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**Polymer brush interfaces for protein biosensing prepared by surface-initiated controlled radical polymerization**

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Thin polymer films that are able to bind proteins are of great relevance for a wide variety of biosensor and biochip applications. Some of these applications require films that have high protein binding capacities while others call for surfaces that allow to control the orientation and preserve the biological activity of the immobilized proteins. Designing protein binding polymer interfaces with properties that are tailored for a specific biosensor or biochip configuration is a creative challenge that provides a host of possibilities to put the assets of modern controlled radical polymerization techniques in practical, technological use. For the preparation of protein binding polymer interfaces, surface-initiated controlled radical polymerization techniques are very attractive since they allow good control over the molecular weight, architecture, grafting density and functionality of the resulting surface-attached polymer assemblies, which are typically referred to as polymer brushes. This review article presents an overview of polymer brush films prepared via surface-initiated controlled radical polymerization, which have been designed as protein binding interfaces. The focus of this article will be on the protein immobilization chemistries that have been explored and the underlying polymer chemistry that is needed to generate these films and control their properties. For the immobilization of proteins on polymer brush films, two principal approaches can be followed; (i) covalent and (ii) non-covalent immobilization. For each of these main approaches an overview of the different polymer brush chemistries will be presented that can be used to covalently or non-covalently bind proteins.

Introduction

Protein biosensors, such as protein biochips are important analytical tools and have gained increasing importance during the last decade, mostly in the field of drug discovery, biomedicine and food safety.1–6 These miniaturized biosensors provide information about the abundance and function of proteins in a high throughput manner. Protein microarrays can be subdivided in three main categories, analytical protein microarrays, functional protein microarrays and reverse-phase protein microarrays.7–16

Analytical protein microarrays provide information about differences in protein expression. This microarray format uses immobilized capture antibodies that are able to selectively recognize and bind the protein of interest from a mixture of proteins or biomolecules. Binding of the protein of interest can be detected either by direct labelling of the target protein (Figure 1A) or by using the “sandwich” detection method (Figure 1B). In the latter case, two antibodies are necessary, one for the immobilization of the target protein and a second reporter antibody for the signalling. The “sandwich” detection method provides an increased sensitivity and specificity compared to the direct labelling of the target protein. Functional protein microarrays use individually purified proteins allowing the measurement of biochemical activities such as protein – protein interactions or interactions between proteins and DNA, lipids, drugs or peptides (Figure 1C). This technique also allows to study post-translational modifications, which helps provide information about protein synthesis and function in the cell. The reverse-phase array format is an alternative to the analytical microarray mentioned above. In a reverse phase array, tissue/cell lysate is immobilized on the chip (Figure 1D).

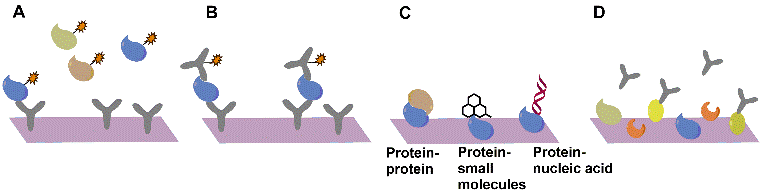


Figure 1. Schematic illustration of an analytical protein microarray format with direct labelling of the proteins (A) or using the “sandwich” assay (B), a functional protein microarray (C) and a reverse-phase protein microarray format (D). (Figure drawn after reference 7).

Protein biochips use various strategies to transduce the protein binding or detection event into a detectable signal. Some of these transduction strategies require the use of labelled proteins. Label dependent methods typically use fluorescence or isotopically labelled proteins.17 Enzymatic methods are also used as they provide a way to amplify the detection signal. As the label can influence protein structure and function, there has been an interest to develop label-free techniques. Examples of signal transduction and amplification schemes that do not require labels are surface plasmon resonance (SPR), localized SPR (LSPR), optical waveguide lightmode spectroscopy (OWLS), quartz crystal microbalance (QCM), electrochemical, thermometric or magnetic techniques.18,19,20 Although the different microarray formats differ greatly in how they transduce protein binding into a measurable signal, they all require sensor chip surfaces that are able to bind the protein of interest or the tissue/cell lysate. The specific characteristics of the protein binding interface depend on the protein microarray format. In some cases, protein binding interfaces are required that provide a high binding capacity while in other cases retention of protein structure and function or reduction of non-specific binding are essential. Polymer chemistry provides a diverse set of tools to fine-tune the properties of protein binding microarray interfaces. Thin polymer films are particularly attractive substrates for protein immobilization as they can provide a high surface concentration of functional groups and their properties can be tuned to prevent non-specific adsorption of proteins and maximize retention of protein structure and function. As compared to planar surfaces modified with protein reactive functional groups, the use of thin polymer films provides a three-dimensional protein binding interface, with a potentially higher binding capacity. One class of thin polymer films that are particularly attractive as substrates for protein binding interfaces are those that are obtained via surface-initiated controlled radical polymerization (SI-CRP).21–25 SI-CRP results in thin polymer films, which are referred to as polymer brushes, in which individual polymer chains are tethered to the underlying substrate via one of their chain ends (Figure 2). Strictly speaking the term polymer brush only applies to systems in which the interchain spacing is much smaller than the radius of gyration of the polymer. The term polymer brush, however, is frequently colloquially used to refer to any chain end tethered surface attached polymer film. The aim of this review article is to provide an overview of the different polymer brushes prepared by SI-CRP that can be used to bind proteins for biosensing applications. The remainder of this article is divided in two parts. First, we will present polymer brushes that are designed to bind proteins covalently. After that, polymer brushes that bind proteins via non-covalent interactions will be discussed.

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Figure 2. Modification of a substrate using surface-initiated controlled radical polymerization and immobilization of a protein.

Covalent protein immobilization.

Polymer brushes prepared by surface-initiated controlled radical polymerization (SI-CRP) techniques have been extensively used to create interfaces for protein binding. A variety of polymer brush films have been used to covalently bind proteins. This section will provide an overview of such polymer brushes. The examples presented in this section are organized in 5 groups, depending on the protein reactive side chain functional group incorporated in the brush film. The most important types of polymer brushes that have been used to covalently immobilize proteins are those that possess carboxylic acid, hydroxyl, epoxide or active ester side chain functional groups. Some other side chain and chain end functional polymer brushes have been used as well, which will be discussed at the end of this part.

Carboxylic acid side chain functional polymer brushes.

Table 1 provides an overview of carboxylic acid side chain functional polymer brushes that have been used to covalently bind proteins.

