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NANOMEDICINE

Optimization of cell receptor-specific targeting through multivalent surface decoration of polymeric nanocarriers

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ABSTRACT

Treatment of tuberculosis is impaired by poor drug bioavailability, systemic side effects, patient non-compliance, and pathogen resistance to existing therapies. The mannose receptor (MR) is known to be involved in the recognition and internalization of *Mycobacterium tuberculosis*. We present a new assembly process to produce nanocarriers with variable surface densities of mannose targeting ligands in a single step, using kinetically-controlled, block copolymer-directed assembly. Nanocarrier association with murine macrophage J774 cells expressing the MR is examined as a function of incubation time and temperature, nanocarrier size, dose, and PEG corona properties. Amphiphilic diblock copolymers are prepared with terminal hydroxyl, methoxy, or mannoside functionality and incorporated into nanocarrier formulations at specific ratios by Flash NanoPrecipitation. Association of nanocarriers protected by a hydroxyl-terminated PEG corona with J774 cells is size dependent, while nanocarriers with methoxy-terminated PEG coronas do not associate with cells, regardless of size. Specific targeting of the MR is investigated using nanocarriers having 0–75% mannoside-terminated PEG chains in the PEG corona. This is a wider range of mannose densities than has been previously studied. Maximum nanocarrier association is attained with 9% mannoside-terminated PEG chains, increasing uptake more than 3-fold compared to non-targeted nanocarriers with a 5 kg mol^{-1} methoxy-terminated PEG corona. While a 5 kg mol^{-1} methoxy-terminated PEG corona prevents non-specific uptake, a 1.8 kg mol^{-1} methoxy-terminated PEG corona does not sufficiently protect the nanocarriers from nonspecific association. There is continuous uptake of MR-targeted nanocarriers at 37 °C, but a saturation of association at 4 °C. The majority of targeted nanocarriers associated with J774E cells are internalized at 37 °C and uptake is receptor-dependent, diminishing with competitive inhibition by dextran. This characterization of nanocarrier uptake and targeting provides promise for optimizing drug delivery to macrophages for TB treatment and establishes a general route for optimizing targeted formulations of nanocarriers for specific delivery at targeted sites.

1. Introduction

Tuberculosis (TB) is an intracellular infection which affects 1 in 3 people worldwide and causes over 1 million deaths yearly [1]. During TB infection, *Mycobacterium tuberculosis* (*M. tuberculosis*) bacilli are phagocytized by host immune cells and the normal enzymatic digestion process is arrested [2], which allows the bacteria to replicate within the host cells [3]. Treatment of the infection is hindered by poor drug permeability, solubility, or biodegradability [4,5] and the increasing threat of multiple drug resistant TB (MDR-TB) has led to a search for improved therapeutics [1]. One approach has been to improve drug localization at the infection site through active targeting with ligands specific for the diseased tissue [6]. In TB infection, the pattern-recognition mannose receptor (MR) binds to mannose caps on the *M. tuberculosis* coat protein, lipoarabinomannan, and also mediates phagocytosis [2,7]. Therefore, designing nanocarriers (NCs) which target drug payloads to cells expressing MR can improve the co-localization of anti-TB drugs with infected cells.

MR-targeted liposomal systems have been studied as therapeutics [8–10] and adjuvants [11]. While liposomes are effective for the delivery of hydrophilic therapeutics, their small hydrophobic volumes make them ineffective for the delivery of hydrophobic actives. Polymeric NCs composed of a drug core stabilized by amphiphilic diblock copolymers can be formed at high drug loadings by Flash NanoPrecipitation (FNP) [12] and are effective candidates for formulating novel hydrophobic TB therapeutics [13,14]. In addition, targeting functionality can be incorporated into the stabilizing block copolymers [15,16]. The success of a targeted formulation is dependent on targeting a pathway specific to the disease pathology. Macrophages, dendritic cells, and other cells of

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the immune system specifically express surface carbohydrate binding proteins, referred to as lectins, which are involved in the phagocytosis of several intracellular pathogens, including *M. tuberculosis* [17]. Classes of lectins recognize common motifs, but ligand specificity between different receptors is determined by the orientation of the carbohydrate binding domain. The MR, which belongs to this class of surface receptors, is a macrophage transmembrane protein possessing multiple carbohydrate binding sites. Binding of terminal mannose and fucose moieties is favored by the MR and the sugar density on ligands is crucial for high affinity binding [18,19], but receptor oligomerization is not required for internalization [19,20]. Since *M. tuberculosis* uptake is mediated by the MR, we target antitubercular drugs to the MR via terminal mannoside moieties on NC surfaces, in a way that mimics lipoarabinomannan coating of TB bacilli.

