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Optimal structural design of mannosylated nanocarriers for macrophage targeting

Peiming Chen [a,1], Xiaoping Zhang [a], Lee Jia [b], Robert K. Prud'homme [c], Zoltan Szekely [a], Patrick J. Sinko [a,*]

[a] Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA
[b] Cancer Metastasis Alert and Prevention Center, College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou 350002, China
[c] Chemical and Biological Engineering, Princeton University, Princeton, NJ 08544, USA

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ABSTRACT

Macrophages are involved in a number of diseases, such as HIV infection/AIDS, tuberculosis, tumor development and atherosclerosis. Macrophages possess several cell surface receptors (e.g., the mannose receptor, MR) that may serve as drug delivery cellular portals for nanocarriers (NCs). In this study, the optimal structural configuration for cell uptake of mannosylated poly(ethylene glycol)-conjugate type NCs was determined. A series of NCs were synthesized to systematically evaluate the effects of the number of mannose units (Man), the PEG carrier size and the mPEG spacer length between adjacent mannose units on NC uptake into MR-expressing J774.E murine macrophage-like cells. Among NCs with 0, 1, 2 or 4 units of mannose, the uptake of $(Man)_2$-NC was the highest, suggesting a trade-off between avidity and NC-MR clustering on the cell surface that sterically hinders endocytosis. This optimal $(Man)_2$-NC configuration was built into subsequent NCs to optimize the other two parameters, PEG carrier size and spacer length. NCs with 0, 5, 12, 20, 30 or 40 kDa linear PEG carriers showed an inverse relationship between PEG size and uptake. The 12 kDa PEG carrier was chosen for investigating the third parameter, the Man-Man distance, since it may represent the best trade off (i.e., tissue penetration vs. systemic clearance) for *in vivo* macrophage targeting. Three $(Man)_2$-PEG_{12kDa} NCs with different Man-Man distances (39, 56 or 89 Å) were synthesized. The uptake of the NC with the 56 Å distance between mannoses was four- and two-fold higher than NCs with 39 Å and 89 Å distances, respectively. Confocal microscopy confirmed that the optimized $(Man)_2$-PEG_{12kDa} NC with the 56 Å Man-Man distance was internalized via endocytosis consistent with temperature-dependent active uptake. In conclusion, the optimal NC structural parameters for targeting the MR on macrophage-like J774.E cells are (i) a small PEG polymer carrier, (ii) two mannose units per NC and (iii) a 56 Å distance between adjacent mannose units.

1. Introduction

Macrophages are primarily involved with innate immune response and tissue homeostasis in mammals. Most tissue macrophages are in the resting state under normal conditions [1]. Upon insults initiated by invading microbes, tissue injuries or tissue stress, they can be activated into different functional phenotypes according to the tissue microenvironment [2,3]. Activated macrophages can be broadly classified into two main groups: classically activated macrophages (or M1) and alternatively activated macrophages (or M2) [4]. The M2 phenotype may be further divided into M2a, b, c subtypes [1]. M1, often regarded as pro-inflammatory, exhibit potent microbicidal properties and promote strong IL-12-mediated Th1 responses, while M2, often regarded as anti-inflammatory, support Th2-associated effector functions and resolution of inflammation and tissue injuries. Activated macrophages remain plastic and respond to the changing environment to modulate their activities [5]. The body response usually results in the clearance of the insult and the return of macrophages to their resting state. However, if these insults persist, pathological conditions can develop. For example, chronic M1 activity can cause collateral damage to normal tissues while chronic M2 activity can lead to tissue fibrosis. Thus, macrophages are involved in various pathological conditions, including obesity, tumor development, atherosclerosis, inflammation and infection with *Mycobacterium tuberculosis*, Leishmania protozoa and HIV [1, 6–10]. The idea of targeting activated macrophages to convert them to a normal phenotype has been proposed; however, in many cases the cell surface biomarkers for abnormal activity and the regulatory effector molecules to revert activated macrophages are yet to be identified [9, 11–13]. By contrast, delivery of drugs to infected macrophages using nanocarriers (NCs) or cell carriers has been extensively explored [14–19].

* Corresponding author. Tel.: +1 848 445 6398.
E-mail addresses: pmchenru@gmail.com (P. Chen), xzhang@rci.rutgers.edu (X. Zhang), pharmlink@gmail.com (L. Jia), prudhomm@princeton.edu (R.K. Prud'homme), zoltan@rci.rutgers.edu (Z. Szekely), sinko@rutgers.edu (P.J. Sinko).
[1] Present Address: CPSC Dophen Corporation, 4070 Truxel Road, Sacramento, CA 95834, USA.

