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***In vivo* Targeting of Alveolar Macrophages via RAFT-Based Glycopolymers**

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Abstract

Targeting cell populations via endogenous carbohydrate receptors is an appealing approach for drug delivery. However, to be effective, this strategy requires the production of high affinity carbohydrate ligands capable of engaging with specific cell-surface lectins. To develop materials that exhibit high affinity towards these receptors, we synthesized glycopolymers displaying pendant carbohydrate moieties from carbohydrate-functionalized monomer precursors via reversible addition-fragmentation chain transfer (RAFT) polymerization. These glycopolymers were fluorescently labeled and used to determine macrophage-specific targeting both *in vitro* and *in vivo*. Mannose- and *N*-acetylglucosamine-containing glycopolymers were shown to specifically target mouse bone marrow-derived macrophages (BMDMs) *in vitro* in a dose-dependent manner as compared to a galactose-containing glycopolymer (30- and 19-fold higher uptake, respectively). In addition, upon macrophage differentiation, the mannose glycopolymer exhibited enhanced uptake in M2-polarized macrophages, an anti-inflammatory macrophage phenotype prevalent in injured tissue. This carbohydrate-specific uptake was retained *in vivo*, as alveolar macrophages demonstrated 6-fold higher internalization of mannose glycopolymer, as compared to galactose, following intratracheal administration in mice. We have shown the successful synthesis of a class of functional RAFT glycopolymers capable of macrophage-type specific uptake both *in vitro* and *in vivo*, with significant implications for the design of future targeted drug delivery systems.

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Appendix. Supplementary data

Synthetic methods and spectroscopic data of glycomonomers and additional spectroscopic data of glycopolymers. This material can be found at .

Keywords

Glycopolymer; RAFT; glycomonomers; macrophage; MRC-1

1. Introduction

The targeted delivery of small molecule drugs and biologics continues to be a major objective towards improving therapeutic efficiency through the mitigation of off-target effects and reduction in required dose [1]. However, few delivery carriers are capable of recognizing cell-specific ligands while avoiding nonspecific cellular uptake. To overcome this limitation, carbohydrate-based materials have been investigated due to their biocompatibility, target specificity, and ability to facilitate receptor-mediated uptake through cell-surface lectins (carbohydrate-binding proteins) [2,3].

Macrophages are an attractive therapeutic target because they play an important role in the inflammatory response and wound healing [4]. A number of macrophage subsets have been described that are associated with distinct phenotypes. Classically activated macrophages (M1) are critical for host defense whereas alternatively activated macrophages (M2) are important in injury resolution and wound healing. In response to acute injury, the predominant macrophage phenotype is pro-inflammatory (M1). However, an overexuberant M1 macrophage response can result in collateral tissue damage and impaired wound healing. Likewise, while M2 macrophages are important for appropriate wound healing, excess M2 response can result in tissue fibrosis. Indeed, dysregulated macrophage function is associated with a wide range of conditions including chronic ulcers, allergic asthma, atherosclerosis, autoimmune disorders, and fibrotic diseases [4]. Therefore, developing a drug delivery platform capable of directly targeting and modulating polarized macrophages has important therapeutic implications, for example, targeting of alveolar macrophage represents a promising strategy for addressing pulmonary inflammatory conditions [5,6].

Carbohydrates are known to play a significant role in the inflammatory response through mediating cell-cell recognition of immune cells, including macrophages [7]. Macrophages are a promising target for carbohydrate-based therapeutics as they express carbohydrate binding receptors which internalize bound material via receptor-mediated endocytosis [8]. One such carbohydrate binding receptor is the macrophage mannose receptor, an endocytic protein that is highly expressed on macrophages, including the alveolar macrophage [9]. The mannose receptor mediates the uptake and internalization of extracellular ligands including potentially harmful extracellular glycoproteins with terminal mannose, fucose, or *N*-acetylglucosamine, and pathogens with high densities of mannose on their surface [10,11]. The murine mannose receptor, MRC-1 (CD206), contains eight extracellular C-type lectin-like domains (CTLDs) [12]. Simple monosaccharides exhibit low affinities towards single MRC-1 CTLDs, with dissociation constants in the low millimolar range [13]. Many natural glycans enhance these weak binding events by clustering multiple glycosides together, thereby allowing multivalent interactions to be made with a multidomain lectin receptor (e.g. the mannose receptor) leading to a significant increase in overall avidity [14]. Ligands capable of exhibiting this multivalent behavior through simultaneous engagement of multiple CTLDs on the mannose receptor are potent facilitators of macrophage-specific uptake [15]. While mannose displays the highest affinity toward mannose receptor CTLDs, other sugars (e.g. fucose, *N*-acetylglucosamine and glucose) are also recognized by these lectin-like domains [16].

