Published in final edited form as:
Curr Opin Biotechnol. 2011 October ; 22(5): 674–680. doi:10.1016/j.copbio.2011.04.001.

Bottom-up tissue engineering

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Abstract

Recapitulating the elegant structures formed during development is an extreme synthetic and biological challenge. Great progress has been made in developing materials to support transplanted cells, yet the complexity of tissues is far beyond that found in even the most advanced scaffolds. Self-assembly is a motif used in development and a route for the production of complex materials. Self-assembly of peptides, proteins and other molecules at the nanoscale is promising, but in addition, intriguing ideas are emerging for self-assembly of micron-scale structures. In this brief review, very recent advances in the assembly of micron-scale cell aggregates and microgels will be described and discussed.

Keywords

Modular scaffold; microgel; tissue engineering; regenerative medicine; hydrogel; self-assembly

Introduction

The development of biomaterials for regenerative medicine is at a crossroads. The goal of producing functional tissues is challenged by the complexity of organ architectures. It is difficult enough to vascularize tissue constructs, let alone to recapitulate the complex arrangement of cells and extracellular matrix (ECM) found in functional organs. Tremendous recent successes achieved using cell-seeded decellularized tissues confirm the important role of a scaffold or structural framework to which cells may adhere [1–3]. On the opposite end of the spectrum, scaffold-less approaches using cell aggregates or cell sheets lead to questions about the importance and necessity of a scaffold.

Despite the tremendous advances using biologically-derived scaffolds, the development of synthetically-derived scaffolds is attractive from practical, regulatory and scientific perspectives. However, will it be possible to engineer cells and materials to recapitulate the complex structures of ECM as it exists in native tissues? Of the many approaches that are currently being investigated, one promising route for the production of such materials is through bottom-up assembly.

In 1959, Feynman described the applicability of (mostly) top-down approaches to nano-manufacturing in his famous lecture, “There s *plenty* of room at the bottom” [4]. A top-down

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approach uses tools to sculpt an object – tools such as knives, saws, brushes, etchants, molds, etc. This holds true even when carried out at the nanoscale, as in photolithography. Later, Drexler described molecular engineering for nano-manufacturing, thus formalizing the bottom-up approach [5]. In regenerative medicine, production of a scaffold in a mold is an example of a top-down strategy, even if the scaffold is made porous by including leachable microparticles, or if structures are introduced post-hoc using optical pattering techniques. A bottom-up approach relies on the self-assembly or directed-assembly of a scaffold from smaller components or modules , potentially with different modules designed to carry out distinct tasks (Fig. 1).

A common characteristic of the current bottom-up strategies for scaffold fabrication is the assembly of molecules into nano- or microscale structures, which then assemble into macroscopic objects. Self-assembling molecules that form nanoscale structures that then form gels are excellent examples of bottom-up assembly. Self-assembly of materials from amphiphilic molecules (proteins, peptides, polymers, etc.) have been recently reviewed and will not be further described here [6]. The focus of the current review will be on the assembly of micron-scale objects into three-dimensional structures.

Modular scaffolds have been investigated by a small but growing number of groups. Previous reviews describe the synthesis of modular scaffolds up to mid-2009 [7, 8]. The current contribution will focus on work subsequent to these reviews.

Assembly of cells and cell aggregates

An exciting development is the DNA-directed self-assembly of multiple cell types. Bertozzi and colleagues have previously developed alkyne derivatives that undergo [3+2] cycloadditions in the absence copper [9–11]. Strained alkynes react readily with an azide, with no detectable cross-reactions with moieties found in cells and tissues. Saccharides such as N-azidoacetylmannosamine added to cell culture medium are taken up by cells and used to produce the extracellular glycocalyx. In a recent study, cell surfaces decorated with azide sugars were reacted with single-stranded oligonucleotides modified with a cyclooctyne derivative. Two batches of Jurkat cells were separately labeled with complementary oligonucleotides. Upon mixing in a 1:1 ratio, the cells assembled into large aggregates. To control aggregation, the ratio of the two types of cells was adjusted to 1:50. In this case, the less common cell type existed as single cells surrounded by rosettes of the other cell type, termed microtissues (Fig. 2). DNA-directed cell assembly was then used to produce a paracrine signaling network between IL-3 expressing CHO cells and an IL-3 dependent untransformed hematopoietic progenitor cell line (FL5.12). FL5.12 cells that were assembled into microtissues with the CHO cells and embedded in agarose gels were able to proliferate even after IL-3 removal. Single FL5.12 cells did not proliferate under these conditions [12].