**Table 1**. Overview of carboxylic acid side chain functional polymer brushes, which have been used for the covalent immobilization of proteins.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer brush** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration / immobilization chemistry)** | **Secondary interaction** | **Ref.** |
| Poly(acrylic acid) (PAA)  X:\Review\Pictures\Poly(AA).tif | SI-ATRP / Au | 9.5 nm  PtBA precursor | 10, 50 and 100 mol% initiator;  0.21 chains/nm2 for 100% initiator | BSA, ~0.8 µg/cm2  EDC/NHS | Anti-BSA  ~0.2 µg/cm2 | 26 |
| SI-ATRP / Au | 85 nm | High[\*] | BSA  EDC/NHS |  | 27 |
| SI-ATRP / Silicon wafer | 30 nm | High | BSA, avidin and BSA-biotin  EDC/NHS | Streptavidin | 28 |
| SI-ATRP / Silicon wafer | 5 to 80 nm | High | Ribonuclease B (RNase A), 5.8 µg/cm2 for 80 nm PAA  EDC/NHS | - | 29 |
| SI-RAFT / Silica nanoparticles ø:80 nm | 68.8 nm | 0.4 RAFT chain transfer agent/nm2 | Streptavidin, 2600 µg/mg  EDC/NHS | - | 30 |
| SI-RAFT / Silica nanoparticles ø:80 nm | 44 nm | High | Horseradish peroxidase (HRP)  677 µg/mg  EDC/NHS | Anti-β-Human chorionic gonadotrophin (anti-β-hCG) | 31 |
| SI-RAFT / Silica nanoparticles ø:85 nm | ~ 145 nm | 0.54 chains/nm2 | BSA, 4200 µg/mg  EDC/NHS | - | 32 |
| SI-ARGET-ATRP / Amino-funct. macroporous monoliths from polymerized high internal phase emulsion (polyHIPE) | - | High | Enhanced green fluorescent protein (eGFP)  Coral derived red fluorescent protein (DsRed)  EDC/sulfo-NHS | - | 33 |
| SI-ATRP / Plasma-polymerized allyl alcohol | - | High | Anti-C reactive proteins (CRP antibody)  EDC/NHS | - | 34 |
| SI-ATRP / Parylene | - | High | Contortrostatin, BSA  (gelatine 30 µg/cm2)  EDC/NHS | - | 35 |
| Poly(methacrylic acid) (PMAA) | SI-ATRP / Silicon nanoparticles  ø:227 nm | - | 1.2 chains/nm2 | 3 peptides  EDC/NHS  (10 amino acids peptides) | - | 36 |
| SI-ATRP / Silicon nanoparticles  ø:~200 nm | - | High | Peptides  EDC/NHS | - | 37 |
| Poly(carboxybetaine acrylamide) (PCBAA) | SI-ATRP / Au | 21 to 55 nm | High | Activated leukocyte cell adhesion molecule antibody (anti-ALCAM)  0.3 µg/cm2  EDC/NHS | ALCAM 0.042 µg/cm2 | 38 |
| SI-ATRP / Au | 13 nm | High | Human chorionic gonadotropin antibody (anti-hcg), 0.34 µg/cm2  Salmonella common structural antigen CSA-1 (Anti-Salm),  0.34 µg/cm2  Polyclonal goat antibodies to *Escherichia coli* (Anti-E-coli),  0.27 µg/cm2  BSA, 0.44 µg/cm2  Streptavidin, 0.16 µg/cm2  EDC/NHS | *Salmonella typhimurium* cells  *E. coli O157:H7* cells | 39 |
| SI-ATRP / Au | 15 to 20 nm | High | Antibody to thyroid stimulating hormone (Anti-TSH), anti-hCG  Anti-ALCAM, 0.009 µg/cm2  EDC/NHS | TSH  0.15 mRIU  hCG  0.22 mRIU  ALCAM  0.2 mRIU | 40 |
| SI-ATRP / Au | 1st layer 7.6 nm.  Regrowth without treatment 13.2 nm.  Regrowth with 2 hours immersion in a solution of sodium azide 17.5 nm. | First layer high, second layer lower grafting density | Anti-human thyroid stimulating hormone (anti-TSH), 0.209 µg/cm2 (one block) and 0.417 µg/cm2 (bimodal brushes)  EDC/NHS | TSH  0.0386 µg/cm2  (one block) and 0.0698 µg/cm2  (bimodal brushes) | 41 |
| SI-PIMP / Au | 1st layer 10.8 nm.  Regrowth without addition of TED 32.1 nm.  Regrowth while using TED 46.1 nm | First layer high, second layer lower grafting density | Anti-human thyroid stimulating hormone (anti-TSH) IgG, 0.253 µg/cm2 (one block) and 0.792 µg/cm2 (bimodal brushes)  EDC/NHS | TSH  0.0421 µg/cm2  (one block) and 0.1288 µg/cm2  (bimodal brushes) | 41 |
| Poly(carboxybetaine acrylamide) (PCBAA) | SI-ATRP / Au | 15 to 20 nm | High | Biotinylated anti-*Salmonella*, 0.4185 µg/cm2  EDC/NHS | Streptavidin | 42 |
| Poly(carboxybetaine methacrylate) (PCBMA) | SI-ATRP / Au | 10 to 15 nm | High | Human chorionic gonadotrophin antibody (anti-hCG), 0.3 ng/cm2  EDC/NHS | Fibrinogen,  lysozyme and  hCG | 43 |
| SI-ATRP / Magnetic iron oxide nanoparticles  ø:231 nm | 25 or 64 nm | High | Human chorionic gonadotrophin (hCG) and HRP-conjugated anti human IgG  EDC/sulfo-NHS | Anti-hCG | 44 |
| Poly(oligo(ethylene glycol) methacrylate–*b*-acrylic acid) (POEGMA-*b*-PAA) | SI-PIMP / Cycloolefin polymer (COP) | - | High | Goat-anti-rabbit immunoglobulin antibody  EDC/NHS | FITC-Ra IgG antigen | 45 |
| Poly(oligo(ethylene glycol) methyl ether methacrylate–*b*-carboxybetaine acrylamide) (POEGMeMA-*b*-PCBAA) | SI-ATRP / Au | 30 nm (POEGMeMA 20 nm) | High | Neutravidin, 0.225 µg/cm2  EDC/NHS | Biotinylated rabbit IgG antibody,  118 ng/cm2 | 46 |

[\*] Throughout the tables, the use of the term “high” to describe the grafting density refers to polymer brushes grown from substrates, which have been modified with 100% initiator or chain-transfer agent.

One of the most straightforward approaches to carboxylic acid side chain functional polymer brush is via direct polymerization of acrylic or methacrylic acid. Qu *et al.* used reversible addition-fragmentation chain-transfer (RAFT) polymerization to modify silica nanoparticles with poly(acrylic acid) (PAA) brushes, which were able to bind 677, 2600 or 4200 µg/mg horseradish peroxidase (HRP), streptavidin (SA) or bovine serum albumin (BSA) using EDC/NHS chemistry.30–32

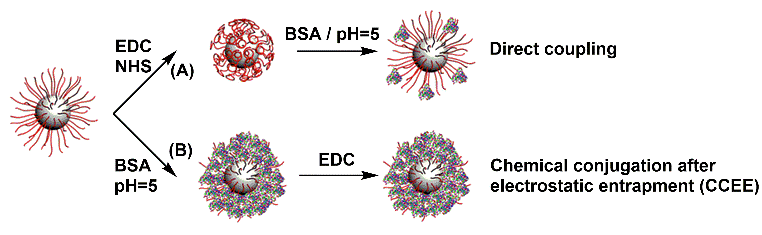


Figure 3. Covalent immobilization of proteins on carboxylic acid side chain functional brushes. (A) Direct EDC/NHS protein coupling and (B) chemical conjugation after electrostatic entrapment (CCEE). (Reproduced from reference 32 with permission from the American Chemical Society).

Akkahat *et al.* used PAA brushes with different grafting densities (10, 50 or 100%), which were used to covalently bind BSA.26 Adsorption of anti-BSA on protein modified PAA polymer brushes with 50 and 100% grafting density provided good specific binding and prevented the non-specific adsorption of streptavidin and fibrinogen, whereas brushes with only 10% grafting density showed poor target binding and suffered from non-specific adsorption. To maximize the protein binding capacity of PAA brushes toward BSA, Xu and coworkers developed the chemical conjugation after electrostatic entrapment (CCEE) technique.32 CCEE involves electrostatically adsorbing the proteins into the brushes before addition of the coupling agents (Figure 3). Using the CCEE method on PAA grafted nanoparticles, they were able to covalently immobilize 868 µg protein per mg particle compared to 335 µg/mg via direct EDC/NHS coupling.

Carboxybetaine functional polymer brushes are attractive candidates for the preparation of biosensing interfaces as they possess both non-fouling properties and present side chain carboxylic acid groups, which can be used to covalently immobilize proteins. Vaisocherová *et al.* demonstrated that poly(carboxybetaine acrylamide) (PCBAA) brushes functionalized with polyclonal goat antibodies to *Escherichia coli* or Salmonella common structural antigen CSA-1 are better able to resist protein fouling than poly(2-hydroxyethyl methacrylate) (PHEMA) brushes functionalized with the same proteins.39 They measured only a fourfold increase in fouling from blood plasma on the protein functionalized PCBAA brushes as compared to an approximate 20 fold increase on the modified PHEMA brushes. The group of Rodriguez-Emmenegger could attach 225 ng/cm2 neutravidin on poly[oligo(ethylene glycol methyl ether methacrylate)-*block*-carboxybetaine acrylamide] (POEGMeMA-*b*-PCBAA) brushes using EDC/NHS, which represents, at saturation, nearly a monolayer of protein.46

By partial termination (SI-ATRP) or via the addition of a reactivator (SI-PIMP, surface-initiated photoiniferter mediated polymerization), Jiang and co-workers prepared bimodal PCBAA brushes composed of densely grafted first PCBAA layer and a lower grafting density top PCBAA block.41 While the high grafting density lower block was designed to impart non-fouling properties, the lower grafting density top layer provided the protein binding interface. The reduced grafting density of the top PCBAA layer allowed to enhance the protein binding capacity. As an example, EDC/NHS mediated immobilization of anti-TSH (anti-human thyroid stimulating hormone) IgG resulted in protein surface concentrations of 253 ng/cm2 for the single layer and 792 ng/cm2 for the two-layer bimodal brushes produced via SI-PIMP.

