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Two-photon polymerization microstructuring in regenerative medicine

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1. ABSTRACT

Two-photon polymerization has developed as a powerful tool for making micro- and nanoscale structures for regenerative medicine applications. This review discusses micro- and nanoscale aspects of tissue engineering, which are followed by a brief description of the two-photon polymerization process and how it has been used thus far in tissue engineering and other regenerative medicine applications. Lastly, potential future applications of two-photon polymerization in regenerative medicine are presented. This review provides a comprehensive summary of the uses of two-photon polymerization thus far in regenerative medicine and a look into how this technique will be used in the future.

2. INTRODUCTION

The fundamental challenges of tissue engineering are ongoing. The issues challenging scientists today have been known since the inception of this field. As stated by Dr. Joseph Vacanti, "The major components of this technique are: 1. the use of biodegradable polymers; 2. cell viability supported temporarily by diffusion; 3. proliferation and organization of cells; 4. vascularization of the growing cell mass; and 5. proper cell function in the context of the new structure" (1). These important factors, known more than 20 years ago, are still the challenges being faced in this field. In the past two decades advances have been made in understanding the importance of some of the underlying factors in tissue engineering, such as

geometry, mechanical cues, and chemical factors. Due to its resolution and geometry control, two-photon polymerization (2PP) as a rapid prototyping technique is capable of addressing these challenges facing tissue engineering. This review will discuss micro-and nanoscale aspects of tissue engineering followed by a discussion of how two-photon polymerization has been used in regenerative medicine thus far. Lastly, potential future applications of 2PP to regenerative medicine will be discussed.

3. MICRO- AND NANOSCALE ASPECTS OF TISSUE ENGINEERING

3.1. Effects of scaffold architecture and surface morphology on tissue engineering

A wide range of architectural factors have an effect on the performance of tissue engineering scaffolds. The native extracellular matrix provides chemical and mechanical cues that induce cells to exhibit adhesion, migration, proliferation, and differentiation that is characteristic of a specific tissue (2). The end goal of tissue engineering scaffold generation from a materials science perspective is to generate a scaffold with the same properties as the natural extracellular matrix of the tissue being engineered, thus producing an environment in which cells will behave in the same manner as in the natural tissue.

It is well-established that cells behave vastly differently in 2-D and 3-D environments (3). Cells grown on 2-D surfaces have a flat, spread morphology; different proliferation rates; inhibited differentiation; and significantly different phenotypes (3, 4). For example, Ma *et al.* found that cells on 2-D surfaces have different cell cycle progression and less differentiation than cells in 3-D surfaces (5). Sahai and Marshall found that cells in 2-D may have different responses to pharmacological agents than cells in 3-D (4). These behavioral differences necessitate that tissue engineering utilizes 3-D scaffold instead of 2-D substrate for generating new tissues.

Another important architectural factor is surface chemistry and morphology. In tissue engineering, cells must have some sort of cell-substrate adhesion in order to survive, since the majority of mammalian cells are anchorage-dependent (6, 7). The quality of cell-substrate adhesion is highly dependent on the surface roughness and surface energy of the scaffold material (8-12). Lampin *et al.* performed cell adhesion and spreading studies on PMMA substrates of varying surface roughnesses that were produced by sand-blasting (8). Increased surface roughnesses resulted in increased hydrophobicity and improved cell adhesion. In a series of studies made by group of Prof. Chichkov, spike structures were produced in various materials to determine the effects of hydrophobicity and surface roughness on cell adhesion (10, 12-14). Spike structures were generated by laser ablation in silicon, titanium and by micromolding in silicone elastomer (10, 12-14). In all of these materials, the presence of a spiked morphology increased the surface roughness and corresponding hydrophobicity of the surface. Interestingly, their studies indicated that the effects of hydrophobicity on proliferation rate was cell-type specific. For example, fibroblasts exhibited decreased proliferation on spiked surfaces while neuroblastoma cells exhibited no change in proliferation rate (10, 12). This finding indicates that surface roughness may be used to select for a certain cell type, for example inhibiting proliferation of fibroblasts, which are responsible for scar tissue formation. Experiments by Ranella *et al.* with similar spiked structures achieved comparable results (15). Mouse fibroblasts, grown on silicon surfaces that were flat and of various spiked surface roughnesses, had increased densities and adhesion for low roughness ratios (<3) and low cell density and adhesion for high roughness ratios (> 3). Dulgar-Tulloch *et al.* examined the effect of the grain size of ceramics, specifically hydroxyapatite, alumina, and titania, on human mesenchymal stem cell, osteoblast, and fibroblast proliferation (16). For all three materials, average grain sizes of 200 nm had the greatest cell proliferation, with grain sizes of 1500 nm having slightly reduced proliferation and grain sizes of 50 nm having significantly reduced proliferation. Numerous studies have shown than surface roughness on the microscale can reduce proliferation and adhesion (10, 12-15), whereas surface roughness on the nanoscale can improve cell adhesion and proliferation (17).