Formation of microtissues may also be performed on larger scales by co-culturing pre-formed cell aggregates. Forgacs and colleagues have demonstrated that spheroids may be formed by cutting cylinders of a cell slurry followed by overnight culture in solution. The spheroids that form are of uniform size and are used as bioink for cell printing [13]. Spheroids of different cell types may be printed into tissue-like structures using a custom 3D printer. This highly promising technique has been very recently reviewed [14]. Morgan and colleagues have demonstrated that if a poorly adhesive surface contained small posts, the cells surrounded the posts, eventually forming a multilayer of cell aggregate with holes after removal from the substrate [15]. More recently, these honeycomb-like structures were formed from ovarian theca cells. Inside the holes, ovarian granulosa cells were added, creating a structure similar to that found in the ovary. Such techniques may allow *in vitro* maturation of oocytes, potentially greatly improving *in vitro* fertilization [16]. The same

group also demonstrated the formation of toroids on poorly-adhesive posts. As the cells contracted around tapered posts, they actually pushed themselves up and off. The toroids proved to be useful building blocks to assemble highly porous tissue-like structures [17]. The group also studied the kinetics of assembly of spheroids in linear troughs. Linear arrays of spheroids tended to fuse into rods and contract over time. The kinetics of fusion and contraction could be manipulated by the length of time the spheroids were allowed to mature, or by blocking cell-cell adhesion [18, 19].

Arrays of spheroids may also be produced. Morgan and colleagues used poorly adhesive multi-wells made from agarose and seeded them with cells to form spheroids. An alginate solution was then poured over the multi-wells and gelled. When removed from the agarose, the alginate gel was decorated with spheroid-laden posts [20]. In a similar spheroid-patterning strategy, Fukada and colleagues filled microwells in a microfluidics device with HepG2 liver cells (the other surfaces of the device were coated with albumin, such that the HepG2 spheroids could only form in the microwells). Then, a solution of polylysine was flowed over the surface, adsorbing to the albumin coating and rendering the surface cell adhesive. Fibroblasts were seeded on the substrate and these cells surrounded the spheroids but did not coat them [21].

Assembly of micron-scale hydrogels

Sefton and colleagues introduced an intriguing approach to modular scaffold formation in 2006 [22]. The approach essentially inverts the architecture of the native vasculature. Cylinders (modules) are produced by cutting a tube collagen gel that is loaded with cells. The modules are then coated with endothelial cells. The modules may be packed into a chamber and subjected to blood flow. Although the thrombogenicity of the construct is low in flowing blood, adhesion of leukocytes was present [23]. This may have resulted from the flow geometry, as a endothelial-lined microfluidic chamber with similar geometry also led to greater adhesion of leukocytes [24]. Collagen/Matrigel modules were formed with embedded rat pup cardiomyocytes. The modules were seeded with endothelial cells and embedded in an alginate gel. The modules contracted in sync with electrical stimulation. However, the endothelial coating did attenuate the response [25]. The modules may be used as vehicles for cell transplantation *in vivo*. Human endothelial-coated collagen modules were implanted in athymic rats. The modules formed hollow channels at their interfaces and the human endothelial cells formed primitive vessels in the channels [26]. Transplanting modules coated with allogeneic rat endothelial cells into immune compromised rats resulted in the outgrowth of the transplanted cells to form functioning blood vessels that persisted for at least 60 days [27]. The modules were also implanted in immune-compromised mice by injecting through a needle. Endothelial apoptosis was noted to be present, which was decreased by coating the collagen modules with fibronectin. The fibronectin-coated modules yielded greater numbers of mature vessels lined with the transplanted endothelial cells [28]. Synthetic materials are also being developed as mechanically stronger substitutes for collagen in the cylinders [29]. A video of the methods for modular scaffold fabrication has been published [30]. The Whitesides lab has also introduced micromolding methods to produce similar, yet smaller, cylinders [31, 32].