Hydroxyl side chain functional polymer brushes.

A second major class of polymer brushes that has been used to covalently immobilize proteins are those that contain side chain hydroxyl functional groups. Table 2 presents an overview of hydroxyl side chain function polymer brushes that have been used to immobilize proteins.

**Table 2**. Overview of hydroxyl side chain functional polymer brushes, which have been used for the covalent immobilization of proteins.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration / immobilization chemistry)** | **Secondary interaction** | **Ref.** |
| Poly(2-hydroxyethyl methacrylate) (PHEMA)  X:\Review\Pictures\Poly(HEMA).tif | SI-ATRP / Au | 18 nm | High | Anti-hcg, 0.2 µg/cm2  Anti-*salmonella*, 0.36 µg/cm2  Anti-*E-coli*., 0.24 µg/cm2  BSA, 0.29 µg/cm2  Streptavidin, 0.01 µg/cm2  DSC/DMAP | *Salmonella typhimurium* cells and *E. coli O157:H7* cells | 39 |
| SI-ATRP / Au | 30 nm | High | Streptavidin, 1 µg/cm2  Biotinylated rabbit-antigoat antibody (bt-Rb-R-Gt)  DSC | Goat-antirat (Gt-α-Rt) antibody and biotinylated  rabbit-antigoat (bt-Rb-R-Gt)  0.13 µg/cm2 | 47 |
| SI-ATRP / Silicon or fused silica wafers | 49 nm to  58 nm after modification | High | IgG  DSC/DMAP | Labelled anti-human IgG | 48 |
| Poly(2-hydroxyethyl methacrylate–*co*-oligo(ethylene glycol) methacrylate) (PHEMA–*co*-POEGMA) (1:1) | SI-ARGET-ATRP / Au | Up to ~400 nm | 0.5% to 10% ATRP initiator | IgG, 6798 RU  EDC/sulfo-NHS | Anti-IgG  1553 RU | 49 |
| Poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) | SI-ATRP/ Au | 18 nm | High | Rabbit antibody  DSC/DMAP | Peptidoglycan-  polysaccharide antigen,  0.1 µg/cm2 | 50 |
| Poly(oligo(ethylene glycol) methacrylate) (POEGMA) | SI-ATRP / Au | 30 nm | High | Chromeon-streptavidin,  460 ng/cm2  Succinic or glutaric anhydride followed by EDC/NHS,  Disuccinimidyl carbonate (DSC), carbonyldiimidazol (CDI), cyanuric chloride and triflic anhydride | Goat-antirat (Gt-α-Rt) antibody and biotinylated  rabbit-antigoat (bt-Rb-R-Gt) antibody  0.09 µg/cm2 (using DSC) | 47 |
| SI-ATRP / Au | - | High | POEGMA-NSC (ARMAPE peptide), 0.56 µg/cm2 and tPA (tissue plasminogen activator)  4-Nitrophenyl chloroformate  (NPC) | Tissue plasminogen activator (tPA) | 51 |
| SI-ATRP / Silicon wafer | 52 or 136 nm | High | FITC conjugated IgG, 3.2 µg/cm2 (52 nm) and 4.8 µg/cm2 (136 m) and human IgG  Succinic anhydride (30% conversion) and EDC/NHS | FITC conjugated anti human IgG | 52 |
| Poly(oligo(ethylene glycol) methyl ether methacrylate–*b*-oligo(ethylene glycol) methacrylate) (POEGMeMA-*b*-POEGMA) | SI-ATRP / Au | 1st block 20 nm  Total 34 (a) or 44 (b) nm | High | Streptavidin  (a)~ 0.06 µg/cm2  (b)~ 0.09 µg/cm2  DSC/DMAP | Biotin-anti Mouse IgG  ~ 0.035 µg/cm2  (a)  ~ 0.04 µg/cm2  (b) | 46 |

Trmcic-Cvitas *et al.* investigated the immobilization of streptavidin (SA) on poly(oligo(ethylene glycol) methacrylate) (POEGMA) brushes using pentafluoropyridine, 3-chloropropionaldehyde diethylacetal, nitrophenyl chloroformate, tresyl chloride, oxalyl chloride, cyanuric chloride, carbonyldiimidazole (CDI) and disuccinimidyl carbonate (DSC) as coupling agents.47 Using fluorescent labelled SA, DSC was found to be the most efficient and reliable coupling agent, followed by cyanuric chloride, CDI and triflic acid. In another set of experiments, the authors evaluated the effect of the ethylene glycol spacer length on the DSC mediated coupling of SA. For these experiments, PHEMA, POEGMA-360 (around 8 times longer than HEMA) and POEGMA-526 (around 12 times longer than HEMA) brushes were used. While the amount of attached SA was similar on the polymer brushes prepared from POEGMA, the amount on PHEMA brushes was more than 4 times higher. Interestingly, the adsorption of a secondary antibody (Gt-α-Rt, goat antirat antibody) after functionalization of the brushes with bt-Rb-α-Gt (biotinylated rabbit-antigoat antibody) showed an inverse trend. The amount of analyte adsorbed was much greater on the POEGMA brushes than on PHEMA. The authors attributed this to an increase in non-specific binding on the POEGMA brushes.

An interesting alternative to PHEMA and POEGMA brushes are poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) brushes. PHPMA brushes show non-fouling properties similar to those of poly(carboxybetaine acrylamide) (PCBAA) upon exposure to blood plasma.50 The hydroxypropyl side chain functional groups of the PHPMA brushes were used to covalently immobilize a rabbit peptidoglycan-polysaccharide antigen via DSC/DMAP coupling. Immobilization of the antibody was not found to result in an increase in non-specific binding.

Ma *et al.* have studied the influence of grafting density and film thickness on the non-fouling properties and protein immobilization capacity of copolymer brushes generated from equimolar quantities of OEGMA and HEMA.49 Copolymer brushes with a thickness of ~17 nm that were grown from gold substrates modified with a solution that contains 0.5% of an ATRP initiator modified thiol and 99.5% of EG3-thiol (11-(mercaptoundecyl) tri(ethylene glycol)) enabled a low non-specific protein adsorption with a high IgG binding capacity. IgG immobilization was achieved via EDC/sulfo-NHS coupling. Reducing the polymer brush thickness or the grafting density results in an increase in non-specific protein adsorption and a decrease in IgG immobilization.

Epoxide side chain functional polymer brushes.

Epoxide side chain functional polymer brushes are attractive matrices for the covalent immobilization of proteins. One of the attractive features of epoxide groups is that they allow direct protein immobilization via reaction with amine groups without the need for additional activation or coupling agents. A drawback of polymer brushes generated from glycidyl methacrylate (GMA) is that they are relatively hydrophobic, which promotes non-specific binding and hampers uptake and penetration of proteins from aqueous media. To this end, GMA is often copolymerized with hydrophilic comonomers. Hu *et al.* investigated the binding of Cy3-labeled anti-goat IgG on brushes prepared via copolymerization of OEGMA and GMA.53 The protein binding capacity of these brushes was found to increase upon increasing the volume % of GMA in the monomer feed from 0 to 0.25% and then reached a plateau value at a GMA monomer feed concentration of 0.5 volume %. SPR measurements indicated that these poly(OEGMA-*co*-GMA) brushes could bind the equivalent of 1.8 monolayers of anti-goat IgG.

Yan and co-workers modified Fe3O4 nanoparticles with copolymer brushes prepared by SI-ATRP of glycidyl methacrylate (GMA) and glycerol mono-methacrylate (GMMA).54 These nanoparticles were used to immobilize Penicillin G acylase (PGA). Nanoparticles coated with brushes obtained from 40/60 or 60/40 monomer ratios possessed a high enzymatic activity as compared to particles modified with brushes synthesized using larger fractions of GMA.

