# CHAPTER U

# Applications of Nanotechnology in Tissue Engineering

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## 1. INTRODUCTION

Tissue engineering, as an emerging and rapidly growing field, has received extensive attention [1–3]. The ultimate goal of tissue engineering as a treatment concept is to replace or restore the anatomic structure and function of damaged, injured, or missing tissue or organs following any injury or pathological process by combining biomaterials, cells, or tissue, biologically active molecules, and/or stimulating mechanical forces of the tissue microenvironment [4–6].

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Biomaterials are fashioned into three-dimensional scaffolds to provide mechanical support and guide cell growth into new tissues or organs. The scaffolds have to be highly porous to allow seeding of cells at high densities and, upon implantation into the body, to facilitate the infiltration and formation of large numbers of blood vessels for nutrient supply of the transplanted cells and the removal of waste products. The extracellular matrix deposited by the cells confers the physical, mechanical, and functional properties of the tissue or organ. Signals originating from the underlying substrate and the surrounding environment govern the response of the cells and their assembly into desired structures [4–6].

However, tissue engineering as a discipline is very young and still in the early developmental stage. Surprisingly, after only approximately 4 decades of growth, the field of tissue engineering is no longer limited to the academic laboratory but is rapidly growing in industry as well. For example, tissue-engineered skin is already available on market shelves in many countries including the United States and the United Kingdom [6]; tissue-engineered cartilage, temporary liver-assistance devices, and tissue-engineered pancreas are all in clinical trials [4, 5]. Up to now, investigators have attempted to grow bone [5], liver [4, 7], arteries [8], bladder [9], pancreas [10], nerves [11], cartilage [5], heart valves [8], corneas [12], and various other soft tissues [13].

To date, most tissue engineering studies are focused on the investigations of macrolevel structures (e.g., supercelluar structures $>100$ $\mu$m and cellular structures $>10$ $\mu$m) to build the essential gross morphology and generate real-size organ systems. However, to ultimately engineer the functional units of the tissue, not only the supercelluar and cellular scale structures but also the subcellular scale structures (0.1–10 $\mu$m) and nanostructures (1–100 nm) need to be constructed to control cellular environment, cell–molecular interactions, and cell–cell interactions. It is quite obvious that the full function of the tissues and organs cannot be recovered without rebuilding the ultrastructures of the tissue itself. The future of tissue engineering is highly dependent upon our profound knowledge of how subcellular and even smaller structures affect cell functions and fabrication of organ scale structures with subcellular resolution and nanoresolution, e.g., integration of functional cells to 3D architectures with nanoresolution for improved tissue functionality. Therefore, engineering tissue toward the miniaturization at the nanolevel to precisely design the components inside the regenerated tissue is one of the most promising directions for tissue engineers. To mimic the native tissue structures in tissue-engineered grafts, biomaterial scaffolds that are designed and fabricated at molecular and atomic resolutions are necessary. Nanotechniques to manipulate materials and devices at the level of atoms, molecules, and supermolecules (1–100 nm in scale) have to be employed. Tissues can be engineered at different scales as shown in Figure 1. Using nanotechnology, tissue engineered products with highly predictable biological and physical properties may be obtained.

## 2. APPLICATIONS OF NANOTECHNOLOGY IN TISSUE ENGINEERING

As we mentioned earlier, there are four basic components in tissue engineering, i.e., biomaterial scaffold, functional cells, biomolecules (e.g., growth factors, ECM, other functional molecules), and dynamic forces. In this chapter, we are going to discuss the applications of nanotechnology in tissue engineering in three aspects: biomaterials scaffolding, cellular engineering, and biomolecular manipulation.

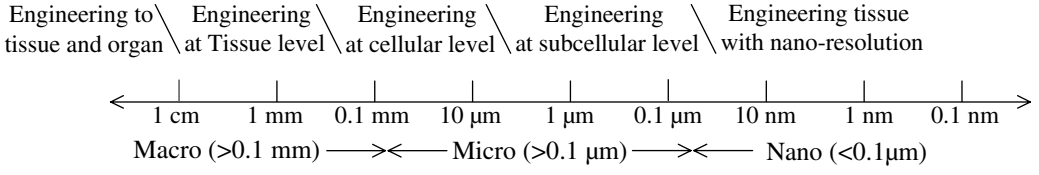


**Figure 1.** Tissue engineering at different levels.

## 2.1. Applications of Nanotechnology in Biomaterial Scaffolds

A biomaterial scaffold creates a milieu within which cells are instructed to form a tissue or organ in a highly controlled way [3, 12]. The principal function of a scaffold is to direct cell behaviors such as migration, proliferation, differentiation, maintenance of phenotype, and apoptosis, by facilitating sensing and responding to the environment via cell–matrix communications and cell–cell communications. Therefore, the desirable physical characteristics of biomaterial scaffolds for tissue engineering applications include high porosity, large surface area, large pore size, and uniformly distributed interconnected porous structures throughout the matrix [7, 8]. In addition, the scaffold has to provide spatial signals to modulate the organization of the cells as well as that of the extracellular matrix derived from them [4–6].

Using nanotechnology, biomaterial scaffold can be manipulated at atomic, molecular, and macromolecular levels and constructed into specific geometrical and topological structures at 1–100 nm scales. Creating tissue engineering scaffolds in nanoscale may bring unpredictable new properties to the material, such as mechanical (stronger), physical (lighter and more porous), optical (tunable optical emission), color, chemical reactivity (more active or less corrosive), electronic properties (more electrically conductive), or magnetic properties (superparamagnetic) and may come up with new functionalities as well, which are unavailable at micro- or macroscales [14–16]. Other advantages of using nanotechnology for scaffold fabrication may include enhancing biocompatibility, improving contact guidance, reducing friction and therefore wear for joint applications, reducing the need for revision surgery, altering physical or chemical characteristics of the scaffold, and promoting tissue growth around the implant. For example, it has been found that nanosculpting the surface of such scaffolds may stimulate cell growth; the cells rapidly follow the nanoscopic etched tracks, resulting in a faster filling of the matrix with the required cells or tissues [17]. More importantly, some complex specific tissue functions cannot be easily mimicked with macroresolution scaffolds.

Nanoscale precision scaffolds can be built in three ways, i.e., atom-by-atom, molecule-by-molecule (also called top-down), or self-assembly (also called self-organization or bottom-up). Top-down means fabricating nanodevices from the microlevel to the nanolevel, for example, stripping a virus particle down to form a viral cage; while bottom-up means obtaining nanodevices from the atoms and small molecules at a level smaller than the nanolevel, for example, building supermolecular architectures from single small molecules, even single atoms [18].

### *2.1.1. Three-Dimensional Nanofibrous Scaffold*

Collagen is a natural extracellular matrix (ECM) molecule found in many tissues such as bone, skin, tendons, ligaments, and other connective tissues. It has a fibrillar structure with a fiber diameter ranging from 50 to 500 nm. Collagen provides a substrate for cellular recognition and promotes cell attachment, proliferation, and differentiated function. Cellular recognition is an advantage for promoting cell adhesion, migration, and proliferation. However, sometimes advantages can be disadvantages. For example, cellular recognition can cause immunogenicity, which is not desirable and a common problem with natural polymers. Other problems with natural polymers are inconsistencies in their mechanical properties, their degradability, and their reproducibility between samples. To mimic the size and morphology of natural extracellular matrix for engineering tissues, such as fibrous collagen matrix, Ma et al. [19] have developed a three-dimensional nanofibrous scaffold using a phase-separation technique from biodegradable synthetic polymers, which avoids the concerns of pathogen transmission and immunorejection associated with collagen from animal and cadaver sources. To further improve the mass transport and new tissue organization, they have built three-dimensional macroporous architectures into the nanofibrous matrices by incorporating water-soluble sugar fibers with special arrangement. Briefly, polymer was dissolved in a solvent to form a homogenous solution. Polymer solutions are cast over the sugar fibers assemblies in a mold and are then thermally phase-separated to form nanofibrous matrices. The sugar fibers are leached out with water to finally form the synthetic nanofibrous scaffold with predesigned macroporous architectures [19]. The overall macroporous architectural design provides channels for improved mass transport and neovascularization

and allows uniform seeding of cells throughout the whole 3D scaffold. The nanofibrous matrices provide a large area for cell attachment, growth, and differentiation.

