Published in final edited form as:
J Tissue Eng Regen Med. 2014 January ; 8(1): 1–14. doi:10.1002/term.1494.

Development of functional biomaterials with micro- and nanoscale technologies for tissue engineering and drug delivery applications

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Abstract

Micro- and nanotechnologies have emerged as potentially effective fabrication tools for addressing the challenges faced in tissue engineering and drug delivery. The ability to control and manipulate polymeric biomaterials on the micron and nanometer scale with these fabrication techniques has allowed for the creation of controlled cellular environments, engineering of functional tissues, and development of better drug delivery systems. In tissue engineering, micro- and nanotechnologies have enabled the recapitulating of the micro- and nanoscale detail of cell's environment through controlling surface chemistry and topography of materials, generating 3D cellular scaffolds, and regulating cell-cell interactions. Furthermore, these technologies have led to advances in high-throughput screening (HTS), enabling rapid and efficient discovery of a library of materials and screening of drugs that induce cell-specific responses. In drug delivery, controlling the size and geometry of drug carriers with micro- and nanotechnologies have allowed for modulation of parameters such as bioavailability, pharmacodynamics, and cell-specific targeting. In this review, we introduce recent developments in micro- and nanoscale engineering of polymeric biomaterials with an emphasis on lithographic techniques, and present an overview of their applications in tissue engineering, HTS, and drug delivery.

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Author Contributions

H.B., H.C., and F.E. contributed equally to this work. H.B., H.C., F.E., A.F.A, S.S., Y.W, and A.K. generated idea and designed the manuscript; H.B., H.C., F.E., A.F.A., J.M.C., S.S., A.K., C.H.K., B.Z., Y.W., and A.K. wrote the manuscript; H.B., H.C., F.E., A.F.A., J.M.C., J.W.N., S.M., Y.W., and A.K. revised the manuscript.

Keywords

Biomaterials; Microtechnology; Nanotechnology; Tissue Engineering; High-throughput screening; Drug delivery

1. Introduction

Tissue engineering and drug delivery are promising approaches to address many current therapeutic shortcomings in the treatment of diseased or damaged tissues and organs.(Langer and Vacanti 1993) However, the clinical applicability of tissue engineering has been limited by a number of challenges including the inability to accurately control the spatial and temporal components of the cell's microenvironment and to recreate biomimetic three-dimensional (3D) cell culture platforms.(Naderi *et al.* 2011) Furthermore, in the pharmaceutical industry, new and existing drugs continue to be scrutinized for their poor specificity, solubility, therapeutic index, and immunogenicity.(Petros and DeSimone 2010) One area of research that has gained traction in terms of addressing these needs has been through the development of polymeric biomaterials.(Peppas *et al.* 2006) With advances in biology, chemistry, and material science, polymeric materials can now be synthetized from a combinatorial array of monomers, oligomers, and polymers with tunable chemical, mechanical, and geometrical properties to create new, biocompatible substances.(Slaughter *et al.* 2009) In the early days of tissue engineering, it was believed that biomaterials simply function as scaffolds for cells; hence, the majority of the emphasis at the time was placed on biocompatibility and mass transport. However, it is now known that the *in vivo* cellular microenvironment contains critical information-rich cues embedded in the extracellular matrix (ECM),(Hynes 2009) neighboring cells, soluble and tethered cytokines, and metabolites that regulate cell survival, adhesion,(Geiger *et al.* 2009) migration,(Petrie *et al.* 2009) and differentiation.(Dolatshahi-Pirouz *et al.* 2011; Edalat *et al.*) Therefore, fabricating biomimetic cell culture systems that resemble the microenvironment of native tissues requires greater control over the micro- and nanometer features of biomaterials.(Ma 2008) In the field of drug delivery, it has been shown that the size and shape—on the order of nano- and micrometers—of drug carriers can affect a drug's circulation time, distribution, cellular internalization.(Petros and DeSimone 2010) Hence, it is not surprising that micro- and nanoscale technologies have emerged as powerful tools for addressing the existing challenges in tissue engineering and drug delivery given their ability to control material properties at the cellular and subcellular length-scales.(Khademhosseini *et al.* 2006; Shi *et al.* 2010) These technologies have been increasingly used to fabricate functional polymeric materials to control cellular behaviors, serve as tools for tissue engineers to develop improved scaffolds, and enhance a drug's pharmacodynamics parameters. In addition, microfabrication has accelerated advances in tissue engineering and drug delivery via the generation of high-throughput assays to facilitate simultaneous screening of thousands of materials,(Hook *et al.* 2010) cytokines, and drugs(Fernandes *et al.* 2009), which has led to miniaturization, cost reduction, and automated analysis.

This paper reviews recent works in micro- and nanoscale technologies that have had significant contribution towards the development of functional biomaterials. In particular,

we will review a variety of micro- and nanoscale fabrication techniques that have been applied to the biomedical field, followed by a discussion of their impact on studying cell-material and cell-cell interactions, development of HTS microarrays, and fabrication of drug carriers of specific sizes and shapes for drug delivery. The prospective contributions of these techniques to future biomedical and pharmaceutical applications will also be discussed.

2. Micro- and nanotechnologies: a preamble

Micro- and nanotechnology refers to a set of techniques used for the fabrication of materials with micron and sub-micron scale features, respectively (Fig. 1).(Gates *et al.* 2005) Recently, the critical threshold for nanotechnological approaches has been redefined to sub-100nm. Although, these technologies were first developed by the electronics industry as a means to increase the density of transistors in integrated circuits, in the past few decades, they have been adapted and expanded for biomedical applications. There remains many newly developed micro- and nanotechnologies, whose potential are yet to be realized in the biomedical field. In this section, we discuss a few conventional and emerging micro- and nanotechnologies that have been widely used or we predict will be utilized in tissue engineering and drug delivery.