**Table 3**. Overview of epoxide side chain functional polymer brushes, which have been used for the covalent immobilization of proteins.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration)** | **Secondary interaction** | **Ref.** |
| Poly(2-(diethylamino)ethyl methacrylate–*co*-glycidyl methacrylate) (PDEAEMA-*co*-PGMA)  GMA 100 / 90 / 75 % | SI-ATRP / Silicon wafer | 50 to 230 nm | High | BSA  PGMA100% 100nm,  1.0 µg/cm2  PDEAEMA10%100nm,  2.2 µg/cm2  PDEAEMA25% 100nm,  3.6 µg/cm2  Lysozyme  PGMA100% 100nm,  1.0 µg/cm2  PDEAEMA10%100nm,  2.8 µg/cm2  PDEAEMA25%70nm,  4.9 µg/cm2  Ovalbumin  PGMA100% 100 nm,  4.9 µg/cm2  PGMA100% 25 nm,  1.7 µg/cm2 | - | 55 |
| Poly(2-(diethylamino)ethyl methacrylate–*co*-glycidyl methacrylate) (PDEAEMA-*co*-PGMA) (1:3) | SI-ATRP / Ta2O5 or silicon wafers | 50 nm or 130 nm on Ta2O5  70 nm on QCM chips | High | Labelled BSA, ovalbumin, goat Fab fragments and human tumour necrosis factor alpha (TNFα)  BSA, 3.5 µg/cm2  Ovalbumin 6 µg/cm2 | Labelled streptavidin, antihuman TNFα and labelled Fab fragments | 56 |
| Poly(ferrocenylmethyl methacrylate–*b*-glycidyl methacrylate) (PFMMA-*b*-PGMA) | SI-ATRP / Au | - | Initiator to  1-undecane thiol molar ratio of  0.1:1 to 1:1 | Anti-TNFα antibody | TNFα | 57 |
| Poly(glycerol monomethacrylate–*co*- glycidyl methacrylate) (PGMMA-*co*-PGMA) | SI-ATRP / Magnetic loaded poly(acrylic acid) nanoparticles ø:520 nm | 95 nm | High | BSA, 27 mg/g | - | 58 |
| Poly(glycerol monomethacrylate–*co*- glycidyl methacrylate) (PGMMA-*co*-PGMA) (3:2) | SI-ATRP / Magnetic loaded poly(acrylic acid) nanoparticles ø:550 nm | - | High | Penicillin G acylase,  46.6 mg/g | - | 54 |
| Poly(glycidyl methacrylate) (PGMA) | SI-ATRP / Ta2O5 or silicon wafers | 50 or 100 nm | High | Labelled BSA, Fab fragments, human TNFα and ovalbumin, 5 µg/cm2 | Labelled streptavidin, antihuman TNFα and labelled Fab fragments | 56 |
| Poly(glycidyl methacrylate–*co*-(2-hydroxyethyl) methacrylate) (PGMA-*co*-PHEMA) (1:10) | SI-ATRP / Glass | 23.6 nm | High | Anti-IgG-Cy3 | - | 59 |
| Poly(glycidyl methacrylate–*co*-oligo(ethylene glycol) methacrylate) (PGMA 0.5% -*co*-POEGMA) | SI-ATRP / Au | 30 nm | High | Anti-goat IgG,  1.675 µg/cm2,  α-Fetoprotein (AFP),  carcinoembryonic antigen (CEA) and hepatitis B surface antigen (HBsAg) | Anti-CEA, anti-AFP and anti-HBsAg antibodies | 53 |
| Poly(glycidyl methacrylate–*co*-oligo(ethylene glycol) methyl ether methacrylate) (PGMA-*co*-POEGMeMA) (7:3) | SI-ATRP / Silica nanoparticles  ø:300 nm | 170 nm  Estimated from the hydrodynamic size measured | High | Biotinylated anti-rabbit IgG and CEA antigen | Cy3-streptavidin | 60 |
| SI-ATRP / PMMA sheets | - | High | Monoclonal anti-CEA | Carcinoembryonic antigen (CEA) | 60 |
| Poly(glycidyl methacrylate–*co*-oligo(ethylene glycol) methacrylate) (PGMA-*co*-POEGMA) (1:10) | SI-ARGET ATRP / Glass | - | High | Anti-rabbit IgG, 250 ug/ml and rabbit or rat or human IgG | Cy3-tagget rabbit IgG, Cy3- anti rabbit IgG, Alexa Fluor 546- anti-rat IgG and Cy3-anti-human IgG | 61 |
| Poly[2-hydroxyethyl methacrylate–*b*-( glycidyl methacrylate–*co*-(2-hydroxyethyl) methacrylate)] (PHEMA-*b*-(GMA-*co*-HEMA)(4:1)) | SI-ARGET-ATRP / Glass | 1st block 27 nm (PHEMA) and 2nd block 60 nm (P(HEMA-*co*-GMA)) | High | Lysozyme, 0.8 µg/cm2 or  1.6 µg/cm2 in water | - | 62 |
| Poly(oligo(ethylene glycol) methacrylate–*b*-glycidyl methacrylate) (POEGMA-*b*-PGMA) | SI-ARGET-ATRP / Au | 1st block 36.7 nm (POEGMA) and 2nd block 11.8 nm (PGMA) | 0.1-100% ATRP initiator concentration; 0.0232 chains/nm2 for 0.5% ATRP initiator  concentration | G-R-IgG, ~ 2750 RU | R-IgG, 1300 RU | 63 |
| Poly(oligo(ethylene glycol) methacrylate–*b*-glycidyl methacrylate) (POEGMA-*b*-PGMA) | SI-ARGET-ATRP / Au | - | 0.1-100% ATRP initiator concentration; 0.0232 chains/nm2 for 0.5% ATRP initiator  concentration | G-R-IgG, ~ 2400 RU | R-IgG, 1400 RU | 63 |
| Poly(oligo(ethylene glycol) methacrylate-*b*-glycidyl methacrylate) (POEGMA-*b*-PGMA) | SI-ARGET-ATRP / Au | - | 0.1-100% ATRP initiator concentration; 0.0232 chains/nm2 for 0.5% ATRP initiator  concentration | G-R-IgG, ~ 3000 RU | R-IgG, 900 RU and 1600 RU | 63 |

The reaction of epoxide groups with primary amines in water at room temperature and at pH 7 can be accelerated by the copolymerization of GMA with monomers containing secondary amine side chain functional groups such as 2-(diethylamino)ethyl methacrylate (DEAEMA).55 Reaction of a PGMA homopolymer brush with an initial dry film thickness of 130 nm with a 1 M solution of propylamine resulted in 40% epoxide group conversion after 24 hours. Using copolymer brushes that contain 10 respectively 25 mol% DEAEMA, the same conversion was measured after 8 respectively 2 hours. Further model experiments with BSA and lysozyme showed that the incorporation of DEAEMA moieties not only enhanced the rate of the immobilization reaction but also augmented the protein binding capacity. These PGMA-*co*-PDEAMA brushes were subsequently successfully used as the protein binding interface on tantalum pentoxide coated optical waveguide based microarray chips.

Active ester side chain functional polymer brushes.

The incorporation of side chain active ester groups is another strategy to allow covalent protein immobilization without the need for additional activation or coupling agents. The direct polymerization of active ester side chain functional monomers, however, can be challenging. Another potential limitation related to the use of side chain active ester functional polymers is the susceptibility of the functional groups towards hydrolysis.

**Table 4**. Overview of active ester side chain functional polymer brushes, which have been used for the covalent immobilization of proteins.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration)** | **Secondary interaction** | **Ref.** |
| Poly(methacryloyl succinimide) (PMSu) | SI-ATRP / Silicon wafer | 1.9 nm | 0.73 chains/nm2 | FITC-Anti HSA (at pH 5.8), 0.5 µg/cm2 and Anti-HSA | FITC-HSA | 64 |
| Poly(pentafluorophenyl acrylate) (PPFPA) 0, 1, 10 or 50% conversion with amino-PEG550 | SI-RAFT / Silica nanoparticles  ø:0.255 µm  or silicon wafer | 12 to 72 kDa (GPC analysis of sacrificial polymer) | 0.07 to 0.15 chains/nm2 | Green fluorescence tagged GFP,  Anti-GFP antibody and Anti-PKR antibody | GFP and PKR (+ TRBP that reacts with PKR) | 65 |

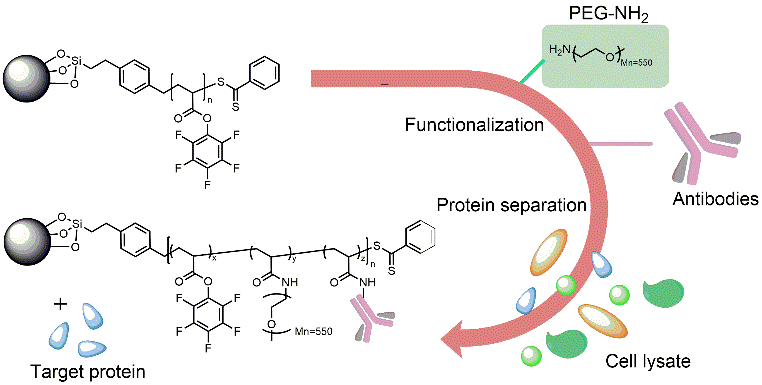


Figure 4. Protein purification using antibody presenting nanoparticles obtained via direct polymerization of pentafluorophenyl acrylate (PFPA). (Figure drawn after reference 65).