### 2.1.2. Electrospinning

Electrospinning is another technique for nanofibrous scaffold fabrication. Polymers are dissolved into a proper solvent or melt before being subjected to a voltage to overcome the surface tension and viscoelastic forces and form different size fibers (50 nm to 30 $\mu$m in diameter), which features a morphologic similarity to the extracellular matrix (ECM) of natural tissue, high porosity, and effective mechanical properties [20]. Such a structure meets the essential design criteria of an ideal engineered scaffold; therefore, the sizes of electrospun fibers represents an attractive size range for tissue engineering, wound healing, and related applications. Electrospun nanofibers have been shown to support cell attachment and proliferation of smooth muscle cells and fibroblasts [21]. Cells seeded on this structure tend to maintain phenotypic shape and guided growth according to nanofiber orientation [20]. Matthews et al. have demonstrated that it is possible to tailor mechanical properties of electrospun nanofiber substrates by controlling fiber orientation [22]. Our group at Clemson University is developing highly aligned ultrathin fiber bundles which mimic the natural fibrous structure in tissue through electrospinning for neuronal regeneration purposes, since it is generally believed that organized neuronal architecture is essential for nervous system development, functioning, and regeneration [23–25].

Adjusting process parameters and polymer solution characteristics can vary fiber sizes and properties. Many polymers are adaptable for an electrospinning process, such as polyethylene oxide, poly(ethylene-*co*-vinyl alcohol), DNA, polyaramids, polycaprolactone, PLA, PGA, polyaniline, and polypeptides [21]. Electrospinning can be used to prepare bioactive nanofibers, which may be used for carrying active biomolecules. For example, enzymes can be immobilized via material engineering and improved stability and activity of the enzymes were observed [26]. The electrospun structure, composed of fibers ranging from 50 to 1000 nm in diameter, features a morphologic similarity to the extracellular matrix (ECM) of natural tissue. A wide range of pore diameter distribution, high porosity, and effective mechanical properties could be obtained through the well-developed electrospinning technique. Adjustable structural and mechanical properties meet the essential design criteria of scaffolds [20].

Huang [27] modified Type I collagen with PEO and fabricated nonwoven nanofibers networks by the electrospinning of a weak acid solution of purified collagen at ambient temperature and pressure. Uniform fibers with a diameter range of 100–150 nm were produced from a 2 wt % solution of collagen–PEO. High-resolution SEM and TEM were used to characterize nanofiber morphology. Their data demonstrated that solution viscosity, conductivity, and flow rate influence nanofiber morphology. Mechanical strength, such as ultimate tensile strength and elastic modulus of the resulting nonwoven fabrics, was dependent upon the chosen weight ratio of the collagen–PEO blend. $^{1}$H NMR dipolar magnetization transfer analysis suggested the superior mechanical properties. This process provides a convenient, nontoxic, nondenaturing approach for the generation collagen-containing nanofibers and nonwoven fabrics that have potential application in wound healing and tissue engineering and as hemostatic agents.

Bioactive nanofibers with immobilized enzymes were prepared from polystyrene by Jia et al. using an electrospinning method. A model enzyme, $\alpha$-chymotrypsin, was attached on the fiber surface by chemical bonding. The enzyme loading and bioactivity were examined by active site titration. Over 27.4% monolayer coverage of the external surface of nanofibers and over 65% activity of that of the native enzyme were achieved. The apparent hydrolytic activity indicates a high catalytic efficiency as compared to other forms of immobilized enzymes. Furthermore, nanofibrous $\alpha$-chymotrypsin exhibited a much-improved nonaqueous activity that was over 3 orders of magnitude higher than that of its native counterpart suspended in organic solvents, including hexane and isooctane. It appeared that the covalent binding also improved the enzyme's stability against structural denaturation, such that the half-life of the nanofibrous enzyme in methanol was 18-fold longer than that of the native enzyme [26].

### 2.1.3. Self-Assembly

Self-assembly, or self-organization, refers to the reversible and cooperative assembly of predefined components into an ordered superstructure. Self-assembly is a key process for life, for example, nucleic acid synthesis, protein synthesis, and energy transduction are associated to self-assembly processes. From a tissue engineering standpoint, self-assembly could be used to engineer different types of surface topography on the nano- and microscale to influence cell adhesion, migration, function, and tissue integration, to establish a controlled local microenvironment (protein and other macromolecular patterning) via surface functionlization of biomaterials to generate micro- and nanoscale mechanical stresses affecting cell–biomaterials interactions, and to position cells precisely on scaffold surface to control cell interactions. However, these structures are not very stable, due to noncovalent interactions, such as hydrogen bonds, ionic bonds, hydrophobic interactions, and van der Waals interactions, among the assembled molecules [28]. Self-assembly can be used to produce a variety of structures, such as films, bilayer, membranes, nanoparticles, fibers, micelles, capsule, tubes, coils, mesophases, or unilamellar and multilamellar vesicles [29]. These structures can further self-organize into the superstructures, such as lamellar, hexagonal, and cubic structures, which are especially attractive for fabrication of zeolite-like nanoporous materials that are superb for scaffold uses. Peptides and proteins are mostly used for fabricating scaffolds by the self-assembly approach, owing to their versatile structures for building suprastructures. For example, natural scaffolds in human tissue are superstructures self-assembled from protein and peptide blocks; these superstructures include collagen, elastin, keratin, etc. The possible applications include the preparation of nanoparticles, the exploitation of biomineralization in bone tissue engineering, the design of nanomotors, the development of functionalized delivery vectors, and templating of nanostructures on scaffold surfaces [30–35].

***a. Templating*** Templating is an interesting phenomena during the normal hard tissue development, organic phase, in which collagen fibers work as a template to guide inorganic phase formation in bone and teeth [36]. Bones and teeth are biocomposites that require controlled mineral deposition during their self-assembly to form tissues with unique mechanical properties. He et al. used dentin matrix protein 1 (DMP1), an acidic protein, to nucleate the formation of hydroxyapatite *in vitro* in a multistep process. The nucleated amorphous calcium phosphate precipitates ripen and nanocrystals form [36]. Pins et al. used a self-assembly process to form collagen fibers, guide the natural hard tissue formation, and engineer the bone tissue through this biomimetic approach [37]. Using this biomimetic idea, protein–hydroxyapatite composites with considerable strength are developed, although the mechanical properties are far from satisfied [38]. In order to obtain real bone tissue with satisfied structure and properties, we must understand the microstructure and molecular-level interactions controlling biomineralization processes [39]. Recently, Hartgerink et al. have used the self-assembly approach to generate a nanostructured gel and mimicked some of the key properties of the human bone extracellular matrix (ECM) [34]. Briefly, they used amphiphilicity of small molecules with a hydrophobic alkyl tail and a hydrophilic oligopeptide head to create micellar structures, which can crystallize into polymeric nanofibrils. The phosphoserine residues on the fibril surfaces can initiate the nucleation and growth of minerals [34]. Approaches based on this process may prove useful in the regeneration of bony tissue [18]. In summary, the templating process involves a number of distinct steps [29]. the first step is design, synthesis, and modification of the self-assembling molecules. The second step is examination of the self-assembly behaviors and characterization of the assembled microstructure. If desired microstructures are obtained, the structures will be used as a template for ruggedization. The templated structures will be characterized and ready for application.

***b. Ionic Self-Complementary Peptide*** Zhang et al. [30] synthesized a 16 amino acid peptide, ~5 nm in size, with an alternating polar and nonpoplar pattern. The peptides can form stable $\beta$-strand and $\beta$-sheet conformations, with side chains with one polar side and one nonpolar side, and then undergo self-assembly to form nanofibers. These nanofibers can form interwoven mats that form three-dimensional hydrogels, with high water contents

(>99.5%), which may by suitable for tissue engineering. The other feature of this peptide is that if the charge orientation is changed, entirely different molecules can be obtained.

***c. Bionanotubes/Lipid Tubules*** Schnur et al. used amphiphilic molecules, which contain distinct hydrophobic and hydrophilic segments, to form self-assembled lipid tubules. These tubules can be used as microvials for long-term release of active agents [40]. However, the lipid tubules are very weak in terms of mechanical properties. Meanwhile, they have very low thermal stability, e.g., a few tens of degrees Celsius will destroy them [29]. To overcome those limitations, Schnur et al. used the lipid tubule as a mold, pattern, or scaffolding to convert them into stronger objects with the same geometry. For example, lipid tubules were subsequently coated with metals or inorganic materials to form scaffolds [29]. This concept is very important for using self-assembly structures for realistic tissue regenerating applications. Using this concept, lipid tubules can be coated using sol–gels to fabricate ceramic rods or hollow cylinders with diameters down to 0.5 $\mu$m, coated with silanes to change the chemical nature of the tubule's surface or coated with metals [29]. Modified tubules as microvials can be used for controlled release applications as well. The use of small hollow cylinders may offer several advantages over a sphere. Because the length and diameter are fixed and not a function of osmotic pressure, they will not change as the contents are released, and these parameters can be easily optimized for the desired release profile [29]. Zhang et al. used surfactant-like peptide, ~2 nm in size, to form nanotubes and nanovesicles with a diameters of ~30–50 nm. These structures can further form an interconnected network similar to what has been found in carbon nanotubes [30]. Perutz et al. used polyglutamines to form water-filled tubules [41].

