycol chitosan; NPG, N-palmitoyl glucosamine; PBS, phosphate buffered saline; TEM, transmission electron microscopy; TF, transferrin.

INTRODUCTION

Actively targeted therapies have demonstrated their potential in the tumour targeting of polymeric gene delivery systems (1), the central nervous system targeting of peptide analgesics (2) and the targeting of oligonucleotides to the liver hepatocytes and macrophages (3). In our laboratories polymeric vesicles for drug delivery have been developed from a specially designed amphiphilic chitosan derivative-palmitoyl glycol chitosan (PGC) (4). While the passive targeting of anti-cancer phospholipid vesicles (liposomes) (5,6) and non-ionic surfactant vesicles (niosomes) (7) to solid tumours has been well documented, liposomes for gene delivery are predominantly passively targeted to the lung endothelium (8,9). Hence, depending on their potential use it will be necessary to elucidate active targeting strategies for vesicular systems. Additionally the targeting of large hydrophilic molecules across the blood brain barrier (BBB) is invariably problematic. Transferrin bearing proteins however may be targeted across the blood brain barrier when administered via the carotid artery (10) and the exploitation of the BBB glucose transporter, GLUT-1 (11,12), using glucose peptide conjugates results in peptide delivery to the central nervous system (2). The GLUT-1 receptor is also over expressed in some tumour tissue (13,14), hence a glucose targeting ligand may be useful for targeting anti-cancer genes to tumour tissue. It is possible that the active targeting of polymeric vesicles for drug/ gene delivery may be accomplished with targeting ligands. This work reports on the preparation and characterisation of polymeric vesicles and niosomes bearing targeting ligands.

MATERIALS AND METHODS

Materials

Palmitic acid N-hydroxysuccinimide, glucosamine, sorbitan monostearate (Span 60), cholesterol, glycol chitosan (Mw = 164,000), concanavalin A gold (Con A-gold, 20nm), β-D-glucopyranosyl phenylisothiocyanate, N-N-diisopropylethylamine, dimethyl-suberimidate (DMSI), triethanolamine, fluorescein isothiocyanate dextran (FITC-dextran), phosphate buffered saline (PBS, pH = 7.4) tablets, iron-saturated human transferrin (TF), Folin Ciocalteu's reagent, uranylformate, sodium carbonate, sodium potassium tartrate and cupric sulphate, were all purchased from Sigma Aldrich Co, UK. Dialysis tubing was obtained from Medicell International, UK. Chloroform, isopropanol, dimethylsulphoxide (DMSO) and diethylether were all purchased from Merck, UK. Cholesteryl poly-24-oxyethylene ether (Solulan C24) was kindly donated by D.F. Anstead, UK. All tissue culture reagents were obtained from Gibco, UK.

Preparation of Glucose-Bearing Niosomes

Synthesis of N-Palmitoyl Glucosamine (NPG)

This was prepared in a similar manner to that described for the glycoside palmitoyl muramic acid (15) and was derived from the method of Lapidot and others (16). Glucosamine (86.3mg) was dissolved in dimethylsulphoxide (15mL) and triethanolamine (93μL). To this was added palmitic acid N-

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hydroxysuccinimide (283mg) dissolved in chloroform (4mL). The mixture was stirred at room temperature for 48 h, protected from light. Chloroform was evaporated off at room temperature and the remaining liquid freeze-dried. The resulting powder was purified by washing consecutively with water (200 ml), chloroform (50 ml), and ether (200 ml) and then was freeze-dried again. NPG (Figure 1) was obtained as a white powder.

^{1}H NMR Analysis of NPG

^{1}H NMR (with integration) and ^{1}H correlation spectroscopy experiments (Bruker AMX 400MHz spectrometer, Bruker Instruments, UK) were performed on NPG solutions in deuterated DMSO.

Mass Spectrometry

Analysis of NPG was carried out by mass spectrometry (fast atom bombardment–FAB on a JEOL AX505 mass spectrometer, Jeol Instruments, UK).

Preparation of NPG Niosomes

Niosomes were prepared by shaking a mixture of NPG (16mg), Span 60 (65mg), cholesterol (58mg) and Solulan C24 (54mg) in water (5mL) at 90°C for 1h, followed by probe sonication (Soniprobe Instruments, UK) for 4 minutes with the instrument set at 20% of its maximum capacity.

