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Secondary ion mass spectrometry and Raman spectroscopy for tissue engineering applications

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Abstract

Identifying the matrix properties that permit directing stem cell fate is critical for expanding desired cell lineages *ex vivo* for disease treatment. Such efforts require knowledge of matrix surface chemistry and the cell responses they elicit. Recent progress in analyzing biomaterial composition and identifying cell phenotype with two label-free chemical imaging techniques, TOF-SIMS and Raman spectroscopy are presented. TOF-SIMS is becoming indispensable for the surface characterization of biomaterial scaffolds. Developments in TOF-SIMS data analysis enable correlating surface chemistry with biological response. Advances in the interpretation of Raman spectra permit identifying the fate decisions of individual, living cells with location specificity. Here we highlight this progress and discuss further improvements that would facilitate efforts to develop artificial scaffolds for tissue regeneration.

Introduction

The microenvironment where stem cells (SCs) reside in the body, which is called the SC niche, presents cellular and matrix cues that modulate whether SCs remain quiescent, self-renew, or selectively differentiate into any lineage found in the body. The ability to recapitulate the SC niche within an artificial culture would enable using SCs to expand specific cell types *ex vivo* for disease treatment. Consequently, much research focuses on developing biomaterial substrates that mimic the physiochemical properties of the extracellular matrix (ECM) within the SC niche.

Efforts to develop biomaterials substrates for directing SC fate *ex vivo* require analytical tools for characterizing both native ECM and engineered biomaterial substrates, and also for accurately identifying the cell responses they elicit. ECM composition is traditionally

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assessed with biomolecule-specific dyes or immunolabeling with antibodies to ECM proteins. Fluorescent antibodies to differentiation-associated cell surface markers and flow cytometry or fluorescence microscopy are often used to determine SC differentiation at the population or single-cell level, respectively. A disadvantage of these approaches is the need for component-specific dyes or antibodies, which increases cost and limits their application to detecting known biomolecules. Ideally, substrate composition and cell fate would be identified without labels and with location specificity. This review focuses on the use of two label-free and location-specific approaches, time-of-flight secondary ion mass spectrometry (TOF-SIMS) and Raman spectroscopy (RS), for assessing biomaterial substrate composition and cell fate decisions.

TOF-SIMS for characterizing substrate composition

TOF-SIMS is an imaging mass spectrometry technique that reveals the chemical composition at the surface (top few nms) of a sample with as high as sub- μm lateral resolution (reviewed in [1,2]). This lateral resolution is sufficient to image molecular distributions on patterned surfaces, which are often used to study the effects of ligand density and spatial confinement on SC fate [3,4]. Unlike other label-free imaging techniques used to characterize tissues and engineered biomaterials, including RS [5–9], TOF-SIMS analyzes the outermost surface of the substrate, which enables identifying protein conformation [10,11] and the chemical moieties that may interact with the cell surface. Such information is essential for understanding the cell-matrix interactions that elicit fate decisions, and for improving biomaterial design for tissue engineering applications [3,4].

TOF-SIMS is performed under ultrahigh vacuum (UHV), so samples must be dehydrated or frozen prior to analysis. Protocols have been developed that preserve protein conformation during the dehydration process [12]. Noteworthy, studies have shown that subsequent exposure to UHV does not alter collagen structure [13].

The macromolecules on the sample surface are fragmented during TOF-SIMS analysis. Though the extent of fragmentation can be reduced by implementing cluster ion beams [1], the spectra acquired from different proteins usually lack individual peaks that are characteristic of a single protein. Consequently, translating the numerous low mass peaks in the spectra into chemical information is challenging. Multivariate analysis (MVA) techniques, such as principal component analysis (PCA), are often used to calculate combinations of mass peaks that capture the variance between samples [14]. This allows discriminating between the spectra of different proteins in the absence of protein-specific mass peaks [14–16]. The compositions of unknown protein samples can be identified by using spectra from protein standards to construct a PCA or partial least-squares discriminant analysis (PLS-DA) model, and then applying this model to the spectra from the unknown sample. For example, the proteins within the ECM that remained on a substrate after cell liftoff were identified by projecting the TOF-SIMS data acquired from the ECM onto a PCA model that was constructed using the spectra from individual proteins absorbed onto substrates [16].

