An optical method to quantify the density of ligands for cell adhesion receptors in three-dimensional matrices

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The three-dimensional matrix that surrounds cells is an important insoluble regulator of cell phenotypes. Examples of such insoluble surfaces are the extracellular matrix (ECM), ECM analogues and synthetic polymeric biomaterials. Cell–matrix interactions are mediated by cell adhesion receptors that bind to chemical entities (adhesion ligands) on the surface of the matrix. There are currently no established methods to obtain quantitative estimates of the density of adhesion ligands recognized by specific cell adhesion receptors. This article presents a new optical-based methodology for measuring ligands of adhesion receptors on three-dimensional matrices. The study also provides preliminary quantitative results for the density of adhesion ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ on the surface of collagen-based scaffolds, similar to biomaterials that are used clinically to induce regeneration in injured skin and peripheral nerves. Preliminary estimates of the surface density of the ligands of these two major collagen-binding receptors are 5775 ± 2064 ligands μm^{-2} for ligands of $\alpha_1\beta_1$ and $17\,084 \pm 5353$ ligands μm^{-2} for ligands of $\alpha_2\beta_1$. The proposed methodology can be used to quantify the surface chemistry of insoluble surfaces that possess biological activity, such as native tissue ECM and biomaterials, and therefore can be used in cell biology, biomaterials science and regenerative medical studies for quantitative description of a matrix and its effects on cells.

Keywords: ligand density; biomaterials; extracellular matrix; collagen scaffolds; multiphoton microscopy; regenerative medicine/tissue engineering

1. INTRODUCTION

1.1. The need for new methods to quantify cell–matrix interactions in three-dimensional matrices

It has become clear that insoluble surfaces are capable of affecting cell phenotypes. Historically, cell regulation was considered to be the domain of soluble regulators (cytokines, growth factors, hormones). In the biological and medical research communities, little attention has been traditionally paid to regulation of cell phenotypes by insoluble regulators (matrices) such as the extracellular matrix (ECM) of tissues or the surfaces of biomaterials. Instead, studies of biologically active insoluble surfaces have originated mostly

from scientists and engineers familiar with synthesis of solid materials, i.e. materials that have well-defined surfaces.

Cells bind to their surrounding matrix at specific sites (adhesion ligands) available on the matrix surface by using their adhesion receptors. Cells can use a particular type of cell adhesion receptor only if the surrounding matrix provides the specific ligands that this receptor recognizes. The resulting cell response depends on the particular set of receptor types that cells use for mediating adhesion (Heino 2000; Larsen *et al.* 2006). Therefore, a major determinant of the microenvironment sensed by a cell is the surface chemistry of the matrix, which is defined in this article as the identity and density of the ligands that the matrix makes available to cells.

The surface chemistry of a matrix, in combination with the geometry and the stiffness of the matrix, regulates the cell–matrix adhesion pattern, which has been shown to affect the way cells sense and respond to their

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microenvironment (Cukierman *et al.* 2002). Experimental data obtained *in vitro* suggest that cells cultured inside porous matrices or gels behave in a significantly different manner than do cells of the same type that are cultured on standard flat surfaces (Maaser *et al.* 1999; Cukierman *et al.* 2002; Sefcik *et al.* 2008). *In vivo* cells are regulated by their surrounding matrix, which they simultaneously remodel. In many important physiological conditions, this bidirectional interaction may need to be prevented (tumour growth and metastasis) or regulated (tissue engineering and regenerative medicine).

The following sections review the role of the matrix as a regulator of cell phenotypes in various biological phenomena, and highlight the current lack of tools for quantifying the surface chemistry of matrices.

1.2. Induced regeneration with collagen-glycosaminoglycan scaffolds

A very small number of biomaterials have been shown so far to possess regenerative biological activity. Such materials are able to induce regeneration (synthesis of new physiological tissue) in injured organs, and prevent the spontaneous adult wound healing response consisting of contraction and scar formation (Yannas 2001, 2005). Porous collagen-glycosaminoglycan scaffolds (CGSs) are a major example of such biologically active materials. CGSs have been used over many years to induce regeneration in adult animals and humans in severely injured dermis (Yannas *et al.* 1982, 1989), peripheral nerves (Chamberlain *et al.* 1998) and the conjunctiva (Hsu *et al.* 2000). The regenerative activity of CGS grafts has been shown to depend strongly on three parameters (referred to as structural determinants): mean pore diameter, degradation rate and chemical composition.

The effect of structural determinants on the wound healing outcome is most simply considered in terms of their effect on the volume density of adhesion ligands D that are available on the scaffold surface, and on the adhesion pattern of cells to the matrix. The chemical composition of CGS affects the set of adhesion receptor types that can be used by cells for binding. Given the scaffold chemical composition, the volume density of adhesion ligands D is inversely proportional to the scaffold mean pore diameter. Scaffolds with relatively small mean pore diameter (30–120 μm) result in extensive cell–scaffold adhesion at several locations all around the cell surface. Recent protocols for synthesis of cardiac tissue make extensive use of such ‘three-dimensional binding’ by culturing neonatal rat cardiac cells inside porous collagen scaffolds (Radisic *et al.* 2008a,b). At one extreme of the pore size range, scaffolds of very small mean pore diameter (less than 20 μm) strongly delay cell migration through the scaffold, possibly accounting for the observation that the scaffold plays no important role during wound healing. At the other extreme, in scaffolds of very large mean pore diameter (more than 200 μm), cells adhere to the scaffold only on one side of their surface, resembling the binding pattern seen on flat dishes.

There is need to develop new methods that can quantify the surface chemistry of porous biomaterials (such as CGS) *in situ* as a function of their structural determinants. Such a detailed description of the biological activity of biomaterials could guide the development of new biomaterials that could possibly induce regeneration in a wider range of injured organs.

