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Research paper

On the synthesis of mucus permeating nanocarriers

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ABSTRACT

The synthesis of nanocarriers with "slippery" surface (i.e., poly(lactide-co-glycolide)–polyethylene glycol (PLGA–PEG) nanoparticles (NPs) and polyelectrolyte complexes (PECs) of polyacrylic acid (PAA) with poly-L-lysine (PLL) and/or polyarginine (PArg)) and of nanocarriers (i.e., PLGA NPs, PLGA–PEG NPs, liposomes) containing a mucolytic agent (i.e., 4-mercaptobenzoic acid (4MBA)) is presented. Depending on the molecular weight (MW) of PEG (i.e., 2, 5 kDa), PLGA–PEG NPs with a "brush" or "dense brush" PEG configuration were prepared. The PLGA–PEG NPs exhibited increased mucus permeability in comparison with non-pegylated PLGA NPs when tested in fresh porcine intestinal mucus. The NPs that were prepared using PEG with a MW equal to 5 kDa and had a "dense brush" PEG configuration, were found to exhibit the highest mucus permeability. The average size and the surface charge of PECs could be effectively tuned by varying the PAA/polycation charge ratio, thus resulting in the synthesis of neutral as well as positively and negatively charged PECs. The PECs with negative surface charges were found to exhibit the highest mucus permeability followed by the neutral and finally the positively charged PECs. Depending on the initial concentration of the mucolytic agent, 4MBA loadings up to 13.65, 13.1 and 18.43 wt% were achieved for PLGA NPs, PLGA–PEG NPs and liposomes, respectively. PLGA and PLGA–PEG NPs were characterized by a rapid release of the mucolytic agent (i.e., >80 wt% of 4MBA was released in 20 min) whereas, its encapsulation in liposomes allowed a more controlled release (i.e., up to 30 wt% of 4MBA was released in 45 min). 4MBA loaded liposomes were found to exhibit increased mucus permeability depending on the composition of the phospholipid bilayer.

1. Introduction

Apart from oral vaccination where the targeted Peyer's patches are not coated by a mucus gel layer, the development of nanoparticulate delivery systems for the mucosal (i.e., oral, nasal, pulmonary, vaginal, etc.) administration of macromolecular drugs (e.g., therapeutic peptides, DNA-based drugs) needs to take into account the crossing of the mucus gel barrier. Today, none of the available nanoparticulate delivery systems is capable of permeating the various mucus gel layers in significant quantities due to a nanoparticle cut-off size of 55–100 nm. Thus, in most cases, the existing nano-delivery systems do not exhibit sufficiently high drug bioavailabilities to justify their commercialization. Therefore, present research efforts are directed toward the development of effective nanocarrier design strategies for the development of mucus permeating drug delivery systems. Promising approaches comprise virus-mimicking nanocarriers with "slippery" surface and nanocarriers allowing the controlled release of mucolytic agents.

Many viruses with a densely charged, yet net neutral surface are capable of diffusing through a mucus layer. Their high surface charge density likely creates a hydrophilic surface that decreases its hydrophobic interactions with mucus and, thus, minimizes the virus entrapment in the mucus layer [1]. By combining high charge density anionic (e.g., polyacrylic acid) and cationic (e.g., poly-L-lysine, polyallylamine) polymers, the formation of polyelectrolyte complexes (PECs) with a "slippery" surface could be achieved. At low ionic strength, the main driving force of the complexation process is the gain in entropy, caused by the release of the low-molecular weight counterions, initially bound to the polyelectrolyte backbone. Hydrogen bonding or Van der Waals interactions are other types of interactions which can also contribute to the ion-pairing process [2–4]. Apart from nanoparticles with a densely charged, yet net neutral surface, an uncharged nanoparticle may be also considered as mucoinert as viral capsids, provided it is sufficiently hydrophilic with a low hydrogen bonding

