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Quantifying the Surface Chemistry of 3D Matrices *in situ*

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ABSTRACT

Despite the major role of the matrix (the insoluble environment around cells) in physiology and pathology, there are very few and limited methods that can quantify the surface chemistry of a 3D matrix such as a biomaterial or tissue ECM. This study describes a novel optical-based methodology that can quantify the surface chemistry (density of adhesion ligands for particular cell adhesion receptors) of a matrix *in situ*. The methodology utilizes fluorescent analogs (markers) of the receptor of interest and a series of binding assays, where the amount of bound markers on the matrix is quantified via spectral multi-photon imaging. The study provides preliminary results for the quantification of the ligands for the two major collagen-binding integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$) in porous collagen scaffolds that have been shown to be able to induce maximum regeneration in transected peripheral nerves. The developed methodology opens the way for quantitative descriptions of the insoluble microenvironment of cells in physiology and pathology, and for integrating the matrix in quantitative models of cell signaling.

Keywords: Biomaterials, Surface chemistry, Adhesion Ligands, Extracellular matrix, Spectral Microscopy

1. INTRODUCTION

Cells perceive their environment using plasma membrane receptors that bind to molecules located in their immediate environment. Binding of a receptor to such a molecule (a “regulator”) induces a cascade of intracellular chemical events (“cell signaling”). Regulators can be classified as soluble (molecules in solution that reach cell receptors via diffusion such as cytokines, hormones, and growth factors), and insoluble (molecules that exist in the solid state and form solid structures where cells can apply forces). The insoluble microenvironment of cells (matrix) refers to either the extracellular matrix (ECM) in tissues, or biomaterials/implants fabricated by humans that interact with cells *in vivo*. ECM is a complex inter-connected network of macromolecules including structural proteins (collagens, elastin), adhesion proteins (fibronectin, laminin), proteoglycans, glycosaminoglycans, and bound soluble factors [1,2]. The composition of biomaterials is usually simpler (few natural or artificial biopolymers) [3]. Several pieces of experimental evidence suggest the critical importance of the matrix in cell regulation. For example, after severe organ injury, the fate of the wound healing process depends critically on whether the defect includes stroma (ECM-rich tissue) [4]. In cancer biology, tumor-associated stroma is associated with carcinogenesis initiation, tumor progression, and metastasis [5]. *In vitro* experiments suggest that stem cell binding to ECM is essential for maintaining stem cell properties and guides asymmetric stem cell division [6]. Despite its importance, the insoluble microenvironment of cells is much more complex to describe compared to the soluble microenvironment. One reason is that it is described by a larger number of properties (including chemical composition, surface density, topology, stiffness, degradation rate, and stress field), several of which can only be measured *in situ* (without matrix digestion).

One of the key properties of the matrix around cells is its surface chemistry, defined as the identity and surface density of adhesion ligands available to cells for binding. The surface chemistry of a matrix defines which adhesion receptors can be utilized by cells for adhesion, controls the signals sensed by cells and the resulting cell response. Despite its importance, there are very few published methods for quantifying the surface density of matrices, and the latter can be applied only to a small class of artificial biomaterials [7, 8].

This paper attempts to fill part of this technology gap and describes a novel optical-based methodology for quantifying the surface chemistry of a matrix. The methodology combines fluorescent protein technology and spectral multi-photon microscopy and can be used to measure *in situ* the density of ligands for particular adhesion receptors in 3D matrices (tissue ECM or biomaterials). It focuses on the methodology and its implementation, and provides preliminary measurements of the surface chemistry of porous collagen scaffolds, similar to the ones used clinically to induce regeneration in transected peripheral nerves.

2. MATERIALS AND METHODS

2.1 Development, Purification, and Characterization of Adhesion Ligand Markers

The proposed methodology utilizes soluble adhesion markers that have similar binding properties (specificity and affinity) as the adhesion receptor of interest. Such adhesion markers include soluble recombinant domains of adhesion receptors that mediate receptor adhesion to its ligands (provided it is feasible to identify and express these domains). Such soluble markers can recognize and bind to ligands present on the surface of a matrix.