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Liposomes and their variants have been extensively used as drug NCs [20–23]. Other commonly used nanoconstructs include micelles, dendrimers, nanospheres, solid lipid particles and polymeric particles. All of these forms of NCs suffer some shortcomings [24]. Conjugation with polyethylene glycol (PEG), or PEGylation, has been used to overcome some of the shortcomings of non-PEG-based NCs, such as low solubility, low stability, immunogenicity and recognition by the phagocytes of the reticuloendothelial system (RES) [25–27]. However, in the case of drug delivery to macrophages, PEGylation can reduce the delivery efficiency due to reduced recognition by macrophages. Therefore, it is desirable to endow PEG NCs with a macrophage-targeting moiety to compensate for the reduced recognition. The combined feature of PEGylation and targeting in a NC can offer prolonged circulation times, cell type-specific delivery and reduction in dosage &/or dose frequency.

There are a number of receptors that can serve as potential cell surface targets on macrophages including scavenger receptors, formyl peptide receptors, integrins, mannose receptors, galactose receptors and Fc-receptors [28]. Among them, the mannose receptor (MR) (also known as MMR, CD206, or MRC1) has attracted the most interest [29,30]. MR is expressed on most tissue macrophages, dendritic cells, hepatic endothelial cells, as well as on selected lymphatic endothelial cells, kidney mesangial cells, tracheal smooth muscle cells and retinal pigment epithelium [29,31]. However, it is not significantly expressed on monocytes, the precursor cells to macrophages. MR mainly recognizes terminal mannose, fucose and N-acetylglucosamine sugars of many glycoproteins on various bacteria and viruses, including that on gp120 of HIV-1. MR also recognizes sugar and non-sugar parts of endogenous ligands, including sulfated glycoprotein hormones, collagen, gelatin, lysosomal hydrolases, tissue plasminogen activator and neutrophil myeloperoxidase. There are other mannose-recognizing receptors, including Endo180, DC-SIGN, L-SIGN, and SIGNR [29], whose cell type expression patterns are similar to that of MR.

Previously, we reported that nanoparticles with surface-displayed mannose were taken up into MR-expressing J774.E cells consistent with active receptor-mediated uptake that was specific and inhibitable [32]. Maximum NC association was attained with 9% mannoside-terminated PEG chains, increasing uptake more than 3-fold compared to non-targeted NCs. However, in view of the lack of information regarding the optimal ligand display pattern properties (i.e, configuration) of a mannosylated PEG NC for targeting the macrophage MR, the present study was undertaken to systematically investigate the effect of the number of mannose units per NC, the distance between the mannose units and the PEG carrier size on NC uptake. A novel NC design was employed to precisely control the geometry of the mannose-targeting moiety on NC. It is found that the optimal NC uptake occurred for a NC displaying two mannose units that are spaced 56 Å apart on a small sized PEG carrier. The polymeric NCs described in this report can either be used as standalone NCs carrying one or more copies of drug/cargo or they can be used as optimized targeting ligands on other carriers such as nanoparticles.

2. Materials and methods.

2.1. Materials

NovaSyn Sieber resin was purchased from Millipore (Bellerica, MA), Fmoc-γ-Abu-OH, Fmoc-Ser-OH, Fmoc-Ser(O^tBu)-OH, Fmoc-Cys(Trt)-OH from Chem-impex (Wood Dale, IL), mPEGx-maleimide (x = 5 kDa, 12 kDa, 20 kDa, 30 kDa, 40 kDa) from NOF (Tokyo, Japan), α-D-mannose pentaacetate, boron trifluoride diethyl etherate, *N,N*-Diisopropylethylamine (DIPEA), NO production kit and NBT reduction kit from Sigma Aldrich (Saint Louis, MO), and trifluoroacetic acid (TFA) from Fisher Chemical (Fair Lawn, NJ). Fmoc-amino-$dPEG_6$-acid, Fmoc-amino-$dPEG_{12}$-acid and Fmoc-amino-$dPEG_{20}$-acid were purchased from Quanta biodesign (Powell, OH). Tetramethylrhodamine B dextran (10,000 M.W.) and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) were purchased from Life Technologies (Grand Island, NY).

