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Direct quantification of dual protein adsorption dynamics in three dimensional systems in presence of cells

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ABSTRACT

Understanding the composition of the adsorbed protein layer on a biomaterial surface is of an extreme importance as it directs the primary biological response. Direct detection using labeled proteins and indirect detection based on enzymatic assays or changes to mass, refractive index or density of a surface have been so far established. Nevertheless, using current methodologies, detection of multiple proteins simultaneously and particularly in a three-dimensional (3D) substrates is challenging, with the exception of radiolabeling. Here using fluorescence molecular tomography (FMT), we present a non-destructive and versatile approach to quantify adsorption of multiple proteins within 3D environments and reveal the dynamics of adsorption of human serum albumin (HSA) and fibrinogen (Fib) on 3D polymeric scaffold. Furthermore, we show that serum starved human articular chondrocytes in 3D environment preferentially uptake HSA over Fib and to our knowledge this represents the first example of direct visualization and quantification of protein adsorption in a 3D cell culture system.

Statement of Significance

The biomaterial surface upon exposure to biological fluids is covered by a layer of proteins, which is modified over a period of time and dictates the fate of the biomaterial. In this study, we present and validate a new methodology for quantification of protein adsorption on to a three-dimensional polymer scaffold from unitary and binary systems, using fluorescence molecular tomography, an optical transillumination technique with picomolar sensitivity. In additional to being able to follow behavior of two proteins simultaneously, this methodology is also suitable for studying protein uptake in cells situated in a polymer environment. The ability to follow protein adsorption/uptake in a continuous manner opens up new possibilities to study the role of serum proteins in biomaterial compatibility.

1. Introduction

Recent decades have witnessed a tremendous increase in the use of biomaterials (natural and synthetic) in the human body for therapeutic purposes [1]. The exposure of biomaterials to plasma invokes an immediate response that involves adsorption of different serum proteins, which regulates future biological events at the implant site such as inflammatory responses, cell migration, attachment[2], infection[3,4] and ultimately the fate of the biomaterial. Considering the importance of this event, gaining more knowledge about the protein adsorption dynamics and competition between the proteins can provide insights for designing more compatible and functional biomaterials. Quantitative information on protein adsorption can be obtained using several direct and indirect techniques. Techniques such as radiolabeling [5,6] and fluorescence labeling (for example OPA (*o*-phthaldialdehyde), fluorescamine, and NanoOrange) [7–9] and enzyme-linked immunosorbent assay (ELISA) offer direct quantification of proteins. Other techniques involve absorbance shift in dyes upon binding to proteins (Bradford assay) or reduction of metal ions in presence of proteins leading to color change (bicinchoninic acid assay (BCA)) that can be measured photometrically [10,11]. Generally, fluorescence-based techniques provide a wider dynamic range, higher sensitivity and higher signal to noise ratio

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than absorbance-based techniques [7]. On the other hand neutron reflectivity [12], quartz crystal microbalance [13], ellipsometry and optical waveguide light mode spectroscopy while capable of providing accurate information on mass and thickness of protein [14] and polymer layers [15] without the need for labels, are however only suitable for thin films, flat two dimensional substrates; and can only sample small areas. Although, simultaneous interrogation of up to three proteins can be achieved by using different radionuclide labels (^{3}H, ^{14}C, ^{125}I), the regulatory and safety issues, and environmental concerns surrounding the use of radioactive tracers have made this approach less practical. Recently a new approach called label-free snapshot proteomics has been used to obtain quantitative time-resolved profiles of human plasma coronas formed on nanoparticles [16]. It is however evident that a non-destructive method, which is capable of measuring protein adsorption within a three dimensional (3D) environment is lacking and such a technique if realized has the potential to further our understanding of protein adsorption processes and can lead to improved forecasting of the protein composition on a biomaterial surface.

Here we report the implementation and validation of a methodology for quantification of the dynamics of adsorption of multiple proteins with the ability to quantify within living systems using a trans-illumination technique - fluorescence molecular tomography (FMT). The primary advantages of FMT are that: (1) it uses near-infrared (NIFR) radiation to excite fluorophores and thus provides high specificity and sensitivity in the picomolar range in the detection of a fluorescent signal within a biological environment, (2) the band width of the NIFR spectral window enables simultaneous visualization of up to four different fluorophores, and (3) being a tomographic technique information can be gained from within a 3D system. We have successfully used FMT to quantify fluorophores within rats with ~95% recovery of fluorescence in 3D reconstructions within 20-mm-thick optical phantoms [17,18]. Furthermore, we have established the linearity of the NIFR fluorescent agents VivoTag 750 over a concentration range of 78–2500 nM [17]. In this study, using human serum albumin (HSA, molecular weight 67 kDa) and Fibrinogen (Fib, molecular weight 340 kDa), two of the most abundant proteins in the human blood plasma (serum concentration: of 35–50 mg/ml and 2–4 mg/ml, respectively) as the model proteins and a commercially available nonwoven surgical-grade fibrous matrix of polyethylene terephthalate (PET), a non-degradable, biocompatible polymer with long history of use in vascular grafts [19] where protein adsorption is eminent; as the model synthetic polymer system, we demonstrate that FMT can provide quantitative information on the adsorption of these proteins from unitary and binary solutions on non-modified, pre-protein-modified and cell-laden PET surfaces.