1.3. Design and characterization of synthetic biomaterials

Although collagen-based scaffolds have a record of clinical accomplishment and are increasingly used in studies of tissue engineering and regenerative medicine (Shih *et al.* 2006; Radisic *et al.* 2008a,b; Sefcik *et al.* 2008), scaffolds based on synthetic polymers have been studied more extensively in experimental protocols (Claase *et al.* 2007; Dong *et al.* 2008; Petrie Aronin *et al.* 2008). A major objective of biomaterials design is to generate appropriate microenvironments that can influence specific cell types to express phenotypes of interest. Applications include tissue engineering, regenerative medicine and stem cell differentiation (Lutolf & Hubbell 2005; Hwang *et al.* 2007; Nakajima *et al.* 2007; Dawson *et al.* 2008; Hillel *et al.* 2008; Keskar *et al.* 2009).

Current research directed towards synthesis of tissues and organs *in vitro* has also been focused increasingly on designing appropriate microenvironments for cells. Authors have repeatedly argued that highly promising *in vitro* environments for synthesis of tissues and organs should possess a well-defined three-dimensional structure (Park *et al.* 2007; Timmins *et al.* 2007; Jakab *et al.* 2008; Radisic *et al.* 2008a,b). These three-dimensional structures are recognized as a necessary adjunct to the presence of cells and growth factors in an appropriate bioreactor environment (Pei *et al.* 2008).

A new generation of synthetic biomaterials is fabricated from elementary ‘building blocks’, which among others include ligands of cell adhesion receptors (Lutolf & Hubbell 2005). The quantity of added ligands is a critical part of the design of a new biomaterial, since it affects the intensity and identity of signals that cells sense owing to adhesion. Nevertheless, there are no established methods today that could be used to quantify the surface chemistry of a biomaterial. If available, such methods could quantify how various fabrication methods affect the surface chemistry of a biomaterial, and how this correlates with the resulting cell phenotypes. Critical quantitative information of this type that is required for the design and characterization of novel biomaterials is not available at this time.

1.4. Cell–matrix interactions between cancer cells and their surrounding matrix

Studies have demonstrated that cancer cell proliferation and migration can be affected by their surrounding matrix (Paszek *et al.* 2005). In a recent study, a CGS (identical to the CGS used for dermis regeneration) was used as a three-dimensional matrix model for studying pancreatic cancer cell proliferation. The study concluded that proliferation of several strains of pancreatic cancer cells requires attachment to the

scaffold via integrin $\alpha_2\beta_1$ (Grzesiak & Bouvet 2007). Another recent study demonstrated that collagenous matrix created *ex vivo* by cancer cells induces cancer-like phenotypes in normal cells *in vitro* (Amatangelo *et al.* 2005). It is known that the connective tissue around tumours is stiffer than normal connective tissue and that cancer cells express different cell adhesion receptors than normal cells (Paszek *et al.* 2005). At this time, tumour-associated connective tissue is analysed using methods of biomechanics, biochemistry and proteomics. There appear to be no published methods that can quantify the surface chemistry of the tumour microenvironment, especially the identity and density of ligands for those adhesion receptors that are upregulated or downregulated during the various stages of tumour progression.

1.5. Computational modelling of cell migration

Cell migration is a key phenotype for biological phenomena of great current interest such as wound healing and cancer metastasis. It has been observed that cell migration speed depends on the cell type and on several properties of the surrounding matrix (Lauffenburger & Horwitz 1996; Zaman *et al.* 2005). Several research groups have developed computational models that attempt to describe and predict how cell migration speed depends on various properties of the surrounding matrix (DiMilla *et al.* 1991; Lauffenburger & Horwitz 1996; Zaman *et al.* 2005, 2006). All proposed models predict that cell migration speed depends strongly on the number and distribution of cell adhesion receptors present on the cell membrane, and on the density of their corresponding ligands available on the surrounding matrix. The numerical values for the density of ligands present on the surrounding matrix used in these models are reasonable estimates based on experiments that provide indirect information. At this time, there appears to be no direct way to verify the validity and accuracy of such models by measuring *in situ* the density of adhesion ligands available on a matrix. If available, experimental measurements of surface chemistry could be integrated into analytical models to improve their accuracy and prediction capability.

1.6. Contribution of this study

This article describes a new optical method to quantify the density of ligands for cell adhesion receptors in three-dimensional matrices. The information obtained thereby can be used to characterize the surface chemistry of a three-dimensional matrix, describe the cell–matrix interactions mediated by particular adhesion receptors and describe the resulting effects on cell phenotypes.

2. CURRENT APPROACHES FOR QUANTIFYING ADHESION LIGAND DENSITY IN THREE-DIMENSIONAL MATRICES

The term ‘ligand’ in this study refers to the small chemical groups that are recognized by cell adhesion

receptors and serve as binding loci on a ‘parent’ biomolecule. The use of this term is distinct from the occasional use of the term ligand in the literature to describe the entire biomolecule.

