INTRODUCTION TO NANOBIOSCIENCE: A TISSUE ENGINEERING PERSPECTIVE

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Contents

Summary

The chapter presents an introduction and applications of nanobioscience in the context of tissue engineering, which is a rapidly developing field that aims at regenerating or repairing diseased or damaged tissues of the human body. There are three key factors that influence the success of engineered tissues, cells, scaffolds, and biomolecules. Scaffolds play a key role in providing a biomimicking support on which cells can grow into corresponding tissues. Therefore, increasing attention is being paid to the *in vitro* simulation of the nano-scale interaction of cells in the body with native extracellular matrix (ECM). In fact, the fabrication of scaffolds with nanofeatures and nanosignals, which mimic the native ECM environment, has a large impact on guiding and directing the cellular behavior for tissue engineering applications. Numerous techniques are now available for the production of nanostructured or nanopatterned surfaces/substrates. In this chapter, we aim to provide an overview of the methods used to fabricate physical and chemical nanopatterns and nanofiber scaffolds as emerging nanofeatured substrates suitable for tissue engineering. Finally, we review and discuss some of their most notable applications in cell and tissue engineering.

1. Introduction

Nanoscience as an interdisciplinary subject is among the most rapidly developing areas of science and technology in the last few decades. The impact of nanoscience, in combination with nanotechnology, is evident in medicine, helping to create functional tissue grafts for tissue engineering and regenerative applications in the last several years (Sharma, Gautam et al. 2011). Millions of people suffer from a variety of tissue/organ diseases or defects throughout the world. Although autografts and allografts help to solve these clinical problems, there is still a shortage of donor tissues and organs. This shortage has drastically increased recently, causing many patients to die while waiting for donor tissues or organs. Therefore, it is essential to develop more biomimetic tissue grafts in the laboratory for the benefit of human health care. For this reason, tissue engineering has emerged as an applied interdisciplinary field, which aims to repair tissue or cause its regeneration by applying the principles and methods of biological, chemical, and engineering sciences (Langer and Vacanti 1993;). The concept of tissue engineering involves isolating cells from a patient or donor, culturing them *in vitro* to increase their number and maintain their distinct phenotypes, seeding them onto a scaffold to create an engineered tissue graft, and finally transplanting the engineered tissue graft back into the patient's body where the tissue regeneration is needed (see Fig. 1).

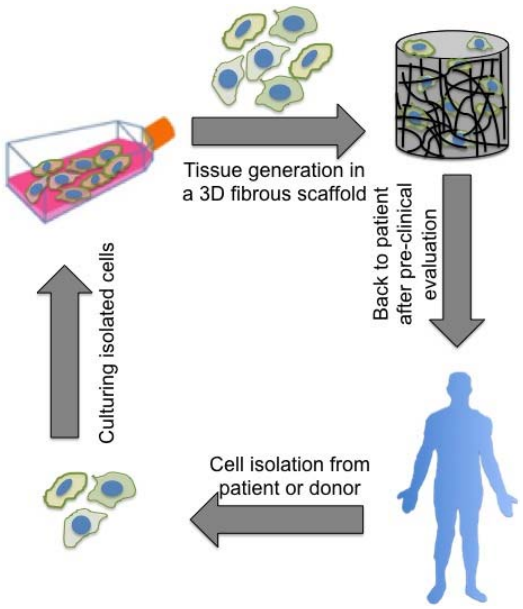

Figure 1. Concept of scaffold-based tissue engineering.

The scaffold provides structural support for cell culture and tissue growth. It also delivers bioactive molecules, such as growth factors, to cells to promote their surface attachment, migration, proliferation, differentiation, and continued growth, all of which ultimately lead to the formation of new tissue. To produce functional tissue grafts, which would successfully integrate into the patient's body and restore the function of lost or damaged tissue, tissue engineering scaffolds should closely mimic the native extracellular matrix (ECM) of the body (Seidi and Ramalingam 2012). ECM consists of various types of proteins exhibiting nanoscale structures and their microscale counterparts, such as fibrils and pillars, which determine cell-matrix interactions that are required for functional tissue development. For example, collagen fibrils, which are one of the most important components of ECM, are approximately dozens of micrometers long and between 50 and 400 nm wide (Bozec, van der Heijden et al. 2007, Murugan and Ramakrishna 2005, Murugan and Ramakrishna 2007). This nanotopographical structure of native ECM has an important role in specific tissue formation through a phenomenon known as contact guidance. Significant attention has also been paid to the development of biomaterials-based three-dimensional (3D) scaffolds, among which nanofiber-based scaffolding systems have shown unique potential for closely mimicking native ECM architecture (Ma and Zhang 1999). Nanofiber scaffolds offer a high surface to volume ratio with a high porosity, which favor the cell adhesion, migration, proliferation, and differentiation. Therefore, there has been an increasing trend toward fabricating nanofiber scaffolds suitable for tissue engineering applications in addition to nanopatterned biomaterial systems. In this chapter, we aim to introduce nanobioscience and its role in tissue engineering as an elegant bottom-up approach for engineering tissues *in vitro*. We focus our attention on the significance of nanopatterns, nanopatterning techniques, nanofiber tissue scaffolds, nanofiber fabrication methods, and their applications to tissue engineering.

