

Bladder tissue engineering through nanotechnology

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Abstract The field of tissue engineering has developed in phases: initially researchers searched for “inert” biomaterials to act solely as replacement structures in the body. Then, they explored biodegradable scaffolds—both naturally derived and synthetic—for the temporary support of growing tissues. Now, a third phase of tissue engineering has developed, through the subcategory of “regenerative medicine.” This renewed focus toward control over tissue morphology and cell phenotype requires proportional advances in scaffold design. Discoveries in nanotechnology have driven both our understanding of cell–substrate interactions, and our ability to influence them. By operating at the size regime of proteins themselves, nanotechnology gives us the opportunity to directly speak the language of cells, through reliable, repeatable creation of nanoscale features. Understanding the synthesis of nanoscale materials, via “top-down” and “bottom-up” strategies, allows researchers to assess the capabilities and limits inherent in both techniques. Urology research as a whole, and bladder regeneration in particular, are well-positioned to benefit from such advances, since our present technology has yet to reach the end goal of functional bladder restoration. In this article, we discuss the current applications of nanoscale

materials to bladder tissue engineering, and encourage researchers to explore these interdisciplinary technologies now, or risk playing catch-up in the future.

Keywords Bladder · Tissue engineering · Regenerative medicine · Nanotechnology · Self-assembly · Supramolecular · Scaffold · Biomaterial · Top-down · Bottom-up · Stem cell

Introduction: bladder augmentation and the need for tissue engineering

The neurogenic bladder remains as a relevant and unresolved target for regenerative medicine. When patients with spina bifida, spinal cord injury, or other bladder insult develop progressive elevated storage pressure, hydronephrosis, or upper tract injury, surgical reconstruction of the bladder may be necessary to reduce storage pressure and prevent further renal injury. Gurocak et al. [1] have provided the most recent review of the current state of bladder augmentation technology. This summary of urologists’ many years of experience with augmentation only provides further evidence of the long-term complications inherent in enterocystoplasty, such as perforations, stone/mucus formation, and cancer risk. Yet, these authors note that, “intestinal cystoplasty still seems to be the gold standard due to the lack of promising alternative options.” These researchers and others continue to look to tissue engineering techniques for answers to the long-standing issues with GI-based augmentations.

The classic tissue engineering model (e.g. [2, 3]) involves the replacement of diseased tissue with a highly porous, three-dimensional scaffold, which mimics natural extracellular matrix (ECM) by providing the necessary

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structure and signals to encourage cell ingrowth and tissue regeneration. Scaffolding materials have been described in detail elsewhere [4], and are separated into two major categories: biologically derived and synthetic. Biologically derived scaffolds are chemically and mechanically decellularized tissues (such as small intestinal submucosa (SIS), bladder acellular matrix (BAM), and others), which offer the benefits of inherent bioactivity and mechanical similarity to native ECM. However, a major disadvantage of these systems is the routine variability in protein composition among batches. There may also be ethical issues regarding their availability, although most naturally derived scaffolds are porcine xenografts. Conversely, synthetic biomaterials, such as the biodegradable polymers poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), and poly(ϵ -caprolactone) (PCL), offer excellent control over batch-to-batch composition and physical properties. Yet, these polymers contain none of the molecular signals that are so relevant in directing cell activity and fate. Importantly, both biologically derived and synthetic scaffolds must meet the necessary mechanical requirements to even allow for their use in surgery. Yet, while macroscale properties, such as substrate stiffness or bulk surface chemistry, can indirectly affect cell behavior, it has become clear that the next generation of biomaterials must “speak” to cells in their native language of cytokines and growth factor signaling. This requires greater control over scaffold synthesis in the size scale of these signaling proteins.

Kanematsu et al. [5] have recently reviewed many of the current trends and ongoing issues specific to bladder tissue engineering research. They note the ongoing debate over the ease and utility of acellular matrices, compared to the potential long-term benefit of pre-seeding with cells prior to implantation. Among other observations, they reach a similar conclusion to ours: that a molecular-based understanding of smooth muscle regeneration has the potential for greater yields than purely surgical-based methods. Scaffold design thus requires materials which can meet researchers’ needs on the molecular scale.

