Cell-repellant Dextran Coatings of Porous Titania Using Mussel Adhesion Chemistry^a

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The resistance of bioceramics against non-specific adsorption of serum proteins is critical for a wide range of biomedical applications. Some polysaccharides serve as natural protein-resistant molecules in extracellular matrices; however, the stable adhesion of polysaccharides to ceramic biomaterials in an aqueous solution is very challenging because chemical linkages at organic/inorganic interfaces are susceptible to hydrolytic degradation. Here, a catechol-grafted dextran, which strongly binds to titania (TiO_2) in an aqueous milieu to effectively suppress cell adhesion through anti-fouling activity against non-specific protein adsorption, is introduced. Catechol is conjugated approximately to 6.7 mol% of glucose units of dextran via a carbamate ester linkage, corresponding to roughly three catechols per dextran chain having an average molecular weight of 6 kDa. Multivalent interactions of catechols with a titanium atom, enabled by the graft-type structure, provide a very stable coating of dextran on this inorganic surface. The adhesion of HeLa cells on the dextran-coated titania surface is reduced by 2.4-fold compared to that on a pristine titania surface. These results suggest that the graft-type incorporation of a small number of catechol moieties along a dextran backbone is an effective means of producing a stable anti-fouling interface on inorganic biomaterials in an aqueous environment.
1. Introduction

Inorganic biomaterials (e.g., hydroxyapatite, titanium, aluminum, zirconium, and their oxides) have received much attention for the preservation, restoration, and replacement of damaged and diseased tissues in musculoskeletal systems due to their good biocompatibility and durability.\(^{1–3}\) In particular, titanium has been widely used as a hard tissue replacement because it is very stable under physiological conditions. Titanium forms a thin surface layer of titania (TiO\(_2\)), ca. 5–10 nm thick, that effectively prevents undesirable metal dissolution and corrosion.\(^{4}\) However, the slightly negatively charged oxide layer also poses a problem when it comes into contact with blood or body fluids because the intrinsic pathway of blood coagulation can be activated by the oxide surface. Serum protein adsorption onto the passive film of a titanium surface can also facilitate the undesirable dissolution of titanium ions and the formation of metal-protein complexes.\(^{5–8}\) This protein-induced breakdown of a passive oxide layer can invoke serious biological responses as the implant materials corrode.\(^{9}\) Therefore, a robust protein-resistant coating is critical when designing orthopedic and dental implants as well as vascular stents or catheters. Protein adsorption also plays a pivotal role in cell adhesion and spreading because the composition and structure of proteins determine the interactions between cell adhesion receptors and substrates.

A commonly used anti-fouling coating method is to reduce the surface energy by immobilizing hydrophilic, flexible polymers onto the surface of biomaterials.\(^{10}\) Various polymers have been investigated to generate a non-fouling surface, including oligosaccharides, phospholipid-mimetic polymers, poly(ethylene glycol) (PEG), and polyacrylates.\(^{11–17}\) Oligo- and polysaccharides have been used to create a biomimetic, non-adhesive glyocalyx layer as in vivo biomolecular interactions are strongly affected by various oligosaccharide chains existing on cell surfaces.\(^{11,12,18}\) Maltose and dextran, typically used oligosaccharides, have a flexible alpha (1→6) linkage between glucose monomers which acts as a steric barrier against non-specific protein adsorption. In particular, dextran has been utilized as an anti-fouling layer on substrates for surface plasmon resonance analytical systems, gene delivery carriers, and superparamagnetic iron oxide nanoparticles for in vivo magnetic resonance imaging applications.\(^{19–21}\)