Takasu *et al.* used SI-ATRP to grow poly(methacryloyl succinimide) (PMSu), poly(amino ethyl methacrylate) (PAEMA), poly(n-butyl methacrylate) (PBMA) and poly(acrylic acid) (PAA) brushes from silicon substrates.64 Experiments with FITC-anti-HSA revealed that at pH 9.6, 5.8 and 4 most protein was bound to the PMSu brushes. At pH 8, electrostatic interactions were found to dominate and protein binding was the highest on the PAEMA and PBMA brushes.

Son *et al*. used silica particles modified with poly(pentafluorophenyl acrylate) (PPFPA) brushes for the immobilization of antibodies, which were subsequently used for protein purification via immunoprecipitation (IP).65 PPFPA brushes were grown on the silica particles using RAFT polymerization. Pentafluorphenyl esters are attractive as they are reactive towards amine groups, but are less susceptible towards hydrolysis as compared to NHS esters. As the PPFPA modified silica particles did not disperse well in aqueous media, part of the active ester side chain functional groups were modified with a low molecular weight amino terminated PEG. While substituting part of the PFPA groups with PEG chains enhanced dispersion in aqueous media, it comes at the cost of a loss of protein reactive groups. This was demonstrated by investigating the PKR (protein kinase R) recovery for PPFPA brush grafted silica nanoparticles, which were modified with 1, 10 and 50% of a 12 KDa PEG (Figure 4). PKR recovery was highest for 10% PEG substituted silica nanoparticles and significantly lower at 1% and 50% PEG substitution. The low PKR recovery with nanoparticles with 50% PEG substitution was ascribed both the low amount of pentafluorophenyl groups available on these particles as well as to the steric barrier that is presented by the PEG chains.

Other protein reactive polymer brushes.

In addition to the examples described in the previous sections, a number of other covalent coupling chemistries have been explored as well for the immobilization of proteins on polymer brushes. Table 5 summarizes some of the other immobilization chemistries. In contrast to the previous section, table 5 does not only include examples of polymer brushes with side chain protein reactive functional groups, but also lists polymer brushes with chain ends that have been modified to allow covalent protein immobilization.

**Table 5**. Overview of other protein reactive polymer brushes, which have been used for the covalent immobilization of proteins.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration / immobilization chemistry)** | **Secondary interaction** | **Ref.** |
| **Side chain functional polymer brushes** | | | | | | |
| Poly(3-acrylaminophenylboronic acid) (PAAPBA)  X:\Review\Pictures\Poly(AAPBA).tif | SI-ATRP / Iron oxide nanoparticles on graphite oxide | - | High | Ovalbumin, 471 mg/g and transferrin, 450 mg/g | - | 66 |
| Poly(glycidyl methacrylate–*co*-sulfobetaine methacrylate) (PGMA-*co*-PSBMA) (1:1 or 1:10) modified with 3-aminophenylboronic acid | SI-ATRP / Polydopamine coated poly(propylene) films | - | High | FITC-goat to rabbit IgG (Go-*to*-Ra IgG) | BSA, FITC rabbit IgG antigen and mouse to goat IgG (Mo-*to*-Go IgG) | 67 |
| Poly(2-methyl-acrylic acid 3-(2,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-3-ylamino)-propylester) (PGMA-G) | SI-ATRP / Silicon microparticles  ø:10 µm | - | High | Concanavalin A, 78.5 µg/mg, wheat germ agglutinin (WGA), 80.0 µg/mg and ricinus  Communis agglutinin (RCA), 55.9 µg/mg | Ribonuclease B (RNase B) | 68 |
| Poly(2-hydroxyethyl methacrylate) modified with benzylguanine | SI-ATRP / Silicon wafer | ~ 100 nm | High | Fluorescent methotrexate labelled *O*6-alkylguanine-DNA-alkyltransferase- dihydrofolate reductase (MTX-Cy5-AGT-DHFR) | - | 69 |
| Poly(oligo(ethylene glycol) methacrylate) (POEGMA) modified with benzylguanine | SI-ATRP / Silicon wafer | ~ 100 or 120 nm | High | Fluorescent methotrexate labelled *O*6-alkylguanine-DNA-alkyltransferase- dihydrofolate reductase (MTX-Cy5-AGT-DHFR) | - | 69 |
| SI-ATRP / Glass | - | High | *O*6-alkylguanine-DNA-alkyltransferase-FKBP (AGT-FKBP) and *O*6-alkylguanine-DNA-alkyltransferase-FRB (AGT-FRB) | Cy3 labelled *O*6-alkylguanine-DNA-alkyltransferase-FKBP (Cy3-AGT-FKBP) and Cy5 labelled *O*6-alkylguanine-DNA-alkyltransferase-FRB (Cy5-AGT-FRB) | 70 |
| **Chain end functional polymer brushes** | | | | | | |
| Poly(*N*-isopropyl acrylamide) (PNIPAM) | SI-RAFT / Indium-tin oxide | 27 nm | High | Azide functinalized polyclonal mesothelin antibody (MSLN) | MSLN antigen | 71 |
| Poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMeMA) | SI-ATRP / Au | 10, 20, 30 nm | High | BSA-maleimide,  0.0838 µg/cm2 (10 nm)  0.0314 µg/cm2 (20 nm)  0.0154 µg/cm2 (30 nm) | Anti-BSA | 72 |
| Poly(sulfobetaine methacrylate) (PSBMA) | SI-ATRP / SixN4 (x>4) | 10 to 20 nm | High | FITC-labelled streptavidin and anti-*salmonella*  Bis-NHS (suberic acid  bis(N-hydroxysuccinimide ester)) | - | 73 |

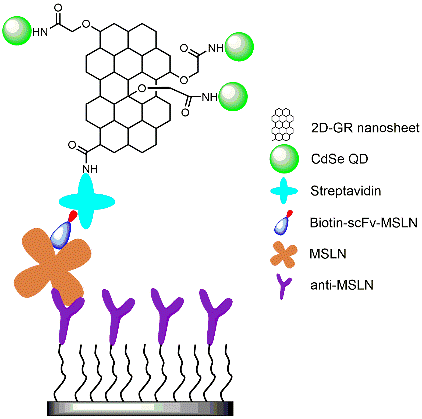


Figure 5. Sandwich type assay for the detection of MSLN based on anti-MSLN chain-ene modified brushes and CdSe decorated graphene oxide nanosheets. (Drawn after reference 71).

Tugulu *et al*. used POEGMA brushes prepared via SI-ATRP for the chemoselective immobilization of *O*6-alkylguanine-DNA-alkyltransferase (AGT) fusion proteins with a defined orientation.69 The POEGMA hydroxyl side chain containing polymer brushes were first modified with *para*-nitrophenyl chloroformate before substitution with *O*6-[4-(13-Amino-2,5,8,11-tetraoxatridecyl)oxymethylbenzyl] guanine. Covalent immobilization of AGT fusion proteins takes advantage of the ability of AGT to transfer the alkyl group of the benzylguanine derivative to one of its cysteine residues. Pan *et al.* prepared glucosamine functionalized silicon microparticles via SI-ATRP of glycidyl methacrylate.68 Oxidation of the vicinal diol groups on the glucosamine residues afforded aldehyde residues, which were used to attach 3 different lectins via reductive amination, viz. concanavalin A (ConA), wheat germ agglutinin (WCA) and ricinus-communis agglutinin (RCA120). These lectin modified particles were used for the enrichment of glycoproteins/glycopeptides from complex mixtures.

Most of the covalent protein immobilization methods presented so far use side chain reactive functional groups to attach proteins to polymer brushes. Alternatively, the chain ends of surface-attached polymers can also be used to couple proteins. Using SI-ATRP, Kuzmyn *et al*. prepared poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMeMA, 300 Da) brushes.72 Reaction of these polymer brushes with nickelocene introduces cyclopentadiene moieties at the chain end, which can be used to attach maleimide functionalized proteins via a Diels-Alder click reaction. Coupling of maleimide modified BSA onto the brushes was studied with SPR. Cyclopentadiene end functionalized brushes with film thickness of 10, 20 and 30 nm were able to bind 838, 314 and 154 pg/mm2, which corresponds to 67, 25 and 12% of a BSA monolayer.

