

Methodology for Measuring Surface Ligand Density

Prof. Ioannis Yannas

What do investigators like to know about the surfaces of their biomaterials? Historically, the focus has shifted from studies of proteins adsorbed on surfaces to assays of surface ligands for cell attachment. Currently, a large number of investigators are interested to find methods for persuading cells to modify their phenotype in order to achieve a useful end result. Typical areas of interest among the biomaterials community are identification of specific substrates that induce a therapy (e.g., block proliferation of cancer cells migrating on substrates) or a tissue-engineering synthesis (e.g., synthesis in vitro of a functional liver organoid) or a desired endpoint in regenerative medicine (e.g., regeneration at the site of a wound, rather than contraction or scar formation).

The motivation behind all these diverse pursuits is the rapidly increasing appreciation that insoluble surfaces are capable of changing cell phenotypes. Traditionally, cell regulation has been considered to be the domain of soluble regulators (cytokines, growth factors, hormones). Although insoluble surfaces were known to be capable of having biological activity from studies completed several years ago, the widespread appreciation of this finding is a relatively new development. In the biological and medical communities little attention has been traditionally paid to regulation of cell phenotypes by insoluble, solid-like surfaces. Most of the studies of regulation of cell phenotype by surfaces appear to have historically originated with biomaterials scientists and engineers.

Assays of proteins adsorbed on biomaterials surfaces have been developed using increasingly sophisticated physicochemical instrumentation. Extensive use has been made of time of flight secondary ion mass spectrometry (Henry et al., 2008; Michel et al., 2005; Wagner, Shen et al., 2003; Wagner, McArthur et al., 2002; Lhoest et al., 2001); X-ray photoelectron spectroscopy (Henry et al., 2008; Michel et al., 2005; Wagner, McArthur et al., 2002); nuclear labels, such as, ^{125}I (Zhang and Horbett, 2008; Jenney and Anderson, 2000); and electron spectroscopy for chemical analysis (Wagner, Horbett et al., 2003), among others.

In these studies the methodological focus appears to be identification of the proteins adsorbed on the surface to which cells adhere, as well as measurement of the protein mass adsorbed, rather than a quantitative study of surface ligand density responsible for cell adhesion. In many studies a major objective of studies of protein adsorption on surfaces has been to improve understanding of *in vivo* response of biomaterials surfaces to substances such as blood. For example, in one study the adhesion of platelets on 2D polystyrene surfaces that had been pretreated to adsorb fibrinogen was studied with methodological emphasis on assaying the mass of adsorbed protein and identification of sites involved in platelet binding (Tsai et al., 2003).

Quantitative methods for measuring the ligand density of surfaces. In a large number of studies, two-dimensional (2D) surfaces were treated to prepare surfaces at various calculated or directly assayed levels of ligand density.

Indirect (calculated) values of ligand density have been published extensively. In these studies a two-dimensional (2D) solid surface is typically incubated with a solution of the ligand and a certain mass of the ligand is thereby adsorbed on the surface. By changing the concentration of ligand adsorbate in solution, the investigators achieve desired degrees of saturation and the ligand density is then simply calculated as the ligand mass adsorbed/area. In several examples from this group of studies, preparations of proteins, known to be ligands for certain cell types, have been adsorbed on 2D surfaces, e.g., nontissue culture plates. An illustrative example of this method involves the binding of platelets on the ligand molecule fibrinogen (Jirouskova et al., 2007). In this study the authors describe preparation of a surface with “low-density fibrinogen”, using a fibrinogen solution of 3 $\mu\text{g}/\text{mL}$ resulting in a surface density of approximately 80 ng/cm^2 or 1.8×10^{11} molecules/ cm^2 ; a “high-density fibrinogen” surface was also prepared using a solution of 100 $\mu\text{g}/\text{mL}$, resulting in a density of approximately 500 ng/cm^2 or 8.9×10^{11} molecules/ cm^2 (Jirouskova et al., 2007). In another study culture plates were exposed to solutions of the ligand molecule (fibronectin) at different concentrations, to produce what were described by the authors as surfaces with different ligand density; migration speed studies of eosinophils were then studied as a function of ligand density of the surface (Holub et al., 2003).

