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One-pot polyglycidol nanogels *via* liposome master templates for dual drug delivery

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ABSTRACT

Polyglycidol-based nanohydrogels (nHGs) have been prepared by optimizing the use of liposome master templates resulting in a high-yielding and more practical one-pot process to provide materials capable of carrying drugs of adverse chemical nature. The nanogels prepared with the one-pot method showed favorable kinetics for the release of either Nile Red (NR) or lysozyme (LYS), loaded with gel precursors such as semi-branched poly(glycidol allylglycidyl ether), PEG dithiol (1KDa), a free radical initiator and liposomal lipids at the liposome formation step. This process is superior to a comparable step-wise traditional approach and circumvents loading of the gel precursors with the hydrophilic drug into preformed liposome templates. A thiol-ene crosslinking reaction accomplishes the formation of the nanonetwork resulting in nHGs prepared in the traditional step-wise (nHG-SW) approach and the one-pot (nHG-OP) process. Both nanogel networks were characterized in terms of particle size and zeta (ζ) potential with average values of 148 nm $\pm$ 39 nm and -25.9 mV $\pm$ 9.2 for the nHG-SW and 132 nm $\pm$ 32 and -23.1 mV $\pm$ 9.7 for the nHG-OPs. Loading efficiency for both of the nanogels with NR was determined by spectrophotometry to be 28% (nHP-SW) and 31% (nHP-OP). The LYS loading was based on the target loading of 10 μg/mg for both nanogels found to be 84% and 86% for the nHG-SW and nHP-OP, respectively. As proof of concept for combination drug delivery, the *in vitro* release of both drug mimics, NR and LYS, were monitored under physiologically relevant conditions by an optimized dialysis method. The implementation of the multi-functional and semi-branched polyglycidol is recognized as the main contributor for the observed highly controlled release of proteins that are otherwise rapidly released from common PEG-based nanogel networks. Furthermore, the one-pot process led to be the most favorable drug delivery system based on the release kinetics pointing to a denser polymer network.

1. Introduction

Combination therapy using dual synergistic drug delivery systems has become a leading approach for treating malignant and drug-resistant cancers [1]. The inability of chemotherapy to eradicate cancer cells can be due to rapid mutations within subgroups of tumor cells which evade cytotoxic drugs [2]. It is well known that a tumor suppresses anti-cancer immune responses within its microenvironment in order to facilitate growth, progression, and metastasis [3]. On the other hand, protein therapeutics, such as cytokines and antibodies, can induce an effective anticancer response by stimulating the immune system. A combined chemo-immunotherapy approach can promote synergy against cancer cells and suppress drug resistance through unique mechanisms of action [4]. Despite promising recent clinical and experimental results, anti-cancer efficacy is still sub-optimal due to short drug and protein half-lives, systemic toxicity, and divergent *in vivo* pharmacokinetics and distribution [5].

Polymeric nanotechnology has opened up unprecedented opportunities to develop controlled delivery devices, which has resulted into a number of benefits to deliver small molecule chemotherapeutic drugs. For example, prolonged blood circulation and tumor accumulation can be achieved by the enhanced permeability and retention (EPR) effect of particles between 10 and 150 nm in diameter to yield more favorable pharmacokinetic profiles. Alternatively, the delivery system can provide enhanced efficacy by utilizing targeting peptides [6]. Despite the versatility of known nanoparticle delivery carriers such as liposomes, star polymers, micelles, nanosponges and PLGA particles, the dual and co-delivery of multiple therapeutic agents, such as hydrophilic large biologicals with small hydrophobic chemotherapeutics from the same nanocarrier can impose challenges. Contributing factors include varying size dimensions of the therapeutics and their adverse solubility [7]. Current designs either separate two drugs in a core-shell structure like in hyaluronic acid (HA) nanogel-enveloped liposomes [8], or use a

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double-emulsion technique with spatial co-localization of oil-water droplets within a larger surfactant stabilized carrier [9]. Designs of delivery systems with no spatial separation of the two therapeutics include porous silicon nanoparticles [10], or lipogels [11], also known as liposome-enveloped nanogels [12] .