2.1. Photolithography

Photolithography is a widely used and well-studied technique for microfabrication, having initially been developed in the semiconductor industry.(Ito and Okazaki 2000) In this technique, a photoreactive material, typically a monomer, oligomer, or polymer, is coated onto a substrate such as a silicon wafer (Fig. 1A). The photoreactive material polymerizes, crosslinks, or degrades upon ultraviolet (UV) light exposure. Selective areas of the material may be exposed to UV via using a mask with micron-scale features designed on a computer-aided design (CAD) software.(del Campo and Arzt 2008) Moreover, mask-less, selective exposure can also be achieved with optical interference techniques such as two-photon absorption(Hahn *et al.* 2006) or stereolithography.(Lee *et al.* 2008) Thereafter, unwanted areas may be dissolved by development in an organic solvent. The resulting pattern can be used on its own or it can act as a bas-relief master. The resolution achieved by photolithography depends primarily on the wavelength of light and the type of mask used, and range from micrometers to 45nm.(Rothschild 2005) Photolithography has been used to pattern a wide range of synthetic and natural polymers for use as 2-dimensional (2D)(Song *et al.* 2011) or cell-encapsulating scaffolds.(Bae *et al.* 2011)

2.2. Soft lithography

Soft lithography is a set of microfabrication techniques that utilizes a soft, flexible material, called an elastomer, to generate micron and sub-micron scale structures or molecules on a surface.(Xia and Whitesides 1998) A master mold fabricated via other lithographic techniques is used to emboss structures onto the elastomer, commonly made from poly(dimethylsiloxane) (PDMS). The elastomer can then be used for molding, printing, or embossing. The most commonly used soft lithography techniques include replica molding, nano- and microcontact printing (μCP),(Li *et al.* 2003) and microfluidics (Fig. 1B-D). In replica molding, a patterned elastomer is used to emboss structures onto other polymers or

soft materials. This technique can be used to generate stencils, which are polymeric membranes containing micron-scale holes of specified geometry and dimension, and have been used to study heterotopic cell-cell interactions.(Folch *et al.* 2000) In μCP, a patterned elastomer is used to transfer "ink" onto a surface via adsorption.(Kaufmann and Ravoo 2010) The choice of "ink" includes proteins, nucleic acids, and cell suspensions.(Perl *et al.* 2009) Finally, microfluidic devices are generated by placing PDMS embossed with channels against a glass substrate to form closed channels.(Whitesides 2006) Microfluidics is characterized by laminar flow and diffusive mixing, and require only pico- to nanoliter volume of reagents.(Burdick *et al.* 2004)

The extension of soft lithography to the third dimension has been achieved via multilayer soft lithographic approaches in which separate structures are assembled on each other on a chip.(Unger *et al.* 2000) These chips can be used to generate robust micromechanical valves and microfluidic channels that minimize cross contamination or leakage between the processes(Hong *et al.* 2004), and have been used for protein crystallization,(Hansen *et al.* 2002) nanoliter-volume polymerase chain reaction,(Liu *et al.* 2002) microfabricated fluorescence activated cell sorting,(Fu *et al.* 2002) and single-cell enzyme screening. (Thorsen *et al.* 2002) Moreover, these techniques are capable of controlling the topography and spatial allotment of molecules on a surface, as well as the subsequent deposition of cells.

2.3. Electron beam lithography

Instead of using photons as in photolithography, electron beam lithography (EBL) uses electron beams to pattern electron-sensitive resists.(Norman and Desai 2006) Due to the low diffraction of electrons, significantly smaller features (3-5nm resolution) can be achieved. (Vieu *et al.* 2000) EBL can be used to fabricate nanopatterns composed of inorganic materials,(Werts *et al.* 2002; Das *et al.* 2009) synthetic polymers,(Peng *et al.* 2003; Idota *et al.* 2009), proteins,(Pesen *et al.* 2007; Christman *et al.* 2009) and self-assembled monolayers. However, one major disadvantage of EBL is the high cost of the equipment and the length of time required to generate a patterned surface. Other weaknesses, such as electrostatic charging, which reduces the smallest feature size, must also be considered. (Egerton *et al.* 2004)

2.4. Nanoimprint lithography

Nanoimprint lithography (NIL) is another high-resolution technique for the fabrication of nanoscale features onto a substrate.(Chou *et al.* 1996) Depending on the type of substrate, NIL is categorized as either a thermal- or light-based process; however, in both cases, a rigid mold is used to transfer patterns onto a material. Thermal NIL begins with the pressing of a mold against a thermoplastic polymer whose temperature is above its glass transition temperature, followed by a cooling process that returns the polymer to a glassy state. In contrast, UV-NIL, otherwise known as step-and-flash imprint lithography, uses UV light and a transparent mold to pattern a photoreactive polymer precursor.(Guo 2007; Schift 2008) NIL has been used to generate structures with resolutions as high as 2nm,(Hua *et al.* 2004) and has been applied for protein patterning,(Hoff *et al.* 2004) nucleic acid manipulation,(Guo *et al.* 2004) and cell alignment.(Subramani *et al.* 2011)

2.5. Direct-write techniques

Direct-write or ejecting technologies include inkjet printing and robotic deposition, and use a nozzle or a printing head to spatially deposit “ink” on a surface (Fig. 1E). Inorganic and organic small molecules, synthetic polymers, proteins, nucleic acids, and cells may be deposited at addressable locations on a surface.(Kim *et al.* 2010; Ker *et al.* 2011) Given the automated nature of these technologies, thousands of different combinations of molecules may be used, which have been utilized to fabricate microarrays for HTS. While in 2D patterning, materials are simply deposited onto a substrate, 3D structures can be formed by a layer-by-layer approach.(Mironov *et al.* 2011) The resolution of inkjet printing is down to 10μm, whereas robotic deposition can achieve resolutions as low as hundreds of nanometers. (Nie and Kumacheva 2008)

3. Functionalizing materials using micro- and nanotechnologies for tissue engineering applications

3.1. Control over cell-material interactions

Mimicking the complexity of the cellular microenvironment, from the structure of ECM to the presentation of cytokines and intracellular signaling, is an essential component of constructing biologically functioning tissues.(Lutolf 2009) For instance, the extracellular milieu contains ECM molecules with nanoscale dimensions (ten to hundreds of nanometers) that act as substrates for cell attachment, and present a host of biochemical and mechanical signals to cells.(Murtuza *et al.* 2009) The latest developments in micro- and nanoscale technologies have focused on modification of biomaterial surfaces, fabrication of substrates with 3D micron- or nanoscale geometric features, and organization of cells in 3D matrices to engineer functional tissues.(Gauvin and Khademhosseini 2011; Gauvin *et al.* 2011)

