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# Engineered hydrogen-bonded glycopolимер capsules and their interactions with antigen presenting cells

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## KEYWORDS

Poly(2-oxazoline), mannose, layer-by-layer, dendritic cells, MDSC, macrophages, PDL-1, CD80.

## ABSTRACT

Hollow glycopolимер microcapsules were fabricated by hydrogen-bonded layer-by-layer (LbL) assembly and their interactions with a set of antigen presenting cells (APCs), including dendritic cells (DCs), macrophages (MACs) and myeloid derived suppressor cells (MDSCs) were investigated. The glycopolymers were obtained by cascade post-polymerization modifications of poly(oligo(2-ethyl-2-oxazoline methacrylate)-stat-glycidyl methacrylate) involving anionic ring-opening of the glycidyl groups with propargylamine and the subsequent attachment of mannose

azide by copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). Multilayer assembly of the hydrogen-bonding pair (glycopolymer/poly(methacrylic acid) (PMAA)) onto planar and particulate supports ( $\text{SiO}_2$  particles,  $d = 1.16 \mu\text{m}$ ) yielded stable glycopolymers films upon cross-linking by CuAAC. Hollow monodisperse capsules were obtained after removal of the silica ( $\text{SiO}_2$ ) particle templates, as demonstrated by fluorescence and scanning electron microscopy. Cellular uptake studies using flow cytometry revealed the preferential uptake of the capsules by DCs when compared to MACs or MDSCs. Mannosylated capsules showed a cytokine independent cis-upregulation of CD80 specifically on DCs and a trans-downregulation of PDL-1 on MDSCs. Thus, the glycopolymers capsules may have the potential as vaccine carriers, as they are able to upregulate costimulatory molecules for immune cell stimulation on DCs and at the same time downregulate immune inhibitory receptors on suppressor APC such as MDSCs.

## INTRODUCTION

Polysaccharides are natural occurring polymers that play important roles in our immune system, e.g. in cellular recognition processes and pathogen infections. Sugar binding proteins, so called lectins are often overexpressed on the surface of antigen presenting cells (APC) and can interact with sugar ligands, (for example like those expressed on bacterial cell walls) and trigger or regulate immune responses.<sup>1,2</sup> In recent years this specific interaction has stimulated the interest in glycomimetic materials with improved therapeutic efficiencies.<sup>3,4</sup> Synthetic glycopolymers have been demonstrated to be superior oligosaccharide mimics because they enable the incorporation of multiple saccharides along a polymer backbone and thus amplification of carbohydrate-lectin/cell interactions,<sup>5,6</sup> also known as the cluster glycoside effect.<sup>7</sup> To date, diverse polymerization techniques have been exploited for the synthesis of defined glycopolymers,<sup>8-12</sup> including but not limited to, reversible deactivation radical polymerizations (RDRPs), ring-opening metathesis polymerization (ROMP), and ring-opening polymerizations (ROPs). The self-assembly of glycopolymers or the surface modification of particles with sugar units give rise to even more sophisticated materials, namely glycoparticles, which not only show carbohydrate-specific interactions but additionally exploit the beneficial physiochemical properties of particulate systems.<sup>13-15</sup> To date, a wide range of glycosylated carriers has been fabricated, such as micelles, polymersomes, and metallic, magnetic, and self-assembled nanoparticles.

Preferential uptake of particles by specific types of antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages (MACs) or myeloid derived suppressor cells (MDSCs), can lead to different immune responses and eventually different therapeutic outcomes. Uptake by DCs is usually a key step to induce adaptive immune responses, and has been a major focus of synthetic vaccine development for infectious diseases and cancer.<sup>16,17</sup> By contrast, uptake by MACs or MDSCs can lead to either activation or conversely suppression of immune responses, and is being explored to develop therapeutics against autoimmune and allergic diseases.<sup>18</sup> The state of activation of the APCs and the type of surface molecules that they upregulate after interacting with particles, will also influence their ability to activate or suppress immune responses. In this context, MACs and MDSCs that express high levels of the programmed death ligand 1 (PDL1) have high capacity to turn off immunity by interacting with programmed death 1 (PD1) molecules present on diverse immune cells such as T cells and NK cells.<sup>19</sup> By contrast,

activated mature DCs that express high levels of molecules that allow them to effectively interact with T cells, such as CD80, are classically strong activators of immunity.<sup>20</sup> Herein, we explore the balance of preferential uptake of glycomimetic layer-by-layer (LbL) microparticles by DCs compared with MACs and MDSCs, as well as the potential change in key regulatory molecules CD80 and PDL1 on these different APC subsets.

Poly(2-oxazoline)s (POx) are an emerging polymer class,<sup>21</sup> which have attracted significant attention in the biomaterials area<sup>22,23</sup> since they combine excellent biocompatibility<sup>24-27</sup> and protein repellence properties<sup>28</sup> along with the ease of synthesizing highly functional materials.<sup>29-32</sup> Recently, we reported the LbL assembly of POx-based systems for the fabrication of low-fouling, redox- and enzyme-degradable and targeted hollow polymer capsules.<sup>33-35</sup> The LbL approach<sup>36,37</sup> has attracted increasing interest for the engineering of particulate delivery systems,<sup>38,39</sup> as it allows tailoring of the physical and chemical properties of the carriers. Previously, particular focus was placed on the design of brush-like POx by RDRPs as they allow for the incorporation of further functional groups that are beneficial for covalent or reversible cross-linking and labelling or modification with targeting units of the capsules post-assembly. Copper-catalyzed alkyne-azide cycloaddition (CuAAC)<sup>33,35</sup> and dynamic thiol-disulfide exchange reactions<sup>34</sup> were employed depending on the desired application, for example for the modification of the capsule surface with a phage-display derived single-chain antibody (scFv) for the specific targeting of activated platelets.<sup>35</sup> Other interesting targeting ligands that are readily available are mono- and disaccharides. Over the last decade, a range of glycopol(2-oxazoline)s has been reported using glycosylated 2-oxazoline monomers<sup>40</sup> or post-polymerization reactions such as thiol-ene chemistry.<sup>41-43</sup> An elegant approach for the synthesis of glycosylated brush-like POx was reported by Weber et al. using telechelic POx macromonomers with  $\alpha$ -sugar and  $\omega$ -methacrylate end groups.<sup>44</sup>