Synthesis of the PGC- Glucose Conjugate

Palmitoyl glycol chitosan (PGC) (Fig. 2) was synthesised by the reaction of glycol chitosan with palmitic acid N-hydroxysuccinimide in a 4:1 sugar monomer, palmitic acid molar ratio and characterised by ^{1}H NMR analysis as previously described (4). PGC - glucose conjugate was prepared using methods described previously (17). PGC (5mg), β-D-glucopyranosyl phenylisothiocyanate (5mg) and N-N-diisopropylethylamine (4μl) were dissolved in DMSO (5mL). The reaction mixture was stirred at room temperature for 24h, diluted with water (25mL), exhaustively dialysed against 1L of water (over a 24h period with 6 changes), and finally freeze-dried.

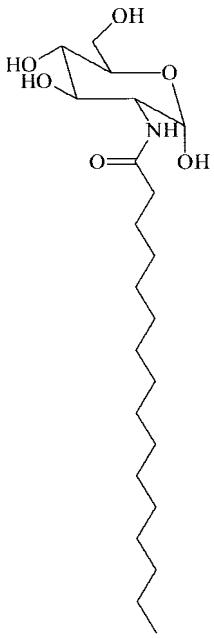

Fig. 1. Chemical structure of N-palmitoyl glucosamine (N-PG).

Preparation of Polymeric Glucose Vesicles

Vesicles were prepared by probe sonicating the PGC-glucose conjugate (4mg) and cholesterol (2mg) in water (4mL) for 4 min with the instrument set at 20% of its maximum output. The temperature of the probe sonicated formulation reached a maximum temperature of ~ 60°C. The vesicle dispersion was then filtered through a 0.45 μm filter.

Preparation Of Control Span 60 Niosomes

Vesicles were prepared by shaking a mixture of Span 60 (73mg), cholesterol (65mg), Solulan C24 (54mg) in water (5mL) at 90°C for 1h followed by probe sonication for 4 minutes with the instrument set at 20% of its maximum capacity.

Transmission Electron Microscopy (TEM)

The presence of glucose on the vesicle surface was evaluated by incubation with a colloidal dispersion of Con A-gold thus exploiting the affinity of the lectin concanavalin A for glucose (18). Glucose bearing vesicles and control plain Span 60 niosome suspensions (0.1mL) were shaken with the Con A-gold dispersion (0.1mL) at 60°C for 1h. To separate unbound Con A-gold from bound Con A-gold, the mixture was then centrifuged (1,000g for 10 min, Beckman L8-55 ultracentrifuge, Beckman Instruments, UK) and the pelleted unbound Con A-gold discarded. Vesicles bearing the bound gold were then imaged by TEM as follows. Droplets of the vesicle preparation were mixed in equal (20 μl) volumes with 1% uranylformate (pH 4.8) on a specimen support grid and immediately dried down using filter paper. The negatively stained grid samples were then imaged on a LEO 902 energy filtering electron microscope at 80 kV.

Preparation of TF-Bearing Polymeric Vesicles Entrapping the Fluorescent Marker FITC-Dextran

Preparation of Plain PGC Vesicles

PGC vesicles were prepared from PGC and cholesterol as previously described (4), by probe sonicating PGC (10mg) and cholesterol (4mg) in PBS (pH = 7.4, 2mL) for 4 min with the instrument set at 20% of its maximum capacity.

Conjugation of TF to PGC Vesicles (TF-PGC)

TF was linked with PGC vesicles by using DMSI as a cross-linking reagent (19) in a similar manner to that reported for TF-coated liposomes (20). To 2mL of the vesicle suspension (2mL), obtained from above (5mg mL^{-1}), was added TF (12mg) and DMSI (24mg) in triethanolamine HCl buffer (pH 7.4, 2mL). The coupling reaction was allowed to take place at room temperature for 2h whilst stirring. Free TF was then removed by ultracentrifugation (2 × 150,000g for 1h). After each ultracentrifugation step the pelleted vesicles were resuspended in PBS (pH = 7.4, 2mL).

