In vitro assessment of bioactive coatings for neural implant applications

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Abstract: Recent efforts in our laboratory have focused on developing methods for immobilizing bioactive peptides to low cell-adhesive dextran monolayer coatings and promoting biospecific cell adhesion for biomaterial implant applications. In the current study, this dextran-based bioactive coating technology was developed for silicon, polyimide, and gold, the base materials utilized to fabricate our prototype neural implants. Chemical composition of all modified surfaces was verified by X-ray photoelectron spectroscopy (XPS). We observed that surface-immobilized dextran supported very little cell adhesion in vitro (24-h incubation with serum-supplemented medium) on all base materials. Inactive nonadhesion-promoting Gly-Arg-Ala-Asp-Ser-Pro peptides immobilized on dextran-coated materials promoted adhesion and spreading at low levels not significantly different from dextran-coated substrates. Arg-Gly-Asp (RGD) peptide-grafted surfaces were observed to promote substantial fibroblast and glial cell adhesion with minimal PC12 (neuronal cell) adhesion. In contrast, dextran-coated materials with surface-grafted laminin-based, neurite-promoting Ile-Lys-Val-Ala-Val (IKVAV) peptide promoted substantial neuron cell adhesion and minimal fibroblast and glial cell adhesion. It was concluded that neuron-selective substrates are feasible using dextran-based surface chemistry strategies. The chemical surface modifications could be utilized to establish a stable neural tissue–implant interface for long-term performance of neural prosthetic devices. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 68A: 177–186, 2004

Key words: biospecific cell adhesion; engineered biomaterials; neural prosthetics; surface modification

INTRODUCTION

A permanent, multisite interface between specific neural populations in the cerebral cortex or spinal cord and the external world is a critical enabling technology for fundamental advances in neurological science and medicine. Although a variety of complex neural implants have been developed over the past 2 decades, only singular examples of long-term functional performance have been reported. Thus, despite the enormous gains in technology over this period, the delivery of a reliable, high-capacity, extended-duration interface with the central nervous system (CNS) remains largely unfulfilled.

One common failure mode of chronic neural implants is an adverse tissue reaction at the neuron–electrode interface. The implant injury response is initiated by disruption of the blood–brain barrier and the activation of blood-borne macrophages. The activated macrophages migrate into the injured brain tissue and send out chemical signals that activate resident brain cells to repair damaged tissues.1–5 Two major resident brain tissue cells involved in the repair process are microglial cells and astrocytes. Injury-induced activation of microglial cells promotes a morphological transition to a migratory ameboid phagocytic cell that actively engulfs and destroys damaged tissues.1–5 Reactive microglia promote secondary tissue injury by releasing neurotoxic factors such as oxygen intermediates, nitric oxide, proteinases, and glutamate.1–5 Astrocytes are the predominant nonneuronal cell type in the brain and display several functions in normal uninjured tissues including: providing a framework for neurons and the blood–brain barrier; insulating postsynaptic surfaces; and providing metabolic intermediates and trophic factors necessary for maintaining homeostasis in the neuronal environment.1–5 In sites of brain tissue injury, astrocytes transform into a proliferative migratory cell programmed for tissue repair. Injury-activated astrocytes synthesize abnormal amounts of extracellular matrix molecules and deposit them into scar tissue. In functional terms, the reactive astrocytes seal off areas of injury and prevent further propagation of damage to adjacent healthy tissues.1–5 This glial scar also can be thought
of as an internal extension of the meninges that protects the surface of CNS structures from the external environment. The downside to this injury response is that the glial scar is an obstacle or barrier to neurite growth and axonal regeneration. As described above, glial scarring around implants is detrimental to device function.