Quantifying the relative abundance of an analyte, such as a protein, with TOF-SIMS is challenging because secondary ion intensities are influenced not only by analyte abundance, but also by sample topography [17,18] and chemical environment, which is called the matrix effect [1,2,19]. Fluctuations in ion intensity can be reduced by normalizing the intensity of the ion signals of interest to that of a ubiquitous ion [20]. Multivariate calibration methods provide a more robust, though also more laborious approach to quantifying analytes with TOF-SIMS. In this approach, a multivariate technique, partial least-squares regression (PLSR), is used to create a calibration model that correlates the TOF-SIMS data from calibration samples of known composition to analyte abundance, and then the model is applied to the spectra from the experimental samples [21]. Caster and coworkers have shown that the PLSR of TOF-SIMS data enables quantifying the relative abundances of different protein components at the surfaces of multicomponent protein films [22,23].

TOF-SIMS is frequently utilized to confirm the composition of engineered biomaterials (Figure 1a), especially when surface modification processes are employed [24–29]. Differences in the superficial protein composition induced by sample treatment have been analyzed by combining TOF-SIMS with PCA [30–33]. This includes analyzing differences in the surface compositions of decellularized ECM scaffolds [30,31] and implantable scaffolds intended for tissue regeneration in the body [33]. TOF-SIMS has been used to show that agarose addition to collagen scaffolds does not alter collagen ligand presentation, indicating the changes in cell response elicited by agarose addition are due to increased matrix stiffness [32]. Though this study focused on glioma cells, this approach could be adapted to investigate the effects of scaffold treatments on ligand presentation and SC response.

The lateral distribution of components at different depths within a biomaterial can be imaged with TOF-SIMS by repeatedly alternating between imaging scans and sputtering scans that remove material from the sample's surface [1]. This three dimensional imaging has been employed to assess the osteogenic potential of various human SC lineages based on the extent of matrix mineralization [34–36]. Noteworthy, like two dimensional TOF-SIMS imaging, the secondary ion signals detected in three dimensional TOF-SIMS analyses are influenced by concentration-independent factors. Therefore, if a PLSR approach cannot be employed for quantitation of three dimensional TOF-SIMS data, peak normalization or a complementary biochemical assay should be used to confirm that differences in the intensities of individual mass peaks correspond to differences in analyte abundance.

An exciting application of TOF-SIMS to the development of tissue engineering scaffolds involves using PLSR to correlate TOF-SIMS peaks, and thus, the surface chemistries they correspond to, with biological response [28,29,37]. Ratner and coworkers previously used this approach to identify the chemical moieties that most strongly influenced bovine aortal endothelial cell growth on plasma-deposited films [37]. More recently, Langer and coworkers used PLSR of TOF-SIMS data acquired from polymeric microarrays to identify the surface chemistries that enable clonally expanding human pluripotent SCs in xeno-free defined medium without feeder cell layers (Figure 1b) [28,29]. This approach will facilitate optimizing the combinatorial development of engineered substrates for *ex vivo* tissue engineering.

Identification of cell fate decisions with TOF-SIMS

The use of microscale culture platforms that minimize the number of rare SCs required to screen cell response to stimuli have created a need for methods to assess the fate decisions of individual SCs with location specificity. Several reports demonstrate that MVA of TOF-SIMS data acquired from the surfaces of intact cells and cell homogenates can be used to discriminate between mammalian cells of differing phenotype (i.e., lineage) [38–42]. This opens the door to using MVA models constructed from calibration sets of cell spectra to identify the fate decisions of individual cells within microscale culture platforms. However, the biochemical differences that occur on the surfaces of differentiating cells may be too subtle to be detected by MVA of TOF-SIMS data, especially if contaminants in the culture environment are a major source of spectral variation between the calibration cell populations. Consequently, most work in identifying cell phenotype for tissue engineering applications has focused on establishing the methodology. For example, Ratner and coworkers developed an approach that employed partial least squares discriminant analysis (PLS-DA) of TOF-SIMS data to identify and spatially locate two types of differentiated mammalian cells in a heterogeneous culture [40]. In this work, sets of mass peaks that were indicative of each cell line were identified by PLS-DA of calibration TOF-SIMS data acquired from known samples. Then the locations of each cell line on the substrate were visualized by color-coding the pixels where the intensities of the cell line-associated peaks exceeded a threshold (Figure 1c).