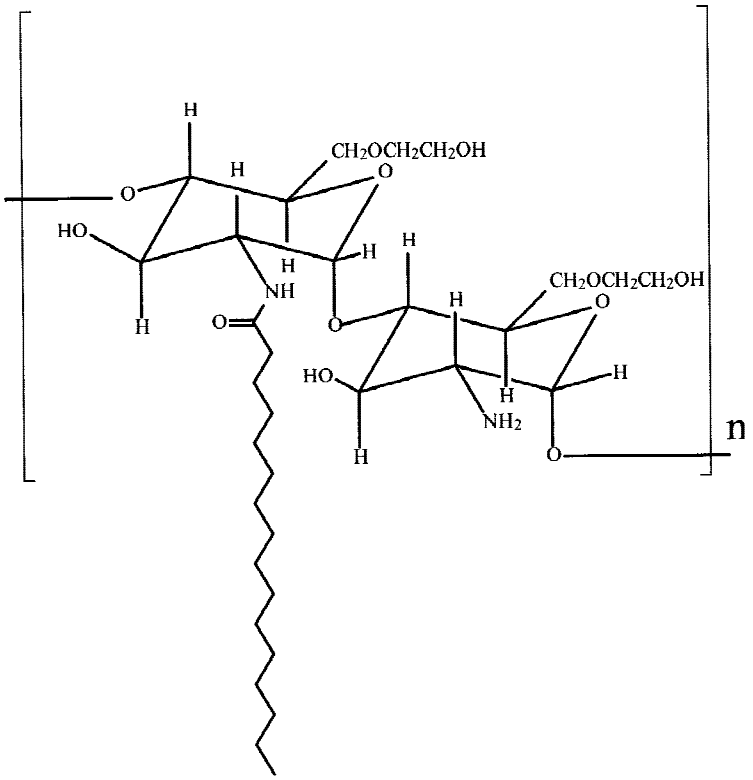

Fig. 2. Chemical structure of palmitoyl glycol chitosan (PGC).

Assay for Conjugated TF

The amount of conjugated TF was determined using the Lowry method (21). TF bearing vesicle suspensions, blank vesicles bearing no TF and vesicle suspensions carried through the TF conjugation reaction but without the cross-linking agent DMSI were used in this assay. The latter vesicles were used to ensure that TF was not merely adsorbed to the vesicle surface. To sodium carbonate solution (25mL, 2%w/v in NaOH 0.1M) was added sodium potassium tartrate (0.5mL, 2%w/v) and cupric sulphate (0.5mL, 1%w/v) with constant stirring to avoid precipitation. To 1mL of this solution (freshly prepared) was added 100μl of each of the vesicle suspensions (5mg mL^{-1} PGC) or the standard TF solutions (0–0.5mg mL^{-1}) and the mixture allowed to stand for 10min. To these samples was then added Folin Ciocalteu's reagent (100μL) with immediate vortexing. All samples were subsequently left to stand for 30min and the colour reaction quantified by measuring the absorbance at 750nm (UV-1, Unicam Ltd., UK). The sample derived from the blank vesicles not containing TF was used in the reference cell, when measuring the absorbance of the vesicle samples. The amount of protein associated with the vesicles was determined with reference to the standard TF solutions.

Loading of FITC-Dextran TF-PGC Vesicles

FITC-dextran loaded TF-PGC vesicles were prepared by probe sonicating on ice TF-coated vesicles, obtained as described above in a solution of FITC-dextran (2mL, 6mg mL^{-1}). Unentrapped FITC-dextran was removed by ultracentrifugation (150,000g × 1h) and the FITC-dextran loaded vesicle pellet resuspended in PBS (2mL).

Assay for the Amount of FITC-Dextran Entrapped by TF- PGC Vesicles

PGC vesicles were disrupted by adding the vesicle suspension (0.1mL) to isopropanol (1mL). This solution was then diluted to 10mL with PBS (pH = 7.4) and the fluorescence measured (Perkin Elmer LS-50 fluorescence spectrometer, Perkin-Elmer Instruments, UK.) at an excitation wavelength of 480nm and an emission wavelength of 560nm. The amount FITC-dextran was computed with reference to standard solutions of FITC-dextran (11μg mL^{-1}–11mg mL^{-1}) in an isopropanol, PBS (pH = 7.4) mixture (10: 90).