2. Nanopatterns and Nanopatterning Techniques

Today's interest in nanomedicine continues to grow because reducing the scale of engineering approaches with the use of nanotechnologies will improve the quality of materials and their interactions with cells and tissues. In fact, the direct cellular

environment is in nanometric scale, including the ECM with which cells are familiar and in which they evolve. Mimicking this natural environment by producing materials with nanofeatures and nanosignals to guide and direct cellular behavior is therefore of the highest importance for tissue engineering applications. Numerous techniques are now available for the production of nanostructures and nanotextured or nanopatterned surfaces, including polymer demixing, nanografting, dip-pen nanolithography, conductive atomic force microscopy, nanocontact printing, nanoimprint lithography, photolithography, laser holography, electron beam lithography, colloidal lithography, UV-assisted capillary force lithography, soft lithography, polymer templating, DNA templating, metal anodization, molecular beam epitaxy, self-assembly, electrospinning, nanophase coating, and glancing angle deposition. Rather than discussing these techniques in detail, we refer those readers who are interested in learning further details to several comprehensive articles (Engel, Michiardi et al. 2008; Dolatshahi-Pirouz, Nikkhah et al. 2011, Seidi and Ramalingam 2012). In the following section, we will focus on the different types of nanopatterns used in basic cell/tissue studies and their effects on cell behavior in terms of cell adhesion, migration, proliferation, differentiation, and continued function, which are all important parameters for the success of functional tissue engineering.

2.1. Physical Nanopatterns

Physical patterning refers to the modification of biomaterial substrates with a pre-defined texture by modulating their size and shape. Physical substrate characteristics, such as stiffness, roughness, and topography, have a significant influence on the regulation of cell behavior, particularly cell adhesion, migration, proliferation, and differentiation. With the introduction of micro/nanofabrication techniques into the life sciences, significant evidence has been gathered that cells sense and respond to microscale and nanoscale features (Evans, Britland et al. 1999). Thus, a recent study showed that myoblasts aligned to 10 – 15 nm diameter cellulose nanowhiskers (Dugan, Gough et al. 2010). The mechanism by which cells sense these physical cues is not clearly known. However, there is some evidence that filopodia are involved in sensing such cues because they extend in front of cells and probe nearby nanotopographic features (Lim, Hansen et al. 2005). These nanopatterns have major effects on cell adhesion, orientation, shape, proliferation, migration, differentiation, signaling cascades, and gene activation (Lim and Donahue 2007). They may have different forms, such as columns, islands, pits, protrusions, or nodes, and can also be anisotropic structures, such as patterns of ridges/grooves or isotropic-like nanoislands, homogeneously covering the substrate surface.

Many studies using patterns of ridges/grooves have shown that cell alignment with the pattern increases with increasing groove depth while decreasing with increasing groove width or pitch, which is the sum of the groove and ridge width (Zhu, Lu et al. 2005). Thus, Yim et al. observed cell and nuclei alignment, elongated cell shape, and reduced cell proliferation when smooth muscle cells (SMCs) were cultured on nanograting with 350 nm line width and depth (Yim, Reano et al. 2005). In addition, a study by Zhu et al. using nanogrooves with a depth of 60 nm reported that mesenchymal stem cell (MSC)-derived osteoblasts cultured on this substrate showed anisotropic alignment and mineralized matrix (Zhu, Lu et al. 2005). If the groove width has a nanometric size, cells tend to bridge over the top of the ridges rather than reside inside the grooves (Teixeira, Abrams et al. 2003). For example, epithelial cells covered the floor of 2 μm wide grooves, whereas they bridged over 950 nm wide grooves. Studies using other nanostructures have shown that fibroblast adhesion was improved on 13 nm-high island nanotextured surfaces but were impaired on 95 nm high islands (Dalby, Giannaras et al. 2004). Lim et al. also observed better adhesion and differentiation of osteoblasts cultured on 11 nm high island nanotextured surfaces than on 85 nm high islands (Lim, Hansen et al. 2005). Although cell adhesion cannot occur across pits, and cells must settle in the inter-pit area, by using