Directly assayed levels of ligand density have been reported. In these studies the mass of ligand adsorbed on the surface was directly assayed by a variety of methods involving use of a characteristic label or biomarker for the ligand site. The quantity of label was eventually assayed by a suitable detection method, which was converted to the mass of ligand present on the surface. In one study the mass of ligand (fibronectin) adsorbed on the surface was assayed using ligand that had been “spiked” with a radiolabel (Valenick and Schwarzbauer, 2006; Maheshwari et al., 2000). In another study the mass of adsorbed collagen on glass plates was assayed by spiking with fluorescent collagen (Engler et al., 2004). Other investigators assayed ligand density on surfaces with high-resolution magic-angle spinning NMR analysis using a novel internal standard (Lucas et al., 2004). A fluorescence resonance energy transfer (FRET) technique was used to quantify the number of bonds formed between cellular receptors and synthetic adhesion oligopeptides coupled to an artificial extracellular matrix (Kong et al., 2006).

Advances in methodology have been based on incorporation of the highly useful and widely used fluorescent labels, followed by development of calibration methods to assay the mass of fluorescently-labeled ligand on the surface (Model and Healy, 2000). Quantitative assay of fluorescent labeling was extended to measurement of the density of ligands on surface of peptide-modified biomaterials. In this protocol specific proteolysis was used to liberate (elute) a labeled probe from an immobilized peptide, followed by assay of the liberated probe by high-throughput fluorometry (Barber et al., 2005). Related developments led to assay of the fluorophore directly on the surface (without prior elution) (Harbers et al., Langmuir, 2005). On the strength of this powerful methodology, it became possible to demonstrate that osteoblast adhesion strength on well-defined surfaces scaled with ligand density (Harbers and Healy, 2005).

Need for a measurement of density of bound ligands in cell-seeded scaffolds. Cells bind on surfaces via receptors. A special class of receptors, the family of integrins, binds to ligand sites on molecules of the natural extracellular matrix (ECM) in tissues. Receptors other than integrins that are involved in cell-ECM interactions include members of the families of cadherins and

selectins. Once receptor-ligand binding is complete there is often induction of intracellular signals that may eventually modify the cell phenotype. Phenotype modification may include changes in migration speed, cell shape, regulation of cell cycle, and others. Clearly, an assay of the density of bound ligands would be valuable to designers of biomaterials surfaces who wish to control cell phenotype.

Although reliable assays for free ligands on surfaces have been established (see section immediately above) very little methodology appears to have been reported for assaying bound ligands. A number of biochemical and immunohistochemical assays have been reported; in these reports, the emphasis is on qualitative identification rather than on quantitative assay (e.g., see Schaff et al., 2007; Mould, 2002). A quantitative assay was recently reported which is based on the use of a spinning disc device in which the mean cell detachment force is reported to be proportional to the number of adhesive bonds (Boettiger, 2007; Gallant and Garcia, 2007; Gallant et al., 2005). This method could mark a very promising development provided it is coupled to a microscopy technique that allows assay of the number of bound ligands.

In this grant application we report preliminary data that can be used to provide an estimate for the density of ligands that are engaged in cell adhesion on a surface. Imaging by two-photon microscopy can provide values of ligand density both on the cell-free and cell-occupied surface (see Methods). Clearly, not all ligands on a surface are eventually engaged in cell adhesion. A suitable assay of a surface to which cells are adhering must therefore break down the total number of ligands measured on the cell-free surface, D_t , into ligands that are engaged in cell-binding (bound ligands), D_b , and those that are not bound (free ligands), D_f .

$$D_t = D_b + D_f \quad (\text{Eq. 1})$$

The total number of ligands, D_t , can be assayed by imaging ligands on the surface of the cell-free surface, as described in Methods and illustrated in Figs. 1 and 2. D_f can be obtained from Eq. 1 by difference provided D_b can be measured or estimated, as described below.

