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INVITED REVIEW PAPER

Nanotechnology in tissue engineering and regenerative medicine

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Abstract−Nanotechnology has advanced to unparalleled heights in biomaterials science. With the clear advantage of utilizing nanotechnology based systems compared to macroscale systems, such as targeted drug delivery, triggered drug delivery, multiple payloads in nanocarriers and immune system compliance, researchers in the field of tissue engineering and regenerative medicine have utilized these to a great extent. Furthermore, with the understanding that nanoscale structures, such as nanofibers, surface topographies and other biomaterial surface features could directly alter the cellular responses, researchers are trying extensively to take advantage of these and bio mimic nature as close as possible to enhance tissue regeneration. The technical advancements in the production of these nanoscale features on a large scale, such as photolithography, has enabled researchers to incorporate these into the existing biomaterials and push forward the bench-to-bedside applications for improving the outcomes of tissue engineering and regenerative medicine. Furthermore, with the evolution of various gel systems, magnetic nanoparticle controllable systems and also the spheroid and organoid cultures, the field of tissue engineering and regenerative medicine is rapidly expanding. This brief review gives a short and basic overview about the various nanotechnological approaches that are being explored and utilized to enhance tissue regeneration.

Keywords: Nanotechnology, Nanosurfaces, Tissue Engineering, Regenerative Medicine, Nanotopography

INTRODUCTION

Tissue engineering aims to reconstruct living tissues for replacement of damaged or lost tissues/organs in order to restore the form and function [1-4]. To achieve normal physiology and aesthetics, researchers utilize cells along with bioactive molecules, and extracellular matrix (ECM) components into or onto which cells will develop, organize, and function as if they are in their native environment. Various strategies have been utilized to develop a suitable microenvironment for the cells by selecting the appropriate ECM mimicking components, such as bio-compatible polymers, decellularized ECM and suitable inorganic components, such as ceramics [5-7]. By compositing these in various ways researchers have been able to design and synthesize various types of ECM mimicking components, such as sponges, fibers, sheets, solid blocks, 3-D printed templates and gels, depending on the specific type of tissue that is being reconstructed [8-12]. Researchers have also been able to construct multi-hierarchical structures which resemble tissue organization more closely [13]. Nanotechnology has proved to be very good in designing effective ECM mimicking components. Since cells rely on various surface receptors and other membrane components to sense the environment and act accordingly, they could very well respond to the nano-scale entities such as porosity, surface texture, topography, and other nanoscale physical cues [14,15]. Researchers have recently begun to understand the relevance of nano scale physical cues in the bio-mechanical modulation of cellular functions [16-18]. With the advancements in photolithography, electrospinning, cryogelation, freeze-drying and other similar scaffold forming techniques, researchers are able to introduce nanoscale features to the ECM mimicking components to enhance the tissue regeneration [11,19-24]. The next requirement in tissue engineering finding various ways in which nutrition and biochemical cues can be effectively incorporated into these ECM mimicking systems. It is also important to control the spatio-temporal release of these nutritive and bioactive molecules as needed by the cells/tissues, so that cellular functions, such as survival, organization and differentiation, can be achieved at efficient time-frames. Nanotechnology has paved the way for incorporation of bio-active molecules into matrix systems, and by formulation of various nano-systems, such as core-shell structures, nanofilms, biodegradable nanoparticles, nano-

