Low-Fouling, Biospecific Films Prepared by the Continuous Assembly of Polymers

Stefanie N. Guntari, Edgar H. H. Wong, Tor K. Goh, Rona Chandrawati, Anton Blencowe, Frank Caruso,* and Greg G. Qiao*

Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

*Corresponding authors: gregghq@unimelb.edu.au, fcaruso@unimelb.edu.au

ABSTRACT

We report that the continuous assembly of polymers (CAP) approach, mediated by ring-opening metathesis polymerization (ROMP), is a facile and versatile technology to prepare engineered nanocoatings for various potential biomedical applications. Low-fouling coatings on biomaterials were obtained by the formation of multicompositional, layered films via simple and efficient tandem CAPROMP processes that are analogous to chain extension reactions. In addition, the CAPROMP approach allows for the efficient post-functionalization of the CAP films with bioactive moieties via cross-metathesis reactions between the surface-immobilized catalysts and symmetrical alkene derivatives. The combined features of the CAPROMP approach (i.e., versatile polymer selection and facile functionalization) allow for the fabrication and surface modification of various types of polymer films, including those with intrinsic protein-repellent properties and selective protein recognition capabilities. This study highlights the various types of advanced coatings and materials that the CAP approach can be used to generate, which may be useful for various biomedical applications.
INTRODUCTION

Over the past few decades, research in biomedical science has focused on developing functional material interfaces that are biocompatible and that can be engineered for specific biointeractions.\textsuperscript{1-4} For example, polymeric thin films have been widely used as coatings for biomaterials and biomedical devices as the film composition, functionality, structure (i.e., thickness and density) and mechanical properties can be tailored depending on the selection of polymers and film fabrication strategy.\textsuperscript{1,5-8} The development of bioactive surfaces aimed at promoting specific interactions while minimizing non-specific interactions (i.e., low-fouling) are central to many biomedical applications, including drug delivery,\textsuperscript{9,10} implantable devices,\textsuperscript{11-13} tissue engineering,\textsuperscript{14,15} biosensors,\textsuperscript{16} diagnostics,\textsuperscript{17} and protein purification.\textsuperscript{18} Of particular importance is the interaction and adsorption of proteins on surfaces when in contact with biological environments, which ultimately governs the biofunctionality of the material and influences cellular interaction, structure and function.\textsuperscript{19,20} As a result, significant effort has been devoted to developing surface coating and modification approaches towards low-fouling bioactive surfaces.\textsuperscript{7,8,21-23} For example, both the grafting-to and -from approaches have been extensively used to prepare low fouling surfaces from hydrophilic and zwitterionic materials, with the low-fouling ability of the surfaces being closely correlated with their hydration layer near the surface.\textsuperscript{24,25} The strength of this surface hydration is preliminary related to the physiochemical properties of the polymers and their surface packing, including film thickness, packing density and chain conformation.\textsuperscript{25} In addition, these grafted surfaces have been further modified with bioactive moieties, to generate anti-fouling coatings that can interact specifically with targeted proteins.\textsuperscript{7,8} However, the application of grafted films to prepare robust, free standing bioactive films has been limited. In contrast, layer-by-layer (LbL) assembly\textsuperscript{21-23} is highly applicable for the generation of stable, free standing bioactive films, including polymeric capsules, which can be generated from or post-functionalised with low-fouling
polymers, and subsequently modified with biofunctional moieties for specific protein interactions.\textsuperscript{26,27} The LbL approach relies on the alternating deposition of polymers with complementary functionalities, thus requiring multiple assembly steps.\textsuperscript{28,29}

Recently, we introduced a facile and versatile bottom-up strategy for the surface-confined, one-step assembly of cross-linked nanoscale films, termed continuous assembly of polymers (CAP), which utilizes the controlled chain growth polymerization of (bio)macromolecules functionalized with pendant polymerizable moieties (referred to as macrocross-linkers).\textsuperscript{30-34} The CAP approach allows for the fabrication of nanoscale films with tailored thickness and composition through variation of the polymerisation time and selection of macrocross-linker(s), respectively. The CAP approach is applicable to both planar and colloidal substrates, thus representing a promising assembly method for the fabrication of tailored nanocoatings on various complex substrates.\textsuperscript{30-32} Furthermore, CAP-derived films can be reinitiated for continued film growth using the unreacted polymerizable groups of the macrocross-linkers that are embedded in the film, providing access to multilayered films through so-called reinitiation reactions with the same or different macrocross-linkers.\textsuperscript{34} These layer thickness extensions or reinitiation reactions do not depend exclusively on the end-group fidelity of polymer brushes/grafts that are inherent and difficult to preserve in the grafting-from approach. Given that these reactions can occur conveniently via the residual polymerizable moieties of the macrocross-linker in the film, the CAP approach thus overcomes the difficulty of the grafting-from approach to prepare higher order multilayer films. In addition, specifically for ring opening metathesis polymerization (ROMP)-mediated CAP, the films can be further post-functionalized via simple and efficient cross-metathesis reactions between the residual polymerizable groups (as well as the end-groups) in the film and symmetrical (bio)functionalised alkenes. As such, biofunctional films, whereby both the outer film
composition and peripheral functionalities play an active role in biological interactions, can be prepared with relative ease.