2. Materials and methods

2.1. Preparation of PET scaffolds

Nonwoven PET meshes (Ø: 4 mm, thickness: 1.2 mm, ~2.50 mg) were cut from PET matrices (Surgical Mesh company PETNF203) with biopsy punch. Samples were soaked in 1% (v/v) tween-20 in DI water and agitated in 60 °C for 35 min in reactor (Buchi Syncore® Reactor R-24) to remove the dirt and fingerprints. Afterwards the solution was discarded and PET matrices were washed with DI water for three times. The hydrolysis process has been done based on the Bide patent [20], PET felts were gone through alkaline hydrolysis process with 2 molar sodium hydroxide in 98 °C for 27 min and afterwards PET felts were washed for three times with DI water, and finally air-dried. That reaction leads to break down of PET felts to terephthalic acid and ethylene glycol, fundamental monomers. Hydrolysis process introduces negative charge to the surface of the polymer mesh and makes it more favorable for protein adsorption and cell attachment. To ensure a hydrophilic surface PET scaffolds were hydrolyzed using sodium hydroxide (2 M, 95° C, 27 min), the presence of carboxylic acid groups was verified by colorimetric (STDB) assay [21] ([COOH]: 70 nmol/cm^2).

2.2. Visualization of HSA on PET fiber

PET felts were coated with HSA750, washed with Dulbecco's phosphate buffered saline (DPBS) to remove unbound and weakly bound proteins and directly imaged with fluorescence microscope (Zeiss Cell Observer-Z1, Germany).

2.3. Fluorescence labeling of HSA and Fib

Free primary amines on HSA and Fib were labeled by NHS-activated near infrared fluorescent (NIFR) dyes VivoTag 645 (VT645, Perkin-Elmer, USA) or VivoTag 750 (VT750, Perkin-Elmer, USA) dyes to be able to be visualized with FMT. For HSA the molar excess of dye/protein for both dyes was fixed to 4 whereas for Fib, the ratio was increased to 20 to compensate for its larger molecular weight (340 kDa) as compared to HSA (67 kDa). In adaptation to Vonwil et al. [17], 1 mg of HSA (96 %, Sigma-Aldrich, Germany, 57.9 nmol) was dissolved in 1 ml DPBS, 100 µl sodium bicarbonate buffer (1 M, pH 8.3), and reacted with 13 µl VT750 (10 mg/ml in DMSO, 57.9 nmol) for 4 h at room temperature. Unreacted dye was separated from the labeled HSA using a 10-kDa cutoff spin column (Amicon Ultra-0.5; Millipore 10 K). Fib (>95%, Merck Millipore, Germany) was reconstituted according to the manufacturers protocol. To 0.9 ml of this Fib solution (5 mg/ml, 9.2 nmol) 180 µl bicarbonate buffer were added and reacted for 4 h with 32 µl VT750 (10 mg/ml in DMSO, 270.5 nmol). Unreacted dye was removed by dialysis of the reaction mixture against a 50 kDa MWCO membrane in citrate buffer (20 mM, pH 7.4). From here on the dye-labeled proteins are referred to as HSA645, HSA750, Fib645 and Fib750, where the number denotes the wavelength of the excitation of the NIFR fluorophores.

2.4. µBCA assay

Samples were analyzed with commercially available µBCA assay protein kit (Thermo Fisher Scientific), following the instruction of the supplier. µBCA assay is routinely used for quantification of proteins and has a sensitivity of detection as low as 0.5 µg/ml of protein. The absorbance was measured at 562 nm using a plate reader (BioTek, Synergy HT) and the total amount of protein adsorbed to PET fibrous mesh was quantified using standard curve of HSA.

2.5. Circular dichroism

Circular dichroism (CD) spectra of HSA and fluorescently labeled HSA were obtained using a J-180 spectro-polarimeter (Jasco, Japan) equipped with a Peltier temperature cell PFD-425S (Jasco). Solutions of HSA and HSA645 and HSA750 with concentration of 0.2 mg/ml were made in 50 mM sodium phosphate buffer at pH 7.5. Samples were placed in a 1 mm path length quartz cell (Hellma, Germany) and CD spectra were recorded at 37 °C. Each spectrum is an average of four independent measurements. The spectra shown were corrected by subtraction of solvent background (50 mm sodium phosphate buffer). The CD spectra were normalized using scaling factors determined from the UV–visible absorbance at 224 nm and offset corrected.