3.1.1. 2-Dimensional control of materials—Current cell culture platforms use glass or polystyrene surfaces coated with ECM-derived proteins. However, these platforms do not recapitulate the biochemical signals present in the cell’s microenvironment. Hence, microtechnological approaches have been used to fabricate natural and synthetic matrices, with tunable chemical properties to more closely resemble *in vivo* conditions. One class of material that closely resembles the structure of ECM is hydrogels, consisting of a network of a crosslinked polymer containing 95-99% water.(Slaughter *et al.* 2009) Hydrogel and other classes of materials are amenable to chemical modification via conjugating or adsorbing cell-adhesion molecules such as arginine-glycine-aspartate (RGD) or growth factors.(Lutolf and Hubbell 2005; Place *et al.* 2009) A substrate can be biochemically altered in a selective fashion to constrain cell adhesion and control cell morphology. The importance of cell morphology is inherent in its role as a regulator of cell processes such as apoptosis(Chen *et al.* 1997) and differentiation.(Kilian *et al.* 2010) For example, the effect of interligand spacing ranging from 55-100nm was studied by patterning a surface with cyclic RGD ligands via micelle lithography.(Huang *et al.* 2009) A critical interligand spacing value of 70nm was found, below which cell adhesion, through integrin clustering and focal adhesion formation, was favored. To impart geometric features onto 2D surfaces, microscale techniques such as photolithography,(Khademhosseini A *et al.* 2006; Karp *et al.* 2007)

stencils,(Moeller *et al.* 2008) and μCP have been developed.(Bauwens *et al.* 2008) These techniques have enabled researchers to pattern cells on 2D substrates to investigate the effect of morphology on cell or tissue function.(Khademhosseini *et al.* 2007) For example, Karp *et al*. fabricated chitosan hydrogels in various geometrical forms—such as squares, circles, triangles, and lanes—using photolithography, as substrates for patterning cardiac fibroblasts, cardiomyocytes, and osteoblasts.(Karp *et al.* 2006). In another example, Yamazoe *et al*. created micropatterned cell adhesive albumin surfaces for fibroblast patterning.(Yamazoe *et al.* 2008) Although albumin in its native form is not conducive to cell attachment, exposure to UV light renders it cell-adhesive. Selective UV irradiation of an albumin-coated surface through a photomask led to the formation of cell-adhesive patterns. Cell sheet engineering is another area where microtechnology has been influential. Cell sheet engineering relies on the formation of cell monolayers and their subsequent manipulation, such as stacking or rolling, for assembly of mechanically robust tissues. However, in this technique, unlike their *in vivo* counterparts, cells lack orientation. μCP has been used to align cellular sheets. (Williams *et al.* 2009; Williams *et al.* 2011) Briefly, fibronectin was selectively stamped onto a poly(*N*-isopropylacrylamide) (PNIPAAm) substrate, forming cell-adhesive lanes. Cells, seeded in serum-free medium on these substrates, attached and elongated on the lanes only. After the addition of serum-containing media, cells grew to confluence in all areas of the substrate, but retained their orientation. The oriented, confluent cellular sheets could then be released from their substrate by lowering of temperature and be transferred to another substrate. While the aforementioned examples demonstrate the benefits of using micro- and nanotechnologies to modulate cell morphology, the potential of these studies are limited given their 2D nature and inadequate representation of *in vivo* conditions.

3.1.2. Topography—ECM is an information-rich scaffold, containing many biological cues such as cell adhesion sites as well as tethered growth factors.(Hynes 2009) In addition to these biochemical cues, ECM presents, through the shape of its structure (*i.e.* topography), physical and geometrical cues that influence many different types of cell behaviors.(Stevens and George 2005) Micro- and nanofabrication techniques have enabled the generation of micro- and nanoscale topographies, mimicking those of ECM.(Lim and Donahue 2007; Dvir *et al.* 2011) Topography can be fabricated in an ordered, symmetrical fashion with techniques such as photolithography, soft lithography, EBL, and NIL, or in a disordered manner with methods such as polymer demixing, phase separation, and electrospinning.(Norman and Desai 2006; Sill and von Recum 2008) Modulating surface roughness, defined as the average distance from the peaks to the troughs of the surface, is one way of introducing topography onto a substrate's surface, and can be achieved with sandblasting, anodic oxidation, and acid-etching.(Sugita *et al.* 2011) One area where surface roughness has been used to promote favorable cell-biomaterial interactions has been in titanium implants for orthopedic applications. For instance, in one study, roughened titanium substrates, compared with smooth titanium surfaces, promoted greater osteoblastic differentiation, alkaline phosphatase activity, and calcium deposition in preosteoblastic cells. (Zhuang *et al.* 2012) Whereas roughened surfaces embody a disordered morphology, nanoscale, geometrically-defined structures, such as grooves, pits, and pillars, can be created (Fig. 2A). In a study by McMurray *et al*., 120nm-diameter polycaprolactone pillars of variable offset spacing, but with a constant average center-to-center spacing, were fabricated

by EBL and used to maintain multipotency of mesenchymal stem cells (MSCs). As the level of offset was reduced, MSCs grown on these nanotopographies were less prone to osteogenic differentiation and retained their MSC markers.(McMurray *et al.* 2011) While the mechanism behind the effect of topography on cell function is not clearly understood, it is believed that it modulates cell attachment through contact-guidance, and produce anisotropic stresses in the cell's cytoskeleton.(Bettinger *et al.* 2009) Control over the nanotopography of scaffolds has been shown to influence cell shape,(Kim *et al.* 2010) adhesion, migration, proliferation,(Ranzinger *et al.* 2009) and differentiation,(Yang *et al.* 2011) and hence provides an additional degree of control in the design of biomaterials used to engineer functioning tissues.

3.1.3. 3-Dimensional cell cultures—In native tissues, cells are exposed to a multitude of biological signals that surround them in a 3D fashion.(Cukierman *et al.* 2001; Doyle *et al.* 2009) Attempts to more precisely mimic the *in vivo* environment have been the driving force behind creating 3D engineered tissues. Our group has demonstrated the feasibility of using gelatin methacrylate (GelMA)(Nichol *et al.* 2010) as a cell-responsive hydrogel for directing 3D cellular behavior (Fig. 2B).(Aubin *et al.* 2010) Nuclear alignment and elongation was demonstrated for cells encapsulated in microfabricated 3D GelMA hydrogel channels. The results demonstrated that cells, which natively elongate and align *in vivo*, will self-organize *in vitro* when confined in these 3D microarchitecture. The versatility of this technique was validated by using a number of different cell types including fibroblasts, myoblasts, cardiac stem cells, and endothelial cells. While in the previous example, a substrate of constant stiffness was used for different cell types, there is evidence that cell function is enhanced when a material with elasticity similar to the cell's *in vivo* substrate is used as a scaffold. (Engler *et al.* 2008) Even though increasing the crosslinking density or the concentration of polymers are often done to increase the stiffness of hydrogels, these methods often compromise other bulk mechanical properties of the material such as porosity, or cell growth and migration. One way of circumventing this problem is to reinforce the hydrogel with carbon nanotubes (CNT). Shin *et al*. showed that CNT-GelMA hybrid hydrogels maintained their porosity and cell growth capacity, while increasing the elastic modulus. (Shin *et al.* 2011) The composite hydrogel was amenable to photopatterning, and showed favorable fibroblast and human MSC proliferation.