In this study, the versatility of the \( \text{CAP}_{\text{ROMP}} \) approach is demonstrated through the synthesis of nanoengineered films on porous and non-porous colloidal substrates, which have low-fouling properties as well as the capability to selectively recognize proteins. These results are anticipated to allow the fabrication of CAP films with tailored biological interactions for applications in biomedical engineering, biosensors and polymer therapeutics.

**Experimental**

**Materials.**

Allyl bromide (99%), 4,4′-azobis(4-cyanopentanoic acid) (≥ 75%), biotin (99%), \( N \)-boc-tyramine (97%), calcium hydride (CaH\(_2\)), \( N,N' \)-dicyclohexylcarbodiimide (DCC, 99%), \( cis \)-3,4-dichloro-2-butene (95%), di(ethylene glycol) vinyl ether (98%), 4-(dimethylamino)pyridine (DMAP, ≥ 99%), 5-norbornen-2-methanol (mixture of \( endo \) and \( exo \), 98%), 5-norbornene-2-carboxylic acid (mixture of \( endo \) and \( exo \), 98%), poly(ethylene imine) (PEI) (\( M_w \) ~ 25 kDa), sodium iodide (NaI, 99.9%), and streptavidin fluorescein 5(6)-isothiocyanate (avidin-FITC) were obtained from Aldrich and used without further purification. The monomers 2-hydroxyethyl acrylate (HEA, 96%) and acrylic acid (AA, 99%) were obtained from Aldrich and past over plugs of inhibitor remover (Aldrich) twice to remove any inhibitors present and stored below 4 °C prior to use. Metathesis catalyst (IMesH\(_2\))(Cl)\(_2\)(C\(_5\)H\(_5\)N)\(_2\)Ru=CHPh (catalyst C\(_1\)) was prepared from the 2\(^{\text{nd}}\) generation Grubbs catalyst (Aldrich), as described in the literature.\(^{35}\) Pyridine was obtained from Scharlau and used without further purification. Magnesium sulphate (MgSO\(_4\), anhydrous), \( n \)-hexane, toluene, isopropanol and ethanol were obtained from Merck and used without further purification. Diethyl ether (DEE), sodium chloride (NaCl), and sodium hydroxide (NaOH)
were obtained from Chem-Supply and used without further purification. \(N\)-(3-dimethylaminopropyl)-\(N'\)-ethylcarbodiimide hydrochloride (EDCI, > 98\%) was obtained from Fluka and used as received. Ammonium hydroxide (NH\(_2\)OH), anhydrous \(N,N\)-dimethylformamide (DMF) and trifluoroacetic acid (TFA) were obtained from Aldrich and used without further purification. Anhydrous \(N,N\)-dimethylacetamide (DMAc) was obtained by distilling from CaH\(_2\) in vacuo. Anhydrous, deoxygenated dichloromethane (DCM), and tetrahydrofuran (THF) were obtained by distillation under argon from CaH\(_2\) and sodium benzophenone ketyl, respectively. Deuterated chloroform (CDCl\(_3\)), methanol (CD\(_3\)OD) and dimethylsulfoxide (\(d_6\)-DMSO) were obtained from Cambridge Isotope Laboratories. High-purity water with a resistivity greater than 18 MΩ.cm was obtained from an in-line Millipore RiOs/Origin water purification system.

