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Active drug encapsulation and release kinetics from hydrogel-in-liposome nanoparticles

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ABSTRACT

Herein, we demonstrate for the first time the use of hydrogel-in-liposome nanoparticles (lipogels) as a promising drug delivery vehicle for the active encapsulation of the anticancer drug 17-DMAPG, a geldanamycin (GA) derivative. This model drug was chosen due to its improved aqueous solubility (4.6 mg/ml) compared to the parent GA (<0.01 mg/ml), and presence of a tertiary amine which readily protonates at low pH. For the design of lipogels, a PAA hydrogel core was formed inside liposomes through UV-initiated DEAP activation and polymerization of AA and BA. We have demonstrated here that electrostatic interactions between drug and gel are critical for active encapsulation and sustained release of 17-DMAPG. We found that optimal loading conditions could be obtained (88% loading efficiency) through control of pH, temperature and incubation time. Dramatic sustained drug release from lipogels was achieved independent of the external solution pH (ca. 54 h to 50% drug release) and confirmed that the lipid bilayer was intact in the presence of the gel core. *In vitro* cell culture studies revealed that at the highest concentration tested, which corresponded to approximately 0.4 mg/ml of material, lipogels did not exert cytotoxicity to cells.

1. Introduction

Liposomes as a template for the preparation of size-controlled hydrogel nanoparticles have recently been reported in the literature [1–14], and show great potential for further development in various biomedical fields [9]. In the few works where such hydrogel nanoparticles have been investigated for drug delivery applications, proteins such as bovine serum albumin (BSA) and lysozyme [13], hemoglobin [6,7], IL-2 [14], or the small molecule TGF-β inhibitor drug [14] relied on the passive encapsulation method for loading into the nanogel systems. Herein, we demonstrate for the first time the use of hydrogel-in-liposome nanoparticles as a promising drug delivery vehicle for the active encapsulation of the anticancer drug 17-DMAPG, a geldanamycin (GA) derivative with enhanced aqueous solubility [15,16].

Liposomes for drug delivery and their preparation techniques are well known in the literature; briefly, liposomes are nanoparticles with an aqueous core surrounded by one or more outer shell(s) consisting of lipids arranged in a bilayer configuration [17]. Liposomes can be prepared by a variety of methods and are classified into various categories based on size and lamellarity: multilamellar vesicles (MLVs, >500 nm), large unilamellar vesicles (LUVs, 100–500 nm), and small unilamellar vesicles (SUVs, <100 nm) [18]. Hydrophilic small molecule drugs are typically passively loaded into liposomes, meaning that drug encapsulation occurs during liposome formation [19]. On the other hand, amphiphilic drugs can be actively encapsulated into liposomes, meaning after liposome formation. Passive encapsulation relies on the ability of liposomes to entrap aqueous buffer containing the water soluble molecule during vesicle formation. Such hydrophilic molecules typically cannot easily cross the lipid bilayer, and this allows for some control over the release kinetics. A major disadvantage of passive encapsulation is that drug loading is typically limited by the captured volume (20–30% maximum) of liposomes and solubility of the drug of interest [18,20]. Conversely, active encapsulation relies on the molecule crossing through the lipid bilayer into the aqueous core, where it then becomes entrapped and cannot diffuse back out. For example, some drugs, lipids, and peptides can be actively entrapped into liposomes through the use of pH gradients with very high loading efficiencies [21,22].

Relatively little is known about the properties of active drug loading into liposomes containing a hydrogel core. Hydrogels, in general, are cross-linked networks of hydrophilic polymers that can adsorb large amounts of water. The cross-linking density of these polymers creates pores and typically allows for loading and controlled diffusion of water soluble drugs [23]. There have been a variety of hydrogel-based delivery systems reported over the years for loading and

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release of small molecule and macromolecular drugs *in vitro* [24–30]. However, hydrogel nanoparticles with unsuitable mechanical properties or poorly-defined dimensions [31] severely limit their usage *in vivo* due to the requirement for sustained release of drugs and a long circulation time in the body; most hydrogel-based drug delivery systems work better for topical applications [32,33]. One advantage of hydrogels for controlled drug release includes their responsiveness to external stimuli. For example, pH-sensitive hydrogels are typically characterized by a variable swelling ratio (weight of adsorbed water to the weight of the sample at a dry state) dependent on pH [34]. This on–off swelling state results from electrostatic repulsions between polymer chains in the hydrogel network [35]. For example, poly(acrylic acid) (PAA) was characterized by a larger swelling ratio at higher pH due to the de-protonation of carboxylic groups, which led to electrostatic repulsions between polymer chains and caused the gel to become more porous to water molecules [36]. When BSA was loaded into PAA hydrogel nanoparticles, it was characterized by a pH-dependent release profile [37]. Similarly, when the cationic anticancer drug doxorubicin (DOX) was loaded into hydrogel microspheres composed of anionic poly(methacrylic acid), drug release was triggered by either a pH change or through addition of excess cations to the solution [38]. pH plays a very important role and can therefore favorably influence hydrogel properties, promote drug interactions with the polymer chains, and influence release kinetics.

In this work, PAA hydrogel in liposomes is of particular interest since a pH gradient and electrostatic/hydrophobic interactions between cationic drug and anionic gel in the liposomal core is used for the active encapsulation of 17-DMAPG. As a model drug, 17-DMAPG was chosen due to (1) its improved aqueous solubility (4.6 mg/ml) compared to the parent GA (~0.01 mg/ml) [16] and (2) presence of a tertiary amine which readily protonates at low pH. It was found that under optimized conditions, consistent drug loading and sustained release from these hydrogel-in-liposomes (lipogels) could be achieved. Whereas previous reports removed the lipid bilayer after forming the hydrogel nanoparticles in the liposomes [39,40], the inclusion of the lipid bilayer here is critical for sustained release and potential surface modifications (i.e. addition of PEG or targeting ligands). This work describes for the first time the preparation of well-defined lipogels for active loading of the model drug 17-DMAPG, its characterizations and release kinetics, and resulting cytotoxicity of the nanoparticles on cancer cells cultured *in vitro*.

