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Small molecule delivery through nanofibrous scaffolds for musculoskeletal regenerative engineering

Erica J. Carbone[1,2,3], **Tao Jiang**[1,2,3], **Clarke Nelson**[1,2,5], **Nicole Henry**[1], and **Kevin W.-H. Lo**[1,2,3,4,*]

[1]Institute for Regenerative Engineering, University of Connecticut Health Center, School of Medicine, Farmington, CT 06030

[2]The Raymond and Beverly Sackler Center for Biomedical, Biological, Physical and Engineering Sciences, University of Connecticut Health Center, School of Medicine, Farmington, CT 06030

[3]Department of Medicine, Division of Endocrinology, University of Connecticut Health Center, School of Medicine, Farmington, CT 06030

[4]Department of Biomedical Engineering, University of Connecticut, School of Engineering, Storrs, CT 06268

[5]Department of Orthopaedic Surgery, University of Connecticut Health Center, School of Medicine, Farmington, CT 06030

Abstract

Musculoskeletal regenerative engineering approach using small bioactive molecules in conjunction with advanced materials has emerged as a highly promising strategy for musculoskeletal repair and regeneration. Advanced biomaterials technologies have revealed nanofiber-based scaffolds for musculoskeletal tissue engineering as vehicles for the controlled delivery of small molecule drugs. This review article highlights recent advances in nanofiber-based delivery of small molecules for musculoskeletal regenerative engineering. The article concludes with perspectives on the challenges and future directions.

Keywords

small molecules; tissue engineering; nanofibers; drug delivery; musculoskeletal disorders

*Corresponding author: Kevin W.-H. Lo, Ph.D., University of Connecticut Health Center, School of Medicine, Farmington, CT 06030, Tel.: 1-860-679-2949, Fax: 1-860-679-1553, wlo@uchc.edu.

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Introduction

Musculoskeletal disorders remain a major cause of discomfort, hospitalization, pain, impaired quality of life, and morbidity. Each year, in the United States, musculoskeletal disorders account for more than 70 million office visits as well as 130 million clinical encounters [1]. Musculoskeletal disorders can affect a variety of tissues including muscles, bones, joints, ligaments, tendons, and nerves. The current gold standards for musculoskeletal repair and regeneration are autologous cell/tissue grafts. For instance, autologous bone tissue (usually taken from the iliac crest), bone-patella tendon-bone graft, and autologous chondrocyte implantation are widely considered the gold standards for bone, ligament, and cartilage tissue repair, respectively [2–4]. Although clinically successful, autografts face certain constraints such as limited tissue supply and donor site morbidity [5]. As an alternative therapy, allogenic grafts that are taken from either a donor or a cadaver have been commonly used by orthopaedic surgeons to repair and regenerate musculoskeletal tissues. Allografts, which have fewer limitations on supply compared to autografts, can generally provide good clinical outcomes in musculoskeletal tissue repair. However, allografts pose the risks of disease transmission and host rejection [6, 7]. Musculoskeletal regenerative engineering approach using advanced biomaterials combined with biological factors has been recently proposed as a viable option for musculoskeletal tissue regeneration and repair [8]. Laurencin and colleagues defined the field of regenerative engineering as “the integration of tissue engineering with advanced material science, stem cell science, and areas of developmental biology” [8, 9]. Certainly, advanced materials will play an important role in facilitating the transition of regenerative engineering technologies into clinical settings [9]. For instance, application of advanced nanofiber technologies makes it possible to produce biomimetic scaffolds that can mimic the extracellular matrix (ECM) for tissue regeneration [10–12]. It should be noted that biological factors play an important role in regenerative engineering. Other than polypeptide-based growth factors such as bone morphogenetic proteins (BMPs) [13], biological factors currently being explored as regenerative therapeutics include micro RNAs [14, 15], short peptides [16–18], metal ions [19, 20], and small molecules [8, 21, 22]. It is worth noting that regenerative engineering utilizing small molecules as pro-regenerative biological factors is emerging, and the related literature is expanding [8, 21, 22], which has prompted us to summarize the recent findings in literature. In this review article, we will first provide a brief overview of small molecules and nanofiber-based scaffolds. We will then discuss the recent advances of nanofiber-based delivery of therapeutic small molecules for regenerating various musculoskeletal tissues. In the last section, we will discuss the challenges in the field and the future directions.