As we have recently described [4], the field of tissue engineering, and specifically its subcategory of regenerative medicine, are well-positioned to benefit from advances in nanoscale synthesis and characterization [6]. Such nano-features span the regime of cellular protein structures, ranging from multimeric assemblies down to receptor–ligand interactions between cells and their substrates. Thus, they offer new, unique possibilities for signaling cells and directing phenotype. The differences between conventional materials and their nanodesigned counterparts may not be immediately apparent, but the great potential for nanoscale materials relies fully on the unique properties which are gained when an object’s dimensions shrink closer and closer to the molecular scale. As we continue to learn how

to mimic nature’s ability to assemble molecules in a Lego-like fashion, we find that nanotechnology sits exactly at the intersection of biology, chemistry, engineering, and medicine.

Why consider nanomaterials?

We broadly define “nanotechnology” as the creation of objects or surfaces whose unique functions are a direct result of their nanoscale dimensions and/or organization. These unique properties may be mechanical, electrical, or photochemical, and are not seen in the bulk materials. “Nanoscale” generally refers to objects 1–100 nm in one or more dimensions (although as seen below, some slightly larger features also demonstrate unique properties). At its lower limit, this definition intentionally excludes individual molecules, which actually define the lower end of nanotechnology—i.e. nano-derived features are as much a function of larger bulk materials approaching the molecular scale as they are a selective change in molecules’ properties as they aggregate.

As one example, conventional fluorophores such as rhodamine, pyrene, and fluorescein-isothiocyanate (FITC) are individual molecules, known to be subject to bleaching and oxidation due directly to their highly conjugated chemistry. In contrast, the recently introduced CdSe/ZnS fluorescent quantum dots are a completely new family of fluorophores, with greatly improved emission and reduced bleaching, whose properties (e.g. emission wavelengths) correlate directly with their 10–20 nm size. As another example, West et al. [7] have demonstrated the utility of gold nanoshells for the localized targeting and ablation of cancerous cells both in vitro and in vivo. Nanoscale shells of gold surrounding a silica core can be selectively tuned to convert near-IR photo energy into thermal energy—a nanofurnace which efficiently targets and destroys cells. While some bulk metals have similar properties, only those with nanodimensions offer such tunable, and deliverable, possibilities.

For regenerative medicine, nanodesigned organic materials offer tunable functionality that may not be easily achieved, or even available, by conventional methods. In fact, collagen itself may be considered as one of the first nanomaterials employed in tissue engineering [8]. Its structure builds in complexity upon itself, beginning in its primary form as a unique triple helix, which forms nanoscale fibrils. Unlike conventional material synthesis, the design of many nano-organized materials is often conceived using similar concepts borrowed from small molecule chemistry and biochemistry, such as non-covalent interactions and secondary structure. This frequently involves the use of “triggers,” such as pH change or ionic gradients, which induce quick changes in aggregation state or surface

display. The use of highly directional non-covalent bonds even makes “supramolecular polymers” possible [9]. Structure, design, and synthetic method are all vital elements in the eventual function of nanobiomaterials.

Methods for nanoscale synthesis: bottom-up versus top-down

The quoted range for nanomaterial sizing (1–100 nm) is made clearer by examining the two common methods for their synthesis: the “bottom-up” and the “top-down” approaches. In one type of “bottom-up” synthesis, individual molecules are triggered to self-assemble into larger objects with nanoscale dimension (Fig. 1). The formation of micelles from individual charged lipids is a classic example of this method. In such systems, aggregate shape and size are pre-programmed through the specific features of the component molecules, often through the inclusion of selectively compatible and incompatible components. When we deliberately create molecules with such opposing segments (e.g. hydrophilic and hydrophobic; rigid and flexible; directional hydrogen-bonding or π - π stacking; etc.),

multiple molecules are forced to reduce their entropic/enthalpic balance through aggregation. Substantial advances have been made in this field of “supramolecular self-assembly” over the past 20 years, and researchers have a continual eye on advances in understanding nature’s own methods for assembling proteins, nucleic acids, and other biomolecules. Peptide-based self-assembling systems in particular have increasingly been explored as scaffolding materials for regenerative medicine, due to their inherent complexity in secondary and tertiary structures [9–11].