A current research emphasis in biomaterials science is focused on developing engineered, physicochemically well-defined, mimetic material surface modifications that are low-protein-adsorbing, intrinsically promote specific cell interactions, and have the potential for promoting tissue integration, minimizing encapsulation, and enhancing long-term biomaterial implant performance including chronically implanted neural prostheses. The lack of precise control of cell and tissue interactions with conventional noneengineered biomaterial surfaces starts with the initial process of biological fluid-borne protein adsorption on biomaterial surfaces. Biological fluids contain hundreds of proteins in solution, which nonselectively adsorb to the biomaterial surface to form a multiprotein monolayer with a random and ill-defined distribution of many types of proteins including various cell-adhesion proteins. Therefore, a provisional matrix that promotes nonselective cell adhesion becomes the interfacial layer between the tissue and biomaterial surfaces. Consequently, at the CNS tissue–implant interface, activated astroglial cells and other inflammatory cells (platelets, neutrophils, and monocytes/macrophages) that arrive at the biomaterial interface soon after implantation adhere to the implant surface, promote glial scar formation around the implant, and ultimately block axonal regeneration. The immediate goal of the engineered biomaterial surface would be to provide a provisional matrix that promotes maximal tissue cell adhesion and axon regeneration/neuron ingrowth with minimal inflammatory cell adhesion and glial scar formation.

In the current study, processes were developed to effectively integrate bioactive molecules within the structure of implantable electrode arrays. Bioactive surface modifications were developed for all materials utilized in the fabrication of our electrodes. Previous studies demonstrated that surface-immobilized dextran reduces cell adhesion and spreading in vitro. Further work developed methods for immobilizing bioactive peptides to low-cell-adhesive dextran monolayer coatings. The substrates were shown to promote biospecific cell adhesion for vascular implant applications. In the current study, the dextran-based bioactive coating technology was developed for silicon (Si), polyimide (PI), and gold (Au), the base materials utilized to fabricate our prototype neural implants.

### MATERIALS AND METHODS

#### Chemical reagents

Dextran, sodium borohydride (analytical grade NaIO₄), and 0.01% poly-L-lysine solution (PLL) were obtained from Sigma; RGD peptides [linear RGD, Gly-Arg-Asp-Ser-Pro (GRGDSP); cyclic RGD, Gly-Pen-Gly-Arg-Asp-Pro-Cys-Ala (GpenGRGDSPCA); inactive sham control peptide, Gly-Arg-Ala-Asp-Ser-Pro (GRADSP)], from Life Technologies; Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg (SRARKQAASIKVA-VSADR) (Bachem), PI precursors (Probimide), and 11-mercaptopoundecylamine (MUAM), from Dojindo Molecular Technologies, Inc.

#### Periodate oxidation of dextran

Dextran was oxidized to produce aldehyde groups via standard periodate methods. Dextran (MW 40 kDa, Sigma), 1g, was first dissolved in 30 mL deionized water. Sodium periodate (NaIO₄, 0.1M) was prepared for immediate use. The NaIO₄ solution was then added to the solution of dextran to make a 50% molar ratio of NaIO₄ to dextran (moles of glucose monomer). The reaction mixture was stirred at 4°C overnight and protected from light by covering the reaction flask with aluminum foil. The solution was then purified by precipitation of unreacted periodate and iodate products using an equimolar aqueous solution of BaCl₂. The purified oxidized dextran solution was then lyophilized and stored (if not immediately used) at 4°C in a 50-mL conical centrifuge tube protected from light. The product was analyzed by Fourier transform infrared (FTIR) spectroscopy. The results showed a peak at 1700 nm, indicating the aldehyde groups within the dextran chain.