2. Experimental

2.1. Materials

Sodium ascorbate, acrylic acid (AA), *N,N′*-methylenebis(acrylamide) (BA), 2,2-diethoxyacetophenone (DEAP), 3-(dimethylamino)-1-propylamine and Triton X-100 were purchased from Sigma–Aldrich. Geldanamycin (GA) was obtained from LC Laboratories. The pH-sensitive fluorescent dye Oregon Green 514 was purchased from Life Technologies. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol were purchased from Avanti Polar Lipids. Thin-layer chromatography was performed using DC-Alufolien Kieselgel 60 F_{254} plates (EMD Chemicals, Darmstadt, Germany) and visualization was achieved by UV light (254 nm) and a ceric molybdate stain activated by heat. For flash chromatography, silica gel 40–63 μm from EMD Chemicals (Darmstadt, Germany) was used. ^{1}H NMR spectra were acquired on Varian Inova 500 spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane, and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). High-resolution mass spectra were obtained on an IonSpec 7 T HiResMALDI FT-Mass Spectrometer at the Analytical Instrumentation Center of University of Wisconsin School of Pharmacy. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBA) and 1% penicillin/streptomycin were purchased from Cellgro. The human PC-3 prostate and human MDA-MB-231 cancer cells were obtained from ATCC and cultured according to recommended protocols.

2.2. Synthesis of 17-[3′-(dimethylamino)-1′-propyl]-amino-17-demethoxygeldanamycin (17-DMAPG)

The water soluble 17-DMAPG was synthesized from GA with 95% yield [15]. Briefly, GA (56 mg, 0.1 mmol) was dissolved in dichloromethane (4 ml) and 3-(dimethylamino)-1-propylamine (25 μl, 0.2 mmol) was added. The reaction mixture was stirred at room temperature for 24 h and TLC analysis revealed complete conversion of the starting material to product. The reaction mixture was loaded onto a silica gel column and eluted with dichloromethane–methanol (stepwise gradient from 95:5 to 90:10). Fractions containing 17-DMAPG were concentrated, and the resulting purple solid was precipitated with hexane (60 mg, 95%). ^{1}H NMR: 9.21 (s, 1H), 7.77 (s, 1H), 7.24 (s, 1H), 6.96 (d, J = 11.4 Hz, 1H), 6.59 (d, J = 11.4 Hz, 1H), 5.92 (d, J = 9.3, 1H), 5.86 (t, J = 10.8, 1H), 5.19 (s, 1H), 4.86 (br s, 2H), 4.51 (br s, 1H), 4.31 (d, J = 10.1 Hz, 1H), 3.77–3.70 (m, 1H), 3.64–3.56 (m, 2H), 3.48–3.44 (m, 1H), 3.37 (s, 3H), 3.27 (s, 3H), 2.78–2.70 (m, 1H), 2.66 (d, J = 13.8 Hz, 1H), 2.45 (t, J = 6.1 Hz, 2H), 2.43–2.36 (m, 1H), 2.27 (s, 6H), 2.03 (s, 3H), 1.80 (d, J = 1.3 Hz, 3H), 1.84–1.68 (m, 5H), 1.00 (d, J = 6.9 Hz, 3H), 0.97 (d, J = 6.4 Hz, 3H). HRMS (ESI): calcd for $C_{33}H_{51}N_4O_8$ $[M + H]^+$ 631.37014, found 631.37199; calcd for $C_{33}H_{50}N_4O_8Na$ $[M + Na]^+$ 653.35209, found 653.35312.

2.3. Preparation of lipogels and nanogels

To prepare lipogels, 80 μmol DPPC and 50 μmol cholesterol were dissolved in chloroform. The solvent was evaporated on a rotary evaporator under vacuum. The resulting dried lipid film was rehydrated with 1 ml of hydrogel precursor solution (100 μl AA, 10 mg BA and 1 μl DEAP dissolved with 900 μl ddH_2O) in a 55 °C water bath with vigorous shaking for 30 min. The resulting multilamellar vesicles were extruded 11 times through a 100 nm polycarbonate filter via a mini-extruder (Avanti Polar Lipids) to generate LUVs. Sodium ascorbate was added to the solution at a molar ratio of 200:1 relative to DEAP. After 15 min of UV irradiation (Black Ray UV lamp, 365 nm, 100 W), polymerization of monomers in liposomes was nearly complete and lipogels of ca. 100–120 nm diameter were obtained. Polymerization outside liposomes was inhibited by the presence of ascorbic acid, a scavenger of free radicals [41]. Next, the lipogel suspension was dialyzed against deionized water to remove un-encapsulated hydrogel precursors and ascorbic acid. Lipogels were concentrated via ultracentrifugation and re-suspended in desired buffer.

To prepare nanogels without the liposomal bilayer, the surfactant Triton X-100 was added to lipogels at a 50:1 surfactant/lipid molar ratio. The solution was heated to 90 °C until it became cloudy (indication that the solution reached the cloud point of the surfactant), then allowed to cool to room temperature. DPPC and cholesterol preferentially form micelles with Triton X-100 at this concentration [4,8], leading to removal of the lipid bilayer from the nanogel core. This mixture was then eluted through a PD-10 column (used as a size exclusion chromatography column) to separate micelles from the resulting nanogels. Fractions were collected for analysis.