A common theme in all of these systems is the implementation of traditional carriers already tested for a small molecule delivery which are improved and modified for a co-delivery application. One of these traditional carriers are liposomes, which are prepared in a facile process and feature a high stability. However, some of the reported disadvantages are prolonged circulation times and incomplete or rapid release rates of therapeutics [13]. Although HA nanogel-enveloped liposomes are recognized as novel drug delivery systems, the liposome component is still the dominant factor in the observed release kinetics, mainly due to the intact liposomal drug delivery core. However, more recently, liposomes are employed as suitable templates to develop carriers, such as liposome-enveloped nanogels or lipogels in which the incorporated nanogel structure plays a greater role in the overall improved release characteristics of this liposome based nanocarrier. One example from the Fahmy group prepares a nanogel from a linear crosslinker (PLA-PEG-PLA) in which the cytokine and the drug loaded acrylate-modified cyclodextran forms a unique incorporated gel using UV-light illumination to achieve the crosslinking of the acrylate functional endgroups [27]. The small molecule and the cytokine delivery kinetics correspond still to a liposomal system but are influenced by the nanogel core component to achieve a more controlled release of the two therapeutics. It has to be noted that the exposure to UV-light and the generated free radicals do not seem to impair the activity of the incorporated cytokines because a high efficacy in *in vivo* studies was observed. The lack of versatile, hydrophilic nanogel components that can be shuttled into liposome templates and provide tailored crosslinking densities for a more controlled release of all types of therapeutics is limiting the clinical relevance for some of these nanogels.

In this work, we sought to utilize a liposomal carrier to form a dual delivery system and aim for a final product with unique features with no resemblance to the native carrier. In other words, the novel system attributes all its features from a created nanonetwork using liposomal templates which are significantly altered in the process. This will be accomplished for one, through the design and synthesis of tailored hydrophilic macromolecular building blocks such as functionalized semi-branched polyglycidols, and also through a developed one-pot technique. We report on the formation and characterization of a novel nanohydrogel (nHG) platform using a thiol-ene click crosslinking system composed of unique allyl-functionalized semi-branched polyglycidols, poly(GLY/AGE), with PEG dithiol (1 kDa). Two preparation methods are presented, one that is in its methodology comparable with traditional stepwise approaches and uses a prefabricated liposome as master template (nHG-SW) to form nanogels, and a second approach in which liposomal lipids are brought together with all components in one pot (nHG-OP) to yield a carrier giving unique characteristics that are not resembling liposomal delivery systems.

2. Materials and methods

2.1. Materials

L-α-phosphatidylcholine (Egg-PC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, poly(ethylene glycol) dithiol (PEG dithiol, M_n 1000 g mol^{-1}), Nile red (NR, technical grade), lysozyme from chicken egg white, Allyl glycidyl ether (AGE), and (2-Hydroxypropyl)-β-cyclodextrin (HBC) were purchased from Sigma, USA. Glycidol (GLY) was also purchased from Sigma and purified *via* Kugelrohr distillation before use. VA-086, 2,2-Azobis(2-methyl-*N*-(2-hydroxyethyl)propionamide), 98% was purchased from Wako Chemicals USA, Inc. and used without further purification. *Micrococcus lysodeikticus* cells (M1) ATCC No. 4698 were purchased from Sigma, USA. HEPES buffer (1M Solution) was purchased from Corning (Corning, NY), diluted to 20 mM in DI water, pH adjusted to 7.4 and supplemented with 200 mM HCB for *in vitro* release studies. Phosphate buffered saline (PBS, 1×, pH 7.4) was obtained from Gibco by Life Technologies for lysozyme *in vitro* drug release studies. Dialysis tubing SnakeSkin® (molecular weight cutoff (MWCO): 10 kD, 16 mm dry I.D.), Spectra/Por® G2 Float-A-Lyzers® (1 mL volume, MWCO: 20 kD and 300 kD) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). All other solvents or reagents were purchased from Sigma Aldrich unless mentioned otherwise and used as received.