The majority of published studies on cell–matrix interactions have used the standard *in vitro* culture system, where cells are incubated on a planar surface coated with a non-porous film of a biomolecule (usually an ECM protein). Researchers describe the density of available ligands indirectly, by estimating the mass of the biomolecule that is physically adsorbed or covalently cross-linked on the surface, and express it in units of biomolecule mass/area (Holub *et al.* 2003; Jirousková *et al.* 2007). A few studies have used three-dimensional *in vitro* systems, such as gels or porous biomaterials. In these studies, researchers describe indirectly the density of available adhesion ligands through the mass fraction of the chemical components that contain adhesion ligands. A few researchers have measured the mass of the biomolecule that is adsorbed or cross-linked on a surface. This was accomplished by detecting the signal emitted by an appropriately labelled adsorbed/cross-linked biomolecule, and then converting the measurement into biomolecule density (mass/area) using a standard calibration curve. Examples include measurements of radiolabelled fibronectin (Maheshwari *et al.* 2000; Valenick & Schwarzbauer 2006) or fluorescently labelled collagen (Engler *et al.* 2004) adsorbed on flat surfaces. Nevertheless, such indirect ways to describe adhesion ligand density are not sufficiently accurate, since the density of the parent biomolecule is not necessarily proportional to the density of adhesion ligands that are available to cells for binding.

A direct method to measure the density of ligands for cell adhesion receptors was recently developed (Barber *et al.* 2005; Harbers *et al.* 2005). In this method, a biologically inert surface is functionalized with peptides that contain the RGD ligand and a fluorophore connected through a linker that contains a chymotrypsin recognition site. Treatment of the material with chymotrypsin releases the fluorophores. The detected fluorescence signal emitted by the released fluorophores is then converted to the total amount of RGD ligands in the entire biomaterial via a calibration curve.

Another recently developed method provides information about the number of bound RGD ligands on cell adhesion receptors (Kong *et al.* 2006; Huebsch & Mooney 2007; Hsiong *et al.* 2008). In this method cells, whose membranes are stained with fluorescein, are incubated inside a gel of alginate molecules attached to peptides that contain the RGD ligand and the rhodamine dye. The number of cell adhesion receptors bound to fluorescently labelled RGD peptides is estimated by detecting the amount of Foerster resonance energy transfer (FRET) between fluorescein and rhodamine. FRET takes place when the distance between the two fluorophores is less than approximately 8 nm, a condition that occurs in the proximity of RGD ligands bound to a cell receptor. The FRET measurement is converted to the number of RGD peptides bound per cell through a calibration curve.

Surface chemistry measurements can be either average measurements over the entire volume of the

matrix (Barber *et al.* 2005), or local high-resolution measurements (Kong *et al.* 2006). High-resolution measurements inside thick matrices (e.g. gels or porous scaffolds) can be acquired using optical methods that possess depth discrimination capability, such as confocal microscopy (Pawley 2006), multiphoton microscopy (Denk *et al.* 1990) and several other methods developed over the past few years (e.g. PALM, STORM; Betzig *et al.* 2006; Rust *et al.* 2006).

The method described in this article combines fluorescent protein technology and multiphoton microscopy to measure *in situ* the density of ligands for particular adhesion receptors in three-dimensional matrices (either connective tissue in an organ or a biomaterial). It quantifies the surface chemistry of a matrix in a cell-centred way that imitates the way cells sense their environment, and fills a critical gap in cell–matrix interaction studies and biomaterial development. Use is made of a library of hybrid collagen–gelatin scaffolds as a means of studying, at one end, either porous surfaces rich in ligands (100% collagen) or, at the other end (100% gelatin), porous surfaces that lack such ligands for particular receptors. Although the results presented in this study focus on quantifying the density of ligands for the two major collagen-binding receptors (integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$) on collagen-based biomaterials (similar to collagen-based scaffolds referred to in Yannas *et al.* 1989), the proposed method can be appropriately generalized to quantify the density of ligands for several types of adhesion receptors in a wide variety of three-dimensional matrices.

3. EXPERIMENTAL METHODS

3.1. Scaffold fabrication

A library of hybrid collagen–gelatin scaffolds (hCGSs), each containing a different mass fraction of collagen relative to gelatin, was fabricated by lyophilizing an appropriate proper mix of collagen and gelatin suspensions. A 0.5% w/w collagen suspension was prepared by suspending type I collagen (Yannas *et al.* 1980) in 0.05 M acetic acid of pH 3.2, and homogenizing the suspension for 30 min in a blender (IKA, Wilmington, NC, USA) while keeping the slurry temperature below 9°C. A 0.5% w/w gelatin suspension was prepared in a similar way; however, during homogenization, the slurry temperature was kept between 42°C and 45°C. Volumes of the two suspensions were mixed and homogenized for an additional 10 min to generate homogeneous suspensions of appropriate collagen mass fraction (ranging from 0 to 100%). The collagen–gelatin slurries were lyophilized according to established protocols that provide 3 mm thick scaffold sheets with homogeneous mean pore diameter of 96 μm (O'Brien *et al.* 2004), and were covalently cross-linked via dehydrothermal treatment at 120°C, 50 mtorr vacuum for 24 h (Yannas & Tobolsky 1967). Cross-linked scaffolds were stored in a desiccated atmosphere at room temperature. The mean pore diameter of the three hCGSs was verified by scanning electron microscopy. Adhesion ligand density measurements

took place in cylindrical samples of hCGSs cut from the scaffold sheet using a 5 or 8 mm tissue punch.

3.2. Design, expression and purification of fluorescent biomarkers for integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$

The cDNA for the I-domains of integrin subunits α_1 and α_2 was a kind gift of the M. Humphries Laboratory, University of Manchester, UK. For each integrin type, two kinds of biomarkers were expressed. The first was the native non-fluorescent I-domain. The second was the I-domain fused to a tetracysteine (TC) peptide WDCCPGCK at its N terminus by polymerase chain reaction (Adams *et al.* 2002). The cDNA of each biomarker was inserted in a pGEX vector (GE Healthcare, Uppsala, Sweden), which was transformed into DH5a bacteria. I-domains were expressed by incubating bacteria initially for 4 h at 37°C (until OD 0.5), and an additional 3 h including 0.4 μM IPTG. I-domains were extracted by careful sonification, purified by affinity chromatography using glutathione-agarose beads (Pierce) and eluted by L-glutathione (Sigma, Saint Louis, MO, USA). The GST tag was removed by thrombin cleavage. Sample concentration into TBS was accomplished by filter centrifugation (Millipore, Billerica, MA, USA). The performance of the purification process was evaluated by SDS–PAGE. The ability of TC-tagged I-domains to bind biarsenical dyes was evaluated by SDS–FlAsH–PAGE (Adams *et al.* 2002). The concentration of the purified I-domain solution was measured by A280 absorption (Pace *et al.* 1995).