varied nanopit sizes, Curtis et al. observed better cell adhesion with 35 nm pits than with 120 nm pits (Curtis, Gadegaard et al. 2004). Fibroblasts cultured on nanocolumn-textured surfaces (160 nm high, 100 nm in diameter, and 230 nm gaps) displayed less spreading, had a rounder shape, had a greater density of filopodia, which probed the cellular environment and attempted to endocytose the nanocolumns, than fibroblasts cultured on non-textured surfaces (Dalby, Berry et al. 2004;). In summary, these nanofeatures can be classified as low- or high-adhesive substrates. Small islands (20 nm), columns (11 nm), nanoposts (pointed columns), and pits (35 nm) promote cell adhesion, whereas cell adhesion decreases when the size of these features increases (Dalby MJ 2007). The scale and type of nanopattern as well as the symmetry of its display (as in orthogonal or hexagonal nanopit arrays) have an effect on cell behavior (Curtis, Gadegaard et al. 2004;Gadegaard, Martines et al. 2006).

Nanostructured surface roughness also influences cell behavior. In fact, several studies of orthopedic implants have shown higher functionalities of osteoblasts cultured on nanotitania rather than on microtitania particle surfaces (Webster, Ahn et al. 2007). To rule out interfering factors, Palin et al. transferred the nanotitania and microtitania pattern roughness to poly(lactic-co-glycolic acid) (PLGA) and observed higher osteoblast adhesion and proliferation with the nanotitania pattern roughness-structured PLGA (Erica,Huinanet al. 2005). With cell adhesion, various signaling cascades and genes are activated by mechanotransduction. Therefore, nanotopography also has important effects on cell differentiation. For example, Yim et al. cultured human mesenchymal stem cells (hMSCs) on nanograting of 350 nm line width and depth and observed cell alignment and elongation along the pattern topography, as well as upregulation of neuronal markers (Yim, Pang et al. 2007). In another study, Yang et al. cultured neural stem cells (NSCs) C17.2 cells on poly(L-lactic acid) (PLLA) electrospun nanofibers and observed that cell differentiation was higher on nanofibers than on microfibers as evidenced by the presence of more extensive neurite-like outgrowths on aligned nanofiber scaffolds (see Fig. 2) (Yang, Murugan et al. 2005). These experimental data and others show that cells are very sensitive to physical nanopatterns, which have an important impact on cell behavior, and this sensitivity must be considered while engineering functional tissues for regenerative applications.

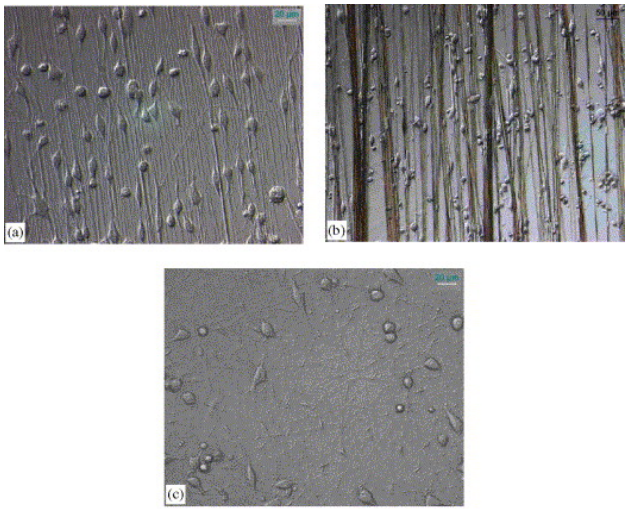

Figure 2. Photographs of phase contrast microscopy showing NSCs on (a) aligned nanofibers, (b) aligned microfibers and (c) random nanofibers after one day of culture. Reprinted with permission (Yang, Murugan et al. 2005).