Small molecules as regenerative medicine

In biomedical sciences, small molecule refers to a non-peptide biologically active organic compound with a molecular size usually less than 1,000 Da. Unlike peptide or protein-based biological factors, utilization of small molecules in biomedical research has a number of benefits since they can be designed to be selective, potent, water soluble, and cell permeable [23]. For clinical applications, generally speaking, small molecule drugs are unlikely to induce unwanted immune responses, and the costs of synthesis are relatively low compared to therapeutic protein-based medications [21, 22, 24, 25]. It is a well-known fact that

recombinant protein growth factors play an important role in musculoskeletal tissue engineering [13, 26, 27]. However, the utilization of small molecules for engineering musculoskeletal tissues has been largely overlooked. This trend is very likely to be reversed as advanced screening technologies have recently generated a large number of novel regenerative small molecule drugs, and advanced tissue engineering scaffolds continue to provide promise for controllable drug delivery to target tissues [8, 22, 28–30].

Advanced nanofibrous scaffolds for controlled drug delivery

Tissue engineering and advanced material science have been the frontiers of biomedical research since the late 1980s aiming to repair or regenerate individual human tissues. As regenerative engineering evolves, the field becomes convergent, bringing together many related disciplines and targeting the regeneration of complex tissues and biological systems, for example, the whole human limb [9]. Specifically, incorporating and building structural and chemical cues that are commonly seen in tissue morphogenesis and developmental biology into a tissue engineering approach has become a key to regenerating functional tissues.

The application of nanofibrous scaffolds has been suggested to be intrinsically advantageous in musculoskeletal regenerative engineering [31]. First, natural musculoskeletal tissues themselves are composed of finely organized extracellular matrices (ECM) in the nanoscale. For example, natural bone itself is a nanocomposite material composed mainly of hydroxyapatite nanocrystallites in an organic collagen-rich matrix [32]. Nanofibrous scaffolds thus can be fabricated to closely mimic tissue-specific ECM and provide important topographical cues to guide cell and tissue growth. Second, nanofibrous scaffolds are characterized by ultra-thin continuous fibers, high surface-to-volume ratio, high porosity, and adjustable pore size distribution [31]. The highly interconnected porous structure of nanofibrous scaffolds provides an appropriate substrate for cell attachment and nutrient transport. In addition, the electrospinning process used to construct nanofibers can be easily adapted to deliver both hydrophilic and hydrophobic drugs. Particularly, nanofibers can be used to efficiently load poorly water soluble drugs and facilitate effective drug release due to the high surface area per unit mass of nanofibers (Figure 1).

Over the past decade, a great number of studies have investigated the use of nanofibers for sustained release of various pharmaceuticals such as small molecule drugs, large bioactive signaling molecules, proteins, genes, and DNAs [33–34][35–38]. Early work focused on the feasibility of loading and delivering these drugs using nanofibrous carriers. Advances in the electrospinning field have recently led to the development of sophisticated nanofibrous structures, overcoming many challenges encountered and providing exciting opportunities in regenerative engineering.

Due to the high surface area and short diffusional path of nanofibers, initial burst release has been considered by many to be a limitation of conventional nanofibrous drug delivery systems. Recent advancements in electrospinning techniques have enabled researchers to fabricate nanofibers exhibiting core-sheath structure, largely preventing the initial burst release phenomenon. Coaxial electrospinning was the first developed technique to produce

core-sheath nanofibers, and has since been widely used to encapsulate drugs into the nanofibers [39, 40]. As the core fluid does not need to be electrospinnable, a variety of proteins, growth factors, and genes which might denature in organic solvents can be dissolved in the core fluid, co-electrospun with the shell solution, and eventually incorporated into the core of the core-sheath nanofibers [41, 42]. While a core-shell nozzle is required for coaxial electrospinning, core-sheath nanofibers can also be obtained using the emulsion electrospinning technique with a conventional single-nozzle setup. A more detailed review on these techniques can be found elsewhere [43]. Research has shown that both techniques can be successfully used to fabricate core-sheath nanofibers with attenuated initial burst release and prolonged drug release profiles [44].

