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The profile of adsorbed plasma and serum proteins on methacrylic acid copolymer beads: Effect on complement activation

Laura A. Wells [a, b, 1], Hongbo Guo [b], Andrew Emili [b], Michael V. Sefton [a, c, *]

[a] Institute of Biomaterials and Biomedical Engineering, University of Toronto, Canada
[b] Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Canada
[c] Department of Chemical Engineering and Applied Chemistry, University of Toronto, Canada

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ABSTRACT

Polymer beads made of 45% methacrylic acid co methyl methacrylate (MAA beads) promote vascular regenerative responses in contrast to control materials without methacrylic acid (here polymethyl methacrylate beads, PMMA). *In vitro* and *in vivo* studies suggest that MAA copolymers induce differences in macrophage phenotype and polarization and inflammatory responses, presumably due to protein adsorption differences between the beads. To explore differences in protein adsorption in an unbiased manner, we used high resolution shotgun mass spectrometry to identify and compare proteins that adsorb from human plasma or serum onto MAA and PMMA beads. From plasma, MAA beads adsorbed many complement proteins, such as C1q, C4-related proteins and the complement inhibitor factor H, while PMMA adsorbed proteins, such as albumin, C3 and apolipoproteins. Because of the differences in complement protein adsorption, follow-up studies focused on using ELISA to assess complement activation. When incubated in serum, MAA beads generated significantly lower levels of soluble C5b9 and $C3a/C3a_{desarg}$ in comparison to PMMA beads, indicating a decrease in complement activation with MAA beads. The differences in adsorbed protein on the two materials likely alter subsequent cell-material interactions that ultimately result in different host responses and local vascularization.

1. Introduction

Methacrylic acid (MAA) copolymer beads and coatings promote a vascular regenerative response after their implantation *in vivo* [1,2]. This response occurs without exogenous growth factors and may involve a variety of signaling pathways [3], gene expression profiles [4,5], and molecules such as osteopontin, sonic hedgehog and SDF1 among others [6]. Studies using smooth coatable MAA copolymers suggest that the chemistry of MAA is critical to the observed responses [5], presumably through the protein adsorbate that is formed a few seconds after implantation or culture *in vitro*. Here we use an unbiased mass spectrometry method to identify many of the proteins that adsorb to MAA copolymer beads from human plasma or serum. This lead us to focus on complement activation as a differentiating feature distinguishing the MAA bead [poly(methacrylic acid − co − methyl methacrylate)] from the control material, poly(methyl methacrylate) (PMMA).

Proteins adsorb to biomaterials immediately after implantation influencing local responses related to inflammation and wound healing, such as coagulation, complement activation, and leukocyte adhesion [7]. Innate immune cells that infiltrate the implant site interact with the proteins that have adsorbed onto the biomaterial surfaces, promoting changes in cellular phenotypes that are dependent on the profile (both amounts and conformations) of adsorbed proteins. Furthermore, the adsorbed proteins activate or inhibit coagulation and complement cascades to variable degrees, which then attract and activate a variety of innate immune cells (mast, neutrophil, and macrophage cells) that will respond to the adsorbed proteins and the local microenvironment. Together, these interactions lead to the observed host response.

Advances in mass spectrometry now allow for the identification of proteins present in plasma and serum [8], as well as proteins that adsorb onto material surfaces [9]. Standard protocols have been established, which include protein desorption, digestion, and separation followed by high resolution tandem mass spectrometry

* Corresponding author. 164 College Street, Suite 407, Toronto, Ontario, M5S 3G9, Canada.
E-mail address: michael.sefton@utoronto.ca (M.V. Sefton).
[1] Current address: Department of Chemical Engineering, Queen's University, Canada.

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[10–12]. Protein database search engines such as SEQUEST are used for protein and pathway bioinformatics analysis. Such proteomic driven studies assess the multitude of proteins that are adsorbed rather than the one or two of classical radiolabel studies [13,14] or the pre-selected proteins in immunoblotting experiments [15]. While the unbiased proteomic method does not generate a surface concentration (moles/cm^2), it does yield a quantitative profile that can include unexpected molecules that may be important in relating material properties to the host response.

Of particular interest here is the activation of the complement system. Complement is part of the innate immune response and its activated components are responsible for the lysis of pathogens, the clearing of apoptotic cells, and the recruitment and activation of inflammatory cells. In the context of biomaterials, complement activation discussions focus on the alternative pathway and adsorbed C3b, which is bound to surface nucleophiles on some biomaterials; other activation processes and pathways including those that proceed through C1q are also relevant [16]. The extent of complement activation by a biomaterial is often a balance between pro-activating components and the variety of complement inhibitors like Factor H and I [17]. The relationship between complement and leukocyte activation is reviewed elsewhere [18].

In this work, proteins that adsorb from fresh human plasma and serum onto 45% MAA beads and PMMA beads (as a control/comparison) were identified by high-resolution shotgun mass spectrometry. Because many complement proteins were identified in the adsorbate, ELISAs for soluble C5b9 (SC5b9) and C3a/C3a$_{desarg}$ were performed on fresh human serum incubated with each bead type.