2.2. Chemical Nanopatterns

Chemical patterns refer to the modification of biomaterial substrates with patterns of different chemicals or biochemical reagents. Chemical nanopatterning can be realized by nanopatterns of chemical moieties (e.g., self-assembled monolayers (SAMs)) or biological moieties (e.g., RGD peptides). Patterning of ECM proteins, such as fibronectin, laminin, vitronectin, or collagen, to promote cell adhesion is of great interest for tissue engineering. Each ECM protein induces a specific binding with integrins (e.g., fibronectin preferentially binds α5β1, vitronectin binds αvβ3, and collagen binds α2β1). It has been proposed that protein deposition plays a role in contact guidance. Following this hypothesis, local micro-nanotopography may interfere with protein adsorption or change of protein's adhesive functionality, leading to discontinuous protein deposition and local protein concentration (van Kooten and von Recum 1999). However, this hypothesis has not been confirmed, and other studies have shown that protein deposition does not localize preferentially with topographic discontinuities, while cells sense topography irrespective of protein deposition (Wilson, Clegg et al. 2005).

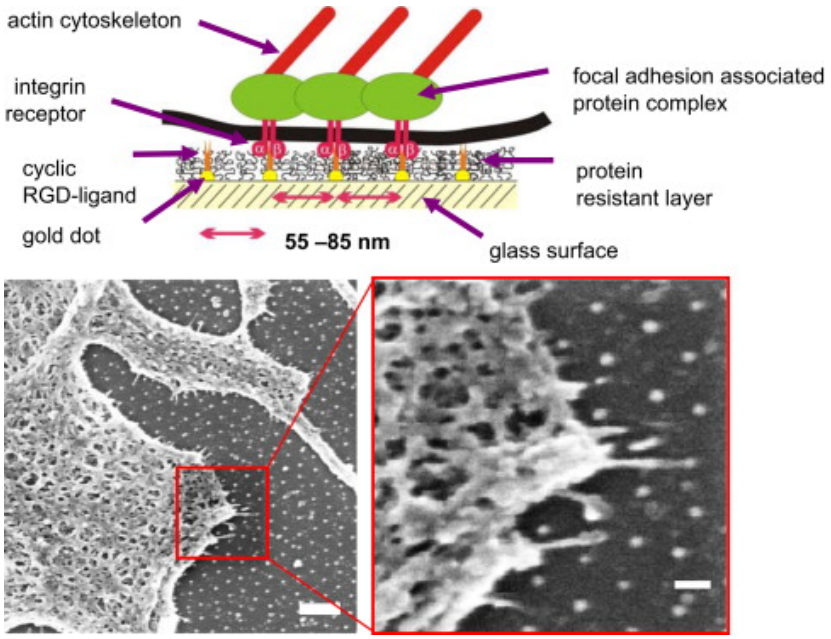

Figure 3. (Upper panel) Schematic showing the biofunctionalization of nanopatterns to control the clustering of integrin: gold dots are functionalized with c(-RGDfK-) thiols whereas the glass areas between the gold dots are covalently bound to polyethylene glycol to prevent non-specific protein adsorption. Thus, cells can only attach on the c(-RGDfK-)-covered gold nanodots. (Bottom panels) Micrographs of MC3T3 osteoblast in contact with a biofunctionalized 80 nm pattern, exhibiting filopodia to sense the environment. Bars: 20 μm (left); 200 nm (right). Reprinted with permission (Hirschfeld-Warneken, Arnold et al. 2008).

As evidenced from different studies, adhesive ligand spacing is also an important parameter regulating cell behavior. For example, Arnold et al. densely packed nanodots on a gold substrate with spacing ranging from 28 to 85 nm. The nanodots were coated with adhesive cyclic peptide RGDfK-thiol, which has a strong affinity for integrin αvβ3, and were <8 nm in size, allowing the binding of only one integrin molecule per dot. When this matrix was used in culture with osteoblasts or other cell types, good cell adhesion and spreading with ligand spacing <70 nm was observed (Arnold, Cavalcanti-Adam et al.