Tissue morphogenesis is typically driven by several growth factors simultaneously. Therefore, a drug delivery system that is capable of loading multiple drugs and enables independent control over the release of each drug is of great importance. The nanofibers with core-sheath structures described above are also applicable in multiple-drug delivery. Jo *et al*. developed a core-sheath nanofiber system containing colloidal arrays in the nanofiber core via single-nozzle electrospinning [45]. Both hydrophilic and hydrophobic small molecule drugs were incorporated into nano-or microscale polymer colloids which were further emulsified with poly(ε-caprolactone) (PCL) solution and electrospun into nano- or microfibers. The drug-containing colloids were confined in the core of the fibers. By choosing polymer colloids with different chemical properties and/or crosslinking density, one can design a system capable of programmable delivery of multiple drugs with high precision [45]. Applying a similar experimental setup, Wang *et al*. fabricated core-sheath nanofibers containing a PCL sheath and a chitosan nanoparticle core. Instead of restricting the drugs only to the core, one drug was encapsulated into the chitosan nanoparticles and another drug was incorporated into the nanofiber sheath (Figure 2A and 2B) [46]. Such core-sheath nanofibers were also able to deliver multiple drugs at distinct rates, with a high drug release rate usually from the drug in the nanofiber sheath due to shorter diffusional path.

When developing drug-encapsulated nanofibrous scaffolds for musculoskeletal regeneration, one has to take into consideration both the drug release profile and the mechanical properties of the scaffolds. If a nanofibrous scaffold serves dual roles of providing mechanical strength and acting as a drug carrier as presented in the aforementioned cases, there might be concerns that the incorporation of drugs into nanofibers deteriorates the mechanical properties of the nanofibers and scaffolds. To solve this problem, Inoescu *et al.* developed microsphere-laden nanofibrous scaffold system in which the nanofibers served solely as a tissue engineering scaffold while the microspheres were to deliver drugs [47]. Drug-encapsulated poly(lactide-co-glycolide) (PLGA) microspheres were introduced into the PCL nanofibrous scaffolds through dual electrospinning of PCL solution and microsphere-containing polyethylene oxide (PEO) solution (Figure 2C). Dissolving away the sacrificial PEO component revealed an anisotropic nanofiber/microsphere composite structure (Figure 2D). The inclusion of microspheres into the nanofibrous mesh did not significantly alter the mechanical properties of the scaffolds. Furthermore, different therapeutic agents can be encapsulated into the microspheres to achieve dual or multiple drug delivery [47].

In addition to the nano- and microstructural design of individual electrospun fiber structure, the macroscopic design of nanofibrous scaffolds may also contribute to the development of drug delivery systems capable of programmed delivery of dual or multiple drugs. Okuda *et al*. fabricated a variety of multilayered drug-loaded nanofiber meshes [48]. In one design, a tetra-layered nanofiber mesh was developed, consisting of a poly(L-lactide-co-ε-caprolactone) (PLCL) top layer containing the first drug, a polymer barrier layer, the second drug-loaded layer, and a basement mesh layer (Figure 2E and 2F). This system exhibited dual drug delivery capability with a fast release profile from the drug in the top layer and a retarded and slower release from the drug in the nanofiber mesh underneath the barrier layer. In addition to the order of the nanofiber layers, the thickness of the drug-containing layers also exerted control over the delivery rate, with thicker layers showing faster drug release [48]

Nanofiber-based delivery of small molecules for engineering musculoskeletal tissue