This study focuses on quantifying the ligands of the two major collagen-binding integrins ($\alpha_1\beta_1$ and $\alpha_2\beta_1$). For each integrin, two kinds of soluble markers are expressed in e-coli and purified by GST affinity chromatography: the non-fluorescent adhesion marker (denoted as α_i I) that consists of the I domain of the α subunit of the corresponding integrin (the domain that mediates binding of a collagen-binding integrin to its ligands [9], Figure 1a), and the fluorescent version (denoted as TC- α_i I) of the adhesion marker that consists of the tetracycline (TC) tag WDCCPGCCK fused at the N-terminus of the non-fluorescent marker. TC-tagged I domains are not intrinsically fluorescent, however they become fluorescent after the small biarsenical dye FIAsh binds to the TC tag [10]. After purification, soluble I domains are characterized by several biochemical methods (SDS-PAGE, protein assays, circular dichroism spectroscopy, BIACORE) in order to verify their purity, measure accurately their concentration, verify that they are correctly folded, and that the presence of the TC entity does not affect binding of I Domains to their ligands.

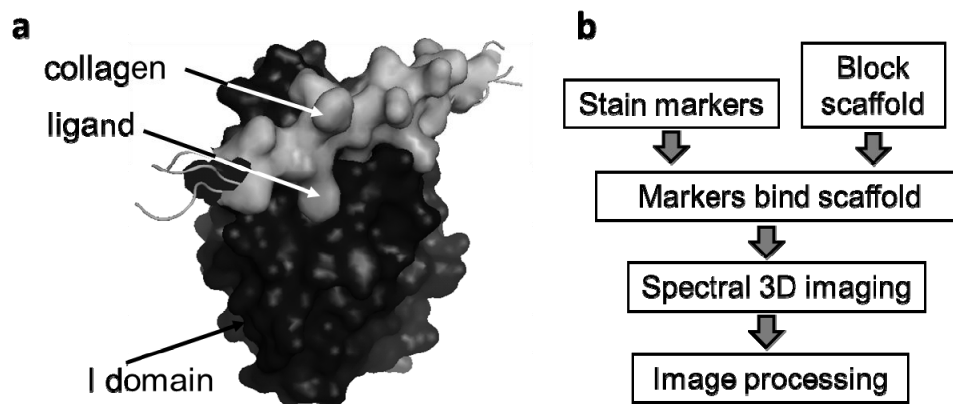


Figure 1. Key features of the proposed methodology for quantifying the surface chemistry of 3D matrices. **a:** Rendering of the I domain of integrin α_2 binding to a collagen-like peptide that contains the GFOGER ligand. A fluorescent version of this molecule expressed in e-coli is used as a fluorescent marker for the ligands of integrin $\alpha_2\beta_1$. Based on structure 1DZI [11] rendered using Pymol 1.0r1. **b:** Flow diagram of the protocol for the binding assay of soluble fluorescent I domains to samples of collagen scaffolds.

2.2 Binding Experiments in Porous Collagen Scaffolds

I domains have been used extensively in studies of collagen-binding integrins [12-15]. However, this is the first case that a fluorescent version of I domains is utilized as a marker in order to measure the surface chemistry of a 3D matrix *in situ*. This was achieved by performing a series of binding experiments where fluorescent markers interact with the matrix and ones that bind on the matrix are detected by 3D multi-photon imaging. Specifically, the matrix samples are blocked (Superblock in PBS, Thermo scientific), and then are treated with solutions of fluorescent markers (in the presence or absence of non-fluorescent markers) of increasing concentration. Solutions of TC-tagged I domains are fluorescently stained by treating them first with 1 mM TCEP reducing agent (to reduce the cysteines of the TC tag) and then with a slight excess (10%) of FIAsh biarsenical dye. The excess unbound FIAsh dye needs to be removed by gel filtration before utilizing the fluorescent markers, because FIAsh is known to bind non-specifically to blocking agents and the resulting background signal would induce large measurement errors. The binding reaction takes place in the presence of either 2 mM Mg+2 (signal experiment) or 10 mM EDTA (control experiment; integrin binding to collagen requires Mg+2). After overnight incubation with fluorescent I domain solutions, the samples are imaged in a 16-channel spectral multi-photon microscope with single-photon counting detection capability (Figure 1b).