Through the presentation of multiple pendant carbohydrates, synthetic glycopolymers provide a promising platform to facilitate mannose receptor-mediated binding and

subsequent endocytosis [2]. While such compounds have been previously synthesized to probe lectin-carbohydrate binding behavior [17], only recently have structurally well-defined, homogeneous glycopolymers capable of multivalent interactions been successfully prepared [18]. For example, multivalent glycopolymers can be synthesized via the functionalization of an individual monosaccharide with a vinyl-containing compound resulting in a glycomonomer [19]. These glycomonomers can be polymerized through free radical polymerization to yield a glycopolymer with pendant carbohydrates [20,21]. Use of reversible addition-fragmentation chain transfer (RAFT) polymerization for this glycopolymer synthesis offers precise control over the reaction, resulting in predictable molecular weights, narrow molecular weight distributions, and the ability to develop complex polymer architectures [22]. Lowe et al. first demonstrated the successful RAFT polymerization of a glycomonomer by using glucosefunctionalized methacrylate monomer in aqueous conditions; the resultant material exhibited a low polydispersity and displayed “living” properties characteristic of the RAFT technique [19]. Additionally, through the modification of the chain transfer agent (CTA) and the incorporation of comonomers, facile telechelic and pendant polymer functionalization is achievable [23-25]. By combining the versatility of the RAFT process with carbohydrate synthetic techniques, structurally complex glycosylated materials capable of mimicking the multivalent binding activity of biological carbohydrate compounds can be realized., e.g. glycopolymer micelles [25,26], stars [18], nanoparticles [27,28], “clickable” constructs [23] [29-31], and glycosylated block copolymers [26] [32-34]. By displaying multiple functional carbohydrates, these materials can be used to target specific cell populations via carbohydrate-dependent uptake mechanisms, allowing for the design of diagnostic and therapeutic glycosylated constructs.

Glycosylation of drug delivery vehicles has been explored as a means to access alveolar macrophages *in vivo*, a cell implicated in the pathogenesis of pulmonary conditions [6]. Chono et al. demonstrated that mannosylating liposomes enhanced their uptake by rat alveolar macrophages *in vivo* following intratracheal administration, work which has been extended by Wijagkanalan et al. [35-37]. In light of this previous work, there has yet to be a study systemically evaluating the uptake of well-defined, multivalent carbohydrate materials by macrophages both *in vitro* and in pulmonary tissue. Herein, we describe the synthesis of glycomonomers and employed RAFT polymerization to develop a family of fluorescently-labeled glycopolymers capable of macrophage-specific targeting.

2. Materials and methods

2.1. Materials

Materials were purchased from Sigma-Aldrich unless otherwise specified. 4,4'-Azobis(4-cyanovaleric acid) (V501) was obtained from Wako Chemicals USA, Inc. 4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) and Pyridyl disulfide ethyl methacrylamide (PDSEMA) was synthesized as described previously [38].

2.2. General synthesis of glycomonomers

TMSOTf (cat.) was added to a mixture of trichloroacetimidate sugar donor [39] (1g, 2.0 mmol) and 2-hydroxyethyl methacrylate (1.2 eq) in dichloromethane at room temperature. The reaction mixture was stirred at room temperature for 20 min and then quenched by the addition of triethylamine. The protected sugar-hydroxyethyl methacrylate was obtained after removal of solvent under reduced pressure, followed by purification via flash silica column chromatography (82-89% yield). The procedure for preparing protected GlcNAc-hydroxyethyl methacrylate is described in Supporting Information. For deprotection, sugar-hydroxyethyl methacrylate was added to 1% sodium methoxide in methanol and the mixture was stirred at 25 °C for 15 min. The reaction mixture was neutralized with glacial acetic

acid. The fully deprotected glycomonomer was obtained after evaporation of solvent under reduced pressure, followed by purification via flash silica column chromatography (70-82% yield).

2.3. Synthesis of glycopolymers

The RAFT polymerization of glycopolymers was conducted in a heterogeneous solvent system of dH₂O/ethanol (3:1 vol:vol) at 70°C with 15 wt% monomer under a nitrogen atmosphere for 4h using ECT and V501 as the chain transfer agent (CTA) and radical initiator, respectively. The initial CTA to monomer molar ratio ($[CTA]_0:[M]_0$) was 50:1, the initial CTA to initiator molar ratio ($[CTA]_0:[I]_0$) was 20:1, and the initial molar feed ratios of glycomonomer to PDSEMA was 9:1. The resultant glycopolymer was isolated by dilution into dH₂O followed by lyophilization. The glycopolymer was further purified by redissolution into dH₂O, chromatographic separation with a PD-10 desalting column (GE Healthcare), and further lyophilization to obtain the final polymer.