A. Skeletal tissue

Thanks to the advances in high throughput screening technologies, over a hundred of osteogenic small molecules have been discovered in the past decade [8, 21, 25, 28, 29, 49, 50]. Of these osteogenic small molecules, a number of them might represent promising candidates for next-generation bone regenerative medicines because their osteogenic activities have been observed in various preclinical animal models [8, 21, 51–54]. For example, the statin family, widely prescribed as a cholesterol-lowering drug, is the most well-characterized group of osteoinductive small molecules for bone repair and regeneration [55–57]. Other common small molecules used for bone regeneration include bisphosphonate, purmorphamine, resveratrol, and oxysterols [8, 58, 59]. Recent research efforts have focused on establishing appropriate delivery methods for these small molecules to bone injury sites [8]. Specifically, research studies have aimed to investigate methods for drug delivery vehicle design and fabrication, drug loading strategy, drug release profile, and the bioactivity of the released small molecule drugs [60, 61]. Zhang *et al.* summarized a variety of delivery methods using biodegradable polymeric nanofibers that has been studied for the delivery of osteogenic growth factors for bone repair and regeneration [62] (Figure 3). There are several types of nanofiber-based drug loading strategies, including physical adsorption, surface covalent immobilization, and encapsulation [62, 63]. It is important to point out that the methods described by Zhang *et al.* can be applied to deliver small molecules via nanofibrous scaffolds for musculoskeletal regeneration [62]

Prior to preclinical animal model studies, *in vitro* and *in vivo* studies are carried out to determine the optimal parameters for intended scaffold design and fabrication, desired drug release profile, and the bioactivity of the scaffold [8]. For instance, Singh *et al*. incorporated the osteoinductive small molecule resveratrol into PCL nanofibers and evaluated the bioactivity of the released resveratrol in an osteoblast cell line [64]. Their data revealed that the nanofibers showed a sustained release of resveratrol without a burst effect. Their data also revealed that the drug-loaded nanofiber showed a significant increase in calcium deposition in osteoprogenitor MC3T3-E1 cells. Similarly, Kouhi *et al.* fabricated PCL

nanofibrous webs combined with bioactive glass in order to improve bioactivity of the nanofiber scaffold [65]. To further improve the bioactivity of their scaffold system, the small molecule simvastatin was incorporated into the nanofibers. Their data revealed that a significant increase in calcium and phosphorous precipitates on the nanofibrous surfaces was observed when the simvastatin-loaded PCL nanofibrous scaffold was incubated with simulated body fluids (SBF) [65]. More importantly, an *in vivo* study conducted by Piskin *et al.* demonstrated that bone formation was significantly enhanced at the critical size cranial defect sites in rats where simvastatin-containing nanofibrous scaffolds had been implanted [66]. Taken together, the results from the *in vitro* and *in vivo* studies demonstrated that the small molecule simvastatin-loaded nanofibrous scaffold system is a promising therapeutic strategy for bone regeneration.

Poly(lactide-co-glycolide) (PLGA) is one of the most widely used FDA approved polymers for a number of biomedical applications [67, 68]. In addition, the utilization of PLGA polymer for nanofiber fabrication is well documented [69, 70]. In fact, several studies indicated that PLGA nanofibers encapsulated with osteoinductive small molecules were able to promote bone formation *in vitro* and *in vivo*. For instance, *in vitro* cell-based studies conducted by Brady *et al.* suggested that incorporation of the osteoinductive small molecule purmorphamine into electrospun PLGA nanofibers improved adhesion, proliferation, and differentiation of human mesenchymal stem cells when the cells were seeded on the purmorphamine-loaded nanofibrous scaffold [71]. In addition, Puppi *et al.* loaded PLGA-based nanofibrous meshes with a natural-derived, osteogenic, small molecule retinoic acid which has been shown to play an important role in osteogenic differentiation and bone formation in a number of cell types including bone marrow-derived stem cells, adipose-derived stem cells, primary osteoblasts, and fibroblasts [72–74]. Specifically, using the electrospinning method, retinoic acid was successfully loaded into 3D PLGA fiber meshes, and such loading method preserved the nanofiber mesh morphology. The bioactivity of retinoic acid was confirmed by various cell-based assays using osteoprogenitor MC3T3-E1 cells [74]. More recently, Das *et al.* loaded PCL/PLGA nanofibrous discs with the small molecule sphingosine-1-phosphate (S1P) analogue, FTY720, which has been shown to stimulate osteoblastogenesis and neovascularization as well as an anti-inflammatory response [75–78]. Their study highlighted that FTY720-loaded nanofiber groups demonstrated significant new bone formation as well as microvascular formation in a rat mandibular defect model at week 12 after the implantation [79].