2.2. Synthesis and characterization of nanocarriers

FITC-Gaba-PEG_{12}-Ser(Man)-Gaba-Gaba-Cys-amide, FITC-Gaba-Ser(Man)-PEG_{12}-Ser(Man)-Gaba-Gaba-Cys-amide, FITC-GABA-Ser(Man)-PEG_{12}-Ser(Man)-PEG_{12}-Ser(Man)-Gaba-Gaba-Cys-amide, and FITC-Gaba-Ser(Man)-PEG_{12}-Ser(Man)-PEG_{12}-Ser(Man)-PEG_{12}-Ser(Man)-Gaba-Gaba-Cys-amide (Fig. 1) were synthesized following standard solid-supported peptide synthetic protocols using a Nautilus 2400 Automated Synthesizer. Fmoc-L-Ser-α-D-[$Man(OAc)_4$]-OH was synthesized as previously described [33]. NovaSynTG Sieber resin was used in the amino acid coupling with a molar ratio of 1:4:4:8 (resin: amino acid:coupling reagent (HATU, HOAt):base (DIPEA)) for 4 h. Fmoc-L-Ser-α-D-[$Man(OAc)_4$]-OH and Fmoc-amino-PEG_{12} acid were conjugated to the peptidic core in the same way as the regular amino acid coupling in a molar ratio of 1:2:2:4 (resin:amino acid:coupling reagents (HATU, HOAt):base (DIPEA)) for 12 h. Fmoc group was removed by 20% piperidine in NMP. MALDI TOF/MS and analytical reverse phase high-performance liquid chromatography (RP-HPLC) were performed after each amino acid coupling to confirm the molecular weight and purity. Five equivalents of fluorescein isothiocyanate (FITC) were dissolved in NMP and reacted to the N terminal of the peptide on solid support in the presence of 10 equivalents of DIPEA for 12 h. The acetyl protecting groups of the mannose moieties were removed by sodium methoxide in anhydrous methanol on the solid support. Peptides were cleaved from the resin by TFA containing scavanger reagents and diluted with DCM (85% DCM, 2% DODT, 1% TIS, 12% TFA) for 1.5 h. The cleaved peptides were then precipitated with cold ether–hexane mixture (50%–50% *v/v*). Semi-preparative RP-HPLC was used to purify the NCs. The fractions were analyzed (HPLC), pooled and lyophilized providing high purity samples (purity >90%). PEGylation was performed in the liquid phase by reacting 5 equivalent mPEGx-maleimide (x = 5, 12, 20, 30, or 40 kDa) to the cysteine moiety of the NCs in 1 ml of phosphate-buffered saline (PBS, pH 7.4). The reaction was stirred overnight at room temperature. Sephadex G-50 or Sephadex G-15 column was used to purify the PEG NCs. Aliquots of the purified PEG NCs were collected and characterized using MALDI-TOF/MS. The collected PEG NCs were then lyophilized. NCs possessing three different Man-Man distances (39, 56 and 89 Å, cf. Fig. 1) were synthesized. The corresponding PEG amino acids were Fmoc-PEG_6-OH, Fmoc-PEG_{12}-OH and Fmoc-PEG_{20}-OH respectively. The mannosylated and labeled building blocks were fully characterized and the PEG carriers were obtained from a commercial source. The molecular weights of the PEG carriers were confirmed by MALDI-TOF mass spectrometry in our laboratory. After conjugation, the products were purified and structural integrity was confirmed by MALDI-TOF MS. Monitoring the differences of the centroid of the *m/z* signals (non-conjugated vs. conjugated) is an accepted method in bioconjugate chemistry for following conjugation reactions of polydisperse precursors. Since the conjugation steps have always been carried out using a 1:1 stoichiometry, the loading efficiency is determined by the chemical reaction, thiol-maleimide coupling. The MALDI-TOF MS spectra provided further verification for the conjugate structures.

2.3. Cell uptake of NC

J774.E cells were a gift from Dr. Philip D. Stahl, Washington University (St. Louis, WA). The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37 °C in 5% CO_2/95% air atmosphere. In uptake experiments, J774.E cells were plated at 2.5×10^4 cells/well to poly-D-lysine-coated 24-well plate to achieve better macrophage differentiation. The cells were incubated with test and control NCs at specified concentrations in culture condition for 1 h at either 37 or 4 °C. The attached cells were