**Measurements.** Polymer molecular weight characterization was carried out via GPC using a Shimadzu liquid chromatography system coupled to a Wyatt DAWN EOS MALLS detector (658 nm, 30 mW) and Wyatt OPTILAB DSP interferometric refractometer (633 nm), and using three Phenomenex Phenogel columns in series (500, 10\(^4\) and 10\(^6\) Å porosity; 5 µm bead size) operating at 30 °C. THF was used as the eluent at a flowrate of 1 mL.min\(^{-1}\). Aliquots (0.5 mL) from each reaction mixture were diluted with an appropriate amount of THF and passed through a 0.45 µm filter and injected into the GPC for analysis. Astra software (Wyatt Technology Corp.) was used to determine the molecular weight characteristics using known \(dn/dc\) values.\(^{36}\)

\(^1\)H and \(^{13}\)C NMR measurements were conducted on a Varian Unity 400 MHz spectrometer at 400 and 100 MHz, respectively, using the deuterated solvent as reference and a sample concentration of ca. 20 mg.mL\(^{-1}\).
Flow cytometry was performed on CAP films coated onto silica particles composed of fluorescently FITC- and RITC-labeled polymers. Fluorescence intensity histograms were acquired with a Partec CyFlow Space instrument using an excitation wavelength of 488 nm and 544 nm for FITC and RITC, respectively. Data analysis was performed with Partec CyflowMax software and mean fluorescence intensities were obtained from the fluorescence intensity histograms.

Fluorescence microscopy images of the CAP particles were taken on an inverted Olympus IX71 microscope equipped with a DIC slider (U-DICT, Olympus), a UF1032 fluorescence filter cube, and a 60× oil immersion objective (Olympus UPFL20/0.5NA, W.D. 1.6).

Zeta-potentials of the CAP particles were analyzed with a Zetasizer Nano ZS (Malvern Instruments). The refractive index was set to 1.453, 1.512 and 1.527 for allyl-PEI, P3 and P4 films, respectively. 700 µL of particles (0.05 wt% solution) was placed into a disposable zeta cell and zeta-potential measurement was conducted at 20 °C. Data analysis was performed with Dispersion Technology Software V5.03 provided by Malvern Instruments.

**Procedures**

*Synthesis of poly(acrylic acid) (PAA):* Acrylic acid (19.8 mmol, 1.5 g) was dissolved in EtOH (10 mL) and 4,4′-azobis(4-cyanopentanoic acid) (0.4 mmol, 111 mg) was added. Nitrogen was bubbled through the reaction mixture for 30 min before the mixture was heated at 60 °C for 2 h under nitrogen. The reaction was subsequently cooled in an ice bath and precipitated dropwise into DEE (100 mL). The precipitate was collected via centrifugation, redissolved in EtOH (5 mL) and the precipitation process was repeated twice before drying the product *in
vacuo to afford PAA as a crystalline solid, 0.51 g (36 %). GPC: $M_n = 48.6$ kDa, $M_w/M_n = 1.3$.

$^1$H NMR (400 MHz, DMSO-$d_6$, 25 °C) $\delta$H 2.05-2.40 ($br$, 1H, CH$_2$CH), 1.20-1.90 ($br$, 2H, CH$_2$CH) ppm.

Synthesis of norbornene-functionalized PAA macrocross-linker P1 (poly((acrylic acid)-ran-(bicyclo[2.2.1]hept-5-en-2-ylmethyl acrylate))): PAA (5.3 mmol –COOH, 0.38 g) was dissolved in anhydrous 1:1 THF:DMac solvent mixture (15 mL) and nitrogen was bubbled through the solution for 30 min. 5-norbornene-2-methanol (1.06 mmol, 0.131 g), EDCI (1.06 mmol, 0.202 g) and DMAP (0.21 mmol, 25.8 mg) were added. The solution was stirred at 25 °C for 20 h. Subsequently, the solution was precipitated into 7:3 DEE:hexane solvent mixture (100 mL). The precipitate was isolated via centrifugation, redissolved in acetone (8 mL) and the precipitation process was repeated twice before drying the product in vacuo to afford P4 as a solid polymer, 0.2 g (53 %). GPC: $M_n = 38.6$ kDa, $M_w/M_n = 1.8$. $^1$H NMR (400 MHz, DMSO-$d_6$, 25 °C) $\delta$H 6.05 ($br$, 1H, CH=C), 5.90 ($br$, 1H, CH=CH), 4.13-4.18 ($br$, 2H, C(=O)OC$_2$H$_5$), 3.53 ($br$, 1H, CH), 2.96 ($br$, 1H, CH), 2.75-2.85 ($br$, 1H, CH), 2.05-2.40 ($br$, 1H, CH$_2$CH), 1.20-1.90 ($br$, 2H, CH$_2$CH) ppm. Pendant norbornene functionality was 16 mol% as determined by $^1$H NMR spectroscopic analysis.