Calibration spectra of cells of known phenotype has also enabled identifying cell types that are studied for tissue engineering applications [41,43]. The phenotypes of macrophages around a hydrogel implant have been tentatively identified by visually comparing their spectra to TOF-SIMS data from the macrophage subtypes involved in biological response to foreign material [43]. We have successfully identified the differentiation states of individual, primary hematopoietic cells from three distinct states of B cell lymphopoiesis with a PLS-DA model of calibration TOF-SIMS data from hematopoietic stem and progenitor cells (HSPCs), common lymphoid progenitors (CLPs), and B cells [41]. While this work demonstrates the feasibility of using TOF-SIMS to objectively identify the fate decisions of individual, unlabeled cells with location specificity, the inability to apply TOF-SIMS to living cells is a major disadvantage.

Identifying SC fate decisions with RS

RS is a label-free technique that uses the inelastic scattering of light to acquire a vibrational spectrum that encodes for the sample's chemical constituents. RS is well-suited for live cell analysis because it can be performed with long wavelength light (785 nm, 633 nm) that has low phototoxicity [44]. Furthermore, the limited spectral contribution of water to Raman spectra, arising from its poor scattering efficiency, allows live biological samples to be analyzed in aqueous medium - a distinct advantage over IR spectroscopy. RS of individual living or fixed cells yields a spectral "fingerprint" of the nucleic acids, proteins, lipids, and carbohydrates in the cell, which may be indicative of the cell type, viability, differentiation stage, cell cycle stage, or metabolism. Advances in surface enhanced and nonlinear RS, such as coherent anti-stokes Raman scattering (CARS) and stimulated Raman scattering (SRS),

enable analyzing and imaging cells with increased resolution and chemical sensitivity [45]. These techniques have been utilized in many of the studies outlined below. While RS-based label-free approaches to high throughput sorting of single SCs have been developed using macro-sampling optical platforms or line-scanning [46,47], this review focuses on microscopy-based RS approaches that enable location-specific cell identification.

RS has been used to analyze and identify the differentiation stages of SCs and their derivatives (Table 1). Information about key biochemical markers of differentiation has been acquired by processing Raman spectra with relatively simple univariate or bivariate approaches that monitor the intensities of a small number of peaks that are distinctive of a cell population. For example, in RS studies of embryonic SC (ESC) differentiation, undifferentiated cells were found to have high ratios of nucleic acid to protein peak intensities, enabling discrimination of ESCs and their mature progeny (Figure 2a) [44,48–51]. The measurement of DNA-related peaks has also been used to assess another important indicator of SC fate, stage of cell cycle, thereby eliminating the need for invasive proliferation assays (Figure 2b) [52,53]. The differentiation of mesenchymal SCs (MSCs) into various connective tissue cells also results in the appearance of phenotypic markers that can be detected by their characteristic Raman peaks [54–56]. Osteogenic differentiation and mineral deposition can be monitored according to the appearance of a hydroxyapatite peak (PO_4^{3-} , $950\text{--}970\text{ cm}^{-1}$) or other mineralized matrix peaks. Furthermore, differences in the positions and shapes of the hydroxyapatite peaks are indicative of subtle changes in mineral crystallinity over time [55]. Similarly, adipose-derived SCs produce Raman-active lipid droplets and ECM components (e.g. collagen) upon adipo- and osteo-induced differentiation, respectively [57].