The “top-down” approach has its foundation in lithographic techniques, in which a bulk material is selectively degraded to produce smaller, often patterned, features. In the case of microlithography, light is shone through a mask to selectively etch either a positive or a negative pattern into a surface, yielding the desired microscopic topography (Fig. 1e). Size reductions to nanolithography are driven in large part by the computer industry, responding to the demand for smaller resistors and stronger computing power. To reach feature sizes <100 nm, researchers rely on shorter wavelengths of light, or even e-beam lithographic techniques. Top-down processing can also be generated through selective chemical etching (Fig. 1f), although this relies much more on

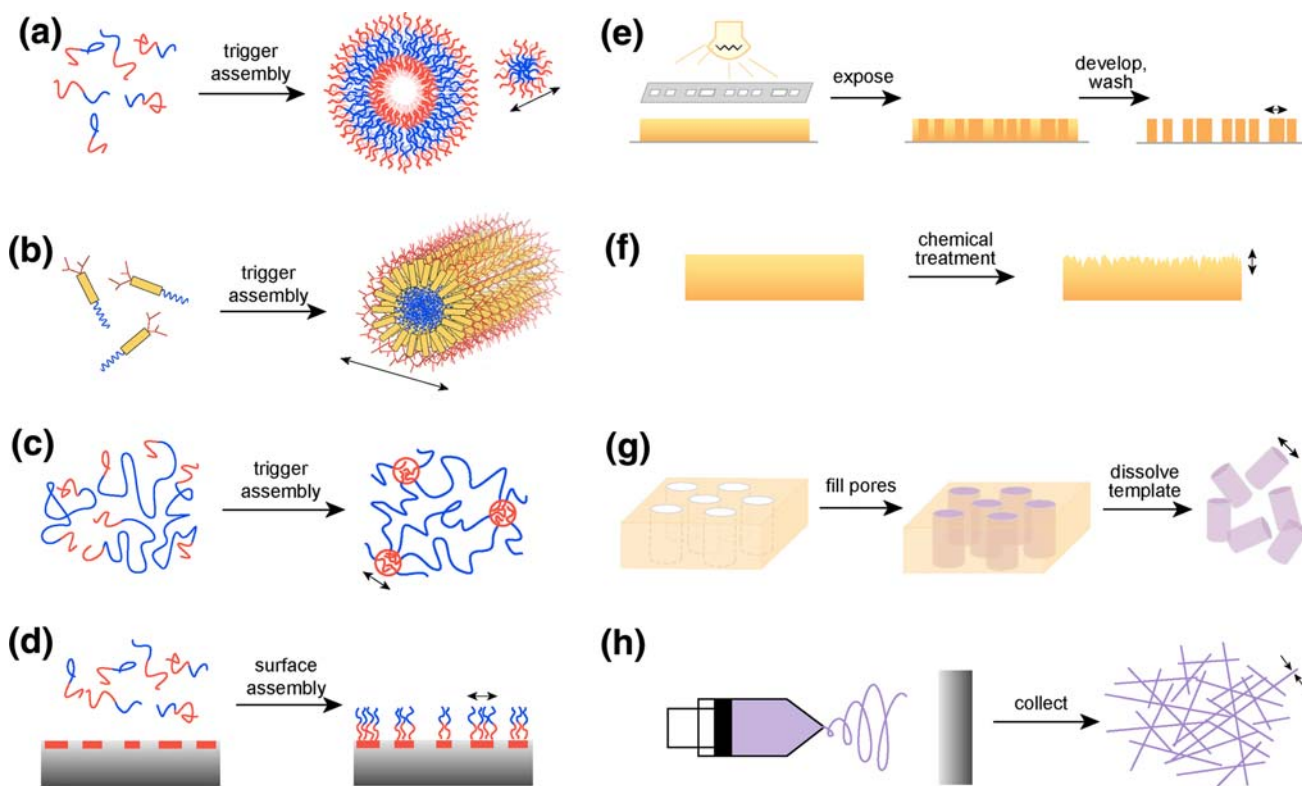


Fig. 1 Methods for synthesis of nanoscale objects and surface features. Nanoscale objects are indicated with a *double-headed arrow*. “Bottom-up” design through **a** self-assembly of individual small molecules into larger supramolecular structures, such as micelles and vesicles; **b** peptide-amphiphile assembly into nanofibers, with β -sheet forming blocks in *yellow*; **c** segregation of polymer segments into

nanoscale domains; **d** surface printing of nanoscale features; “Top-down” design through **e** lithographic patterning of bulk surfaces; **f** selective chemical etching to produce nanoscale features; intermediate methods of bulk confinement through **g** template-assisted patterning; **h** electrospinning of micro- and nano-fiber scaffolds

the initial properties of the bulk material. Recent reports demonstrate the biomimetic application of top-down processing for reproducing the mechanism of gecko adhesion to surfaces, through the formation of submicron polymer pillars [12].

Experts predict a convergence between the top-down and bottom-up methodologies within the next 5–10 years. Some techniques already seem to lie midway between the two categories, by using mechanical means to confine bulk materials into nanoscale dimensions. Template-assisted technology uses existing nano objects as a “mold” to either fill or surround with another material (Fig. 1g). After filling, the template is dissolved, leaving the desired material with nanoscale dimension. The formation of nanorods using anodic aluminum oxide is a classic example of this technique [13]. Electrospinning (discussed in detail below) is a variation on polymer extrusion, forcibly creating polymer fibers with diameters of 100–3,000 nm (Fig. 1h). As the fibers entangle and settle onto a collector, they can form an open, porous scaffolding, reminiscent of non-woven PGA microfiber scaffolds. In all of these cases, the resulting nanoscale shapes often provide a range of unexpected and potentially useful properties.