While there continues to be intense research invested in the development of new biomaterials, the existing, developed polymers are being used in a variety of applications. Cell-based actuators is one such application; these actuators contain living biological components that help power synthetic components by the conversion of chemical to mechanical energy.(Chan *et al.* 2012) For instance, a cardiomyocyte-driven actuator was constructed by cardiac cells seeded on a poly(ethylene glycol) (PEG) diacrylate and acrylic-PEG-collagen composite hydrogel. With the aid of stereolithography, a micron-scale cantilever, embedded with cardiomyocytes, was fabricated and powered by the cells. With the rapid pace of progress in using materials as 3D cellular scaffolds, future challenges that needs to be addressed include appropriate crosslinking conditions as to not harm encapsulated cells, adequate gas and nutrient exchange, and control over mechanical properties approximately those of cell's nature environment.(Lutolf *et al.* 2009)

3.2 Controlling cell-cell interactions

Cells are in contact, or in close proximity, with many neighboring cells of the same or different type in a highly organized manner *in vivo*, and the cross-talk between these adjacent cells governs many important biological processes.(Engler *et al.* 2009; Huh *et al.* 2010) Therefore, controlling cell-cell interactions can improve the proper functioning of tissue-engineered constructs by mimicking the architecture and geometry of native tissues. Microscale technologies that have been used to investigate and characterize cell-cell interactions include micromolding, μCP,(Nelson and Chen 2003) stencils,(Wright *et al.* 2007) interdigitating micromachined plates,(Hui and Bhatia 2007) stereolithography, (Zorlutuna *et al.* 2011) robotic deposition, and dielectrophoresis.(Albrecht *et al.* 2006)

Patterning of different cell types at addressable locations on a substrate has been used to generate patterned co-culture systems to investigate cell-cell interactions. One method of fabricating such systems is to use stimuli-responsive polymers. These polymers are a class of materials that respond to external stimuli via conformational or chemical changes.(Stuart *et al.* 2010) These stimuli may include temperature, chemical, mechanical, radiation, electrical, or magnetic field changes. PNIPAAm is a temperature-responsive hydrogel with a lower critical solution temperature of 32 °C, above which it shrinks and below which it swells. Using PNIPAAm as a bas-relief master, Tekin *et al*. were able to generate patterned hydrogel microstructures containing different cell types (Fig. 3). Briefly, the PNIPAAm master was filled with agarose gel at room temperature and crosslinked at 4 °C. The master mold was then incubated at 37 °C to shrink the PNIPAAm molds, creating space between the molds and the agarose gel. A second gel precursor was used to fill the newly created space, and upon further incubation at 37 °C, crossliking of the second precursor occurred. Patterened co-cultures of 3T3/human umbilical vein endothelial cells (HUVECs) and HepG2/HUVECs were created using the abovementioned technique. Microfabricated stencils have also been used to pattern cells in a co-culture system. For example, micropatterns of hepatocytes, embryonic stem cells (ESC), and fibroblasts were generated by using a parylene-C stencils.(Wright *et al.* 2008)

A disadvantage of the aforementioned works on cell-cell communication is the static nature of the culture platforms. However, it is well known that dynamic cell-cell communication are important for understanding a number of biological phenomena, such as wound healing and morphogenesis.(Kaji *et al.* 2011) To recreate a dynamic cellular environment, a silicon platform consisting of two interdigitating pieces was fabricated by micromachining, enabling the adjustment of the distance between the interdigitating plates—containing different cell types—and facilitating dynamic manipulation of the cell-cell interactions.(Hui and Bhatia 2007) Using this device, the dynamics of intercellular communication between hepatocytes and stromal cells was assessed, revealing that short distances between cells (<400μm) are likely to be required for the maintenance of hepatocytes. As mentioned above, a variety of microscale technologies have been introduced to regulate the degree of cell-cell contacts, allowing greater control over generation of spatially organized tissue constructs.

4. High-throughput screening microarrays

Despite significant efforts made by the pharmaceutical industry towards drug discovery, a handful of drugs get approved annually.(Chung *et al.* 2007) Each year, only a few of the thousands of developed or discovered compounds proceed to human clinical trials, which then takes years to complete. Therefore, HTS systems using microscale technologies have been developed to miniaturize the drug discovery process, enabling a dramatic increase in the number of screenable drug candidates while reducing reagent consumption and cost. (Fernandes *et al.* 2009) The HTS traditionally used in the pharmaceutical industry has been expanded to other applications such as testing of cellular responses to various biomolecules. Moreover, as mentioned previously, cells grown in 3D culture more closely resemble their *in vivo* counterparts than traditional 2D systems. Such an implication—demonstrated in gene expression, cell adhesion and migration,(Cukierman *et al.* 2001) epithelial morphogenesis, (Grant *et al.* 2006) tumor biology,(Mueller-Klieser 2000) and developmental biology,(Hove *et al.* 2003)—could mean that more effective material and drug screening needs to take place in 3D platforms. In this regard as well, micro- and nanoscale technologies have provided powerful tools to generate miniaturized HTS systems through techniques such as soft lithography, robotic spotting,(Kwon *et al.* 2011) and inkjet printing(Sele *et al.* 2005; Park *et al.* 2007). These cell-based assays can be used to perform thousands of tests in parallel and are valuable tools to analyze cell-material and cell-cell interactions in a rapid and reproducible manner, both in 2D and 3D.

2D monolayers of a broad range of molecules can be printed on a glass surface using robotic spotting technology(Mei *et al.* 2010). In the case of polymeric materials, the polymers can either be synthesized prior to their deposition or the polymerization may be initiated on the substrate. Subsequently, cells can be seeded across the array and their behavior analyzed using various detection methods. For example, Mei *et al*. fabricated a combinatorial synthetic material microarray for testing of the self-renewal capability of human pluripotent stem cells.(Mei *et al.* 2010) Their array contained 496 different combinations of 22 acrylate monomers that were robotically deposited and polymerized via UV light. The material properties of each substrate, such as elastic modulus, topography, surface chemistry, and wettability were quantified in a high-throughput manner. Substrates with high acrylate content favored maintenance of pluripotency. Other studies have generated combinatorial libraries of synthetic materials,(Anderson *et al.* 2005) ECM proteins,(Flaim *et al.* 2005) and ECM/growth factors.(Flaim *et al.* 2008) One of the disadvantages of these systems are susceptibility to region-to-region contamination, caused by the lateral diffusion of molecules between test spots.(Fernandes *et al.* 2009) To overcome this problem, Wu *et al*. developed a sandwich HTS platform in which cells were seeded in a microwell array and separately, chemical compounds were printed on microposts. Finally, the posts and wells were aligned leading to the formation of isolated reaction chambers where the effect of a test compound on cells could be studied without risk of cross-contamination.(Wu *et al.* 2010)

