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Photopolymerizable zwitterionic polymer patterns control cell adhesion and guide neural growth.

Braden L. Leigh¹, Elise Cheng², Xu Linjing², Corinne Andresen¹, Marlan R. Hansen^{2,3}, and C. Allan Guymon¹

¹Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA 52242, USA.

²Department of Otolaryngology Head and Neck Surgery, University of Iowa, Iowa City, IA 52242, USA.

³Department of Neurosurgery, University of Iowa, Iowa City, IA 52242, USA.

Abstract

Developing materials that reduce or eliminate fibrosis encapsulation of neural prosthetic implants could significantly enhance implant fidelity by improving the tissue/electrode array interface. Here, we report on the photografting and patterning of two zwitterionic materials, sulfobetaine methacrylate (SBMA) and carboxybetaine methacrylate (CBMA) for controlling the adhesion and directionality of cells relevant to neural prosthetics. CBMA and SBMA polymers were photopolymerized and grafted on glass surfaces then characterized by X-ray photoelectron spectroscopy, water contact angle, and protein adsorption. Micropatterned surfaces were fabricated with alternating zwitterionic and uncoated bands. Fibroblasts, cells prevalent in fibrotic tissue, almost exclusively migrate and grow on uncoated bands with little to no cells present on zwitterionic bands, especially for CBMA-coated surfaces. Astrocytes and Schwann cells showed similarly low levels of cell adhesion and morphology changes when cultured on zwitterionic surfaces. Additionally, Schwann cells and inner ear spiral ganglion neuron neurites aligned well to zwitterionic patterns.

Keywords

neural prosthetic; zwitterionic polymer; foreign body response; photopolymerization; neural regeneration

Introduction

The foreign body reaction is observed after virtually all medical device implantation.¹ In many cases, particularly for neural implants such as cochlear implants and deep brain or cortical electrodes, encapsulation by fibrotic tissue compromises the function of the implant.

Corresponding Author: C. Allan Guymon Tel.: +1 319 335 5015; fax: +1 319 335 1415. allan-guymon@uiowa.edu, Mailing Address, 4133 Seamans Center, Iowa City, Iowa, 52242.

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In some cases, fibrotic tissue formation also interferes with the normal function of the surrounding native tissue.² A number of different approaches to reducing foreign body response have been investigated in recent years with many focusing on using glucocorticoids to modulate the immune and/or inflammatory response.^{3,4} These drugs have been used systemically for many years and have more recently been used in a number of topical and local controlled-release formulations.^{3,5} Other immunomodulatory drugs have also been studied in control-release formulations, such as IL-1 antagonist.⁶ However, the drawback to all drug-eluting systems is that eventually the drug will deplete, which could potentially allow for a delayed foreign body response.

Nonspecific protein adsorption to implant surfaces often trigger the foreign body response, i.e. the inflammatory response evoked by the presence of a foreign object in host tissue.^{1,7,8} Macrophages recognize these nonspecifically-protein labeled implants as foreign, attach to the surface, and attempt ingestion. Because the implant is large and not biodegradable, these macrophages fuse together and form foreign body giant cells, which secrete inflammatory cytokines and induce fibroblast adhesion.⁹⁻¹¹ After about three weeks of implantation, adhered fibroblasts fuse together to form an avascular collagenous capsule around the implant which permanently isolates the device and renders the implant less effective or even nonfunctional.¹⁰

Engineering materials that resist nonspecific protein adsorption, the theorized primary step in the foreign body response,^{1,7,8} provide an alternative approach to drug treatment. The most commonly investigated materials to achieve this goal are hydrophilic polymers such as poly(ethylene glycol) (PEG) or poly(2-hydroxyethyl methacrylate) (HEMA). However, both only partially reduce nonspecific protein adsorption and PEG is additionally subject to oxidative degradation *in vivo*.¹⁰ More recently, zwitterionic polymers, or net neutral polymers containing positive and negative charges on the same repeat unit, have emerged as excellent alternatives.^{12,13} It has been proposed that the proximity of intramolecular positive and negative charges strongly structures surrounding water molecules in a hydrogen-bonded network, making adsorption of any other molecules energetically unfavorable.¹⁴ Among these polymers, carboxybetaine methacrylate (CBMA), sulfobetaine methacrylate (SBMA), and phosphorylcholine chemistries have proven effective as ultra-low-fouling materials (Figure 1).¹⁵ For example, Jiang et al. reported both CBMA and SBMA polymer brushes grown on gold surfaces demonstrated ultra-low nonspecific protein adsorption (0.4 and 9.1 ng/cm² respectively) from 100% human plasma.^{15,16} Additionally, CBMA and SBMA brushes dramatically reduce cell adhesion and biofilm formation when compared to unmodified surfaces.^{12,17,18} Therefore, great interest has developed in the use of zwitterionic materials in implantable and insertable devices, including urinary catheters and intravascular devices.^{19,20} CBMA and SBMA materials have also been examined in a wide range of applications including glucose sensing in 100% blood serum,^{21,22} antigen detection in complex media,²³ and prolonging the blood circulation time of coated drugs²⁴ among others.^{25,26} Most important to biomedical implant research, promising results have been reported in animal models showing that a bulk CBMA hydrogel inhibits fibrotic capsule formation,¹³ which demonstrates the ability of CBMA materials to not only prevent protein adsorption *in vitro*, but also fibrosis *in vivo* where many other materials with low nonspecific protein adsorption fail.