Khademhossenei and colleagues have intensively investigated the assembly of cell-laden microgels to produce scaffolds (Fig. 3). The microgels have been assembled primarily by manipulating the hydrophilicity of surfaces and interfaces. For example, a PDMS stamp was used to pattern a hydrophobic silane onto a glass slide. Cell-laden microgels were added to the glass substrate in PBS. After pouring off the excess solution, the buffer solution remained in the hydrophilic regions of the slide, trapping some of the microgels in the remaining liquid [33]. A mathematical model of microgel assembly in oil-like media has been developed [34].

Hexagonal microgels were recently produced by the photopolymerization of PEG or the calcium-induced gelation of alginate. The microgels were assembled in two-dimensional arrays and pores were generated by chelating calcium to dissolve the alginate microgels, with little impact on cell viability within the PEG microgels. Multiple layers could be stacked to produce three-dimensional structures [35]. Microgels of various shapes were also assembled while floating on the solvent perfluorodecalin. Cell-laden microgels aggregated and were crosslinked by a second photopolymerization with little loss of cell viability [36]. PEG microgel rings were also synthesized, which stacked into tubes in mineral oil. By varying the number of holes in the rings, branching structures could be introduced. Concentric rings were also produced by sequential photopolymerization with different photomasks. Endothelial cells were polymerized in the inner ring, while smooth muscle cells were polymerized in the outer ring, which could be assembled into blood vessel-like structures. [37]. The group also introduced the concept of micro-masonry . PEG microgels containing HepG2 cells were formed in various shapes (usually bricks). The microgels were suspended in a solution of PEG-methacrylate with photoinitiator and added to a PDMS template (e.g., a cylinder). When excess solution was removed, capillary forces brought the bricks together around the template. Photopolymerization was used to crosslink the solution-phase PEG-methacrylate, serving as the mortar that bound the bricks together in the shape of the template [38].

In another strategy from the same group, mouse embryoid bodies were formed and deposited into microwells on a substrate. Photopolymerizable PEG was added and photopolymerized in roughly half of the microwell. Another mask was used to photopolymerize gelatin in the other half of the microwell. Thus, half of each embryoid body was encapsulated in a block of PEG gel, and the other half was encapsulated in a block of crosslinked gelatin. Vasculogenic cells sprouted from the embryoid body into the gelatin matrix, introducing polarity into the construct [39].

Harada et al. recently used the differential affinities of cyclodextrins to selectively assemble hydrogels. Adamantane or tert-butyl groups bind strongly to β-cyclodextrin, while n-butyl groups bind strongly to α-cyclodextrin. When the various chemical groups were added to poly(acrylamide) gels, the gels self-segregated based on the specific interactions (Fig. 4A) [40]. Although the gels were in the millimeter size regime, the approach may prove to be useful for the assembly of microscale hydrogels.

In the author s lab, we have recently produced macroporous scaffolds by the assembly and chemical reaction of hydrogel microspheres. Microspheres with different functions (structural, drug delivery and porogenic) were synthesized from reactive PEG derivatives and proteins. The method that was developed for microsphere fabrication relied a thermally induced phase separation of PEG in the presence of sodium sulfate [41]. The microspheres retained reactive groups, allowing crosslinking upon mixing the microspheres and centrifugation, forming a scaffold. The crosslinking was mild enough to be performed in the presence of living cells. The porogenic microspheres dissolved with two days in culture to produce porous scaffolds without affecting cell viability [42]. The density of the crosslinks in the microspheres affected their buoyancy relatively independently of their size, allowing the formation of gradients in microspheres upon centrifugation (Fig. 4B) [43]. Microspheres used for scaffold assembly may also be produced by the precipitation photopolymerization of PEG-diacrylate [44].

A number of groups are investigating aggregates consisting of cells and drug-delivering hydrogel microspheres. The overall hypothesis is that growth factor diffusion into spheroids is a limiting factor in the differentiation of stem cells. Hyaluronic acid microspheres containing perlecan for controlled release of BMP-2 were added to mesenchymal stem cells

in a micromass to promote chondrogenesis [45]. Others have developed chondroitin sulfate, agarose and gelatin microspheres were developed to release growth factors to affect cell differentiation. The microspheres were incorporated within mouse embryoid bodies, with good viability and some effects on stem cell differentiation [46, 47]. Gelatin/chitosan microspheres have been similarly used to promote the differentiation of adipose derived stem cells [48].