***d. Nanometer-Thick Coating*** Using self-assembly, chemical, physical, and biological properties (e.g., surface texture, functional groups, integrins, and adhesive motifs) of a scaffold surface can be modified to promote specific cell adhesion, differentiation, migration, and orientation [30].

***e. Switchable Surface*** As reported recently in *Chemical Engineering News* [42], the properties of a surface can be controlled by changing the conformation of molecules formed by self-assembly. Langer et al. used electrical potential to reversibly switch an alkanethiolate monolayer between hydrophilic and hydrophobic states [43]. The molecules in the monolayer have large globular end groups with a free carboxylate end group on each molecule. When an electrical potential is applied to the underlying gold substrate, the carboxylate is attracted to the surface, bending the alkane chains and exposing them to the surroundings. Such surfaces have potential as drug delivery controllers.

***f. Three-Dimensional Scaffold*** In order to engineer functional tissues and organs, three-dimensional scaffolds are very necessary. The major difficulty with the self-assembly approach is to fabricate a three-dimensional scaffold with reproducible gross and microstructure and satisfied mechanical properties. Most three-dimensional scaffolds formed by self-assembly are the hydrogel type. By exposing self-assembling peptides to a salt solution or a physiological media, small amounts of macroscopic structures, mostly membranes, may be obtained for studying the cell behavior on self-assembled peptides [44]. Macroscopic structures are formed with self-assembled nanofibers with diameters ~10 nm and pores ~5–200 nm [30]. To improve mechanical properties of the scaffolds, artificial amphiphilic protein scaffolds with over 200 amino acids are synthesized. These scaffolds can resist high temperatures, over 90 °C, as well [45]. One major direction in this area is fabricating scaffolds with controllable/reproducible macroscopic and microscopic structures and mechanical properties.

### 2.1.4. Molecular Biomimetics

One promising approach to fabricate tissue engineering scaffolds is to mimic the ability of specific cells to synthesize and organize hierarchical materials with very fine features or resolution at the nanometer level. However, based on the knowledge we have currently on natural molecule synthesis and tissue remodeling/organization, it is very difficult to achieve this objective. A hybrid approach called molecular biomimetics can be used to synthesize

scaffolds with required mechanical properties and functional characteristics for engineering specific tissues from synthetic materials. Highly functionalized scaffolds with bioactive properties or some natural polymers, such as proteins, polysaccharides, and lips, can be obtained. Molecular biomimetics uses microstructures and functional domains of organismal design principles to synthesize new materials or structures. Nanotechnology offers great opportunities to combine physical and biological knowledge together to generate hybrid materials with potentials of assembling scaffolds at the molecular level. One good example is using recognition properties of proteins to control the organization and specific functions of scaffolding materials. To ensure precise control over the molecular structure and organization, inorganic surface-specific polypeptides/proteins can be designed through genetics. Inorganic-binding peptides/proteins may be designed using a theoretical molecular approach; however, it is currently impractical because it is extremely expensive and time consuming [46]. One interesting approach is using extracted biomineralizing proteins from hard tissue to mimic the binding between minerals and proteins in normal tissue. There are limitations with this approach as well. One is that there are multiple proteins involved in inorganic and protein binding; the other is that tissue extracted proteins are very specific as to the types of inorganics the bind and are of limited practical use [46]. Due to lack of knowledge on natural protein folding prediction and surface-bonding chemistry [47], customized peptides and proteins, obtained from massive libraries of synthetic polypeptides, would be a preferred route to find a specific sequence for binding specific inorganics. Surface-specific proteins are used as linkers to bind synthetic nanostructures, such as functional polymers, onto the templates. This type of structure can be used to specifically promote a certain wanted cell type to attach and proliferation on the scaffold surface but ward off the unwanted cell types. One typical example is for neuronal regeneration, to engineer the guidance bridge surface with matrix and cell surface adhesion molecules/ligands that are able to activate signaling pathways within the growth cone and have selectivity to allow the attachment of axons and growth cones yet ward off other cell types of inhibitory nature (Fig. 2A). This also called immobilization. Inorganic-binding proteins could potentially be used as linkers to bind two inorganic particles together as well (Fig. 2B).

### 2.1.5. Smart Scaffolds

Response to stimulus is a basic process of living systems. By adopting the features from nature biological system, scientists have been designing tissue engineering scaffolds that respond to external stimuli such as temperature, pH, light, electric field, magnetic field, chemicals, and ionic strength. These responses can be any of the followings: shape/position, structure, surface characteristics, solubility, integrate sensing, actuation (secretion), formation of an intricate molecular self-assembly, or a sol-to-gel transition [48]. The scaffolds, which are able to respond with dramatic property changes to a very small changes in their environment, are called stimuli-responsive scaffolds or "smart scaffolds". These kinds of scaffolds can be very useful for engineering functional bioartificial tissues and organs. Takezawa et al. prepared a collagen-conjugated thermoresponsive polymer, poly-*N*-isopropyl acrylamide (PNIPAAm), which solidifies above its lower critical solution temperature

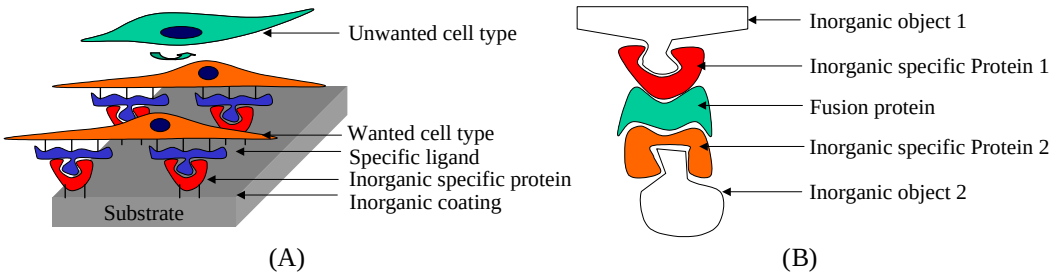


**Figure 2.** (A) Specific ligands could be immobilized on inorganic substrate to selectively promote wanted cell type attach and ward off unwanted cell types. (B) Inorganic-binding proteins could potentially be used as linker to bind two inorganic particles together.

(LCST, about 30 °C) and instantly dissolves into the culture medium below its LCST. They used this polymer to prepare spheroids containing two types of cells (hetero-spheroid), fibroblasts, and hepatocytes. TEM study showed the presence of structures morphologically similar to the Disse's space and the bile canaliculus in normal liver [49].

### 2.1.6. Patterning

***a. Microfabrication*** Cells not only can respond to microscale topography but also may respond to nanoscale topography. Highly reproducible nanotopography with several nanometers resolution can be obtained by advanced techniques such as electron beam lithography (EBL). Using such a method, very uniform surface textures can be obtained, although the fabrication process is very costly and time consuming, especially if large areas are needed [50]. Such surfaces can be used as very important scientific tools to systematically study the cell respond to different size topography from several nanometers to several hundred nanometers. The behaviors of different cell types on different nanoscale textures provide fundamental information about sensitivity of cells to topography. Microfabrication technologies are a sophisticated way to obtain reproducible delicate structures. Depending on the applications, nanostructure can be fabricated either through bulk micromachining (building texture out of bulk materials) or surface micromachining (building the structure on a surface). Photolithography, etching, and deposition are mostly utilized. More precise techniques such as nanolithography can be used to arrange nanoparticles or holes in a pattern and dip pen nanolithography can align magnetic nanoparticles using an AFM cantilever [51]. Etching is one popular way to generate an organized texture on a scaffold surface. Etching can be wet or dry, depending on the chemical used. If a liquid chemical is used, it is called wet etching. If a gas-phase chemistry is applied, it is called dry etching. For a thin film, silicon, and glass substrates, both wet and dry etching will work very well. Plastics are usually sputter etched. Some materials, such as photoresist and parylene C can be etched in oxygen plasma [52].

Using nanofabrication methods, our group is developing aligned channels on scaffolds for tissue engineering application to mimic the bands of bungner structure in peripheral nerves and honeycomb architecture fashioned by bees. Scaffolds with highly aligned channels may provide excellent guidance, which is especially important for nervous tissue regeneration, for the formation of three-dimensional architecture. Wen and Zhang are collaborating on the design and fabrication of honeycomb thin sheets for the regeneration of retina as well. Paralleled multichannels, schematically shown in Figure 3A, are fabricated from biodegradable polymers with average channel size around 5–10 $\mu$m; the rod photoreceptor cells (95%) and cone photoreceptor cells (5%) cells are seeded into the "honeycomb" channels (Fig. 3B). By use of a similar approach, bipolar cells are seeded onto the honeycomb sheet to form an aligned monolayer; retina ganglion cells are seeded onto the sheet to form the third layer. By stacking three layers together, a tissue-engineered retina structure is expected: retina ganglion cells form the outmost layer, photoreceptor cells constitute the innermost layer, and the biopolar cell layer is sandwiched in the middle.

