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Hemocompatibility and biocompatibility of antibacterial biomimetic hybrid films

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ABSTRACT

In previous work, we developed novel antibacterial hybrid coatings based on dextran containing dispersed Ag NPs (~5 nm, DEX-Ag) aimed to offer dual protection against two of the most common complications associated with implant surgery, infections and rejection of the implant. However, their blood-material interactions are unknown. In this study, we assess the hemocompatibility and biocompatibility of DEX-Ag using fresh blood and two cell lines of the immune system, monocytes (THP-1 cells) and macrophages (PMA-stimulated THP-1 cells). Glass, polyurethane (PU) and bare dextran (DEX) were used as reference surfaces. PU, DEX and DEX-Ag exhibited non-hemolytic properties. Relative to glass (100%), platelet attachment on PU, DEX and DEX-Ag was 15%, 10% and 34%, respectively. Further, we assessed cell morphology and viability, pro-inflammatory cytokines expression (TNF-α and IL-1β), pro-inflammatory eicosanoid expression (Prostaglandin E_2, PGE_2) and release of reactive oxygen species (ROS, superoxide and H_2O_2) following incubation of the cells with the surfaces. The morphology and cell viability of THP-1 cells were not affected by DEX-Ag whereas DEX-Ag minimized spreading of PMA-stimulated THP-1 cells and caused a reduction in cell viability (16% relative to other surfaces). Although DEX-Ag slightly enhanced release of ROS, the expression of pro-inflammatory cytokines remained minimal with similar levels of PGE_2, as compared to the other surfaces studied. These results highlight low toxicity of DEX-Ag and hold promise for future applications in vivo.

Introduction

Current complications associated with implant surgery involve post-operative infections and implant rejection (Sharples et al., 1991). Once the patient develops an infection, the microorganisms are extremely resistant to antibiotic therapy and most infections cannot be fully resolved until the biomaterial is removed. The rejection of medical devices is associated with a natural response of the body toward foreign materials. When in contact with blood, foreign materials promote the rapid formation of thrombus that can either adhere to the surface of the material (i.e., a local effect) and disrupt its performance or be detached and carried downstream and eventually occlude a blood vessel (i.e., thromboembolism). These complications aggravate a patient's recovery and result in prolonged hospital stay, need for further medical interventions, increased healthcare cost, and even mortality. Present precautions often require patients to take antibacterial and anticoagulant drugs during for the duration of the implant's use. Hybrid biomimetic films having both antibacterial and antithrombogenic properties are an alternative approach to limiting complications of use and reducing the need for additional therapies.

In previous work, we developed antibacterial biomimetic hybrid films (DEX-Ag) intended for blood contacting devices such as implants and catheters (Ferrer et al., 2012). DEX-Ag coatings were inspired in the endothelial glycocalyx, an irregular brush-like layer that lines the blood vessels and protects them from non-specific interactions. The endothelial glycocalyx is made of a complex blend of polysaccharides and proteins. Within polysaccharides, dextran, known to limit cell and protein adhesion, was the polymer of choice (Eckmann et al., 2003; Massia et al., 2000; Ombelli et al., 2011). The DEX-Ag coatings were prepared by grafting dextran to a substrate embedded with silver nanoparticles (Ag NPs). The methodology involved two steps, the synthesis of Ag NPs in situ in the presence of oxidized dextran followed by simultaneous grafting of dextran and the trapping of Ag NPs within the layer. The resulting film displays dextran features as well as individual Ag NPs (5 nm) and aggregates, which are embedded within the film. The antibacterial properties of the film were demonstrated against gram positive bacteria, *Staphylococcus aureus*, the most common microorganism causing surgical site infections (O'Grady et al., 2011). The hybrid films showed reduction in bacteria colonization when compared to control surfaces. Relative to silicon and bare dextran, the hybrid coating strongly reduced bacteria adhesion by 93% and 78%, respectively.

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The hemocompatibility and biocompatibility of dextran and derivatives of dextran has been longer known. Clinical uses of dextran (40,000–100,000 Da) include plasma volume expansion and blood flow improvement whereas dextran derivatives are used for instance, as anticoagulant (sulfate ester of dextran) and as oral iron supplementation (iron dextran complex) (Naessens et al., 2005). In contrast, the hemocompatibility and biocompatibility of Ag NPs remains controversial. While several reports focus on the benefits of Ag NPs as an antibacterial, antifungal, anti-viral and anti-inflammatory agent (Zhang and Webster, 2009), others alert of their toxicity (Nair and Laurencin, 2007). In vitro exposure of Ag NPs in different cell lines has been associated with disruption of mitochondrial function and increase in reactive oxygen species (ROS) levels, which may lead to cell apoptosis (Braydich-Stolle et al., 2005; Hussain et al., 2005). Although the exact mechanisms by which AgNPs alter mitochondrial function are unknown, Ag NPs react with the thiol groups of proteins and enzymes including key components of the cell's antioxidant defense mechanism such as glutathione, thioredoxin, SOD and thioredoxin peroxidase. As a consequence, Ag NPs may deplete the cell antioxidant defense mechanism which can lead to an accumulation of ROS. Excess of ROS is associated with various human diseases. For instance, ROS play a role in diabetes and neurodegenerative diseases. Furthermore, ROS influences central cellular processes such as proliferation, apoptosis and senescence which are implicated in the development of cancer (Waris and Ahsan, 2006).