Synthesis of poly((2-hydroxyethyl)acrylate) (PHEA): HEA (30.3 mmol, 3.5 g) was dissolved in EtOH (25 mL) and 4,4'-Azobis(4-cyanopentanoic acid) (0.61 mmol, 159 mg) was added. Nitrogen was bubbled through the reaction mixture for 30 min before the mixture was heated at 60 °C for 4 h under nitrogen. The reaction was subsequently cooled in an ice bath and precipitated dropwise into DEE (200 mL). The precipitate was collected via centrifugation, redissolved in EtOH (15 mL) and the precipitation process was repeated twice before drying the product in vacuo to afford PHEA as a viscous polymer, 3.1 g (89 %). GPC: $M_n = 35.1$ kDa,
$M_w/M_n = 1.8$. $^1$H NMR (400 MHz, DMSO-$d_6$, 25 °C) $\delta_H$ 4.76 (br, 1H CH$_2$OH), 4.00 (br, 2H, C(=O)OCH$_2$), 3.55 (br, 2H, CH$_2$OH), 2.15-2.40 (br, 1H, CH$_2$CH), 1.35-1.80 (br, 2H, CH$_2$CH) ppm.

Synthesis of norbornene-functionalized PHEA macrocross-linker $P2$ (poly((2-hydroxyethyl)acrylate-ran-(2-(5-norborn-2-oxo)ethyl acrylate) (P(HEA-co-NOEA))): PHEA (6.8 mmol –OH, 0.79 g) was dissolved in anhydrous DMAc (10 mL) and nitrogen was bubbled through the solution for 30 min. 5-norbornene-2-carboxylic acid (0.68 mmol, 83 µL), DCC (2 mmol, 0.42 g) and DMAP (0.2 mmol, 24.9 mg) were added. The solution was stirred at 25 °C for 7 h. Subsequently, the precipitated urea was removed by filtration and the filtrate was precipitated into DEE (100 mL). The precipitate was isolated via centrifugation, redissolved in EtOH (8 mL) and the precipitation process was repeated twice before drying the product in vacuo to afford $P3$ as a viscous polymer, 0.2 g (26 %). GPC: $M_n = 35.2$ kDa, $M_w/M_n = 1.7$. $^1$H NMR (400 MHz, DMSO-$d_6$, 25 °C) $\delta_H$ 6.15 (br, 1H, CH=C), 5.88 (br, 1H, CH=C), 4.75 (br, 1H, CH$_2$OH), 4.14-4.22 (br, 2H C(=O)OCH$_2$), 4.00 (br, 2H, C(=O)OCH$_2$), 3.55 (br, 2H, CH$_2$OH), 2.15-2.40 (br, 1H, CH$_2$CH), 1.35-1.80 (br, 2H, CH$_2$CH) ppm. Pendant norbornene functionality was 12 mol% as determined by $^1$H NMR spectroscopic analysis.