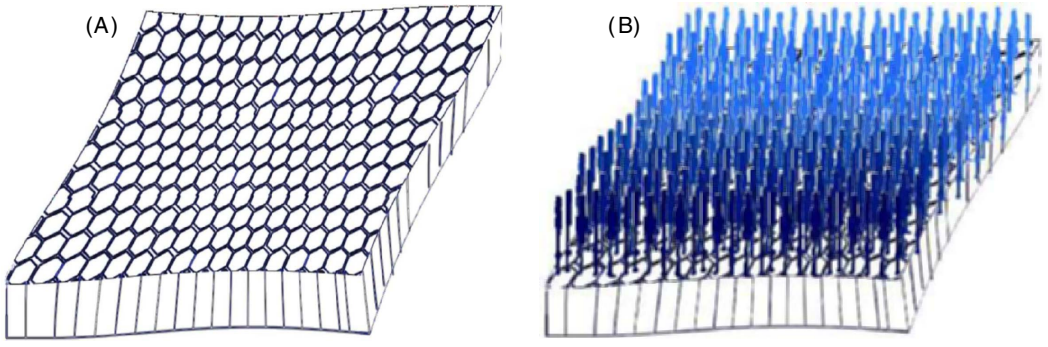


**Figure 3.** (A) Hone-cone like scaffold fabricated with nanotechnology; (B) photoreceptor cells are seeded into the channels to form aligned monolayer sheet for tissue-engineered retina.

Scanning probe microscopes (SPM), atomic force microscopy, and scanning tunneling microscopy are useful tools for the construction of nanosystems with 3D precision at up to 0.01-nm resolution. However, these tools require manual manipulations, which are time-consuming and barely reproducible. Nanoplanning system is an automating 2D assembly tool for fabrication of nanoobjects. One advantage is that it possesses the possibility of using artificial intelligence to control molecular level manufacturing [53].

***b. Soft Lithography (Nonphotolithographic Techniques)*** Deposition and patterning biomolecules and cells are an important application. There are three types of methods to accomplish this job. One is ink-jet printing, or may be called "protein printing", "cell printing", "tissue printing", or even "organ printing". However, the patterns generated by ink-printing lack resolution. A second method is based on protein adsorption. Since only physical adsorption of a protein from a solution to a substrate occurs, patterns created lack the resolution in handling living cells and biomolecules. A third type of patterning using stamps and channels to transfer patterns on polymers has been developed, including microcontact print, microfluid channels, and laminar fluid patterning. Microcontact print, which is excellent for patterning cells and fragile molecules [54], relies on an elastomeric stamp which is cast from a textured surface made by photolithography, tooling, or molding [55]. The stamps can be used to print cell and biomolecular favorable zones on scaffold surfaces. The shapes and degrees of spreading of cells can be controlled: for example, well-spread cells can be placed next to rounded cells, and square cells can be placed next to circular cells [56]. The advantages of microcontact print include that it utilizes cell and biomolecule friendly conditions (e.g., no exposure to harsh chemical or physical treatments, printing occurs under ambient conditions, and postprinting steps occur in aqueous solutions that do not denature proteins) and it is an operation friendly process (e.g., allows patterning large areas, is easy to operate, and has low cost) [55]. Nagaoka et al. investigated the effect of surface nanomodification of aromatic fluorinated polyimide (6FDA-6FAP) derived from 2,2′-bis(3,4-dicarboxyphenyl)hexafluoropropane dianhydride (6FDA) and 2,2′-bis(4-aminophenyl)hexafluoropropane (6FAP) on the interaction with proteins and cells. Nanoordered stripes were created by a surface-rubbing technique [57]. The fibroblasts formed multicellular spheroids on the modified surface and expressed a large amount of collagen similarly *in vivo*, while on the nonmodified surface the cells formed two-dimensional monolayers and the collagen production was negligible [57]. Tjia et al. have shown that collagen "ligand associated microdepots" (LAMs) developed using nanotechnology at polymer substrates can significantly enhance cell migration [58]. They also suggested that improved cell migration may be due to cell activation via increased tyrosine kinase activity [59]. Self-assembly of rodlike protein polymers, use of polymerized liposomes, and production of self-assembled monolayers and other nanostructures on surfaces assist in guiding tissue regeneration [60, 61]. Unfortunately, biomolecular patterning using nanotechnology is limited to two dimensions and is currently used only as a model system to study the basic cell biology question. Scaling up to three-dimensional porous scaffold for tissue engineering is a challenge [18].

***c. Polymer Demixing*** In order to produce large areas of nanotopography at low cost and high efficiency, Muller-Buschbaum et al. [62] developed a polymer demixing method to produce organized topographic features such as pits, islands, or ribbons of varying height or depth. Briefly, polystyrene and poly(4-bromostyrene) blends spontaneously undergo phase separation during spin casting onto silicon wafers. The scale of the textures can be adjusted by controlling polymer ratio and polymer concentration. By varying the ratio of the polymers, topography shape can be controlled; by varying the concentration of the polymer, topographic features can be changed. They studied endothelial cells and fibroblasts response to this type of topography and found that 13-nm-high islands increase cell spreading and proliferation when compared with 95-nm-high islands for both cell types. Cytoskeletal conformation, filopodia formation, lamellapodia formation, proliferative gene expression, G-protein receptors, G-protein regulators, and other G-protein related genes are influenced by nanoscale topography.

***d. Selective Cell Attachment*** In order to achieve desired cell adhesion but repel unwanted attachment, scaffold surfaces have been made nonadherent with hyaluronan (HA), poly(ethylene oxide) (Pluronics) [63], PEG, Glycocalyx [64], antibacterial with silver or N-alkylated poly(vinylpyridine) coatings, and bioadhesive with RGD peptide insertion, growth factor attachment, other bioactive groups decoration, plasma etching, or other chemical modifications [65]. With designed patterns, the spatial organization of the cells in two and three dimensions may be obtained [61]. Cell morphology and cellular activities can be managed by patterning as well. By creating specific patterns of surface chemistry and/or texture, cell behaviors can be confined with physical or chemical ultrastructures, which can be used to control cellular activity [61].

### 2.1.7. Composite Materials

Many natural tissues possess a composite microstructure, such as bone and teeth. These complex composite structures play critical roles for the physical and biological properties of the tissue. To mimic the natural tissue structure, biomedical polymers, bioceramics, and other inorganic materials are to be combined for superior properties. Polymer–inorganic nanoparticle composites can be used as scaffolds for tissue-engineered bone. When compared with conventional polymer composites, **nanoparticle filled polymers** have many superior properties, such as less weight, better mechanical properties, improved durability, and bioactive interface, which is a key factor to a successful and long-term use of prostheses. **Nanostructured polylactic/glycolic acid (PLGA) and titania composites**, created by chemically treating micrometer-structured PLGA with NaOH and mixing with nanometer grain size titania, were investigated for bone engineering using osteoblasts (bone-forming cell) and chondrocytes (cartilage-synthesizing cell). Increased osteoblast and chondrocyte adhesion were observed on polymer surfaces of nanometer scale roughness when compared with that of micrometer scale roughness [66]. Data suggest that nanostructured composites may possess the ability to simulate surface and/or chemical properties of bone and cartilage, respectively, to allow better regeneration [66]. **Nano-HAp/Collagen Porous Scaffolds**: A nano-HAp/collagen composite was fabricated into a three-dimensional scaffold from nano-HA powders and natural collagen by simply blending the two components together [67]. Osteogenic cells were seeded on this composite and observed three-dimensional distribution and growth of cells within the porous scaffold. **Nano-HA/Biodegradable Polyurethane Hollow Fiber Membranes**: In order to tackle the problems with most degradable polyesters, such as that they are brittle and stiff (not very flexible), our group is developing nano-HA/biodegradable polyurethane composites. To mimic the normal bone microstructures, we have developed a multichannel scaffold assembled from different sized nano-HA/biodegradable polyurethane hollow fibers. To further improve blood vessel formation in the tissue-engineered grafts, we specially fabricated pores on the hollow fiber membrane wall. The macrostructure of the scaffold closely mimicks the natural bone architecture and nano-HA in the scaffold is highly favorable for bone regeneration as schematically shown in Figure 4.

The other interesting composite is **nanocarbon reinforced polymer** composite. Two forms of pure carbon, fullerenes and nanotubes, have been obtained. The soccer ball shape is called a fullerene (C60, ~1 nm), and the cylinder shaped is named a nanotube. In addition to applications in life sciences, materials science, and electronics, nanotubes are very useful in biomaterials and tissue engineering in strengthening and lightening synthetic bone implants, artificial joints, and tissue engineering scaffolds due to their unique characteristics, such as that they are 100 times stronger than and about one-fifth the weight of steel.