Applications of nanotechnology to the bladder: diagnostics and delivery

Current discoveries in nanotechnology have been applied to bladder research in several ways, however the majority of these involve applications in basic science and cancer, including increasing use in diagnostics for bladder cancer [14, 15]. Applications in imaging the topography of the bladder epithelium [16] and tracking cell lineage and fate [17, 18] have also been reported. Nanoparticles are also widely used as delivery agents, whose properties are once again specific to their combination of unique composition and nanoscale dimension, such as drug delivery agents [19], cancer-killing agents [20–22], and gene delivery tools [23].

The majority of these reports involve inorganic nanoparticles, used either as sensors or as delivery agents. While these applications do fall under the broadest definition of tissue engineering, most current uses of nanotechnology in urology remain confined to these areas [24]. Specific uses in regenerative medicine require organic, preferably degradable, materials, which can incorporate into tissues and interact with cells to direct their fate. Below, we focus on recent efforts at the interface of nanotechnology and regenerative medicine in the bladder.

Bladder regeneration through nanotechnology

A large segment of work in nanoscale scaffolds for the bladder (and other tissues) has been published through the

collaborations of Haberstroh and Webster. In their reports, polymer films [PLGA, PCL, polyurethane (PU)] were treated with NaOH (or HNO₃ for PU), for varying times and concentrations, to selectively degrade the polymer surface (Figs. 1f, 2a) [25]. This “top-down” degradation process created topographies with nanoscale features (50–100 nm) which enhanced bladder smooth muscle cell adhesion [26]. Such chemical degradation inevitably changes surface chemistry, and the authors used ESCA (also known as XPS, X-ray Photoelectron Spectroscopy) to analyze the elements present on the polyester surfaces and document the expected increase in oxygen concentrations (from the likely formation of surface hydroxyls and carbonyls). They were able to eliminate the contributions of surface chemistry by using silastic molds to reproduce only the nanoscale textures in untreated polymer samples, again leading to improved cell adhesion. Application of this technology to a porous three-dimensional scaffold yielded similar improvements in SMC adhesion, and measurable increases in total elastin and collagen [25]. While the mechanism behind these improvements remains unclear, the potential for nanotextured surfaces to affect the adsorption and display