Vesicle Sizing

Vesicle sizing was performed by photon correlation spectroscopy on a Malvern Zetasizer 1 (Malvern Instruments, UK.).

Cellular Uptake of FITC-Dextran Loaded TF- PGC Vesicles

The A431-human epidermoid carcinoma cell line (ATCC CRL-1555) (22,23) was grown as a monolayer culture at 37°C in 5% CO_2 and maintained by regular passages in Dulbecco's medium supplemented with 10% foetal bovine serum, L-glutamine (1%w/v). Plated A 431 cells (10^5 cells/well) were incubated (37°C for 4 h) with transferrin-bearing vesicles (0.2 mL, 104μg mL^{-1} PGC) loaded with FITC-dextran (24μg mL^{-1}), blank FITC-dextran vesicles or with FITC-dextran solution. The concentration of FITC-dextran was the same for all the samples.

For the microscopic studies cells were grown and exam-

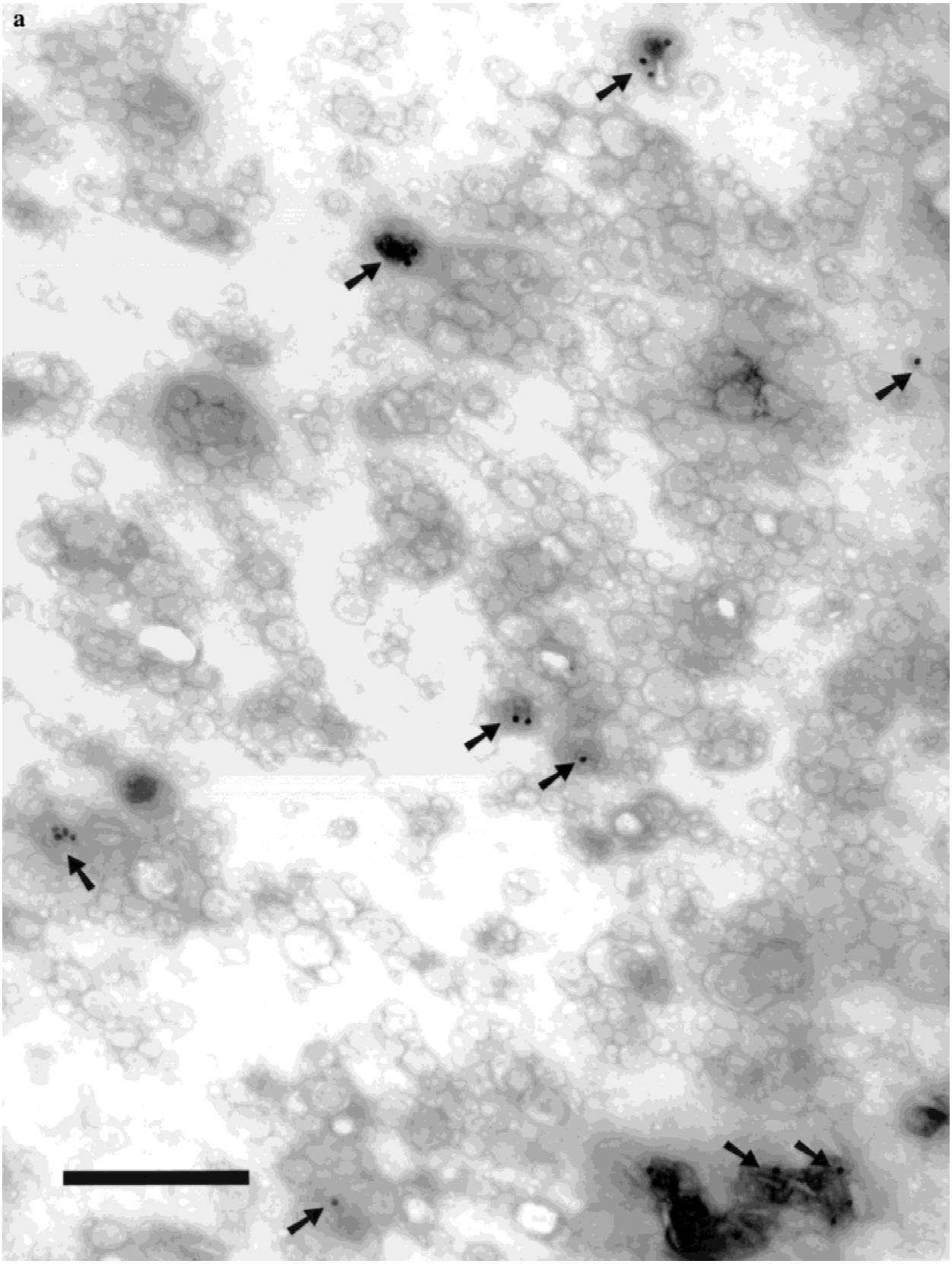

Fig. 3. Continued on facing page.