To obtain the number of bound ligands, D_b , it is proposed to use a library of scaffolds with gradually decreasing density of ligands, D_t , on the surface of its members. This library (referred to as Library #1 below) is prepared by progressive masking of ligand sites, using as

masking agent the highly specific I-domain molecules (prepared as clones of a subunit for the $\alpha1\beta1$ and $\alpha2\beta1$ integrins). The I-domain molecules bind only to the ligand sites on the collagen surface that are specific for the $\alpha1\beta1$ integrin or the $\alpha2\beta1$ integrin (the ligand sites are collagen oligopeptides with known structure; see Methods). The masking reaction is a covalent crosslinking process, leading to securely masked ligands, not affected by ionic strength or dilution. The library will therefore be designed as a calibrated scale of D_t values that gradually decrease along the series.

The density of bound ligands, D_b , can then be estimated as the ligand density of the library member which shows minimal cell adhesion, D_c :

$$D_b \approx D_c \quad (\text{Eq. 2})$$

In other words, D_c is the ligand density of the library member that is sufficiently high to just secure adhesion of cells onto its surface. The critical library member will be identified as the D level (or short range of D values) along this calibrated scaffold series at which metrics related to adhesion change significantly compared to a scaffold with no ligand masking. These metrics include the cell aspect ratio, I (a metric for cell shape), average scaffold area occupied per cell, S, and density of bound cells, C, all of which will be assayed at each D level. The estimate will probably be a high value which can be refined at will by reducing the increments in D along the series close to the critical masking range where cell adhesion fails.

This approach has similarities with the spinning disk method described in a preceding paragraph (Boettiger, 2007; see above). However, unlike the spinning disk, the method described here is complemented with direct assaying of ligand density by two-photon microscopy at all levels of the test sequence.

B5. Need for a three-dimensional (3D) description of cell-scaffold interactions. Much of current methodology for surface characterization supports studies of cells on two-dimensional (2D) surfaces. There is, however, an increasing amount of data showing clearly that cells express different phenotypes on 3D compared to 2D surfaces. For example (only a few cases are reported here for purpose of illustration), integrin expression profiles for two cell types have been shown to be different when cultured on 3D substrates

presenting RGD groups than in 2D culture (Hsiong et al., 2008). In other studies differences between cell phenotype expression in 3D and in 2D were observed with cell adhesion (Cukierman et al., 2001), response to electric stimulation (Sun et al., 2004) and cell migration (Maaser et al., 1998; Zaman et al., 2006). There is widespread agreement that 3D surfaces are much closer to surfaces of physiological matrix (Alavi and Stupack, 2007; Tsai et al., 2007). It has been also conceded recently that available tools for characterizing cell cultures in 2D versus 3D environments are difficult to compare (Ochsner et al., 2007). This may explain why very few, if any, rules have been developed to date comparing cell behavior in 2D and 3D environments.

Scaffolds are archetypical 3D surfaces. A cell inside a scaffold with average pore size somewhat larger than the cell diameter is in contact with surfaces (scaffold “struts”) almost all around its outer surface and bound ligands are therefore distributed around the entire cell surface. In contrast, on a 2D surface a cell is making contact mostly on one side of its surface only. Advanced protocols for synthesis of tissues, such as those for cardiac tissue engineering (Radisic et al., 2008), make extensive use of the three-dimensional nature of the environment by culturing cardiac cell populations on porous scaffolds. Collagen-GAG scaffolds, well-known for their 3D structures, have been used over many years to induce regeneration of the dermis, peripheral nerves and the conjunctiva in animals and humans (Yannas, 2001, 2005). There is little doubt that scaffolds of one kind or another will be used more and more extensively in future applications of tissue engineering and regenerative medicine. This expectation increases the urgency with which investigators seek to identify methodology for the quantitative characterization of 3D surfaces, especially scaffold surfaces.