Despite the well-known anti-fouling activity of dextran, the immobilization of dextran to the surface of inorganic materials is very challenging. Several approaches have been proposed to immobilize dextran chemically onto various inorganic substrates, though these efforts are often limited by several factors, including the use of complicated and expensive procedures, the narrow substrate specificity, and the high level of susceptibility to hydrolytic cleavages. Recently, a mussel-inspired design of functional adhesive polymers incorporating multiple catechol moieties into the polymer backbone was proved to be a versatile, cost-effective, and facile method to modify a broad range of surfaces with pre-synthesized polymers.\(^{13,16,22–26}\) This simple surface modification process was expected to be of great value in designing anti-fouling interfaces. Initial attempts have been made by attaching trimeric catechol groups to one end of a PEG chain or an anti-fouling peptide.\(^{13–15}\) These studies demonstrated that polymer/peptide mimics of mussel adhesive proteins can be useful for attaching dense protein-resistant polymers on surfaces. Our recent studies also showed that a graft-type modification of PEG with catechol groups can effectively bind to various types of material surfaces presumably due to more effective multivalent interactions with substrates.\(^{16}\)

In this work, we introduce a new type of dextran derivative, catechol-grafted dextran (denoted “dextran-g-ct”), as a glyocalyx-mimetic anti-fouling coating material with a very strong affinity towards inorganic surfaces. The graft-type design was expected to be beneficial for stable adhesion, as the repeated occurrence of the catechol moieties provides each dextran chain with multiple binding sites.\(^{26}\) In addition, in the graft structure, catechols have a structural orientation in a polymer backbone that differs from the end-chain modification, which can increase the binding affinity of catechol-modified polymers.\(^{26}\) We used a relatively low grafting number of catechols to avoid any complicated interactions between the excess unbound catechols and the proteins. The adsorption of serum proteins and the degree of cell adhesion were then determined as part of the effort to investigate the anti-fouling activities of dextran-g-ct on titania substrates.

2. Experimental Section

2.1. Synthesis and Characterization of Dextran-g-ct

Dextran (8.31 × 10\(^{-3}\) mol based on the hydroxyl groups, molecular weight ≈ 6 kDa, from *Leuconostoc spp.*, Sigma-Aldrich) was dissolved in 5 mL anhydrous dimethyl sulfoxide (DMSO). 1,1’-Carbonyldiimidazole (1.42 × 10\(^{-3}\) mol, CDI, Sigma-Aldrich) in anhydrous DMSO (2 mL) was added to the dextran solution, followed by incubation at room temperature for 30 min. Dopamine hydrochloride (9.45 × 10\(^{-4}\) mol, Sigma-Aldrich) dissolved in anhydrous DMSO (1 mL) was then added to the CDI-activated dextran solution. 4-tert-Butylcatechol (11.67 × 10\(^{-3}\) mol, Sigma-Aldrich) was also added to prevent the oxidation of dopamine during the reaction. The conjugation reaction was carried out with magnetic stirring at about 300 rpm at room temperature overnight. The product was isolated by repeated
precipitation in a 6:4 mixture of 1-propanol and diethyl ether at 4 °C. 1H NMR (300 MHz, DMSO-d$_6$, 8 ppm): 2.88 (t, 2H, –NH–CH$_2$–CH$_2$–), 3.23 (t, 2H, –NH–CH$_2$–CH$_2$–), 3.52 (t, 1H, dextran C4-H); 3.58 (t, 1H, dextran C2-H); 3.71 (t, 1H, dextran C3-H); 3.73–3.78 (q, 1H, dextran C5-H); 3.89–4.01 (d, 1H, dextran C6-H); 4.97 (d, 1H, dextran C1-H); 6.75 (d, 1H, arom.), 6.85 (s, 1H, arom.), and 6.91 (d, 1H, arom.).

2.2. Preparation and Characterization of Titania Films

Titania films were prepared using the procedures described in a previous study with some modifications.[27] The titania nanoparticles (100 mg, Degussa P25, Evonik Industries, Essen, Germany) were ground with a pestle in a mortar with deionized water (1 mL) containing acetylacetone (100 μL). Additional deionized water (1.7 mL) was slowly added to the paste with continuous grinding. One drop of Tirton-X100 was added to the paste and ground until a homogeneous titania paste was obtained. Approximately 2 mm of the edge of a microscope glass slide (Marienfeld, Germany) was covered with Scotch tape (thickness ≈ 62.5 μm, 3M, MN, USA). The titania paste (100 mL) was placed on the substrate and spread using a razor blade (DORCO, Seoul, Republic of Korea). The titania paste spread on the glass slide was then dried at 70 °C for 10 min, followed by incubation in a furnace at 400 °C for 45 min. The glass samples were cut into pieces 2.5 cm × 2 cm in size. The surfaces were analyzed using Atomic Force Microscopy (AFM) on a PSIA XE-100 AFM system and Scanning Electron Microscopy (SEM) on a Hitachi S-4800 (Japan).

