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Self-assembled nanoplatform for targeted delivery of chemotherapy agents via affinity-regulated molecular interactions

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ABSTRACT

Site-specific delivery of drugs while minimizing unwanted distribution has been one of the pursued goals in cancer therapy. In this endeavor, we have developed targeted polymeric nanoparticles called amphiphilic urethane acrylate nonionomer (UAN) for encapsulation of diverse water-insoluble drugs and diagnostic agents, as well as for simple and reproducible surface conjugation of targeting ligands. Using monoclonal antibodies or lymphocyte function-associated antigen-1 (LFA-1) I domain engineered for varying affinities to intercellular adhesion molecule (ICAM)-1, we were able to deliver UAN nanoparticles to human cancer cells with the efficiency dependent on the strength of the molecular interactions and the degree of ICAM-1 expression on cell surface. Compared to non-specific uptake of free drugs, targeted delivery of UAN nanoparticles carrying equal amount of drugs produced more potent cytotoxicity. Notably, without the targeting ligands attached, UAN nanoparticles were largely precluded from non-specific uptake by the cells, resulting in much lower toxicity. The versatility of our UAN nanoparticles in both payload encapsulation and presentation of targeting ligands may facilitate developing a robust platform for evaluating various combinations of cancer drugs and molecular interactions toward developing effective cancer therapy formulations.

1. Introduction

Nanoparticles are emerging as a powerful platform for delivery of imaging [1] and therapeutic entities [2], particularly due to their potential applications in cancer therapy for early detection, enhanced therapeutic potency, and reduced side effects [3]. For example, microspheres, liposomes, nanoshells, dendrimers, and biodegradable polymers have been utilized as specific drug delivery systems [4]. Compared to the conventional methods of delivering therapeutic agents directly via intravenous or enteral routes, or as molecular conjugates, nanoparticle-mediated delivery may protect the therapeutic agents from chemical and enzymatic degradation and circumvent the need for chemical modification of therapeutic agents, which often leads to reduced potency and stability of drugs [5,6]. Yet, a successful targeted delivery of nanoparticles needs to address the challenges in delivering hydrophobic payloads, controlling drug release, and accomplishing specific delivery while minimizing adverse immune response [7,8].

Recently, polymeric nanoparticles have been reported to exhibit characteristics of sustained release, effective solubilization of hydrophobic drugs, and reduced interactions with the reticuloendothelial system [6,9,10]. In order to achieve selective targeting by nanoparticles, targeting ligands are often attached to the surface of the particles. The high surface-to-volume ratio in these nanoparticles increases the surface density of ligands, leading to a multivalent, high affinity binding effect [11]. Specific targeting is additionally improved by the enhanced permeation and retention effect in tumor environments due to a leaky vasculature structure, allowing appropriately sized nanoparticles to extravasate [12]. Nanoparticles of certain size range (10–100 nm) have been shown to accumulate more readily in tumors through the retention effect compared to the larger ones, making them optimal for tumor penetration.

To take advantage of these unique characteristics of polymeric nanoparticles, we have developed a versatile nanoparticle platform using amphiphilic urethane acrylate nonionomer (UAN) [13] with two functional layers. The hydrophilic outer shell is coated with a high density of Nickel-nitrilotriacetic acid (Ni-NTA) for simple conjugation of tumor-specific targeting molecules [14] and the

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hydrophobic core has a large capacity for water-insoluble chemotherapy drugs and imaging agents, where the encapsulation process occurs through the diffusion of hydrophobic molecules from water into the hydrophobic core [14,15]. Through this approach, we demonstrate efficient encapsulation of water-insoluble fluorescent dyes, 9,10-diphenylanthracene (DPA) and fluorescein isothiocyanate (FITC), and cytotoxic drugs, such as camptothecin, a topoisomerase inhibitor [16], and celastrol, a proteasome inhibitor [17].

Some of the current approaches in targeting tumors include ligands specific to Her2 [18], transferrin receptor [19], epidermal growth factor receptor [20], folate receptor [21], integrin $\alpha_V\beta_3$ [22], hyaluronan receptor [23], and chondroitin sulfate [24]. As the target molecule in the present study, we have chosen intercellular adhesion molecule (ICAM)-1. ICAM-1 is present in low concentrations in the membranes of endothelial cells, and epithelial cells, and a subset of immune cells but is greatly upregulated in response to inflammatory signals [25,26]. In addition, constitutive over-expression of ICAM-1 has also been found in many carcinomas, such as breast, colon, non-small cell lung, renal-cell, pancreas, and gastric carcinomas compared to the respective normal epithelial cells [27–32] as well as in the tumor microenvironments, wherein an inflammatory milieu promotes angiogenesis and tumor growth [33].