Methods to synthesize zwitterionic polymer brushes have focused largely on surface initiated atom transfer radical polymerization (ATRP), which utilizes a copper bromide catalyst.^{27,28} This technique provides precise control over molecular chain length (molecular weight) and molecular weight distribution. Alternatively, photopolymerization offers a unique set of advantages over conventional polymerization methods in biomaterials fabrication. Among these advantages are precise spatial control over the rate of polymerization and mild reaction conditions (i.e. aqueous and ambient).²⁹ Photopolymerization initiates only in areas exposed to a light. Accordingly, patterns can be generated using a photomask with features on the same size scale as cell bodies.^{30,31}

Neural prosthetic devices, such as the cochlear implant or developing retinal implant, rely on communication between stimulating elements and primary neural receptors.³² The effectiveness of these devices is not only reduced by fibrous tissue on the surface, but also by the distance the electrode current must traverse in order to stimulate the neural cells.^{33–35} Because of this separation, nonspecific excitation of adjacent neural cells, induced by current spread, significantly decreases stimulation specificity. Consequently, neural prosthetics fail to effectively relay the intricate information required for a high-fidelity device. Strategies to close this gap have focused largely on directing the regeneration of proximate neurites toward stimulating elements to narrow this distance or even contact electrode arrays.³⁶ Recent advances in microfabrication technology have allowed for a variety of approaches to direct neural processes including aligned microfibers,^{37,38} parallel micro- and nano-channel topography,^{30,39–42} chemo-attractive gradients,^{43–45} electrical fields,^{46,47} oriented glial cells,⁴⁸ biomolecular patterning,⁴⁹ and chemo-repellents.^{50–52} Many of these studies have focused on elucidating the fundamental mechanisms behind neurite pathfinding to improve the tissue/prosthetic interface. The chemo-repulsive properties of zwitterionic surfaces could potentially be used to guide neural cell in addition to reducing the foreign body response.⁵³

In this work, we aim to develop materials that could improve the interface between biomaterials and neural prosthetics. Our approach is to develop patterned zwitterionic surfaces using photopolymerization to resist cell adhesion and fibrosis and control the outgrowth of neural cells among other cell types. To the best of our knowledge, this research represents the first use of a patterned zwitterionic substrate to control the adhesion and direct the growth of several cell types relevant to implant materials, including neuronal cells. We demonstrate that CBMA and SBMA polymers can be covalently grafted and patterned from glass surfaces via a one-step photopolymerization reaction. Surface coverage with the zwitterionic polymer is investigated by varying the solution concentration of the CBMA and SBMA monomers to generate surfaces with variable levels of atomic composition and protein adsorption. Due to the inherent spatial control afforded by the photopolymerization reaction, parallel patterned substrates with 100 μm periodicity were fabricated and used to study the ability of zwitterionic stripes to locally control cell adhesion. Fibroblasts, the cell type primarily comprising fibrotic tissue found on the surface of biomedical implants,^{1,9} were grown on these substrates to understand their efficiency in preventing cell attachment and fibrotic tissue formation. Alignment behavior of spiral ganglion neuron (SGN) neurites, the cells responsible for transmission of hearing response to the brain, to CBMA and SBMA stripes was evaluated to understand the ability of these patterns in directing neural cells. The

alignment of Schwann cells was also tested due to the supportive role they play in neural regeneration.^{54,55} Additionally, astrocytes were cultured to evaluate the effectiveness of these surfaces in repelling central glial cells that encapsulate brain and spinal cord prostheses.^{56,57} These findings provide fundamental understanding of the biomaterial/cell interface and lay the framework for next generation neural prosthetic devices.

Experimental

Materials and synthesis

2-(N,N'-dimethylamino)ethyl methacrylate (DMAEM), β -propiolactone, triethyl amine, hydroquinone, [2-(Methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (SBMA), mouse anti-CD20, rabbit anti-S100 β , mouse anti-NF200, 3-(trimethoxysilyl)propyl methacrylate, paraformaldehyde, collagenase, dulbecco's modified eagle medium (DMEM), neurotrophin-3 (NT3), insulin, human fibrinogen, human anti-fibrinogen, and all organic solvents were purchased from Sigma Aldrich. Rabbit anti-vimentin and rabbit anti-glial fibrillary acidic protein (GFAP) antibodies were purchased from Abcam (Cambridge, MA). Hank's balanced salt solution (HBS), fetal bovine serum (FBS), poly-L-ornithine, trypsin-EDTA dissociation reagent, DAPI-containing mounting medium, were purchased from Gibco (Carlsbad, CA). Laminin was purchased from Labtek (Campbell, CA). Brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were purchased from R&D systems (Minneapolis, MN).

2-Carboxy-N,N-dimethyl-N-(2'-(methacryloyloxy)ethyl)ethanaminium inner salt (carboxybetaine methacrylate, CBMA) was synthesized as previously described.⁵³ Briefly, DMAEM was dissolved in acetone and cooled to 0° C. β -propiolactone was dissolved in acetone and added to the DMAEM solution dropwise under inert atmosphere. The reaction was stirred overnight after which 10 mg of hydroquinone inhibitor was added and the solvent was removed. The residual oil was dissolved in methanol and trimethylamine was added to quench any side reactions. The solution was then precipitated into chilled diethyl ether and filtered to yield a white solid. The product was dried under vacuum and used without further purification. The NMR spectrum was recorded on a Bucker spectrometer (Avance 300). ¹H NMR (D₂O, 300 MHz), δ 6.06 (s, 1H, =CH), δ 5.68 (s, 1H, =CH), δ 4.55 (t, 2H, OCH₃), δ 3.70 (t, 2H, NCH₂), δ 3.59 (t, 2H, NCH₂), δ 3.10 (s, 6H, NCH₃), δ 2.64 (t, 2H CH₂COO), δ 1.84 (s, 3H =CCH₃).

Zwitterion-functionalized glass substrate fabrication and characterization

Standard 2.54 $\times$ 7.62 cm glass microscope slides were functionalized with a 3-(trimethoxysilyl)propyl methacrylate (silane coupling agent) as previously described⁵⁸ to allow covalent grafting of zwitterionic polymers from glass surfaces. Zwitterionic polymers were grafted from glass surfaces represented schematically in Figure 2. Aqueous monomer mixtures of zwitterion (CBMA or SBMA at 0.1, to 20 wt%) in phosphate buffered saline (PBS) containing 0.05 wt% 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (HEPK, photoinitiator) were pipetted onto activated glass surfaces. The liquid was then dispersed evenly across the sample using a 2.54 $\times$ 2.54 cm glass-chrome Ronchi rule photomask for patterned samples or a cut glass slide of the same dimensions for

uniform samples. Substrates were subsequently illuminated for 10 min at 16 mW/cm² measured at 365 nm wavelength using a mercury vapor arc lamp (Omniculture S1500, Lumen Dynamics, Ontario, Canada). Samples were then copiously washed with distilled H₂O and dried using a nitrogen stream, then kept in a sealed container until use.