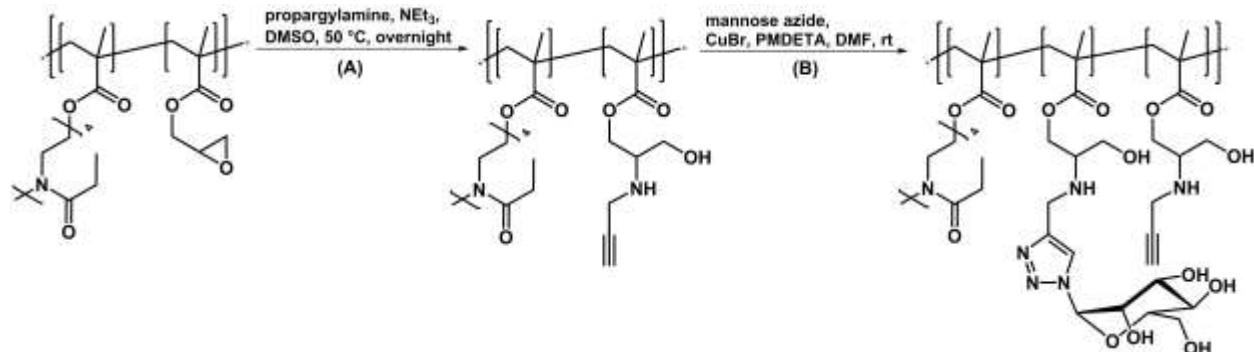
In this work, we demonstrate the fabrication of LbL assembled glycopol(2-oxazoline)-based capsules. To the best of our knowledge, the formation of hollow multilayered capsules by hydrogen-bonded LbL of glycopolymers has not yet been reported. The glycopolymer was obtained by a combination of living/controlled polymerization techniques and highly efficient post-polymerization modifications. Alkyne functionalities introduced during this process were then exploited for the modification with mannose moieties, labeling and multilayer crosslinking. The mannosylated films and capsules showed specific interactions with the lectin concanavalin

A (ConA). All capsules types tested were clearly preferentially taken up by DCs when compared to MACs or MDSCs. Moreover, the functional characteristics of the microparticles are further investigated in their capacity to downregulate PDL1 on multiple APC types, including professional suppressor APCs such as MDSCs. Only mannosylated capsules were capable of selectively upregulating CD80 specifically on DCs (but not MDSCs or MACs). The mechanism by which these molecules are controlled was further explored by the use of fluorescently labeled microparticles, providing evidence for the existence of two mechanisms: cytokine independent cis-activation for CD80 up-regulation specifically on DCs, and broad trans-downregulation of PDL1 across multiple cell types (balanced against particle uptake induced up-regulation). Together these results point to useful novel dual properties for mannosylated particles, making them suitable vaccine carriers, by virtue of being taken up by DCs and downregulating broadly immune inhibitory receptors on suppressor APCs such as MDSCs, while selectively being able to upregulate costimulatory molecules for immune cell stimulation on DCs.

## RESULTS AND DISCUSSION

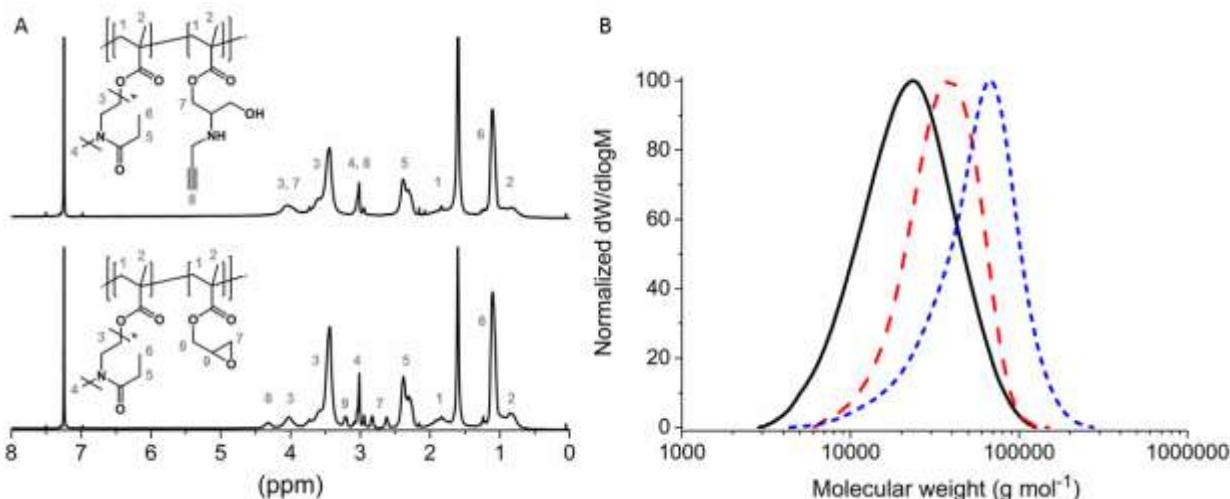
**Copolymer synthesis.** Mannosylated brush-like PEtOx-based copolymers ( $\text{PEtOxMA}_{\text{Man}}$ ) were prepared by a combination of cationic ring-opening polymerization (CROP), atom transfer radical polymerization (ATRP), efficient ring-opening reaction of glycidyl side chains and copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC, Scheme 1). The PEtOx macromonomer, namely oligo(2-ethyl-2-oxazoline methacrylate) ( $\text{OEtOxMA}$ ), was synthesized by CROP of 2-ethyl-2-oxazoline (EtOx) initiated with methyl tosylate and terminated with methacrylic acid (Scheme S1).<sup>45</sup> The copolymerization of  $\text{OEtOxMA}$  with glycidyl methacrylate (GMA) by ATRP yielded highly functional copolymers ( $\text{PEtOxMAGMA}$ ), which can be efficiently modified through ring-opening reaction with nucleophiles. In this way, brush-like PEtOx with pendant alkyne moieties were prepared ( $\text{PEtOxMA}_{\text{Alk}}$ ), which were further functionalized with mannose azide in a CuAAC reaction to yield  $\text{PEtOxMA}_{\text{Man}}$ .

**Scheme 1.** Synthesis of mannosylated poly(oligo(2-ethyl-2-oxazoline methacrylate)) brush copolymer ( $\text{PEtOxMA}_{\text{Man}}$ ). (A) Base-catalyzed ring-opening reaction using propargylamine ( $\text{PEtOxMA}_{\text{Alk}}$ ). (B) Introduction of mannose moieties by CuAAC ( $\text{PEtOxMA}_{\text{Man}}$ ).



$^1\text{H}$  NMR spectroscopy was used to determine the degree of polymerization (DP) and functionalization (DF). EtOx was polymerized with a monomer-to-initiator ratio of 6:1 to full conversion of the monomer before the living chain ends were terminated with methacrylic acid to give the  $\text{OEtOx}_6\text{MA}$  macromonomer. ATRP of  $\text{OEtOxMA}$  and GMA provided functional PEtOx-containing copolymers with 17 mol% glycidyl groups, as determined by the ratio of the integrals of the GMA peaks (2.5–2.9 ppm) and the  $\text{CH}_2$  peak of the PEtOx side chain (2.1–2.5 ppm). The GMA groups were quantitatively converted into alkyne groups by treatment with propargylamine (Figure 1A, top), as can be seen by the disappearance of the respective  $^1\text{H}$  NMR

peaks (2.5–2.9 ppm, 4.2 ppm). The modification of PEtOxMA<sub>Alk</sub> with mannose azide via CuAAC resulted in the formation of triazole groups, which was confirmed via a <sup>1</sup>H NMR signal at 8.1 ppm (Figure S1). The degree of functionalization was determined by comparing the integral ratio of the triazole and the CH<sub>2</sub> PEtOx signal, which revealed an overall mannose content of 6 mol%. Thus, PEtOxMA<sub>Man</sub> still contains unreacted alkyne groups, which can be used for further post-polymerization reactions, such as cross-linking reactions and labeling. Size exclusion chromatography (SEC) measurements revealed the synthesis of defined copolymers with dispersity ( $\bar{D}$ ) values < 1.4. A shift to higher molar masses was observed by both post-polymerization modifications, the ring-opening reaction with propargyl amine and CuAAC with mannose azide (Figure 1B).