2.3. Protein Adsorption on Titania Films

The prepared dextran-coated titania film was tested for the anti-fouling effect of dextran against fetal bovine serum (FBS). Each set of pristine and dextran-coated titania film was immersed in phosphate buffered saline (PBS) at pH 7.4 without and with FBS (5 vol% and 10 vol%) at 37 °C for 5 h with gentle shaking at about 50 rpm. The films were washed with deionized water for three times to remove unbound proteins, after which they were lyophilized. Protein adsorption on the titania films was analyzed using X-ray Photoelectron Spectroscopy (XPS) on a Sigma Probe (Thermo VG Scientific, West Sussex, UK). In addition, the anti-fouling effect of the dextran coating of titania against bovine serum albumin (BSA; Sigma-Aldrich) was quantitatively evaluated. The titania nanoparticles (80 mg) were homogeneously dispersed in 12 × 10$^{-3}$ m PBS (20 mL). BSA (20 mg) was dissolved in a 12 × 10$^{-3}$ m PBS (20 mL, pH 7.4). A 1:1 (v/v) mixture of the protein solution and the titania suspension was incubated with rotary mixing at about 100 rpm; at pre-determined time intervals, the titania nanoparticles were isolated using a bench-top centrifuge (Eppendorf 5804) at 12 000 rpm for 10 min. The protein concentration of the supernatant was determined using UV-Vis absorption spectroscopy at 280 nm. The calibration curve was obtained using a protein standard solution (1 mg mL$^{-1}$ BSA, Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.4. Stability of Dextran-coated Titania

To determine the remaining amount of dextran attached to the titania after incubation in PBS and a serum-containing medium, using the procedures described in a previous study with some modifications.[28] The dextran-coated titania nanoparticles were dispersed in PBS and 10 vol% FBS solution at a concentration of 4 mg mL$^{-1}$, and then 500 μL of each solution was transferred to an Eppendorf tube, followed by incubation at 37 °C. The samples were centrifuged at 12 000 rpm for 10 min and re-dispersed in deionized water. These procedures were repeated for three times. The rinsed dextran-coated titania nanoparticles were re-dispersed with 500 μL of deionized water and mixed with 500 μL of 95% sulfuric acid containing 0.1 wt% of an anthrone (Sigma-Aldrich). The mixed sample was incubated at 95 °C for 7 min and centrifuged at 12 000 rpm for 10 min. The light absorption at 625 nm was measured by using UV-Vis spectrophotometry.

2.5. Cell Adhesion on Dextran-coated Titania Films

The pristine and dextran-coated titania films (area: 5 cm$^2$) were placed into each well of a six-well tissue culture plate. The Green Fluorescent Protein (GFP)-expressing HeLa cells (human cervical cancer cells) were seeded on the plate at a density of 4 × 10$^5$ and 8 × 10$^5$ cells per well and were incubated for 2 h at 37 °C in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10 vol% FBS. After washing with PBS twice, the cells were fixed with 1 wt% paraformaldehyde at 4 °C for 1 h. The fluorescence images were obtained using a fluorescence microscope (Nikon Eclipse 80i, Japan) with an excitation filter of 450–490 nm, a dichromic mirror of 505 nm, and an emission filter of 520 nm. The GFP intensity of each image was evaluated using ImageJ software (National Institute of Health, USA). The GFP expressing HeLa cells were seeded on the pristine and dextran-coated titania films as described above. After incubation at 37 °C for 2 h, the cells were washed with PBS two times and lysed with 1% Triton-X100. The GFP expression level was measured using a spectrophotometer (Shimadzu, Japan) at an excitation wavelength at 490 nm and an emission wavelength at 510 nm. For SEM observation, the fixed cells were gradually dehydrated in a series of ethanol solutions (25, 50, 60, 70, 80, 90, and 100 vol% ethanol) for 5 min each. The dehydrated cells were lyophilized and coated with platinum at about 3 nm using a sputter coater (BAL-TEC SCD005, CA, USA).