X-ray Photoelectron Spectroscopy (XPS) analysis was performed using Kratos Axis Ultra x-ray photoelectron spectrometer with concentric hemispherical electron energy analyzers combined with the established delay-line detector. The incident radiation monochromatic Al K α X-ray (1486.6 eV) at 150 W (accelerating voltage 15 kV, emission current 15 mA) was projected 45° to the sample surface and the photoelectron data was collected at a takeoff angle of $q = 90^\circ$. Survey scans were taken at a pass energy of 160 eV, and carried out over a 1200 eV $\sim$ 5 eV binding energy range with 1.0 eV steps and a dwell time of 200 ms. All spectral analyses were conducted using CasaXPS software. Shirley type calculations were used to account for the background spectrum when quantifying percent atom composition.⁵⁹ Relative sensitivity factors were used to normalize peak intensity for elemental quantification. The C 1s peak at 285.0 eV was used to calibrate all spectra before data analysis.

Water contact angles were measured using the sessile drop method on a Ramé-Hart Model 190 Goniometer. Five areas of each substrate were tested in triplicate and the average contact angle was taken as the mean value of all fifteen measurements.

Protein adsorption

Immunofluorescence was used to measure protein adsorption to substrates. The glass samples were first incubated in PBS (room temperature) for 30 minutes prior to protein exposure. Human fibrinogen (1 mg/mL) was pipetted onto substrates and dispersed by placing a glass coverslip on the solution followed by a 1 hour incubation at room temperature. The samples were then soaked in PBS for at least three hours with the PBS being changed every hour. After the rinsing step, the substrates were incubated in paraformaldehyde (4%) for 15 min at 2° C. The samples were then rinsed three times with PBS followed by a blocking buffer solution being applied to block the areas unoccupied by fibrinogen for 30 min at room temperature. A horseradish peroxidase conjugated anti-fibrinogen antibody was applied to the samples overnight at 2° C. The substrates were then rinsed 3 times with PBS and the secondary antibody was applied for one hour at room temperature. The samples were washed three times with PBS and coverslips were applied before epifluorescent imaging. Digital epifluorescent images were captured on a Leica DMIRE2 microscope (Leica Microsystems, Bannockburn, IL) with Leica DFC350FX digital camera and Metamorph software (Molecular Devices, Silicon Valley, CA). Images were taken at each sample condition and gray-scale measurements were used to evaluate relative fluorescence intensity in Image J software (NIH, Bethesda, MD). All samples were made in triplicate and five representative images were taken for each condition.

Cell culture and immunofluorescence

Dissociated spiral ganglion (SG) culture from p4–7 perinatal rat cochleae were performed as previously described.^{60,61} Dissociated fibroblast cultures were obtained from p4–7 perinatal

rat skin. The tissue was scraped to remove subcutaneous fat and digested in 0.125% trypsin with EDTA and 0.2% collagenase for one hour at 37°C followed by gentle trituration. Astrocyte and Schwann cell cultures were prepared and maintained from p4–7 perinatal rat cerebral cortex and sciatic nerve, respectively, as previously described.^{30,62,63} All cells were plated onto glass substrates without prior coating of protein.

SGN cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with N2 additives, 10% fetal bovine serum (FBS), NT-3, (50 ng/ml) and BDNF, (50 ng/ml). Fibroblast, Schwann cell, and astrocyte cultures were maintained in DMEM with 10% FBS. All cultures were fixed with 4% paraformaldehyde in PBS after 48 hours of cell culture before immunostaining.

Spiral ganglia cultures were immunostained with anti-neurofilament 200 (NF200) antibodies to label neurons and their neurites. Astrocytes and Schwann cell cultures were immunostained with anti-GFAP or anti-S100 antibodies, respectively. Fibroblast cultures were immunostained with anti-vimentin antibodies. Coverslips with DAPI-containing mounting medium were placed onto the stained cultures to label nuclei.

Quantification of cell density and alignment to micropatterns

Cell density was determined by counting the total number of nuclei of each cell type (identified by immunostaining as described above) from digital images of randomly selected 20X microscopic fields using the cell counting feature of MetaMorph software package. At least 10 fields were taken for each culture and each culture was before on at least 3 substrates per experiment. The experiments were repeated at least three times.

SGN total neurite length was determined from digital images captured using the scan slide feature in MetaMorph by measuring the longest process of randomly selected neurites from each condition using the measurement tool in Image J (NIH, Bethesda, MD) as previously described.⁶⁴ Neurite alignment was calculated as previously published by our group.^{58,65} Briefly, alignment to the pattern was defined as a ratio of total length per aligned length. The ratio is represented as $[T_L/A_L]$, where T_L is total neurite length and A_L represents aligned length in the pattern direction. Aligned length (A_L) was determined by measuring the distance from the neuronal cell body to the neurite terminus in a straight line in the direction of the micropattern. The pattern direction was always set horizontally prior to measurements. The total length to aligned length ratio is referred to as the alignment ratio throughout the text. A ratio close to one represents a neurite that closely follows the pattern along its entire length. A wandering neurite, which does not strongly align to the pattern would be represented by a high alignment ratio. Schwann cell orientation to the pattern was determined as previously described by drawing the outline of the cell using Image J software and fitting an ellipse to the cell outline.^{58,65} The angle made between the major axis of the ellipse and the pattern (θ) was measured in Image J as Schwann cell alignment.

Statistics

Statistical analysis was conducted using Graphpad Prism 7.01 software. To compare cell density between uncoated, CBMA-coated, and SBMA-coated and between striped groups on patterned substrates, a one-way ANOVA with *post hoc* Tukey test was used. A two-tailed t-

test was used to compare cell density between coated and uncoated regions on striped substrates. Analysis of alignment of Schwann cells and SGN neurites was conducted using a one-way ANOVA by a *post hoc* Kuskal-Wallis analysis of variance on ranks and a Dunn's test.