### 2.1.8. Nanogels

Nanogels refer to hydrogels in nanoscale; micrometer scale hydrogels are called “microgels”, in order to tell the difference. Nanogels are formed by collapse of swollen hydrophilic polymer gels. Drugs can then be attached to the nanogels through weak bonds (such as, electrostatic interactions, hydrogen bond, and hydrophobic interactions) and cleavable covalent bonds between the drug moiety and the gel surface [68]. McAllister et al. [69] utilized

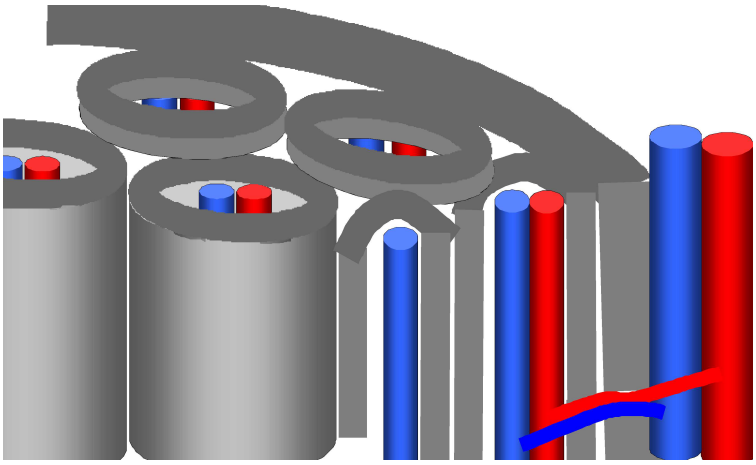

**Figure 4.** Different diameter nanoHA-degradable polyurethane hollow fiber membranes are used to construct a macro-structure highly mimic the natural bone structure with pores to promote vascular formation throughout the whole graft.

inverse microemulsion polymerization to synthesize biocompatible monodisperse nonionic and cationic nanogels with controlled size (ranging from 40 to 200 nm in diameter), morphology, and composition. The chemical composition, size, polydispersity, stability, and swelling behavior of the nanogels were investigated by NMR, light scattering, TEM, and AFM. The cell viability, uptake, and physical stability of nanogel–DNA complexes were evaluated under physiological conditions. Cationic nanogels formed monodisperse complexes with oligonucleotides and showed enhanced oligonucleotide uptake in cell culture. Vinogradov et al. [68] developed nanosized cationic nanogels of cross-linked poly(ethylene oxide) (PEO) and polyethyleneimine (PEI). Nanogel materials could be formed by the interaction of anionic amphiphilic molecules or oligonucleotides with PEO–PEI nanogels to form distinct hydrophobic polyion-complex regions and hydrophilic PEO chains. This system allows for immobilization of negatively charged biologically active compounds such as retinoic acid, indomethacin, and oligonucleotides (bound to polycation chains) or hydrophobic molecules (incorporated into nonpolar regions of polyion–surfactant complexes) on the nanogels. To allow for the targeted delivery of nanogels in the body, the nanogel surface can be modified with various biospecific ligands. Various coupling strategies can be employed for this purpose, including covalent attachment of the ligand moiety to the free amino groups of PEI segments. For example, biotin moieties can be attached to the surface by using biotin *p*-nitrophenyl. The biotinylated nanogel can be further reacted with drugs or oligonucleotides and attached with recognizable moieties, such as antibodies. Enhanced receptor-mediated delivery can be achieved this way [68]. From the drug delivery perspective, the size of the particles is particularly important. Size variation at nanoscale can affect the blood circulation and bioavailability of the particles within the body [68]. In general, particles with diameter less than 10 nm can be rapidly cleared through extravasation and renal clearance. Particles over 10 nm cannot be removed through extravasation and renal clearance, and sizes smaller than 70 nm can easily penetrate and flow in all size blood vessels. Therefore, 10–70 nm particles may offer the most effective distribution in the tissue [68]. Less than 100-nm size range particles can easily get into the targeted cells via endocytosis. Particle size larger than 200 nm are usually captured through mechanical filtration by the spleen and are eventually removed by the phagocyte system. Particles in the range 70–200 nm demonstrated the most prolonged circulation time [68]. Therefore, 10–100 nm particles are preferred for intracellular delivery, and 10070–200 nm 200 nm particles are preferred for delivery through the circulation system. Vinogradov et al. also demonstrated efficient cellular uptake and intracellular release of oligonucleotides immobilized in PEO–PEI nanogel, and antisense activity of an oligonucleotide in a cell model was elevated as a result of formulation of oligonucleotide with the nanogel [68]. The nanogels synthesized in these studies demonstrate potential utility as carriers of oligonucleotides and DNA for antisense and gene delivery.

### 2.1.9. DNA Nanotechnology

The use of DNA nanotechnology can be used for tissue engineering for two purposes, one is to construct a scaffold from specific geometrical and topological DNA structure, for example, the use of DNA structure to assemble a tissue engineering scaffold using synthetic or natural biomaterials; the other is to drive nanomechanical devices through DNA transitions [70]. Nanomechanical devices may be used for drug delivery, growth factor release, and cellular engineering. Although most studies related to DNA nanotechnology are for electronics applications, such as memory-device construction [71], there are possibilities of using this technology for tissue engineering scaffold development. Many of the DNA nanotechnologies are based on branched DNA motifs, which permit guidance of the formation of long lines, circles, knots, catenanes, networks, or close mimicking natural ECM structures through nanotechnology. The advantages of using DNA nanotechnology for tissue engineering scaffold fabrication include readily programmed and reliably predicted DNA structures, availability of arbitrary sequences because of convenient solid support synthesis [72], stable structures, and easy manipulation and modification of DNA structures. DNA nanotechnology has been explored for a long time; however, its application is mainly at the stage of ideas and relatively far from reality. Tissue engineering applications may bring DNA technology into reality sooner.

### 2.1.10. Angiogenesis

One vital requirement for tissue engineered grafts to survive is the sufficient supply of oxygen and nutrients and removal of carbon dioxide and waste. Both the supply and removal process involve the transport of substances in the tissue possibly in all forms, gas, liquid, and solid. Transport in tissue engineering has two main issues: to design tissue with a well-perfused transport network and to create tissues that would have the function of transport, such as blood vessels. To properly perfuse large organs, such as kidney and liver, diffusion and convection alone cannot meet the requirements. A well-established vascular network is essential. However, techniques to grow a blood vessel network throughout the tissue have not been developed. Using nanofabrication, different size scale textures and structures can be obtained from a few nanometers to tens of micrometers. These structures are good substrates for learning the endothelial cell behaviors on different structures. For example, Dike et al. showed that endothelial cells could switch between proliferation, apoptosis, and differentiation, when attached on different size scale textures and vascular-like tubular structures could form on certain texture range surfaces [73]. Kaihara et al. applied micromachining to generate branched channels to promote vascular formation and ultimately to enhance the transport in tissue-engineered liver [74]. Our group is studying vascular formation using well-defined pores at microscale and trying to find out the critical size for varscularization both in vitro and in vivo. At the same time, blood vessel formation is monitored with time-lapse microscopy in vitro, and the effect of perfusion and shear forces (straight flow vs rotation) on the vascularization and endothelial cell behaviors will be obtained, analyzed, and modeled using a computer. Moldovan and Ferrari [75] developed a silicon-based "angiogenesis assistant device", which is a nanofilter-based, drug delivery silicon capsules. The nanofilter with controlled size ranging from 10 to 200 nm for angiogenic growth factor delivery is jointed with a millimeter-scale silicon frame with an endothelial cell coating for blood compatibility and vascularization. The nanofilter is created with micromachining and sacrificial layer techniques. This device would possess several advantages in both research and clinical applications, such as a controllable delivery if there is a combined sensor and remote control device in the design, a supply of endothelial cells for better blood compatibility, a delivery of angiogenic factors for improved vascularization, and ease of implantation.

The microscopic networked tubes with branches, which mimic the circulatory system in the body, can be designed by computer and fabricated with nanotechnology. Kaazempur-mofrad's approach is to create two half-pipes on silicone, which serves as a mold for making degradable polymer tubes, using nanofabrication techniques. This technique has potential applications in tissue engineering liver and kidney, which require a significant amount of

blood vessels to provide oxygen and nutrients to the engineered grafts. The data were recently presented at the American Society for Microbiology Conference (July, 2003).

#### 2.1.11. Nanosensor

To fully mimick organ functions, for example, secretagogue-triggered hormone release, miniaturization of sensors to nanodimensions may decrease the typical time constant down to the milliseconds time scale [76], which is very close to the trigger system in a normal human body. With huge advantages in ultrasmall size, nanosensors can be easily embedded into the scaffolds, even the cell membrane, to establish smart/responsible implantable tissues grafts.