In addition to affecting cell adhesion and proliferation, surface features may also be used to control cell orientation. Rebollar *et al.* produced grooves of varying periodicities (200-430 nm) and depths (30-100 nm) in polystyrene by polarized KrF laser light (17). Human embryonic kidney cells, Chinese hamster ovary cells, human myoblasts, and rat skeletal myoblasts all aligned parallel to the nanoscale grooves. These modified surfaces also demonstrated enhanced adhesion and proliferation for human embryonic kidney cells. Teixeira *et al.* produced grooves in silicon with pitches of 400, 800, 1200, 1600, 2000, and 4000 nm for examining the effects of surface topography on keratocytes (18). Smooth silicon has random orientation of cells; the 400 nm pitch surface had approximately 50% of the cells oriented parallel to the grooves; and the rest of the surfaces had greater than 65% of the cells oriented parallel to the grooves. Patterned surfaces also had significantly greater cell elongation than the smooth surface. Cells on the nanoscale surfaces exhibited fewer stress fibers and focal adhesions than the microscale surfaces. Cells on smooth silicon had random stress fiber orientation, cells on the 4000 nm surface were strongly aligned parallel to the grooves, and cells on the 400 nm surface were less strongly aligned. Miller *et al.* demonstrated that laser-micromachined lines in silicon may be used to control alignment of human aortic-vascular smooth muscle cells; on these surfaces the cells were aligned perpendicular to these grooves which were spaced approximately 100 μm apart (19). These findings indicate that cell orientation can be controlled and is highly dependent on the size scale of the surface patterning.

3.2. Mass transport in tissue engineering scaffolds

Mass transport is essential for a successful tissue engineering scaffold. Without a means of receiving

nutrients and removing wastes, cells in a scaffold cannot survive (20). In native tissue, mass transport is achieved via the vasculature. However, producing vascularized *in vitro* scaffolds has been challenging so an alternative approach has generally been preferred. The predominant technique for enabling mass transport in tissue engineering scaffolds has been via porosity. Scaffolds with a high porosity enable nutrients and waste to be transported via diffusion (21). Research has indicated that 80-90% porosity is ideal for mass transport in scaffolds (22). However, there is a trade-off between high porosity and mechanical integrity of the scaffold, so an appropriate balance between the two must be maintained.

An alternative solution to the mass transfer issue is to develop scaffolds with vasculature. As native tissue uses vasculature to achieve mass transport, this native transport mechanism can be used in tissue engineering scaffolds. An additional cause for producing pre-vascularized scaffolds is that vascularization aids scaffolds in integrating with the host tissue (23). It has been established in the literature that endothelial cells in a 3-D environment will spontaneously form tube-like structures (24-28). Kelm *et al.* produced a vascularized macrotissue structure by assembly of pre-generated microtissues (29). Myoblast microtissues produced by gravity-enforced self-assembly were coated with vascular endothelial cells, which resulted in development of a vascular structure initiated by the fibroblast VEGF production. A macrotissue was then produced by casting of these microtissues in an agarose mold. Tsuda *et al.* constructed pre-vascularized tissue by creating multiple alternating monolayers of fibroblasts or patterned endothelial cells (30). Unger *et al.* showed that human endothelial cells and osteoblasts in co-culture on scaffolds will distribute and form structures resembling bone tissue, including microcapillary-like structures (31). *In vivo* implantation of these scaffolds into immune-deficient mice confirmed integration with the host vasculature and formation of chimeric microcapillaries (containing both human and mouse cells). Both outgrowth of human cell microcapillaries into host tissue and ingrowth of mouse vasculature into the scaffold were observed. In contrast, scaffolds with solely endothelial cells and unseeded scaffolds exhibited poor ingrowth.

4. TWO-PHOTON POLYMERIZATION IN REGENERATIVE MEDICINE

Two-photon polymerization is a light-based rapid prototyping process that has numerous attributes which make it appealing for generation of tissue engineering scaffolds. The most obvious advantage is that, like all rapid prototyping techniques, two-photon polymerization enables complete control of scaffold geometry (e.g., porosity, pore location, size, and shape). Additionally, two-photon polymerization has the ability to precisely control the surface features of scaffolds. Light-based rapid prototyping generally facilitates higher resolution structures than other rapid prototyping processes. The ability to control many of the factors that affect the performance of tissue engineering scaffolds makes two-photon polymerization an important tool in advancing the field of tissue engineering.

