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Development of a diagnostic polymersome system for potential imaging delivery

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ABSTRACT

In order to enhance visualization of soft tissues, a dual-imaging diagnostic polymersome system featured with highly hydrated multilamellar wall structure capable of simultaneously embedding a hydrophobic near-infrared fluorophore, Cy5.5, and a paramagnetic probe, gadolinium (Gd(III)) cations was developed. The polymersomes were obtained from the self-assembly of lipid-containing copolymer, poly(acrylic acid-co-distearin acrylate), in aqueous solution. The Cy5.5 and Gd(III) species were loaded into polymersomes *via* hydrophobic association (loading efficiency of Cy5.5 *ca* 74%) and electrostatic complexation (Gd(III) 83%), respectively. The Cy5.5/Gd(III)-loaded polymersomes (CGLPs) have shown excellent payload confinement, reduced dilution effect on assembly dissociation and decreased protein/salt-induced colloidal aggregation. Owing to the highly hydrated structure of vesicular membrane, the superior contrast enhancement of CGLPs in magnetic resonance (MR) imaging was obtained as a result of prolonged rotational correlation time of Gd(III) cations and fast water exchange from Gd(III) to bulk solution. The CGLPs exhibit a 15-fold higher longitudinal relaxivity value (*ca* 60 mM^{-1} s^{-1}) than that (4 mM^{-1} s^{-1}) of the commercial contrast agent, Magnevist, in phosphate buffered saline. The *in vivo* characterization demonstrates that CGLPs exhibit a signal-to-noise ratio in T_1-weighted MR image contrast similar to that of Magnevist, yet with a Gd dose 5-fold lower. An excellent contrast in NIR imaging at tumor site was attained following the intravenous injection of GGLPs into Tramp-C1 tumor-bearing mice (C57BL/6). Along with their non-toxicity at the dose used, these results demonstrate the great potential of the CGLPs as an advanced diagnostic nanodevice.

1. Introduction

For improving cancer therapy efficacy, imaging contrast agents are usually required to assist evaluating disease states and highlight differences between normal and abnormal tissues prior to the therapy process [1–4]. Along with the advance in medical imaging technology, a variety of noninvasive imaging modalities have been utilized to facilitate the early detection of cancer [5]. Magnetic resonance (MR) imaging is one of the powerful and non-invasive imaging techniques to obtain high resolution, three-dimensional body images [6–8]. However, small gadolinium (Gd) ion complexes serving as T_1-weighted contrast agents in clinical MRI diagnosis frequently suffer from rapid renal clearance, non-specific tissue accumulation, and low contrast efficiency. The use of supramolecular Gd(III) complexes in MR imaging applications has shown many practical advantages, such as high contrast in blood-pool imaging [9] and long blood circulation half-life [10], over that using small Gd(III) complexes [11]. Another popular imaging technique is optical imaging that is often utilized for accurately detecting the accumulation of nanoparticulate delivery systems at target sites by using a near-infrared (NIR) fluorophore with emission wavelengths larger than 620 nm where the auto-fluorescence is minimized [12]. Nevertheless, there are still several limitations in every modality such as low sensitivity, low spatial resolution and inaccurate diagnosis due to undesired accumulation of diagnostic agents to non-target organs [13]. Multi-modal imaging delivery systems bearing each individual benefits may be advantageous in overcoming the limitations associated with the stand-alone systems [14].

To develop a multi-modal imaging delivery system, polymer assemblies are attractive due to their unique assembly

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architectures wherein the hydrophobic domains can serve as a depot for hydrophobic cargos while ionic payloads can be entrapped within oppositely charged polyelectrolyte outer layer by electrostatic complexation. The polymer assemblies can thus readily act as a supramolecular contrast agent by pertinent loading with paramagnetic components or optical luminescent probes within assembly structures. In our previous works [15–18], the pH-responsive polymersomes obtained from spontaneous assembly of the lipid-containing copolymers, poly(acrylic acid-co-distearin acrylate) (poly(AAc-co-DSA)) have shown the capability of loading cationic species into polymeric vesicle membrane *via* charge interaction with ionized AAc residues. With a high negative charge density within polymer multilemellar walls, hydrophilic cationic species can preferentially penetrate into the interior structure of vesicle membrane and subsequently attach onto hydrated vesicular multilayers by virtue of ionic complexation with ionized AAc residues.

Herein, we propose a novel dual-imaging diagnostic polymersome system in which excellent NIR/MR imaging contrasts are attained by NIR fluorescence-emitting Cy5.5 and paramagnetic Gd(III) species. The hydrated multilamellar structure renders the Cy5.5/Gd(III)-loaded polymersomes (CGLPs) from poly(AAc-co-DSA) capable of firmly confining diagnostic payloads by hydrophobic interactions of close-packed lipid bilayer islets with Cy5.5 and electrostatic association of ionized AA residues within hydrated interlayer with Gd(III) (Fig. 1). The multilamellar assembly architecture of CGLPs also exhibits several characteristics pertinent to enhanced colloidal stability against large volume dilution and protein/salt-induced aggregation, prolonged rotational correlation time of the embedded Gd(III) cations, and promoted water exchange from Gd(III) to bulk solution.

