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Mucoadhesive maleimide-functionalised liposomes for drug delivery to urinary bladder

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ABSTRACT

Intravesical drug administration is used to deliver chemotherapeutic agents *via* a catheter to treat bladder cancer. The major limitation of this treatment is poor retention of the drug in the bladder due to periodic urine voiding. In this work, maleimide-functionalised PEGylated liposomes (PEG-Mal) were explored as mucoadhesive vehicles for drug delivery to the urinary bladder. The retention of these liposomes on freshly excised porcine bladder mucosa *in vitro* was compared with conventional liposomes, PEGylated liposomes, two controls (dextran and chitosan), and evaluated through Wash Out$_{50}$ (WO$_{50}$) values. PEG-Mal liposomes exhibited greater retention on mucosal surfaces compared to other liposomes. The penetration abilities of conventional, PEG-Mal-functionalised and PEGylated liposomal dispersions with encapsulated fluorescein sodium into the bladder mucosa *ex vivo* were assessed using a fluorescence microscopy technique. PEGylated liposomes were found to be more mucosa-penetrating compared to other liposomes. All liposomes were loaded with fluorescein sodium salt as a model drug and the *in vitro* release kinetics was evaluated. Longer drug release was observed from PEG-Mal liposomes.

1. Introduction

Bladder cancer (BC) is caused by uncontrolled growth of tumour cells in the urinary bladder. It has the 9th highest incidence globally, with an estimated 430,000 newly diagnosed cases in 2012 (Stewart and Wild, 2014). The prevalence of this malignancy of the genitourinary tract tends to increase with economic development and males are more likely to develop this condition than females (Torre et al., 2015). The most common type of BC is transitional cell carcinomas that comprise over 90% of tumours, while squamous cell carcinomas and adenocarcinomas represent about 5% and 1% of the reported cases, respectively.

Intravesical drug delivery (IDD) is a direct administration of therapeutic agents into the bladder *via* insertion of a urethral catheter (Au et al., 2001; Malmström, 2003; Kolawole et al., 2017). This allows localised treatment, minimises adverse effects and improves the exposure of the diseased tissues to therapeutic agents. Also, the oral route of the drug intake is undesirable in the therapy of BC due to absorption, metabolism and renal excretion, resulting in poor drug bioavailability in the bladder.

IDD has intrinsic limitations related to the substantial chemotherapy dilution and wash out due to urinary voiding, low permeability of the urothelium, and intermittent catheterisations (GuhaSarkar and Banerjee, 2010). Additionally, the procedure is relatively unpleasant for the patients and may cause inflammatory reactions and infections. To counteract the limitations associated with low drug permeability, mucoadhesive formulations offer great promise. The ability of mucoadhesive materials to adhere to the bladder epithelium and withstand wash out effect could improve drug bioavailability by prolonging the residence in the bladder. Mucoadhesive formulations for IDD must fulfill the following criteria: the dosage form should have rapid and efficient adhesion to the bladder mucosa; must not interfere with the normal physiology of the bladder; and should be able to stay adhered *in situ* for a few hours even after urination (Tyagi et al., 2006).

A number of mucoadhesive formulations have been researched, including the use of hydrophilic polymers of both natural and synthetic type, such as chitosan, carbomers and cellulose derivatives (Hombach and Bernkop-Schnürch, 2010; Khutoryanskiy, 2011). The adherence of these polymers is due to the ability to interact with mucin glycoproteins *via* non-covalent bonds such as hydrogen bonds, electrostatic

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interactions and chain entanglements, diffusion and interpenetration (Khutoryanskiy, 2011; Davidovich-Pinhas and Bianco-Peled, 2014). In a comparative study, chitosan was found to exhibit greater mucoadhesion to pig vesical mucosa compared to carboxymethylcellulose and polycarbophyl, thus resulting in a slower drug release and longer residence time (Burjak et al., 2001).

In recent years, various chemical approaches have been used to improve mucoadhesive properties of polymers by introducing specific functional groups such as thiols (Bernkop-Schnürch, 2005; Davidovich-Pinhas et al., 2009; Cook et al., 2015), acrylates (Davidovich-Pinhas and Bianco-Peled, 2011; Brannigan and Khutoryanskiy, 2017), and catechols (Kim et al., 2015). Some studies reported the use of chemically modified mucoadhesive materials for IDD to urinary bladder. Thiol-modified chitosan nanoparticles (NPs) have been used for IDD in an *in vitro* study using porcine urinary bladder (Barthelmes et al., 2011). It was found that chitosan functionalised with thiol groups demonstrated superior mucoadhesion, greater stability and controlled release compared to the unmodified chitosan NPs. In a different study, the retention of thiolated chitosan NPs on rat bladder mucosa *in vivo* was approximately 170-fold greater compared to the polymer-free fluorescent marker (Barthelmes et al., 2013). Mun et al. (2016) developed and evaluated the retention of thiolated and PEGylated silica NPs on porcine urinary bladder mucosa *in vitro* through use of a novel Wash Out_{50} (WO_{50}) quantitative method. It was shown that the retention of these NPs on bladder mucosa depended on both their thiol content and dimensions.