Two-photon polymerization (2PP) utilizes excitation of photoinitiator molecules to induce polymerization of a resin, similarly to stereolithography. Unlike conventional stereolithography, where the photoinitiator is excited by single-photon absorption, in 2PP excitation occurs via multi-photon absorption. Ultra-short (generally femtosecond) laser pulses can induce nearly simultaneous absorption of photons. Upon absorption of multiple photons an excited state is achieved, which creates an effect similar to single-photon absorption at ½ of the laser wavelength (32, 33). Two-photon absorption results in a non-linear energy distribution that is radially dependent perpendicular to the axis of propagation and dependent on distance from the focal point along the axis of propagation; the energy distribution is quadratic in shape in both of these directions (34, 35). This energy distribution results in a volume approximately in the shape of a bicone (two cones joined at their base) where the energy is high eneough for excitation of photoinitiator molecules. Polymerization occurs in this volume, known as a voxel, where the threshold excitation energy is achieved (36, 37). Due to the nonlinear nature of two-photon absorption, the resolution of the polymerization voxel can be beyond the diffraction limit (36, 37). Therefore, in contrast with other laser direct write techniques (e.g., stereolithography, selective laser sintering, laser bioprinting), 2PP can produce sub-micron features (e.g., on the same length scale as subcellular organelles). Resolutions of 30 nm have been reported (38). While exceptionally high resolutions are capable with 2PP, the resolution is scalable, which facilitates tuning the resolution to one's needs and thus minimizing the fabrication time (39). Since many medical devices do not require sub-100 nm or even sub-micron resolution, tailoring the resolution to the application minimizes production time and cost (34). The resolution of 2PP is dependent on a wide range of factors. The laser spot size, wavelength, energy, pulse width, pulse duration, pulse frequency, and pulse peak intensity all affect the voxel size. From a structuring perspective, scanning speed, rastering spacing, and layer spacing affect the resolution of a structure. Further, the optical properties of the resin, photoinitiator type and concentration, and additional of radical quenchers can influence the resolution.

Two-photon polymerization has been used to process a wide range of materials, including Ormocer® (33,34,40,41-43), polyethylene glycol diacrylate (44-47), biodegradable polycaprolactone and polylactic acid based copolymers (48), Zr-based sol-gel composites (49, 50), Ti-based sol-gel composites (51), metal ion-doped acrylates (52), bovine serum albumin (53, 54), fibrinogen (53), and collagen (54). The biocompatibility and more importantly the nonfouling properties of polyethylene glycol diacrylate make this material appealing for tissue engineering scaffolds, particularly devices where biofouling may lead to scaffold failure (46, 47). The ability to crosslink natural polymers with 2PP is interesting in that it may enable production of tissue engineering scaffolds from natural materials (53, 54)

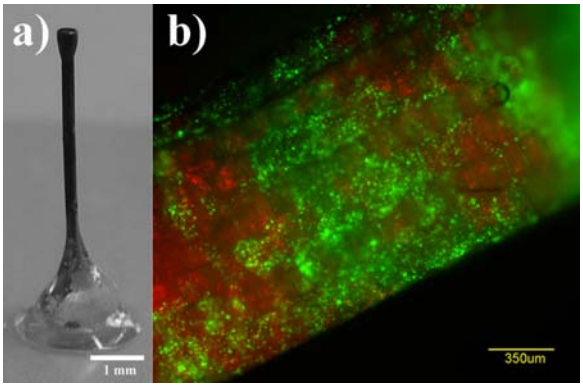

Figure 1. (a) A total ossicular prosthesis produced by two-photon polymerization of the shell of the structure and subsequent polymerization of the core by a UV lamp. (b) Cross-sectional view of a scaffold made by 2PP that was seeded with cells by laser bioprinting. The scaffold is composed of interconnected rings, which provide various pore sizes, and is seeded with alternating sections of adipose-derived stem cells (green) and endothelial cells (red).

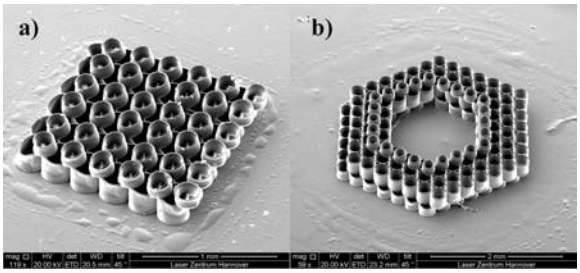

Figure 2. Lego™ scaffolds for solid tissue (a) and vascular tissue (b) engineering made by 2PP.