A univariate or bivariate approach cannot be used to assess SC fate if the change in cell phenotype is not accompanied by a change in the abundance of one or two cellular components (e.g., bone minerals or lipid) that have distinctive Raman signatures. In this case, MVA is used to identify combinations of multiple spectral features that are characteristic of a single cell population. MVA of Raman spectra may reveal biochemical changes during differentiation or between closely-related SCs [58]. PCA and linear discriminant analysis (LDA), a technique for identifying linear combinations of features that discriminate samples/classes, of Raman spectra have enabled accurately classifying human ESCs and their cardiac derivatives based on variance in combinations of peaks common to multiple cell lines (Figure 2c, 2d) [59,60]. Similar techniques have been used to monitor the differentiation status of neural SCs and alveolar epithelial type II cells, and to identify abnormal ESCs and MSCs in culture [61–63]. MVA of MSC spectra has also been used to extract spectral features that improve the accuracy of cell classification over using phenotypic markers (i.e., lipid droplets or minerals) alone, and for comparison of closely-related MSCs from different age groups [64,65]. Notably, the accuracy of identification and sensitivity to early stages of differentiation depends on the subcellular location from which the spectra are acquired (cytosol vs. nucleus). In addition, CARS enabled high-resolution localization of MSC functional markers to their respective subcellular organelles [64]. The concentrations of biomolecules within the nucleoli of fibroblasts and induced pluripotent SCs have been estimated by biomolecular component analysis (BCA) of Raman spectra [66]; such quantitation is seldom achieved with other MVA techniques.

While there has been significant progress in characterizing SCs and their progeny with RS, much of this work studied cells on traditional culture substrates or relatively Raman-inactive substrates (CaF₂, fused silica, gold or silver mirrors, etc.) and not complex engineered tissue scaffolds. The choice of the coating on the substrate can significantly influence cell physiology and, consequently, the cell's Raman spectroscopic signal; therefore, the substrate coating should be carefully considered [67]. A major concern in obtaining and analyzing spectra of cells seeded on complex substrates is that Raman scattering from the substrate can overwhelm the weaker signal from the cell. This is especially problematic for screening SC response in microscale culture platforms with RS because variations in the matrix properties that affect SC differentiation, such as thickness, stiffness, and porosity, may alter the Raman spectra acquired from the cell and decrease the accuracy of identifying cell fate. Complete and precise subtraction of background signals without interfering with cellular signatures is challenging. Optimal experimental conditions for reducing the background signal in tissue samples have been evaluated and may be applied to analyzing individual cells on tissue engineering substrates [68]. Spectral acquisition using a 785 nm laser, which is popular for cell analysis because it minimizes photodamage, in water immersion yields spectra with the lowest background fluorescence signal, with a time-dependent reduction in background signal due to photobleaching [68].

Recent work has demonstrated the potential for analyzing single cells within complex, heterogeneous environments with RS. We have identified the lineages of individual cells from four mature cell lines that were seeded in cocultures using PLS-DA models constructed from Raman spectra of cells in separate monocultures [69]. This use of spectra from cells in separate cultures for PLS-DA model construction helps ensure that the cell identifications were not biased by spectral contributions from the substrate, medium, or surrounding cells. Many studies have focused on characterizing and imaging tissues, including bone, epithelial, muscular, nervous, lung, skin, and breast tissue [9], and some have demonstrated the feasibility of identifying cell phenotypes in these complex environments. For example, murine hair follicle SCs have been identified and characterized within mouse hair tissue by LDA of their Raman spectra [70]. Notable progress has been made in analyzing the Raman spectra of mature and progenitor cell behaviors in artificial tissues to track cellular growth, material production, and interaction with the ECM (Figure 2e) [71–76]. The sensitivity of RS in detecting changes in SC fate decisions within tissue scaffolds on the single cell level remains to be determined.

Conclusions and future directions

TOF-SIMS enables characterizing the surface chemistries on biomaterial scaffolds, which is critical for confirming the outcomes of surface treatments and for understanding the biointerfacial interactions that elicit cell response. The implementation of MVA to correlate TOF-SIMS data with biological response has the potential to accelerate the development of engineered culture platforms that permit directing the stem cell fate *ex vivo*. A next step would be to extend this MVA and TOF-SIMS approach to more complex biomaterials, including native ECM, in order to identify the surface biochemistries in the SC niche that direct SC fate. Ongoing efforts to develop microfluidic analysis chambers for TOF-SIMS address this technique's greatest limitation, its inability to analyze hydrated, unfrozen

samples [77,78]. Such work may eventually enable imaging the surface chemistries on hydrated biomaterials with TOF-SIMS.