Results and Discussion

Zwitterion-functionalized substrate fabrication and characterization

Surface properties are a key component when engineering low fouling materials because the surface comes into direct contact with host fluid, blood protein, and other immunogenic factors. These biomolecules and their nonspecific adsorption to implant surfaces play an integral role in fibrous tissue formation. A common approach to achieve nonfouling properties involves introducing functional groups onto the surface, which then act as anchors for polymer growth.²⁸ Glass was used as a proof of concept substrate because it can be easily modified with a variety of functional groups using silane coupling chemistry.⁶⁶ While glass has been used as a substrate for other low-fouling surfaces,⁵³ our system uses photopolymerization as a simple method to covalently graft and pattern zwitterionic polymers on the surface. Our systems were used to investigate adhesion of cell types relevant to neural regeneration (astrocytes, Schwann cell, and spiral ganglion neurons), which have not been previously studied on these zwitterionic-coated substrates, in addition to fibroblasts, critical in fibrotic tissue formation. To fabricate these substrates, glass samples were first exposed to oxygen plasma to generate hydroxyl surface groups followed by reaction with a silane-containing coupling agent incorporating a reactive methacrylate group. Zwitterionic methacrylates were then covalently attached to glass surface methacrylates by illuminating a monomer- and photoinitiator-containing solution in contact with the substrate. UV-light was used to induce the polymerization reaction and covalently build a grafted zwitterionic polymer from the substrate (Figure 2). During UV-illumination, radicals in the solution are generated from photoinitiating molecules absorbing light that dissociate into initiating species. These initiating radicals then react with methacrylates on the surface and in the solution to generate a propagating species that builds the zwitterion zwitterionic polymer from the surface of the glass slide. For our experiments, SBMA and CBMA (Figure 1) were used as zwitterionic monomers to alter the surface properties of functionalized glass substrates. The concentration of zwitterionic monomer in solution was varied between 0.1 wt% and 20 wt% to change surface coverage and probe the physicochemical properties of the antifouling polymer-grafted surface.

Grafting of the methacrylate silane coupling agent and zwitterionic SBMA and CBMA polymers was characterized, as shown in Figure 3, through X-ray photoelectron spectroscopy (XPS) analysis, which provides elemental composition of the first 5–10 nm of a surface. The carbon 1s peak, located at 285 eV, was used to confirm the grafting of the methacrylate reactive group. The silane-activated sample exhibited a much stronger band than the bare glass control at 285 eV, indicating higher carbon content at the surface as would be expected for a carbon-containing silane coupling agent (Figure 3A). The presence of the characteristic nitrogen 1s quaternary ammonium band found around 402 eV was used to confirm and quantify the successful photo-induced grafting of SBMA and CMBA

polymers from the glass. Both the bare glass and silanized glass controls exhibited no peak at 402 eV, while the SBMA- and CBMA-coated samples (20 wt% uniformly coated sample depicted) showed distinct peaks in this region (Figure 3A). Additionally, the sulfur 2s and 2p peaks located at 231 and 168 eV respectively were used to confirm the grafting of the SBMA polymer, with its inherent anionic sulfate functional group. The silicon 2s and 2p peaks located at 153 and 102 respectively were orders of magnitude lower in area for the 20 wt% samples confirming good surface coverage and thickness nearing the XPS depth penetration (5–10 nm).

To understand how the elemental composition of the surface changes as a function of SBMA and CBMA solution concentration, percent nitrogen was quantified using the 402 eV nitrogen peak of the XPS spectra (Figure 3B). At low SBMA solution concentrations (less than 1 wt%), the atomic nitrogen content is less than 1%, indicating low levels of grafting and surface coverage with the polymer. As the SBMA solution concentration increases, the atomic composition increases and approaches the theoretical maximum (5.6%) of a pure SBMA polymer, calculated using atomic percent. A similar trend was also observed in the CBMA-coated samples with slightly higher percent nitrogen due to the higher theoretical maximum (6.25% as calculated by atomic percent of the pure polymer). The nitrogen percent once again remains small at low concentrations then approaches the theoretical maximum at a solution concentration of 20 wt%. These observations imply that the surface coverage of the SBMA and CBMA polymers is strongly dependent on the concentration of monomer in the solution. Further, for both the SBMA- and CBMA-coated samples, the percent silicon decreased with increasing solution concentration of monomer and showed very low signal at higher concentrations.

These observations are consistent with increases in thickness with higher monomer solution concentrations. On the basis of the XPS data collected, it is estimated that the thickness of the polymer layers measured using all monomer solution concentrations is less than the penetration depth of XPS data collection. XPS only examines the atomic composition the first 5–10 nm of the surface, and thus it is possible that changes (a few nanometers) in film thickness and/or changes in surface coverage of zwitterionic polymer occur on the surface giving rise to changes in nitrogen percent. Film thicknesses are likely less than the penetration depth of XPS causing atomic composition of the glass (Si and O) to be evident in the spectrum even at higher solution concentrations. As the solution concentration of zwitterion increases a corresponding decrease in silicon and oxygen percent is observed. This decrease is caused by more zwitterionic polymer on the surface. With more zwitterionic monomers in solution, the probability of molecular collisions and reaction between methacrylate groups on the surface is increased before termination, thereby increasing the length of the polymer chains grafted from the surface. The longer polymer chain length induce greater coverage of zwitterion on the surface, increasing the percent nitrogen measured through XPS.