3. Results and Discussion

Dextran-g-ct was synthesized by the conjugation of dopamine to dextran, as described in Scheme 1. The hydroxyl groups of dextran reacted with CDI to produce an imidazolyl carbamate. The primary amine of dopamine was coupled to the active intermediate, forming a carbamate ester linkage between catechol and dextran. The 1H NMR spectrum of the prepared dextran-g-ct shows the presence of the 3,4-dihydroxyphenyl group, while the
characteristic peaks of dextran appeared in the range of 3.5–4.1 ppm (Figure 1A). The FT-IR analysis also showed that two additional peaks, the C–N bond around 1250 cm$^{-1}$ and the aromatic group around 1560 cm$^{-1}$, appeared in dextran-g-ct, indicating the successful conjugation of dopamine to dextran (Figure 1B). The absorbance of catechol at 280 nm was used to determine the number of catechol units grafted to a dextran backbone (Figure 1C). A calibration curve was determined using dopamine as a standard molecule, as described in the Supporting Information (Figure S1). Dextran has no significant absorption at the same wavelength. The measured molar ratio of glucose (the monomer unit of dextran) to catechol was about 93:7. This ratio corresponds to 6.7 mol% in terms of the grafting.
density of catechol. A low grafting density of catechols is desirable for aqueous coatings because the solubility of dextran-g-ct in an aqueous solution decreases with an increase in the grafting density of catechol. In addition, effective anti-fouling coatings require that no residual catechol groups exist which are not bound to the titania surface, as free catechol groups can be chemically reactive towards amine groups on peptides, proteins, and other biomolecules. This chemical reactivity of catechols can induce the irreversible, chemical immobilization of biomolecules on titania, which needs to be avoided for anti-fouling surface modifications unless additional surface functionalization is required.

Catechol can bind to titania through its coordination with the Ti atom (Scheme 2). It was reported that the highest adhesion densities of catechol-modified PEGs to titania can be achieved under critical point conditions at elevated temperatures and ionic strength levels, implying that the processing conditions are important for the binding of the catechol-modified polymers. We determined whether the binding of catechol to titania depends upon the functional group of catechol present at the carbon 1 position. Three different catechols were used: tert-butyl catechol, dopamine and 3,4-dihydroxyphenylacetic acid (DHPA). Equivalent volumes of catechol solutions (1×10⁻³ M) and 4 mg mL⁻¹ titania nanoparticles were mixed in three different buffered solutions (Figure S3, Supporting Information). In acidic and neutral conditions, dopamine and DHPA showed similar adsorption behaviors despite the fact that the number of dopamine adsorbed per unit area was larger than that of DHPA. The saturated adsorption levels in PBS were about 2.2×10⁻³ mol per m² for dopamine and 1.5×10⁻³ mol per m² for DHPA. The tert-butyl catechol showed the lowest level of binding to the titania surface among all of the buffers used in the study, indicating that a single catechol molecule does not strongly bind to titania without assistance from redox-active functional groups. Carboxylic acid can form a hydrogen bond with the hydroxyl group of titania, which could provide an additional binding site. The primary amine of dopamine can initiate the polymerization of catechols and thus facilitate the multivalent binding of the molecules. PBS was chosen as a buffer for the dextran-g-ct coatings of titania because the carbamate bond is more stable at a neutral pH. The subsequent protein adsorption experiments were also performed using the same buffer. Dextran-g-ct was able to bind to titania with a surface density of 4.3×10⁻³ mol catechols per m², which was 1.7 times higher than that of individual dopamine molecules despite the absence of primary amine groups. This result suggests that multivalent adhesion motifs grafted to a dextran backbone could be significantly related to the strong binding of the polymer to titania. Previous studies using catechol-grafted PEG revealed that the binding affinity of the catechol-modified PEG is strongly correlated with the number of catechol residues. XPS also indicates the presence of nitrogen from the amide linkage in the dextran-coated titania surface, while pristine titania did not show any nitrogen peak (Figure 2A). Dextran-coated titania showed a UV-Vis absorption spectrum that differed slightly from that of pristine titania, indicating that the interaction with catechol could affect the oxidation states of Ti atoms and the surface oxygen vacancies (Figure 2B). To examine the cell-repellant properties of dextran-g-ct, titania films on a slide glass were prepared using the doctor-blade technique. Figure 3A shows that the titania nanoparticles were annealed together to form a uniform film with a surface roughness.
of about 2.89 nm (Figure 3B). The thickness of the titania film was about 9 μm.