2004). Other studies have also shown that cell activation is increased when ligand spacing decreases over the range of 70 to 10 nm (Cavalcanti-Adam, Volberg et al. 2007). Gradients of ligand density have also been used to investigate cellular adhesion, spreading, and migration. For example, Hirschfeld-Warneken et al. cultured osteoblasts on a gradient of spaced nanodots (see Fig. 3). These nanodots had a diameter of 6 nm and were coated with cyclic RGDfK. The researchers observed an increase in cell adhesion and spreading with decreased nanodot spacing, while nanodot spacing >73 nm reduced cell adhesion and spreading (Hirschfeld-Warneken, Arnold et al. 2008). As the gradient of binding site spacing had a strength of Δ15 nm/mm and induced cell polarization, by analyzing cell morphology, they were able to determine that an osteoblast cell can sense a 1 nm spacing difference between its front and back (Hirschfeld-Warneken, Arnold et al. 2008). During displacement, a cell binds to a new position at its front while unbinding from positions at its back; thus, adhesive ligand spacing is also important for cell motility. Maheshwari et al. showed that cell migration depends not only on the spacing between binding sites but also on the number of adhesive ligands on the site (Maheshwari, Brown et al. 2000). Experimental studies with gradients of adhesive ligand density have shown that cell migration increases toward higher ligand density and adhesiveness up to a threshold point, above which it reaches a plateau or decreases (Smith, Elkin et al. 2006).

Hydrogels, a type of biomaterial that has a network of hydrophilic polymer chains, have also been used to study cell behavior, particularly 2D and 3D cell adhesion, largely because the number and repartition of adhesive ligands presented to cells can be tuned. Huebsch et al. showed that hMSCs encapsulated in an alginate hydrogel containing RGD peptides mechanically reorganized ligand presentation near the integrins at a nanoscale level when the flexibility of the substrate allowed such behavior (Huebsch, Arany et al. 2010). Nanopatterns can also be realized by self-assembly, and networks of nanofibers made of self-assembled amphiphilic peptides can be functionalized to direct cellular behavior (Khademhosseini, Rajalingam et al. 2010). For example, Silva et al. synthesized a hydrogel with incorporated peptide isoleucine-lysine-valine-alanine-valine (IKVAV), which promotes neurite outgrowth. When murine neural progenitor cells (NPCs) were encapsulated, the researchers observed rapid stem cell differentiation into neurons and repression of astrocyte development (Silva, Czeisler et al. 2004). Wang et al. functionalized RADA16-I peptide by coupling it with RGD peptide to increase endothelial cell adhesion and with vascular endothelial growth factor (VEGF) mimicking motifs to induce angiogenesis. Cultured with HUVECs, this self-assembled nanofiber scaffold enhanced endothelial cell adhesion, migration, proliferation, and induced capillary formation (Wang, Horii et al. 2008). Self-assembly technology has also been applied *in vivo* to the release of basic fibroblast growth factor (bFGF). When amphiphilic peptide and bFGF solution were injected subcutaneously into a mouse, a clear hydrogel formed that induced prolonged bFGF release with significant angiogenesis (Hosseinkhani, Hosseinkhani et al. 2006). Other studies have used self-assembly to culture synthetic dermis and synthetic skin (Kao, Kadomatsu et al. 2009), for bone regeneration (Yoshimi, Yamada et al. 2009), neural regeneration (Ellis-Behnke, Liang et al. 2006; Gelain, Bottai et al. 2006), hemostasis (Ellis-Behnke, Liang et al. 2006) and other biomedical applications (Ellis-Behnke and Jonas 2011). Another nanopattern type currently used in orthopedic applications is made using nanophase deposition. Nanophase coating by sol-gel reaction or pulse electrodeposition is currently used to enhance the osteointegration of a material by coating it with hydroxyapatite (Caruso and Antonietti 2001;Saremi and Golshan 2007). Delivering chemical and topographical signals together to the targeted site is desirable for tissue engineering applications. Some studies have showed that chemical and topographical cues can compete or have synergetic effects on cells. For example, Britland et al. showed that BHK cells preferred to align on chemical rather than topographical cues when cultured on groove/ridge patterns overlaid orthogonally with continuous adhesive protein strips (Britland, Morgan

et al. 1996). However, when both cues were presented to cells in parallel, cell alignment was synergistically enhanced. In contrast, when Charest et al. cultured MC3T3-E1 osteoblasts on embossed groove/ridge patterns overlaid orthogonally with discontinuous printed fibronectin lanes, they observed that cells aligned on the topographical cues (Charest, Eliason et al. 2006).