2.4. Glycopolymer characterization

Monomer conversion and incorporation were determined by ¹H-NMR (500 MHz, D₂O). Conversion was determined to be greater than 99% due to the absence of resonances associated with vinyl protons on the glycomonomer (δ 6.07) and PDSEMA (δ 5.77) following polymerization. Copolymer composition was calculated from an aromatic PDSEMA proton (δ 8.40) and a methylene proton vicinal to the ester group (δ 4.15 Man) or methine proton on the pyranose ring (δ 4.36 Gal and δ 4.51 GlcNAc). Molecular weights (M_n) and polydispersity indices (PDI) were determined by gel permeation chromatography (GPC) using a Viscotek GPCmax VE2001 and refractometer VE3580 (Viscotek) with Tosoh TSK-GEL α -3000 (2X) and α -4000 columns connected in series (Tosoh Bioscience). HPLC-grade N,N-dimethylacetamide (DMAc; 0.03% w/v LiBr; 0.05% w/v BHT) was used as the eluent at a flow rate of 0.85 mL/min while column temperature was maintained at 50°C. Absolute number average molecular weights were calculated from dn/dc values that were determined for each glycopolymer (pManEMA: 0.101, pGalEMA: 0.100, pGlcNAcEMA: 0.130). PDSEMA incorporation was further validated by reduction of the glycopolymers in the presence of Bond-Breaker TCEP solution (~150 molar excess per polymer at 50 mM; Thermo Scientific) followed by spectroscopic measurement of liberated pyridine-2-thione ($\epsilon_{343} = 8080 \text{ M cm}^{-1}$).

2.5. Fluorophore labeling of glycopolymers

Glycopolymers (~10 mg mL⁻¹, in 0.1 M sodium phosphate pH 7.4 with 0.15 M NaCl buffer) were incubated in immobilized TCEP disulfide reducing gel (~10 molar excess per polymer; Thermo Scientific) for 2h followed by elution with PBS. Alexa Fluor 488 (AF488) C5 maleimide (10 mg mL⁻¹ in DMSO) was added to the reduced polymer in solution resulting in an approximately equimolar amount of fluorophore to reduced PDS groups and an overall polymer concentration of ~2 mg mL⁻¹. The reaction proceeded overnight at room temperature followed by removal of excess fluorophore by a PD-10 desalting column and lyophilization. Fluorophore labeling efficiency was determined by spectroscopic measurement of the trithiocarbonate species on the glycopolymer end group ($\epsilon_{310} = 20,000 \text{ M cm}^{-1}$) and the Alexa Fluor 488 ($\epsilon_{495} = 73,000 \text{ M cm}^{-1}$).

2.6. Lectin agglutination assay

The ability of the glycopolymers to bind a mannose-specific lectin, Concanavalin A (ConA), was assessed by an agglutination assay. 1 μ M ConA was mixed with 10 μ M glycopolymer (based on number of carbohydrate repeats) and the solution turbidity was measured by UV-Vis spectroscopy at 350 nm at one minute intervals for 30 minutes.

2.7. In vitro glycopolymer uptake assay

Animal protocol was approved by University of Washington Institutional Animal Care and Use Committee. Primary mouse lung fibroblasts (MLF) were isolated and cultured as previously described (Choi 2009). The murine lung epithelial cell line MLE12 was obtained from American Type Culture Collection (ATCC). MLF and MLE12 uptake assays were performed in colorless DMEM supplemented with Glutamax (Life Technologies) and Nutridoma-SP (Roche). Bone marrow derived macrophages (BMDM) were isolated from femurs and tibias of 8-12 week-old C57BL6 mice as previously described [40] and cultured in RPMI-1640 containing 10% Fetal Bovine Serum and 30% L929-conditioned medium for 7-10 days. To obtain differentiated macrophages, BMDMs (M0) were treated with 50 ng mL⁻¹ *E. coli* 0111:B4 LPS (Sigma) for 24 hours (M1) or 20 ng mL⁻¹ each of IL-4 and IL-13 (Life Technologies) for 48 hours (M2). Cells were seeded in 12-well plates and allowed to adhere overnight. Cells were rinsed with DPBS, and then incubated with indicated concentration of Alexa488-labeled glycopolymers in colorless RPMI supplemented with Glutamax and Nutridoma-SP. For competition experiments, unlabeled glycopolymer was added 15 min prior to addition of labeled glycopolymer. At the end of incubation, the cells were washed and lifted with cold DPBS aided by cell scrapers. Internalized polymer was measured as fluorescence intensity of the cells using Guava EasyCyte Plus System (Millipore) and data analyzed using CellQuest 2.0 (BD Biosciences). All experiments were performed in triplicate and repeated at least 3 times.

2.8. In vivo glycopolymer uptake assay

8-12 week-old C57BL6 mice (Jackson Laboratories) underwent intratracheal instillation with 10 μ M Alexa-488 glycopolymers or control (unlabeled) polymer in 50 μ L DPBS. After 15-30 min, the lungs were lavaged with 1 mL of DPBS containing 0.6 mM EDTA. Bronchoalveolar lavage cells were centrifuged and rinsed to remove unincorporated polymers and resuspended in PBS for flow analysis by Guava as above [41]. In some cases, BAL cells were spun onto microslides, and nuclei were counterstained with DAPI. Images were obtained using a Nikon Eclipse 80i microscope with a DS Camera Head DS-5M for fluorescent microscopy.

2.9. Statistical analysis

Means of more than two groups of data were compared using one-way analysis of variance (ANOVA) for analysis of one independent variable or two way ANOVA, for analysis of two independent variables, followed by Tukey's honestly significant difference (HSD) post hoc test. Student T-test was used for comparison of paired parametric data. For non-parametric data, Mann-Whitney's U test was performed. All tests were two-tailed and p values ≤ 0.05 were considered significant.