#### Preparation of base materials

Because the bioactive coating methods were being developed as an integral component in the device fabrication process, the base implant materials—Si, Au, and PI—were prepared in the cleanroom, utilizing implant fabrication methods. The Si wafers used in the current study, for bioactive coating processes, were prepared from P-type cubic zirconia (CZ) wafers, 150-mm–diameter CZ crystals with (100) n-type orientations. Wafers were loaded into a quartz tube (stabilized at 700°C for 10 min) of a horizontal thermal reactor. After wafer introduction and N₂ purge, the temperature was ramped from 700°C to 800°C and then to 1050°C. Oxidation was performed at saturated humidity (0.45 L/min of N₂ through an H₂O bubbler along with 6 L/min O₂) at 1050°C for 90 min to get 0.5-µ-thick oxides (as measured by an ellipsometer). Gold substrates were created by sputter coating in the cleanroom, using an electron beam TorrVac device, operating at 10 kW and 10⁻⁶ Torr. Polyimide films were prepared as follows: PI precursor, Probimide 7520, was spin-coated on 4-inch Si wafers at 2800 rpm for 30 s. The PI-coated substrates were then soft
baked for 10 min at 80°C and then 10 min at 110°C to remove solvents. Substrates were then cured (polymerized) in an N2-purged furnace with a ramp-up temperature from room temperature to 350°C for 1 h.

**Oxygen plasma surface treatment**

Oxygen plasma treatment was used to improve PI surface adhesion and eliminate the organic contamination of Si and Au substrates. Polymer films coated on Si wafers were cut in small pieces (10 mm x 10 mm) were exposed to oxygen plasma at 200–300 mTorr for predetermined periods of time (typically 2 min for PI and 15 min for Au and Si oxide), using a glow discharge reactor (Teal Asher) in the clean room.

**Surface amination of base materials**

All base material samples were cut into small squares (1 cm x 1 cm). Surface-oxidized Si wafers were immersed in a 2% solution of 3-aminopropyltrimethoxysilane (APS) in 95% ethanol for 2 min, washed with 95% alcohol, and cured at 110°C in an explosion-proof oven (Fig. 1). Gold-coated Si substrates were surface-aminated by forming self-assembling monolayers (SAMs) with aminoalkanethiols using a published method.19 Immediately after oxygen plasma, substrates were soaked in alcoholic solution (1.0 M) of MUAM for at least 16 h (Fig. 2). Samples then were rinsed with deionized (DI) water several times following a rinse with 0.1 M phosphate buffer at pH 7–8.5. Preparation of aminated PI surfaces was performed by exposing the substrates to oxygen plasma followed by immediate immersion in 0.01% aqueous PLL solution and then incubating overnight (Fig. 3).

**Covalent coupling of oxidized dextran to surface-aminated base materials**

Oxidized dextran, prepared as described above, was dissolved in 0.2M sodium phosphate buffer (pH 9, 0.02 g/mL). Immediately following surface amination procedures in the cleanroom, oxidized dextran solution (2 mL) was added to six-well multiwell dishes containing surface-aminated substrates (Fig. 4). The substrates were allowed to incubate at room temperature for 16 h on a rocker platform and protected from light. Following incubation, the reaction mixture was decanted from the culture wells and replaced by fresh 0.1M solution of sodium borohydride, NaBH₄, to reduce Schiff bases formed and to quench any free unreacted aldehyde groups present on the oxidized dextran chain. The substrates were allowed to incubate for 2 h on the rocker platform. The NaBH₄ solution was then decanted and the substrates were rinsed gently several times with deionized water to remove unbound dextran (Fig. 4). Samples were not allowed to dry during transport from the cleanroom to the laboratory and before coupling of peptides.

**Covalent coupling of bioactive peptides to dextran-coated base materials**

Four synthetic peptides were utilized in these studies. For cell–tissue-adhesive surfaces, the peptide GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) (Life Technologies) was employed. GRADSP (Gly-Arg-Ala-Asp-Ser-Pro) (Life Technologies) was used as an inactive control for nonspecific peptide-induced cell adhesion. For surface coatings with neurite growth-promoting activity, the laminin-based peptide SRARKQAASIKVAVSADR (Bachem) was utilized. Studies by Thanos et al.20,21 showed that this peptide is very effective in promoting neurite growth in vitro.