Another example of the use of chain end protein reactive brushes was reported by Trau and coworkers.71 The aim of this study was to develop an electrochemical immunoassay. To this end, an electrode surface was modified with a poly(*N*-isopropyl acrylamide) (PNIPAM) brush using RAFT polymerization. Conversion of the trithiocarbamate polymer end group into a thiol group via aminolysis followed by reaction with *N*-propargyl maleimide generated an alkyne-terminated surface-anchored brush. Via click chemistry, azide functionalized polyclonal mesothelin antibody (anti-MSLN) was coupled to the polymer brush. The anti-MSLN was used for the detection of the MSLN antigen, which is an ovarian and pancreatic cancer biomarker (Figure 5). Binding of the antigen was registered following a sandwich approach using graphene oxide nanosheets modified with CdSe quantum dots and recombinant single chain fragment toward MSLN, which allowed for electrochemical detection using square-wave anodic stripping voltammetry (SWASV). In another example, Nguyen *et al.* modified the bromine end groups of poly(sulfobetaine methacrylate) (PSBMA) brushes, which were grown via SI-ATRP from silicon nitride, first with tris(2-aminoethyl)amine and then with suberic acid bis(N-hydroxysuccinimide ester).73 These chain end reactive brushes were used to immobilize FITC labelled streptavidin and anti-*Salmonella* antibody.

Non-covalent protein immobilization.

The second part of this article will present an overview of polymer brushes prepared by SI-CRP, which have been designed for the non-covalent immobilization of proteins. For each of the different approaches that can be used to non-covalently immobilize proteins, examples of polymer brush based interfaces prepared via SI-CRP will be discussed.

Electrostatic interactions.

A very straightforward strategy for the non-covalent immobilization of proteins on polymer brushes is based on the use of electrostatic interactions. Table 6 provides an overview of polymer brushes prepared via SI-CRP that have been used for the non-covalent immobilization of proteins using electrostatic interactions.

**Table 6**. Overview of polymer brushes, which have been used for the non-covalent immobilization of proteins using electrostatic interactions.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration)** | **Secondary interaction** | **Ref.** |
| Poly(acrylic acid) (PAA) | SI-ATRP / Au coated glass slides | 9.5 nm  (PtBA) | 10, 50 or 100 mol% ATRP initiator; 0.21 chains/nm2 for 100 mol% | lysozyme, up to 24 pmol/cm2(100% initiator), streptavidin, up to 1.7 pmol/cm2 (10%), BSA up to 1.7 pmol/cm2(10%) and fibrinogen, up to 1.3 pmol/cm2(10%) | - | 26 |
| SI-ATRP / Regenerated cellulose | 250 nm | High | Lysozyme, 98.5 (static) or 71.2 (dynamic) mg/mL | - | 74 |
| Poly(acrylic acid–*co*-(n-butyl) methacrylate–*co*-N-isopropyl acrylamide) (PAA –*co*- PBMA -*co*- NIPAM) | SI-ATRP / Silica microparticles ø:5 µm | Mn 12.4 kDa | 0.13 chains/nm2 | Lysozyme | - | 75 |
| Poly(2-aminoethyl methacrylate) (PAEMA) | SI-ATRP / Quartz surface | 1.4 nm | 0.37 chains/nm2 | Anti-HSA, 1.55 µg/cm2 | - | 64 |
| Poly(2-aminoethyl methacrylate–*b*-(2-methacryloyloxyethyl) phosphorylcholine) (PAEMA-*b*-PMPC) | SI-ATRP / Quartz surface | 6.5 nm PMPC  1.4 nm PAEMA  7.3 nm total | 0.36 chains/nm2 | BSA, FITC-HSA and anti-HSA, 1.1 µg/cm2 | - | 64 |
| Poly(carboxybetaine methacrylate–*co*-(2-(dimethylamino) ethyl methacrylate)) (PCBMA-*co*-PDMAEMA) degree of quaternization (QD); 60, 80 or 100% | SI-RAFT / Silica nanoparticles ø:100 nm | 60, 80,70 nm | 0.1 chain/nm | Percentages adsorbed protein;  QD60, BSA 38%, Lys 13%  QD80, BSA 6%, Lys 3%  QD100, BSA 0.5%, Lys 4% | - | 76 |
| Poly(2-(dimethylamino) ethyl methacrylate) (PDMAEMA) | SI-ATRP / Poly(divinyl benzene-*co*-chloromethyl styrene) (P(DVB-co-CMS)) (16.5 nm) coated magnetic iron oxide nanoparticles  ø:235 nm | 5 nm | High | BSA, 665 mg/g | - | 77 |
| Poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) | SI-ARGET-ATRP / Au nanoparticles ø:10 nm | 65 nm | High | C-reactive protein, 50 ng/mL | - | 78 |
| Poly(2-(methacryloyloxy)ethyl succinate) (PMES) | SI-ATRP / Au coated silicon wafer | 55 nm | High | Lysozyme, 14.4 µg/cm2 | - | 79 |
| SI-ATRP / Porous nylon membranes 1.2 and 5 µm pores | - | High | Lysozyme, up to 118 mg/cm3 (1.2 µm pores) and lysozyme from egg white up to 120 mg/cm3 (5 µm pores) | - | 80 |

One example of a polymer used for the adsorption of proteins using electrostatic interaction is poly(acrylic acid) (PAA). At pH values above 4.5, PAA is negatively charged and can interact with positively charged proteins. The isoelectric point of lysozyme is 11 and below this pH this protein is positively charged. Singh *et al*. showed that PAA grafted cellulose membranes at a pH of 7 were able to adsorb efficiently lysozyme.74 The lysozyme binding capacity of the membranes was found to increase with the reaction time used to produce the PAA brush coating. Maximum binding capacities were obtained with a polymerization time of 1 hour and were 98.5 mg/mL (static) respectively 71.2 mg/mL (dynamic). Hoven and co-workers used SPR to study binding of lysozyme (Lys), streptavidin (SA), BSA and fibrinogen (Fib) on PAA brushes at pH 7.26 Depending on the grafting density, around 9 to 24 pmol/cm2 Lys was bound, approximately 10-20 times more as compared to the other proteins, which were all negatively charged at pH 7.4. Baker, Bruening and co-workers developed an alternative carboxylic acid side chain functional protein binding brush interface, which was obtained via SI-ATRP of (2-methacryloyloxy)ethyl succinate (MES).79 Gold coated silicon wafers modified with a 55 nm PMES brush were able to bind the equivalent of ~70 monolayers of Lys. Application of these PMES brushes on porous nylon membranes allowed to bind 118 ± 8 mg/cm3 Lys.80



Figure 6. Schematic illustration of the selective non-covalent binding of C-reactive protein to PMPC brush modified gold nanoparticles. (Reproduced from ref. 78 with permission from the American Chemical Society).

In many biosensing experiments, it is crucial to maintain the structure and activity of the immobilized proteins. Poly(methacryloyloxyethyl phosphorylcholine-*b*-amino ethyl methacrylate) (PMPC-*b*-PAEMA) diblock copolymer and PAEMA homopolymer brushes have been shown to efficiently immobilize anti-HSA antibody.64 At pH 8, the adsorption of the antibody on the PAEMA homopolymer brush was slightly higher as compared to the PMPC-*b*-PAEMA diblock copolymer brush, but much higher as compared to covalent binding on poly(methacryloyl succinimide) (PMSu) brushes. The authors also found that protein structure and activity were least affected when PMPC-*b*-PAEMA diblock brushes instead of PAEMA homopolymer brushes were used.

Chen *et al.* investigated protein adsorption onto poly((dimethylamino)ethyl methacrylate) (PDMAEMA) brush modified silica nanoparticles, which were obtained via RAFT polymerization.76 By controlled post-polymerization modification with propiolactone to introduce carboxybetaine moieties it was possible to endow the nanoparticles with tunable adsorption toward both acidic and basic proteins.

Electrostatic interactions are generally nonspecific. An interesting exception is the binding of C-reactive protein (CRP) to phosphorylcholine groups.81,82 PMPC brushes are well-known to very efficiently prevent non-specific protein adsorption, but enable the selective binding of CRP in presence of Ca2+ ions. Using LSPR (localized surface plasmon resonance), Kitayama *et al.* showed that the limit of detection for the binding of CRP on PMPC brush grafted gold nanoparticles was around 50 ng/mL, which is comparable to antibody-based CRP sensors currently used (Figure 6).78 The affinity of CRP to PMPC layers in presence of calcium ions remained high even when in presence of diluted human serum albumin (HSA).

Biotin-streptavidin interactions.