Recently we have demonstrated for the first time that polymers functionalised with maleimide groups exhibit excellent mucoadhesive properties to conjunctival tissues *ex vivo* and the ability of these materials to retain on mucosal tissues was comparable to well-known mucoadhesive chitosan (Tonglairoum et al., 2016). This excellent mucoadhesive performance of maleimide-functionalised polymers is due to their ability to form covalent linkages with thiol-groups present in mucins. More recently, Shtenberg et al. (2017) reported the functionalisation of alginate with maleimide-terminated polyethyleneglycol to achieve superior mucoadhesive properties towards porcine intestine mucosa.

Liposomes are microscopic vesicles composed of phospholipid bilayers with the size range from 30 nm up to several microns that have attracted a lot of interest over the past four decades as pharmaceutical carriers. Conventional liposomes and liposomes coated with mucoadhesive polymers previously were used for transmucosal drug delivery (Sasaki et al., 2013; Berginc et al., 2014; Adamczak et al., 2017). Some liposome-based formulations were also reported for intravesical drug delivery (Chuang et al., 2009, 2014; Kawamorita et al., 2016). Recently, Oswald et al. (2016) reported the preparation and characterisation of maleimide-functionalised liposomes; however they did not demonstrate any application of these systems for drug delivery.

In this study, we explored the mucoadhesive properties of maleimide-functionalised liposomes and compared their retention on urinary bladder mucosa with conventional liposomes and PEGylated liposomes. We also have studied the physicochemical properties of different liposomes, their penetration into the bladder mucosa and drug release profiles.

2. Materials and methods

2.1. Materials

Soybean L-alpha-phosphatidylcholine (PC) was purchased from Alfa Aesar (Heysham, UK). [*N*-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanol-amine, sodium salt] ($MPEG_{2000}$-DSPE) was a generous gift from Lipoid GmbH (Ludwigshafen, Germany). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] ammonium salt (PEG_{2000}-DSPE-Mal) was purchased from Avanti Polar Lipids (Alabaster, USA). Cholesterol (CHO), chitosan (low molecular weight; Mw 62.3 kDa, PDI 3.42 as reported by Symonds et al. (2016)), fluorescein isothiocyanate dextran (FITC-dextran, M_W 3000–5000 Da), fluorescein isothiocyanate (FITC) and fluorescein sodium salt (NaFI) were purchased from Sigma Aldrich (Gillingham, UK). All other chemicals were of analytical grade and were used as received.

Table 1
The composition (%) of lipid nanocarrier formulations.

Liposome formulations	PC	CHO	$MPEG_{2000}$-DSPE	PEG_{2000}-DSPE-Mal	NaFI
Conventional	0.773	0.077	–	–	0.2
PEGylated	0.773	0.077	0.075	–	0.2
PEG-Mal	0.773	0.077	–	0.075	0.2

PC – soybean L-alpha-phosphatidylcholine; CHO – cholesterol; $MPEG_{2000}$-DSPE – [*N*-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanol-amine, sodium salt]; PEG_{2000}-DSPE-Mal – 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] ammonium salt; NaFI – fluorescein sodium salt.

Phosphate-buffered saline (PBS) was composed of 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 (pH 7.4). The buffer solution was made with deionised water (total volume 1000 mL).

2.2. Preparation of liposomes

The liposomal formulations containing fixed amounts of PC, CHO and PEGylated lipids at molar ratios of 10:2:0 and 10:2:3 mM (Table 1) were prepared using thin film hydration and sonication method (Rangsimawong et al., 2016). In brief, a mixture of PC, CHO and PEGylated lipids dissolved in chloroform-methanol (2:1, v/v) in test tubes. The organic solvent was evaporated under a stream of nitrogen and a thin film of lipid was formed inside the test tubes. The test tubes were then placed under vacuum at least 6 h to remove any residual solvent. Then, solution of NaFI in PBS (pH 7.4) was added to the dried lipid films to generate hydrated liposome vesicles and the tubes were left for 1 h at room temperature. The tubes were vortex-mixed vigorously for 30 min and these liposome dispersions were then sonicated in a sonication bath (FS200b, Decon Laboratories Ltd., UK) for 30 min to reduce the size of the liposomes. Excess lipids were separated from the vesicle formulations by centrifugation at 14,000 rpm (8765 × *g*) at 4 °C for 30 min. The supernatants were collected and stored in a fridge overnight prior to characterisation.

2.3. Synthesis of fluorescently-labelled chitosan

FITC-chitosan was synthesised according to the procedure described previously (Cook et al., 2011; Symonds et al., 2016). Briefly, 1 g of chitosan was dissolved in 100 mL of acetic acid (0.1 M) and left stirring overnight. 100 mg of FITC was dissolved in 50 mL of methanol (MeOH) and subsequently was added to the chitosan solution and stirred for 3 h in the dark at room temperature. The modified chitosan was then precipitated in 1 L of 0.1 M sodium hydroxide and filtered. The resulting product was redissolved and purified by dialysis against deionised water in the dark to remove any unreacted FITC before lyophilisation. FITC-chitosan was kept wrapped in aluminum foil to avoid exposure to light and stored in a fridge for further use.

2.4. Particle size and zeta potential analysis

The size of liposomes, their polydispersity index (PDI) and zeta potential values were determined using dynamic light scattering (DLS) with a Zetasizer Nano-ZS (Malvern Instruments, UK). Each formulation was diluted 100-fold with ultrapure water. A typical liposome refractive index of 1.45 and absorbance of 0.1 were used in all measurements. Each sample was analysed three times at 25 °C and the mean ±