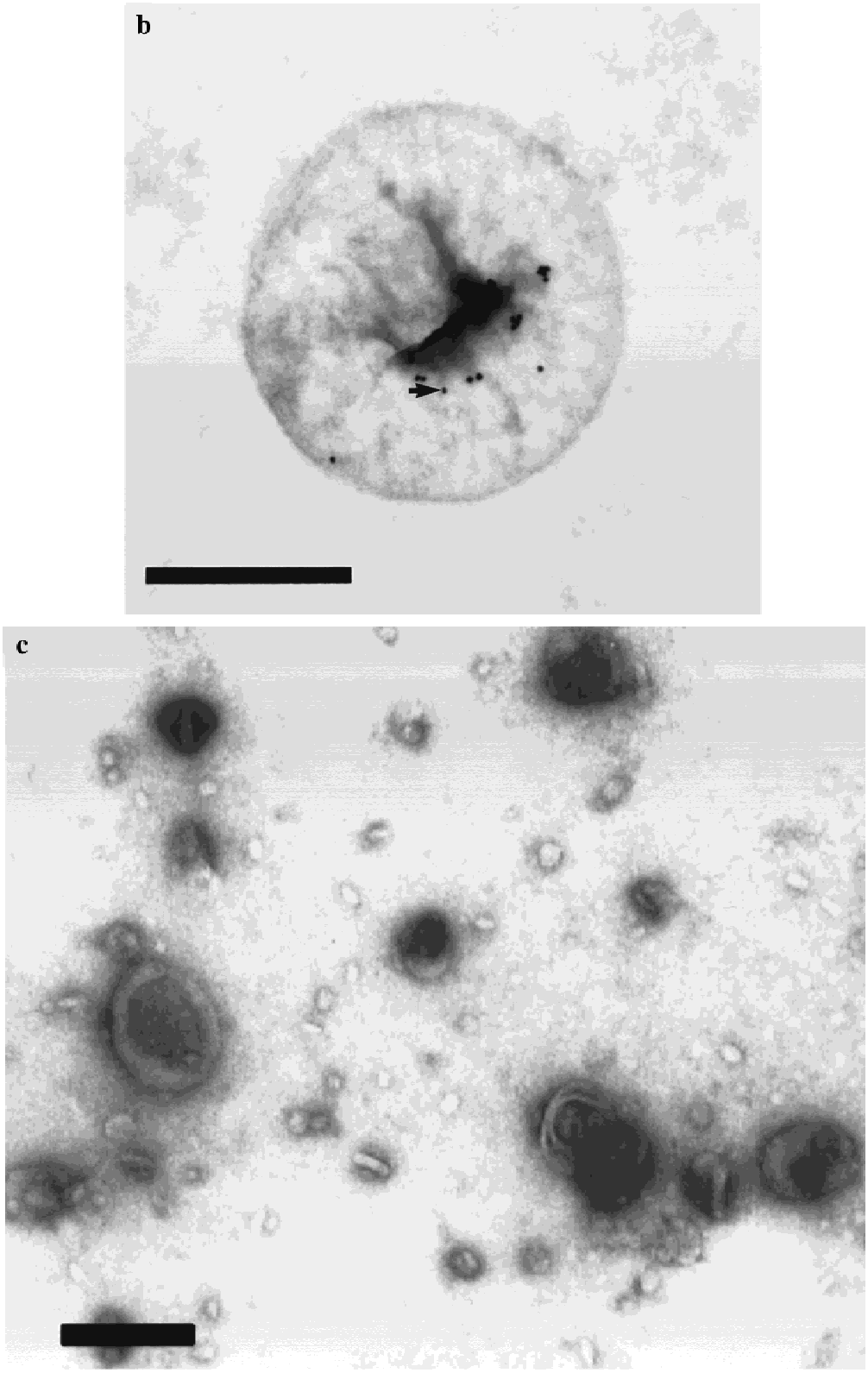

Fig. 3. Transmission electron micrographs with negative staining of Con A-gold (20 nm) associated with a) N-PG vesicles, b) PGC- glucose vesicles, and c) plain Span 60 niosomes. Gold particles are indicated by arrow , bar = 100nm.