These strategies are intermediate between scaffold-based and scaffold-free approaches, and evolved from techniques using non-hydrogel microspheres made from degradable poly(esters) [49–54].

Conclusions

The self-assembly strategies discussed here may evolve to allow the formation of spatially organized tissues. The ability to control the architecture of 3D cell/material hybrid structures may prove to be critical for the development of highly advanced scaffolds for cell transplantation that are better mimics of natural ECM.

Acknowledgments

This work was supported by NIH R01HL085364.

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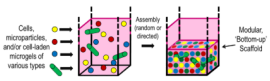

Figure 1.

Modular approach to the production of scaffolds. Nano- or microscale modules consisting of cells, cell aggregates, particles or gels are allowed to assemble. The assembly process may be guided by specific interactions or may be random. Following crosslinking in some manner, a three-dimensional construct is available for *in vitro* expansion of cells or for *in vivo* transplantation.

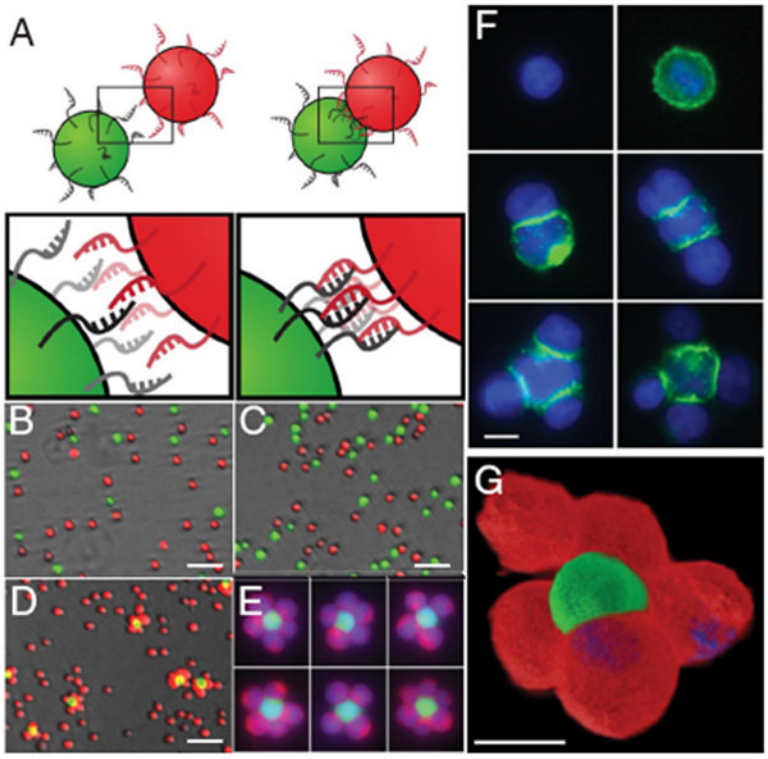

Figure 2.

(A) Complementary oligonucleotides were covalently coupled to the surfaces of different cells by click chemistry. (B–E) Two non-adherent cell types were mixed, and did not aggregate if their surfaces were modified with: (B) no oligonucleotides, (C) non-complementary oligonulceotides. However, specific aggregation was observed if the cell surfaces were modified with complementary oligonucleotides (D&E) . (F) Aggregation of DAPI stained cells (blue), with the central cell modified with fluorescein-conjugated oligonucleotides (green). (G) 3D reconstruction of an aggregate of Texas Red-labeled (red) and fluorescein-labeled cells (green). From Gartner ZJ, Bertozzi CR. “Programmed assembly of 3-dimensional microtissues with defined cellular connectivity”, Proceedings of the National Academies of Science, USA, 106:4606–10. Copyright 2009 to the authors, permission for reuse not required.