#### 2.1.12. Sweeping Vehicle

Some researchers have proposed that nanorobots can be used as sweeping vehicles in the human body, such as teeth cleaning robots that collect harmful bacteria from the mouth, blood vessel cleaning robots that remove extra fat from the circulation system, and lung cleaning cleaning robots that can be used in the lungs to clean dust, which mimic the natural macrophages in alveoli [77]. However, natural macrophages are unable to metabolize foreign particles like fibers of asbestos and reverse the toxic effects of smoking in the lungs. With robots, these particles may be completely cleaned out of the lung. A similar idea may be used in biomaterials and tissue engineering. For example, ultratiny robots may be used to remove biomaterial debris, which may come from degradation or wear at joint surfaces, from the transplanted grafts or implanted artificial implants for better integration and function.

### 2.2. Applications of Nanotechnology in Cellular Behavior and Engineering

Cells harvested from a variety of tissue sources can be used for tissue engineering purposes. Examples include autologous cells, from the same individual, allogeneic cells, from a different individual but the same species, or xenogeneic cells, from a different species. The major issue with cells in tissue engineering applications is that they have to be isolated, cultured properly, and expanded to achieve sufficient numbers of cells of a single phenotype to recover the lost function in the damaged areas [4–6].

In terms of cells for tissue engineering, nanotechnology can be used in two ways. One is to engineering cells for better regeneration, and the other is to study cell behavior, such as cell movement, migration, proliferation, tracing, and identification.

#### 2.2.1. Nanotechnology for Cellular and Genetic Engineering

***a. Self-assembly*** The self-assembly approach is not only good for tissue engineering scaffold fabrication but is also very attractive for cellular engineering. With self-assembly, membranes with special channel structures can be obtained for engineering cell membranes, and virus structures can be achieved for genetic engineering functional cells as well. Channel-forming proteins, such as porins, can be inserted into lipid bilayers to control transport across the cell membrane [78].

***b. Cell Surgery*** Cells can be engineered through a nanosized fiber optic probe as well. For example, this probe can be used to locate the target molecules or genes and turn them on or off using a nanoparticle.

***c. Gene Delivery/Engineering*** The challenge in gene delivery is how to precisely control plasmid penetrating into the specific cells, across the karyotheca, get into the nucleus, and insert to the specific DNA site. With nanotechnology, recognition structures can be attached onto the plasmids for specific cells. Negative charges could be applied on the plasmids, which will facilitate plasmids across the karyotheca and insert into the DNA. Another interesting tool called a gene gun, developed by Dr. Sanford, made it easier to inject genetic materials into a cell.

***d. Cell Life*** Drs. Rzigalinski and Seal found that nanoparticles have unexpected roles in tripling or quadrupling the life of brain cells. They have received grant support from NIH (National Institutes of Health) and NIA (National Institute on Aging) to study the mechanisms behind the reaction and possible future applications.

### 2.2.2. Cell Behavior Studies with Nanotechnology

***a. Nanomachined Structure for Cell Behavior Study*** Most of studies on cell behavior are using a large population of cells, and the results are the average responses of the whole population. Using a conventional approach, we are unable to know how the local environment affects cell behavior. To understand individual cell respond to local environmental cues, such as chemical, physical, biological, and mechanical cues, approaches based on nanotechnology are utilized [55]. One way is to engineer the local microenvironment on the cellular and even smaller scale by patterning mechanical, physical, and biochemical signals to individual cells in spatial and temporal arrangements [55]. Nanomachined structures are used to interrogate and manipulate cells. When comparing manipulating the scaffold structure and manipulating biomolecules, manipulating cells is more challenging because of the need to maintain cell viability. Although it is challenging, much exciting research is being conducted. Dogterom et al. and Holy et al. used microfabricated chambers to study the cytoskeletal elements. Dogterom et al. [79] investigated the force–velocity relation for growing microtubules inside the cell body. Holy et al. [80] examined the assembly and positioning of microtubule asters in constrained geometry. Kricka et al. used micromachined glass–glass microchips to study the cell motility, especially on sperm motility, fertilization, and other functions [81].

Micro- and nanofabricated structures are used to measure the forces generated by individual cells. Using nanofabricated cantilever force transducers, Neumann et al. have measured the mechanical properties of isolated thick filaments from muscle. The single thick filament was suspended between the tip of a flexible cantilever and the tip of a stiff reference beam. Axial stress was placed on the filament, which bent the flexible cantilever. Cantilever tips were microscopically imaged onto a photodiode array to extract tip positions, which could be converted into force by using the cantilever stiffness value [82].

Not only forces generated by cells can be measured, cell deformability [83] and flow behavior of cells [84] can also be examined. Ogura et al. micromachined a very thin (0.4 micrometer) filter that consists of a single micropore (diameters down to 1 micrometer) on a $Si_3N_4$ film. An individual RBCs deformability is evaluated by filtration, by measuring the cell pore passage time, which simulates the deformations encountered in the recticuloendothelial system (in particular the spleen) [83]. Sutton et al. developed small micromachined channels to study the microrheology of erythrocytes as they flow through channels of dimensions similar to human blood capillaries. Briefly, with micromachining, an array of precise and highly reproducible microfluidic flow channels with different sizes is fabricated. They characterized each individual cell in a sample of ca. 1000 in terms of its volume and flow velocity profile during its transit through a channel [84].

Micromachined sensors could be used for both invasive [85] and noninvasive analysis of cell and tissue signaling and enzymatic metabolism [86]. Ocvirk et al. studied single cell enzymatic metabolism using a microchip through lysing of single cells and identifying their galactosidase activities [85]. Wolf et al. used a modular arrangement of various planar and nonplanar sensor elements surrounding small cell culture chambers monitored invasively by high-resolution light microscopy and spectrophotometric techniques. The system was originally designed for biomedical research in chemotherapy and pharmacology, but it proved to be an effective device for monitoring cellular signaling and metabolism, as well as toxicological and environmental research [86]. With a similar concept, micromachined sensors could be used to monitor cell secretion of certain factors. Bratten et al. micromachined a titer chamber containing an integrated electrochemical sensor, capable of measuring analytes produced by a single cell. Metabolites were detected via the amperometric detection of enzymically generated hydrogen peroxide and measured at a platinized microelectrode. The amount of analyte produced by the cell can be quantified by the addition of a known amount of calibrant [87].

Micromachined electrodes can be used to record and stimulate electrogenic cells [88]. The microelectrode array, fabricated on a silicon substrate perforated by multiple holes, can be implanted between two stumps of a surgically severed nerve. This electrode array can be used not only for recording electrophysiological signals from regenerating axons but also for stimulating axons which promote axonal outgrowth. Kovacs et al. found that regenerating tissue fixes the electrodes securely in place and a stable mapping of electrophysiological behaviors can be obtained by measuring and stimulating individual regenerated axons according spatial distribution. Such arrays, implanted in the peroneal nerves of rats, were used to for recording and stimulating for up to 13 months [88].

Micromachined electrical biosensors are used to continuously track morphological changes of adherent cells and provide quantitative data from both sparse and confluent cultures [89]. More importantly, this method is capable of detecting vertical motion of cells of the order of 1 nm [89]. Hagedorn et al. used perforated Si membranes to clarify cell locomotive ability. The motility and impedance of the growing cells are monitored simultaneously by a time-lapse video system and electrical biosensors, respectively. The correlations between cell activities and impedance events are classified. Their method is very sensitive and allows discrimination between signals arising from translocation of single cells and those arising from filopodia activities [90].

Using micropatterned substrates, Chen et al. found that human and bovine capillary endothelial cells switch from growth to apoptosis by decreasing texture size to progressively restrict cell extension. They also found that cell spreading highly depends on texture size. Their conclusion is that cell shape is one important factor governing whether individual cells grow or die. Through local geometric control, cell growth and viability can be controlled for better engineering of functional tissue grafts [91].

The interactions between a cell and its underlying cues and between neighboring cells, binding of cells with soluble factors, and mechanical forces sensed by cell bodies can be achieved through nano- and microfabrication technologies with laser tweezing techniques that allow us to precisely control these interactions and the microenvironment. Our research group is combining nanofabrication technology with laser tweezing techniques to study a single neuron's respond to different diameter fibers with diameter down to 50 nm. We expect to discover the optimal diameter range of fibers for greatest axonal outgrowth rate and highest directionality. Meanwhile, we will be able to dig out the possible mechanisms of controlling neurite outgrowth and directionality through this model.

The traditional approach for analysis of cell/particle samples usually requires a rather large amount of sample and sample preparation. Miniaturized dielectrophoresis or electrorotation allows Lab-on-a-Chip analysis, which requires less sample and samplepreparation. Cells and large biomoelcules can be considered as dielectric particles. When a dielectric particle is suspended in a spatially nonuniform electric field, a dipole can be induced in the particle; the interaction of the induced dipole with the electric field generates a force. Due to the presence of a field gradient, these forces are not equal and there is a net movement. If the particle is more conductive than the medium around it, the dipole aligns with the field and the force acts up the field gradient toward the region of highest electric field. If the particle is less polarizable than the medium, the dipole aligns against the field and the particle is repelled from regions of high electric field [92]. The force is dependent on the induced dipole and is unaffected by the direction of the electric field, responding only to the field gradient. Since the alignment of the field is irrelevant, this force can also be generated in ac fields, which have the advantage of reducing any electrophoretic force to zero. By using a miniaturized chip, one can move cells and particles and distinguish between different types by applying electric fields. Furthermore, it is possible to characterize cells and discriminate between different stages of their life cycle [92].