Though most molecular targeting approaches have been based on antibodies [34] and small peptides [35], herein, we demonstrate ICAM-1 targeting with a native ligand, called the inserted (I) domain derived from lymphocyte function-associated antigen (LFA)-1. LFA-1 is a member of the integrin family, expressed on all leukocytes [36], that mediates leukocyte adhesion to endothelial cells and transmigration into inflamed tissues [37]. We used the I domains that have previously been engineered by various activating mutations (F292A, F292G, F265S/F292G) to increase the affinity to ICAM-1 in a step-wise manner [38]. Using ICAM-1 expressing HeLa cells [39] as a model cancer cell line, we demonstrate specific delivery of UAN encapsulating hydrophobic dyes and therapeutic agents to the cells, in a manner dependent on the affinity between the LFA-1 I domains and ICAM-1, as well as the expression level of ICAM-1.

2. Materials and methods

2.1. Synthesis and characterization of NTA-coupled UAN nanoparticles

The synthesis of UAN was previously described [40]. In brief, glycerol propoxylate (*n*5, MW ~1000, Sigma Aldrich) was mixed with toluene diisocyanate (TDI, Sigma Aldrich) to react the hydroxyl groups of glycerol propoxylate and the isocyanate (−N=C=O) groups of TDI (Fig. 1A). The remaining isocyanate groups in TDI were reacted with the hydroxyl groups of 2-hydroxyethyl methacrylate (2-HEMA, *n*34, Sigma Aldrich) and polyethylene glycol (PEG, MW = 1500, Sigma Aldrich). The final product, urethane acrylate nonionomer, consisted of a 1:3:2:1 M ratio of glycerol propoxylate:TDI:HEMA:PEG. 50 mg of the UAN monomers were then cross-linked by the reaction between the vinyl groups (−CH = CH_2) of HEMA. This polymerization was accomplished by 2 mg of azobisisobutyronitrile (AIBN, Sigma Aldrich), which creates free radical initiators when heated. The cross-linking step was carried out in 10 ml of dimethyl sulfoxide (DMSO, Sigma Aldrich) at 65 °C overnight with vigorous stirring (Fig. 1B). The average molecular weight of the synthesized UAN chains was 6700 (MALDI Micro MX, Waters) with a polydispersity of 2.0 measured via ambient temperature gel permeation chromatography (GPC, Waters). To create the NTA moiety on the hydroxyl end of the PEG groups in polymerized UAN, 10 mg of N_α,N_α-Bis(carboxymethyl)-L-lysine hydrate (NTA analog, Sigma Aldrich) in 0.1 ml DMSO was mixed with 6.4 mg of TDI (molar ratio of NTA to TDI is 1:1) for 2.5 h at room temperature to prepare NCO-terminated NTA-TDI complexes. Then NTA-TDI was mixed with 50 mg of cross-linked UAN overnight at room temperature to covalently link NCO of NTA-TDI to OH groups of UAN chains. In order to form nanoparticles from these monomers, the UAN chains dissolved in DMSO were diluted in distilled water (dH_2O) at a volume ratio of 1:10 (DMSO:dH_2O). The payloads such as FITC, camptothecin, and celastrol were added to UAN chains in DMSO at 0-20% of UAN weight prior to dilution with water.

Dynamic light scattering (DLS, Malvern Instruments) was used to measure the average size of the UAN nanoparticles after synthesis, after NTA conjugation, and after payload encapsulation. Transmission electron microscopy (TEM; FEI Tecnai™) images of the UAN particles were taken after staining with 1% uranyl acetate. 5 μl of the sample was placed on a glow-discharge grid for 2 min, after which excess solution was removed with filter paper. 5 μl of 1% uranyl acetate was then placed on the grid for 1 min and removed with filter paper. Images were taken after air-drying the grid for 30 min.

2.2. His-peptide column and Ni-NTA functionality test

His-peptide column was made by covalently attaching 2 mg of His-tag peptide (NH_2-Cys-Gly_4-Trp-Ser-His_6-COOH; subscripts denote the number of repeats) to 1 ml of aldehyde-activated agarose resin (AminoLink Coupling Resin and Kit, Pierce). The Schiff base formed between the amine and aldehyde groups was then reduced by cyanoborohydride according to the manufacturer's protocol.