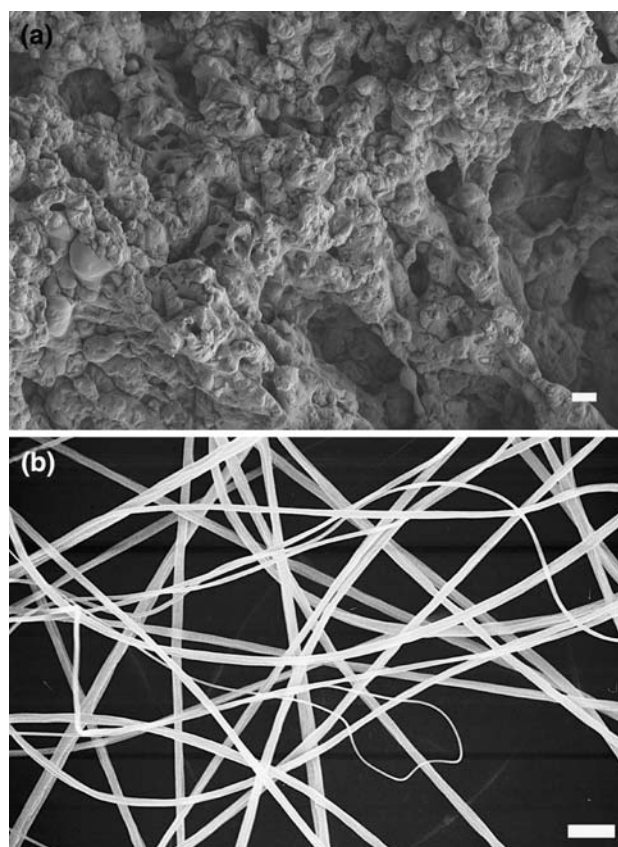


Fig. 2 **a** Porous PLGA scaffold, treated to create surfaces with nanoscale roughness. Scale bar 10 μ m (reproduced with permission from Elsevier [25]); **b** electrospun polystyrene scaffold, with nanoscale fibers. Scale bar 2 μ m (Image courtesy of S. Baker, J. Southgate)

of adhesive proteins (fibronectin, vitronectin) remains a likely hypothesis [27], and a potentially fruitful avenue for further research.

One processing method that lies somewhere between top-down and bottom-up is the extrusion of polymers under an electric field, referred to as “electrospinning.” Highly porous electrospun scaffolds have been widely explored [28], with fiber diameters ranging from hundreds of nanometers to microns in size (Fig. 2b). In a recent application to bladder tissue engineering, Baker et al. [29] reported on bladder SMC attachment and growth on polystyrene electrospun scaffolds. Other examples of electrospun scaffolds for bladder regeneration have also been recently reported using fibrinogen [30] and cellulose acetate [31], although the latter work employed micron-sized fibers. The potential for all of these systems lies in the researcher’s control over fiber alignment and diameter. Unlike conventional microfiber polymer scaffolds, nanofiber scaffolds may offer greater influence over the cytoskeletal arrangement of adherent cells, and thus direct phenotype expression. Additional research with other polymers (e.g. biodegradables) is surely underway for these systems.