3.3. Competitive binding of fibroblasts and I-domains to a collagen scaffold

Primary human dermal fibroblasts (Lonza Allendale, NJ, USA), passage 7–9, were cultured in plastic T75 flasks, in a 5 per cent CO₂ humidified incubator maintained at 37°C, supplemented with DMEM medium plus 10 per cent FBS, 1 per cent streptomycin/penicillin (Invitrogen, Carlsbad, CA, USA). At 80 per cent confluence, cells were detached using 0.05 per cent trypsin/EDTA solution (Invitrogen). Cells were then suspended in 50 μl DMEM (1500 fibroblast μl^{-1}) supplemented with 5 μM of each non-fluorescent I-domain. Fibroblasts were seeded into scaffolds by placing a 50 μl cell suspension on an agarose-coated Petri dish. The scaffold was then gently brought in contact with the top of the drop, which resulted in aspiration of the cell suspension into the scaffold owing to capillary action. The seeded scaffolds were incubated at 37°C, 5 per cent CO₂ for 1 h to allow cell binding. After 1 h, scaffolds were washed twice with PBS to remove cells that did not attach and then incubated with 1 μM calcein AM dye (Invitrogen) in PBS for 25 min to stain cells that had become bound to the scaffold. The number of cells that managed to attach to the scaffold was estimated by measuring the fluorescence (excitation: 480 nm; emission: 530 nm) emitted by the stained cells. The detected fluorescence intensity was converted into number of attached cells using a

calibration curve, acquired by measuring the fluorescence signal emitted from scaffolds freshly seeded with a known number of pre-stained cells (scaffolds were not washed prior to imaging).

3.4. Multiphoton microscopy

Three-dimensional images of scaffolds were acquired using a custom-made multiphoton microscope (Buehler *et al.* 2005). The specimen was mounted on an inverted microscope (Axiovert 100TV, Zeiss, Jena, Germany) and excited by focusing a laser beam (generated by an MIRA 900 Ti-sapphire laser, Coherent Inc., Santa Clara, CA, USA) inside the specimen through a Zeiss 40 $\times$ C-apochromat 1.2 NA objective. A three-dimensional image of the specimen was acquired by raster scanning the focus point inside the specimen using a computer-controlled pair of scanner mirrors (6350, Cambridge Technology, Lexington, MA, USA) and a piezoelectric objective actuator (P-721.00, Physik Instrumente, Karlsruhe, Germany). The emitted light was collected via the epi-luminescence path in a de-scanned configuration. The signal was separated from the laser beam via a dichroic mirror (675DCSX, Chroma Technology, Rockingham, VT, USA), filtered via a BG39 band-pass filter, spectrally resolved via a spectrograph (MS125-77 414, Oriel Instruments, Stratford, CT, USA), and detected by a 16-channel PMT (R59000U-00-L16, Hamamatsu, Bridgewater, NJ, USA). The PMT output was processed and transmitted to a control PC by custom-made electronics (Buehler *et al.* 2005). The samples were excited by a 780 nm laser beam of 6 mW mean power (at the sample). The pixel sampling time was 80 μ s, the field of view was 50 $\times$ 50 and each image consisted of 256 $\times$ 256 pixels, 16 channels per pixel. Each PMT channel counted photons within an average 13.3 nm range of the spectrum, so that the 16 channels of the PMT detected photons in the range [370, 582] nm.

3.5. Labelling and measuring the density of ligands in porous collagen-based scaffolds

Dry scaffold samples (5 mm diameter, 3 mm height) were treated with 100 μ l blocking buffer (TBS, 2% BSA, 5 mM MgCl₂) in a 96-well plate and incubated for 2 h at 37°C. The blocking buffer was then aspirated and I-domain solution was added (0.01–10 μ M TC-tagged I-domain in TBS, 5 mM MgCl₂, 4 μ M FAsH (Invitrogen), 25 mM TCEP). Samples were incubated at 4°C for 2 h to let I-domains bind to their ligands. Unbound I-domain molecules were removed by washing the scaffold samples four times with wash buffer (TBS, 5 mM MgCl₂). Non-specific binding of FAsH was minimized by incubating the scaffolds with 0.5 $\times$ BAL buffer containing 5 mM MgCl₂ (Invitrogen) for 5 min. The scaffolds were washed twice with wash buffer for 5 min, sandwiched aseptically between a microscope glass and a coverslip separated by a 1 mm silicon spacer (Invitrogen), mounted on the multiphoton microscope and imaged. Typical acquisition volumes were 100 $\times$ 100 $\times$ 50 μ m with spatial spacing of 0.2 μ m in the x – y -direction and 1 μ m in the z -direction.