2.3. 'Escape' kinetics assay

The release kinetics of the payloads encapsulated in UAN were indirectly measured using dialysis tubes. A highly water soluble dye, 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS, Anaspec) was used as a comparison with hydrophobic agents such as FITC (Pierce), celastrol (Cayman Chemical), and camptothecin (MP Biomedicals) to demonstrate UAN's ability to delay the 'escape' or the rate of diffusion of hydrophobic molecules through dialysis tubes. 1 ml of each UAN (payload) sample was placed in separate dialysis tubes (MWCO 7000 Da, Fisher) and subsequently placed in separate 2 L water-baths with stirring. After overnight dialysis, the concentrations of the payloads encapsulated within UAN were measured and the samples were subsequently placed in separate fresh dialysis tubes. 2 μl of each of the samples was taken out from the dialysis tubes and the absorbance levels were measured at different time points using a spectrophotometer (Nanodrop™ 2000) set at appropriate wave-lengths (HPTS: λ_{max} = 454 nm, FITC: λ_{max} = 495 nm, celastrol: λ_{max} = 424 nm, camptothecin: λ_{max} = 365 nm). Since DPA (Sigma Aldrich) was completely water-insoluble, DPA precipitated out of solution did not diffuse through the dialysis membrane. Therefore, UAN(DPA) was placed in a 1.7 ml microcentrifuge tube, and the supernatant after removing the DPA pellet that was released from UAN was measured for absorbance (λ_{max} = 413 nm). The extinction coefficient and water solubility of each of the molecules are as follows: HPTS: 20,800 M^{-1} cm^{-1} and highly water soluble, FITC: 70,000 M^{-1} cm^{-1} and 100 μg/ml, celastrol: 10,063 M^{-1} cm^{-1} and 2.33 μg/ml, camptothecin: 42,282 M^{-1} cm^{-1} and 1 μg/ml, and DPA: 14,000 M^{-1} cm^{-1} and water-insoluble. The same procedure was carried out to evaluate the escape kinetics of free materials with the initial concentrations of the payloads set to be equal to those encapsulated in UAN. The percent escape was plotted as 100% $[C_0 - C(t)]/C_0$, where $C(t)$ and C_0 refer to the measured payload concentration inside the dialysis tube at time = t and at time = 0 h, respectively. The rate of escape from dialysis tubes (τ) was then calculated by assuming the first-order diffusion model, $C(t) = C_0 \exp(-t/\tau)$ and curve-fitting the resulting equation, $100\%\,[1 - \exp(-t/\tau)]$, to the percent escape data.

2.4. Production of I domains, protein A, and R6.5 antibody

LFA-1 I domains and immunoglobulin G-binding recombinant protein A (17 kDa fragment) with a His-tag at the C-terminal were produced in *Escherichia coli BL21 (DE3)* (Invitrogen) using pET20b and pET28a vectors, respectively. To produce protein A, a 1 L bacteria culture with OD600 of 0.4–0.5 was induced with 1 mM isopropyl-b-D-thiogalactoside (IPTG, Gold Biotechnology) for 4 h, after which it was centrifuged (3000 rpm, 4 °C, 10 min) to pellet the cells. The soluble fraction of protein A was extracted by sonication and was purified using a Ni-NTA column (Novagen). Elutions from the column were further purified by size exclusion in a liquid chromatography column (Akta, GE Healthcare). Recombinant LFA-1 I domains (Asn-129 to Tyr-307) were produced as described previously [38]. Briefly, after protein induction, inclusion bodies were resuspended in 10 ml of the washing buffer (50 mM Tris (pH 8.0), 23% w/v sucrose, 0.5% w/v Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA)), sonicated, and centrifuged again to wash the inclusion body. This process was repeated three times. Then, the inclusion bodies were solubilized with 20 ml of the solubilization buffer (50 mM Tris (pH 8.0), 6 M Guanidine-HCl) for 1.5 h at 4 °C with stirring. This solution was diluted with 2 L of refolding buffer (50 mM Tris (pH 8.0), 10% glycerol, 1 mM $MgCl_2$) and stirred slowly at 4 °C overnight to initiate protein refolding. Finally, the refolded solution was concentrated down to 1–5 ml by centrifugation (Amicon 5 kDa MWCO Millipore), filtered through 0.45 μm, and purified by gel filtration chromatography. Monoclonal antibody (mAb) R6.5 was produced from hybridoma (ATCC), and purified by a Protein A column (Pierce) followed by size exclusion.

2.5. Cell culture and labeling with UAN

HeLa cells were grown in Advanced DMEM (Gibco) containing 10% FBS (Atlanta Biologicals) and 2 mM L-glutamine (GlutaMAX™, Gibco) at 37 °C in a 5% CO_2 humidified incubator. HeLa cells in 96 well plates (~90% confluency) were washed twice with 100 μl of the labeling buffer (phosphate buffer saline (PBS), pH 7.4, 5% bovine serum albumin (BSA), 5 mM $MgCl_2$) and incubated with UAN (250 μg/ml)