2.2. Characterization

^1H and ^{13}C NMR spectra of poly(glycidol allylglycidyl ether), poly(GLY/AGE), were obtained from a Bruker AV600 Fourier transform spectrometer with deuterated methanol as the solvent. Gel permeation chromatography-size exclusion chromatography (GPC-SEC) of the GLY/AGE copolymer was performed in DMF (with 1 mg/mL LiBr) at 45 °C with a flow rate of 1.0 mL/min (Waters 1525 binary HPLC pump); columns: 7.8 × 300 mm; Styragel HR 5 DMF, Styragel HR 4E, and Styragel HR 3: molecular weight range 50,000 to 4 × 106, 50 to 100,000, and 500 to 30,000 g/mol. GPC detection was accomplished using a Waters 2414 refractive index detector at 410 nm. Molecular weights (M_n and M_w) and polydisperisity were determined from PEG standards provided by Varian. We documented in SEC-MALDI correlation studies that PEG standards are also applicable for the semi-branched polyglycidols in this contribution (See ESI section 7). The average size and size distribution (polydispersity index, PDI) of the nanogels were analyzed *via* dynamic light scattering (DLS), and the zeta potential (ζ) was determined *via* analysis of electrophoretic light scattering on a Malvern Zetasizer Nano ZS apparatus with Malvern Instruments DTS software (v.6.0d1) (Malvern Instruments, UK). Measurements were collected on solutions of nanogels and liposomes in DI water at 25°C. Temperature stability measurements of nanogels were conducted in PBS (1×, pH 7.4) at varying temperatures. The mean hydrodynamic diameter (d_h) was computed from the intensity of the scattered light using Malvern software based on Brownian motion and the Stokes-Einstein equation. Transmission electron microscopy (TEM) images were captured using a FEI Technai Osiris FEI operating at 200 kV. Grids were prepared by dipping an Ultrathin Carbon Type-A 400 Mesh Copper Grid (Ted Pella, Redding, CA) into the sample solution (0.01 mg/mL) twice, followed by addition of 1 drop (~3 μL) of 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) to the carbon side of grid, and excess solution was wicked away after 3 min using a piece of filter paper. Grids were allowed to dry for 3 h on the benchtop at room temperature before imaging. Nile red was detected *via* UV–vis absorbance at 550 nm (DMSO) or 580 nm (HBC-HEPES media) after 2 μL of sample was loaded on a NanoDrop 2000c spectrophotometer (Thermo, Wilmington, DE, USA) using instrument software NanoDrop 2000/2000c (v1.4.1). Blank samples of solvent, liposome and nanogels were analyzed to ensure no other compounds absorbed in this region. Lysozyme was quantified with Pierce BCA® Assay and a Synergy HT Microtiter Plate reader (BIOTEK) at 562 nm. The retained activity of lysozyme was determined by an activity assay with a protocol adapted from Worthington (see Supplementary Information). The cytocompatibility of liposomes and the nHG-OP was performed according to a protocol described in the Supplementary Information.

2.3. Synthesis of poly(glycidol allylglycidyl ether)

Copolymerization of allyl glycidyl ether (AGE) and glycidol (GLY) was performed by cationic ring-opening polymerization with a 25/75 feed of AGE/GLY. $Sn(OTf)_2$ (5.2 mg; 8.52×10^{-6} mol; 0.00035 eq) and 3-methyl butanol (54 μL; 3.33×10^{-4} mol; 0.066 eq) were added to a N_2-purged, flame dried 25-mL round bottom reaction flask with small magnetic stir bar and lowered in to an ice bath at 0 °C. After 15 min of