To investigate biomimetic 3D microenvironments, a number of HTS technologies have been developed for creating 3D cell-laden microgel arrays.(Fernandes *et al.* 2010) In this approach, arrays of murine ESC-laden alginate hydrogels were created to study the interactions between cells and soluble factors in a 3D environment. Such an array

demonstrated an efficient method of studying the expansion or neural commitment of ESCs, and the effects of fibroblast growth factor-4 (FGF-4) on pluripotency. Microtechnological approaches can also be used to fabricate polymeric microwell arrays with defined dimensions for controlling supracellular interactions and cell aggregation.(Khademhosseini *et al.* 2006; Moeller *et al.* 2008) For instance, soft lithography and laser micromachining have been used to generate an array of PEG(Moeller *et al.* 2008), PNIPAAm,(Tekin *et al.* 2010) and polyester microwells,(Selimovic *et al.* 2011). These microwell arrays exhibit low shear stress inside the wells, which allows for cell docking and positioning. This method of cell seeding is a useful research tool for generating uniform ESC aggregates, called embryoid bodies (EBs), by controlling the size of the microwells (Fig. 4A).(Hwang *et al.* 2009) In one study, modulating the EB size via control of microwell size (150, 300, and 450μm) led to size-dependent endothelial and cardiac cell differentiation in the EBs. In smaller EBs endothelial cell differentiation was enhancd, while cardiogenesis was favored in larger EBs. Furthermore, non-canonical Wnt molecules that were differentially expressed as a function of EB size were identified. While the abovementioned microwells provide a high-throughput platform, they do not allow for rapid screening of cues that affect cells. To overcome this limitation, Gobaa *et al*. designed a microwell array with each well having its own unique biochemical properties.(Gobaa *et al.* 2011) A microfabricated silicon stamp, onto which different proteins at various concentrations had been deposited with a DNA spotter, was pressed against an incompletely cross-linked PEG hydrogel to make microwells with unique biochemical cues (Fig. 4B). By changing the concentration of the PEG prepolymer, varying degrees of substrate stiffness in the range of 1-50 kPa was obtained. This microwell array platform showed that adipogenic differentiation is favored in microwells containing a greater number of MSCs; further, osteogenesis occurred to a greater extend in microwells with higher elastic moduli.

5. Micro- and nanotechnologies in drug delivery

From the structural simplicity of a virus to the complexity conferred by a bacteria or a eukaryotic cell, the size and shape of these species partly dictate the nature of their interactions with other biological entities.(Young 2010) For example, the discoid shape of inactivated platelets allows them to adhere or roll on the vascular endothelium, and the biconcave disk-shape and elasticity of erythrocytes enables them to squeeze through capillaries, avoid filtration in the spleen, and maximize surface area for gas exchange. Thus, in biology, size and shape are essential determinants of functionality within the body. In the field of drug delivery, the size and shape of drug carriers have emerged as important design criteria in the pursuit of the next generation of therapeutic delivery systems. Significant research in the area of drug delivery is focused on discovering new chemical and molecular recognition patterns for improved control over pharmacokinetic and pharmacodynamic properties of drugs such as half-life, solubility, release rates, and toxicity.(Mitragotri 2009; Mitragotri and Lahann 2009) A major focus in this area has been on size, material chemistry, and particle surface characteristics of drug carriers. Gaining micro- and nanoscale control over particle size has helped researchers study the effects of size on various *in vivo* functions such as immunogenicity,(Champion *et al.* 2008) circulation times,(Decuzzi *et al.* 2009) uptake, intracellular trafficking,(Rejman *et al.* 2004; Gao *et al.* 2005; Sant *et al.* 2008)

extravasation,(Stolnik *et al.* 1995) targeting, degradation,(Glangchai *et al.* 2008) and blood flow (Fig. 5).(Goldsmith and Turitto 1986; Lamprecht *et al.* 2001; Patil *et al.* 2001) For instance, tumors are known to accumulate nanometer-scale particles such as liposomes and nanoparticles (NPs), due to their leaky vasculature and undeveloped lymphatic drainage, a phenomenon known as the enhanced permeability and retention (EPR) effect.(Matsumura and Maeda 1986; Yuan *et al.* 1995; Hobbs *et al.* 1998) Hence, drug carriers for cancer therapeutics have been designed to be in the range of 10-100nm which demonstrate the EPR effect.(Moghimi *et al.* 2005)

Apart from size, particle geometry has been shown to be an important parameter in the biodistribution, phagocytosis, and intracellular trafficking of NPs.(Gratton *et al.* 2008) In particular, developing methods to simultaneously control shape and size have been challenging. Traditional particle synthesis methods vary from emulsion polymerization(Clark *et al.* 1999), self-assembly,(Moghimi *et al.* 2005) and jet breaking, (Berkland *et al.* 2001) while more recently developed methods include soft lithography, (Rolland *et al.* 2005) microfluidics,(Dendukuri *et al.* 2006) self-assembly,(Manoharan *et al.* 2003) and electrospinning.(Bhaskar *et al.* 2010) Despite decades of experience with these techniques, emulsion and nanoprecipitation methods for particle synthesis can produce only spherical particles with little control over their shape and size. Direct extension of microfluidic and lithographic techniques to drug delivery has enabled researchers to precisely control the size, shape, particle rigidity, biological cargo, and surface properties of these nanocarriers. Using these methods, distributions obtained are highly homogenous and allow more complex study of shape-specific interactions. In this section, we will highlight the applications of micro- and nanofabrication approaches to the control of the size and shape of polymeric drug delivery systems along with brief descriptions of the fabrication processes.

Researchers have found that the shape of particles influence their biodistribution, as well as their pharmacokinetics and pharmacodynamics.(Champion *et al.* 2007; Mitragotri 2009) Mathematical models have described receptor-mediated endocytosis,(Decuzzi and Ferrari 2008) adhesive behaviour,(Decuzzi and Ferrari 2006) and margination dynamics of non-spherical particles,(Gentile *et al.* 2008; Decuzzi *et al.* 2009) allowing the study of the transport, internalization and vascular dynamics of these particles. Theoretical studies using these models have predicted that oblate particles will result in more efficient adherence to the vascular endothelium compared to spherical particles of comparable volume. Particle geometry has been shown to be one of the crucial parameters in cell internalization pathways as well. It has been experimentally shown that oblate particles with their high aspect ratio have the ability to induce internalization when they contact macrophages along their length.(Champion and Mitragotri 2006) Despite evidence demonstrating the need to control geometry for drug delivery applications, progress in control of shape has been limited by product yield and non-homogeneity.