Surface grafting of peptides to dextran-coated substrates was achieved via a previously reported method.17 Dextran-coated substrates committed for peptide grafting were oxidized with 0.1M sodium periodate to activate substrate surfaces for covalent immobilization of peptides (Fig. 5). Following surface oxidation, samples were rinsed with DI water and peptide stock solutions (0.1 mg/mL in 0.2M dibasic sodium phosphate, pH 9.0) were added to each sample well. A rocker table agitated the plates for 24 h while being protected from light with an aluminum foil covering. The
peptide solution was decanted at the end of the 24-h duration, and the modified substrates were then rinsed with DI water. Following peptide coupling, the substrates were incubated (2 h, room temperature) in dibasic sodium phosphate (0.2 M, pH 9.0) containing 0.1 M sodium borohydride, NaBH₄ to reduce Schiff bases formed and to quench any free unreacted aldehyde groups present. The substrates were allowed to incubate for 2–3 h on the rocker platform. The substrates were rinsed with PBS and immediately were employed for *in vitro* experiments (Fig. 5).

![Diagram of surface chemistry]

**Figure 2.** Introduction of surface amines on Au-coated Si wafers by the formation of self-assembled monolayers with an aminoalkanethiol.

![Diagram of surface chemistry]

**Figure 3.** Introduction of surface amines on PI-coated Si wafers by the adsorption of poly-l-lysine.

![Diagram of surface chemistry]
Surface chemical analyses

For all materials synthesized for these studies, XPS spectra were acquired using a Kratos XSAM800 instrument. Wide-range and high-resolution C1s spectra were obtained for each sample using a 45° takeoff angle. The high-resolution spectra were further resolved into individual Gaussian peaks using software provided by the manufacturer.

Cell lines

The cell cultures investigated in this study were 3T3 fibroblasts (ATCC #CRL-6476), neuronallike PC12 cells (ATCC, cat #30-2004), and a gliallike (glioblastoma) T98-G cell line (ATCC #CRL-1690). The 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), PC12 cells were maintained in F-12
K medium (containing 15% horse serum, 1% antibiotic/antimyotic (ABAM) and 2.5% FBS), and T98-G cells were maintained in MEM with 10% FBS. All cell culture media and reagents were obtained from Life Technologies, Inc.

Cell seeding

Six-well culture plates were initially coated with a 0.5% poly-hydroxyethylmethacrylate (pHEMA) (in 95% ethanol) solution to reduce cell attachment to well surfaces. Approximately 2 mL of cell suspension in media (15,000 cells/mL for 3T3s and T98-Gs; 100,000 cells/mL for PC12s) was added to each well of the culture dish. The cultures plates were then incubated at 37°C, 5% CO2 for 24 h.

Cell adhesion assay

Cell area coverage assays were performed to assess the extent of 3T3, T98-G, and PC12 cell adhesion and spreading on all substrates. The 3T3, T98-G, and PC12 cells were seeded as previously described and incubated for 24 h. Following incubation, samples were fixed (3.8% formaldehyde in phosphate-buffered saline, PBS, for 5 min) and (0.1% aqueous toluidine blue for 5 min). Stained cells were then examined using phase contrast or stereomicroscopy (Leica) at 100× magnification. Three random 100× fields were selected for each substrate for analysis. The extent of cell adhesion was determined for each captured digital image by calculating a percentage of cell area coverage using digital image analysis software. Final data were presented as a percentage of control adhesion. The percentage of control adhesion was calculated by multiplying the ratio of percent area coverage on dextran-coated and peptide-grafted substrates to percent cell area coverage on aminated glass by 100. The average percentage of control adhesion was determined from duplicate independent experiments. Comparisons between samples groups were made using ANOVA.

Neurite growth assay

The extent of neurite growth in PC12 cultures was assessed on each substrate. Cells were incubated, fixed, stained, and imaged as described above. Three random 100× fields were selected for each substrate for analysis. The number of cells with neurites was determined on each captured digital image. The mean percentage of cells with neurites was calculated by multiplying the ratio of cells with neurites to total cells per three random fields by 100. The average percentage of cells with neurites was determined from duplicate independent experiments for each substrate. Comparisons between samples groups were made using ANOVA.