In this section, we have highlighted different aspects of nanopatterns and nanopatterning techniques. Physical features, such as topography, size, symmetrical order or disorder, roughness, and 2D or 3D scaffolding environments, and chemical features, such as adhesive ligand number and spacing, SAMs, protein or nanophase coating, and functionalized peptides, all have a strong impact on cell behavior and must be considered for tissue engineering applications. *In vivo*, native ECM is a 3D map full of biophysical and biochemical cues that guide and regulate such cell behaviors as adhesion, proliferation, differentiation, and gene expression. Mimicking ECM with respect to the way in which information is delivered to cells is still far from being accomplished. Therefore, mimicking the environmental complexity familiar to cells by integrating ECM nanofeatures into scaffolding systems is necessary and will enhance cell functionality. For example, increasing cell adhesion or favoring stem cell differentiation by the use of nanopatterned surfaces is already advantageous from a tissue engineering perspective. Nanofeature-patterned surfaces may also be more easily accepted by host tissues, reducing fibrous encapsulation. Combining physical and chemical cues on nanopatterned scaffolds offers a way to induce multiple synergetic cellular signaling responses and heterotypic cell stimulation. Mixing micro- and nanofeatures will increase the scaffold's potential. To mimic 3D natural matrix more thoroughly, the adjunction of temporal control on signal delivery will be required. With advances in nanoscience and nanotechnology, patterning techniques are currently evolving toward biomedical applications that could help to build biomimetically functional matrix suitable for tissue engineering. In the following sections, current methods of fabricating nanofiber scaffolds as promising nanobiomaterials for tissue engineering applications will be discussed.

3. Methods of Fabricating Nanofiber Scaffolds

There are three major techniques currently utilized for the fabrication of nanofiber scaffolds: electrospinning, self-assembly, and phase separation. Among them, electrospinning is the most widely used technique for fabricating tissue engineering scaffolds.

3.1. Electrospinning

Electrospinning is a versatile technique for producing nanofibers from polymers and their composites. As explained in Murugan and Ramakrishna 2006, this technique was introduced almost a century ago. At that time, it was named "electrostatic spray" or "electrostatic spinning" and was later renamed as "electrospinning" in the 90's. However, only a decade ago, this method attracted a great deal of attention from researchers fabricating scaffolds for tissue engineering applications. The particular merit of this technique is that it allows building scaffolds with structural features quite similar to those of native ECM. It is also a more versatile technique than other conventional scaffold methodologies. For example, nanofibers with spatial orientation, high aspect ratios, and large surface areas can be produced, while allowing control over pore geometry. These favorable characteristics directly influence the cell adhesion, migration, contact guidance, and transportation of oxygen and nutrients to the cells. On this base, electrospun nanofibers could serve as an optimal tissue scaffold providing spatial environments for the growth of new functional tissue with appropriate physiological metabolic functions.

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Biographical Sketches

Dr. Serge Ostrovidov received his Ph.D. degree in biology and health from Nancy University, France, for which he worked on the antioxidative properties of new selenated molecules. From 1998 to 2001, he worked in immunology at Northwestern University, Chicago, USA, on the role of oxidative metabolites in altering the immune response. He moved then to Japan where he worked on BioMEMS during several years at the University of Tokyo. During 5 years he was a scientist at Pentax Corporation working mainly on biomaterials as hydroxyapatite and polyethylene terephthalate. He is currently working on biomaterials and tissues engineering in the Khademhosseini laboratory at WPI-Advanced Institute for Materials Research in Tohoku University, Sendai, Japan.

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Dr. Ali Khademhosseini is an internationally recognized bioengineer regarded for his contributions and research in the area of biomaterials and tissue engineering. Currently he is an associate professor at Harvard University and holds appointments at the Harvard-MIT Division of Health Sciences Technology, Brigham & Women's Hospital and Tohoku University. Also he is an associate faculty of the Wyss Institute and Harvard Stem Cell Institute. His research is based on developing micro and nanoscale technologies to control cellular behavior, developing microscale biomaterials and engineering systems for tissue engineering, drug discovery and cell-based biosensing.

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Tissue Engineering, and the *Journal of Bionanoscience*. He is a recipient of several prestigious fellowships and awards, including CSIR Fellowship (India), SMF Fellowship (Singapore), NRC National Academies Fellowship (USA), Nationale Professeur des Universités (France), Fellow of Institute of Nanotechnology (UK) and Fellow of Royal Society of Chemistry (UK).