3. Results and discussion

3.1. Glycopolymer synthesis and characterization

Carbohydrates are attractive ligands for imparting biological targeting functionality to polymeric systems. Carbohydrate-ligands can be easily synthesized in large scale [42], are stable to indefinite storage at ambient temperatures [43], and can leverage low-affinity binding interactions through multivalency [44]. Well-defined glycopolymers were prepared via the RAFT polymerization of synthesized glycomonomers (Scheme 1). We selected mannose (Man) and *N*-acetylglucosamine (GlcNAc) because of their known mannose receptor binding and galactose (Gal) due to its lack of mannose receptor interactions [16,45]. Each monosaccharide was functionalized with ethyl methacrylate (EMA), yielding the glycomonomers: ManEMA, GlcNAcEMA, and GalEMA (Supplementary Fig. S1). These

glycomonomers were polymerized via the RAFT technique. A pyridyl disulfide comonomer, pyridyl disulfide ethyl methacrylamide (PDSEMA), was incorporated into the polymerization (at a 10% molar feed ratio) to provide a conjugatable handle for a maleimide-containing fluorophore. Glycopolymers (subsequently referred to as pManEMA, pGlcNAcEMA, and pGalEMA) were successfully synthesized under controlled conditions with consistent size and composition as determined by gel permeation chromatography (GPC) and ^1H -NMR spectroscopy (Table 1, Supplementary Fig. S2 and S3). The glycopolymers exhibited narrow molecular weight distributions with polydispersity indices (PDI) of 1.2 and resultant block lengths of 11400 – 13400 g mol $^{-1}$. The monomer incorporations of pyridyl disulfide groups per polymer were also similar between the glycopolymers (3.3 – 5.2). These findings demonstrate the first direct RAFT polymerization of the ManEMA and GlcNAcEMA glycomonomers used here while the GalEMA glycomonomer has been previously polymerized via this technique [28,46].

A maleimide-functionalized fluorophore (Alexa Fluor 488, AF488) was conjugated to the glycopolymers through the pyridyl disulfide (PDS) groups following reduction with tris(2-carboxyethyl)phosphine (TCEP). Labeling efficiency was similar among the three glycopolymers as determined by UV-Vis spectroscopy: 0.93 – 1.2 fluorophores/polymer, and there was no measurable increase in solution turbidity suggestive of a lack of glycopolymer aggregation (Supplementary Fig. S4). Moreover, dynamic light scattering (DLS) measurements of the glycopolymers following labeling demonstrated there is no significant aggregation of the materials into larger, macromolecular assemblies as mean particle diameters of less than 8 nm were observed (approximately 4 – 7 nm; Supplementary Fig. S5). To initially determine lectin-binding activity, each glycopolymer was incubated with Concanavalin A (ConA) [47], a mannose-specific-binding lectin known to bind mannose and glucose, but not galactose or N-acetylglucosamine [48]. pManEMA was found to agglutinate ConA, as measured by an increase in solution turbidity, showing that the material is capable of multivalent CRD-binding, a prerequisite for mannose receptor engagement (Supplementary Fig. S6). pGlcNAcEMA and pGalEMA did not induce ConA agglutination, demonstrating that the glycopolymer binding activity is carbohydrate-specific.

3.2. In vitro macrophage uptake of glycopolymers

We first examined whether the glycopolymers were differentially internalized by murine bone marrow-derived macrophages (BMDMs), a cell type known to express the mouse mannose receptor, MRC-1. BMDMs were incubated with increasing doses of AF488-labeled glycopolymers for varying lengths of time and cell uptake was assessed by flow cytometry and fluorescent microscopy (Fig. 1 and 2). We found that pManEMA and pGlcNAcEMA, but not pGalEMA, were internalized efficiently by BMDMs in a time dependent fashion. The uptake of pManEMA and pGlcNAcEMA were 30- and 19-fold higher than pGalEMA, respectively. Significant uptake occurred by 15 min and increased up to 6 hours (Figure 2 and data not shown). Mannose-binding protein, a soluble multi-domain C-type lectin in the same family as MRC-1, is known to exhibit carbohydrate-binding specificities defined by interactions with the vicinal, equatorial hydroxyl groups, C-3 and C-4, that are shared between several sugars [49]. Therefore, the ability of polymerized Man and GlcNAc to target macrophages is not surprising. BMDMs demonstrated a cytoplasmic punctate distribution of internalized pManEMA and pGlcNAcEMA, consistent with localization in the endosome/lysosome vesicles. No cell fluorescence above background was observed with pGalEMA. We also observed a dose-dependent response on uptake for both pManEMA and pGlcNAcEMA with saturation of pManEMA uptake occurring at 0.5 μM (Fig. 2); no significant dose-dependent effects were observed for pGalEMA uptake.