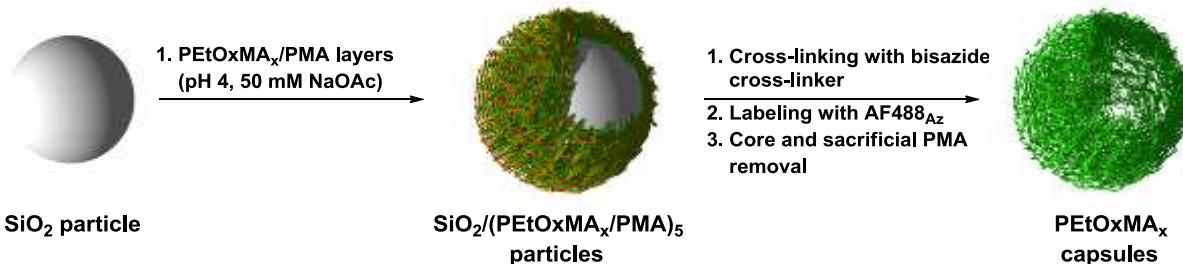


**Figure 1.** (A) <sup>1</sup>H NMR spectra (400 MHz, CDCl<sub>3</sub>) of poly[oligo(2-ethyl-2-oxazoline methacrylate)-stat-glycidyl methacrylate] (PEtOxMA<sub>GMA</sub>) synthesized by ATRP (bottom), which was modified with propargylamine (PEtOxMA<sub>Alk</sub>) in a base-catalyzed ring-opening reaction (top). (B) SEC traces (solvent: DMAc/LiBr) of PEtOxMA<sub>GMA</sub> (—), PEtOxMA<sub>Alk</sub> (—), PEtOxMA<sub>Man</sub> (---).

**Multilayer Assembly on Planar and Particle Supports.** Quartz crystal microbalance (QCM) and flow cytometry were used to monitor the assembly of PEtOxMA<sub>Alk</sub>/PMA and PEtOxMA<sub>Man</sub>/PMA multilayer films via hydrogen bonding, respectively. Polymer solutions were prepared at 1 mg mL<sup>-1</sup> (50 mM NaOAc, pH 4) and five PEtOxMA<sub>x</sub>/PMA bilayers were iteratively deposited. For QCM studies, an additional PEI (1 mg mL<sup>-1</sup>, 0.5 M NaCl) layer was

initially deposited onto the gold surface of the QCM crystal prior to the layer buildup. PMA was allowed to adsorb for 15 min before washing the film in NaOAc buffer. Subsequently, PEtOxMA<sub>x</sub> was adsorbed for 20 min followed by the next washing step. This procedure was repeated four more times. The formation of hydrogen-bonded PEtOxMA<sub>x</sub>/PMA multilayer films was confirmed by a frequency decrease with increasing number of polymer layers (Figure S2A). The deposition of the PEtOxMA<sub>Man</sub>/PMA bilayer system showed only a slightly higher frequency change compared to the PEtOxMA<sub>Man</sub>/PMA bilayer system, suggesting a similar hydrogen-bonding interaction of both systems.

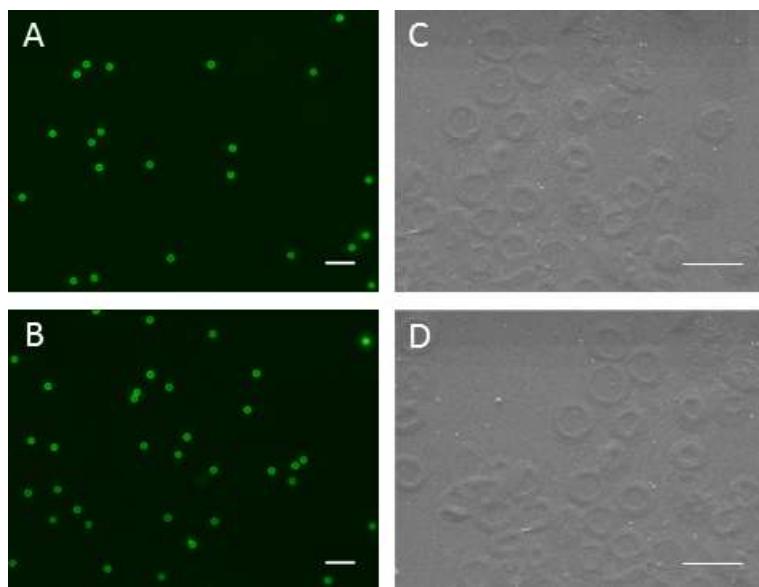
To examine the multilayer film formation on particle supports, PEtOxMA<sub>x</sub> and AF488-cadaverine labeled PMA (PMA<sub>488</sub>) were deposited alternatively onto monodisperse SiO<sub>2</sub> particles (1.16  $\mu$ m in diameter) at pH 4. The particles were incubated in the respective polymer solutions for 15 min before washing them via three centrifugation/re-dispersion cycles, as detailed in the Experimental Section. After the deposition of each bilayer the fluorescence intensity of the core/shell particles was measured by flow cytometry. For both bilayer systems a regular and linear layer buildup was observed (Figure S2B) similarly to the QCM studies.



**Scheme 2.** Preparation of PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> capsules via hydrogen-bonded LbL assembly of PEtOxMA<sub>Alk</sub> (non-mannosylated) or PEtOxMA<sub>Man</sub> (mannosylated) and PMA.

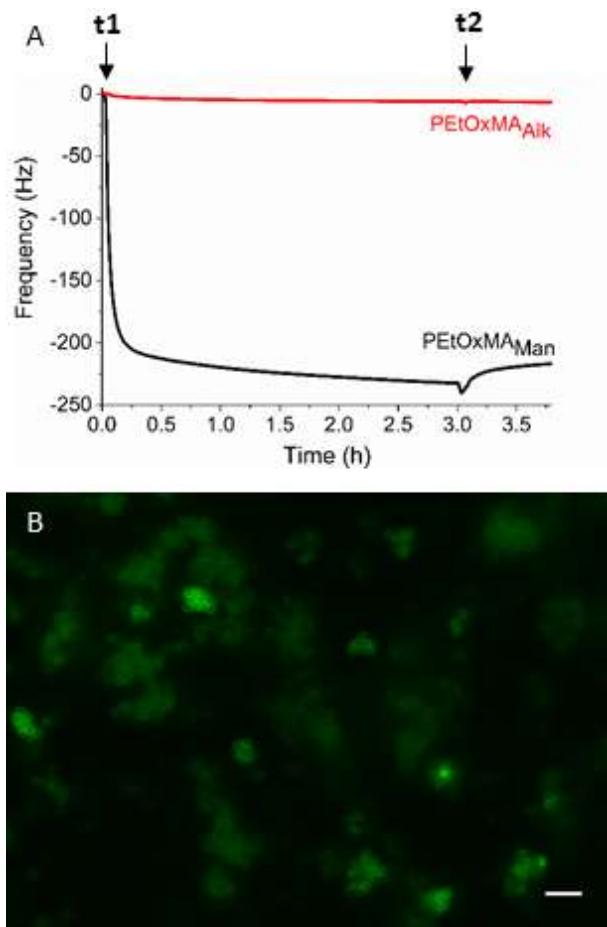
For the fabrication of hollow mannosylated and non-mannosylated polymer capsules, five PEtOxMA<sub>Man</sub>/PMA and PEtOxMA<sub>Alk</sub>/PMA bilayers, respectively, were deposited onto monodisperse SiO<sub>2</sub> particles (1.16  $\mu$ m) and cross-linked with a non-degradable bisazide cross-linker similar to a procedure reported for other CuAAC "click" capsules previously (Scheme 2).<sup>46</sup> Remaining alkyne groups in the multilayer films were further modified with azide-functionalized Alexa Fluor 488 (AF488<sub>Az</sub>) to provide a suitable fluorescent tag for subsequent cell studies. After removal of the particle template by hydrogen fluoride (HF) treatment and the