This study provides preliminary measurements of the surface density of ligands for integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ on cylindrical samples (3mm diameter, 3mm thick) of porous collagen scaffolds (0.5% solid fraction, approximately 96 μm mean pore diameter) fabricated by freeze-drying a suspension of micro-fibrillar collagen I (Integra Life Science Ltd) in 0.05M acetic acid and cross-linked by dehydro-thermal treatment (48h at 120°C at 50 mTorr vacuum) [16]. The scaffolds are fabricated in 60×60×3 mm sheets and are cut into cylindrical samples using biopsy pouches. This biomaterial is identical to the one that recently showed optimal ability to induce regeneration in severely injured peripheral nerves [17].

2.3 Spectral Imaging and Image Processing

Three-dimensional images of the scaffolds that have been treated with solutions of fluorescent I domains were acquired using a custom made spectral multi-photon microscope. Specimen were excited by a femtosecond pulse laser (Spectra Physics Tsunami) of 4.8 mW power and 775nm wavelength through a Zeiss 40× C-apochromat 1.2 NA objective. 3D imaging (40 μs pixel acquisition time, 021 μm pixel size) was achieved by raster scanning using scanning mirrors (Cambridge Technology 6350) and a piezoelectric objective actuator (Physik Instrumente P-721.00). Emission was collected via the epi-luminescence path in de-scanned configuration, filtered by a dichroic mirror (Chroma Technology T700DCSPXRUV) and a band-pass filter (Chroma Technology ET680SP-2P8), spectrally resolved in a spectrograph (Oriol Instruments MS125-77 414), and detected by a 16-channel PMT (Hamamatsu R59000P-00-L16). The PMT outputs were processed and transmitted to a control PC by custom-made photon counting electronics [18]. Each channel of the 16-channel sensor detects photons within a 13.3 nm wide range of the spectrum, and that the 16 PMT channels detected photons in the (380, 592) nm range.

The acquired data were processed using custom-made software in MATLAB (Tzeranis et al., under preparation) due to the spectroscopic nature of the detector and the low signal level (less than 120 photons per pixel). Briefly, the emission of two fluorophores (FIAsH, collagen) present in the image is separated by spectral unmixing. The image pixels are then segmented into two classes: “scaffold” (contains collagen and FIAsH emission) and “solution” (contains only FIAsH emission). It is possible to reliably segment the pixels of the two classes due to the extra collagen emission in the scaffold pixels, as well as the fact that the emission rate of FIAsH in solution (less than 5 photons per pixel) is at least one order of magnitude less compared to the fluorescence emission of FIAsH in scaffold pixels. The large FIAsH signal inside the scaffold struts corresponds to FIAsH molecules bound to I Domains bound to ligands in collagen. For each experimental condition, the bound fluorescent signal is calculated based on the mean FIAsH intensity of the scaffold pixels that lie close (within 2 pixels) to the scaffold-solution interface. Measurements are taken in regions of interest are picked manually for consistency reasons (due to the random 3D nature of the scaffold struts it is necessary to take measurements at locations of similar geometry).

3. RESULTS

3.1 Purification, and Characterization of Adhesion Ligand Markers

Figure 2 shows a SDS-PAGE of the four purified fractions of non-tagged (α_1 , α_2) and TC-tagged ($T\alpha_1$, $T\alpha_2$) I domains. The same gel has been stained in two ways. First, all I domain samples were treated with FIAsH in the presence of TCEP [Adams et al. 2002] before electrophoresis. The top image shows a fluorescent picture of the gel, which reveals that only TC-tagged I domains bind to FIAsH and become fluorescent. The bottom image shows a picture of the gel after coomassie blue staining, revealing all protein content in the gel. The small difference in the size of non-tagged and tagged I domains correspond to the 1 kDa tetracysteine (TC) tag. The gel also reveals the presence of a second band around 48 kDa, that corresponds to I domains fused to the GST tag (after purification the GST entity is cleaved using thrombin, however this process is not 100% efficient).