A number of studies have shown that the small molecule zoledronic acid can enhance *in vivo* bone regeneration due to its ability to reduce bone resorption rate [80–82]. Recently, Lu *et al.* took a unique approach to encapsulate small molecule zoledronic acid by creating a novel “sandwich structure-like” nanofibrous mesh with zoledronic acid incorporated into the middle layer mesh [83]. Their work highlighted that the drug release profile could be easily controlled by adjusting the thicknesses of the drug-loaded mesh and the electrospun nanofiber barrier mesh [83]. However, *in vitro* and *in vivo* studies of the zoledronic acid-loaded nanofiber scaffold were not reported. Nevertheless, their results provide a promising platform for the creation of not only zoledronic acid-loaded nanofibrous scaffolds, but also a variety of drug/polymeric nanofibers with extended and controllable drug release kinetics.

To date, many research studies have focused on the establishment of novel fabrication methods to produce the desired drug-loaded scaffolds, whereas the differentiation of human adult stem cells on these new scaffold systems has not yet been systematically characterized. In addition, in order to bring the technologies closer to clinical applications, more appropriate pre-clinical animal studies should be carried out to assess the efficacy of the scaffold system in bone repair and regeneration.

B. Other musculoskeletal tissues

Other than bone tissue, musculoskeletal tissues also include cartilage, tendons, muscles, nerves, joints, and ligaments. Although the utilization of small bioactive molecules for regenerating muscle, cartilage, tendons, and nerves has been reported [22, 84], to our knowledge, only a very few studies have been conducted to investigate the feasibility of combining small molecules with nanofiber-based scaffolds for regenerating these tissues.

It is interesting to note that certain osteoinductive small molecules described in the above section can also be applied to regenerate musculoskeletal tissues other than bone tissue. For instance, a mixture of purmorphamine and retinoic acid has been shown to induce the differentiation of neuronal stem cells (NSCs) into motor neurons [85]. In fact, the Jolicoeur group recently fabricated a polymeric mat consisting of co-electrospun nanofibers of poly-l-lactic acid (PLLA) and gelatin, where the gelatin mesh was loaded with a mixture of purmorphamine and retinoic acid [86]. Their results demonstrated that the mechanical properties of the co-electrospun nanofibers were similar to those of peripheral nerve tissue, and addition of the small molecule drugs did not alter the morphology of the nanofibers. More importantly, NSC cultured on this novel scaffold system could proliferate and differentiate into motor neurons as well [86]. Similarly, while small molecule cyclic AMP analogs have been shown to induce bone formation *in vitro* and *in vivo* [87–89], a study conducted by Niu *et al*. demonstrated that the small molecule dibutyryl cyclic AMP (db-cAMP) analogue has a significant effect on neural differentiation [90, 91]. In addition, Zhu *et al*. incorporated the small molecule rolipram, a phosphodiesterase 4 inhibitor that can enhance cAMP activity, into PLLA/PLGA electrospun nanofibrous scaffolds [92]. Their pre-clinical study demonstrated that the rolipram-loaded scaffolds were able to promote axon growth through the scaffolds in a hemisection lesion in athymic rats.