ined on coverslips. At the end of the incubation period, cells were washed with PBS (pH = 7.4), transferred to a holder where the cells were immersed in PBS (pH = 7.4) and examined using confocal microscopy (λ_{ex} = 488 nm, Biorad 600 confocal microscope, Biorad, UK). For flow cytometry studies, the cells were trypsinised after the incubation period, washed (PBS , pH = 7.4) and pelleted (1,000g) twice. Cellular FITC-dextran uptake was examined on a FACStar flow cytometer (Becton-Dickinson Instruments, UK). The forward scatter (FSC) and sideward scatter (SSC) of a control cell suspension was used to discriminate cells and debris. 20,000 cells (gated events) were counted for each sample. FITC–dextran fluorescence was detected with logarithmic settings (FL1, λ_{em} = 515–545 nm). Cells were counted as positive when their fluorescence (FL1) was higher than that of 95% of cells from an untreated cell suspension, (i.e. channel 198 to 1024)

RESULTS

Glucose Bearing Vesicles

NPG Niosomes

The characterisation of PGC was as previously described (4). Proton assignments by ^{1}H NMR of NPG were as follows: δ 0.86 ppm = CH3 (palmitoyl), δ 1.25 ppm = CH2 (palmitoyl), δ 1.89 ppm = CH2 (palmitoyl shielded by carbonyl), δ 2.14 ppm = CH2 (adjacent to carbonyl protons), δ 2.71 ppm = CH (C2 sugar proton), δ 3.3–4.0 ppm = non-exchangeable sugar protons. Mass spectrometry data yielded one main peak corresponding to the mass ion 418 (100%, M^+) and further minor peaks 400 (72.75 %, M^+ -OH) and 432 (24.43%, M^+ + OH). These results indicate that NPG was successfully prepared.

Stable vesicles could be formed from NPG, sorbitan monostearate, cholesterol, Solulan C24 (10: 40: 40: 10 mole%). Higher levels of NPG resulted in unstable formulations with the NPG crystallising out of the formulation within hours. Glucose niosomes prepared from NPG had a z-average mean diameter of 164nm.

Glucose-PGC Vesicles

Polymeric (PGC) glucose bearing vesicles had a z-average mean diameter of 155nm.

Con-A Gold Binding

Both types of vesicles effectively bound Con A-gold while the control plain vesicles (devoid of glucose) did not (Figure 3), indicating the presence of accessible glucose units on the surface of these niosomes and polymeric vesicles.

Transferrin Bearing PGC Vesicles

Transferrin was successfully conjugated to PGC in these vesicles, as determined by the Lowry assay at a level of 0.60 ± 0.18g of TF per g polymer (50 ± 15% of the initial transferrin used). FITC-dextran was entrapped in the transferrin bearing vesicles (0.23g per g polymer, corresponding to 10% of the initial FITC-dextran) and in the plain chitosan based vesicles (0.32g per g polymer, corresponding to 13% of the initial FITC-dextran). Plain PGC vesicles had a z-average mean diameter of 420nm, while TF-PGC vesicles had a z-average size of 458nm and TF-PGC vesicles loaded with FITC-dextran had a z-average size of 740nm.

Cellular Uptake of Fluorescently Labelled TF-PGC Vesicles

Fluorescence microscopy images showed a brighter fluorescence (more fluorescently labelled vesicles) associated with cells incubated with the transferrin bearing vesicles when compared to the plain vesicles (Figure 4). Flow cytometry data (Fig. 5) also indicated that there was the greater percentage of positive cells when the transferrin bearing vesicles were incubated with the cells. However it is interesting to note that the polymeric vesicles without TF also associated to a greater degree with the cells than the fluorescent marker (FITC-dextran) in solution. This is indicative of the fact that these latter vesicles are also taken up by the cells or at least enhance the uptake of the fluorescent polymer.