Significant advances have been made in identifying the phenotypes of unlabeled living cells with location specificity and on a single cell level using RS. MVA approaches enhance the ability to identify cell differentiation state, especially in the absence of Raman peaks characteristic of phenotypic markers. Importantly, PLS-DA of RS has the potential to significantly reduce the inter-user variability of identifying SC fate decisions, which would improve the reproducibility of assessing cell response in screening applications. However, further work is necessary to realize the full potential of RS for identifying cell fate decisions. While existing data processing tools reduce the substrate's contributions to cell spectra, additional techniques that minimize substrate-related spectral variation are needed to permit accurately identifying the phenotypes of cells on substrates with spatial variations in composition. Additionally, research must address discriminating and identifying individual living cells from the immature, undifferentiated populations that arise during the stepwise differentiation of SCs in order to permit detecting early SC fate decisions with RS. New MVA techniques may be required to unmask the subtle spectral changes that denote small biochemical differences between temporally related cell populations in the presence of intra-population spectral variance related to cell cycle and the culture environment. Advances in instrumentation are also necessary to address certain limitations of RS, such as nonlinear techniques to minimize acquisition times, and spatially offset Raman spectroscopy (SORS) to analyze cells in 3D environments. Finally, these optimized experimental and data processing methods must be integrated into a single approach that permits identifying the differentiation states of individual, living cells in microscale culture platforms for screening cell response. The resulting label-free RS technique for accurately identifying the phenotypes of individual living cells in engineered cultures with location specificity would facilitate identifying the extrinsic cues that permit directing SC fate decisions *ex vivo*.

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Highlights

- TOF-SIMS and Raman spectroscopy are label-free chemical imaging techniques.
- Biomaterial surface chemistry can be imaged and identified with TOF-SIMS.
- MVA of TOF-SIMS data can correlate surface chemistry with cell response.
- Raman spectroscopy can identify the differentiation stages of single, living cells.
- The stage of cell cycle can be determined with Raman spectroscopy.

Figure 1.

Application of TOF-SIMS to tissue engineering applications. **(a)** TOF-SIMS images show the distribution of Ca^+ and $\text{C}_6\text{H}_9\text{O}_3^+$ ions characteristic of hydroxyapatite crystals and carboxymethylcellulose polymer, respectively, at the surface of a composite material for bone tissue engineering. Reproduced with permission from [27]. Copyright © 2013 Wiley Periodicals, Inc. **(b)** PLSR was used to correlate TOF-SIMS data from 48 different polymer substrates to the frequency of SC colony formation [28]. Left: The experimental frequency of colony formation on each polymer is plotted against that predicted by the TOF-SIMS data. Right: The regression coefficient, which is a measure of the ion's contribution to colony formation frequency, is listed for secondary ions associated with various polymer functionalities. Larger values indicate stronger correlation. Negative values indicate the ion inhibits colony formation, positive values indicate a supportive effect. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Mater.* 2010, **9**:768–778. **(c)** Pixels in the TOF-SIMS image are color coded to show the intensities of the peaks associated with esophageal epithelial cells (left) or fibroblasts (middle). Peak associations were identified by PLS-DA of TOF-SIMS data from each cell type. The overlay of the two images (right) shows the relative locations of the esophageal epithelial cells (green) and fibroblasts (purple) in the culture [40]. Adapted with permission from *Anal. Chem.* 2012, **84**:893–900. Copyright 2012 American Chemical Society. **(d)** Identification plots for a PLS-DA model of calibration TOF-SIMS data from HSPCs, CLPs, and B cells [41]. Cells that exceed the

classification threshold (red dashed line) are identified as the indicated cell type. Reprinted with permission from *Anal. Chem.* 2012, **84**:4307–4313. Copyright 2012 American Chemical Society.

Figure 2.