Synthesis of (Z)-bis-1,4-(4-(2-biotin ethyl)phenyloxy)but-2-ene ($M1$): $M1$ was synthesised in 3 steps using a similar procedure to that previously reported.$^{37}$ N-boc-tyramine (5.01 g, 0.021 mol), K$_2$CO$_3$ (4.86 g, 0.035 mol) and NaI (0.132 g, 0.001 mol) were dissolved in anhydrous DMF (30 mL) under argon. 1,4-dichlorobut-2-ene (0.93 mL, 0.009 mol) was added to the mixture and the mixture was heated at 90 °C for 4 h then cooled to room temperature for 12 h. The solvent was removed in vacuo (0.05 mbar, 70 °C) and the residue was dissolved in DCM (120 mL), then washed with water (2 x 100 mL) followed by saturated NaCl (100 mL). The
organic phase was dried over MgSO₄, filtered and concentrated \textit{in vacuo} (0.05 mbar, 70 °C) to afford viscous brown oil as a crude product. The product was purified by silica gel chromatography, eluting with DCM (1.4 L), followed by DCM – EtOH, 99:1 (500 mL) and DCM – EtOH, 97:3 (900 mL). The pure fractions were concentrated \textit{in vacuo} to give (Z)-bis-1,4-(4-(2-((O-t-butyl)-N-carbamoyl)ethyl)phenyloxy)but-2-ene \textbf{M2}, 2.49 g (60\%): \textbf{1H NMR} (400 MHz, CDCl₃) δH 7.25 (AA’BB’, ArCH, 4H), 6.85 (AA’BB’, ArCH, 4H), 5.93 (t, =CH, 2H), 4.66 (d, CH₂O, 4H), 4.55 (br s, NH, 2H), 3.33 (t, CH₂N, 4H), 2.73 (t, CH₂, 4H), 1.43 (s, CH₃, 18H) ppm. \textbf{M2} (2.49 g, 0.005 mol) was dissolved in DCM (30 mL) under argon. TFA (6.08 mL, 0.079 mol) was added dropwise to the mixture and the mixture was stirred at room temperature for 36 h. DCM (50 mL), water (50 mL) were added to the reaction mixture followed by addition of concentrated NH₄OH (30\% in water) until pH 12 was achieved. The organic phase was removed and the aqueous phase was washed with DCM (3 x 30 mL). The organic extracts were combined, dried over MgSO₄, filtered and concentrated \textit{in vacuo} to afford (Z)-bis-1,4-(4-(2-aminoethyl)phenyloxy)but-2-ene \textbf{M3} as a pale brown viscous oil, 1.08 g (66\%): \textbf{1H NMR} (400 MHz, CDCl₃) δH 7.09 (AA’BB’, ArCH, 4H), 6.85 (AA’BB’, ArCH, 4H), 5.90 (t, =CH, 2H), 4.63 (d, CH₂O, 4H), 2.89 (t, CH₂N, 4H), 2.66 (t, CH₂, 4H) ppm. Biotin (0.48 g, 1.95 mmol), EDCI (0.46 g, 2.38 mmol) and DMAP (0.01 g, 0.087 mmol) were dissolved in anhydrous DMF (10 mL) under argon. \textbf{M3} (0.242 g, 0.741 mmol) was dissolved in anhydrous DMF (5 mL) and added to the mixture. The clear pale yellow reaction mixture was allowed to stir at room temperature for 4 d under argon. The solvent was then removed \textit{in vacuo} (0.05 mbar, 60 °C) to afford a white solid. The residue was suspended in water (90 mL) and stirred in the dark for 24 h to afford a milky solution. The mixture was centrifuged and supernatant was removed. The residue was suspended in 0.1 M K₂CO₃ solution (40 mL), vortexed and centrifuged. This process was repeated twice. The residue was then suspended in saturated NH₄Cl – water, 1:1 (40 mL), vortexed and centrifuged. The supernatant was removed
and the solid was resuspended in water (40 mL), vortexed and centrifuged. The residue was collected and suspended in THF (40 mL), vortexed and centrifuged. This step was repeated twice. The residue was dried in vacuo (0.35 mbar, 30 °C) for 24 h to afford M1 as a white solid, 0.35 g (59%): $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta_H$ 7.82 ($t$, NH, 2H), 7.09 ($AA'BB'$, ArCH, 4H), 6.89 ($AA'BB'$, ArCH, 4H), 6.43 ($s$, NH, 2H), 6.38 ($s$, NH, 2H), 5.83 ($t$, =CH, 2H), 4.70 ($d$, CH$_2$O, 4H), 4.29-4.33 ($m$, CH$_2$, 2H), 4.10-4.13($m$, CH$_2$, 2H), 3.20-3.27 ($m$, CH$_2$N, 4H), 3.04-3.12 ($m$, CHCH$_2$S, 2H), 2.80-2.85 ($m$, CHCH$_2$S, 2H), 2.55-2.69 ($m$, (CH)$_3$, 6H), 2.03 ($t$, CH$_2$CH$_2$C=O, 4H), 1.22-1.65 ($m$, CH(CH$_2$)$_3$CH$_2$, 12H) ppm.

**Synthesis of hyperbranched poly(N-allyl ethylene imine) (allyl-PEI):** This compound was prepared according to a previously published procedure.$^{30}$ $^1$H NMR (400 MHz, CD$_3$OD) $\delta_H$ 5.85 ($br s$, CH$_2$=CHCH$_2$N), 5.17-5.22 ($m$, CH$_2$=CHCH$_2$N), 3.11-3.20 ($m$, CH$_2$=CHCH$_2$N), 2.57 ($br s$, N(CH$_2$)$_2$N) ppm. Allyl functionality was 30% as determined by $^1$H NMR spectroscopic analysis.