3.6. Image processing and statistics

The acquired images were processed using custom-made software written in MATLAB (Mathworks, Natick, MA, USA). The detected signal was decomposed at each pixel into the photon contributions of the signal sources (collagen fluorescence, second-harmonic (SH) emission, FAsH) using non-negative least squares (Lawson & Hanson 1974). Each pixel was classified into one of the domains ‘matrix’ and ‘void’ via a maximum-likelihood calculation. It was assumed that each domain contained a known number of known emission sources (collagen–gelatin, FAsH). The first step of the classification was a pixel-based calculation that considered only the detected signal from a 3 $\times$ 3 pixel neighbourhood centred at the pixel of interest. The classification was then refined by maximizing the conditional probability that pixel i belongs to domain j given neighbour pixels belong to domain k (Richards & Jia 2006). The probability coefficients used in this step had been estimated based on the mass fraction of the scaffold. The FAsH-emitted signal corresponding to the pixels associated with the matrix domain and located at the boundary of the void domain was taken to be directly proportional to the ligand density. The mean FAsH intensity from several locations in the scaffold was converted into a probability distribution of surface density of ligands (ligands μ m^{−2}) by assuming that ligands lie on a plane of homogeneous density located normal to the optic axis. The FAsH brightness (probability of detecting a photon emitted by a FAsH molecule per laser pulse) was obtained by imaging a series of FAsH solutions, whose concentrations were verified by fluorescence correlation spectroscopy (Elson & Magde 1974; Hess *et al.* 2002).

4. RESULTS

4.1. Expression of biomarkers for ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$

The ligands of a specific adhesion receptor could be quantified optically by labelling them with a fluorescent biomarker. The biomarkers used to label the ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were fluorescently labelled I-domains of the α subunit of the corresponding integrin. It is known that binding of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ to their ligands is mediated entirely via the I-domain of the α subunit (Lee *et al.* 1995; Hynes 2002).

Two versions of each biomarker were expressed. The fluorescent version (referred to as ‘ α_1 -TC’ and ‘ α_2 -TC’) was fused to a small TC tag at its N terminus and became fluorescent following binding of a biarsenical molecule (Griffin *et al.* 1998). Addition of the small (10 amino acid) TC tag in the N terminus of an I-domain is not expected to alter significantly the specificity and affinity of the I-domain for its ligands owing to the small size of the tag and the fact that the critical C terminus is not modified (Emsley *et al.* 1997). The non-fluorescent version (referred to as ‘ α_1 ’ and ‘ α_2 ’) lacked the TC tag. Figure 1 shows two images of a polyacrylamide gel that contained the four biomarkers following treatment with FAsH (Adams *et al.* 2002).

Figure 1. Images of a polyacrylamide gel containing the two non-fluorescent biomarkers (α_1 and α_2) and the two fluorescent biomarkers (α_1 -TC and α_2 -TC) for the ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$. The biomarkers were incubated with 5 μ M FAsH before loading and running the gel (Adams *et al.* 2002). (a) The image was acquired by fluorescence imaging immediately after running the gel. (b) The image was obtained after overnight staining with the general-purpose protein stain Coomassie Blue.

Figure 2. Number of human dermal fibroblasts that adhere to a CGS within 1 h after cell seeding in the absence ('unmasked' CGS) and presence ('masked' CGS) of saturated amounts of non-fluorescent biomarkers α_1 and α_2 for ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$.

prior to removing the GST tag by use of thrombin. The first image (figure 1a) was obtained by fluorescence imaging, while the second (figure 1b) was obtained after incubating the gel with a generic protein stain (Coomassie). Figure 1 demonstrates that TC-tagged I-domains are slightly larger than non-fluorescent I-domains (about 1 kDa) and can become fluorescent upon FAsH treatment.

4.2. Ligand density biomarkers compete with human fibroblasts for binding to CGSs

The functional activity of the expressed biomarkers was verified by a competitive binding assay of fibroblasts and I-domains to CGSs. The interaction between fibroblasts and CGS has been shown to be a central aspect of the regenerative biological activity of CGS (Yannas 2001, 2005). Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the most commonly studied collagen-binding integrins (Hynes 2002) and both of them are expressed in fibroblasts. Well-folded biomarkers for ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ should be able to compete for the available ligands on the surface of the CGS with the integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ that are present on the cell membrane. Figure 2 shows that indeed the number of human dermal fibroblasts that adhere to a CGS within 1 h is significantly less (almost by four times) in the presence of a saturating amount (5 μ M) of biomarkers α_1 and α_2 .

4.3. Measuring the density of ligands on CGS

The density of ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ on the surface of porous collagen-based scaffolds was measured

by treating a series of aliquot scaffold samples (3 mm diameter, 3 mm thick) with solutions of fluorescent biomarkers α_1 -TC or α_2 -TC, respectively, of increasing concentration, washing away the unbound I-domains and imaging the treated scaffolds in a multiphoton microscope. The detected signal contained the signal of interest (the fluorescence emitted by the FAsH-labelled I-domains bound to integrin ligands on the matrix) as well as signals emitted by collagen (autofluorescence, SH emission). The signal emitted by FAsH-labelled I-domains was separated from the collagen autofluorescence by exploiting the spectral information provided by the 16-channel sensor and the known emission spectra of FAsH and collagen (measured by imaging the pure compounds). Signal separation was implemented by the non-negative least-squares algorithm, which constrains the photon counts that are attributed to each source (collagen, FAsH) to be positive numbers. Scaffolds mostly consist of an interconnected network of struts. The FAsH emission originating from struts was localized mainly in the outer surface of the struts, and this can be seen when the imaging plane of the multiphoton microscope sections a strut longitudinally (figure 3).