A production method combining photolithography and soft lithography, called Particle Replication In Non-wetting Templates (PRINT), was developed by DeSimone and colleagues representing a major step towards improved control of particle geometry (Fig. 6). (Gratton *et al.* 2008) This method is used to obtain monodispersed particles of controlled

shape and size by means of creating patterns on a silicon master template, which is subsequently used in creating cavities on a fluorinated mold. The particle pre-polymer is then used to fill these cavities by means of capillary filling favoured by the fluorinated polymer's higher surface energy. These molds have been used with different substrate materials to make particles of specific geometries.(Rolland *et al.* 2005) The PRINT technology is capable of controlling particle size (20nm to >100μm), shape (spheres, discs, cylinders, toroidal), composition (solid/porous, organic/inorganic), mechanical properties (deformable, stiff,), cargo (hydrophilic or hydrophobic compounds, oligonucleotides, siRNA, imaging agents), surface properties (cationic/anion charges, targeting peptides, aptamers, antibodies, stealth PEG chains), and in a simultaneous and independent manner. (Gratton *et al.* 2008; Gratton *et al.* 2008) The difference between PRINT and traditional soft lithography is that instead of using silicone-based polymers, PRINT uses low surface energy, non-wetting perfluoropolyethers, which overcomes scum layer formation.(Rolland *et al.* 2005) By using this robust method, studies were carried out on the biodistribution of particles (Gratton *et al.* 2007; Gratton *et al.* 2008); also, it was observed that particles with higher aspect ratio were internalized more readily.(Gratton *et al.* 2008) It was also possible to modulate the surface charge of shape-controlled particles to study the effect on cellular internalization mechanisms.(Gratton *et al.* 2008) It was observed that positively charged particles were internalized more efficiently than negatively charged ones, which could be used to improve the targeting function of such particles. Furthermore, the mechanism of the cellular uptake of positively-charge 1μm cylindrical particles was predominantly clathrin-mediated endocytosis and macropinocytosis. More recently, this technology has been applied in colloidal chemistry giving anisotropic chemical properties to the particle.(Bhaskar *et al.* 2010) While microfabrication techniques like PRINT can be used to control various parameters such as shape and size, greater targeting specificity and understanding of the biological mechanism behind shape-specific uptake of drug carriers are needed.

6. Conclusions and future perspectives

In the past, developments in the biomedical and pharmaceutical fields was hindered by limitations of traditional methodologies such as inaccurate, macroscopic control of cellular behaviors and labor intensive, expensive testing of cellular responses to pharmaceutical agents in low-throughput systems. Currently, due to the rapid growth of micro- and nanoscale technologies combined with advances of biomaterials, new solutions have been proposed. As discussed in this review, micro- and nanoscale technologies demonstrate the feasibility to regulate the spatial and temporal aspects of the cell microenvironment in biomimetic scaffolds by precisely controlling cell-material and cell-cell interactions; these advances will pave the road for fabrication of functional cellular tissue constructs for regenerative medicine purposes. In addition, the development of HTS systems using microfabrication techniques demonstrates the ability to dramatically enhance screening efficiencies in drug target validation and preclinical toxicology processes at considerably lower cost. Furthermore, the control of size and shape of drug carriers with technologies such as PRINT has allowed for a modulating of pharmacological properties. In conclusion, current and future biotechnologies will be further advanced by the continued development of

micro- and nanoscale technologies presenting a bright future for tissue engineering and drug delivery.

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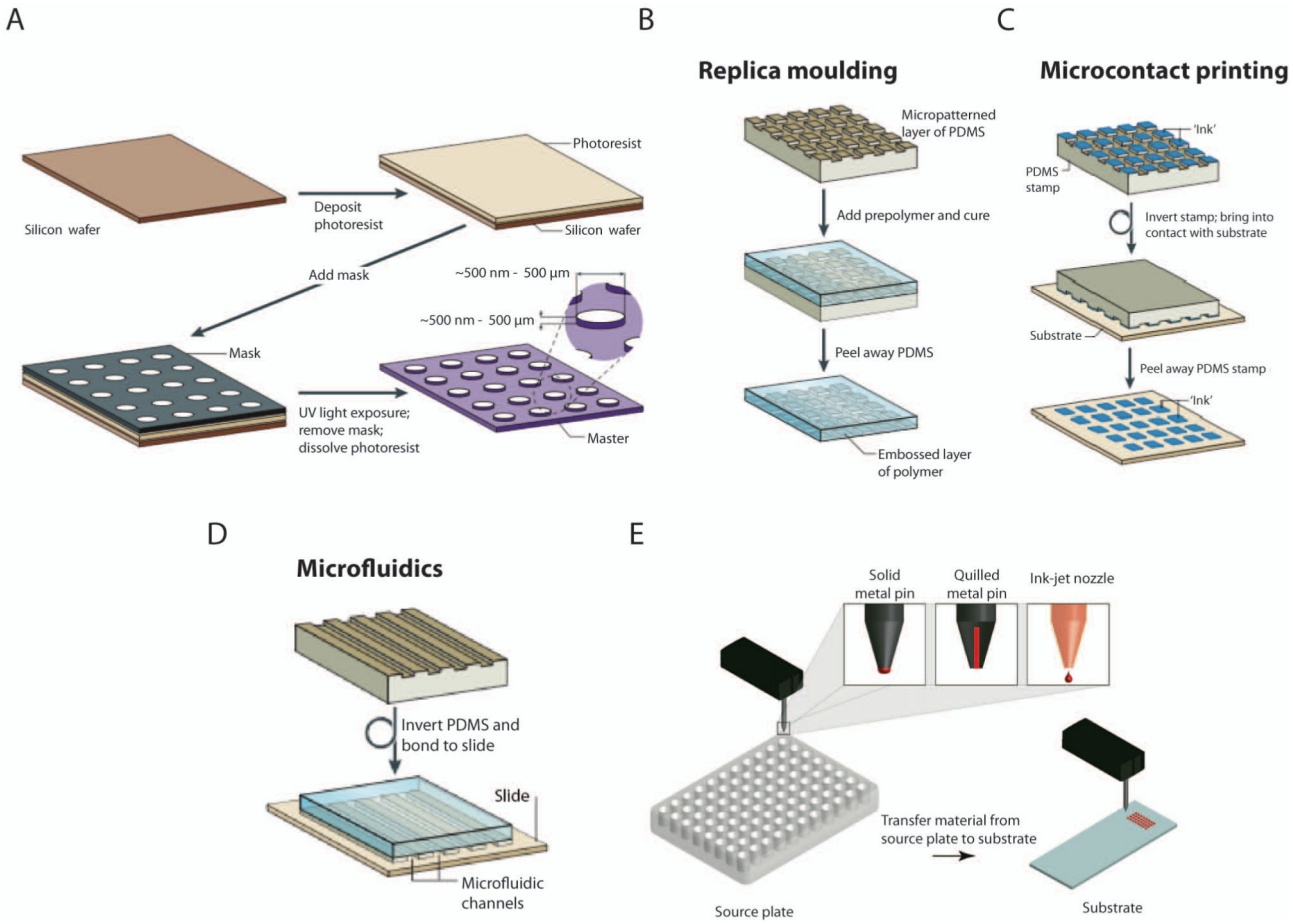

Figure 1.