While the use of electrostatic interactions represents a very effective strategy to immobilize proteins on polymer brushes, this approach generally does not allow selective binding of specific proteins. One possibility to selectively immobilize protein is to use biotin-streptavidin interactions. A variety of well-established protocols are available that can be used to derivatize specific functional groups in proteins with biotin moieties.83 Table 7 summarizes examples of biotin functionalized polymer brushes that have been prepared via SI-CRP.

**Table 7**. Overview of polymer brushes, which have been used for the non-covalent immobilization of proteins using biotin – streptavidin interactions.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration)** | **Secondary interaction** | **Ref.** |
| Poly(acrylic acid) (PAA)  X:\Review\Pictures\Poly(AA) with biotin structure.tif | SI-ATRP / Silicon wafer, nanoparticles or Au coated SPR chips | 9.5 nm | 0.32 chains/nm2 | FITC-streptavidin, 0.7342 µg/cm2 | - | 84 |
| Poly(acrylic acid) (PAA)  X:\Review\Pictures\Poly(AA) with biotin.tif | SI-ATRP / Au coated glass slides | 9.5 nm  (PtBA) | 10, 50 or 100 mol% initiator; 0.21 chains/nm2 for 100 mol% | 0.5 µg/cm2 biotin binds ~0.8 µg/cm2 streptavidin | - | 26 |
| Poly(oligo(ethylene glycol) methacrylate) (POEGMA) | SI-ATRP / Au | 4 nm | High | Streptavidin, 0.6485 µg/cm2 | - | 85 |
| SI-ATRP / Au | Up to 31 nm depending on polymerization time and grafting density (Reaction times 15, 30 and 90 minutes) | 20, 40, 60, 80 or 100 mol% ATRP initiator | Thickness of the brushes with immobilized streptavidin  increased with 4-5 nm | - | 86 |
| Poly(oligo(ethylene glycol) methacrylate) (POEGMA) | SI-ATRP / Au | 30 nm | High | Streptavidin, 0.46 µg/cm2 | - | 47 |
| Poly(oligo(ethylene glycol) methacrylate) (POEGMA) | SI-ATRP / Silicon wafer | 100 nm | High | Labelled streptavidin | - | 87 |
| Poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMeMA) | SI-ARGET-ATRP / Au | 70 nm | High | Streptavidin, fluorescence intensity | - | 88 |
| Poly(oligo(ethylene glycol) methacrylate) (POEGMA) (functionalization with aminoethanol and biotin amine (1:1) | SI-ATRP / Polydopamine coated magnetic iron oxide nanoparticles ø:20 nm | - | High | Streptavidin, fluorescence intensity | - | 89 |
| Poly(oligo(ethylene glycol) methyl ether methacrylate–*b*-oligo(ethylene glycol) methacrylate) (POEGMeMA–*b*-POEGMA) | SI-ATRP / Au | 1st Block 15 nm  2nd block 20 nm | High | Streptavidin, 0.021 µg/cm2 | - | 90 |
| Poly(oligo(ethylene glycol) methyl ether methacrylate–*b*-glycidyl methacrylate) (POEGMeMA-*b*-PGMA) | SI-ATRP / Au | PMeOEGMA 20 nm  PGMA 6 or 20 nm | High | Streptavidin, 0.28 µg/cm2 (6 nm PGMA) or 0.27 µg/cm2 (20 nm PGMA) | - | 91 |
| Poly(oligo(ethylene glycol) methyl ether methacrylate–*b*-glycidyl methacrylate) (POEGMeMA-*b*-PGMA) | SI-ATRP / Au | 1st block 20 nm (PMeOEGMA) and 2nd block up to ~40 nm (PGMA) | High | Streptavidin, 0.14 µg/cm2 | - | 91 |
| Poly(sulfobetaine methacrylate) (PSBMA) | SI-ATRP / Indium-tin oxide | 20 nm | High | Fluorescent labelled streptavidin, fluorescence measurement | - | 92 |

Akkahat *et al.* prepared biotin functionalized brushes with various grafting densities by post-polymerization modification of PAA brushes with biotin-NH2.26 Protein binding on these brushes was studied using SPR. The biotin functionalized PAA brushes were very effective in binding SA and at the same time resisted non-specific binding of proteins such as bovine serum albumin (BSA) or fibrinogen (Fib). PAA brushes with a grafting density of 50% were found to possess the highest SA binding capacity. This may be due to steric hindrance at higher grafting densities, which limits the accessibility of the polymer brushes to the proteins. De Los Santos *et al*. used the photomediated nitrile imine-mediated tetrazole-ene cycloaddition (NITEC) to attach a maleimide functionalized biotin derivative onto tetrazole modified poly(oligo(ethylene glycol) methyl ether methacrylate)-*b*-oligo(ethylene glycol) methacrylate) (POEGMeMA-*b*-POEGMA) diblock brushes.90 The use of this photomediated post-polymerization modification protocol is attractive as it provides spatial control over the presentation of the biotin moieties. These brushes were able to bind up to 21 ng/cm2 streptavidin. In another article, the authors used catalyst-free strain-promoted alkyne-azide cycloaddition (SPAAC) to introduce biotin moieties either at the chain end of POEGMeMA brushes or into the side chains of poly(OEGMeMA-*b*-GMA) diblock brushes.91 While the chain end functionalized polymer brushes only showed a very low streptavidin immobilization capacity, diblock copolymer brushes modified with DBCO-biotin (dibenzocyclooctyne) were able to bind an amount of streptavidin, which corresponds to 83% of a protein monolayer. Benetti et al. combined UV-assisted imprint lithography and SI-ATRP to generate nanostructured PPEGMA brushes that were composed of line pattern with line widths of 520 to 80 nm or pillars with diameter of 130, 140 or 200 nm. The hydroxyl side chain functional groups of these patterned brushes were modified with biotin-PEG (10) NH2 to allow the immobilization of streptavidin.87

Metal affinity interactions.

Metal affinity interactions represent another powerful approach for the non-covalent immobilization of proteins. In particular complexes of nitrilotriacetate (NTA) with Ni2+ or Cu2+ are widely used as they are able to selectively bind to short oligohistidine sequences. Amongst other advantages that are characteristic of this non-covalent protein immobilization strategy, the high selectivity of the NTA-Ni2+ or NTA-Cu2+ interaction is attractive as it allows to control the orientation of the bound proteins.93 Table 8 provides an overview of polymer brushes prepared via SI-CRP that have been used to immobilize proteins via metal-affinity interactions.

**Table 8**. Overview of polymer brushes, which have been used for the non-covalent immobilization of proteins using metal affinity interactions.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration)** | **Secondary interaction** | **Ref.** |
| Poly(acrylic acid) (PAA) | SI-ATRP / Au | 55 nm | High | BSA, 5.1 to 6.7 µg/cm2, lysozyme, 3.3 µg/cm2, myoglobin, 7.7 µg/cm2 and anti-IgG, 9.6 µg/cm2 | - | 27 |
| Poly(2-hydroxyethyl methacrylate) (PHEMA) | SI-ATRP / Silica coated magnetic nanoparticles ø:43 + 8 nm | 50 nm | High | NTA-Cu2+; BSA, 220 mg/g  NTA-Ni2+; HisU 245 mg/g and HisCRALBP | - | 94 |
| Poly(methacrylic acid) (PMAA) | SI-ATRP / Silicon nitride | 177 nm | High | His-tagged green fluorescent protein and His-mCherry-NaChBac | - | 95 |
| Poly(2-(methacryloyloxy)ethyl succinate) (PMES) | SI-ATRP / Au | 55 or 85 nm | High | NTA-Cu2+; BSA, 6.8 µg/cm2 (55 nm) and 7.2 µg/cm2 (85 nm) | - | 79 |
| SI-ATRP / Porous nylon membrane (pore sizes 1.2 and 5 µm) | - | High | NTA-Cu2+; BSA, up to 82 mg/cm3 (1.2 µm pores) | - | 80 |
| SI-ATRP / Porous nylon membrane (pore sizes 1.2 and 5 µm) | - | High | NTA-Ni2+; BSA, up to 55 mg/cm3 (1.2 µm pores), His tagged Ubiquitin, 60 mg/cm3 and lysozyme 100 mg/cm3 | - | 80 |
| SI-ATRP / Porous alumina membrane | 51 nm | 1 g/cm3 PHEMA | NTA-Ni2+; His-tagged Ubiquitin, 120 mg/cm3 | - | 96 |
| SI-ATRP / Porous alumina membrane | 51 nm | 1 g/cm3 PHEMA | NTA-Cu2+; BSA, 110 mg/cm3 and myoglobin, 83 mg/cm3 | - | 96 |

Xu *et al*. investigated the binding of His-tagged proteins on magnetic nanoparticles that were modified with an NTA-Ni2+ functionalized PHEMA brush coating. These NTA functionalized brushes were prepared by SI-ATRP of HEMA, followed by post-polymerization modification of the hydroxyl side chain functional groups with succinic anhydride and aminobutyl NTA. Ni2+-NTA PHEMA brush coated nanoparticles were able to bind 220 and 245 mg BSA and His-tagged ubiquitin(HisU)/g of particle, which was 5.5 to 200 times more than the binding capacity of several commercially available particles that were studied for comparison. In an earlier study, these researchers had reported the effect of film thickness of these polymer brushes on the HisU binding capacity.96 It was found that the protein binding capacity increased with increasing film thickness and reached a plateau for brushes with a thickness of 115 nm. NTA-Ni2+ functionalized PHEMA brushes of this thickness were able to bind the equivalent of about 23 monolayers of HisU.