sacrificial PMA layers by washing the capsules into PBS (pH 7.4), stable hollow capsules were obtained. Analysis of the capsules using fluorescence microscopy showed spherical capsules with diameters consistent with the used template for both the non-mannosylated (Figure 2A) and mannosylated (Figure 2B) capsules. In addition, capsule morphology and size were analyzed using SEM (Figure 2C/D), which showed spherical structures with folds and creases typical for stabilized LbL polymer capsules.



**Figure 2.** Fluorescence (A, B; scale bar = 5  $\mu\text{m}$ ) and scanning electron (C, D; scale bar = 2  $\mu\text{m}$ ) microscopy images of PEtOxMA<sub>Alk</sub> (A, C) and PEtOxMA<sub>Man</sub> (B, D) capsules. Multilayer assembly was performed on sacrificial SiO<sub>2</sub> particles (1.16  $\mu\text{m}$  diameter) at pH 4 (NaOAc, 50 mM). The core-shell particles were treated with HF to remove the core and washed into PBS to remove sacrificial PMA. The capsules were labeled with azide-functionalized Alexa Fluor 488 (AF488<sub>Az</sub>).

**Concanavalin A (ConA) binding studies.** Lectins, which are carbohydrate-binding proteins play an important role in biological processes including cell proliferation and death. Glycopolymers or glycopolymer assemblies are capable of multivalent binding to lectins, a process known as the "cluster-glycoside effect".<sup>7</sup> Concanavalin A (ConA) is a lectin, which specifically binds to mannose and glucosyl residues. ConA possesses four binding sites, which enable the binding of up to four sugar groups resulting in protein aggregation when a multivalent carbohydrate or glycopolymer is employed.

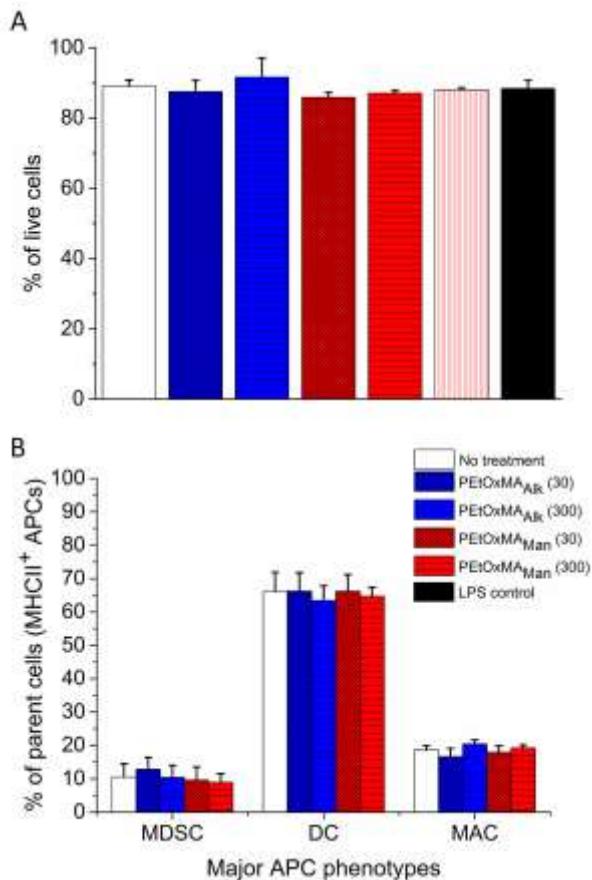


**Figure 3.** Concanavalin A (ConA) binding studies on, (A) PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> planar films, as monitored by QCM, t1: deposition of ConA ( $0.2 \text{ mg mL}^{-1}$ , HBS buffer), t2: wash with HBS buffer. PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> surfaces were obtained by sequential deposition of PEI ( $1 \text{ mg mL}^{-1}$ ), PMA<sub>Alk</sub> ( $1 \text{ mg mL}^{-1}$ ) and PEtOxMA<sub>x</sub>/PMA ( $1 \text{ mg mL}^{-1}$ ); (B) PEtOxMA<sub>Man</sub> capsules assessed by fluorescence microscopy. Scale bar =  $5 \mu\text{m}$ .

QCM measurements were performed to provide evidence of the binding of the glycopolymer films to ConA. To this end, the gold surfaces of the QCM chips were coated with PEI and PMA<sub>Alk</sub>. The latter was used to deposit PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> via hydrogen bonding interaction, respectively, and stabilize the film at physiological pH using CuAAC chemistry. Upon exposure of the PEtOxMA<sub>Man</sub> film to ConA, a significant decrease in frequency was observed. In contrast, the PEtOxAlk film showed only marginal frequency changes (Figure 3a). This is indicative of a specific interaction between the mannose containing film and the carbohydrate-binding protein. Non-mannosylated and mannosylated capsules were also

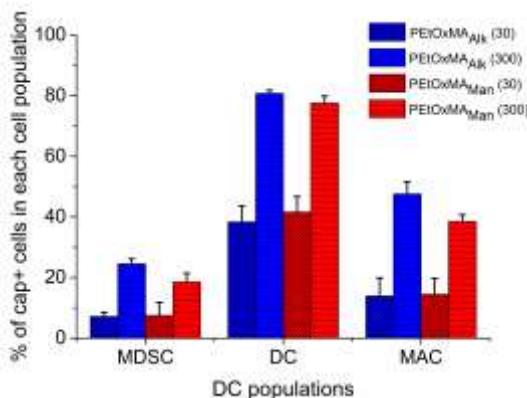
investigated for their potential to interact with ConA. Both capsule types were incubated with AF647 labeled ConA and examined using fluorescence microscopy. PEtOxMA<sub>Alk</sub> capsules remained individually dispersed (as shown in Figure 2A), whereas PEtOxMA<sub>Man</sub> aggregated significantly (Figure 3b). The overlay of the AF488 and AF647 channels further proved the ConA-driven aggregation (Figure S3).