Schematics of common micro- and nanotechnologies. (A) Photolithography. (B) Replica molding. (C) Microcontact printing. (D) Microfluidics. (E) Inkjet Printing and Robotic Deposition. (A-D): (Weibel *et al.* 2007) Adapted with permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology], copyright (2007). (E): (Hook *et al.* 2010) Adapted by permission from Elsevier: [Biomaterials], copyright (2010).

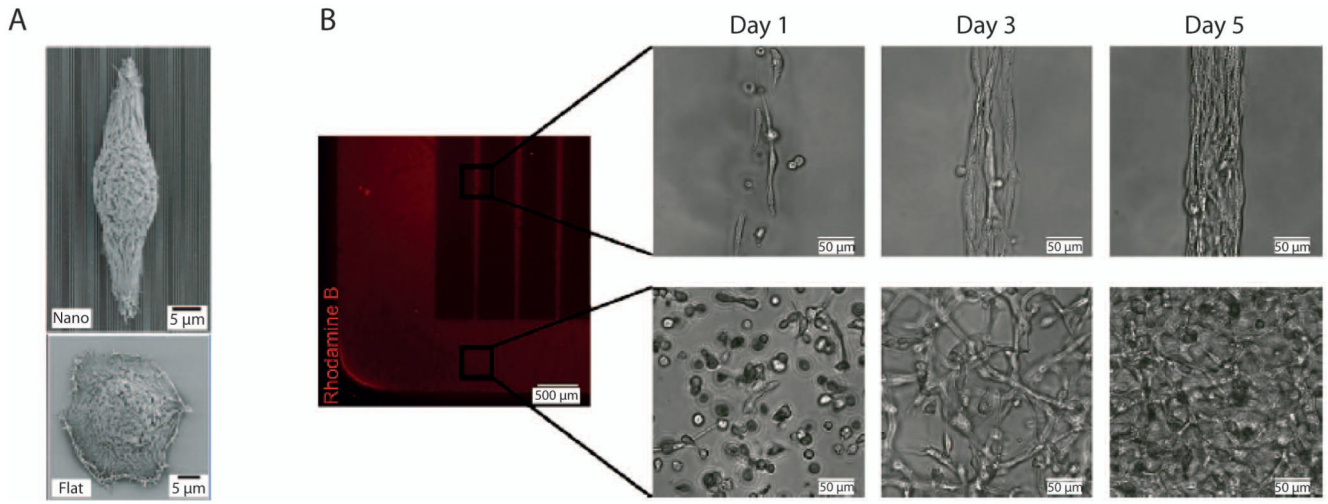

Figure 2.

Cell-Material Interactions. (A) Scanning electron microscopy images of a corneal epithelial cell on a nanograting topography (top) and flat surface (bottom).(Teixeira *et al.* 2003) Adapted with permission from Company of Biologists Ltd: [Journal of Cell Science], copyright (2003). (B) Fibroblast morphology and organization in patterned, 50μm-width rectangular (top) and unpatterned (bottom) gelatin methacrylate constructs.(Aubin *et al.* 2010) Adapted with permission from Elsevier: [Biomaterials], copyright (2010).

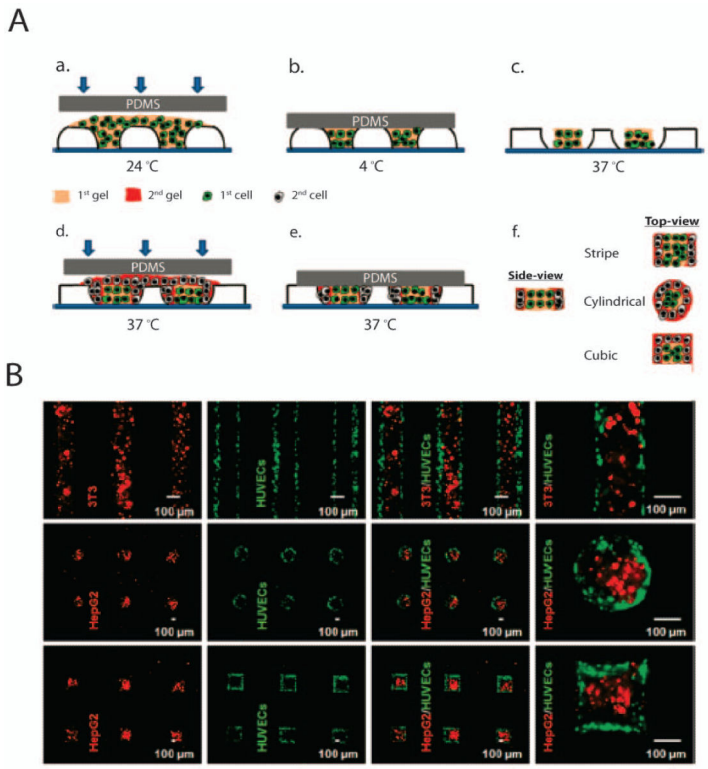

Figure 3.

Generation of organized heterotopic cell co-cultures. The sequential patterning of hydrogels is illustrated in the schematic (A). Patterning of differerent cell types encapsulated in microgels (B). Adapted with permission from (Tekin *et al.* 2010). Copyright (2011) American Chemical Society.