The FAsH signal detected at the strut surface was then converted into density of fluorescent biomarkers bound on ligands on the scaffold surface via a standard curve. A standard curve was obtained by imaging a series of FAsH-stained I-domain solutions. The detected signal per pixel can be expressed as $\lambda_{\text{sol}} = \lambda_0 \cdot V \cdot \rho_V$, where ρ_V is the concentration of stained I-domains in solution, V is the focal volume and λ_0 is a constant that depends on the imaging

Figure 3. Estimation of surface density ρ_S of ligands for integrin $\alpha_1\beta_1$ on three collagen scaffolds treated with increased concentration of FAsH-labelled α_1 -TC I-domains. The signal emitted by labelled α_1 -TC bound on the outer surface of the scaffolds increases as the concentration of the α_1 -TC solution that is added increases between 100 and 1000 nM. (a) 30 nM α_1 -TC; (b) 300 nM α_1 -TC; and (c) 3 μ M α_1 -TC. Scale bar, 20 μ m.

conditions and the fluorophore properties. In order to convert FAsH intensity into surface concentration, it was assumed for simplicity that I-domains were homogeneously distributed with surface density ρ_S on a plane normal to the imaging axis. In this case, the signal emitted per pixel was expressed as $\lambda_{\text{surf}} = \lambda_0 \cdot S \cdot \rho_S \exp(-(z/\omega_z)^2)$, where λ_0 is the same constant that appears in λ_{sol} , S is the cross-section area of the focal volume normal to the optic axis, ω_z is the $1/e^2$ radius of squared intensity inside the focal volume (Zipfel *et al.* 2003) and z is the position of the plane that contains the ligands with respect to the optic axis ($z = 0$ at the focus centre). The constant λ_0 was calculated from the standard curve of I-domain solutions. However, since λ_{surf} depends on the position z , which cannot be detected by ordinary optics, there are infinite combinations of z and ρ_S that result in the same value of λ_{surf} . Therefore, z was treated as a random variable that follows uniform distribution in the range $[-\omega_z, \omega_z]$, and a measurement λ_{surf} was converted to surface density by the approximation $\rho_S = \lambda_{\text{surf}} \cdot E[\exp((z/\omega_z)^2)]/(\lambda_0 \cdot S)$, where E refers to the expected value of a random variable.

As scaffold samples were treated with solutions of fluorescent biomarker of increased concentration, the measured signal increased. This increase was almost undetectable for I-domain concentrations less than 100 nM, and was clearly detectable when the concentration of the I-domain solutions was in the 100–1000 nM range. This result agrees with previously published results that estimated the dissociation constant of collagen-I-domain binding to be around 200 nM (Calderwood *et al.* 1997; Xu *et al.* 2000; Tulla *et al.* 2001, 2008; Kim *et al.* 2005). Measurements of ligand density therefore took place by treating the scaffolds with 3000 nM stained I-domain solutions, since any additional signal detected when treating the scaffold with higher concentration of I-domains would be attributed to non-specific binding.

Figure 3 shows the fluorescence signal emitted by FAsH-labelled α_1 -TC biomarkers bound to ligands of integrin $\alpha_1\beta_1$ on the surface of collagen scaffold samples. Figure 3 shows several cases where the imaging plane of the multiphoton microscope sections a scaffold strut, highlighting the fact that FAsH emission is localized on the outer surface of struts. The mean detected

signal originating from α_1 -TC biomarkers located on the outer surface of scaffold struts corresponded to the signal that would be emitted by an α_1 -TC solution of concentration $\rho_V = 2.63 \pm 0.94 \mu\text{M}$. Similar experiments with α_2 -TC resulted in mean signal at the scaffold surface equivalent to $\rho_V = 7.79 \pm 2.44 \mu\text{M}$. Based on the approximation described above, these concentrations can be converted into estimated surface density of ligands of $\rho_S = 5775 \pm 2064 \text{ ligands } \mu\text{m}^{-2}$ for $\alpha_1\beta_1$ and $\rho_S = 17\,084 \pm 5353 \text{ ligands } \mu\text{m}^{-2}$ for $\alpha_2\beta_1$.

The majority of the FAsH fluorescent signal is localized on the outer surface of the scaffold struts. This is clearly visible when the imaging plane sections the centre of a strut longitudinally. The diffuse FAsH signal originating from the bulk of the strut is probably out-of-focus signal (owing to the somewhat elongated point-spread function (PSF) of the optics in the z -axis) as well as from estimation errors during the separation of the FAsH signal from the collagen autofluorescence. The localization of the FAsH-labelled I-domains on the outer surface of the scaffold struts is shown in the images of figure 4, where the regions where the image plane sections a strut longitudinally have been highlighted with an asterisk. Figure 4 also demonstrates the repeatability of the imaging results, since images were obtained from different scaffolds (the same day) using the same protocol. The variation of the signal detected on the outside surface for scaffolds of the same type that have been stained and imaged based on the same protocol is of the order of 25 per cent.

4.4. Measuring the density of ligands for integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in hCGSs

Specific binding of receptors to their ligands depends among other factors on the conformation of the ligand. It is known (Knight *et al.* 2000) that integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind to native collagen but not to denatured collagen (gelatin). In order to demonstrate that the developed fluorescent biomarkers simulated the binding properties of the corresponding matrix as closely as possible, the fluorescent biomarker α_2 -TC was used to measure the density of ligands of integrin $\alpha_2\beta_1$ in a series of hCGSs that were characterized by the same mean pore diameter and total mass fraction

Figure 4. Images of three different scaffolds, treated with either (a) 3 μM FIAsh-stained $\alpha 1\text{-TC}$ or (b) 3 μM FIAsh-stained $\alpha 2\text{-TC}$ using the same staining and imaging protocol. Asterisks are used to highlight locations where the imaging plane sections a strut longitudinally. The field of view of each image is $55 \times 55 \mu\text{m}$.