**Effects of capsules on viability and composition of bone marrow cultures.** Subsequently, the interaction of the capsules with a range of cells derived from bone marrow was investigated. For potential use in vaccine delivery the capsules need to possess negligible toxicity. This was investigated by the assessment of the effect of capsules on the viability of bone marrow derived dendritic cells (BMDCs) designed to induce functionally relevant APCs such as DCs, MACs and MDSCs. Overall, cell viability in these cultures was not negatively affected by any of the capsules, independent of the capsule type and concentration (30 vs. 300 capsules per cell, referred to ‘30’ or ‘300’ in the figures) tested. Soluble PEtOxMA<sub>Man</sub> at the corresponding doses to those used on the capsules was also found to be not toxic. Lipopolysaccharide (LPS), used in subsequent studies as a positive control stimulatory agent was also confirmed to be non-toxic at the standard dose. Similar results were observed after 1 h (data not shown) or 18 h of incubation (Figure 4A), with no differences in cell viability across all treatment groups compared to the no treatment group. Additionally, there were no changes in the proportional frequency of DCs (CD11c+Gr1-), MACs (CD11c-Gr1-) or MDSCs (CD11c-Gr1+) over 1 h (not shown) or 18 hrs of co-culture with the different formulations (Figure 4B).



**Figure 4.** Cell viability of BMDC cultures (A) and antigen presenting cell (APC) phenotypes (B) after 18 h co-culture with different capsule types. The effect of capsules on cells were tested on BMDCs cultures. BMDCs were co-cultured with PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> capsules at 30 or 300 capsules/cell or control agent (LPS) for 1 or 18 h, at which time point cells were harvested and stained with various antibodies that define the phenotypes of the cells, e.g., MDSCs (CD11c-Gr1+); DCs (CD11c+Gr1-), MACs (CD11c-Gr1-), and analyzed by flow cytometry. Cell viability (A) were determined by live/dead staining; and the effect of capsules on the APCs in major APC phenotypes were determined by the percentage of MHCII expression in each type of immune cells. N=4 for each treatment conditions.

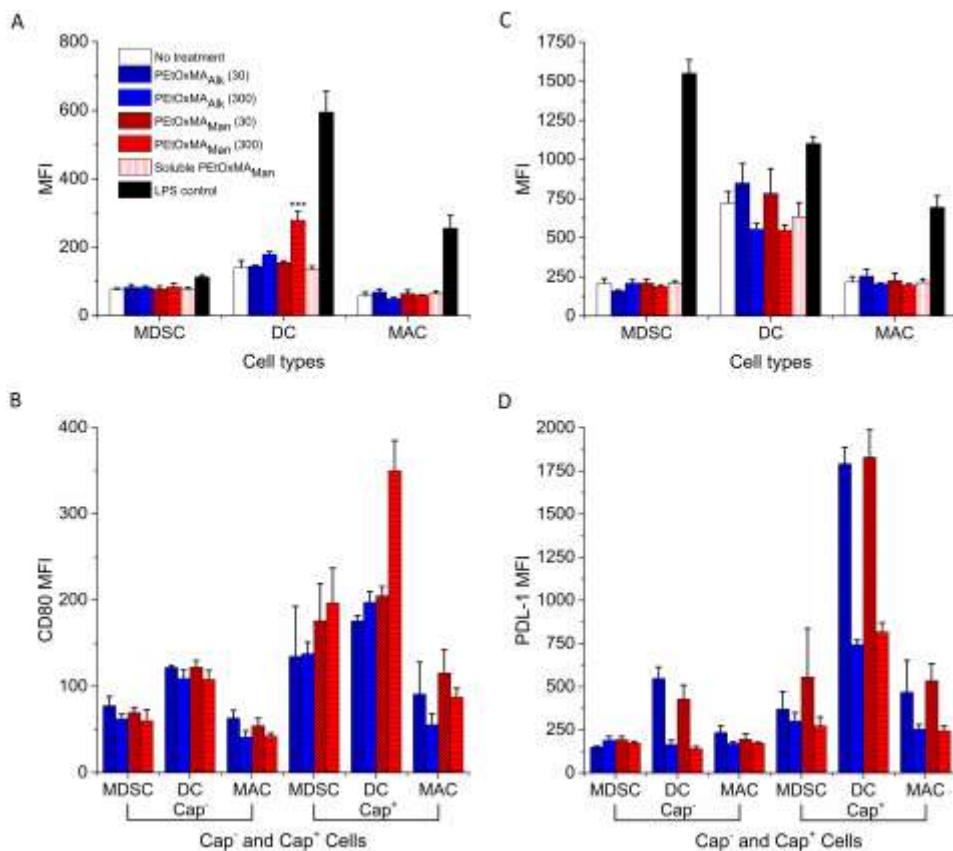
**Capsule uptake by different APC subsets.** All capsule types, regardless of surface modification by mannose, showed preferential uptake by stimulatory rather than inhibitory APC subsets. Hence, as shown in Figure 5, capsules were preferentially internalized by conventional DCs (CD11c+Gr1-), MACs (CD11c-Gr1-) and then by MDSCs (CD11c-Gr1+) after 18 h co-culture. The effect was titratable, with more cells taking up capsules at higher capsule doses.



**Figure 5.** Capsule uptake by different APC populations. Capsules (PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub>) uptake was tested on BMDC cultures at 30 or 300 capsules/cell, and determined by the percentage of capsule positive cells in each APC population after 18 h of co-culturing particles and BMDCs as detailed in the Experimental Section. N=4 for each treatment conditions.

**DC and MAC activation and maturation induced by the capsules.** Stimulation via pathogen recognition receptors (PRRs), such as toll like receptor 4 (TLR4) for LPS or the mannose receptor, activates and matures APC and enables them to perform important functions, such as naïve T cell priming by ‘licensed’ DCs. The molecule CD80 is a vital functional marker for this process. Expression of CD80 was analyzed on all three key types of APCs, DCs, MACs and MDSCs (Figure 6A) after 18 h co-culture with the different types of capsules or controls, including LPS as a positive control. As expected, CD80 expression was significantly enhanced in LPS stimulated cells on both DCs and MACs, and to a lesser degree on MDSCs (Figure 6A). PEtOxMA<sub>Alk</sub> capsules did not induce CD80 expression on any cell type tested. In contrast, PEtOxMA<sub>Man</sub> capsules showed large and significant increases in CD80 expression on DCs compared to the other groups, including the treatment group with soluble PEtOxMA<sub>Man</sub> at an equivalent concentration to that present on the capsules. To further determine whether CD80 upregulation was due to a direct (cis) interaction between the capsules and DCs or to an indirect (trans) effect mediated by soluble cytokines, we analyzed the cells which had either internalized or not internalized the capsules in the same culture. Figure 6B shows clearly that only DCs that had taken up capsules (Cap+ cells) also up-regulate CD80 expression.

PDL-1 is an important negative modulator of immune function, and is associated with a tolerogenic DC phenotype.<sup>47</sup> Expression of PDL-1 was determined on the three types of APC cell types studied after treatment with the control LPS, soluble PEtOxMA<sub>Man</sub>, PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> capsules. LPS treatment significantly increased PDL-1 expression (Figure 6C) in all cell types studied (DCs, MDSCs and MACs). By contrast, high doses of capsule particles decreased PDL-1 expression on DCs across the cultures, independent of the type of capsule. An analysis of cells that had taken up capsules compared to those that did not, revealed PDL-1 downregulation on DCs in the absence of particle uptake, suggesting significant trans effects for PDL-1 downregulation (Figure 6D). By contrast, overall PDL-1 expression, found also preferentially on DCs was highest on cells that had taken up particles, consistent with uptake induced upregulation.