The effect of glycopolymer competition on uptake was assessed by incubating BMDMs with labeled glycopolymer in the presence of excess unlabeled glycopolymer. Competition with

unlabeled pManEMA was more efficient than pGlcNAcEMA at attenuating uptake of labeled pManEMA and pGlcNAcEMA, suggesting that the binding affinity of MRC-1 towards pManEMA is higher than pGlcNAcEMA (Fig. 3). This finding is consistent with the mannose receptor's stronger affinity for Man residues over GlcNAc [8]. We presume the minimal uptake of pGalEMA by BMDMs was due to nonspecific macropinocytosis, supported by the lack of competition from any of the glycopolymers.

3.3. *In vitro* glycopolymer uptake by polarized macrophages

We tested whether the polarization state of macrophages altered the uptake of pManEMA. Macrophages can be polarized into a classic “pro-inflammatory” (M1) or alternative “pro-resolution” (M2) state, depending on the local environment of cytokines and other immune-stimulating compounds. Macrophages can be differentiated *in vitro* by LPS to the M1, “classically” activated state which results in the secretion of pro-inflammatory cytokines. Incubation with IL-4 and IL-13 leads to differentiation into M2, or “alternatively” activated macrophages, which are considered a pro-resolution and anti-inflammatory phenotype [50]. M2 macrophages secrete pro-fibrotic cytokines, such as tumor growth factor β (TGF β) and platelet-derived growth factor (PDGF), that act on nearby fibroblasts to promote a fibroproliferative response. M2 macrophages also secrete matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) that regulate matrix remodeling. Additionally, they produce chemokines that attract other inflammatory cells (e.g. monocytes and dendritic cells) that subsequently clear apoptotic cells and debris, dampening the inflammatory response [51,52]. Due to the orthogonal roles played by the M1 and M2 macrophage phenotype, differential targeting of activated macrophages is attractive for therapeutic drug delivery applications. Human alveolar macrophages adopting a M2 polarization are believed to play an important role in the pathogenesis of pulmonary fibrosis, highlighting this phenotype as a potential clinical target [5].

BMDMs were cultured in either LPS or IL-4/IL-13 to obtain either the M1 or M2 polarization, respectively. Macrophages activated by the alternative pathway (M2) showed increased internalization of pManEMA whereas classically activated macrophages (M1) had similar internalization of pManEMA as naïve macrophages (M0) (Fig. 4). These differences were retained over a two-hour time course. At this later time point, M2 macrophages had internalized pManEMA at an approximately 3- and 5-fold higher level than M0 and M1 macrophages, respectively. M2 macrophages have higher expression of mannose receptor [53], providing further support that this receptor is facilitating glycopolymer uptake.

3.4. *In vitro* cell-specific glycopolymer uptake

To determine cell-type specificity, we compared uptake and internalization of labeled glycopolymers by BMDMs, primary mouse lung fibroblasts (MLF) and the murine lung epithelial cell line, MLE-12. The latter two cell-types were selected as they are representative of the cell phenotypes encountered in the lung, the target site of our *in vivo* study. Neither MLF nor MLE-12 had significant internalization of any of the glycopolymers at any timepoint examined (Fig. 5), whereas BMDMs had significant uptake of pManEMA and pGlcNAcEMA. These results are consistent with mannose receptor-mediated uptake of the glycopolymers by macrophages.

3.5. *In vivo* macrophage uptake of glycopolymers

To determine whether macrophage internalization of glycopolymers retained the same carbohydrate specificity *in vivo*, we administered Alexa 488-labeled glycopolymers intratracheally in normal mice and measured uptake by alveolar macrophages. Bronchoalveolar lavage (BAL) was performed at different timepoints following administration, and BAL cells were analyzed for uptake of the glycopolymers. We verified

that greater than 90% of the BAL cells were alveolar macrophages by staining with a macrophage cell surface marker (F4/80, data not shown). We found that alveolar macrophages had similar *in vivo* uptake of glycopolymers as BMDM uptake *in vitro*: pManEMA and pGlcNAcEMA were readily internalized by alveolar macrophages as early as 30 minutes after instillation whereas pGalEMA had minimal internalization (Fig. 6). Flow cytometric analysis showed that uptake of pManEMA was up to 6-folds higher than the pGalEMA at 15 min (data not shown). *Ex vivo* examination of alveolar macrophage demonstrated a similar punctate distribution pattern as observed in BMDMs (Fig. 6 inset). These findings are consistent with previous work examining the uptake of mannosylated liposomes by alveolar macrophages following intratracheal administration in rats. Chono et al. demonstrated that adding mannose to liposomes resulted in a 2.2 fold increase in uptake over a 24 hour period as compared to bare liposomes [35]. Wijagkanalan et al. supported these findings by showing a significant increase in the internalization of mannosylated liposomes versus unmodified liposomes after two hours [36]. The results presented here demonstrate that these functional glycopolymers are active within the complex biological milieu found within the lung. Local delivery via intratracheal instillation also has the advantage of less systemic toxicity and off target effects, lower doses, and better drug stability. For example local delivery of therapeutics to the lung is a feasible option in intubated patients suffering from acute respiratory distress syndrome.