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fibers, protein constructs and liposomal formulations, researchers have been able to use these structures as carriers for bio-active molecules [25-29]. When site specificity, degradation stimuli, degradation time and release kinetics of these nanocarriers are tuned, researchers are able to achieve efficient loading, site specific delivery, spatio-temporal release and also on demand release [30-33]. Such systems prove to be very advantageous over systemic delivery in terms of bioactive molecule loading, bioactive molecule protection from body metabolisms, dosing and specificity, leading to less dose of bioactive molecules used, which in turn reduces the cost for the patients. These advantages are mainly attributed to the high surface area-to-volume ratios that are observed in the nano regime, thus increasing the ligand binding and interactions in the biological systems [34,35]. Nanotechnology has also enabled researchers to establish combinatorial approaches such loading of nanoparticles in to the nanofibers or into other nano-features, thereby establishing a two-pronged approach in the field of tissue engineering. When cells are combined with these nano-architectured ECM mimicking matrices and the controllable bio-active molecules delivery systems, it is possible to achieve various functional organoids *in vitro* that could be greatly used for molecular screening studies as well as for regenerative applications [3,36,37]. Furthermore, magnetic nanoparticles have also been developed and utilized to greatly improve the guided delivery and activation of bioactive molecules responsible for enhancing tissue regeneration through external magnetic fields. This short review will give an overview and update on how nanotechnology helps in the advancement of the above-mentioned components for greatly enhancing tissue regeneration.

NANOSCALE FEATURES FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE

The microenvironment of cells plays an important role in controlling cellular behavior and function. There have been many efforts to investigate changes in cellular behavior, depending on the microenvironment by examining extracellular matrix (ECM) or geometric cues *in vitro*. Investigating the effect of nanoscale features on cellular behavior has drawn interest ever since researchers were able