**Figure 6.** Activation/maturation of immune cells (A, B) and effect of the immune-regulatory functions (C, D) by capsule particles. (A) Overall activation molecule CD80 expression; (B) differential CD80 expression in cells taken up capsules (Cap<sup>+</sup>) and no capsule cells (Cap<sup>-</sup>); (C) overall immune-regulatory molecule PDL-1 expression; and (D) differential PDL-1 expression in

cells taken up capsules ( $\text{Cap}^+$ ) and no capsule cells ( $\text{Cap}^-$ ). BMDC were cultured with PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> capsules at 30 or 300 capsules/cell or control agent (LPS) for 18 h, at which time point cells were harvested and stained antibodies specifically for activation molecules such as CD80 (A, B) and PDL-1 (C, D). Data were presented as mean fluorescence intensity (MFI) of CD80 expression. N=4 for each treatment conditions.

## CONCLUSIONS

Mannosylated hollow poly(2-oxazoline)-based capsules were successfully prepared by hydrogen-bonding LbL assembly of a mannosylated brush poly(oligo(2-ethyl-2-oxazoline)methacrylate). The copolymer was synthesized in a three-step cascade employing a combination of cationic ring-opening polymerization, controlled radical polymerization and efficient post-polymerization modifications. The mannosylated multilayer films were prepared on both planar and particle supports and showed interactions with a mannose-specific lectin in contrast to non-mannosylated films. A preferential uptake of the capsules in DCs as compared to MACs and MDSCs was observed, independent of the mannosylation. However, only mannosylated capsules triggered a significant upregulation of costimulatory molecules for immune cell stimulation on DCs, without upregulation of inhibitory receptors such as PDL-1. This finding contrasts conventional ‘danger signal’ activation via TLR4 by LPS, which massively upregulates PDL-1, particularly on the immune-suppressive MDSC subset. The results obtained demonstrate the potential of these mannosylated capsules as future vaccine carriers, highlighting some potential beneficial properties of particulate glycopolymer systems, while also noting the challenges associated with the translation of lectin-glycopolymers interactions to *in vitro* cell studies.

## EXPERIMENTAL PART

### Materials and Instrumentation

Silica particles ( $1.16\text{ }\mu\text{m}$ ) were purchased from MicroParticles GmbH (Germany) as a 5 wt% suspension and were used as received. Poly(methacrylic acid, sodium salt) (PMA, 30 wt%),  $M_w$  15 kDa, was purchased from Polysciences (USA). 2-Ethyl-2-oxazoline, methyl tosylate, and triethylamine were purchased from Sigma-Aldrich, distilled to dryness and stored under argon. Acetonitrile, anisole, glycidyl methacrylate (GMA), ethyl  $\alpha$ -bromoisobutyrate (EBIB), N,N,N',N'',N'''-pentamethyldiethylenetriamine (PMDETA), copper(I) bromide (CuBr), dimethylsulfoxide (DMSO), propargylamine, phosphate buffered saline (PBS) tablets, and buffering salts were purchased from Sigma-Aldrich and used as received. HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, pH 7.4) containing 1 mM  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  was prepared by mixing the appropriate salts. Alexa Fluor 488 azide (AF488<sub>Az</sub>) was obtained from Life Technologies, Inc. High-purity water with a resistivity greater than  $18\text{ M}\Omega\text{ cm}$  was obtained from an in-line Millipore RiOs/Origin water purification system (Milli-Q water). Mannose azide was prepared according to a literature procedure.

Nuclear magnetic resonance ( $^1\text{H}$  NMR) spectra were recorded in deuterated solvents ( $\text{CDCl}_3$ , DMSO-d6) using a 400 MHz Varian INOVA system at  $25\text{ }^\circ\text{C}$ . SEC analyses of polymer samples were performed using a Shimadzu modular system comprising a DGU-12A degasser, an SIL-20AD automatic injector, a  $5.0\text{ }\mu\text{m}$  bead-size guard column ( $50 \times 7.8\text{ mm}$ ) followed by three KF-805L columns ( $300 \times 8\text{ mm}$ , bead size:  $10\text{ }\mu\text{m}$ , pore size maximum:  $5000\text{ }\text{\AA}$ ), a SPD-20A ultraviolet detector, and an RID-10A differential refractive-index detector. The temperature of columns was maintained at  $40\text{ }^\circ\text{C}$  using a CTO-20A oven. The eluent was *N*, *N*-dimethylacetamide (DMAc, HPLC grade, with 0.03% w/v LiBr) and the flow rate was kept at  $1\text{ mL min}^{-1}$  using a LC-20AD pump. A molecular weight calibration curve was produced using commercial narrow molecular weight distribution polystyrene standards with molecular weights ranging from 500 to  $2 \times 10^6\text{ g mol}^{-1}$ . Polymer solutions at approx.  $3\text{ mg mL}^{-1}$  were prepared and filtered through  $0.45\text{ }\mu\text{m}$  filters prior to injection.

Fluorescence microscopy measurements were performed with an inverted Olympus IX71 microscope equipped with a DIC slider (U-DICT, Olympus) with a  $60\times$  objective lens (Olympus UPLFL20/0.5 N.A., W.D. 1.6) and used to visualize the capsules. A CCD camera (Cool SNAP fx, Photometrics, Tucson, AZ) was mounted on the left hand port of the microscope.

Fluorescence images were illuminated with an Hg arc lamp using a UF1032 filter cube. Scanning electron microscopy (SEM) images were obtained using a FEI Quanta 200 field emission scanning electron microscope operated at an accelerating voltage of 10 kV.

### Polymer synthesis and post-modifications

*Oligo(2-ethyl-2-oxazoline methacrylate) (OEtOxMA):* The OEtOxMA macromonomer (Scheme S1) was synthesized as follows: a solution of methyl tosylate (I; 1.86 g; 10 mmol) and 2-ethyl-2-oxazoline (EtOx; 4 g; 40 mmol) was prepared in acetonitrile (3.8 g; 92.6 mmol). The total monomer concentration was adjusted to 4 M with a [EtOx]:[I] ratio of 4:1. The sealed vial was heated to 85 °C in an oil bath for 60 min. After cooling, methacrylic acid (1.29 g; 15 mmol) and triethylamine (2 g; 20 mmol) were added to the polymerization mixture and stirring was continued overnight at 50 °C. Subsequently, the polymer was purified by washing with sodium bicarbonate and brine. The combined organic phases were dried over magnesium sulfate, filtered and the solvent was removed under reduced pressure. OEtOxMA was obtained after drying the polymer for 2 days. <sup>1</sup>H NMR analysis demonstrated the quantitative conversion of the monomer and quantitative functionalization with methacrylic acid. SEC (DMAc, PS standard):  $M_n = 1\ 020\ g\ mol^{-1}$ ,  $D = 1.13$ .