Nanofabrication significantly enhances the performance of existing devices, enables extremely high resolution in surface texture, and miniaturizes the size of devices. Miniaturization/small size can either enhance or enable characteristics. For example, an electrorotation device can be used to manipulate cells and large molecules by generating gradient forces. Miniaturizing the space between driving electrodes can increase force gradients and thus greatly enhanced the performance [92]. As device size goes down, their surface area relative

to volume increases significantly. When the scale is down to nanoresolution, this leads to a situation where surface effects dominate volume effects. Remarkable physical enhancements result [52]. With the electrorotation device as an example, as the device is miniaturized, the driving voltages necessary to generate gradient forces decrease from a few hundred volts to several volts. Thus heat removal is not a problem. Meanwhile, higher electric fields than in conventional systems can be used without adverse heating effects. This gives larger forces for the dielectrophoresis and electrorotation devices [52]. For the study of cellular behaviors, nanofabrication allows one to pattern geometries from macroscopic down to nanoresolution. Bhatia et al. [93] precisely controlled the spatial organization of hepatocytes and fibroblasts using micromachined textures. This made it possible to eliminate variations present in random cocultures, such as amount of heterotypic interface between two cells types [52]. They evaluated liver-specific function by measuring albumin and urea synthesis and found that albumin production is localized to hepatocytes at the heterotypic interface [93]. Such as study would be impossible to perform without highly precise fabrication [52].

***b. Labeling*** Nanoparticles, such as quantum-dot (CdSe, ~8 nm), can be used to label a variety of cellular targets. For example, allowing cells to ingest quantum-dot dyes, the *cell movement* can be monitored for days without photobleaching the quantum-dot dyes [94]. *Cell migration* behavior can be studied by watching the quantum-dot ingestion behavior, so that the cell types may be distinguished. Invasive cancerous cells and immotile nontumor cells can be differentiated by seeding them on a quantum-dot bed. Cells ingest quantum-dot and leave a so-called phagokinetic track behind. Cancer cells exhibit diverse uncertain behavior, and some nontumor cells show specific endocytosis and migration patterns. Quantum-dot can also be used to trace cells for up to 10 generations, because quantum-dots are distributed during dividing. Therefore, dots can be used to trace cancer cells and stem cells.

***c. Cell Purification*** Using nanotechnology, we are able to measure the electric properties of a single individual cell. Then we can sort/purify cells by their different intrinsic electric properties. This application will be very important in stem cell purification. Currently, we do not have the techniques to get purified stem cells; with the aid of nanotechnology, large quantities of purified stem cells may be obtained in the very near future.

***d. Detection*** Arrays of nanowires, about a thousand wires, each about 8 nm in diameter, coated with different antibody or nucleic acid, can be used to detect thousands of genes in a cell and proteins synthesized by a cell. Silicon nanowires tethered with biomolecules specifically bond to a protein expressed by cancer cells. When there is bonding, an electrical signal can be detected. The detectors made of nanowires are very sensitive and required minimal sample preparation; several bonds can be precisely detected. Moreover, the detectors are very cheap and patients can use them at home. However, the ions in the media for maintenance of the normal life of the cell may interfere the biochemical reactions.

***e. "Suicide Bombing"*** Despite significant progress of tissue engineering in both academic research and in industry, a number of issues have arisen that have forced the research progress and commercial procedures to slow down. The biggest setback that the field of tissue engineering faces now is the unknown fate of tissue-engineered analogues inside the human body. That is why the FDA has approved very few tissue-engineered products so far. Before any further breakthroughs can be made, the following questions must be answered. Where will the transplanted cells go? How will they grow? What will they differentiate into? How will they be eliminated if an unexpected event occurs, such as tumor genesis? Unfortunately, most scientists are concentrating on regenerating all kinds of tissue and organs but are ignoring the control issues for proper regeneration and the fate of tissue engineered grafts *in vivo*. Before these questions to be completely answered, one temporary solution called "suicide bombing" may be used. A "smart" agent in nanoscale can be taken up by all cells in the tissue-engineered grafts. The agent is inactive during normal condition and is activated only by specific enzymes that are expressed in the cell under certain pathologic states. The activation of the agent will kill the abnormal cells. This idea is not only good for killing the abnormal cells but also suitable for treatment of specific diseases if a particular therapeutic agent is used.

## 2.3. Applications Nanotechnology in Biomolecular Manipulation

Biologically active molecules, such as cell adhesion molecules (fibronectin, laminin, collagen, and many others) and soluble factors (vascular endothelial growth factor, nerve growth factor, transforming growth factor, and so on), furnish the biomaterial surfaces with adhesion properties that are permissive for the attachment of particular cell types [9]. In contrast, other cell-derived matrix molecules such as the family of sulfated proteoglycans are found to be inhibitory for cell growth, e.g., axonal outgrowth [10]. Biologically active molecules interact with cells via cell surface receptors or initiating intracellular cell signaling pathways, which trigger the expression or repression of genes and alter the protein products that regulate cell behavior. Selective incorporation of such biologically active molecules into the tissue engineering constructs makes the fine-tuning of cell behavior possible [11, 12].

Cells and tissues respond to bioactive molecules such as cytokines, growth factors, angiogenic factors, and drugs during development and tissue remodeling. Therefore combining these molecules during the tissue engineering process will accelerate the tissue regeneration process. Using nanotechnologies, nanoparticles, nanofibers, nanopores, nanotubes, and dendrimers loaded with these molecules can be embedded into the scaffolds.

### *2.3.1. Nanodelivery System*

To speed up functional tissue/organ development, a localized bioactive agent delivery system is desirable. The ideal delivery system would be targeted, precisely controlled, and very small in size. For example, design an insulin delivery system with the ability to detect the blood glucose level, release insulin accordingly, and precisely control the blood glucose level at normal. Nanomaterials can be used as drug and gene carrier: drugs and genes can be either encapsulated inside the nanoparticle or attached onto the material surface. Nanocarriers, such as lipid particles [95], albumin [96], or polymer nanosystems [97] have been studied for transporting a wide variety of agents. The major advantage of using nanocarriers for delivery is that, due to the their extrasmall size, nanocarriers can easily penetrate and flow in all size blood vessels and be taken into cells. However, ultrasmall size causes low drug loading capacity and relatively complicated preparation procedures. In order to further enhance the performance, recognition sites can be introduced on the particles to have targeted drug delivery. For example, with a conjugate-specific antibody on the surface, the particles can selectively bond to a specific cell type. Targeted local delivery can decrease the system toxicity significantly. Nanocarriers deliver agents into specific cells by diffusion, fusion, or internalization through component (lipid–lipid) exchange and convective flux, biolistics, or some combination of these mechanisms. One interesting case is the delivery of active agents after nanoparticle binding to the target cellular epitopes by a so-called "contact facilitated drug delivery" [98]. Nanoparticle or nanocapsule can be used not only for targeted biomolecular delivery but also for imaging the delivery process. Therefore, one major advantage of using nanotechnology is image-based delivery and dose monitoring systems for drugs, trophic factor, or gene delivery powered by the detectable nanobiomarker. The opportunity to confirm drug delivery and dose by imaging represents a novel feature for nanosystems that provides controlled drug release [99]. When paramagnetic nanoparticles are used, quantitative differentiation among a variety of simultaneous agents can be detected and quantified by NMR spectral analysis [98]. This unique nanodelivery system ultimately might allow rational dosing on the basis of quantification of the local concentrations of the agent, eventually permitting more precise titration of especially potent bioactive agents that would otherwise exhibit unacceptable toxicity if employed at high serum levels [99].

***Controlled Channel Delivery*** To mimic the function of cell membranes, channel forming proteins, such as porins, are inserted into the lipsomes formed by a lipid bilayer [78]. The channeled liposome has great potential in encapsulation and drug delivery applications. For example, by combining the recognition system, such as biotin–streptavidin complex [100], on the bilayer to control the opening of the channels, a drug delivery system with physiological feedbacks can be developed. One major problem using nanoscale delivery systems is that the particles are eventual recognized and cleared by macrophages of the reticuloendothelial system [101]. The pharmacokinetics as well as the tissue distribution of such so-called

"stealth" nanoparticles are also altered after repeated intravenous injection. An understanding of immunological and pathological factors that control the pharmacokinetic and biological behavior of long-circulating particles after repeated administration is therefore crucial for the design of a system with an optimal diagnostic and/or therapeutic performance [101]. In order to overcome this obstacle, antigen–antibody specific binding, a temperature- and pH-sensitive scaffold, polymer–protein conjugates with temperature-switchable ligand binding or mechanical properties, and cell-adhesive delivery systems are developed for controlled and targeted delivery of biomolecules [61].