4. Conclusions

Synthetic glycopolymers provide a promising platform to leverage mannose receptor-mediated endocytosis for the intracellular delivery of therapeutic cargo into macrophage populations in the lung. In this study, structurally well-defined glycopolymers were synthesized via RAFT polymerization and shown to specifically engage macrophages both *in vitro* and *in vivo* in a carbohydrate-dependent manner. We demonstrate for the first time that macrophages activated by the alternative pathway (M2) showed increased internalization of mannose glycopolymers and, to a lesser degree, *N*-acetylglucosamine glycopolymers, whereas classically activated macrophages (M1) exhibit similar internalization of glycopolymers as naïve macrophages (M0). Demonstration of specific uptake of pManEMA by alveolar macrophages *in vivo* is promising for future therapeutic applications. While not explored in this work, the pyridyl disulfide handle on these glycopolymers could be conjugated to other maleimide- or thiol-functionalized compounds, e.g. small molecule drugs and biological macromolecules. Coupled with the versatility of RAFT-based polymer synthesis, glycopolymers are a promising strategy for the design of targeted polymeric drug delivery systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- [1]. Allen TM. Ligand-targeted therapeutics in anticancer therapy. *Nat Rev Cancer*. 2002; 2:750–63. [PubMed: 12360278]
- [2]. Ting SRS, Chen G, Stenzel MH. Synthesis of glycopolymers and their multivalent recognitions with lectins. *Polym Chem*. 2010; 1:1392–412.

- [3]. Fleming C, Maldjian A, Da Costa D, Rullay AK, Haddleton DM, St John J, et al. A carbohydrate-antioxidant hybrid polymer reduces oxidative damage in spermatozoa and enhances fertility. *Nature*. 2005; 1:270–4.
- [4]. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 2011; 11:723–37. [PubMed: 21997792]
- [5]. Pechkovsky DV, Prasse A, Kollert F, Engel KMY, Dentler J, Luttmann W, et al. Alternatively activated alveolar macrophages in pulmonary fibrosis--mediator production and intracellular signal transduction. *Clin Immunol*. 2010; 137:89–101. [PubMed: 20674506]
- [6]. Zhang-Hoover J, Sutton A, Van Rooijen N, Stein-Streilein J. A critical role for alveolar macrophages in elicitation of pulmonary immune fibrosis. *Immunology*. 2000; 101:501–11. [PubMed: 11122454]
- [7]. Esko, J.; Kimata, K.; Lindahl, U.; Varki, A.; Cummings, V.; Esko, J., et al. *Essentials of glycobiology*. 2nd ed. Cold Spring Harbor Laboratory Press; Cold Spring Harbor (NY): 2009.
- [8]. Taylor M, Bezouska K, Drickamer K. Contribution to ligand binding by multiple carbohydrate-recognition domains in the macrophage mannose receptor. *J Biol Chem*. 1992; 267:1719–26. [PubMed: 1730714]
- [9]. Ezekowitz R, Williams D, Koziel H, Armstrong M, Warner A, Richards F, et al. Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. *Nature*. 1991; 351:155–8. [PubMed: 1903183]
- [10]. Stahl PD, Rodman JS, Miller MJ, Schlesinger PH. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proc Natl Acad Sci U S A*. 1978; 75:1399–403. [PubMed: 274729]
- [11]. Stahl P, Schlesinger PH, Sigardson E, Rodman JS, Lee YC. Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell*. 1980; 19:207–15. [PubMed: 6766809]
- [12]. Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. Macrophage receptors and immune recognition. *Annu Rev Immunol*. 2005; 23:901–44. [PubMed: 15771589]
- [13]. Lee RT, Ichikawa Y, Kawasaki T, Drickamer K, Lee YC. Multivalent ligand binding by serum mannose-binding protein. *Arch Biochem Biophys*. 1992; 299:129–36. [PubMed: 1280063]
- [14]. Mammen M, Choi SK, Whitesides GM. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew Chem Int Ed*. 1998; 37:2755–94.
- [15]. Lee RT, Lee YC. Affinity enhancement by multivalent lectin-carbohydrate interaction. *Glycoconj J*. 2000; 17:543–51. [PubMed: 11421347]
- [16]. Shepherd VL, Lee Y, Schlesinger PH, Stahl PD. L-Fucose-terminated glycoconjugates are recognized by pinocytosis receptors on macrophages. *Proc Natl Acad Sci U S A*. 1981; 78:1019–22. [PubMed: 6940120]
- [17]. Hoppe CA, Lee YC. Accumulation of a nondegradable mannose ligand within rabbit alveolar macrophages. Receptor reutilization is independent of ligand degradation. *Biochemistry*. 1984; 23:1723–30. [PubMed: 6326807]
- [18]. Bernard J, Hao X, Davis TP, Barner-Kowollik C, Stenzel MH. Synthesis of various glycopolymer architectures via RAFT polymerization: from block copolymers to stars. *Biomacromolecules*. 2006; 7:232–8. [PubMed: 16398520]
- [19]. Lowe AB, Sumerlin BS, McCormick CL. The direct polymerization of 2-methacryloxyethyl glucoside via aqueous reversible addition-fragmentation chain transfer (RAFT) polymerization. *Polymer*. 2003; 44:6761–5.
- [20]. Ladmiral V, Melia E, Haddleton DM. Synthetic glycopolymers: an overview. *Eur Polym J*. 2004; 40:431–49.
- [21]. Miura Y. Design and synthesis of well-defined glycopolymers for the control of biological functionalities. *Polym J*. 2012 doi:10.1038/pj.2012.4.
- [22]. Moad G, Rizzardo E, Thang SH. Living radical polymerization by the RAFT process. *Aust J Chem*. 2005; 58:379–410.