The surface was then modified with dextran-g-ct by a simple immersion process, as described above. The protein adsorption behaviors on the pristine and dextran-coated titania substrates were compared using FBS. FBS contains many different blood proteins, including serum albumins, globulins, fibrinogens, regulatory proteins, and clotting factors. Serum albumin make up about 55% of plasma proteins, corresponding to 35–50 g L⁻¹, and contributes to the transport of hydrophobic insuline molecules. Globulins comprise about 38%, fibrinogen about 7%, and other proteins less than 1%. In serum, most of fibrinogen and clotting factors are removed, but there are some cell adhesive proteins, including fibronectin and vitronectin, that facilitate cell binding to various substrates. The adsorption of serum proteins onto the substrates was analyzed using XPS, as the nitrogen peak from the proteins would increase with an increase in the amount of proteins adsorbed. Compared to pristine titania film, the dextran-coated surface exhibited a significant reduction of the nitrogen peak (Figure 4A). However, a quantitative comparison using XPS data was not straightforward due to the difficulty of establishing an appropriate calibration curve for the amount of proteins adsorbed. Therefore, we carried out an additional quantitative analysis of the adsorption of serum proteins using purified BSA on the dextran-coated titania surface. Serum albumin accounts for more than one half of the plasma proteins, and
its non-specific adsorption onto implanted materials is of particular interest with regard to thrombus, fibrosis, and scar tissue formation. Accordingly, there have been very extensive studies of the adsorption of serum albumin on various substrates. Therefore, an effective non-fouling coating to prevent the adsorption of albumin at solid–liquid interfaces is of great importance for practical applications of biomaterials. BSA is negatively charged at a neutral pH because the isoelectric point is about 4.8.

The initial concentration of BSA was fixed at 0.5 mg mL\(^{-1}\) for all experiments in this study. BSA was adsorbed on the pristine titania surface with the typical saturation binding kinetics (50% saturated in 90 min), as shown in Figure 4B. The amounts of BSA adsorbed per unit area were calculated using the nominal surface area of P25/50 m\(^2\) g\(^{-1}\), which was provided by the manufacturer. A previous study showed that the maximum density of BSA adsorbed on titania was about 100 ng per cm\(^2\) of the titania surface. The protein adsorption on the dextran-coated titania dramatically decreased, only showing about 1.8% of the amount of protein adsorbed on pristine titania surface. The Langmuir regression curves of the adsorption isotherm of the protein on the pristine and dextran-coated titania are shown in Figure 4C. The total amounts of BSA adsorbed per unit area of titania (\(I_{\text{max}}\)) were 1.83 mg m\(^{-2}\) for pristine titania and 0.16 mg m\(^{-2}\) for dextran-coated titania. This result indicates that dextran-g-ct could effectively shield the apparent binding sites on titania; thus, the dextran coating dramatically decreased the maximum adsorption per unit area to only 8.7% of the value for pristine titania. The calculated Langmuir equilibrium constant (\(K\)) values were 4.32 L g\(^{-1}\) and 3.23 L g\(^{-1}\) for pristine and dextran-coated titania surfaces, respectively. The decreased equilibrium constant for the dextran-coated surface also indicates that dextran significantly decreases the molecular interactions between BSA and titania. Next, we also evaluated the stability of the dextran coatings in an aqueous milieu. The remaining amounts of dextran on the titania surface were determined by using an anthrone-based colorimetric method with a calibration curve of free dextran (Figure S5a, Supporting Information). The time-course analysis of the remaining amount of dextran in PBS and 10 vol% FBS showed no significant change at 37 °C for at least 5 d (Figure 4D and Supporting Information, Figure S5b). Further analysis of long-term stability of the dextran coatings is now under way.

