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The surface molecular functionality of decellularized extracellular matrices

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ABSTRACT

Decellularization of tissues and organs is a successful platform technology for creating scaffolding materials for tissue engineering and regenerative medicine. It has been suggested that the success of these materials upon implantation is due to the molecular signals provided by the remaining scaffold extracellular matrix (ECM) components presented to probing cells *in vivo* as they repopulate the surface. For this study, decellularized matrices were created from esophagus, bladder, and small intestine harvested from adult male Fischer 344 rats. The three decellularized matrices (each originating from source tissues which included an epithelial lining on their luminal surfaces) were immunostained for collagen IV and laminin to determine basement membrane retention. Scanning electron micrographs of the surfaces were used to provide insight into the surface topography of each of the decellularized tissues. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) was used to generate high-resolution mass spectra for the surfaces of each scaffold. This surface-sensitive technique allows for detailed molecular analysis of the outermost 1–2 nm of a material and has been applied previously to thin protein films and secreted ECM proteins on poly(N-isopropyl acrylamide) (polyNIPAAM) surfaces. To extract trends from within the complex ToF-SIMS dataset, a multivariate analysis technique, principal component analysis (PCA), was employed. Using this method, a molecular fingerprint of each surface was created and separation was seen in the PCA scores between the decellularized esophagus and the decellularized small intestine samples. The PCA scores for the decellularized bladder sample fell between the previous two decellularized samples. Protein films of common extracellular matrix constituents (collagen IV, collagen I, laminin, and Matrigel) were also investigated. The PCA results from these protein films were used to develop qualitative hypotheses for the relationship of the key fragments identified from the PCA of the decellularized ECMs.

1. Introduction

Decellularized tissue matrices are widely used for tissue engineering and regenerative medicine [1–4]. It is suggested that their success is due to embedded biospecific signals found within their protein structures. These materials have been created from a variety of source tissues both allogeneic and xenogeneic [5]. The extracellular matrix (ECM) proteins which comprise the bulk of these materials, are highly evolutionarily conserved [6–8]. This sequence similarity helps to explain their favorable immune response upon implantation. The cellular components of tissue are primarily responsible for the antigenicity and adverse response when implanted in non-autologous hosts [5].

It is probable that the body may take embedded signaling cues from the biomolecular structures that comprise these decellularized extracellular matrices and uses these cues to direct the *in vivo* remodeling process. Acellular tissues have been explored in numerous applications including full heart constructs [8], cardiovascular grafts [9], heart valve [10,11], nerves [12], skeletal muscle [13], liver [14], bladder [15], esophagus [1,2], and skin [16] among others.

Decellularized tissue scaffolds present a particularly challenging characterization problem due to their complex arrangement of

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interacting ECM components and the probable alterations of these structures during the decellularization process. Many different chemical and mechanical decellularization techniques have been employed and each method has the potential to alter the native three-dimensional ultrastructure of the ECM uniquely [5,17]. However, largely, these ECM scaffolds induce a constructive remodeling response and favorable clinical outcome. To date, characterization methods for decellularized matrices have included mechanical property testing along with imaging techniques such as scanning electron microscopy and immunohistochemistry [5,17].

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) represents a powerful surface-sensitive analytical method for the characterization of implantable decellularized materials. Using an energetic primary ion beam to eject surface species, the resulting secondary ions are collected and detected with a time-of-flight mass analyzer to yield an information-rich spectrum. This technique can detect all elements with masses ranging from hydrogen to molecules up to several thousands of Daltons without the need for specific markers or the addition of an analysis matrix. However, due to bombardment with highly energetic ions, the analyte is subject to fragmentation, which can complicate data interpretation. Each ToF-SIMS spectrum thus represents a complete molecular fingerprint of the outermost 1–2 nm of the surface under analysis. For every spot analyzed, hundreds of individual spectral features can be identified. Multiple spots (spectra) are taken per sample and the spectra are overlaid so that comparisons of individual peak variance relationships of within-group and between-group changes can be assessed. To understand such complex data sets, multivariate analysis (MVA) techniques have been applied to statistically reduce the complexity to manageable patterns of captured variance [18–21]. For this study, principal component analysis (PCA) was employed. In PCA, a set of new variables called principal components (PCs) are calculated which represent new axes within the data space [22,23]. These new axes, or PCs, now bisect areas of variance within the original dataset. The first PC will capture the largest percentage of the variance in the dataset while each subsequent PC is orthogonal to its predecessor and captures sequentially less variance until there are no longer identifiable trends. Specifically, PCA has been used previously to determine a set of protein-related peaks within ToF-SIMS data sets which can be used to compare amino acid related structures [18,19,24]. Variations in the amino acid fragment intensities can be related to the identity, conformation and orientation of surface bound proteins [25].

ToF-SIMS characterization of decellularized ECM-based scaffolds is analytically challenging because of the scaffolds' surface chemical and structural complexity. This surface represents the first contact a given cell would encounter once seeded (*in vitro*) or repopulated (*in vivo*). With this in mind, characterizing surface chemistry precisely could provide vital insight into the mechanisms behind the *in vivo* success of decellularized matrices. ToF-SIMS has the power to assess the molecular chemistry and some aspects of the molecular organization associated with the outer layer. The challenge lies in the interpretation of the data. Previously in the characterization of biomaterials, this technique has been used to study adsorbed model protein films [26]. In a recent study, ECM proteins were analyzed after cell lift-off from poly(N-isopropyl acrylamide) (polyNIPAAM) [19,20]and compared to an adsorbed protein film model. With the detailed molecular characterization of the surfaces investigated in the present study, researchers will have an enhanced understanding of the cellular interactions that occur upon implantation. This knowledge could help to improve functionality of future generations of these naturally-derived materials as well as guide the production of successful synthetic mimetics that incorporate molecular specificity [21].