Studies that demonstrate improved uptake of various mannosylated NCs relative to non-targeted formulations, including liposomes [8,11,21,22], gelatin nanoparticles [23], and oil in water emulsions [24] have been published. The binding and internalization of NCs by a MR mediated pathway are influenced by the relative ligand surface density [8,9,24,25] and the length of the PEG spacer between mannoside and the particle surface [26]. Prior reports for liposomes indicate increasing cellular association with increasing mannoside surface density, but the surface densities explored in these studies were limited to 30 and 60% [8], or 2.5%, 5%, and 7.5% [9,24]. The phagocytosis of PEGylated 5 μm microspheres by macrophages in vitro was increased only at 34 and 45% mannosylated PEG chains, relative to 100% methoxy-terminated PEG chains, which were the highest compositions tested [25]. In these reports, the number of mannose moieties is not reported, and so a direct comparison of the ligand surface density cannot be made. There have not been reports of a polymeric NC system in which surface mannosylation is controlled to find the optimal NC composition to maximize cellular association via the MR. Recent studies have systematically explored the importance of ligand surface density on NC association with cell surface receptors, and results indicate that binding and uptake do not increase monotonically with increasing surface ligand density. It has been found that there exists an optimum surface density for binding and uptake of NCs which have been modified with peptide [27] and folate [28–30] targeting ligands.

In this work, we formulate and characterize MR targeted NCs in order to optimize association with macrophages expressing the MR. PEGylated NCs with 7 different ligand surface densities and 2 different methoxy-terminated PEG molecular weights are prepared by rapid precipitation and directed assembly of amphiphilic diblock copolymers in FNP. The FNP process enables the assembly of functional NCs with variable surface properties in a quantitative and efficient manner. The association with macrophages in vitro is then characterized as a function of incubation time and temperature, NC size, dose dependence, and polymer structure. In these experiments we use the murine macrophage J774 cell line, which was chosen since it has been extensively studied [31–33], multiple clones have been isolated [34] with quantified mannose receptor expression [33], and it has been used in previous studies of NC uptake [23,35–38]. We obtained two J774 clones: the J774E clone which over-expresses the mannose receptor (98,200 binding sites per cell in suspension), and the J774A.1 clone which has approximately half the number of receptors [33]. The J774A.1 clone has been previously used to study nanocarrier interactions with cells *in vitro* and for *in vitro* TB models, and we find the cellular association of PEG protected NCs with a single methoxy or hydroxyl terminal group on PEG demonstrates an unexpected sensitivity to the terminal group and NC size. With the J774E clone, we show that targeting the MR triples NC uptake relative to the methoxy-terminated PEGylated NCs. We present the first results indicating that uptake of mannoside functionalized NCs by macrophages does not saturate with increasing mannose functionalization, but has a relatively sharp maximum. These findings have significant implications for enhanced localization of therapeutic TB drugs.