3.2 Imaging Data

Figure 3 shows representative images from the binding experiments after spectral unmixing. Images correspond to optical sections of cylindrical samples from the same collagen scaffold that have been treated with various concentrations of fluorescent (FIAsH-stained TC-tagged) I domains in the presence or absence of Mg^{+2} cations. Optical sections were acquired 30 μm inside the collagen scaffold using the 3D imaging capability of the multiphoton microscope. Data reveal that in all samples, the signal intensity of collagen autofluorescence is similar (on the order of 5-8 photons per pixel). However, the intensity of FIAsH emission depends strongly on the concentration of fluorescent I domains and the presence of Mg^{+2} . Particularly, the presence of 10 mM EDTA removes Mg^{+2} cations and reduces significantly the observed FIAsH emission, which corresponds to fluorescent I domains bound on the scaffold. The

dependence of I domain binding on collagen on the presence of Mg^{+2} supports that I domains are well folded and that they bind specifically on the collagen surface. The observed FIASH emission is localized in the whole volume of the scaffold struts (each strut is on the order of 5 μm thick), suggesting that the scaffold strut is permeable to soluble molecules of 25 kDa size. The detected FIASH intensity can be converted into equivalent FIASH concentration (therefore I domain concentration) using a standard curve.

Figure 4 shows quantification results from binding experiments where collagen scaffold samples have been treated with solutions of fluorescent I domains (TC- $\alpha 1$ and TC- $\alpha 2$) of 0.75, 1.5, 3 and 6 μM concentration in the presence and absence of Mg^{+2} . In order to remove any non-specific binding, results from three experiments in the absence of Mg^{+2} are subtracted from results from three experiments in the presence of Mg^{+2} . FIASH emission has been converted into equivalent bound I domain concentration using a standard curve. In all cases, the detected signal in the presence of Mg^{+2} is always significantly larger compared to the detected signal in the absence of Mg^{+2} . These data, combined with an appropriate binding model and biochemical characterization of the elementary I domain-collagen binding can be used to estimate the total concentration of binding sites for I domains (therefore the total adhesion sites of the corresponding integrin) in the scaffold volume (Tzeranis et al. in preparation).

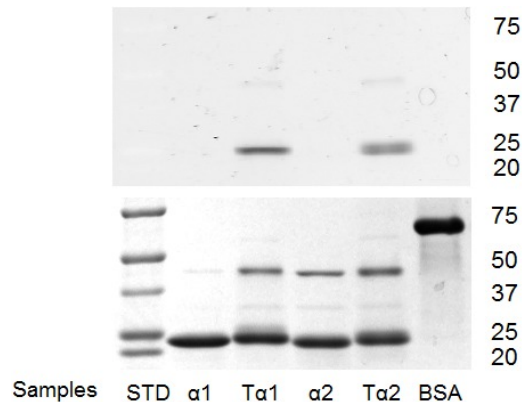


Figure 2. SDS-PAGE of purified fractions of non-tagged ($\alpha 1$, $\alpha 2$) and TC-tagged ($T\alpha 1$, $T\alpha 2$) I domains. All I domain samples were stained with FIASH in the presence of TCEP before electrophoresis [10]. Top image: fluorescent image of the gel reveals that only TC-tagged I domains become fluorescent. Bottom image: coomassie blue staining of the gel reveals all protein content. STD refers to protein standards (see scale on the right in kDa) and BSA refers to a bovine serum albumin sample (not FIASH stained).

4. DISCUSSION

The surface chemistry of the matrix is a critical property of the insoluble microenvironment of cells because it defines which adhesion receptors can be utilized, what kind of stimuli are provided by the matrix to the cell, and therefore how the matrix controls cell function. Despite its importance, there are very few and application-limited methods to measure the surface chemistry. Such measurements are hard due to the 3D topology of the matrix, and the complexity of its structure. Existing proteomic methods that could quantify the chemical composition of a matrix require digesting and homogenizing the matrix [1]. However, matrix digestion destroys the 3D structure of the matrix and provides average macroscopic entities that are not directly sensed by individual cells. On the other hand, *in situ* measurements can provide a more realistic description of the micro environment felt by cells.

This study presents a novel methodology that combines fluorescent protein technology and 3D imaging to quantify the surface chemistry of a 3D matrix *in situ*. The method is demonstrated in preliminary data for the two major-collagen binding integrins in porous collagen biomaterials used to induce regeneration in peripheral nerves [17]. Although the data presented focus on collagen and collagen-binding integrins, the methodology can be applied to study other kinds of matrices (e.g. biomaterials, ECM from decellularized tissues [19]) or ligands of other adhesion receptors. The proposed methodology is a step towards obtaining a quantitative description of the insoluble microenvironment around cells. Combined with quantitative mechanistic models of cell signaling, such information can reveal the mechanism of how different matrix properties regulate cell behavior. This can help to explain the role of ECM in physiology and pathology, and guide the principle-based development of novel biomaterial-based treatments for disease and regenerative medicine.