2. Materials and methods

2.1. Decellularized ECM preparation

Fischer 344 adult male retired breeder rats were obtained (Taconic Labs, Inc.) and euthanized with CO_2. The esophagus, bladder, and central portion of the small intestine were excised and placed in phosphate buffered saline (PBS, Sigma–Aldrich) plus 1% antibiotic/antimicotic (A/A, Gibco) and shaken gently on an orbital shaker at 25 °C for 10 min. Tissues were then transferred into a 10 mM Tris(Sigma–Aldrich), 5 mM ethylene-diaminetetraacetic acid (EDTA, Fisher Scientific), 0.2 mM phenylmethanesulfonyl fluoride (PMSF, Sigma–Aldrich) and 1%(v/v) A/A in deionized water (dH_2O) solution at pH 8.0 for 48 h at 4 °C shaken gently on an orbital shaker. For the main decellularization step, tissues were soaked in a Tris/EDTA/PMSF dH_2O solution with 0.1%(w/v) sodium dodecyl sulfate (SDS, Fischer) for 24 h at 25 °C and shaken gently on an orbital shaker. After 24 h, PBS was used to rinse the sections three times each for 10 min with gentle shaking. Tissues were then rinsed in a solution of 0.1%(v/v) peracetic acid (Sigma–Aldrich) in a 4%(v/v) ethanol in dH_2O solution for 24 h at 4 °C. Sterile PBS was used to rinse the previous solution from the tissue sections (3× each for 10 min at 4 °C with gentle shaking) and the tissue sections were critical point dried and stored under nitrogen for no longer than three weeks at 4 °C before use. The nomenclature scheme used in this work is: decellularized esophagus (dESO), decellularized bladder (dBLAD), decellularized small intestine (dSI) and general decellularized ECM (dECM).

2.2. Immunohistochemistry

To assess degree of decellularization and protein retention after decellularization, immunohistochemistry was performed. Samples were fixed in Methyl Carnoy's fixative (60% methanol, 30% acetic acid, 10% chloroform) overnight at 4 °C and embedded in paraffin. Serial 5 μm thick sections were cut. Deparaffinization of slides was performed as follows: xylenes (3 × 5 min), 100% ethanol (2 × 3 min), 95% ethanol (1 × 3 min), 70% ethanol (1 × 3 min), 50% ethanol (1 × 3 min), phosphate buffered saline (PBS) (2 × 2 min). One set of slides was stained by a standard hematoxylin and eosin protocol (Sigma–Aldrich). For the immunohistochemistry, sections were rinsed in a 3% hydrogen peroxide in methanol solution for 30 min and subsequently rinsed in PBS (3 × 5 min). For antigen retrieval, a 0.01 M citrate buffer pH 6 was made and heated to boil in a microwave oven. Slides were placed in the hot buffer for 10–15 min and subsequently rinsed in PBS (3 × 5 min). Sections were circled on the slides with a hydrophobic pen and blocked overnight at 4 °C in 4% normal goat serum in 0.25% BSA. The following day, sections were incubated with rabbit anti mouse polyclonal collagen IV antibody (1:500 dilution, Abcam) and rabbit anti mouse polyclonal laminin antibody (1:25 dilution, Abcam) for 1 h at 25 °C in the 4% blocking solution. Sections were subsequently rinsed in PBS (3 × 5 min). The secondary horse anti-mouse (Vector Labs) was diluted (1:200) in 2% blocking serum and used for incubation of each section for 30 min at 25 °C and subsequently rinsed in PBS (3 × 5 min). An avidin–biotin kit (ABC, Vector Labs) was used as per manufacturer instructions for 30 min at 25 °C and sections were subsequently rinsed in PBS (3 × 5 min). The peroxidase substrate, 3,3′-diaminobenzadine (DAB, Sigma–Aldrich) was prepared as per manufacturer instructions and sections were incubated while being visualized under a microscope to time the color change for subsequent section staining intensities. Tissues were rinsed in PBS (3 × 5 min). Sections were dipped in hematoxylin (Sigma–Aldrich) for 10 min for a nuclear counterstain and subsequently rinsed in PBS (3 × 5 min). Sections were dehydrated as follows: 70% ethanol (quick rinse), 95% ethanol (1 min), 100% ethanol (2 × 1 min), Xylene (1 min), Xylene (3 min), Xylene (5 min). Sections were coverslipped with Permount (Sigma–Aldrich). For the additional location of collagen IV and laminin in the alternate fixative solutions, intact rat organs (esophagus, bladder, small intestine) were explanted and fixed overnight at 4 °C in both 10% neutral buffered formalin and zinc fixative solution (both from Sigma–Aldrich). Then, the immunohistochemistry procedure from above was repeated with a primary incubation step overnight at 4 °C (as opposed to 1 h at 25 °C) with no other changes.

2.3. Scanning electron microscopy

Dried samples were sputtered with gold and viewed with an FEI Sirion SEM at the UW NanoTech User Facility. SEM images were taken at 70× magnification for gross identification of structures and 1000× magnification for studying the fine structures.

2.4. Preparation of extracellular matrix protein films on mica

The extracellular matrix proteins collagen I, collagen IV, laminin, and a basement membrane extract (Matrigel) were adsorbed onto mica at 37 °C for 2 h in their respective buffers (all proteins were purchased from BD Biosciences). Briefly, mica was cut into 1 cm squares and cleaved with tape. Laminin and Matrigel were diluted in 50 nM tris buffered saline (TBS, Invitrogen Corp.) pH 7.5 and collagen I and collagen IV were diluted in 0.05 N HCl. All proteins were reconstituted at a concentration of 100 μg/ml. Once protein adsorption was complete, each of the samples were washed