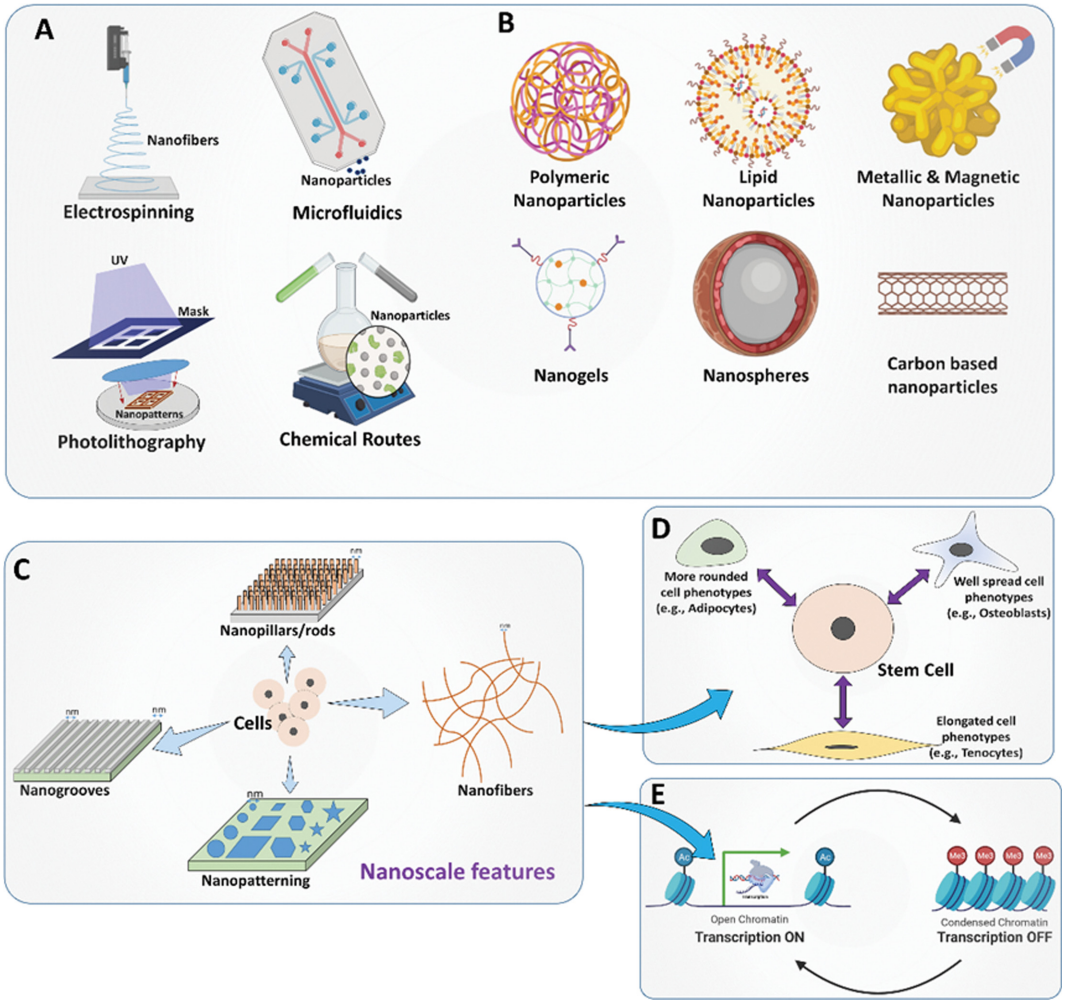


Fig. 1. Schematic showing the utilization of nanotechnology for tissue engineering and regenerative medicine. (A) Various methods to prepare nanoparticles and nanoscale features; (B) Various nanoparticle systems for efficient drug/molecule delivery; (C) different nanoscale features onto which cells can be grown, which can lead to change in cellular phenotypes (D) and also can cause epigenetic changes (E). Partly created with BioRender.com.

to develop nano-sized features on biomaterials and culture systems. These nanoscale features include nanopillars, nanoscale 2-D patterning, nanowires, nano-grooves, nanotubes, nano topographies such as concave and convex surfaces, and various physical modifications to surfaces [38,39] (Fig. 1). Various studies have showed that such nanoscale features affect the cells to change size, shape, and various cellular functions. It has also been established now that, the mechanical traction forces sensed by the protrusions and receptor on cell membrane can even orchestrate the nuclear mechanics [40]. Researchers have been able to stimulate the differentiation of bone marrow mesenchymal stem cells into osteogenic lineage by introducing nanopatterns onto the scaffolds surface [41,42]. This has also been extended to titanium implants which have shown enhanced bone regeneration *in vivo* [43]. It has been known that several cell types have their own predetermined shapes. Several researchers believe that by inducing stem cells to morph similar to shape of committed cells, the cellular functions of actual committed cells could be easily achieved [44]. This has been utilized to induce neuronal differentiation, chondrocytic differentiation, tenogenic differentiation, regulating macrophage phenotypes and several other cell phenotypes from stem cells [45-48]. Most of these observations are due to the fact that ECM organization that has been altered on nano-patterns and nanotopography has directly/indirectly affected cellular behavior through focal adhesion and integrin signal transduction, which results in varied mechanotransduction [49]. The control of cell alignment also alters cell-matrix interactions as well as cell-cell interactions, thereby changing the cellular functioning leading to better regeneration [50]. Controlling the stem cell functions through nanoscale features has emerged into new era for its limitless possibility in tissue engineering and regenerative medicine. With the current advancements, researchers are even able to attach functional groups or bioactive molecules to the nanotopographies, so that stem cells can be directed towards specific lineages [51,52]. Further, when polymeric nanofibers are utilized to provide nanoscale cues to the cells, several bio-active molecules such as drugs, proteins, and various other ligands can be incorporated into the nanofiber itself. This helps in the direct stimulation of cells both mechanically as well as biologically and, further, by controlling the degradation profiles of these polymers/polymeric blends, one can easily tune the release of these bio-active factors [52-56]. Researchers have also made microfibers with aligned nanogrooves on the fiber surface through electrospinning methods. This nanotopology has enhanced cellular adhesion and improved wound healing [57]. A more extensive discussion about the utilization of nanofibers in different tissue engineering applications will be provided in later sections. Nanogrooved microdiscs have been utilized to promote aggregation of stem cells and endothelial cells, thereby leading to macroscale constructs which have a potential for osteogenesis as well as endothelial vessel-like formation. This system effectively utilizes the mechanosignalling cues from the nanogrooves to achieve the regenerative potential [58]. Researchers have begun to understand/map many of the mechanosignalling pathways and proteins that actively take part in the signal transduction of nanotopographies and other mechanical cues. Some of them include integrins, Piezo, FAK, Ras/Raf, RhoA, Rock, Yap/Taz, ROCK, Filamin, Actin, lamins and myosin. Signal transduction from the nanoscale surface features through these components can eventually lead to transcription of various genes, leading to preservation of stemness, survival, proliferation and/or controlled and regulated differentiation of one type of cell to another [59]. Thus, by taking advantage of these systems, one can enhance or support tissue regeneration majorly through the tuning of nanoscale features of biomaterials.