- [23]. Abdelkader O, Moebs-sanchez S, Queneau Y, Bernard J, Fleury E, Lyon D. Generation of well-defined clickable glycopolymers from aqueous RAFT polymerization of isomaltulose-derived acrylamides. *Polymer*. 2011; 49:1309–18.
- [24]. Boyer C, Davis TP. One-pot synthesis and biofunctionalization of glycopolymers via RAFT polymerization and thiol-ene reactions. *Chem Commun (Camb)*. 2009:6029–31. [PubMed: 19809633]
- [25]. Xiao N-Y, Li A-L, Liang H, Lu J. A well-defined novel aldehyde-functionalized glycopolymer: synthesis, micelle formation, and its protein immobilization. *Macromolecules*. 2008; 41:2374–80.
- [26]. Wang Y, Li X, Hong C, Pan C. Synthesis and micellization of thermoresponsive galactose-based diblock copolymers. *J Polym Sci, Part A: Polym Chem*. 2011; 49:3280–90.
- [27]. Deng Z, Li S, Jiang X, Narain R. Well-defined galactose-containing multi-functional copolymers and glyconanoparticles for biomolecular recognition processes. *Macromolecules*. 2009; 42:6393–405.
- [28]. Spain SG, Albertin L, Cameron NR. Facile in situ preparation of biologically active multivalent glyconanoparticles. *Chem Commun (Camb)*. 2006:4198–200. [PubMed: 17031430]
- [29]. Hetzer M, Chen G, Barner-Kowollik C, Stenzel MH. Neoglycopolymers based on 4-vinyl-1,2,3-triazole monomers prepared by click chemistry. *Macromol Biosci*. 2010; 10:119–26. [PubMed: 19731270]
- [30]. Slavin S, Burns J, Haddleton DM, Becer CR. Synthesis of glycopolymers via click reactions. *Eur Polym J*. 2011; 47:435–46.
- [31]. Kumar J, McDowall L, Chen G, Stenzel MH. Synthesis of thermo-responsive glycopolymers via copper catalysed azide-alkyne ‘click’ chemistry for inhibition of ricin: the effect of spacer between polymer backbone and galactose. *Polym Chem*. 2011:1879–86.
- [32]. Albertin L, Kohlert C, Stenzel M, Foster LJR, Davis TP. Chemoenzymatic synthesis of narrow-polydispersity glycopolymers: poly(6-O-vinyladipoyl-D-glucopyranose). *Biomacromolecules*. 2004; 5:255–60. [PubMed: 15002981]
- [33]. Albertin L, Stenzel M, Barner-kowollik C, Foster LJR, Davis TP. Well-defined glycopolymers from RAFT polymerization: poly (methyl 6-O-methacryloyl- α -D-glucoside) and its block copolymer with 2-hydroxyethyl methacrylate. *Macromolecules*. 2004:7530–7.
- [34]. Lowe AB, Wang R. Synthesis of controlled-structure AB diblock copolymers of 3-O-methacryloyl-1,2:3,4-di-O-isopropylidene-D-galactopyranose and 2-(dimethylamino)ethyl methacrylate. *Polymer*. 2007; 48:2221–30.
- [35]. Chono S, Tanino T, Seki T, Morimoto K. Uptake characteristics of liposomes by rat alveolar macrophages: influence of particle size and surface mannose modification. *J Pharm Pharmacol*. 2007; 59:75–80. [PubMed: 17227623]
- [36]. Wijagkanalan W, Kawakami S, Takenaga M. Efficient targeting to alveolar macrophages by intratracheal administration of mannosylated liposomes in rats. *J Control Release*. 2008; 125:121–30. [PubMed: 18037185]
- [37]. Wijagkanalan W, Kawakami S, Higuchi Y, Yamashita F, Hashida M. Intratracheally instilled mannosylated cationic liposome/NF κ B decoy complexes for effective prevention of LPS-induced lung inflammation. *J Control Release*. 2011; 149:42–50. [PubMed: 20035809]
- [38]. Moad G, Chong YK, Postma A, Rizzardo E, Thang SH. Advances in RAFT polymerization: the synthesis of polymers with defined end-groups. *Polymer*. 2005; 46:8458–68.
- [39]. Upreti M, Ruhela D, Vishwakarma RA. Synthesis of the tetrasaccharide cap domain of the antigenic lipophosphoglycan of *Leishmania donovani* parasite. *Tetrahedron*. 2000; 56:6577–84.
- [40]. Manicone AM, Birkland TP, Lin M, Betsuyaku T, van Rooijen N, Lohi J, et al. Epilysin (MMP-28) restrains early macrophage recruitment in *Pseudomonas aeruginosa* pneumonia. *J Immunol*. 2009; 182:3866–76. [PubMed: 19265166]
- [41]. Choi JE, Lee S, Sunde DA, Huizar I, Haugk KL, Thannickal VJ, et al. Insulin-like growth factor-I receptor blockade improves outcome in mouse model of lung injury. *Am J Respir Crit Care Med*. 2009; 179:212–9. [PubMed: 19011156]
- [42]. Zhang J, Wu B, Liu Z, Kowal P, Chen X, Shao J, et al. Large-scale synthesis of carbohydrates for pharmaceutical development. *Curr Org Chem*. 2001; 5:1169–76.