**Assembly of CAP$_{ROMP}$ films on particles:** All particle experiments were conducted in individual 1.5 mL Eppendorf tubes. Particles (0.5 wt% solution) functionalized with catalyst C1 (details of this functionalization are provided in the previously published literature$^{30}$) were combined with a 1 mL CAP-active macrocross-linker P1 or P2 solution (1 mM in 50 mM CuSO$_4$ solution/isopropanol mixture (50/50) or 50 mM CuSO$_4$ solution, respectively) in an Eppendorf tube. The mixture was agitated with an orbital shaker at room temperature for a 25 h and the CAP$_{ROMP}$ process was terminated by the addition of excess di(ethylene glycol) vinyl ether (100 µL). After 5 min the particles were isolated by centrifugation, washed with methanol (3 $\times$ 1 mL) followed by Milli-Q water (3 $\times$ 1 mL), soaked in Milli-Q water (1 mL) for 12 h, and
washed with Milli-Q water (3 × 1 mL) prior to analysis. Reinitiation experiment was performed via repetition of the above procedure.

Post-functionalization of CAP-ROMP films via cross-metathesis: CAP-coated particles (0.5 wt% solution in Milli-Q water) were subjected into gradient washing with Milli-Q water (1 mL), followed by degassed THF (3 x 1 mL) and degassed DCM (2 x 1 mL), prior to functionalization with catalyst C2 (the functionalisation step was identical to the CAP-ROMP reaction). Then, the particles were exposed to a symmetrical alkene molecule M1 solution (5 mM in degassed toluene/methanol mixture (75/25)) for 18 h. The particles were then isolated by centrifugation, washed with methanol (5 x 1 mL), followed by DMSO (2 x 1 mL) and Milli-Q water (3 x 1 mL) prior to avidin incubation.

RESULTS AND DISCUSSIONS

The first part of this study demonstrates how protein-repellent properties can be imparted on fouling surfaces by the introduction of low-fouling layers using CAP reinitiation reactions. In the second part, the ability of CAP-derived films to undergo further chemical functionalization via cross-metathesis reactions that allow for specific bioconjugation reactions is demonstrated. The combination of both parts demonstrates how the CAP approach can be used to prepare nanoengineered coatings with both low-fouling and selective protein recognition capabilities.

For the CAP-ROMP process, the surface of the substrate is firstly functionalized with initiators, followed by exposure to a solution of macrocross-linker, which results in one-step assembly of the nanoscale cross-linked films mediated via controlled polymerization across the pendent norbornene groups of the macrocross-linker. In this study, the substrates were initially functionalized with an allyl-modified poly(ethylene imine), followed by cross-metathesis with the bispyridine modified 2nd generation Grubbs catalyst C1 (1 mM in dichloromethane) to form
the initiating prelayer (Scheme 1). The macrocross-linkers (P1 or P2), which contain 16 and 12 mol% pendent norbornene functionalities, respectively, were employed at a concentration of 1 mM (with respect to the polymer) and selected based upon the desired final characteristics of the resulting film (Scheme 1). The CAPROMP reactions were terminated by addition of di(ethylene glycol) vinyl ether. Reinitiation reactions were performed by refunctionalizing the prefabricated films with C1, followed by exposure to a fresh solution of macrocross-linker for further film growth. The post-modification of the prefabricated CAPROMP films was performed by functionalizing the films with the 2nd generation Hoveyda-Grubbs catalyst C2 (1 mM in dichloromethane), followed by cross-metathesis with the symmetrical alkene M1 (5 mM in 4:1 toluene:methanol) (Scheme 1).
Scheme 1. Schematic illustrating film formation and surface modification via the CAP<sub>ROMP</sub> approach, and structures of the compounds used in this study.