Surface grafting of a polymer or other molecule is accompanied by a corresponding change in surface properties including surface energy that can be evaluated using water contact angle measurements. Higher contact angles indicate a more hydrophobic surface as water avoids interaction with the surface. Conversely, low contact angles indicate hydrophilic

surfaces as water spreads to maximize interactions with the substrate. The silanized glass was relatively hydrophobic with a contact angle of over 57 degrees as would be expected given the nonpolar nature of the coupling agent used to functionalize the glass substrates. Conversely, zwitterionic polymers are hydrophilic, due to the presence of charged atoms along the polymer side chains and are expected to decrease contact angles. As shown in Figure 4A, the contact angles of the SBMA-coated samples decreased sharply with increasing SBMA wt% in solution. At higher concentrations, the contact angles remained unchanged at approximately 15 degrees. A similar trend was also observed above 1 wt% for the CBMA-functionalized substrates with the contact angle leveling around 11 degrees with higher concentrations (Figure 4B). The marked decrease in contact angle for both SBMA- and CBMA-coated surfaces indicates increases in hydrophilicity and further corroborates that the surface was successfully functionalized with the zwitterionic polymer. These decreases in contact angle are consistent with the results of an SBMA polymer grafted from silicon wafers using thermally-initiated polymerization.⁶⁷ The observation that the contact angle of the substrates remain constant at higher concentrations (Figure 4) implies that at a solution concentration of 1 wt% the surface has reached a maximal coverage: i.e. virtually all accessible methacrylate groups on the surface have reacted with the zwitterionic monomer. At lower concentrations, many of the reactive surface methacrylate groups are unreacted due to an insufficient number of zwitterions in the solutions available for functionalization. As solution concentrations increase above 1 wt%, the surface coverage of the glass does not change significantly evidenced by the relatively constant contact angle measurements. Water contact angles remain unchanged because the surface energy is directly related to the ability of the surface to bind water molecules which no longer changes once a sufficient grafting density has been reached. These findings further support the claim that changes in surface coverage could explain the variations in surface chemistry as measured by XPS for solution concentrations above 1 wt%.

With large changes in surface properties, it is reasonable to believe that the zwitterionic polymer will affect adsorption of molecules including proteins. As mentioned previously, the first step in foreign body response is nonspecific protein adsorption, such as would occur when a surface comes into contact with serum during implantation.^{1,10} Hydrophilic, net neutral surfaces such as zwitterionic polymers have been shown to effectively prevent the nonspecific adsorption of protein.¹⁵ Fibrinogen is of particular interest as adsorption would be expected to trigger an early foreign body response. We therefore quantified fibrinogen adsorption by first incubating substrates prepared from solutions of varying monomer concentration with a 1 mg/mL solution of fibrinogen in PBS. The surface was then fixed and stained with antibody to quantify the amount of protein (Figure 4). Five representative images were taken for each sample using epifluorescent microscopy followed by gray scale measurements to quantify levels of protein adsorption. The relative fluorescence is normalized to that from the glass control. As shown in Figure 4A, SBMA protein adsorption correlates to water contact angles with sharp decreases up to solution concentrations of 0.5 wt% SBMA. At higher concentrations, protein adsorption drops to values less than 10% of the level observed with the uncoated control. CBMA-coated substrates performed similar to that of the SBMA samples with protein adsorption correlating to the water contact angles (Figure 4B). The CBMA samples exhibited lower, although not statistically significant,

levels of protein adsorption reaching about 6% of that observed in the control. These observations suggest that, similar to the contact angle, a maximum in graft density is reached at low solution concentrations. The protein adsorption appeared to reach a minimum at slightly lower concentrations than observed with the contact angle (0.5 wt% compared to 1 wt%) implying that even less polymer is necessary to repel protein than to maximize surface energy. These results are consistent with those conducted by Jiang *et al.* who showed SBMA- and CBMA-coated gold surfaces resisted the nonspecific adsorption of fibrinogen equally well.¹⁶ The protein adsorption data, in combination with the XPS data, suggest that only a very thin film (a few nanometers) of the zwitterionic polymer film is necessary in order to achieve a significant decrease in protein adsorption and water contact angle.

To determine the ability of zwitterionic-functionalized glass to spatially control protein adsorption and direct cell growth, micropatterned substrates were fabricated with alternating zwitterion-coated and uncoated bands. Due to the inherent spatial control afforded by photopolymerization, areas of functionalization can be precisely defined through use of a photomask. In regions that are shielded from irradiation by the opaque bands of the photomask, the glass substrate remains chemically unchanged because no photoinitiating molecules are locally excited by photons. Conversely, in illuminated areas, absorption of photons by the photoinitiator induces polymerization causing covalent grafting of the polymer from the activated glass surface. Accordingly, alternating transparent and opaque parallel bands of with a periodicity of 100 μm (defined as the distance between repeating features in the pattern) were used in generating micropatterns of SBMA and CBMA polymers. Characterization of the micropatterns was determined by first adsorbing protein to the surface to the zwitterion-patterned surface followed by immunostaining to visualize areas with high protein adsorption. As described in the previous section, protein adsorption was greatly reduced by SBMA- and CBMA-coated surfaces. Representative images of both SBMA- and CBMA-striped substrates are shown in Figure 5. Green bands indicate areas where protein is adsorbed, as would occur on an unfunctionalized surface. Conversely, dark regions represent areas that have been successfully functionalized through photopolymerization, which result in significantly lower levels of protein adsorption. The width of the green bands precisely matches the width of the transparent and opaque bands of the photomask used to generate the zwitterionic pattern.

Fibroblast and astrocyte adhesion

Fibroblasts are the primary cell type that contribute to fibrosis on the surface of implanted devices.¹ Therefore, determining the effectiveness of these zwitterion-coated substrates in preventing adhesion of fibroblasts could serve as an effective *in vitro* measure of the ability of these materials to resist fibrosis. Accordingly, fibroblasts were seeded in equal densities onto uncoated (Figure 6A), uniformly-coated with SBMA (Figure 6B), coated with 100 μm periodicity SBMA stripes (image not shown), uniformly-coated CBMA (Figure 6C), and coated with 100 μm periodicity CBMA stripes (Figure 6D). Cell cultures were fixed after 48 hours followed by immunostaining and epifluorescent microscopy. Vimentin immunostaining revealed that the morphology of fibroblasts was markedly altered on coated surfaces. On uncoated glass, fibroblasts showed a characteristic elongated multipolar shape, while they showed fewer cytoplasmic extensions and exhibited a round morphology on

zwitterion-coated substrates (Figure 6A-C). Further, noticeably fewer cells adhered to CBMA-coated substrates compared to SBMA-coated substrates. Random images were analyzed and cell nuclei counts were performed to determine cell density. Analysis of fibroblast cell density on uniform substrates reveals a statistically significant reduction in adhered cells to SBMA-coated surfaces with less than half of the cell density when compared to uncoated glass. Very few fibroblasts adhered on CBMA-coated surfaces with less than one percent of the cell density as compared to the uncoated glass (Figure 6E). To test the stability of the SBMA- and CBMA-coated surfaces to extended culture, fibroblasts were seeded a second time after 48 hours and cultured for 24 more hours. These experiments showed consistency results from the 48 hour culture study and the results are shown in Figure S1.