BIOPHYSICAL REGULATION OF THE EPIGENOME USING NANO-SUBSTRATES

The role of mechanotransduction on the regulation of cellular behavior has been extensively investigated and reported on [60-64], clearly demonstrating the critical role of mechanical and topographical properties of a cell's microenvironment in the development and function of different cellular phenotypes. Cues from this microenvironment are sensed by the cell through focal adhesions, integrins and cadherins, and these signals are transduced throughout the cytoskeleton and ultimately to its nucleus [65] (Fig. 2). The instructional source for all cellular behavior originates from the nucleus via the precise orchestration of gene transcription in space and time, which governs how a cell will develop, function and survive. Gene transcription is regulated largely through heritable epigenetic mechanisms which cause structural changes to chromatin organization and gene accessibility without changing the DNA sequence itself [66]. Thus, the precise orchestration of gene transcription regulation necessary for controlling cellular behavior is largely achieved through epigenetic mechanisms, and an increasingly comprehensive understanding of the epigenome will enable increased control over cellular behavior.

Although many studies have investigated what genes are influenced by defined properties of biocompatible materials, there have been very few studies on the upstream epigenetic mechanisms that dictate how these genes are regulated, in the context of biomaterials. These upstream events include posttranslational chemical modifications to histone tails, DNA methylation and the activity of chromatin remodeling protein complexes, all of which cause structural alterations to chromatin, subsequently changing downstream gene transcription patterns.

Methylation of DNA is bound to occur when there is high repetition of cytosine nucleotides next to guanine nucleotides, which are termed CpG islands [67]. If CpG-devoid areas exhibit hypomethylation, then one can expect the promotion of an "open" chromatin conformation, called euchromatin. Conversely, silencing of those genes occurs when there is hypermethylation of CpG islands. Such a state at the promoters of key developmental genes results in a "closed" chromatin conformation termed as heterochromatin [68, 69] (Fig. 3).

Apart from the above discussed methylation, many other chemical modifications can occur to different amino acid residues on histones, including acetylation, phosphorylation, ubiquitylation, sumoylation and biotinylation [70,71]. Such chemical modifications are called posttranslational modifications (PTMs). Modifications to histones can reduce their positive charge, thereby leading to decreased force of attraction between the core histones and their surrounding DNA phosphate backbone which has a negative charge. This leads to loosening of the DNA around the histones towards