*Poly(oligo(2-ethyl-2-oxazoline methacrylate)-stat-glycidyl methacrylate) (PEtOxMA<sub>GMA</sub>):* PEtOxMA<sub>GMA</sub> was prepared by atom transfer radical polymerization (ATRP). OEtOxMA (1 g; 2 mmol) and PMDETA (19.9 mg; 0.11 mmol) were dissolved in anisole (3 mL) and degassed for 30 min. In parallel, GMA (187.4 mg; 1.32 mmol) and EBIB (6.5 mg; 0.033 mmol) were dissolved in anisole (1 mL), CuBr (15.5 mg; 0.11 mmol) was added and the suspension was degassed for 30 min. The total monomer concentration was adjusted to 0.6 M with a [OEtOxMA]:[GMA]:[EBIB] ratio of 60:40:1. The OEtOxMA solution was added to the CuBr suspension and then reacted at 60 °C for 22 h. After cooling to room temperature, the polymer was precipitated in ice-cold diethyl ether, re-dissolved in dichloromethane and passed through a neutral alumina column. Subsequently, the polymer was dialyzed against Milli-Q for 3 days (MWCO = 3.5 kDa) and recovered by freeze-drying. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>,  $\delta$ ): 4.5 – 4.2 (COO-CH<sub>2</sub> GMA), 4.2 – 3.3 (N-CH<sub>2</sub> OEtOxMA), 3.3 – 3.1 (CHO GMA), 3.1 – 2.9 (CH<sub>3</sub>N OEtOxMA), 2.9 – 2.5 (CH<sub>2</sub>O GMA), 2.5 – 2.1 (CH<sub>2</sub> OEtOxMA), 2.0 – 1.4 (CH<sub>2</sub> methacrylate

*backbone*), 1.3 – 0.9 ( $\text{CH}_3 \text{ OEtOxMA}$ ), 0.9 – 0.6 ( $\text{CH}_3 \text{ methacrylate backbone}$ ); SEC (DMAc, PS standard):  $M_n = 17\,500 \text{ g mol}^{-1}$ ,  $D = 1.37$ .

*Post-polymerization modification of PEtOxMA<sub>GMA</sub> with propargylamine (PEtOxMA<sub>Alk</sub>)*. PEtOx<sub>GMA</sub> (150 mg; 0.0085 mmol), propargylamine (76 mg; 1.37 mmol) and triethylamine (138 mg; 1.37 mmol) were dissolved in dry DMSO (2 mL) and the solution was stirred at 50 °C overnight. Subsequently, the solvent was removed under reduced pressure. The polymer was re-dissolved in methanol and dialyzed against methanol for 1 day and subsequently against Milli-Q for 2 days (MWCO = 3.5 kDa). PEtOxMA<sub>Alk</sub> was recovered by freeze-drying.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 4.3 – 3.2 (N- $\text{CH}_2 \text{ OEtOxMA}$ , COO- $\text{CH}_2 \text{ GMA}$ ), 3.1 – 2.9 ( $\text{CH}_3\text{N OEtOxMA}$ ), 2.5 – 2.1 ( $\text{CH}_2 \text{ OEtOxMA}$ ), 2.0 – 1.4 ( $\text{CH}_2 \text{ methacrylate backbone}$ ), 1.3 – 0.9 ( $\text{CH}_3 \text{ OEtOxMA}$ ), 0.9 – 0.6 ( $\text{CH}_3 \text{ methacrylate backbone}$ ); SEC (DMAc, PS standard):  $M_n = 30\,200 \text{ g mol}^{-1}$ ,  $D = 1.28$ .

*Post-polymerization modification of PEtOxMA<sub>Alk</sub> with mannose azide (PEtOxMA<sub>Man</sub>)*. PEtOx<sub>Alk</sub> (20 mg; 0.0011 mmol), mannose azide (2.6 mg; 0.011 mmol) and PMDETA (3.65 mg; 0.02 mmol) were dissolved in DMF (0.5 mL) and the solution was deoxygenated for 30 min before the addition of CuBr (3 mg; 0.02 mol). The reaction mixture was subsequently stirred at room temperature for 19 h. To remove unreacted compounds and CuBr/PMDETA the polymer was dialyzed against Milli-Q (MWCO = 3.5 kDa) for 3 days and passed through a NAP-25 column. PEtOxMA<sub>Man</sub> was recovered by freeze-drying.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-d}_6$ ,  $\delta$ ): 8.25 – 7.9 (triazole), 6.0 – 4.5 (mannose), 4.25 – 3.0 (N- $\text{CH}_2 \text{ OEtOxMA}$ , COO- $\text{CH}_2 \text{ GMA}$ ), 3.0 – 2.8 ( $\text{CH}_3\text{N OEtOxMA}$ ), 2.4 – 2.0 ( $\text{CH}_2 \text{ OEtOxMA}$ ), 2.0 – 1.25 ( $\text{CH}_2 \text{ methacrylate backbone}$ ), 1.25 – 0.75 ( $\text{CH}_3 \text{ OEtOxMA}$ ), 0.75 – 0.5 ( $\text{CH}_3 \text{ methacrylate backbone}$ ); SEC (THF, PS standard):  $M_n = 42\,900 \text{ g mol}^{-1}$ ,  $D = 1.4$ .

## Film and capsule preparation

*Multilayer Assembly on Planar Supports*: Gold-coated 5 MHz AT-cut crystals (Q-Sense AB, Västra, Frölunda, Sweden) were cleaned with piranha solution (7:3 v/v sulfuric acid/hydrogen peroxide) for 20 min followed by extensive rinsing in water, drying with nitrogen, and exposure (20 min) to UV (Bioforce NanoScience, USA). *Caution! Piranha solution is very corrosive. Extreme care should be taken when handling piranha solution, and only small quantities should be prepared.* QCM measurements were conducted using a QCM-D E4 device with four flow

cells (Q-Sense AB, Västra, Frölunda, Sweden). All frequency values quoted are for the third overtone. The other overtones measured (fifth and seventh overtones) followed the same trend. According to the Sauerbrey equation, a frequency change of 1 Hz corresponds to a film mass of  $17.7 \text{ ng cm}^{-2}$  for the 5 MHz QCM crystals used. The temperature was kept constant at  $23^\circ\text{C}$  for the entire deposition process. After initially depositing a layer of PEI ( $1 \text{ g L}^{-1}$  in 0.5 M NaCl, 10 min; washed with Milli-Q and subsequently with 20 mM NaOAc pH 4), PMA ( $1 \text{ g L}^{-1}$  in 20 mM NaOAc, pH 4) and PEtOxMA<sub>x</sub> ( $1 \text{ g L}^{-1}$  in 20 mM NaOAc, pH 4) were sequentially adsorbed to the films until a total number of 9 layers was obtained. After each adsorption step, the films were washed with 0.5 mL NaOAc (20 mM, pH 4).

*Multilayer Assembly on Particle Supports:* PEtOxMA<sub>x</sub> and PMA were directly dissolved in NaOAc (20 mM, pH 4) to a concentration of  $1 \text{ g L}^{-1}$  and pH adjusted. 100  $\mu\text{L}$  silica particles (5 wt%, 1.16  $\mu\text{m}$ -diameter) were washed three times with 0.5 mL NaOAc (20 mM, pH 4) by centrifugation. To form the multilayers, 200  $\mu\text{L}$  of PEtOxMA<sub>x</sub> ( $1 \text{ g L}^{-1}$  in 20 mM NaOAc, pH 4) was added to the particle suspension for adsorption (15 min) with constant shaking. The particles were then washed via three centrifugation (1000  $g$ , 1 min)/redispersion (300  $\mu\text{L}$ ) cycles. As second layer, 200  $\mu\text{L}$  of PMA ( $1 \text{ g L}^{-1}$  in 20 mM NaOAc, pH 4) was added to the particle suspension for adsorption (10 min) with constant shaking. The particles were then washed via three centrifugation (1000  $g$ , 1 min)/redispersion (300  $\mu\text{L}$ ) cycles. This adsorption process was repeated with a further four bilayers of PEtOxMA<sub>x</sub> and PMA ( $1 \text{ g L}^{-1}$  in 20 mM NaOAc, pH 4), followed by one protective capping bilayer of PVPON and PMA ( $1 \text{ g L}^{-1}$  in 20 mM NaOAc, pH 4).