2. Materials and methods

2.1. Materials

Tetrahydrofuran (THF, 99.9%), dichloromethane (DCM, 99.5%), dimethylsulfoxide (DMSO), hexane, ethyl acetate, anhydrous ethyl ether (ether) and sodium bicarbonate were purchased through Fisher Scientific (Pittsburgh, PA). Vitamin E (VE, 97%) and dextran (Mw = 60–80 kg mol^{-1}) were purchased from Sigma-Aldrich (St. Louis, MO). Anhydrous magnesium sulfate ($MgSO_4$) was from EMD Chemicals Inc. (Gibbstown, NJ). Prior to use, water was purified by 0.2 μm filtration and four stage deionization to a resistivity of 17.8 MΩ or greater (NANOpure Diamond, Barnstead International, Dubuque, IA). The block copolymers are designated:"XXX_m–PEG_n–YY", where XXX designates the hydrophobic block type, m is the Mw of the hydrophobic block in g mol^{-1}, n is the Mw of the PEG block in g mol^{-1}, and YY is the terminal group on PEG (methoxy: OCH_3, hydroxyl: OH, mannoside: MAN). Poly-D,L-lactide-*b*-polyethylene glycol ($PLA_{3.8k}$-*b*-PEG_{5k}-OCH_3) was kindly provided by Evonik, Inc. (Birmingham, AL). $PS_{1.6k}$-*b*-$PEG_{1.8k}$-OH was purchased from Polymer Source (Dorval, QC, CAN) and the synthesis of $PS_{1.5\ k}$-*b*-PEG_{5k}-OH has been reported previously [39]. For the latter polymer, prior to polymerization of PEG, a fraction of the $PS_{1.5k}$-OH was set aside and used as a hydrophobic filler in the NC formulations. J774A.1 murine macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA); J774E cells were provided by Dr. Philip Stahl (Washington University, St. Louis, MO). Dulbecco's modified Eagle medium (high-glucose with sodium pyruvate and L-glutamine), fetal bovine serum (FBS), and penicillin–streptomycin (Penicillin — 5000 IU mL^{-1}, Streptomycin — 5 mg mL^{-1}) were from Fisher Scientific.

2.2. Conjugation to form $PS_{1.5k}$-*b*-PEG_{5k}-MAN (**5**) [40]

The syntheses of precursor compounds **1**–**4** and the TBTA catalyst are detailed in the Supplemental Information (SI). **3** (0.138 g) and **4** (0.5 g) were dissolved in 26 mL of degassed anhydrous THF. TBTA (0.0035 g) and CuBr (catalytic, Alfa Aesar, Ward Hill, MA) were dissolved in 15 mL of dry THF and transferred to the solution of **3** and **4**. The reaction was stirred for 2 days and was then dialyzed (regenerated cellulose, MWCO: 3.5 kD, Spectra/Por) against THF (350 mL×3) for 1 day followed by dialysis in DI H_2O (2 L×4) for 2.5 days. The product was dried by lyophilization and 0.13 g was recovered (yield: 24%).

2.3. Synthesis of PS_m-*b*-PEG_n-OCH_3 (**6**)

This procedure was based on Aoyama and Shioiri [41], and details on the adaptation are available in the SI. PS_m-*b*-PEG_n-OH (0.147 mmol, $PS_{1.5k}$-*b*-PEG_{5k}-OH or $PS_{1.6k}$-*b*-$PEG_{1.8k}$-OH) was dissolved in 19.75 mL DCM and cooled in an ice bath. After adding aqueous trifluoroboric acid (0.025 mL, 48%, Alfa Aesar), 0.88 mL of trimethyl silyldiazomethane (2 M in Hexane, $TMSCHN_2$, Alfa Aesar) was added dropwise over 4 min. In subsequent 20 min intervals, 0.44 mL, 0.22 mL, and 0.22 mL of $TMSCHN_2$ were added and gas was evolved. The mixture was stirred overnight at 4 °C. The reaction solution was extracted once with 20 mL brine, the organic phase was dried over $MgSO_4$ and concentrated to 3 mL. The polymer was precipitated in ether, chilled by dry ice, and isolated by centrifugation (15 min, 1000 ×*g*, 4 °C). The end group conversion was determined by NMR analysis (see SI, S.3.3).

2.4. Nanocarrier formulation

A multi inlet vortex mixer (MIVM) geometry [12,42] was used to prepare Formulation 1.1 (Table 1) at high supersaturation with mixing times on the order of 1.5 ms (SI, Fig. S.6a). In this mixing scheme, a THF stream containing a solution of $PS_{1.5k}$-OH (7.5 mg mL^{-1}), EtTP5 (2.5 mg mL^{-1}), and $PS_{1.5k}$-*b*-PEG_{5k}-OH (10 mg mL^{-1}), was fed into