DISCUSSION

This work is the first report of the preparation of polymeric chitosan based vesicles bearing targeting ligands. In addition the synthesis of a new surfactant NPG is described. The synthesis is a simple one-step procedure unlike the synthetic methods previously reported (24). Stable vesicles could not be produced from this surfactant alone or in the presence of cholesterol and the incorporation of more than 10mole% NPG into the bilayer of niosomes resulted in NPG crystallising out within hours. 6-O-alkanoyl-α-D glucose amphiphiles also crystallise out of niosomes prepared with these agents and cholesterol within 3–4 weeks (25). Small niosomes in the colloidal size range may be formed with this new amphiphile, NPG. 1-alkyl glucosides have been reported to form large unilamellar niosomes by Kiwada and others (26). These latter niosomes are 1μm in diameter and were reported to be stable when stored in the dark for up to 25 weeks. The production of colloidal dispersions of the 1-alkyl glucoside niosomes was not however reported by these authors. To our knowledge this is the first report of the production of sub-micron glucose vesicles in which the glucose units are found to be recognisable by the glucose specific lectin-con A. Con A served as a model for the glucose specific receptor.

The PGC-glucose conjugate produced vesicles with a smaller z-average mean diameter than vesicles produced from plain PGC (155nm vs 420nm). The conjugation of glucose to PGC probably resulted in an increase in the size of the hydrophilic portion of the molecule relative to the hydrophobic portion of the molecule. An increase in the hydrophilic head group of an amphiphile or mixture of amphiphiles would result in an increase in vesicle curvature and hence a decrease in vesicle size (27). It appears that with amphiphilic pendant like polymers, a similar increase in the hydrophilic head group area also decreases vesicle size. Vesicles incorporating only 10 mole % of NPG are able to bind Con A, as do vesicles prepared from the PGC-glucose conjugate (Figure 3). This indicates that the glucose units were accessible on the vesicle surface and may be accessible to glucose receptors in vivo. Because human cancer cells have an enhanced need for glucose and hence frequently over express the GLUT receptors