Applications of RS in identifying and visualizing stem cells. **(a)** Average Raman spectra of differentiated and undifferentiated ESCs (top) and classification of cells based on DNA/protein peak ratios and PCA (bottom) [44]. Adapted with permission from *Anal. Chem.* 2010, **82**:5020–5027. Copyright 2010 American Chemical Society. **(b)** Determination of cell cycle in ESCs based on DNA (783 cm⁻¹) peak intensity [52]. Reprinted with permission from *Anal. Chem.* 2013, **85**:8996–9002. Copyright 2013 American Chemical Society. **(c)** PCA was used to monitor cardiac differentiation of ESCs using CARS, specifically of beating and non-beating embryoid bodies. PC1, top, was used to map differentiation in single cells, bottom. Reprinted from [60] with permission from Elsevier. **(d)** Cardiac differentiation of ESCs was also monitored by PCA of spontaneous confocal RS (difference spectra on left) [59]. Reprinted with permission from *Anal. Chem.* 2009, **81**:1324–1331. Copyright 2009 American Chemical Society. **(e)** Integration of smooth muscle cells, visualized using CARS (orange), in artificial compact (i,ii) and porous (iii,iv) cellulose scaffolds, visualized using second harmonic generation imaging (blue) [71]. Images were taken at different focal depths, with a close-up in (iv) from white box in (iii) showing individual cellulose fibers. Reproduced from Brackmann C, Esguerra M, Olausson D, Delbro D, Krettek A, Gatenholm P, Enejder A: Coherent anti-Stokes Raman scattering microscopy of human smooth muscle cells in bioengineered tissue scaffolds. *J. Biomed. Opt.* 2011, **16**:021115.

Table 1

Reported analysis and identification of stem cells using RS.

Year	First author	Instrumentation	Stem cell types analyzed	Analytical techniques	Ref
2004	Nottingham	RS	murine ESC	PCA, HCA	[49]
2004	Nottingham	RS	murine ESC	UniV	[48]
2005	Nottingham	RS	murine ESCs, foetal human bone cell	CLS fitting	[58]
2007	Konorov	CARS	murine ESC	UniV	[51]
2008	Jell	RS	human foetal osteoblast	PCA, LS fitting	[79]
2008	Kim	RS	rhesus monkey MSC	PCA	[65]
2008	Swain	RS	primary human alveolar type II cell	PCA, LS fitting	[62]
2009	Chan	RS	human ESC	PCA-LDA	[59]
2009	Chiang	RS	human MSC	UniV	[56]
2010	Schulze	RS	human ESC	UniV, PCA	[44]
2011	Downes	RS	human ESC, murine blastocyte, human MSC, human ADSC	UniV	[50]
2011	McManus	RS	human MSC	UniV, PCA	[55]
2012	Brackmann	CARS, SHG*	murine osteoprogenitor cell	UniV	[73]
2012	Ghita	RS	murine neural stem cell	UniV, PCA, LDA	[61]
2012	Mortati	CARS, SHG*	human MSC	UniV	[75]
2012	Mouras	CARS, TPEF, SHG	human ADSC	UniV	[57]
2013	Hung	RS	primary human mesenchymal stromal cell	UniV	[54]
2013	Pascut	RS	human ESC	PCA	[60]
2013	Pliss	RS	induced pluripotent stem cell (iPSC) from human dermal fibroblasts	BCA, LCSM	[66]
2014	Lee	BCARS	human MSC	UniV, PCA	[64]
2014	Tsai	RS	murine hair follicle stem cell	PC-LDA	[70]

Legend

RS – Raman spectroscopy (confocal)

(B)CARS – (broadband) coherent anti-stokes Raman

SHG – second harmonic generation imaging

TPEF – two-photon-excited fluorescence

ESC – embryonic stem cell

MSC – mesenchymal stem cell

ADSC – adipose-derived stem cell

* Imaging of surrounding matrix

PCA – principal component analysis

HCA – hierarchical cluster analysis

UniV – univariate (includes bivariate)

LS – least squares

CLS – classical least squares

LDA – linear discriminant analysis

BCA – biomolecular component analysis

LCSM – linear combination spectral modeling