In contrast to these top-down methods, we have described [32] a bottom-up approach to forming bio-scaffolds with nanoscale features, focusing in particular on self-assembling peptide-amphiphile (PA) systems, which have been explored in detail by Stupp et al. [10, 33]. PAs are designed for self-assembly through the covalent attachment of hydrophobic (alkyl) and highly charged hydrophilic (peptidic) segments. Within the peptide segment, amino acid residues are chosen which promote β -sheet formation among the individual molecules. The terminal end of each PA can include a biologically relevant epitope for signaling cells, binding other biomolecules, or yielding other functionality. Individual PA molecules in solution can be triggered to self-assemble through screening of the terminal charges via pH or ionic adjustment, inducing a

hydrophobic collapse. The resulting supramolecular structures are nanofibers, 6–8 nm in diameter, and hundreds of nanometers in length (Fig. 1b). At sufficiently high concentrations, the nanofibers interact to form a self-supporting aqueous gel.

In our own contributions to nanoscale bladder tissue engineering [32], we described a family of “branched” PA molecules, which employed G2 lysine dendrons (Fig. 1b). A variety of molecules were designed, with selected lysine branches terminating in the RGDS and PHSRN sequences, known to mediate integrin-based cell adhesion. Building from our previous work with other self-assembling systems [34, 35], we coated conventional PGA microfiber scaffolds with a thin layer of these PA molecules. When these PAs were triggered to self-assemble, the resulting nanofibers displayed a surface of positively charged lysine moieties, interspersed with the above adhesion sequences. The combination of these features resulted in improved SMC adhesion, infiltration throughout the scaffold, and matrix deposition.

In the above example, PA molecules were used as thin coatings, to promote SMC adhesion onto PGA scaffolds. However, PAs may also be used as hydrogels, to encapsulate cells within the open pores of these scaffolds. We are currently developing such PA systems in this model, for the delivery of specific beneficial growth factors to the regenerating bladder tissue [36]. Specific binding sites for these growth factors may be added to the terminus of these PAs. As shown schematically in Fig. 3, solutions of PAs, growth factor, and SMCs can form a solid gel around a PGA scaffold. We then have the option of repeating the process on the opposite face with urothelial cells (UC), which have been shown to contribute necessary signals to SMCs during regeneration. While this is reminiscent of other models using Matrigel as a gelation agent, the use of PAs allows for exact control over the gel composition and displayed surface chemistry.