RESULTS

Surface analysis

The XPS analyses were performed on all substrates. High-resolution C1s spectra revealed an increased percentage of the total C1s spectrum in higher-energy components (286–289 eV) when the dextran and RGD peptide were immobilized on PI and Si substrates (Table I). In contrast, the coatings promoted modest increases in the 286–289-eV energy spectrum on Au substrates, whereas a dominant 285-eV component persisted for all surface modifications (Table I).

Cell adhesion

Cell adhesion was determined on all substrates and expressed as a percentage of control adhesion on aminated base materials. Surface immobilization of dextran on aminated materials significantly reduced adhesion of all cell types (Table II; Figs. 6–8). Surface-grafted inactive (nonadhesive) control peptide

### Table I

<table>
<thead>
<tr>
<th>C1s spectrum component (eV)</th>
<th>Amine</th>
<th>Dextran</th>
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<td></td>
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GRADSP on dextran-coated substrates promoted cell adhesion at levels not significantly different from immobilized dextran and significantly lower \((p < 0.05)\) than substrates containing surface-grafted linear GRGDSP (Table II; Figs. 6–8). The PC12 cell adhesion was significantly lower \((p < 0.05)\) than 3T3 and T98-G cell adhesion on RGD substrates (Table II; Figs. 6–8). Surface-grafted laminin-based SIKVAV peptide promoted low 3T3 and T98-G cell adhesion and significantly higher \((p < 0.05)\) PC12 adhesion (Table II; Figs. 6–8).

**Neurite growth**

The PC12 neurite growth was assessed and expressed as a percentage of total cells with neurites on all substrates. No significant neurite growth was observed on nonadhesive dextran-coated PI (0 ± 0%) aminated control PI), Si wafer (0 ± 0%), and Au-coated Si wafer (0 ± 0%) (Fig. 9). No significant neurite growth was observed on linear RGD peptide-grafted PI (0 ± 0%) and Au (0 ± 0%) substrates (Fig. 9). A low level of neurite growth was observed on linear RGD peptide–grafted Si wafer substrates (14.0 ± 2.4%) (Fig. 9). Extensive neurite growth was observed on SIKVAV peptide–grafted substrates; PI (51.7 ± 1.6% total adherent cells), Si wafer (57.7 ± 2.3%), and Au-coated Si wafers (52.3 ± 2.1%) (Fig. 9).

**DISCUSSION**

In previous work, we developed methods for immobilizing bioactive peptides to low-cell–adhesive dextran monolayer coatings.\(^\text{17}\) The substrates were shown to promote biospecific cell adhesion for vascu-
lar implant applications. In the current study, this bioactive coating technology was developed for Si, PI, and Au, the base materials utilized to fabricate our prototype neural implants.

Chemical composition of all modified surfaces was verified by X-ray photoelectron spectroscopy (XPS). High-resolution C1s spectra of PI and Si revealed a chemical shift from predominantly C—C bonds (283–285 eV) on untreated PI or silanized Si to predominantly C—O/C—N bonds (286 eV) on peptide-grafted, dextran-coated PI and Si substrates (Table I). This is in support of an increased presence of C—O and C—N bonds in the coated substrates. High-resolution C1s spectra of aminoalkanethiol SAMs on Au substrates revealed a predominant C—C (283–285 eV) peak with no chemical shift on surfaces containing immobilized dextran and peptides (Table I). Minor 286–288 eV peaks were present on SAM, dextran-coated SAM, peptide-grafted, and dextran-coated SAM substrates (Table I). The results suggest that C—O, C=O, C=S, and C=N from thiols, peptides, dextran, etc., are present as minor peaks and the predominant C—C peak is due to aminoalkanethiols in the SAM.

Cell adhesion and spreading assays verified the chemical analysis results for each substrate and provided insight into how three different prototypical CNS tissue cell types responded to each substrate surface modification. Cell lines were chosen over primary cell culture because they can be propagated easily in culture and respond to biomaterials similar to their primary culture counterparts. Our cell culture methods were developed as a rapid screening process for coatings that will be evaluated in neural implants in vivo. Isolation and maintenance of primary implants would slow down the screening process tremendously. Correlation of any in vitro results with outcomes in vivo has not yet been determined. If the cell lines do not correlate well with in vivo results, then screening methods with primary cell cultures will be considered.