Analysis of cell density on striped substrates showed a distinct preference for cell adhesion to uncoated glass compared to adjacent SBMA- or CBMA-coated stripes. A statistically significant difference in cell density was found between stripes coated with SBMA and uncoated bands with a nearly 40% reduction in fibroblast adhesion (Figure 6F). While reductions in cell density were seen on SBMA stripes, in many regions cells were found bridging across SBMA-coated bands (Figure S2). For CBMA-coated stripes, a distinct border is visible between coated and uncoated regions with fibroblasts growing up to the edge, but not extending into the coated regions. Remarkably, zero fibroblasts were observed adhered to CBMA stripes while uncoated regions had a similar cell density to the uncoated regions of the SBMA-striped substrates. A significant difference was found in cell density between SBMA and CBMA stripes, further corroborating the effectiveness of CBMA over SBMA films in preventing fibroblast cell adhesion.

To demonstrate the versatility of zwitterion-coated surfaces in controlling and directing a variety of cell types, glial cells of both the peripheral and central nervous system (CNS) were seeded onto the bare and both uniformly coated and patterned zwitterionic surfaces. Astrocytes, a glial cell of the CNS, provide trophic support to migrating neuronal cells in addition to guiding the formation and maintenance of neuronal pathways.^{68,69} Furthermore, astrocytes function as immune effector cells in the CNS, and play critical roles in CNS inflammation and the formation of glial scar around implants.^{56,57} Thus, zwitterion-coated surfaces were evaluated to determine if these substrates control astrocyte adhesion and growth. Cortical astrocytes were passaged from cell culture flasks that had been processed to remove oligodendrocyte precursor cells and microglia.⁷⁰ Anti-GFAP immunostaining confirmed >98% purity of the astrocyte cultures. Astrocytes seeded onto uncoated surfaces adapted a typical stellate astrocyte morphology as shown with anti-GFAP immunostain in green (Figure 7A). Few astrocytes were observed on SBMA- and CBMA-coated surfaces. Additionally, the cells that were present took on circular morphology with few cytoplasmic extensions (Figure 7B-C). Quantification of cell density on uniformly-coated surfaces revealed a 13 fold reduction in astrocyte density on SBMA-coated substrates versus plain glass substrates (Figure 7E). This effect was more pronounced than that seen with fibroblasts (Figure 6E) implying that astrocytes may be more sensitive than fibroblasts to SBMA. As observed for fibroblasts, the resistance to CBMA-coated substrates is even more pronounced with an astrocyte density decrease of over 90 times.

On SBMA-striped substrates a 75% reduction in astrocyte cell density was observed in coated stripes compared to uncoated regions (Figure 7F). The uncoated bands showed similar cell density to the uncoated controls implying that the presence of the stripes does not disrupt cell adhesion to the bands. On the CBMA-striped sample, astrocyte cytoplasmic extensions were observed parallel to the pattern with few cytoplasmic extensions bridging across the striped pattern (Figure 7D). Cell density on CBMA-coated regions decreased about 20 fold in cell density as compared to the uncoated glass. Even though the magnitude of decrease was less for astrocytes when compared to fibroblasts, the cell density on CBMA-coated regions of the striped substrates was similar to that of uniformly-coated CBMA substrates. Astrocyte morphology was significantly altered on CBMA-striped substrates. On uncoated glass they exhibited a stellate shape, while on CBMA-striped substrates they became fusiform, failing to extend their cytoplasmic processes into coated regions.

Schwann cell adhesion and alignment

Schwann cells are the principal myelinating cell type of the peripheral nervous system and function to support the growth and development of neurons.⁷¹ It is therefore important to understand how these cells interact with zwitterion-coated substrates especially for potential neural prosthesis applications. Similar to the above-mentioned experiments, uncoated, SBMA- and CBMA-coated uniformly, and 100 μm periodicity (both SBMA and CBMA) substrates were examined for Schwann cell growth. Representative images of Schwann cells seeded onto these substrates are found in Figure 8A-D. Cell morphology on uncoated substrates was visibly different for SBMA- and CBMA-coated surfaces with cells spreading and interacting with adjacent cells (Figure 8A). Conversely, Schwann cells grown on SBMA-functionalized substrates appeared less dense and showed a rounded morphology (Figure 8B). Few cells were visible in CBMA-coated substrates with very little spreading (Figure 8C). Quantification of cell density on uniformly SBMA-coated substrates revealed more than a 40 fold decrease in Schwann cell density compared to uncoated substrates, a larger effect than seen for fibroblasts or astrocytes. Similar to the findings with fibroblasts and astrocytes, CBMA-coated surfaces experienced more than double the reduction in cell density with over 80 times decrease in Schwann cell density on CBMA-coated substrates compared to uncoated controls.

Analysis of Schwann cell growth on patterned substrates showed similar results with a repulsion of cell adhesion on coated regions as opposed to uncoated areas. Schwann cells grown on substrates with both SBMA and CBMA stripes elongated parallel to the zwitterionic bands. Quantification of cell density on SBMA stripes showed a relatively modest 13 fold reduction in cell density on coated bands as opposed to uncoated bands, significantly less than for uniformly-coated substrates but still much greater than for most other cells. Conversely, on CBMA-striped substrates, Schwann cell density decreased over 150 fold over coated regions compared to uncoated regions. This repulsion was even greater than that observed for uniformly-coated surfaces. While the CBMA-coated substrates yielded a larger decrease in cell density, both SBMA and CBMA polymer films showed a significant reduction when compared with the adjacent uncoated band ($p < 0.001$, t-test).