2. Materials and methods

2.1. Copolymer bead synthesis

Poly (MAA-co–MMA) ("MAA", Poly(methacrylic acid co-methyl methacrylate), 45 mol % MAA) beads were synthesized by free radical polymerization suspended in sodium chloride, using ethylene glycol dimethacrylate (EGDMA) as a crosslinker, benzoyl peroxide as an initiator, and tricalcium phosphate as a dispersion agent [1,4]. The beads were sieved to a diameter range of 150–250 μm. MAA content was determined by back titration of NaOH solutions incubated with the beads. PMMA beads of the same diameter were purchased from Polysciences (Warrington, PA). To account for the approximately 3× swelling of MAA beads [1], 3 times the mass of PMMA beads were used in all experiments, so that bead volume was the same; this is discussed further below.

Beads were washed in either 95% ethanol (MAA beads) or 1 N HCl (PMMA beads) repeatedly and then rinsed five times in LAL reagent water (MJS Biolynx Inc., Brockville, ON, Canada) prior to use. Analysis with a limulus amebocyte lysate (LAL) pyrochrome endotoxin test kit (Cape Cod Inc., Falmouth, MA) indicated that beads contained <0.25 EU/100 mg. Elemental surface composition analysis (ThermoFisher XPS, Surface-Interface Ontario, University of Toronto) showed minimal Si contamination (~0.07%) and that measured surface composition (atom%) was close to the theoretical expectation. MAA beads had a rough, porous surface, were negatively charged and did not degrade over time *in vivo*; PMMA beads were smooth [4,5].

2.2. Isolation of plasma and serum

Human blood (with or without heparin) was obtained from volunteers who had no medications for the previous 7 days, in accordance with an approved Human Ethics Research Protocol at the University of Toronto. To isolate plasma, whole blood (5 units/mL heparin) was centrifuged at 1000 rpm for 10 min to obtain platelet rich plasma (PRP). Platelet poor plasma (hereafter referred to as plasma) was obtained by centrifuging the PRP at 2000 g for 15 min. Fresh plasma from three different donors was used for the protein adsorption experiments.

To isolate serum, human whole blood without anti-coagulant was transferred to opened, silicone-coated plastic vacutainers (Becton Dickinson, Mississauga, ON) and allowed to clot for 30 min at room temperature. The clotted blood was centrifuged at 1500 g for 10 min, and the top layer (serum) was recovered. Fresh serum from three different donors was used for the protein adsorption and three different donors were used for complement activation experiments.

2.3. Protein adsorption

For serum experiments, 10 mg (dry) of MAA beads or 30 mg of PMMA beads were incubated in 200 μL of serum for 90 min at 37 °C (on rocker). For plasma experiments, 20 mg (dry) of MAA beads or 60 mg of PMMA beads were incubated in 800 μL of plasma for 90 min at 37 °C (on rocker). After incubation in serum or plasma, the serum or plasma was removed, and the beads were rinsed 5× with phosphate buffered saline (PBS) [Gibco (Grand Island, NY), 1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic and 0.16 M sodium chloride], including one incubation overnight (16 h). The small vials holding the beads were also changed to ensure that only proteins adsorbed to the beads were identified by mass spectrometry.

Complement studies typically use serum because anticoagulants like heparin have confounding effects on activation and inhibition and calcium is a necessary cofactor (precluding the use of citrate or EDTA). On the other hand, the *in vivo* environment is often better reflected by plasma, since the absence of coagulation factors and low fibrinogen (in serum) limits how well the *in vitro* system can mimic *in vivo*. Both plasma and serum were investigated here and differences in adsorption profiles were noted.

2.4. Mass spectrometry

The proteins on the surface of the beads for each sample were solubilized in 50 μL of 8 M urea with 50 mM ammonia bicarbonate. They were then reduced by 2 mM DTT for 1 h and 5 mM iodoacetamide for 45 min in the dark and at room temperature. The samples were diluted to 200 μL to a final concentration of 2 M urea before adding 4 μg trypsin for an overnight digestion. After centrifugation, the supernatant was acidified to 1% using formic acid. The peptides were then desalted on Toptip C-18 column (Glygen) and lyophilized to dryness.

As described previously [19], all peptides were desalted in C18 25 mm x 75 silica capillary trap column and separated in 100 mm × 75 μm silica capillary analytical column packed with 5 μm Luna C18 stationary phase (Phenomenex) using the EASY-nLC system (Proxeon). A 60 min gradient was used for separation at a flow rate of 300 nL/min from 98% buffer A (5% acetonitrile with 0.1% formic acid) to 90% buffer B (95% acetonitrile with 0.1% formic acid). Eluted peptides were directly sprayed into an Orbitrap Velos mass spectrometer (ThermoFisher Scientific) with collision induced dissociation (CID) using a nanospray ion source (Proxeon). 16 ms/ms centroid mode CID data-dependent scans were acquired simultaneously with one profile mode full scan mass spectra. The full scan was performed in 60,000 resolutions. An exclusion list was enabled to fragment again for 22.5 s in order to increase the possibilities of determining low abundant ions.

The resulting RAW files were extracted from the mass spectrometry data with the ReAdW program and submitted to a database search using SEQUEST v2.7 and a UniProt/SwissProt protein