Figure 5. Optical quantification of collagen mass fraction in a series of hybrid collagen–gelatin scaffolds (hCGSs) based on the contribution of second-harmonic (SH) emission to the total detected signal. (a) Normalized spectra detected from five hCGSs of increasing collagen mass fraction. Only pixels classified as ‘matrix’ were considered. (b) Contribution of the SH emission to the total detected signal. The SH emission contribution is strongly correlated to the nominal collagen mass fraction of the hCGSs. (a) Solid black line, 100% collagen; dashed line, 75% collagen; dotted line, 50% collagen; dashed dotted line, 25% collagen; solid grey line, 0% collagen. The arrow indicates SH emission.

but differed in their content of mass fractions of collagen and gelatin.

The mass fraction of collagen in hCGSs was verified optically by quantifying the contribution of SH emission to the detected signal. SH is a coherent optical phenomenon that takes place in large-scale assemblies of structural proteins, such as collagen fibres in connective tissue, myosin in sarcomere patterns found in muscle tissue and microtubules (Campagnola & Loew 2003). SH is emitted by collagen fibrils in which the collagen molecules are arranged in space with high order

(Yannas 1972). SH is not emitted by gelatin owing to the random spatial organization of gelatin molecules. Figure 5 shows the detected emission spectra from five hCGS samples, and also shows the strong linear correlation ($R^2 = 0.9654$) between the nominal collagen mass fraction of the hCGSs and the mean contribution of SH emission to the total detected signal (the mean was calculated from four different samples). The small SH emission detected in the 100 per cent gelatin hCGSs is probably an artefact caused by the assumption that the whole signal detected in channel 2 is SH

Figure 6. Fluorescent signal originating from FIAH-labelled α_2 -TC biomarkers bound on the surface of three hybrid collagen–gelatin scaffolds (hCGSs) of increasing collagen mass fraction, after the hCGSs have been treated with FIAH-labelled α_2 -TC. The detected signal (mean $\pm$ s.d.) is normalized to the mean signal detected on the surface of a 100% hCGS.

emission, while in reality this channel detects also some autofluorescence. The results suggest that both collagen and gelatin are autofluorescent (if only collagen was autofluorescent, then probably the contribution of SH emission would be constant) and that they have common emission spectra.

Figure 6 shows the measured fluorescent signal from FIAH-stained α_2 -TC biomarkers that are bound on the outer surface of three hCGSs. The detected fluorescent signal is analogous to the surface density of ligands for integrin $\alpha_2\beta_1$ on these materials. This figure shows that the measured surface density of ligands is directly proportional to the fraction of collagen component present in the hCGS. These results agree with biochemical experiments that suggest integrin $\alpha_2\beta_1$ binds to collagen but not to gelatin (Knight *et al.* 2000). The small signal detected in the 0 per cent collagen hCGS was attributed to the photon noise present in the detection system that induces uncertainty in the separation of FIAH emission from collagen emissions.

5. DISCUSSION

The ability of an insoluble matrix to affect particular cell phenotypes of interest depends on the identity and density of available ligands for specific cell adhesion receptors, i.e. its surface chemistry. There are currently no general-purpose methods to quantify the surface chemistry of three-dimensional matrices in a way that could be used to determine their biological activity. This article reviews the need for such a method and provides preliminary results for an optics-based method that can quantify the surface density of ligands for particular cell adhesion receptors on the surface of a three-dimensional matrix.

The proposed method was demonstrated by measuring *in situ* the density of ligands of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ on the surface of porous scaffolds made of collagen and gelatin (hCGS). The ligands of each integrin are labelled by a fluorescent biomarker that is based on the I-domain of the α subunit of the corresponding protein. There is significant prior work on characterizing the

binding of α_1 and α_2 I-domains to collagen (binding affinities, number of binding sites, specificity). I-domains have been used extensively in the literature to identify binding partners for integrins, to identify the ligand motifs that are recognized by each integrin and to quantify the affinity of each integrin for particular binding partners (Tuckwell *et al.* 1995; Calderwood *et al.* 1997; Xu *et al.* 2000; Tulla *et al.* 2001, 2008; Kim *et al.* 2005). Here, the small TC tag CCPGCC (Adams *et al.* 2002) was added on the N terminus of each I-domain, rendering the biomarker fluorescent upon treatment with small biarsenical dyes (shown in figure 1). The TC tag system was chosen to fluorescently label the I-domains, owing to its small size that would not be expected to prevent the necessary conformation changes that regulate I-domain binding affinity to their binding partners (Emsley *et al.* 1997, 2000).

Two pieces of evidence show that the fluorescent biomarkers are functionally active and resemble the binding properties of the corresponding integrins. The first consists of the finding that the biomarkers compete with cell integrins for binding to ligands present on the surface of a collagen scaffold, resulting in significantly less adhesion within 1 h (figure 2). The second piece of evidence is based on the finding that the expressed fluorescent biomarkers bind to native collagen but do not bind to gelatin, a result that agrees with previous findings (Knight *et al.* 2000). Figures 5 and 6 show that the measured density of ligands is proportional to the mass fraction of collagen in the hCGSs.