- [43]. Kallin E, Lönn H, Norberg T. New derivatization and separation procedures for reducing oligosaccharides. *Glycoconj J*. 1986; 3:311–9.
- [44]. Wolfenden, ML.; Cloninger, MJ. Carbohydrate recognition: biological problems, methods, and applications. 1st ed. John Wiley & Sons Inc; 2011. Multivalency in carbohydrate binding; p. 349-70.
- [45]. Taylor ME, Bezouska K, Drickamer K. Contribution to ligand binding by multiple carbohydrate-recognition domains in the macrophage mannose receptor. *J Biol Chem*. 1992; 267:1719–26. [PubMed: 1730714]
- [46]. Cameron NR, Spain SG, Kingham JA, Weck S, Albertin L, Barker CA, et al. Synthesis of well-defined glycopolymers and some studies of their aqueous solution behaviour. *Faraday Discuss*. 2008; 139:359–68. discussion 399-417, 419-20. [PubMed: 19049006]
- [47]. Edelman GM, Cunningham BA, Reeke GN, Becker JW, Waxdal MJ, Wang JL. The covalent and three-dimensional structure of concanavalin A. *Proc Natl Acad Sci U S A*. 1972; 69:2580–84. [PubMed: 4506778]
- [48]. Glycan binding protein database. Available from: <http://www.functionalglycomics.org>
- [49]. Weis WI, Drickamer K, Hendrickson WA. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. *Nature*. 1992; 360
- [50]. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008; 8:958–69. [PubMed: 19029990]
- [51]. Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis*. 2010; 30:245–57. [PubMed: 20665377]
- [52]. Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond)*. 2003; 104:27–38. [PubMed: 12519085]
- [53]. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003; 3:23–35. [PubMed: 12511873]

Fig. 1. *In vitro* BMDM glycopolymer uptake

Representative histograms (left) and fluorescence microscopy images (right) of BMDMs incubated *in vitro* with indicated AF488-glycopolymer (1.5 μ M) for 4 hr. Black line in histograms represents no polymer control. Inset shows a higher magnification image of the AF488-pManEMA treated cells.

Fig. 2. Dose dependent internalization

BMDMs were incubated with AF488-glycopolymers for 15 min at 37°C. Data are reported as mean fluorescence intensity $\pm$ standard deviation from three independent experiments.

Fig. 3. Competitive glycopolymer uptake

BMDMs were preincubated with 15 μ M unlabeled glycopolymer for 15 min followed by 1 μ M AF488-glycopolymer for 30 min at 37°C. NC represents no competition. Data are reported as mean fluorescence intensity $\pm$ standard deviation from three independent experiments.

Fig. 4. Time course of mannose uptake in stimulated macrophages
AF488-pManEMA (1 μ M) uptake by unstimulated (M0), LPS-stimulated (M1), or IL-4/
IL-13 stimulated (M2) BMDM's (n=3).

Fig. 5. Cell specificity of glycopolymer internalization

Internalization of AF488-glycopolymers (1.5 μ M) by BMDMs, mouse lung epithelial cells (MLE-12), or primary mouse lung fibroblasts (MLF) after 2h (A) or 24h (B) incubation.

Fig. 6. Internalization of glycopolymers by alveolar macrophages *in vivo*
Mice were given 10 μ M AF488-glycopolymers intratracheally. Bronchoalveolar lavage (BAL) was performed after 30 min and BAL cells were analyzed by flow cytometry. Data are reported as mean fluorescence intensity $\pm$ standard deviation from three independent experiments. * $p < 0.01$ compared to no polymer.

Scheme 1. RAFT-mediated glycopolymer synthesis and subsequent fluorophore conjugation and proposed mechanism of MRC-1-mediated macrophage uptake of the glycopolymers.

Table 1

Molecular weights, compositions, conversions, and labeling efficiency of glycopolymers.

Glycopolymer	M_n^a (g/mol)	PDI ^a	%Conversion ^b	PDS/ polymer ^c	Alexa488/ polymer ^c
pManEMA	11400	1.2	>99	5.2	0.93
pGalEMA	12200	1.2	>99	4.5	0.95
pGlcNAcEMA	13100	1.2	>99	3.3	1.2

^a Absolute number average molecular weights and polydispersity index (PDI) as determined by gel permeation chromatography (GPC).

^b Determined by ¹H-NMR

^c Determined by UV-Vis spectroscopy; ratio represents number of pyridyl disulfide (PDS) groups per polymer chain