Carbohydrate - protein interactions.

Carbohydrate-protein interactions provide an alternative non-covalent strategy to selectively immobilize proteins.97,98,99 Table 9 summarizes examples of carbohydrate-functionalized polymer brushes prepared via SI-CRP, which were used to non-covalently bind proteins.

**Table 9**. Overview of side chain carbohydrate functional polymer brushes, which have been used for non-covalent immobilization of proteins using carbohydrate- protein interactions.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Polymer** | **Polymerization method / surface** | **Film thickness [nm]** | **Grafting density** | **Protein (highest protein concentration)** | **Secondary interaction** | **Ref.** |
| Poly(2-O-(*N* -acetyl-β-D-glucosamine)ethyl methacrylate) (PGlcNAcEMA**)** | SI-ATRP / Silicon wafer | 5.2 nm | High | Lectins: Bandeiraea Simplicifolia GS-II and Erythrina Cristagalli Lectin (ECL) | - | 100 |
| Poly(2´-acrylamidoethyl-α-D-mannopyranoside) (PAAEM) | SI-ATRP / Silicon wafer | 41.5 nm | 0.16 chains/nm2 | Concanavalin A | - | 101 |
| Poly(2**´**-acrylamidoethyl-*β*-D-  glucopyranoside) (PAAEGlc) | SI-ATRP / Silicon wafer | 42.5 nm | 0.16 chains/nm2 | Concanavalin A | - | 101 |
| Poly(2**´**-acrylamidoethyl-*β*-D-  Galactopyranoside) (PAAEGal) | SI-ATRP / Silicon wafer | 42.1 nm | 0.12 chains/nm2 | Concanavalin A | - | 101 |
| Poly(2´-acrylamidoethyl-β-D-galactopyranoside–*co*-(2´-acrylamidoethyl)-α-D-mannopyranoside) (PAEEGal-*co*-PAEEM) | SI-ATRP / Au | Thickness up to 24.4 nm, amount mannose between 0 and 100% | Grafting density from 0.006 to 0.1 chains/nm2 | Concanavalin A | - | 102 |
| Poly(D-gluconamidoethyl methacrylate) (PGAMA) | SI-ATRP / Au | Up to 63 nm | From 0.5 to 0.9 chains/nm2 | Concanavalin A and ricinus communis agglutinin (RCA120) | - | 99 |
| Poly(D-gluconamidoethyl  acrylamide) (PGAA) | SI-ATRP / Silicon wafer | 37.6 nm | 0.3 chains/nm2 | Concanavalin A | - | 101 |
| Poly(2-hydroxyethyl methacrylate–*co*-(2-lactobionamidoethyl) methacrylate) (PHEMA-*co*-PLAMA)  40 to 100% LAMA | SI-ATRP / Au | ~ 4 to 12 nm | 40, 60, 80 and 100% ATRP initiator | Ricinus communis agglutinin (RCA120) | - | 103 |
| Poly(N-isopropyl acrylamide–*co*-(2-lactobionamidoethyl) methacrylate) (PNIPAM-*co*-PLAMA) | SI-ATRP / Glass | 6.8, 7.3 and 7.6 nm for 1.1, 5.2 and 9.1 mol% LAMA | 0.32 to 0.39 chains/nm2 | FITC conjugated RCA120 and biotinylated ricinus communis agglutinin (RCA120) | - | 104 |
| Poly(N-isopropyl acrylamide–*b*-(2-lactobionamidoethyl) methacrylate) (PNIPAM-*b*-PLAMA) | SI-ATRP / Glass | PLAMA 8.2 nm | 0.33 chains/nm2 | FITC conjugated RCA120 and biotinylated ricinus communis agglutinin | - | 104 |
| Poly(2-lactobionamidoethyl methacrylate) (PLAMA) | SI-ATRP / Au | Up to ~ 30 nm (100% ATRP initiator) | 10, 40, 60 or 100% ATRP initiator | Ricinus communis agglutinin (RCA120) | - | 103 |
| SI-ATRP / Au | Up to 40 nm | From 0.5 to 1.2 chains/nm2 | Concanavalin A and ricinus communis agglutinin (RCA120) | - | 99 |
| SI-ATRP / Track-edged PET membrane | Up to 45.2 nm | High | Peanut agglutinin (PNA);  Static, up to 7.7 mg/cm3 and dynamic, up to 8.4 mg/cm3 | - | 105 |
| Comb-like poly(2-lactobionamidoethyl methacrylate) (PLAMA) | SI-ATRP / Track-edged PET membrane | PHEMA up to 45.2 nm  + PLAMA up to 147.8 nm | High | PNA; Static up to 11.4 mg/cm3 and dynamic up to 23.6 mg/cm3 | - | 105 |
| Poly(3-*O*-methacryloyl-D-  glucofuranose) (PMAGlc) | SI-ATRP / Silicon wafer | 30.5 nm | 0.25 chains/nm2 | Concanavalin A | - | 101 |

In an attempt to explore carbohydrate-protein interactions for the immobilization of proteins on polymer brush surfaces, Yu and Kizhakkedathu prepared three different glycopolymer brushes by direct SI-ATRP of the corresponding acrylamide functionalized pyranosides (Figure 7).101 Poly(2´-acrylamidoethyl-*β*-D-glucopyranoside) (PAAEGlc) brushes showed very low levels of non-specific BSA and fibrinogen adsorption while retaining their ability to selectively bind concanavalin A (ConA). Yang and Ulbricht grafted linear and comb-like poly(2-lactobionamidoethyl methacrylate) (PLAMA) brushes from PET membranes surfaces by ATRP.105 Both brush surfaces were effective in preventing non-specific binding of BSA. These glycopolymer brushes, however, provide a high binding capacity interface for the selective immobilization of peanut agglutinin (PNA). For the comb-like PLAMA brushes a PNA binding capacity of up to 23.6 mg/cm3 was achieved.

X:\Review\Pictures\Figure 7.tif

Figure 7. Preparation of carbohydrate containing side chain functional polymer brushes via direct polymerization of the unprotected carbohydrate monomer.

Conclusions.

Surface-initiated, controlled radical polymerization techniques are powerful tools to generate polymer brush films consisting of chain-end tethered polymers with well-controlled molecular weights, architectures, composition and grafting density. This article has presented the use of surface-initiated controlled radical polymerization techniques to generate polymer brushes that can serve as the protein-binding interface in protein biosensors and biochips. Main focus of this article has been on the chemical approaches that have been used to bind proteins to polymer brushes. To this end, the article was subdivided in two parts, which presented covalent, respectively, non-covalent protein immobilization chemistries. For each of these strategies, a variety of specific polymer brush chemistries was presented and discussed together with details regarding the protein binding capabilities of these films. The examples highlighted in this article underline the potential of surface-initiated controlled polymerization reactions for applications in protein sensor and biochip technologies. By judicious engineering of film thickness, polymer architecture, grafting density and brush chemistry, protein binding can be tailored towards the biosensor or biochip configuration of interest. The current emergence of alternative controlled radical polymerization techniques in particular metal free and/or photomediated ATRP and RAFT procedures will provide access to polymer brush films under conditions that are less susceptible to oxygen and which can be uniformly produced over large surfaces, which further aids the practical use of these chemistries for sensing and diagnostic applications.

Conflicts of interest

The authors declare that the writing of this review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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X:\Review\Pictures\Graphical table of content III.tifThis article discusses protein-binding polymer brushes and the various strategies that can be used to immobilize proteins on these films.