Due to the many properties that are conducive to medical device production, a variety of 3-D micro-structured medical devices for regenerative medicine have been generated by 2PP, such as small prosthetics (55) and tissue engineering scaffolds (13, 33, 47, 56-58). Small prostheses, such as ossicular prostheses, are another class of medical devices that may be produced by 2PP. An example of a TORP produced by 2PP is presented in Figure 1a. This device was produced by structuring a shell of the structure with 2PP and polymerizing the liquid core with a lamp after washing, which significantly reduces the fabrication time. Ovsianikov *et al.* produced TORPs (Total Ossicular Replacement Prostheses) out of Ormocer® by 2PP. Insertion and removal of the Ormocer® TORP from the intended site of use in a frozen cadaver head without fracture of the prosthesis was demonstrated (55). The flexibility of 2PP may enable TORPs to be produced with dimensions that are specifically tuned to the patient. Recently, Schizas *et al.* have produced microscale valves via 2PP, indicating that 2PP may be used to produce prosthetic valves for vasculature (59).

Two-photon polymerization has been used to create tissue engineering scaffolds with complex geometries (12, 47, 48, 58, 60-63). Scaffolds containing pores of multiple sizes may allow preferential transport of cells versus smaller molecules (e.g., nutrient, waste, growth factors). For example, Tayalia *et al* produced scaffolds with pores ranging from 12 to 110 μm; cells were able to penetrate the larger pores but not the smaller ones (63). Stackable Lego™-like tissue engineering scaffolds have also been created by 2PP, which enable assembly of larger constructs fabrication (33, 60-62). The primary advantage of this technique is that the "Lego™ pieces" can be mass produced by soft lithography and assembled to form the final structure. This assembly technique greatly reduces the production time and allows the customization of the end product. Also, the components may be seeded prior to assembly in order to improve cell distribution throughout the larger scaffold. In one Lego™-like design, 2PP was used to produce scaffolds with raise cylinders on one side and cylindrical wells on the other (33, 62). Porous Lego™-like scaffolds have also been produced, which consist of multilayer scaffolds of rings of different sizes. These rings can interlock to form scaffolds that can be arranged together while still maintaining a high porosity. Scaffolds made by this porous Lego™ concept have been produced for solid tissue as well as vascular tissue engineering (Figure 2) (60, 61).

Two-photon polymerization has also been used in combination with laser bioprinting to produce vascular scaffolds (58). High-porosity scaffolds produced by 2PP are conducive to cell seeding by laser bioprinting. Some advantages of combining these two techniques are that high cell densities can be achieved and multiple cell types can be deposited in a controlled configuration. For example, a scaffold can be seeded with alternating sections of adipose-derived stem cells (in green) and endothelial cells (in red), as shown in Figure 1b.

5. FUTURE APPLICATIONS OF TWO-PHOTON POLYMERIZATION IN REGENERATIVE MEDICINE

In the past decade, two-photon polymerization has developed as a powerful tool for tissue engineering. The flexibility in controlling geometries and fabricating scaffolds with feature sizes ranging from sub-micron to tens of microns makes this technique particularly appealing. While 2PP has already been used extensively as a tool in regenerative medicine, there are still many new opportunities for applying 2PP to this field. Future applications of 2PP include production of micro-scale wound closure systems, multibeam writing, production of prevascularized scaffolds, and micromolding replication with natural materials.

One of the factors that are limiting widespread use of 2PP for fabrication of tissue engineering scaffolds is production time. As 2PP involves rastering the laser beam over the entire volume of the structure, fabrication can be a lengthy process, especially for high-resolution structures. A technological advancement that has recently been proposed for decreasing 2PP building time is multibeam fabrication (64). Obata *et al.* used computer-generated holographic

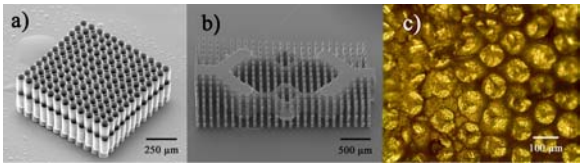

Figure 3. Scanning electron micrographs: (a) of a tissue engineering scaffold produced by 2PP with four beams writing simultaneously and (b) of 3-D scaffolds with microfluidic systems mimicking the natural capillary network. (c) Optical microscopy image of a fibrin high-porosity scaffold made by micromolding replication of a 2PP-generated master structure.