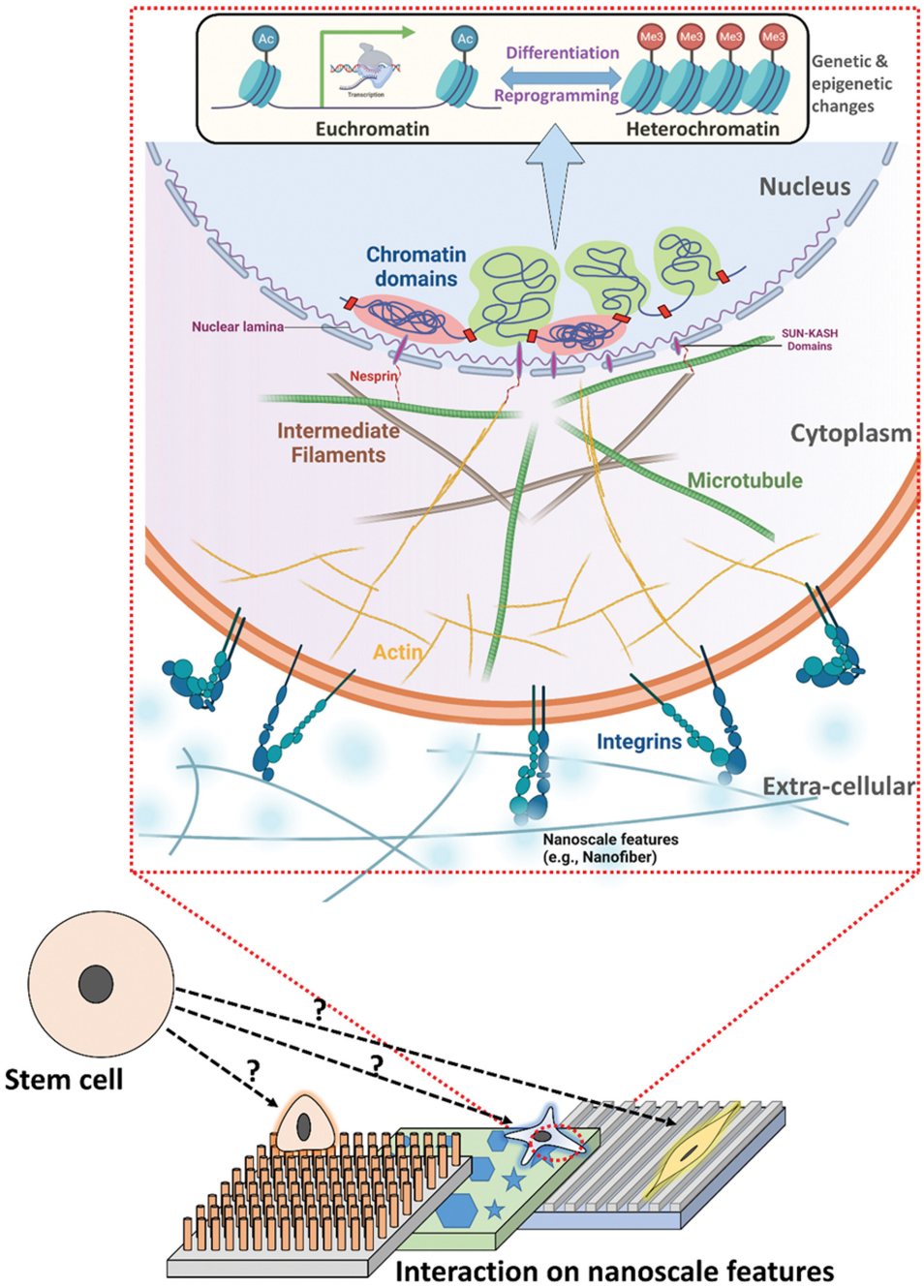


Fig. 2. Schematic showing the interaction of cell with the nanoscale features and some of the subsequent signal transduction. Biophysical cues are detected at the cell surface and ultimately manifest in organizational changes in the nucleus. Starting from the nanomaterial/cell interface, cells sense the various properties of the immediate microenvironment via integrins, cadherins and focal adhesions. These signals are transduced throughout the cytoskeleton and relayed to the nucleus, where subtle structural changes to chromatin cause further gene transcription changes and also epigenetic changes. Partly created with BioRender.com.

euchromatin, which is more accessible to transcriptional machinery and switches to a transcriptionally active form. In contrast, PTMs can also restore or increase the positive charge of the core histones, leading to a totally opposite situation as discussed above, leading to a transcriptionally silent state [72] (Fig. 3).