A description of ligand density applicable to a 2D surface needs to be replaced by a description that applies to a 3D scaffold surface. The scalar quantity Γ for the ligand density of flat surfaces, occasionally expressed in the literature in units of pmoles/cm² (e.g., Barber et al., 2005), must be replaced by a function that describes the distribution of bound ligands around the entire cell surface. We propose use of the vector distribution function $\mathbf{J}(\mathbf{r}, \boldsymbol{\theta}, \mathbf{z})$ for bound ligands that are distributed around the center of mass of a cell (centroid). This function can be determined for each cell by two-photon microscopy, as described in Methods. Data for a large

number of cells can then be processed to derive a description for the average cell inside the scaffold.

B6. Choice of microscopy method for study of cell-seeded surfaces of biomaterials. In the presence of cells it becomes necessary to use specialized microscopic methods for structural characterization of the cell-surface interaction. A commonly used approach for studying cell-substrate interactions is based on confocal microscopy.

We provide two arguments supporting the notion that two-photon microscopy provides superior data compared to confocal microscopy, especially when cell-seeded surfaces are being studied (So et al., 2000). First, confocal microscopy uses a UV laser which damages cells more than does two-photon microscopy that is based on use of an IR laser. (Rubart, 2004; Nikolenko et al., 2003; Schwille et al., 1999; So et al., 1998; Yu et al., 1996). Among other advantages, the reduced photodamage available with two-photon microscopy enables long-term imaging of living preparations to study kinetics of processes. Second, in the proposed quantitative study of the entire cell within the 3D environment of a scaffold (rather than on a 2D surface) the depth of penetration available with two-photon microscopy (100-120 μm) adequately encompasses a cell and nearest-neighbor scaffold struts compared to the less-than-adequate depth of penetration available by confocal microscopy (30-40 μm) (Rubart, 2004; Konig, 2000).

Although we propose to occasionally use both microscopy methods (confocal and two-photon) in the early part of the study, we are clearly interested in developing long-term methodology based on the less-well-developed but more promising two-photon microscopy. Our ongoing collaboration with the MIT laboratory of Dr Peter So, one of the pioneers of two-photon excitation fluorescence imaging microscopy, provides us with a strong basis for adapting this methodology to solve the problems of interest. Preliminary data ([see below](#)) show clearly that two-photon excitation fluorescence imaging microscopy can provide unprecedented quantitative data on cell-substrate interactions, including the total ligand density of cell-free surfaces, D_t , estimates of the average bound ligand density per cell, D_b , and 3D views of cell-scaffold systems that lead to description of the distribution of bound ligands around the cell surface, $\mathbf{J}(\mathbf{r}, \boldsymbol{\theta}, \mathbf{z})$.

B7. Significance of proposed study.

I. New methodology for quantitative design of biomaterials surfaces.

Biomaterials scientists are becoming increasingly adept at the design of synthetic surfaces that modify cell phenotype. As a result, they now require two kinds of information. First, there is need for increasingly quantitative information about the specific surface features that induce phenotype modification, especially with cell-seeded surfaces. Second, since highly porous scaffolds are increasingly being used, there is additional need for assays that can describe the bound ligand density in 3D, i.e., the distribution of bound ligands around the center of mass of the cell.

The growing urgency for studying cells quantitatively in three-dimensional structures is illustrated in recent research leading to synthesis of tissues and organs in vitro. Highly promising in vitro environments for synthesis of tissues and organs are expected to possess a well-defined 3D structure (Jakab et al., 2008; Radisic et al., 2008; Park et al., 2007; Timmins et al., 2007). These 3D structures are recognized as a necessary adjunct to the presence of cells, growth factors and an appropriate bioreactor environment (Pei et al., 2008).