The adsorption of serum proteins on the surface of substrates is critical for cell adhesion because it is the proteins that recognize cell adhesion receptors, determining the extent of cell spreading. In this work, HeLa cells

Figure 5. Fluorescence microscopy images of GFP-expressing HeLa cells seeded on the A) pristine and B) dextran-coated titania films (scale bars: 100 μm); C) The number of cells bound to the pristine and dextran-coated titania film (n = 4). D) Green fluorescence intensity evaluated using the ImageJ software (n = 4).
expressing GFP were employed to examine the anti-cell adhesion activity of dextran-g-ct on titania surfaces. The cells were seeded onto pristine and dextran-coated titania films at a density of 4 \times 10^5 and 8 \times 10^5 cells per well and were then incubated for 2 h at 37 °C in DMEM supplemented with 10 vol% FBS. After washing with PBS and fixing with paraformaldehyde, the green fluorescence signal from the bound cells was observed using fluorescence microscopy (Figure 5A,B). The number of cells bound on the pristine titania film was apparently greater than that on the dextran-coated titania film. The mean GFP intensities, which were determined in an image analysis using the ImageJ software, were also compared. The pristine titania film exhibited nearly six-fold greater fluorescence intensity than the dextran-coated titania film, indicating excellent anti-cell binding activity. However, the number of cells bound to the pristine titania surface was only about 2.4-fold higher than the number bound to the dextran-coated titania film (Figure 5C,D). This result indicates that the metabolic activities of the cells bound to the different surfaces were not similar. In particular, the size of the fluorescent dots, representing individual cells in Figure 5A, B, was relatively large on the pristine titania film. The SEM analysis showed that the cells formed a more extended morphology on the pristine titania surface, indicating the increased extent of cell spreading due to the higher density of the binding sites (Figure 6A,B). The relative degree of cellular membrane adhesion on the pristine and dextran-coated titania films was evaluated by calculating the ratio of the peripheral area to the cell body area of the cells on the SEM images; as a result, the ratio of the area of the cells on the dextran-coated titania film was about 2.5-fold higher than that of pristine titania film (Figure 6C).

4. Conclusion

In conclusion, this study has demonstrated that catechol-grafted dextran can be used as a stable protein-resistant coating material in an aqueous milieu. Dextran-g-ct containing about 6.7 mol% catechol moieties strongly binds to a titania substrate at a neutral pH through multivalent Ti-catechol coordination interactions. The dextran coatings of titania effectively suppressed the non-specific adsorption of serum proteins at a neutral pH. As regards BSA, the total protein adsorption per unit area of the dextran-coated surface dramatically decreased to only 1.8% of that of the pristine titania, indicating the very effective reduction of the accessibility of BSA to the substrate. The cell adhesion on the dextran-coated titania surface was also greatly reduced with a cellular morphology that was much less extended compared to the pristine titania surface. These results suggest that catechol-grafted dextran can be used for very effective serum protein- and cell-resistive coatings of various inorganic surfaces of the types used in biomedical applications.

Acknowledgements: This work was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (2012M3A7B4049802) and the Interdisciplinary R&D Program of Medicine and Technology (2012-0006085).

![Figure 6. SEM images of HeLa cells on the A) pristine and B) dextran-coated titania films (scale bars = 2 μm). C) The ratio of the peripheral area to the cell body area of HeLa cells seeded on the pristine and dextran-coated titania films (n = 5).](https://www.mbs-journal.de)
Received: May 5, 2013; Revised: June 21, 2013; Published online: July 31, 2013; DOI: 10.1002/mabi.201300224

Keywords: anti-fouling; catechol; cell adhesion; dextran; titanium dioxide