The proposed method estimates the surface density of ligands on the surface of collagen scaffolds to be of the order of 5700 ligands μm^{-2} for $\alpha_1\beta_1$ and 17 000 ligands μm^{-2} for $\alpha_2\beta_1$. Assuming all ligands lie on a single plane, these results suggest that the average distance between ligands of $\alpha_1\beta_1$ is 13.25 nm and the average distance between ligands of $\alpha_2\beta_1$ is 7.67 nm. These distances seem small compared with the existing knowledge of tropocollagen organization in collagen fibrils (Orgel *et al.* 2006). The large observed surface density is probably caused by the fact that the stained ligands are not located on an ideal flat and smooth surface as assumed above for simplicity of calculation. Each collagen molecule (approx. 300 nm long and 4 nm wide) contains more than one ligand for each one of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Sweeney *et al.* 2008), some of these ligands bind to both integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ with different affinities (Xu *et al.* 2000; Tulla *et al.* 2001). Furthermore, it has been shown that the number of ligands on collagen-coated surfaces depends on the degree of collagen crystallinity and that native collagen fibres bind to fewer α_1 and α_2 I-domains compared with swollen collagen fibres (Jokinen *et al.* 2004). This dependence of I-domain binding to collagen on the collagen crystal structure is expected to affect the effective number of ligands for the collagen-based scaffolds in this study because the acetic acid treatment of collagen during scaffold fabrication results in collagen swelling. The collagen in the scaffolds showed a reduced fraction of collagen fibres possessing the characteristic 67 nm collagen banding, although the triple helical structure of collagen in the scaffold was identical to that in native collagen (Yannas *et al.* 1989).

Measurement errors in the use of the methodology described here can be grouped into three classes. One potential source of error is related to the basic biochemical interactions involved. The quantification of the ligand density depends on the accuracy of measuring the concentration of functionally active biomarkers (biomarkers that can bind to their ligands with strong affinity). Furthermore, biomarker–ligand binding is in dynamic equilibrium, so after washing away the unbound biomarkers, the amount of fluorescent biomarkers bound to ligands progressively decreases over time. Tighter binding between the biomarker and the ligand, possibly by a cross-linking method, will improve the precision and accuracy of the measurements. Transport of biomarkers inside thick samples (of the order of millimetres or hundreds of micrometres) may further complicate measurements. A second source of error is related to the optics used. Measurement of ligand density inside three-dimensional matrix systems requires the use of three-dimensional optics, whose resolution depends on the ability to focus the excitation light in as small a volume as possible (described by the excitation PSF) inside the sample. Because three-dimensional matrices are optically inhomogeneous systems, the shape of the PSF will be position dependent. As measurements take place deeper inside a three-dimensional matrix, variations in the PSF may affect the accuracy of the system. Ligand density measurement is based on measuring the intensity emitted by the biomarkers, which is therefore sensitive to the optical inhomogeneity of the sample. The third source of error is computational. The total detected signal needs to be processed so that the useful signal (fluorescence emitted by the FLAsH-labelled biomarkers) is distinguished from the background collagen signal (mainly autofluorescence). The accuracy of this calculation depends mostly on the ratio of FLAsH signal to background collagen signal, and therefore is improved in scaffolds treated with biomarker solutions whose concentration is larger than 100 nM. Finally, the surface density of ligands is estimated from the detected intensity of FLAsH-labelled biomarkers, and the accuracy of the results depends on the simple geometric assumptions that were used, and can be improved by including spectral and geometric information from several neighbouring pixels in the estimation algorithm. A detailed study that aims to specify the importance of each potential error source is currently under way.

Although the proposed method described in this article focuses on measuring the ligands of the two major collagen-binding integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, it is possible to apply the methodology to assay ligands for a wider range of adhesion receptors. This method is based on the expression of soluble fluorescent biomarkers that resemble very closely the binding properties of the corresponding receptor, and therefore can bind the same set of ligands with approximately the same level of affinity. Such molecular specificity can be achieved by designing biomarkers that consist of one or more domains of the receptor of interest using the available information on the molecular mechanism of ligand binding by the receptor of interest

(e.g. crystal structure, domain homology). Significant information about the structure and function of several adhesion receptors is available in the literature.

This is a cell-centred approach, designed to imitate the process by which cells recognize and bind to their environment. It focuses on the number of ligands for a particular receptor rather than on the number of a particular kind of a ligand in the matrix. It must be kept in mind that a given receptor typically binds to more than one kind of ligand. This viewpoint differs from that used in previous methods (Harbers *et al.* 2005; Hsiong *et al.* 2008) where the authors focused on measuring the density of a particular kind of ligand without considering the adhesion receptor(s) that binds to this ligand. However, in matrices that contain only one ligand for a particular receptor, the proposed method can be used to measure the density of this particular ligand. Some potential applications include the following.

- Measuring the density of the RGD ligand in an artificial biomaterial using a fluorescent biomarker based on one of the integrins that bind to RGD ($\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_s\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_{IIb}\beta_3$; Plow *et al.* 2000; Hynes 2002). A soluble fluorescent biomarker for RGD can be based on the construct that Marciano and colleagues have developed in order to identify binding partners for integrin $\alpha_s\beta_1$, which included RGD peptides (Marciano *et al.* 2007).
- Measuring the density of glycosaminoglycans on biomaterials using fluorescently labelled lectins. These are adhesion receptors that recognize specific sugar residues (Hocdé *et al.* 2009) found on the surface of bacteria or mammalian liver cells.

Three-dimensional matrix environments can be studied by this method. Materials in this class include biomaterials made of either natural monomers (proteins, sugars) or artificial monomers functionalized with appropriate adhesion ligand building blocks. The method can also be applied to quantify the density of ligands in *ex vivo* matrices or explanted solid tissues, after removing cells by standard biochemical methods. The potentially wide application range of this method is the second difference with the existing published methods for ligand density measurement, since such earlier methods can be applied only with specific artificial biomaterials that contain specially labelled ligands.

The results of the proposed method can help a wide range of researchers who wish to quantify the insoluble microenvironment sensed by cells inside three-dimensional matrices. Specifically, such results can be used to relate the surface chemistry of the matrix to the short-term (signal transduction) and long-term (protein expression) responses that features of the insoluble surface induce in cells that interact with it. This approach, uniquely applicable to insoluble surfaces that regulate cell function, has the potential to yield information of a similar basic interest to information that is currently available for soluble regulators.

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