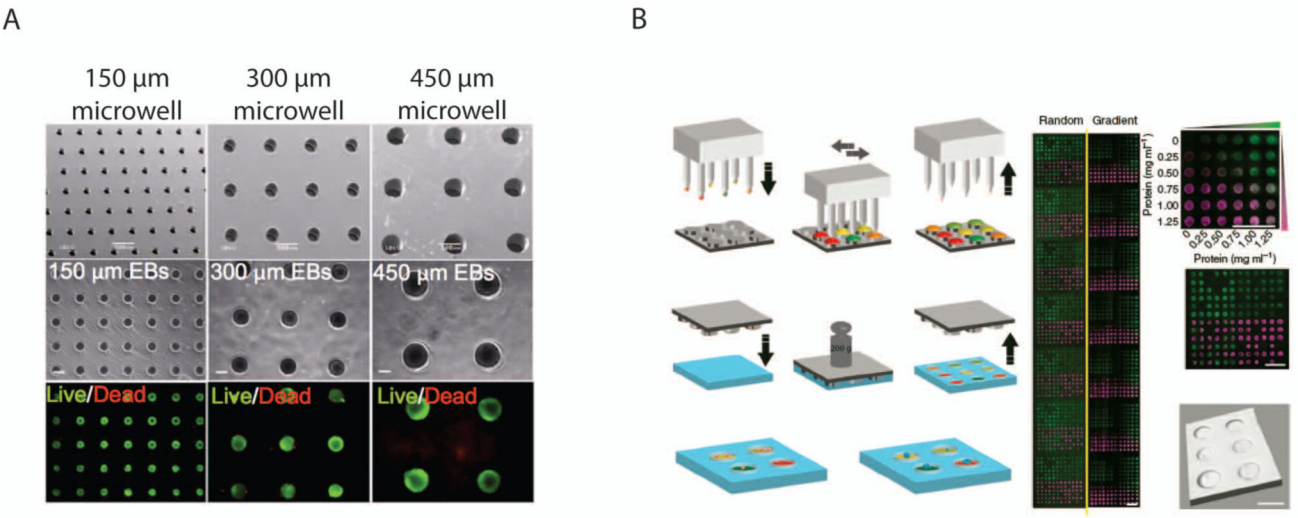

Figure 4.

High-throughput systems. (A) A poly(ethylene glycol) microwell array for generating uniformly sized embryoid bodies.(Hwang *et al.* 2009) Copyright (2009) National Academy of Sciences, USA. (B) A method for creating a high-throughput microarray with different biochemical signals. Different proteins (represented by the different colors) are deposited onto a microfabricated stamp via a DNA spotter (left). The stamp is then pressed against a partially cross-linked hydrogel to transfer the proteins and generate microwells. A microarray of a combinatorial gradients of two fluorescently labeled proteins is shown (right).(Gobaa *et al.* 2011) Adapted with permission from Macmillan Publishers Ltd: [Nature Methods], copyright (2011).

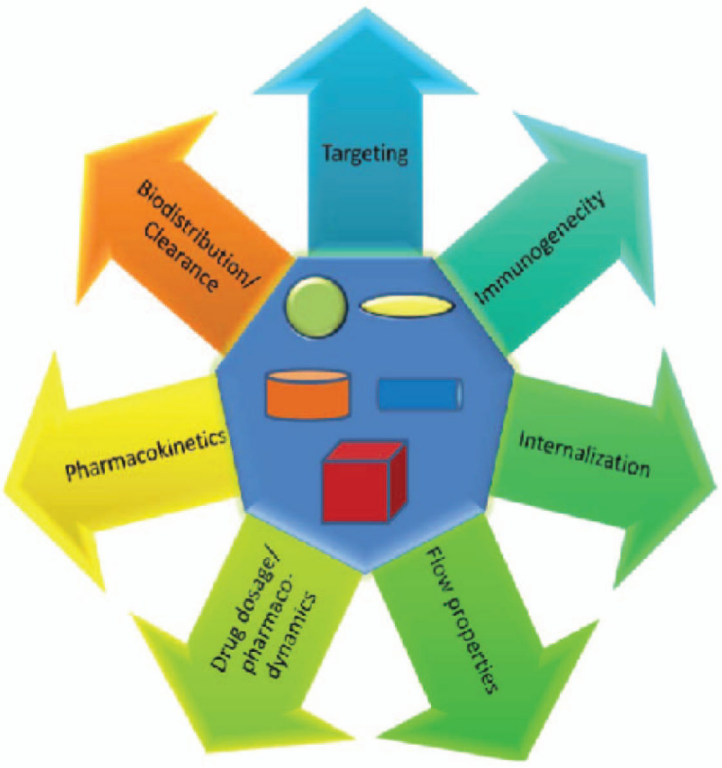

Figure 5.
Schematic illustration of some of the parameters of drug delivery that may be affected by shape and size of particulate drug delivery agents.

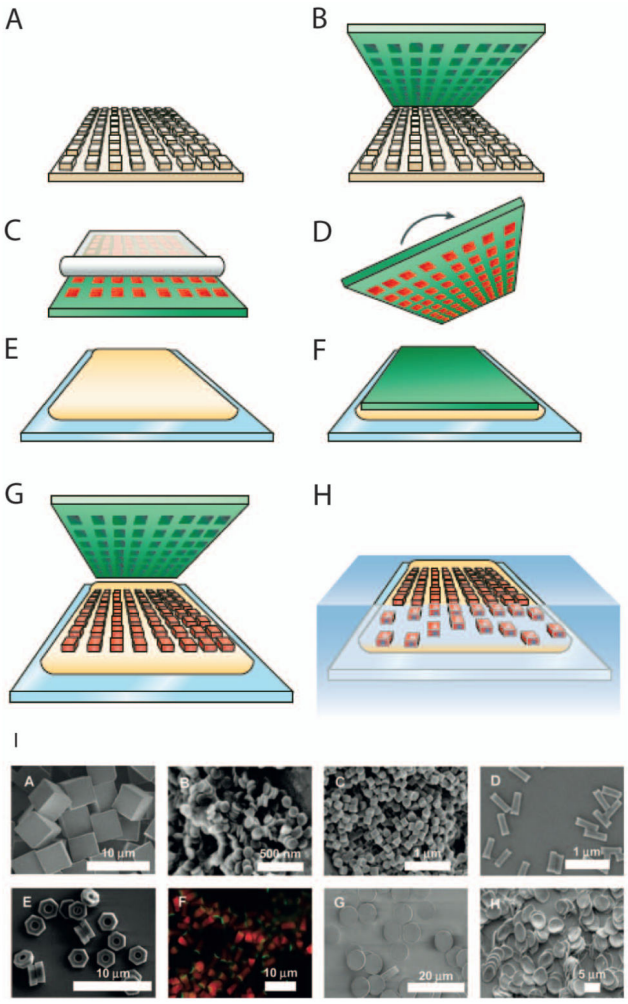

Figure 6.

Diagram of the Particle Replication In Non-wetting Templates (PRINT) process: A silicon master (A) is used as a master template to make perfluoropolyether molds (green) (B); capillary filling of the molds with liquid precursors (red), followed by their solidification (C) generates particles that can be harvested with an adhesive film. Alternatively, the solidified particles can be obtained by turning over the mold (D) onto a liquid harvesting layer (yellow) (E,F); the harvesting layer is then cured, trapping the particles, and the mold is peeled away (G). Finally, the harvesting layer is dissolved and individual particles are generated (H).(Petros and DeSimone 2010) Adapted with permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery], copyright (2010). (I) PRINT Particles varying in size and shape (A-H), surface chemistry (F), and deformability (G,H). Adapted with permission from (Gratton *et al.* 2008). Copyright (2008) American Chemical Society.