The goal of this study was to test whether DEX-Ag elicits specific blood-contact reactions, thrombosis and inflammation, that could limit its potential for in vivo applications. A hard inorganic surface (glass), a commercially available biomaterial (PU) and dextran (DEX) surfaces were selected as reference surfaces. The hemocompatibility of the surfaces was assessed by hemolysis and thrombogenicity (quantification of platelet adhesion and activation). The biocompatibility of the surfaces was assessed using monocytes and macrophages, cellular components of the immune system that play a critical role in biological responses to materials (Anderson, 2001), e. g., mediate inflammation. Monocytes circulate freely in the body and can maturate into macrophage-like adherent cells to simply replenish macrophages or as a response to inflammation. These two cell types cover three main functions in the body, phagocytosis, antigen presentation and cytokine production. Because of these functions, monocytes and macrophages are commonly used to evaluate biocompatibility of materials. In particular, we used THP-1 cells, a human monocytic cell line, and matured THP-1 cells (macrophage-like) following stimulation with phorbol 12-myrstate 13-acetate (PMA). We assessed their morphological changes and cytotoxicity, monitored the release of three markers of inflammation, TNF-α, IL-1β and PGE_2, and examined the release of two ROS, superoxide and hydrogen peroxide, after incubation with the surfaces.

Materials and methods

Materials and instruments

All solvents and reagents were of analytical grade and used as received. Dextran from *Leuconostoc* ssp. (Mw = 100 kDa) was purchased from Fluka Chemie (Buchs, Switzerland). 3-aminopropyl triethoxysilane (APTES), sodium periodate ($NaIO_4$), sodium cyano borohydride ($NaBH_3CN$), silver nitrate ($AgNO_3$), dihydroethidium (DHE), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) and ethidium homodimer were purchased from Sigma-Aldrich (St. Louis, MI, USA). Square glass slides (25 × 25 mm^2), round glass slides (1 cm diameter), phosphate buffered saline (Ca^{2+} and Mg^{2+} free) (PBS), Cyanmethemoglobin Standard and Drabkin's reagent were purchased from Fisher Scientific (Hampton, NH, USA). Tecothane TT-1055D (PU), medical grade aromatic polyether-based thermoplastic polyurethane, was kindly donated by Lubrizol (Wilmington, DE, USA). Calcein, Calcein Violet AM and 7-amino-actinomycin D (7-DAA) were purchased from Invitrogen (Carlsbad, CA, USA). Prior to analyses, the surfaces were sterilized with ethanol.

Epifluorescence microscopy was performed using an Olympus IX70 microscope (Olympus, Melville, NY, USA) outfitted with a Chroma Photofluor metal halide light source (89 North, Burlington, VT, USA). Images were captured using a SensiCam QE camera (The Cooke Corp., Romulus, MI, USA) (2 × 2 binning, 688 × 520). IPLab software was used for image acquisition and to control the LUDL programmable filter wheels, shutters, and focus (Ludl Electronic Products, Hawthorne, NY, USA). ImageJ (NIH, Bethesda, MD, USA) was used for image analysis. Confocal microscopy was performed on an Olympus IX81 with Fluoview FV1000 controller. Fluoview 1.6 was used for image acquisition and ImageJ was used for analysis. Plate reader assays were performed using CHAMELEON™V (Hidex, Turku, Finland). Flow cytometry was performed using BD FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA, USA). Two-color analysis was performed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cleaning of glass surfaces

The glass slides (round for blood incubation studies and square for all other studies) were cleaned prior further modification using "piranha" solution (70% H_2SO_4 and 30% H_2O_2) for 20 min at 80 °C, extensively rinsed with deionized water, blown dry with compressed N_2 (g) and exposed to ultraviolet light in a UVO-cleaner (UVO Cleaner model 42, Jelight Co. Inc., Irvine, CA, USA) for 10 min.

Preparation of DEX and DEX-Ag surfaces

DEX and DEX-Ag surfaces were prepared as previously reported (Ferrer et al., 2012). Briefly, dextran was oxidized using $NaIO_4$ (1:1 molar ratio) at a concentration of 50 mg/mL for 6 h, dialyzed for at least 3 days and lyophilized. Ag NPs were synthesized in an aqueous solution of oxidized dextran (2 mL, 1 g/L) using $AgNO_3$ as a precursor agent (1 mL, 2 mM) at 70 °C for 30 min. Prior grafting the coatings, surface amination was carried out on glass using the vapor deposition method for 3 h at 70 °C. The aminated surfaces were then submerged in 3 mL of aqueous solution of oxidized dextran (1 mg/mL) or freshly made silver nanoparticles in oxidized dextran containing 75 μL of aqueous solution of $NaBH_3CN$ (100 mg/mL) and were allowed to react in dark conditions overnight. After immobilization, the grafted surfaces were removed from solution, submerged in deionized water for 5 min, rinsed and blown dry with compressed N_2 (g).

Preparation of PU surfaces

PU surfaces were prepared as described somewhere else (Ferrer et al., 2010). Briefly, 250 μL solution of PU (tetrahydrofuran/dichloroethane 1:1, 0.75 wt.%) was spun coated (2000 rpm, 15 s) on glass. The coated surfaces were allowed to dry overnight in the hood.

Blood collection and preparation of platelet rich plasma

Following the UPENN Institutional Review Board protocol, blood was drawn from healthy volunteers by venipuncture into acid citrate (hemolysis assay and blood incubation test) or acid citrate dextrose (platelet adhesion test) as anticoagulant. Platelet-rich plasma (PRP) was isolated from fresh blood by centrifuging at 3500 rpm for 20 min.

Hemolysis

The hemolysis assay was performed in agreement with standard ASTM F56-08 practice, a colorimetric assay that measures the release of cyanmethemoglobin in solution (ASTM-F756–08, 2000). According