**Low-Fouling Coatings.** The ability of the CAP<sub>ROMP</sub> approach to generate multilayered films with different compositions was demonstrated on particles, which have potential in a range of biomedical applications. For example, the CAP<sub>ROMP</sub> approach could be employed to induce low-fouling properties to (bio)materials by the addition of ultrathin coatings that reduce protein adsorption. Specifically, films were fabricated on 7 µm-diameter (D40/D90 < 1.35) mesoporous silica particles via CAP<sub>ROMP</sub> with the poly(acrylic acid) (PAA) macrocross-linker P<sub>1</sub> (Scheme 1), followed by reinitiation and further film growth using the poly(2-hydroxyethyl acrylate) (PHEA) macrocross-linker P<sub>2</sub> to generate a low-fouling outermost layer. The CAP<sub>ROMP</sub> reactions were performed in degassed 1:1 isopropanol (IPA):aqueous 50 mM CuSO<sub>4</sub> (pH 7) solution for macrocross-linker P<sub>1</sub> and in an aqueous 50 mM CuSO<sub>4</sub> (pH 7) solution for P<sub>2</sub>. After formation of each polymer layer the zeta-potential of the coated particles was measured at pH 5 and the fouling behaviour was studied via incubation of the coated particles at pH 5 with bovine serum albumin (BSA) protein tagged with the fluorescent marker Alexa Fluor 488. PAA-based films are reported to be predominately protonated at pH 5, which facilitates binding of BSA via hydrogen bonding, and possibly non-specific hydrophobic interactions. In comparison, PHEA-based films are known to possess low-fouling behaviour towards BSA regardless of the pH conditions. The initial zeta-potential measurement of the particles after deposition of the allyl-PEI initiator prelayer was 33 mV, which decreased to -3 mV after the CAP<sub>ROMP</sub> reaction with PAA macrocross-linker P<sub>1</sub>, before increasing again to +3 mV after subsequent CAP reinitiation and film growth with the PHEA macrocross-linker P<sub>2</sub> (Figure 1a). Fluorescence microscopy images and the fluorescence intensity (as determined by flow cytometry) of the particles incubated with BSA indicate that the most significant
protein adsorption was observed on the allyl-PEI coated particles (ca. 200 a.u., normalized), followed by the PAA P1 films (ca. 100 a.u., normalized) (Figure 1b and 1c). In contrast, BSA adsorption on the PHEA P2 film was negligible (ca. 2 a.u., normalized). These results are expected as allyl-PEI is strongly positively charged and therefore attracts the negatively charged BSA through electrostatic interactions. Furthermore, the zeta-potential value of -3 mV for the P1 film confirmed that the PAA is predominantly protonated and attracts the BSA via hydrogen bonding, resulting in fouling of the surface. In comparison, the addition of the PHEA-based P2 film to the P1-coated particles changes the zeta-potential to neutral and imparts low-fouling properties to the particles. These results illustrate the versatility of the CAPROMP approach to continuously refunctionalize surface-confined films effectively – regardless of the substrate morphology – with different functional polymers to afford specifically tailored properties.
Figure 1. (a) Zeta-potential measurements (recorded at pH 5) of the PEI initiator-functionalized 7 µm-diameter mesoporous silica particles and the particles after subsequent CAP reactions with PAA \( \text{P1} \) and PHEA \( \text{P2} \). (b) Illustrations and fluorescence microscopy images of particles coated with the PEI initiator layer and \( \text{P1} \) and \( \text{P2} \) CAP layers followed by incubation with fluorescently-tagged BSA at pH 5, and; (c) the corresponding fluorescence intensity of the particles, as determined by flow cytometry. Scale bars = 3 µm.

**Biomacromolecule Recognition.** A unique feature of the \( \text{CAP}_{\text{ROMP}} \) approach is the ability to post-functionalize the films via cross metathesis between the Ru alkylidenes on the film with
symmetrical alkene derivatives. This feature of \textit{CAPROMP}, which allows for efficient (bio)molecule conjugation akin to click reactions,\textsuperscript{40,41} is useful for biotechnology applications, particularly protein conjugation for biochemical assays.\textsuperscript{42} This concept was demonstrated using the highly specific biotin-avidin system. Firstly, \textit{CAPROMP} films were assembled on both nonporous and mesoporous silica particles using a rhodamine isothiocyanate (RITC)-tagged PHEA macrolinker \textit{P2} and any remaining pendant norbornyl functionalities in the films were subsequently converted to Ru alkylidenes by exposing the coated particles to a fresh solution of catalyst \textit{C2}. Biotin was then conjugated to the coated particles via cross-metathesis between the surface bound Ru alkylidenes and the symmetrical alkene biotin-derivative \textit{M1} (\textbf{Figure 2a}). To demonstrate the successful conjugation of biotin, the functionalized particles were incubated with fluorescein isothiocyanate (FITC)-tagged avidin in PBS buffer at pH 7.4. Fluorescence microscopy images revealed that the particles fluoresce at both red and green wavelengths (upon excitation at $\lambda_{ex} = 544$ and 488 nm, respectively), which correspond to the rhodamine-labeled \textit{P2} film alone, and subsequent biotin functionalization and conjugation with the fluorescein-tagged avidin, respectively (\textbf{Figures 2b and 2c} for nonporous and mesoporous silica particles, respectively). The fluorescence intensity, as measured by flow cytometry, revealed a large increase in green fluorescence for both nonporous and mesoporous silica particles after avidin attachment to the biotin-functionalized surface compared to the unmodified films. To discount any possibility that the avidin conjugation is merely due to nonspecific interactions, a control experiment was performed whereby particles only coated with \textit{P2} films (i.e., in the absence of the biotin immobilization step) were subjected to the avidin incubation procedure, which revealed that there was negligible intensity from fluorescein tagged avidin. In addition, the fluorescence intensity of the control sample was very similar in intensity to the \textit{P2} films alone. These results clearly demonstrate that the avidin adsorption to the substrate surface is purely due to the selective binding abilities of the biotin-conjugated
films. This experiment emphasises the versatility of $\text{CAP}_\text{ROMP}$ films to undergo further conjugation through grafting-to reactions via facile cross-metathesis reaction of surface bound Ru alkylidene groups with symmetrical alkene derivatives.