As expected, cell adhesion and spreading was very low in serum-supplemented medium on dextran-coated surfaces for all cell types on all base materials (Table II; Figs. 6–8). Inactive, nonadhesion-promoting GRADSP peptides immobilized on dextran-coated materials produced adhesion and spreading at levels comparable to (not significantly different from) dextran-coated substrates (Table II; Figs. 6–8). The results suggest that peptides (without cell adhesion-promoting activity) grafted to dextran-coated substrates did not alter the nonadhesive properties of dextran. Therefore, the adhesion-promoting activity of RGD and SIKVAV peptides grafted to dextran-coated surfaces was intrinsic to the adhesive signals presented by the surface-grafted peptides without contributions from adsorbed serum-derived adhesion proteins. Adhesion and spreading on GRADSP-grafted substrates was lower than previously reported values. We attribute this result to utilizing a cleanroom environment to clean base materials and initialize surface modifications in the current work. Also, substrates were never exposed to ambient air during all surface modification procedures. In the previous studies, all cleaning and surface modification procedures were performed in ambient air in the laboratory, with air drying of surfaces between all modification steps. Therefore, airborne surface conta
minants (mostly hydrophobic in nature) could have partially masked the effects of surface-immobilized dextran and peptides, leading to higher adhesion levels on dextran-coated and GRADSP-grafted, dextran-coated substrates than observed in the current study.17

The RGD peptide-grafted surfaces were observed to promote substantial fibroblast and glial cell adhesion with minimal neuronal cell adhesion, indicating a substrate that selects against PC12 adhesion (Table II; Figs. 6–8). Lower PC12 adhesion may not be representative of neuron cell adhesion to RGD peptide-grafted substrates in general, because PC12 cells have long been reported to be loosely adherent cells in culture, requiring special extracellular matrix (ECM) coatings on cell culture substrates to enhance cell adhesion.22 The RGD peptide-coating technology may be utilized for neural implants where tissue anchorage is desired to limit injurious micromotion at the tissue–implant interface.

The IKVAV peptide-grafted surfaces promoted substantial neuronal cell adhesion and minimal fibroblast and glial cell adhesion, indicating a substrate that is selective for neuron adhesion and selective against fibroblast and glial cell adhesion (Table II; Figs. 6–8). The IKVAV-grafted surfaces also promoted neurite growth on all substrates (Fig. 9). The results are in agreement with other reports in the literature.20,21 Applications for the IKVAV peptide-grafted coating technology include developing tracts for guidance of neurite growth on implant surfaces and coating areas around recording/stimulation sites to promote neurite growth near the sites for optimal signal transfer between neurons and implants.

The current study demonstrates that dextran-based bioactive coating methods can be developed for microfabrication processes including fabrication of microelectronic neural prostheses. It is confirmed that surface-immobilized dextran effectively reduces cell adhesion and spreading on base materials utilized for fabricating our prototype neuroprosthetic implants. This low cell-binding surface was then further modified with surface-grafted bioactive molecules using nontoxic aqueous chemistry. The peptide-grafted substrates promoted biospecific interactions with minimal levels of competing nonspecific interactions. With specific peptides, it is demonstrated that cell-type selective interactions could be achieved with these coating technologies. Recent in vitro studies showed that the coatings are stable to multiple-cell passages (proteolytic environment) and thus show promise for stability in an implant environment.23 In the specific case of neural prosthetic implants, the coating technologies could be utilized to establish a stable neural tissue–implant interface for long-term device performance. In general, the dextran-based surface coating methods could lead to the development of well-defined surface modifications that allow for the precise control of cellular interactions at the tissue–biomaterial interface and ultimately improved performance of long-term biomaterial implants.

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