2. Materials and methods

2.1. Materials

Synthesis and characterization of the poly(AAc-co-DSA) copolymer are described in detail in Supplementary Data (SD). $GdCl_3$ was obtained from Aldrich–Sigma. Human cervical tumor cell line (HeLa cell) was purchased from the Food Industry Research and Development Institute of Taiwan. The Dulbecco's modified Eagle medium (DMEM) was purchased from Invitrogen. AlamarBlue was purchased from AbD Serotec. Deionized water was produced by the Milli-Q Synthesis System (18 MΩ, Millipore). All chemicals are reagent grade and used as received.

2.2. Preparation of CGLPs

Poly(AAc-co-DSA) (10.0 mg) and Cy5.5 (400 μg) were dissolved in 1 mL THF. The Cy5.5/copolymer solution (0.1 mL) was added dropwise into acetate buffer (pH 5.0, 3.0 mL, ionic strength 0.01 M). The mixture was subjected to ultrasonication at 25 °C for 10 min. After repeated ultrafiltration (Amicon 8010 with a Millipore PBMK membrane, MWCO 100k) with pH 5.0 acetate buffer to remove THF, the polymer solution was concentrated to a final concentration of *ca* 1.67 mg/mL. The Cy5.5-loaded polymersomes were thus attained. The aqueous solution of $GdCl_3$ (0.01 M, 62 μL) was added directly into the polymersome suspension (pH 7.4, 2.0 mL) to a final concentration of 2×10^{-4} M. The suspension was then stirred at 4 °C for 12 h to complete the Gd(III) loading process. To eliminate the unbound Gd(III) species, the CGLP suspension was further dialyzed (Cellu Sep MWCO 12,000–14,000) against pH 5.0 buffer for 3 days. The loading quantification is described in SD.

2.3. Laser light scattering measurements

In this study, the ratio of the gyration and hydrodynamic radii (R_g/R_h) of the polymer assemblies was used to assess the assembly morphology as a function of pH. The medium pH was adjusted by either NaOH or HCl aqueous solution. The mean hydrodynamic radius (R_h) was attained by dynamic light scattering (DLS) on a Brookhaven BI-200SM goniometer equipped with a BI-9000 AT digital correlator using a solid-state laser (30 mW, λ = 637 nm) at 90°. The CONTIN algorithm method was employed to confirm the absence of bimodal particle size distribution of polymeric assemblies with much enhanced reliability [16,19]. The weight-average molar mass (M_w) and R_g of polymeric assemblies were evaluated by static light scattering from the Berry treatment of the angular dependent measurements of the time-averaged intensity of scattered light for a series of concentrations of polymeric assemblies (75–120 μg/mL) in aqueous suspensions [20,21]. The dn/dc values of the aqueous phases as the media to disperse polymer assemblies were obtained by a BI-DNDC differential refractometer (λ = 620 nm).

2.4. SAXS measurements

For the SAXS experiments, samples were run at beamline 23A1 at the National Synchrotron Radiation Research Center, Hsinchu, Taiwan. The energy of X-ray source was 14 keV. A small amount of the polymersome suspension (10.0 mg/mL) at the preset pH was placed in the center of a steel washer and closely sealed with Kapton tape and run for 5 min. The scattering signals were collected by MarCCD detector with a pixel resolution of 512 × 512. The scattering intensity profile was depicted from the scattering intensity, $I(q)$, versus the scattering vector, $q = (4\pi/\lambda)\sin(\theta/2)$ (where θ is the scattering angle), after corrections for solvent background, sample transmission, empty cell transmission, empty cell scattering and the detector sensitivity [15].

2.5. The longitudinal relaxation time (T_1) measurements of CGLPs

The relaxation parameter (T_1) was determined by a relaxometer (Bruker Minispec mq20) at 37 °C. The inversion recovery method was adopted in order to obtain longitudinal relaxivity (r_1) from Eq. (1) shown below. The observed relaxation rate $(1/T_1)_{obs}$ of water protons is comprised of both diamagnetic and paramagnetic contributions. The paramagnetic contribution is linearly related to the concentration of paramagnetic species, in this event the Gd(III) cations present in the system [2,22].

$$\left(\frac{1}{T_1}\right)_{obs} = \left(\frac{1}{T_1}\right)_{dia} + r_1[\mathrm{Gd}] \quad (1)$$

The measurements were performed with CGLP suspensions in the pH 7.4 buffer and in phosphate buffered saline (PBS), respectively. The Gd(III) concentration in the CGLP suspension was determined by ICP-MS prior to longitudinal relaxation time measurements.

2.6. Cellular uptake and cytotoxicity

HeLa cells were cultured in DMEM with 1.0% penicillin–streptomycin (Pen–Strep) and 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C and 5% CO_2 for 2 days. Cells were harvested by addition of a 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution, re-suspended in DMEM, and placed in a 96-well plate (8000 cells/well) for 24 h prior to the cytotoxicity analysis. The culture medium was replaced with DMEM (10% FBS, 1% Pen–Strep) containing $GdCl_3$ or CGLPs at equivalent Gd(III) concentrations for 24 h at 37 °C. The medium was removed and