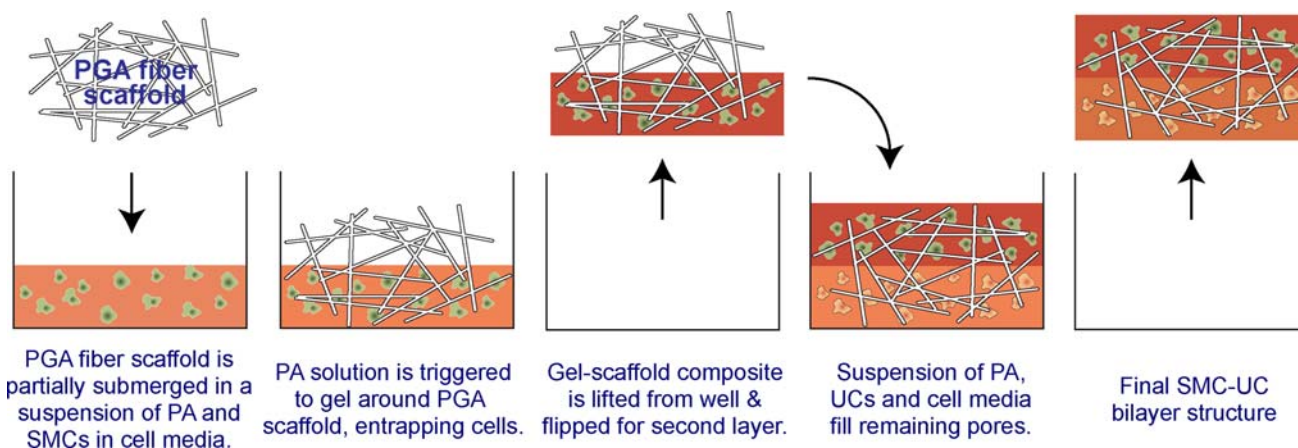


Fig. 3 Schematic of gelation process for making PA-PGA composite scaffolds

As one example of this work, we embedded human bladder SMCs and UCs in a PA-scaffold composite with bFGF, which has been shown to modulate SMC proliferation and matrix production [37, 38]. After 3 weeks of *in vivo* incubation in a subcutaneous nude rat model, we found that the embedded human cells were not only retained, but composed the majority of the scaffold cellular content (Fig. 4a). We also found that a distinct UC layer could be maintained in the SMC-UC bilayer system (Fig. 4b). Finally, systems which used cells + PGA + PA + bFGF demonstrated higher levels of phenotypic α -smooth muscle actin (α -SMA) than control scaffolds using just cells on PGA (Fig. 4c, d). In summary, these PA systems demonstrate great promise for localized placement of cells within a scaffold, and directed growth factor delivery during regeneration.

It should be noted that examples of bottom-up nanoscale features are not isolated to small molecule assembly. Guvendiren and Shull [39] have recently demonstrated a biocompatible triblock polymer system, composed of hydrophobic poly(methyl methacrylate) (PMMA) and hydrophilic poly(methacrylic acid) (PMAA) blocks. Under aqueous conditions, their (PMMA)–(PMAA)–(PMMA) system self-organizes to confine the PMMA blocks into discrete nanoscale domains (Fig. 1c). These domains serve as physical crosslink points for the resulting triblock hydrogel, and as depots for storing hydrophobic fluorophores or other deliverable molecules. We have recently used these triblock copolymers to form porous three-dimensional NaCl-leach scaffolds, with conventional pore sizes of $>200\ \mu\text{m}$ (Fig. 5a, b). Figure 5a demonstrates the cells' distribution on the scaffold surface with a DAPI stain, while

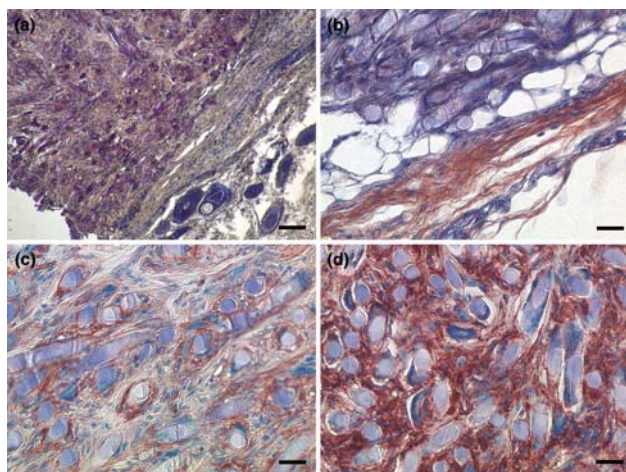


Fig. 4 Immunostaining of PA-PGA composite scaffolds after 3 weeks *in vivo*. **a** MHC staining, demonstrating the retention of human SMCs (red); **b** uroplakin staining, demonstrating the formation of a UC layer (red), and the retention of a SMC-UC bilayer; **c** α -SMA staining of SMCs on a plain PGA scaffold, and **d** cells on a scaffold with PA and bFGF. More intense α -SMA staining is observed on the sample in **d**. Scale bars **a** 100 μm ; **b–d** 25 μm

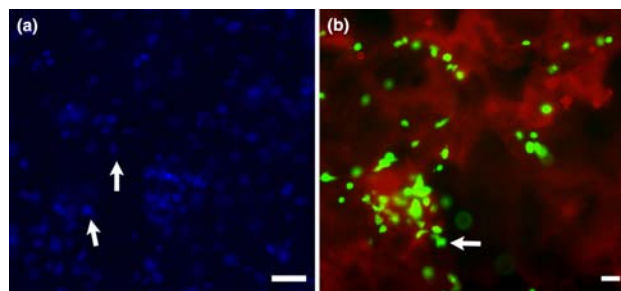


Fig. 5 Fluorescence microscopy of human bladder SMCs seeded onto a porous NaCl-leach (PMMA)–(PMAA)–(PMMA) scaffold. White arrows point out representative cells in each image. **a** Confocal image of DAPI-stained nuclei, showing a high density of cells adherent to a pore wall; **b** epi-fluorescence image of lower-density SMCs stained with calcein green AM to demonstrate cell viability on scaffold (red). Scale bars 50 μm