Future prospective and conclusion

Although there have been considerable advances in the development of biomaterial paradigms for musculoskeletal regeneration, application of these concepts to human patients remains elusive. Due to the high surface energy and low cell-surface specificity for synthetic polymers, cells begin secreting ECM proteins upon contact. This deposition of ECM, while serving the purpose of functionalizing the surface, decreases intimate contact between the cells and surface, and therefore substrate-guided differentiation becomes less controllable [93]. In order to more accurately guide cell behavior, investigators have looked to optimize combined material, small molecule, and ECM-mimetic attributes in a single scaffold. Although currently there are no completed clinical trials with nanofibers, studies are recruiting patients and promising work has been done to optimize separate scaffold components both *in vitro* and *in vivo* as shown in previous sections of this paper. The field

will remain stagnant, however, unless studies can be accomplished that take not only individual scaffold components into account, but also the entire scaffold construct as shown in Figure 4. The ability of investigators to learn from past mistakes regarding certain specific scaffolds components, especially growth factors in the form of full length proteins, will be critical in advancing new therapeutics to market. While applied *in vivo* to human patients currently, the FDA-approved method of full length protein administration, including BMP2 and BMP7, requires exposure of complex, multi-lineage tissue interfaces in a stage of healing to supraphysiological doses of potent biological factors and can lead to a multitude of complications [94]. These tissue interfaces that occur, for instance, at the site of a degenerated vertebral disc, cannot be adequately reproduced in preclinical studies and therefore properly evaluated in relevant laboratory models. Learning from the drawbacks of these full length protein applications, a number of groups have looked into small molecule alternatives and found promising results that indicate these small molecules may be more appropriate for targeted activation of certain signaling schemes in a complex tissue interface [22]. Fast-tracking the most promising of these small molecule candidates for inclusion in combinatorial scaffold paradigms will be critical to decreasing the regulatory path of progressive, multi-component bone graft substitutes. Not only will the right combination of scaffold components need to be evaluated, but these scaffolds need to be evaluated *in vivo* using animal models that can extract as much information as possible. Paraffin embedding histological evaluation and mechanical evaluation are techniques from the previous century. In the 21st century, animal models will include transgenic organisms that can highlight or report crucial developmental signaling schemes at tissue interfaces or responses of the immune system. Bringing these together with more traditional 20th century assessments can play a tremendous role in reducing the amount of wasted efforts for combinations of scaffold elements that work well on the bench but poorly in the patient.

While this paper highlights preliminary work of combinations of nanofibers and small molecules, there is still a considerable amount of work that needs to be done. Questions of immediate concern are whether small molecules can improve on the target specificity and activity of full length proteins in humans, and whether or not nanofibers are the optimum therapeutic delivery vehicle. This will require cooperation between scientists of many fields, including immunology, materials science, developmental biology, and genetics to allow a true convergence of paradigms. Only by working as a team will the field achieve its goal: a low cost, readily available, biomaterial construct for precisely targeted regeneration of lost or damaged musculoskeletal tissues.

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Abbreviations

BMP bone morphogenetic protein

db-cAMP	Dibutyryl cyclic adenosine monophosphate
ECM	Extracellular matrix
NSC	Neuronal stem cells
PCL	Poly(ε-caprolactone)
PEO	Polyethylene oxide
PLCL	Poly(L-lactide-co-ε-caprolactone)
PLGA	Poly(lactide-co-glycolide)
PLLA	Poly-l-lactic acid
SBF	Simulated body fluids
TPPS	5,10,15,20-tetraphenyl-21H,23H porphinetetrasulfonic acid disulfuric acid