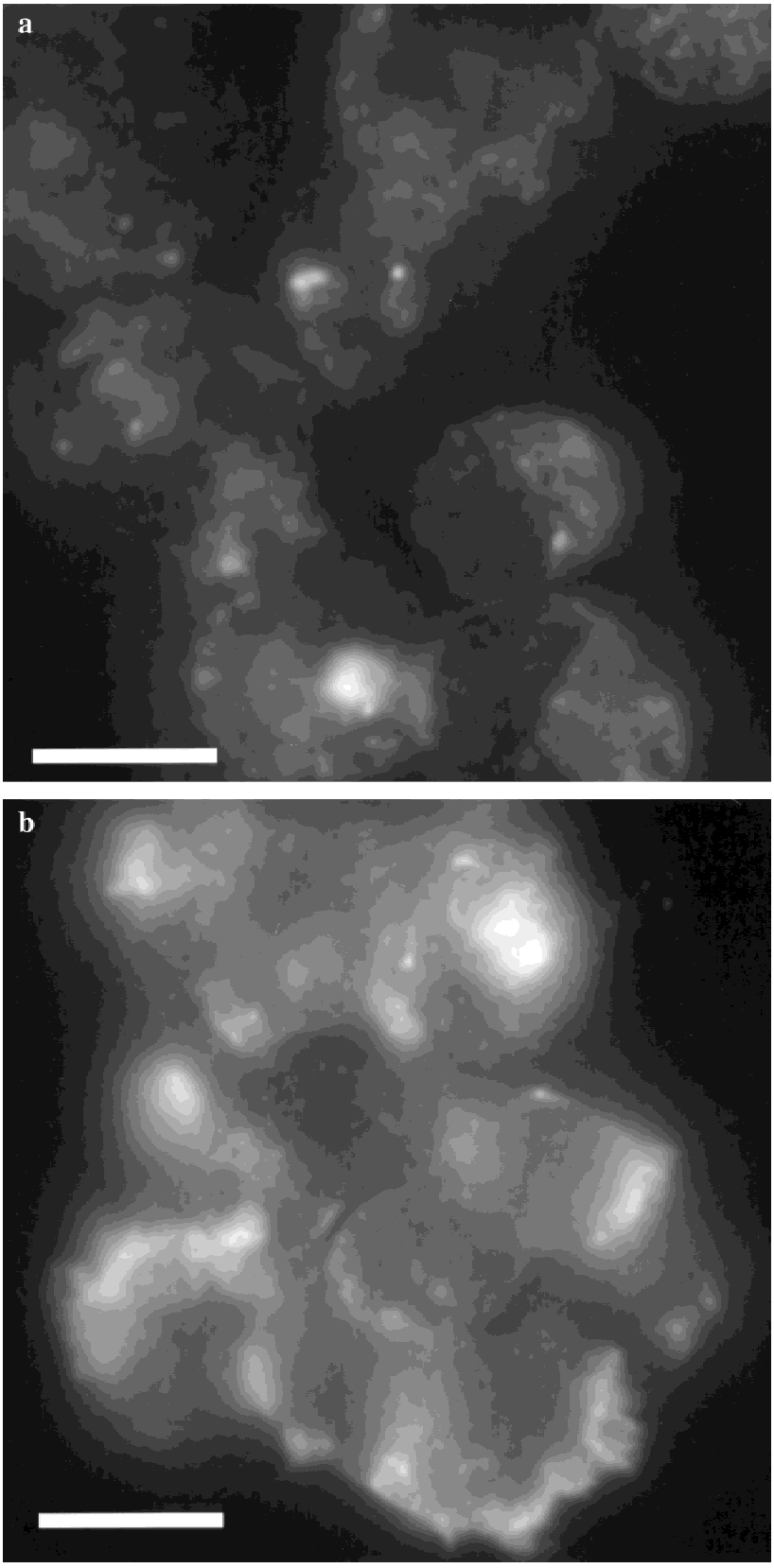

Fig. 4. Fluorescence micrographs of A 431 tumour cells treated with a) plain FITC-dextran loaded vesicles (without transferrin) and b) transferrin bearing FITC-dextran loaded vesicles, bar = 100 nm.

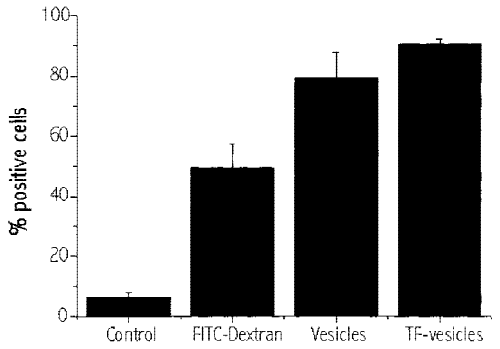

Fig. 5. FACS analysis of the uptake of FITC-D solution ("solution"), FITC-D vesicles without Tf ("vesicles"), and Tf-bearing vesicles loaded with FITC-D ("TF vesicles"), by A 431 cells. "Control": untreated cells. Cells were counted as FITC positive when their fluorescence was higher than that of 95 % of cells from an untreated cell suspension. n = 3.

(14), these glucose bearing vesicles may prove useful as gene targeting agents to tumour cells over-expressing the GLUT receptor isoforms. In addition the presence of GLUT 1 receptors at the BBB (28) may potentially be exploited with these carriers, causing them to increase the transfer of large hydrophilic molecules across the BBB.

Transferrin was coupled to the surface of the polymeric vesicles and appeared to be accessible to the TF receptor in the A431 cell line (Figures 4 and 5). Transferrin receptors are also over expressed on the surface of many proliferating cells (29) and their presence may be exploited for the targeting of gene expression to tumours (1). In addition the administration of transferrin-protein conjugates via the carotid artery resulted in the enhanced transfer of large proteins across the BBB (10). These transferrin bearing vesicles may thus find a use in the targeting to the central nervous system.

CONCLUSION

Glucose niosomes and glucose or transferrin bearing polymeric vesicles have been successfully prepared for drug targeting. The accessibility of these targeting ligands to the glucose specific lectin Con A or to the transferrin receptor have been demonstrated. In addition the encapsulation of FITC-dextran within polymeric vesicles has been shown to promote the uptake of the fluorescent marker. Further studies on the usefulness of these new vesicles in vivo are planned for the very near future.

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