Schwann cells provide trophic support to neurons, myelinate axons and guide axon regeneration when peripheral nerves are disrupted. Regenerating neural cells have been shown to closely align with Schwann cells in culture,^{72,73} suggesting that the ability of our substrates to cause Schwann cell alignment might allow for control of axonal pathfinding. To determine the degree of Schwann cell alignment to striped substrates, Schwann cells were seeded onto 100 μm periodicity SBMA and CBMA stripes coated on glass. Alignment was evaluated by fitting an ellipse to the cell bodies using Image J software then measuring the angle of the long axis of the ellipse relative to the zwitterionic pattern (Figure 9A). An angle of 0° indicates the long axis of the cell was parallel to the pattern whereas an angle of 90° shows that the long axis of the cell was perpendicular to the pattern; a randomly oriented set of cells would be expected to yield an average angle of 45° . Representative images of Schwann cells grown on uncoated glass (Figure 8A), SBMA-striped (Figure 9A), and CBMA-striped (Figure 8C) substrates demonstrate the effectiveness of these patterns to induce alignment along the zwitterionic bands. Schwann cells grown on uncoated glass substrates were randomly oriented with a mean angle of about 42° (Figure 9B). By comparison, SBMA-patterned substrates were aligned parallel to the stripes with an average angle of less than 6 degrees. Cells on CBMA substrates were even more closely aligned to the stripes with an average angle of just under 2 degrees. The alignment of the Schwann cells appeared to occur because the cells were repelled by the zwitterionic stripes. Schwann cells cultured on substrates with CBMA stripes almost never crossed into the zwitterionic regions forming distinct borders between coated and uncoated areas (Figure 8D). Conversely, SBMA stripes were less effective at repelling cell adhesion and as such Schwann cells were observed forming bridges across zwitterionic bands. Thus, while SBMA appeared to strongly influence Schwann cell alignment, CBMA demonstrated a superior ability to direct growth.

SGN neurite alignment to zwitterionic micropatterns

Spiral ganglion neurons (SGNs) are the afferent neurons of the inner ear that innervate hair cells in the organ of Corti in the hearing ear. In deafened cochleae with a cochlear implant, SGNs are the target neural elements that are stimulated by cochlear implant electrodes. However, a number of factors (including scar tissue formation and distance between SGNs and cochlear implant electrodes) compromise the fidelity with which cochlear implants mimic native hearing.^{74,75} If patterned substrates affect the alignment of SGN neurites, these coatings may form the basis for an implant with an improved interface with its target neural elements. Accordingly, neonatal rat spiral ganglia were plated directly onto both plain glass and zwitterion-patterned substrates. Representative images of uncoated, SBMA-patterned, and CBMA-patterned samples are shown in Figure 10A-C. SGN neurite growth on uncoated substrates (Figure 10A) reveals random growth analogous to SGN neurites grown on glass and polymer substrates uniformly coated with laminin.^{30,58,64} Alignment of SGN neurites along SBMA-coated substrates (Figure 10B) shows a preference to grow on the uncoated regions. However, neurites are occasionally observed crossing over SBMA-coated bands together with non-neuronal cells (nuclei depicted in blue) that have formed a bridge between SBMA-coated regions. Alignment of SGN neurites to CBMA-coated patterns revealed neurite segments that extend along the middle of the uncoated bands and almost never

deviate from parallel patterns, in contrast to the occasional extension of a neurite across an SBMA-coated stripe.

To quantify alignment of SGN neurites to the zwitterionic patterns, alignment ratios were calculated by dividing the total length along the path of the neurite by the aligned length (T_L/A_L) as measured using the trace tool in Image J where the aligned length (A_L) is the distance the neurite travels in the direction of the pattern. Therefore, an alignment of 1 indicates perfect alignment while larger values indicate greater deviations from the parallel path. SGN neurites evaluated on glass showed an alignment ratio of over 2.2, whereas neurites on SBMA-striped substrates showed an alignment ratio of approximately 1.5. Neurites on CBMA-striped substrates exhibited an amazingly low alignment ratio of 1.09. A statistical difference was found between all samples groups ($p < 0.001$, one way ANOVA). CBMA samples direct alignment almost perfectly to the pattern, while SBMA stripes also showed a noticeable effect on neurite alignment. Previous experiments in our lab have shown similar alignment ratios of SGN neurites grown on polymer microchannels and demonstrate the efficacy of these zwitterionic patterns compared to previously studied substrates with physical microtopographical patterns.³⁰ These results suggest that zwitterionic coatings may be used to not only reduce undesirable cell adhesion, but also could be used to guide neurite extension toward a target such as a neural prosthetic device. Similarly, photopatterning could allow for directed fibroblast or glial adhesion and growth in circumstances where patterning of these cells is desirable.

Conclusion

Engineering surfaces that prevent the nonspecific adsorption of protein to biomedical implant surfaces may allow significant mitigation of fibrosis and directing of cell growth. Accordingly, we described a facile photopolymerization method to covalently graft two zwitterionic polymers from glass surfaces. These zwitterionic polymer thin films showed increases in surface composition with increasing solution concentration as measured by XPS. Nonspecific protein adsorption and contact angle also decreased sharply at solution concentrations much lower than the saturation observed using XPS indicating that a very thin coating of zwitterionic polymer is needed to induce significant changes in surface properties. We also directly compared the ability of SBMA- and CBMA-coated surfaces to reduce adsorption of fibrinogen and adhesion of various cell types. Fibroblasts, astrocytes, and Schwann cells all showed dramatic decreases in cell density on SBMA- and CBMA-coated surfaces as compared to uncoated substrates. Fibroblasts, the primary cell type in fibrotic tissue also exhibited a significant difference in cell density between SBMA- and CBMA-coated surfaces, with CBMA-coated systems showing orders of magnitude decreases in adhesion. Using the inherent spatial control of photopolymerization, zwitterionic parallel line patterns were generated to evaluate the cellular adhesion of coated compared to uncoated glass regions. All cell types grown on patterned substrates showed elongation along the both the SBMA and CBMA bands. However, CBMA stripes showed fewer cell crossings along the pattern and greater cellular repulsion along film edges. Alignment of Schwann cells and SGN neurites was evaluated on both SBMA and CBMA surfaces with CBMA directing alignment to a greater extent for both cell types. Therefore, CBMA-coated surfaces demonstrated a superior ability to resist cell adhesion and to direct

cell growth. The described photopolymerizable surfaces enabled investigation of cell-material interactions that could enable engineering of surfaces that resist fibrotic tissue formation. Additionally, generated micropatterns provide potential in directing regeneration of neurites for neural prosthetic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:
Molecular structures of zwitterionic monomers, methacrylate coupling agent, and photoinitiator.