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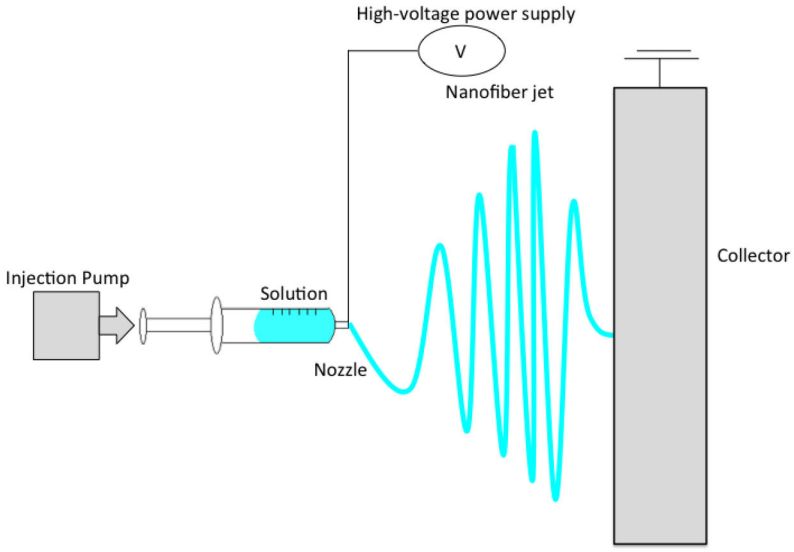

Figure 1.

Schematic representation shows a typical setting of electrospinning equipment. The polymer solution is released from a syringe, using an injection pump to control the flow rate. A high-voltage power supply is used to apply an electric field to the nozzle of the syringe, inducing a charge on the surface of the liquid. When the electrical force overcomes the force of the surface tension of the polymer solution, a fibrous jet is ejected from the nozzle. As the nanofiber jet travels through the electric field, the solution becomes thin and elongated, and the solvent evaporates. The resulting nanofibers are collected on a grounded collector that exhibits the opposite polarity of the polymer solution. Figure was modified from [63].

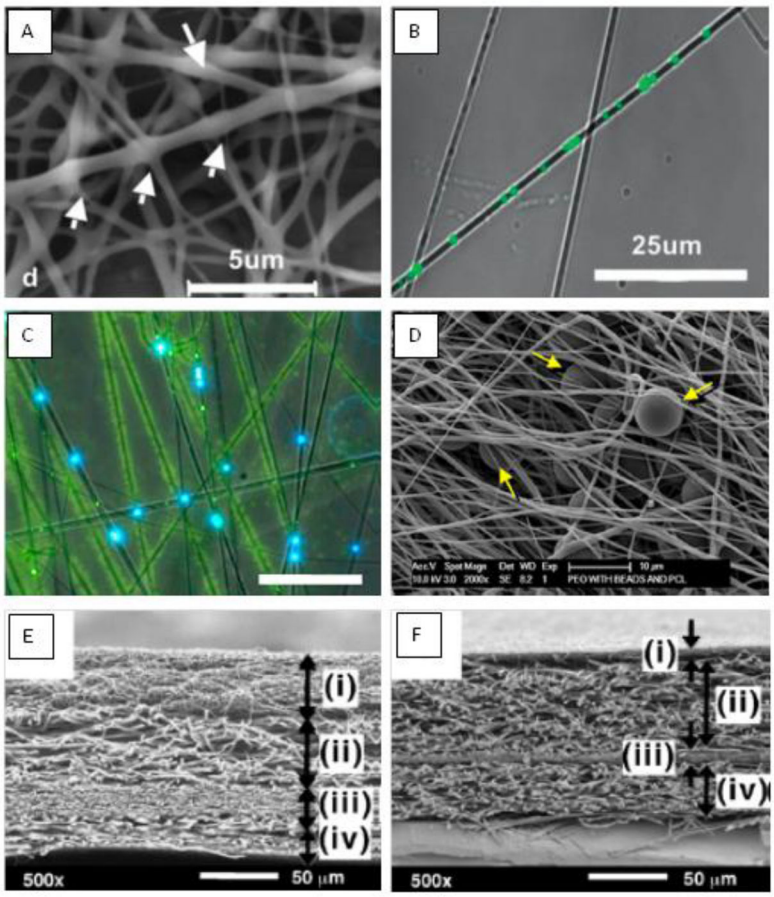

Figure 2.