Fig. 5b shows that the SMCs (green) seeded onto these scaffolds (red) were adherent, viable, and proliferative. Few elastomeric materials are available for regenerative medicine needs, especially with the potential tunability of this triblock system. We continue to explore options which make greater use of their amphiphilicity, as well as the incorporation of biodegradable segments in similar systems.

Potential future applications in bladder regeneration

For reasons that remain unclear, technological advances in materials design and processing for tissue engineering are often slow to reach urologic applications. Electrospun scaffolding is one immediate example: reports of these scaffolds tested in other tissue systems were published several years before the handful of recent papers addressing their application to urinary structures. Similarly, a broad library of literature has developed regarding micro- and nano-lithography, both in the ordered patterning of surface textures and surface chemistries. Yet, despite researchers' efforts in understanding cell behavior on these surfaces (e.g. alignment, motility, focal adhesion formation), virtually none of these concepts have been explored with bladder-derived cells, or applied to bladder regeneration. Nanoscale (or near-nano) systems for drug delivery may also offer abundant opportunities for either intravesicular delivery, or gradual release from an implanted scaffold. With bladder SMCs and UCs readily available, these are open areas for exploration by urology researchers.

These same comments apply to the increasing interest in using progenitor stem cells as cell sources for bladder tissue engineering. Derived from tissues of varying developmental stages ranging from embryo through adulthood, stem cells have the capability to either self-renew, differentiate

upon lineage committed pathways, or transdifferentiate into distinct cell/tissue types. The differentiation potential of stem cells can therefore be harnessed and utilized for bladder tissue engineering purposes. Jack et al. [40] have described the isolation and characterization of a subpopulation of adipose derived mesenchymal stem (aMSCs) cells capable of transdifferentiation into an SMC phenotype. Disease states such as spina bifida that effect overall structural and physiological bladder function may be partially treated by utilizing autologous aMSCs for bladder regeneration in lieu of traditional augmentation procedures. Other functional based studies described by Kanematsu et al. [41] and Chung et al. [42] eloquently demonstrate the role of bone marrow derived stem cells in the restructuring of bladder tissue. Utilizing in vivo bladder injury models, both groups describe similar phenomena in which bone marrow cells home to partially cystectomized or augmented bladders with the aid of biologic matrices. These cells were shown to distinctly contribute to the architectural reconstitution of the bladder based upon immunohistochemical analyses.

Our group and others have explored the potential utility of these cells (from a variety of sources) in bladder engineering, and the coupling of such multipotent cells to nanotechnology is a natural and inevitable result. Recent examples demonstrate the utility of both the bottom-up approach (in which a well-defined self-assembling nanofiber system was used to culture neural stem cells) [43] or a top-down approach (in which differentiation was solely controlled by nanotexture size) [44]. Clearly, the complex regenerative process involving the bladder will rely upon the contribution of stem cells complemented with nanodesigned matrices that can reliably mimic the extracellular milieu of target tissues.

Conclusions

Despite continual progress in advancing our ability to regenerate bladder tissue, the field has yet to produce a complete solution that offers both the ability to store, and expel, urine in a safe and controllable manner. Researchers continue to strive toward scaffolds which signal cells directly, via their own biochemical language, rather than through indirect means. Increasingly, the many faces of nanotechnology appear to be immediately relevant to this need. For future efforts toward incorporating nanotechnology into bladder tissue engineering, urologists should make steps toward teaming with chemists and engineers whose primary interests are in nanomaterial design. Given the complexity of the task, such interdisciplinary teams are the key to building a better bladder—either from the top-down, or the bottom-up.

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