### 2.3.2. Biomolecular Patterning

Three-dimensional molecular chemical imprinting can be used to develop highly stable and biospecific surfaces with nanometer resolution for inducing specific cellular arrangement and tissue organization. To mimic the extremely complex 3D topography of extracellular matrices (ECM) that cells are adherent to in vivo, Goodman et al. created an inverse replica of the subcellular ECM surface in the micrometer to nanometer range resolution by gentle removal of adhered cells and precise coating of a biomedical polyurethane. A replica of ECM morphology at macromolecular level can then be obtained on a polymeric scaffold surface that has a topography similar to that of cell adhesion substrates found in biological tissues. Endothelial cells cultured on the ECM replicas spread more rapidly and had a three-dimensional appearance and spread areas at confluence which appeared more like endothelial cells in native arteries, compared with cells cultured on untextured control surfaces [102]. Shi et al. used molecular imprinting techniques to create specific recognition sites in polymers by using template molecules to mimic molecular recognition, which is attributed to binding sites that complement molecules in size, shape, and chemical functionality. They used radio frequency glow-discharge plasma deposition to form polymeric thin films around proteins coated with disaccharide molecules. With a covalent attachment of disaccharides to the polymer film, polysaccharide-like cavities that exhibit highly selective recognition for a variety of template proteins, including albumin, immunoglobulin G, lysozyme, ribonuclease, and streptavidin, are created. Direct imaging of template recognition is achieved by patterning a surface at the micrometer scale with imprinted regions [103]. Biomolecular patterning with signal molecules, such as cell adhesion molecules and growth factors, has potential to regulate cellular functions and their precise behaviors. Data suggested that immobilized biomolecules are more active than diffusible ones [76].

### 2.3.3. Nanomachined Structure for Biomolecular Manipulation

As discussed earlier, using micro- or even nanofabrication approaches, structures with nanoresolution could be obtained. The ability of these structures to interrogate and manipulate biomolecules is rapidly emerging [52]. These applications include, but are not limited to, ultrasmall channels for molecular separation and analysis [104], piercing structures for DNA injection or delivery [105].

### 2.3.4. Dendrimers

Dendrimer is a compact, synthetic polymer branching out like a tree with a large surface area, which provides a good substrate for building blocks and carrier molecules at the nanometer level, and it has a unique spherical molecular shape, which is advantageous as a coating. The term "dendrimer" was first used and patented by Donald Tomalia et al. in 1979 [106]. A new generation of hyperbranched dendrimers, biodegradable nanoparticles, can be formed from many materials, such as polyamidoamines, amino acids, DNA, sugars, organosilicons, and organic/inorganic hybrids [61]. The unique properties of dendrimer molecules are the holes in the core structure and folded branches of molecules that create cages and channels that can be used to accommodate either water-soluble or water-insoluble agents. Therefore, dendrimers are useful in biomolecular and gene delivery. Dendrimer research is a fast growing field. From a patent search we can observe an explosion in dendrimer patenting and academic publishing. A drug called a nanodecoy, developed by Donald Tomalia, can bind to viruses like a trap before they enter cells and cause disease. Therefore, it may be

able to fight viruses (influenza virus) before infection occurs, and it has been approved by the FDA (United States Food and Drug Administration) for clinical use, which is the first time for a nanodendrimer [107, 108]. Another dendrimer based nanodrug called VivaGel, received clearance by the FDA in July 2003 to commence clinical trials. This drug can be used to inhibit the HIV infection process by preventing the attachment of the virus to healthy cells. Studies in monkeys have shown complete inhibition of HIV infection [107, 109]. These dendrimers can be used to improve the health of transplanted cells inside the engineered grafts.

### 2.3.5. Oxygen Delivery

The transport of substances in all forms, such as gas, liquid, and solid, throughout tissue and organs is very important for maintaining normal living cells in engineered grafts. A nanodevice not only can deliver drugs and biomolecules but also can deliver oxygen. A nanorobot called a "respirocyte" is able to provide oxygen in the oxygen-deficient region [110]. If oxygen supply is low and tissue damage can occur, this artificial red blood cell can release oxygen using ultrasound signals. This tiny system has bidirectional transport, i.e., oxygen is released into the tissue and, at the same time, carbon dioxide can be eliminated from the tissue to keep the tissue and organs alive. The devices are made from carbon arranged in a diamond lattice and act as a tiny pressure tanks that are filled with up to nine billion oxygen or carbon dioxide molecules. These gases are released from the tank when needed [110]. The techniques to grow a well-perfused blood vessel network throughout the large tissue have not been developed yet. In order to properly perfuse large engineered tissues, this tiny device may be incorporated to achieve better oxygen supply.

### 2.3.6. Studying the Mechanical Behavior of a Single Molecule

Currently, for most natural materials, the mechanical properties have been investigated only at the gross level. However, owing to the low reproducibility of prepared biological samples, the data lack reproducibility. With nanotechnology, the mechanical properties of single biomolecules can be directly measured with an optical tweezer technique. Fujii et al. studied the mechanical properties of a single hyaluronan molecule, which is a major component of the extracellular matrix and plays an important role in the mechanical functions of the extracellular matrix and stabilization of cells. The knowledge of the mechanical behavior of a single molecule may help us to understand the mechanical roles in the extracellular matrix infrastructure and cell attachment and to design tissue engineering systems where the mechanical functions of biomolecules are essential [111].

### 2.3.7. Study of the Bonding Force between Biomolecules

By attaching nanoparticles to a substrate and an AFM cantilever, the bonding force between individual molecules can be measured, as shown in Figure 5. For example, the bonding force between a growth factor and its receptor can be precisely measured.

### 2.3.8. Detection

Single nanometer-sized pores (nanopores) can be embedded into scaffolds or membranes as a new class of nanosensors for rapid electrical detection and characterization of biomolecules. These tiny nanopores with single-nanometer precision can provide direct visual feedback [17].

### 2.3.9. Diagnosis and Treatment

Nanoparticles containing antibodies can be injected to detect tumors. At the same time, drugs can be attached to the particles to treat diseases. One advantage is that very localized delivery causes minimum side effects. One unique feature of nanoparticle drugs is the ability to penetrate the blood–brain barrier, which will be very helpful for the treatment of brain tumors and other CNS diseases. Nanotechnology also allows incorporating a very tiny biocomputer chip into the scaffold design; for example, nanoscale transistors and sensors can be used to mimic a brain circuit for the treatment of Alzheimer's diseases.

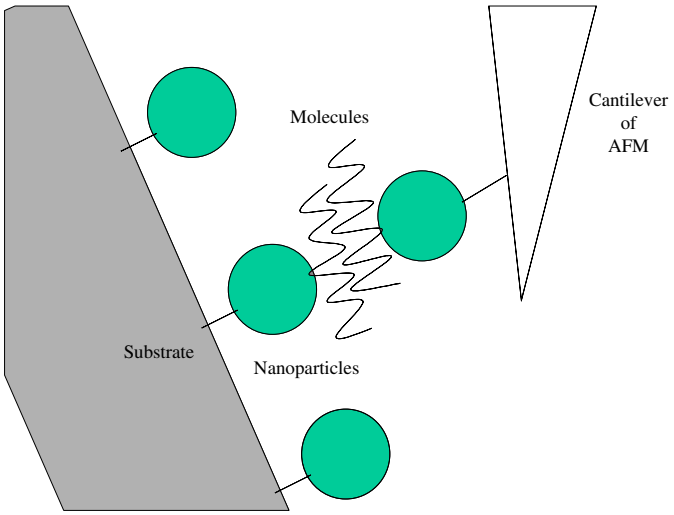


**Figure 5.** Measurement of the bonding force between molecules using nanoparticles and AFM. Nanoparticles are tethered on the AFM cantilever and substrate.

## 3. SUMMARY

Nanotechnology for tissue engineering applications is just started, and most of the applications are still in the idea stage. To date, the applications are limited to the following: design of biomaterial scaffolds to provide controlled cell adhesion, modulating the spatial organization of cells in the scaffold, regulating the biomolecule in the local environment, self-assembling scaffolds, using a "lab-on-a-chip" for a better understanding the mechanisms of cell differentiation and propagation and tissue development and formation; combination of biomolecule functionality with synthetic or natural biomaterial scaffold for better regeneration; surface modification at nanoscale level for enhanced biological compatibility and activity; manipulation and detection of a single molecule; controlled and targeted drug delivery, the fabrication of highly organized scaffold, and cellular and genetic engineering of functional cell types. Although there are a lot of unknowns, nanotissue engineering is not just a dream but quickly becoming reality. There is plenty space for the practical applications of nanotechnology in tissue engineering. Tissue engineering will be a perfect fields for exploring the practical applications of nanotechnology.

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