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capability (e.g., polyethylene glycol (PEG), cyclodextrin coatings) [1,5]. Wang et al. [5] covalently modified the surfaces of polystyrene nanoparticles with CH_3O-PEG-NH_2 of various molecular weights (MW = 2, 5, 10 kDa) and showed that low PEG MW and high PEG surface coverage are both required for rapid mucus penetration of coated NPs. More specifically, Wang et al. [5] observed that there exists a critical MW between 5 and 10 kDa where dense PEG coatings transition from being mucoinert to mucoadhesive. Cu and Saltzman [6] also demonstrated that the incorporation of PEG onto the surface of PLGA particles can improve their diffusion in cervical mucus and that the diffusion is dependent on PEG MW and density. More recently, Yu and coworkers [7] presented novel biodegradable mucus-penetrating particles (MPP) based on PLGA–PEG. These NPs were found to diffuse rapidly through fresh, undiluted human CVM with an average speed only 8-fold lower than their theoretical speed in water. With respect to polyelectrolyte complexes, Laffleur et al. [8], prepared neutral polyacrylic acid/polyallylamine (PAA/PAM) PECs with a diffusion efficiency in intestinal mucus 2.5 and 18-fold higher than PAM and PAA NPs respectively.

Mucolytic agents are commonly used to reduce the bulk viscoelasticity of cystic fibrosis (CF) sputum by cleaving sputum constituents and, therefore, improve sputum clearance and lung function [9]. Commonly used mucolytic agents include recombinant human DNase (rhDNase, also known as dornase alpha or Pulmozyme®) and N-acetyl cysteine (NAC; Mucomyst®). NAC, a mucolytic agent that cleaves disulfide bonds of mucin fibers, has been shown to reduce the viscosity of mucus/sputum in vitro and in vivo [10]. In vivo studies have shown that the diffusion of non-viral gene vectors across the hyper-viscoelastic sputum could be increased when co-administered with mucolytic agents (i.e., NAC or NAC + rhDNase), resulting in an improved gene expression [11]. Suk and co-workers [10] investigated whether NAC, in combination with polyethylene glycol (PEG) coated carboxyl-modified polystyrene (PS) nanoparticles, could synergistically enhance particle penetration across fresh undiluted CF sputum. It was shown that NAC facilitated the rapid diffusion of PEG-coated, mucoinert nanoparticles in CF sputum. However, in all these approaches, the mucus layer was nearly completely degraded due to the considerably high amounts of co-administered mucolytic agents. A promising approach could be based on the development of nanocarriers releasing comparatively low amounts of a mucolytic agent, as they move through the mucus gel layer, thus, enabling the local only disruption of the mucus. To reach this goal, controlled release of a mucolytic agent (e.g., NAC, glutathione, 4-mercaptobenzoic acid) needs to be achieved.

This paper addresses the development of nanocarriers with "slippery" surface (i.e., PLGA–PEG NPs, and PECs consisting of polyacrylic acid (PAA) and poly-L-lysine (PLL) or polyarginine (PArg)) and nanocarriers (i.e., PLGA NPs, PLGA–PEG NPs, liposomes) loaded with the mucolytic agent 4-mercaptobenzoic acid (4MBA). The PLGA–PEG NPs were prepared by the method of double emulsion following the synthesis of PLGA–PEG copolymers via a carbodiimide reaction between PLGA (e.g., RG502H, RG752H) and various types of functional PEG (e.g., NH_2-PEG-NH_2 (MW 3 kDa), CH_3O-PEG-NH_2 (MW 2 and 5 kDa)). The effect of PLGA (i.e., MW, lactide:glycolide ratio) and PEG (i.e., MW, functional groups) type on the conjugation efficiency of PEG as well as on the properties of the produced NPs (i.e., average size, encapsulation efficiency of the mucolytic agent, release profile of 4MBA from the nanoparticles, mucus permeability) was examined for the first time. Polyelectrolyte complexes (e.g., PAA/PLL, PAA/PArg) were synthesized by ionic complexation. The effect of various process parameters (e.g. PAA/polycation charge ratio, polyelectrolyte concentration, PAA molecular weight, mixing order) on the PECs size and zeta potential was examined. Pegylated and non-pegylated liposomes containing 4MBA were prepared by the hydration/extrusion method. The effect of the liposome composition (i.e., lipid combination and molar ratios) on the properties of the produced liposomes (i.e., average size, encapsulation efficiency of the mucolytic agent, release profile of 4MBA from the liposomes, mucus permeability) was examined. The ability of the PLGA–PEG NPs, PAA/PLL complexes and 4MBA loaded liposomes to diffuse through fresh porcine intestinal mucus was experimentally assessed. To our best of knowledge, experimental results on the ionic complexation of PAA with PLL or PArg and on the encapsulation of 4MBA in PLGA or PLGA–PEG NPs and liposomes have not been reported in the open literature.