Figure 2: Schematic of the photografting/patterning process. UV-light is used to initiate polymerization and grafting of zwitterionic monomers forming polymer thin films in exposed regions. Functionalization is limited to illuminated regions, which in turn repel protein and cell adhesion.

Figure 3:

Characterization of zwitterion-coated glass surfaces using XPS. A) Representative XPS spectra for glass, activated glass, SBMA-coated glass (20 wt% monomer solution), and CBMA-coated glass (20 wt% monomer solution). The zwitterion-coated glass curves both show a characteristic N1s peak at 402 eV and the SBMA-coated sample exhibit S2s and S2p peaks at 231 and 168 eV respectively. B) Percent nitrogen as a function of wt% monomer for both SBMA- and CBMA-coated glass. Nitrogen percent increases for both zwitterions with monomer wt% and is shown to approach the theoretical maximum (pure polymer composition) at 20 wt%.

Figure 4: Tuning of physicochemical properties of (A) SBMA- and (B) CBMA-functionalized glass surfaces through varying the wt% monomer in solution. Relative protein adsorption (bars) refers to the left axis and is measured compared to an activated glass slide control. Contact angles (•) refer to the right axis. A strong correlation is observed between contact angle and relative protein adsorption.

Figure 5:

Epifluorescent images of protein (human fibrinogen) adsorption to zwitterionic stripes on glass substrates: A) SBMA stripes with a 100 μm periodicity. B) CBMA stripes with 100 μm periodicity. Protein adsorbs to uncoated regions as observed by the green bands, while protein does not adsorb to zwitterion-coated bands as seen in black.

Figure 6:

Representative epifluorescent images of fibroblasts grown on (A) glass, (B) uniformly SBMA-coated, (C) uniformly CBMA-coated, and (D) CBMA-striped surfaces and immunolabeled with anti-vimentin antibody (green). Nuclei are labeled with DAPI (blue). E) Fibroblast cell density on uniformly coated surfaces. A significant difference was observed between all groups (SBMA, CBMA, and uncoated glass) with CBMA exhibiting the lowest cell density (* $p < 0.001$ one way ANOVA). F) Fibroblast cell density on striped substrates, both on and off the zwitterionic pattern. A significant difference was determined between coated and uncoated regions for both SBMA- and CBMA-coated substrates (** $p < 0.001$ t-test). Cell density was shown to be statistically different between SBMA- (image not shown) and CBMA-coated stripes (* $p < 0.001$ one way ANOVA). Zwitterionic micropatterns used for cell culture have a periodicity of 100 μm. Error bars represent standard error of the mean.

Figure 7:

Representative epifluorescent images of astrocytes grown on (A) glass, (B) uniformly SBMA-coated, (C) uniformly CBMA-coated, and (D) CBMA-striped surfaces and immunostained with anti-GFAP antibody (green). Nuclei are labeled with DAPI (blue). E) Astrocyte cell density on uniformly coated surfaces. A significant difference was observed between the glass control and SBMA- and CBMA-coated surfaces (* $p < 0.001$ one way ANOVA). F) Astrocyte cell density on striped substrates, both on and off the zwitterionic pattern. A significant difference was determined between coated and uncoated regions for both SBMA- and CBMA-coated substrates (** $p < 0.001$ t-test). Cell density was shown to be statistically different between SBMA- (image not shown) and CBMA-coated stripes (* $p < 0.001$ one way ANOVA). Zwitterionic micropatterns used for striped substrates have a periodicity of 100 μm . Error bars represent standard error of the mean.

Figure 8:

Representative epifluorescent images of Schwann cells cultured on (A) unmodified glass, (B) uniformly SBMA-coated, (C) uniformly CBMA-coated, and (D) CBMA-striped substrates and immunostained with anti-S100 antibody (green). Nuclei are labeled with DAPI (blue). E) Schwann cell density on uniformly coated substrates. A statistical difference was found between Schwann cells cultured on coated (both SBMA and CBMA) and uncoated (plain glass) surfaces (* $p < 0.001$ one way ANOVA). F) Schwann cell density on SBMA- and CBMA-striped substrates. Analysis of cell density showed significantly fewer cells adhered to both SBMA- and CBMA-coated stripes than the uncoated regions (** $p < 0.001$ t-test). Zwitterionic micropatterns used for striped substrates have a periodicity of 100 μm. Error bars represent standard error of the mean.

Figure 9:

Representative image of Schwann cells cultured on 100 μm SBMA-striped substrates (A). Schwann cells elongate and extend between SBMA stripes occasionally forming bridges between SBMA bands. Cell alignment was determined by measuring the angle (θ) of an ellipse fitted to the major axis of the cell relative to the pattern. B) Average angle of Schwann cell alignment on uncoated and zwitterion-striped surfaces. A significant difference was found across all groups (* $p < 0.001$ one way ANOVA) with CBMA exhibiting the lowest average angle from the micropattern. The number inside or above each column represent the total number of cells evaluated. Cultures were immunostained with anti-S100 antibody (green). Nuclei are labeled with DAPI (blue). Error bars represent standard error of the mean.

Figure 10:

Alignment of SGN neurites on glass (A), SBMA-striped (B), and CBMA-striped (C) surfaces. Cultures were immunostained with anti-NF200 antibody (green) to label neurons. Nuclei are labeled with DAPI (blue). SGN neurites extend randomly on unpatterned glass substrates (A), but elongate and extend between SBMA (B) and CBMA (C) bands. D) Alignment ratios of SGN neurites grown on uncoated and zwitterion-striped surfaces. Quantification of alignment ratios confirms statistical difference between all groups (* $p < 0.001$ one way ANOVA). CBMA-coated surfaces exhibited the strongest alignment. The number inside or above each column represent the total number of neurites measured. A periodicity of 100 μm was used for patterned samples. Error bars represent standard error of the mean.