(A) A scanning electron micrograph of core-sheath nanofibrous mesh consisting of a chitosan nanoparticle core and PCL sheath. A small molecule model drug Rhodamine B was encapsulated into the chitosan nanoparticles in the core. A second small molecule drug Naproxen was incorporated into the nanofiber sheath. White arrows indicate the locations of the chitosan nanoparticles. (B) A fluorescent image of FITC-conjugated chitosan nanoparticle/PCL core-sheath nanofibers. The green fluorescent dots are chitosan nanoparticles labelled with FITC. (C) A microsphere-laden nanofibrous scaffold with sacrificial PEO component. Blue shows PLGA microspheres, green shows PCL nanofibers, and black shows sacrificial PEO nanofibers within the composite structure. (D) A scanning electron micrograph shows the anisotropic PCL nanofiber/PLGA microsphere composite structure after the PEO component is removed. Yellow arrows indicate the drug-encapsulated PLGA microspheres. (E) A scanning electron micrograph showing the cross-sectional observation of a tetra-layered nanofiber mesh. A small molecule model drug chromazurol B was incorporated into the top nanofiber layer (i). A second small molecule 5,10,15,20-tetraphenyl-21H,23H-porphinetetrasulfonic acid disulfuric acid (TPPS) was incorporated into nanofiber layer (iii) underneath the barrier polymer layer (ii). A base nanofiber layer (iv) lies at the bottom. (F) The tetra-layered nanofiber mesh with thinner drug containing layer (i) and layer (iii), a strategy to manipulate the release rate of the encapsulated drugs. Reprinted with permission from Ref. [46] Copyright © 2010 John

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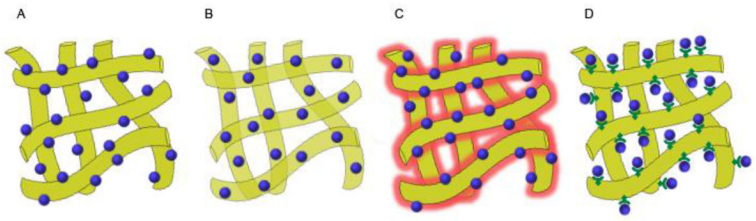

Figure 3.

Schematic representation showing various nanofiber-based drug delivery strategies: A) simple physical adsorption onto the nanofibers; B) coaxial electrospinning into the nanofibers; C) pore surface immobilization of drug-loaded micro/nanoparticles; and D) surface covalent immobilization of the target drug. Figure was modified from [63].

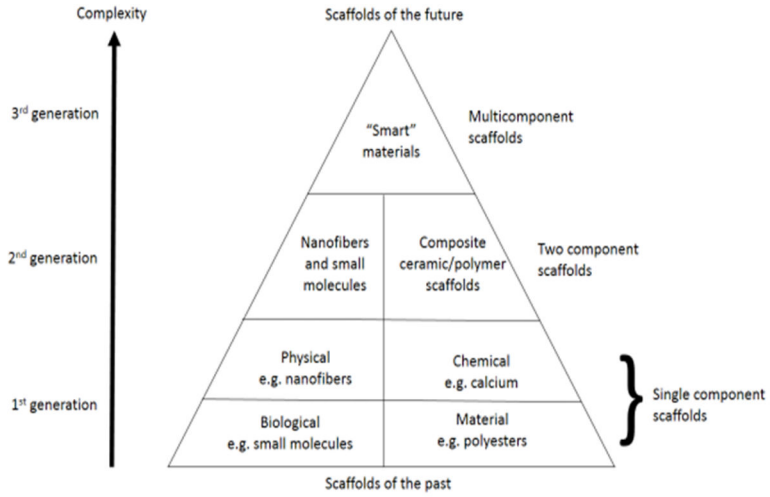

Figure 4.

Diagram of the evolution of scaffolds from simple, single-component structures (1st generation) to advanced, multi-component materials capable of specific, targeted tissue regeneration (3rd generation). Progression from the bottom of the pyramid to the top will be slow unless investigators can learn from the mistakes of the past including the use of supraphysiological doses of full length proteins that may inhibit regulatory approval.