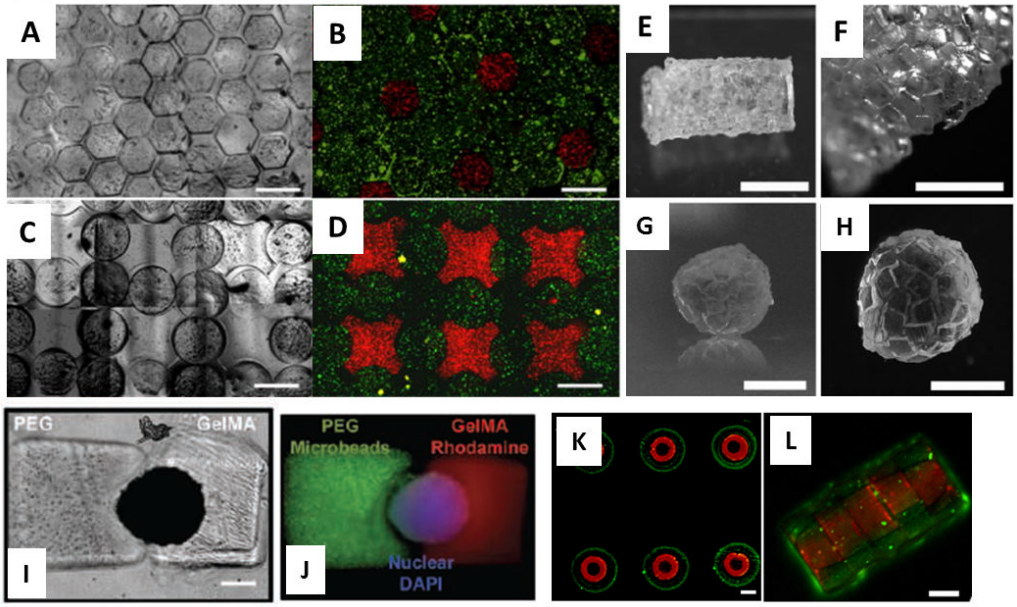

Figure 3.

(A) Assembly of hexagonal PEG microgels floating on a perfluorinated solvent. (B) Fluorescence image of liver cells (red) in some of the hexagonal microgels and fibroblasts (green) in the rest. (C) Assembly of lock-and-key microgels. (D) Fluorescence image of liver cells (red) in the lock microgels and fibroblasts (green) in the key microgels. From: Zamanian B, Masaeli M, Nichol JW, Khabiry M, Hancock MJ, Bae H, et al. "Interface-directed self-assembly of cell-laden microgels", Small, 2010, 6:937–44. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission. (E–H) Micromasonry , using surface tension of a polymerizable solution of PEG to assemble microgels, followed by photopolymerization. (E&F) Tube formed around a PDMS cylinder. (G&H) Sphere formed in a non-aqueous solvent. From: Fernandez JG, Khademhosseini A. "Micro-Masonry: Construction of 3D Structures by Microscale Self-Assembly", Advanced Materials. 2010;22:2538–41. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission. (I&J) Photomasks were used to sequentially polymerize PEG (left) and gelatin (right) around a cell cluster (center). Qi H, Du Y, Wang L, Kaji H, Bae H, Khademhosseini A. "Patterned differentiation of individual embryoid bodies in spatially organized 3D hybrid microgels", Adv Mater. 2010;22:5276–81. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission. (K) Concentric rings formed by sequential photopolymerizations using different masks. (L) The rings stack to form tubes in mineral oil. Du Y, Ghodousi M, Qi H, Haas N, Xiao W, Khademhosseini A. "Sequential assembly of cell-laden hydrogel constructs to engineer vascular-like microchannels", Biotechnol Bioeng. 2011; In press. Copyright John Wiley & Sons. Reproduced with permission.