The scaffolds that we propose to study are analogs of the ECM rather than synthetic polymers. Analogs of this class are in wide use today (see section immediately below). They are often based on naturally occurring polymers, usually proteins, such as collagen, elastin and fibrin, with occasional participation of proteoglycans and glycosaminoglycans. The surfaces of these proteins provide a rich source of well-defined ligands for many important cell receptors, particularly integrins. Cell-surface interaction in scaffolds is a demonstrably 3D phenomenon that can only be understood definitively by 3D imaging methodology. We conclude that scaffolds based on analogs of the ECM are an important class of biomaterials surfaces because they provide well-defined ligands in 3D environments.

The opportunity to base a new quantitative methodology for surface characterization of cell-seeded 3D surfaces on these naturally occurring scaffolds cannot be missed. If this opportunity is pursued successfully, there will generation of new paradigms in methodology that will provide the basis for assays with other biomaterials as well. The new data that will emerge will be shaped

into rules for the rational design of new cell-seeded surfaces based both on synthetic polymers as well as natural polymers.

II. Improved understanding of the molecular mechanism by which collagen-GAG scaffolds modify cell phenotypes in four cases. The materials focus of this study is collagen-GAG scaffolds (CGS). It has been known for years, from studies conducted in the laboratory of the PI, that certain scaffolds of this class can induce partial regeneration of the dermis in animals and humans (Compton et al., 1998; Yannas et al., 1982, 1989), peripheral nerves in animals and humans (Harley et al., 2004; Chamberlain et al., 1998) and the conjunctiva in animals (Hsu et al., 2000). In our Preliminary Results we report the first measurement of ligand density on the surface of a collagen-GAG scaffold.

At least two FDA-approved devices have been based on collagen-GAG scaffolds (CGS). The first is IntegraTM, a dermis regeneration template, first synthesized and tested with animals in the laboratory of the PI (Yannas, 1981; Yannas et al., 1981, 1982, 1989). It is currently used to induce synthesis of the dermis in burn patients (Klein et al., 2005), in patients undergoing plastic surgery of the skin (Blanco et al., 2004) and in patients with chronic skin wounds and skin ulcers (Gottlieb and Furman, 2004). The second is NeuragenTM, a CGS scaffold-based tube that induces reconnection of peripheral nerves across gaps of unprecedented length. Rules for design of tubular scaffolds that induce regeneration of peripheral nerves across unprecedented gaps have been described in detail (Yannas, 2001; Yannas et al., 2006). There is evidence that early fetal regeneration, a spontaneous process in mammals, is an appropriate model for induced regeneration of organs in adults by CGS (Yannas, 2005; Yannas et al., 2007).

Although a general mechanistic explanation of the biological activity of certain scaffolds at the cell-biological level has been reported (Yannas, 2001, 2005), there has been little quantitative characterization of the detailed molecular features of their 3D surface that account for the unusual biological activity of these scaffolds. For example, the maximum in dermis regenerative activity of CGS in the pore size range 20-140 μm (Yannas et al., 1989) is not understood. **It is hypothesized that the maximum in contraction-blocking activity corresponds to a maximum in engaged ligand density.**

In a second example the maximum engaged ligand density that fibroblasts require in order to start and the minimum required to stop wound contraction in the presence of a CGS are not known. Blocking of wound contraction by CGS is a known requirement for induced regeneration (Yannas, 2005). Knowledge of this type would allow design of improved scaffolds and possibly small-molecule approaches to regenerative activity. Contraction force

A third example is based on the recent use of CGS as a model substrates for study of cancer cell proliferation and metastasis. In this model it is known that proliferation of certain types of cancer cells requires attachment to CGS surface in an $\alpha2\beta1$ integrin-specific adhesion in order to proliferate (Grzesiak and Bouvet, 2007) but it is not clear just what kind of surface modification can block cancer cell proliferation.

Finally, it has been shown that an increase in stiffness of CGS scaffold struts accelerates migration of certain cell types through the scaffold but the mechanism for such an effect is not understood (Harley et al., 2008). Cell migration is a basic property that affects all studies of cell-scaffold interactions.

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