To follow the assembly of multilayers on particle supports fluorescently labeled PMA (PMA<sub>488</sub>) was used for the buildup to monitor the sequential assembly with flow cytometry. PEtOxMA<sub>x</sub> and PMA<sub>488</sub> were alternately adsorbed onto 100  $\mu\text{L}$  silica particles (5 wt%, 1.16  $\mu\text{m}$ -diameter), as described above, until 5 bilayers were deposited (i.e., PMA as the terminating layer) onto the particle supports. After each adsorption step, the core-shell particles were re-suspended in 20  $\mu\text{L}$  NaOAc (20 mM, pH 4) and a 3  $\mu\text{L}$  sample of the particles was taken and analyzed using the flow cytometer.

*Crosslinking of the Multilayers:* After completion of the assembly and extensive washing, the core–shell particles were re-dispersed in 0.5 mL NaOAc (20 mM, pH 4) and incubated in 600  $\mu\text{L}$

bisazide linker ( $1\text{ g L}^{-1}$ ) in the presence of  $200\text{ }\mu\text{L}$  sodium ascorbate ( $4.4\text{ g L}^{-1}$ ) and  $200\text{ }\mu\text{L}$  copper sulfate ( $1.75\text{ g L}^{-1}$ ) (all in  $20\text{ mM NaOAc, pH 4}$ ) overnight. After washing, the crosslinked core–shell particles were re-dispersed in  $200\text{ }\mu\text{L}$  of NaOAc ( $20\text{ mM, pH 4}$ ). To this suspension,  $200\text{ }\mu\text{L}$  of ammonium fluoride ( $8\text{ M}$ ) and  $100\text{ }\mu\text{L}$  of hydrofluoric acid ( $2\text{ M}$ ) were added for core dissolution, followed by three centrifugation ( $3200\text{ g, 5 min}$ )/re-dispersion ( $200\text{ }\mu\text{L}$ ) cycles. *Caution! Hydrofluoric acid and ammonium fluoride are very toxic. Extreme care should be taken when handling HF solution, and only small quantities should be prepared.*

*Labeling of the Capsules:* The crosslinked capsules were re-suspended in  $200\text{ }\mu\text{L}$  of NaOAc ( $20\text{ mM, pH 4}$ ) and incubated with a mixture of  $3\text{ }\mu\text{L AF488}_{\text{Az}}$  ( $1\text{ g L}^{-1}$ ),  $100\text{ }\mu\text{L}$  sodium ascorbate ( $4.4\text{ g L}^{-1}$ ), and copper sulfate ( $1.75\text{ g L}^{-1}$ ) (all in  $20\text{ mM NaOAc, pH 4}$ ). After  $5\text{ h}$  of constant shaking, the labeled capsules were washed extensively in NaOAc buffer to remove unreacted dye and subsequently in PBS (pH 7.4) to remove the sacrificial/capping PVPO and PMA.

### Glycopolymers film-ConA interaction studies

*Planar supports:* PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> films (4 bilayers) were assembled using QCM as described above. Crosslinking of the multilayered films was performed by incubation for  $24\text{ h}$  with nondegradable bisazide linker ( $1\text{ g L}^{-1}$ ), sodium ascorbate ( $4.4\text{ g L}^{-1}$ ), and copper(II) sulfate ( $1.75\text{ g L}^{-1}$ ). Subsequently, the films were washed into PBS (pH 7.4) to remove sacrificial PMA and prior to the ConA studies into HBS (pH 7.4). The films were incubated in ConA ( $0.2\text{ mg mL}^{-1}$ , HBS) for  $3\text{ h}$  and washed into HBS (pH 7.4) to remove unbound ConA.

*Particle supports:* PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> capsules (AF488 labeled) were incubated with ConA (AF647 labeled) for  $3\text{ h}$  under constant shaking. After washing cycles with HBS (pH 7.4) the capsules were investigated by fluorescence microscopy.

### Generation of bone marrow derived DC, MAC and MDSC cultures *in vitro*

Bone marrow cells from femurs and tibias of C57BL/6 mice ( $n=2$ ) were collected and treated with ACK lysis buffer ( $155\text{ mM NHCl}_4$ ,  $10\text{ mM Na-EDTA}$ ,  $100\text{ mM KHCO}_3$  pH 7.2) for  $3\text{-}5$  minutes at room temperature to lyse erythrocytes. Cells were pooled, washed and cultured at  $5 \times 10^5$  cells/ml in a 24-well plate (1 ml/well) with complete media [RPMI 1640,  $2\text{ mM L-glutamine}$  (Sigma–Aldrich, Louis, MO, USA),  $20\text{ mM HEPES}$  (Sigma–Aldrich, Louis, MO, USA),  $0.1\text{ mM 2-mercaptoethanol (2-ME)}$  (Sigma–Aldrich, Louis, MO, USA),  $100\text{ U/ml Penicillin}$ ,  $100\mu\text{g/ml}$

of Streptomycin (Sigma–Aldrich, Louis, MO, USA) and 10% of heat-inactivated Foetal Calf Serum (FCS) (Sigma–Aldrich, Louis, MO, USA)]) at 37°C, 6% CO<sub>2</sub>. Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF; PeproTech, Rocky Hill, NJ, USA) (10 ng/ml) was added to induce DC generation for 5 days (with a change of culture media at day 3). At day 5, PEtOxMA<sub>Alk</sub> and PEtOxMA<sub>Man</sub> capsules were added to cells at the final concentration of 30 or 300 capsules/cell, each condition was tested in quadruplicates. Matching quantities of mannosylated polymers on the capsules were also test as controls. The lipopolysaccharide (LPS) was used as positive control in the assay. After 18 h co-culture with capsules or control agents, cells were harvested and stained with antibodies as shown below.

### **Flow cytometric analysis**

Cultured cells were harvested and labelled with anti-CD11c V450 (HL3) (BD Biosciences, Franklin Lakes, NJ, USA) to identify DC. To study the activation marker expression, cells were labelled with anti-CD80 BV650 (BD Biosciences, Franklin Lakes, NJ, USA) and anti-MHCII APC-Cy7 (M5/114.15.2) (BD Biosciences, Franklin Lakes, NJ, USA). Dead cell exclusion was by LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Life Technologies Australia Pty Ltd, Victoria Australia). Antibodies were prepared in flow cytometry staining buffer (PBS, 2% FCS) and cells were stained for 20 min on ice. Samples were acquired with LSRII (BD Biosciences, Franklin Lakes, NJ, USA) at AMREP Flow-Cytometry Core Facility (Melbourne, Australia). Data was analyzed with FlowJo Flow Cytometry Analysis Software (TreeStar).

**Supporting Information.** This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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## Engineered hydrogen-bonded glycopolymer capsules and their interactions with antigen presenting cells

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