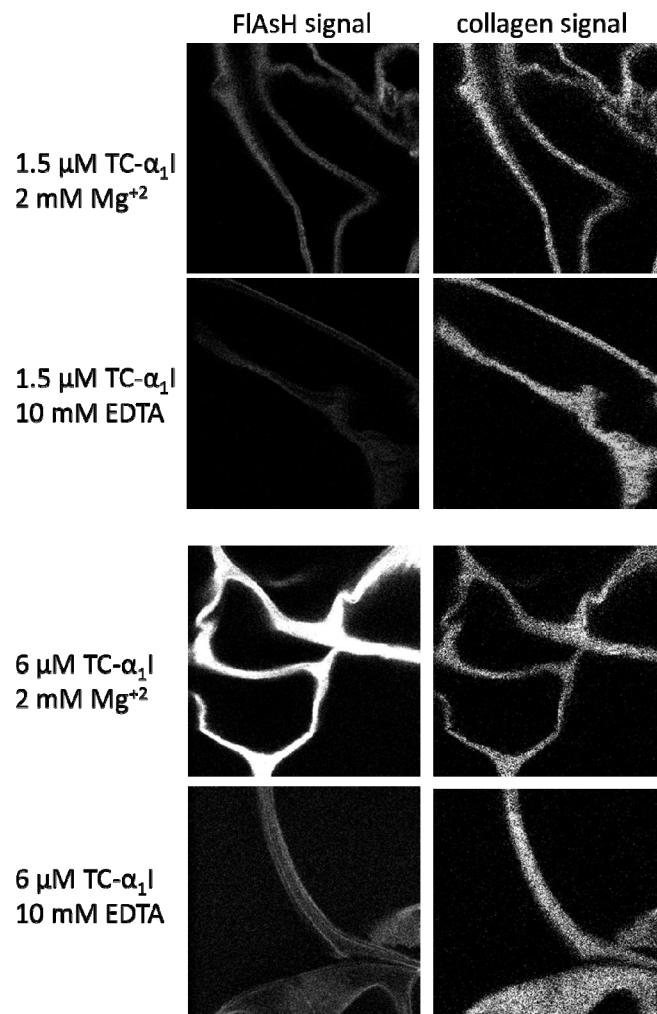


Figure 3. Representative images of collagen scaffolds treated with 1.5 or 6 μM FLAsH-stained TC-tagged $\alpha_1\text{I}$ domains in the presence or absence of Mg^{+2} cations after spectral unmixing. The first column shows the fluorescence emission of FLAsH. The second column shows the fluorescence emission of collagen. All images display a $55 \times 55 \mu\text{m}$ area. All collagen images are scaled in the 5 – 70 photons/pixel range. All collagen images are scaled in the 0 – 10 photons/pixel range.

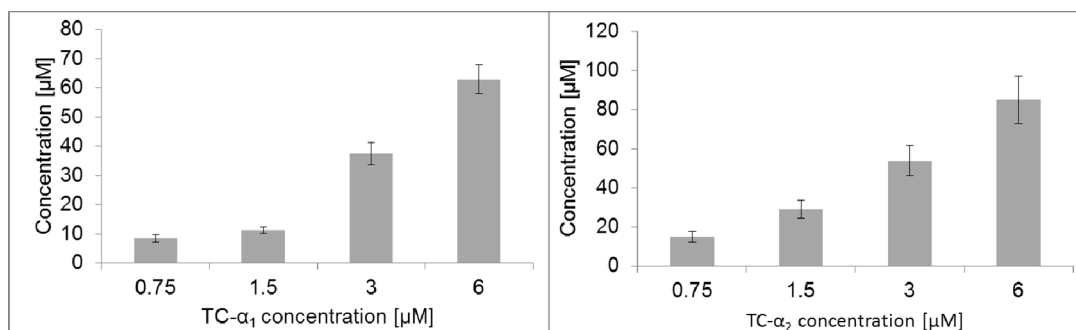


Figure 4. Mean concentration of FLAsH-stained TC-tagged I domains that bind to the collagen scaffold volume as a function of I domain concentration. Data correspond to the difference of the mean of three experiments in the presence of Mg^{+2} minus the mean of three experiments in the absence of Mg^{+2} cations. Error bars correspond to the sum of the standard deviations of the mean of the two groups.

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