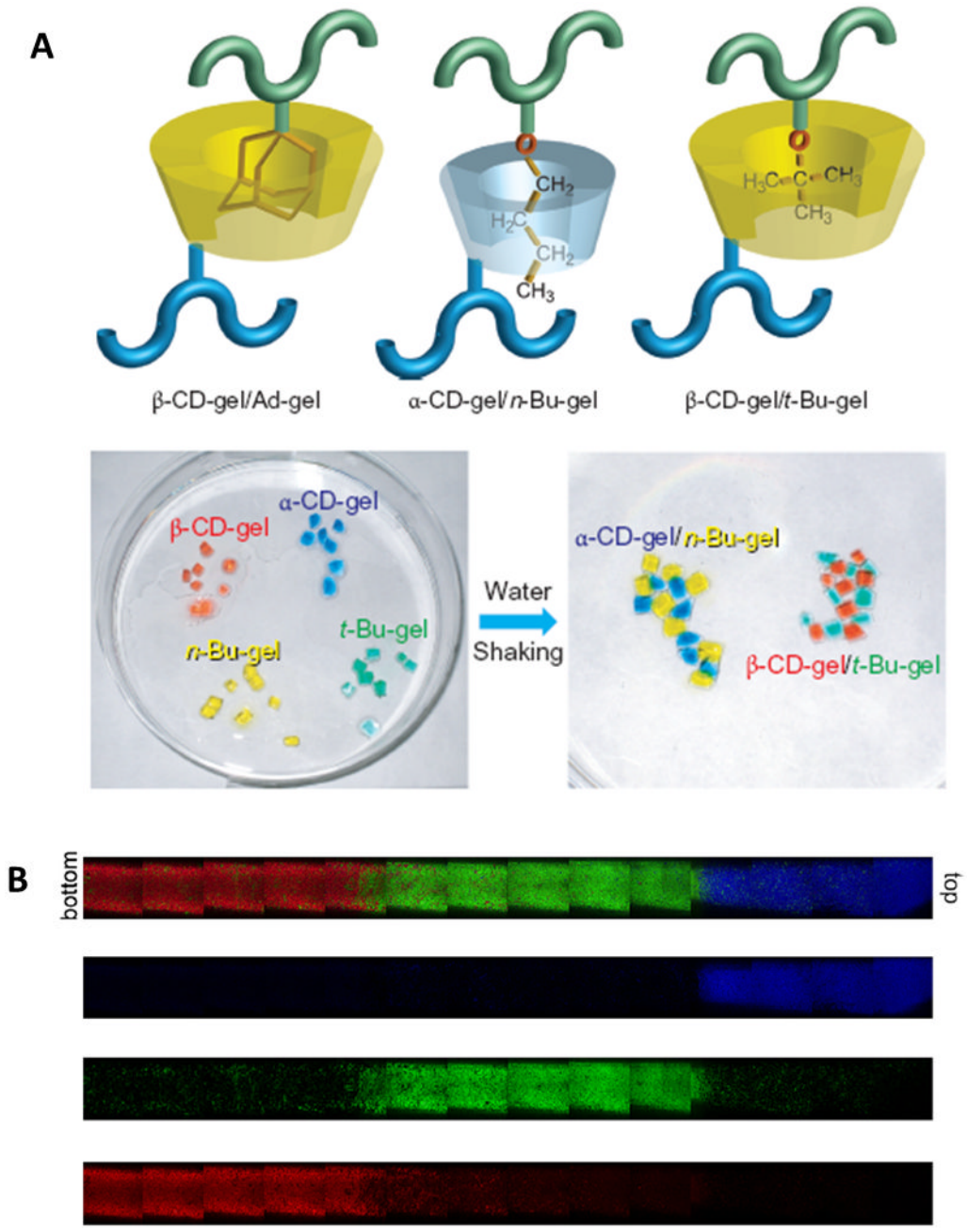

Figure 4.

(A) Millimeter-sized gels decorated with α- or β-cyclodextrins, or n-butyl or tert-butyl groups aggregate upon shaking, but only with gels containing their appropriate binding partners. Reprinted by permission from Macmillan Publishers Ltd.: Nature Chemistry, 3:34–37. Copyright 2011. (B) PEG microspheres were formed with identical chemistries and similar size, but with different buoyancies. Upon centrifugation microspheres separated out into distinct layers based on the buoyancy differences to produce graded structures. From: Roam JL, Xu H, Nguyen PK, Elbert DL., "The Formation of Protein Concentration Gradients Mediated by Density Differences of Poly(ethylene glycol) Microspheres", Biomaterials. 2010;8642–8650. Copyright Elsevier, used with permission.