**Figure 2.** Film modification with symmetrical alkene-functionalized biotin M1: (a) General scheme of cross metathesis between the surface bound Ru alkylidene groups of the RITC-tagged $\text{P2}$ $\text{CAP}_\text{ROMP}$ film with M1, followed by incubation with FITC-tagged avidin in PBS buffer. Fluorescence microscopy images (left = red fluorescence; right = green fluorescence) of RITC-tagged $\text{P2}$ films after biotin modification and incubation with FITC-tagged avidin on (b) nonporous and (c) mesoporous silica particles. Fluorescence intensity measurements before
and after biotin-avidin modification (as well as the control experiment), as measured by flow cytometry for (d) nonporous and (e) mesoporous silica particles. Scale bars = 2 µm.

**Low-fouling CAP Films with Selective Protein Recognition Capability.** The specific protein recognition capabilities of the CAP films demonstrated for the biotin-avidin system were subsequently combined with the low-fouling coatings. Dual layered CAP films consisting of an inner PAA (P1) layer and an outer PHEA (P2) layer, which provides protein-repellent properties, were post-functionalized via cross-metathesis with the symmetrical alkene-functionalized biotin M1 (Scheme 1). The resulting biotin-functionalized mesoporous silica particles were found to repel BSA protein, as expected, however, most importantly they retain their specific binding ability to the protein avidin. Fluorescence microscopy was used to image the biotin-functionalized particles after incubation in BSA protein-AlexaFluor 488 or avidin-FITC at pH 5 (Figure 3a and b) or PBS buffer at pH 7.4 (Figure 3c and d). At both pH 5 and pH 7.4, the particles incubated in avidin solution showed a high intensity of green fluorescence, indicating avidin adsorption on the film (Figure 3b and d, respectively), whereas those incubated in BSA solution displayed negligible fluorescence (Figure 3a and c, respectively). These results were supported by flow cytometry analysis, which revealed a significant difference between the green fluorescence intensity of the avidin-FITC (shown in green) and BSA-AlexaFluor 488 (shown in orange) (Figure 3e). This study further highlights the versatility of the ROMP-mediated CAP system for the generation of low-fouling films that simultaneously retain selective protein recognition capabilities.
Figure 3. (a-d) Fluorescence microscopy images of dual layer CAP P1 (inner) and P2 (outer) films after biotin modification and incubation with (a,c) AlexaFluor 488-tagged BSA or (b,d) FITC-tagged avidin at (a,b) pH 5 or (c,d) PBS buffer at pH 7.4 environment. (e) Fluorescence intensity measurements after biotin modification and incubation in BSA and avidin at both pH environments, as measured by flow cytometry. Scale bars = 2 µm.

CONCLUSION

In this study, the versatility of the CAP\textsuperscript{ROMP} approach to construct functional, engineered nanocoatings on various particle substrates for potential biomedical applications was
demonstrated. The formation of multicomponent films was performed on mesoporous colloidal substrates using two macromolecules with entirely different fouling properties. CAPROMP of the low-fouling PHEA macrocross-linker P2 was used to generate an outermost layer on a previously prepared PAA P1 film to impart low-fouling properties, which was validated via protein binding studies with BSA. In demonstrating another unique feature of the CAPROMP approach, functional films were prepared via efficient conjugation of bioactive molecules using cross metathesis between the surface bound Ru-alkylidene groups distributed throughout the film with symmetrical alkene derivatives containing biotin (M1). When applied to the low-fouling coating system, fluorescence measurements confirmed the selective binding of avidin to the biotin-modified films. From a synthetic perspective, the CAP methodology offers an efficient alternative to thin film formation strategies and as shown by these studies, the CAP approach serves as a promising platform technology for a wide range of applications, including coatings for implantable devices, tissue engineering constructs, and drug delivery vectors.
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Low-Fouling, Biospecific Films Prepared by the Continuous Assembly of Polymers

Stefanie N. Guntari, Edgar H. H. Wong, Tor K. Goh, Rona Chandrawati, Anton Blencowe, Frank Caruso,* and Greg G. Qiao*

Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

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