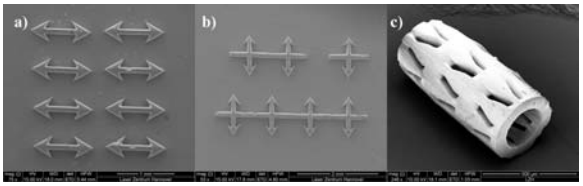

Figure 4. 2PP-generated devices for regenerative medicine. (a, b) Barbs for wound closure and (c) a polyethylene glycol stent.

(CGH) patterns to separate a laser beam into multiple foci. Production of tissue engineering scaffolds via structuring with multiple beams can greatly reduce the time required for producing a scaffold. For example, a scaffold produced with four beams, as shown in Figure 3a, can be generated in one fourth of the time of conventional single-beam 2PP.

As previously mentioned, producing pre-vascularized scaffolds is one means of achieving mass transport. Vascularization as a means of mass transport is preferential to high porosity since it results in scaffolds with greater structural integrity and also better mimics the mass transport mechanism of natural tissue. In order to engineer tissues which are more than a few mm in size, vascularization of the generated tissue is essential. While making a pre-vascularized scaffold is preferable, this approach has not been largely pursued due to difficulties in making a scaffold with a vascular network. The spatial control and high resolution of 2PP make it a technique that could be used to produce a vascular network. Two-photon polymerization-generated microfluidic systems have been in use for several years (33, 39, 65). However, this technique has not yet been used for producing microfluidic networks within a tissue engineering scaffold. First 3-D scaffolds with microfluidic systems mimicking the natural capillary network, such as that shown in Figure 3b, have been recently fabricated by 2PP in the group of Prof. Chichkov. This approach can be used to produce pre-vascularized tissue constructs in the near future.

Micromolding replication has become well established as a technique for mass fabrication of structures produced by 2PP. This technique, which involves making a silicone elastomer mold of the 2PP-generated structure and "stamping" replicas, has been used to produce a range of structures from a variety of materials. It has been previously demonstrated that micromolding replication is capable of replicating complex 3-D structures, including tissue engineering scaffolds with overhanging features (13, 60). Another advantage of micromolding replication is that it can make replicas out of materials that are not compatible with 2PP. Previous examples include opaque photopolymers and pharmaceutical-doped polymers (44, 46). A new application of this technique is to use 2PP-micromolding replication to produce scaffolds from natural extracellular matrix (ECM) materials. For example, micromolding replication can be used to produce high-porosity, 3-D scaffolds from fibrin (66) (see Figure 3c). The ability to produce scaffolds from natural ECM materials while still taking advantage of the structural control of the 2PP process could enable next-generation scaffolds with both complex structures and enhanced bioactivity (66).

Two-photon polymerization can be applied for production of additional microscale devices for regenerative medicine beyond tissue engineering scaffolds. First prototypes of such devices have been fabricated by 2PP in a joint project between the groups of Prof. Narayan and Prof. Chichkov. In Figures 4a,b, microscale barbs for wound closure generated by 2PP are shown. These barbed structures may serve as an alternative to sutures or glues for bonding tissues. Two-photon polymerization can also be used to produce stents. The resolution of 2PP could allow generation of stents with finer details than conventional stents made by machining. Also, problems with machining of stents, such as burr formation and melting of temperature sensitive materials, do not occur in 2PP. Further, 2PP can be used to produce stents from better suited materials. Stents could be produced from the nonthrombogenic material polyethylene glycol, as shown in Figure 4c or from biodegradable polymers.

6. CONCLUSIONS

This work has reported on the current status and potential future applications of two-photon polymerization to regenerative medicine. Numerous regenerative medicine devices can be produced by two-photon polymerization, including tissue engineering scaffolds, small bone prosthetics, stents, and wound closure systems. The development of rapid prototyping via two-photon polymerization and UV soft lithography has been used to produce scaffolds with complex overhanging geometries from a range of materials, including components of the extracellular matrix. Two-photon polymerization structuring with multiple foci may greatly reduce the fabrication time of these regenerative medicine devices. The research reported throughout this work provides a comprehensive review of the applications of two-photon polymerization towards regenerative medicine.

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Abbreviations: 2PP: Two-photon polymerization; UV: ultraviolet; 2-D: two-dimensional; 3-D: three-dimensional; PMMA: Polymethyl methacrylate; VEGF: vascular endothelial growth factor; TORP: Total ossicular replacement prostheses; CGH: computer-generated holograph; ECM: extracellular matrix.

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