2. Materials and methods

2.1. Materials

PLGA (Resomer RG752H, MW: 4–15 kDa; RG502H, MW: 7–17 kDa), PEG (NH_2-PEG-NH_2, MW: 3 kDa; CH_3O-PEG-NH_2, MW: 2 and 5 kDa), PAA (MW: 100, 250 kDa), PLL (linear PLL with MW: 3–15 kDa), PArg (MW: 15–70 kDa), polyvinyl alcohol (PVA) (MW: 30–70 kDa, 87–90% hydrolyzed), ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS, 10x, pH 7.4), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide 98% (NHS), N,N-diisopropylethylamine purified by redistillation 99.5%, sodium phosphate dibasic, sodium phosphate monobasic, sodium hydroxide, 4-mercaptobenzoic acid (4MBA), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, phase transition temperature T_m = 41 °C), 1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DMPG, T_m = 23 °C), didecyldimethylammonium bromide (DDAB, T_m = 16 °C), cholesterol (CHOL) and fluorescein diacetate (FDA) dye were purchased from Sigma–Aldrich (Vienna, Austria). PAA (MW: 1.8, 50 kDa) was purchased from Polysciences Europe GmbH (Eppelheim, Germany). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC, T_m = 23 °C) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were of analytical grade and commercially available. Dialysis sacks (MWCO 12 kDa) were also purchased from Sigma–Aldrich (Vienna, Austria). Purified porcine intestinal mucus was kindly provided by the Institute for Cell and Molecular Biosciences, Newcastle University (Newcastle upon Tyne, United Kingdom).

2.2. Synthesis and characterization of PLGA–PEG copolymers

PLGA–PEG copolymers were synthesized via the carbodiimide method [12,13]. More specifically, NHS and EDC were added into 2 mL of a PLGA (e.g., RG752H, RG502H) solution in anhydrous dichloromethane (200 mg/mL), at molar ratios equal to 1:10, in order to activate the carboxyl acid groups of PLGA and convert it to the semi-stable amine-reactive activated NHS-ester (PLGA-NHS). The reaction mixture was magnetically stirred overnight at room temperature under nitrogen. PLGA-NHS was collected by precipitation with cold diethyl ether. The NHS-ester was subsequently dissolved in anhydrous dichloromethane, re-precipitated with diethyl ether and washed in cold diethyl ether and methanol (50:50) in order to remove excess of EDC, NHS and remaining by-products. It was then dried under vacuum and lyophilized (*ScanVac Freezedryers CoolSafe 55-9*). PLGA-NHS (100 mg/mL) was subsequently reacted with PEG (i.e., NH_2-PEG-NH_2, CH_3O-PEG-NH_2 in molar ratios 1:1, 1.2:1, 2:1) in 2 mL of anhydrous chloroform in the presence of N,N-diisopropylethylamine (PEG:DIPEA molar ratio equal to 1:2). The reaction mixture was kept under magnetic stirring for 24 h at room temperature. PLGA–PEG was collected and purified by three successive precipitation–centrifugation

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