In addition to chemical modifications to chromatin, nucleosome can also be modified by multi-protein complexes referred to as chromatin remodelers [73]. These are powered by ATP hydrolysis, and are able to non-covalently manipulate chromatin structure by expelling histones to regulate access to the DNA [74]. Thus, chromatin remodeling complexes, posttranslational modifications to histones and DNA methylation are the major epigenetic mechanisms that direct the dynamic nucleosome, which subsequently specifies and regulates required patterns of gene transcription [75]. Therefore, to maximize the precision for the exogenous control of cellular behavior, it is important to understand how these epigenetic modifica-

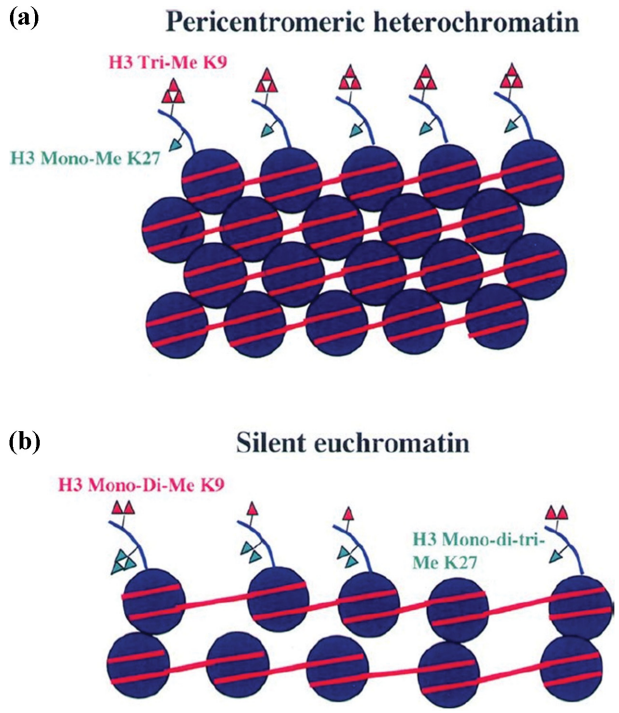


Fig. 3. Simplified diagram illustrating the chromatin structural states which occur as a result of histone acetylation and DNA methylation. The nucleosomes of pericentromeric heterochromatin are arranged into a regular array with a reduced linker length between adjacent nucleosomes. (a) This type of organization may facilitate packaging into a structure that is highly condensed compared with silent euchromatin, (b) although the details of how thisstructure is organized have not been determined. Pericentromeric heterochromatin is marked by the presence of tri-methyl lysine 9 and mono-methyl lysine 27 residues of histone H3. The major histone modifications in silent euchromatin are mono- and di-methyl H3 lysine 9 and mono-, di- and tri-methyl H3 lysine 27. Reproduced with permission from [72].

tions are influenced by defined features of a cell's microenvironment.

To date, there have been scant studies reporting on how defined biophysical cues influence epigenetic modifications. One recent study investigated the influence of biophysical cues on select epigenetic marks in the context of cellular reprogramming [76]. In this study, mouse fibroblasts were reprogrammed to a pluripotent state on topographical microgrooves, where a direct correlation was made between narrower microgrooves and increased reprogramming efficiency. Chromatin Immunoprecipitation (ChIP) was then used to assess how these microgrooves cause changes in specific post-translational modifications associated with key pluripotency maintenance genes [76], thereby beginning to elucidate how reprogramming efficiency is improved by these microgrooves from an epigenetics perspective. However, since the scale of the grooves is in the micron range (i.e., 10 μm), their topography is more influential to the growth and development of colonies of cells rather than single cells. Thus, topographic influences on cells may be better elucidated by studying topographic patterns on the nano-scale.

In a similar fashion as microgrooves, nano-grooves can also be fabricated via the soft lithography of biocompatible polymers. One particular substrate, fabricated from polyurethane acrylate, is able to systematically vary the spacing and widths of topographic nano-grooves from 350 nm to 2,000 nm. Human mesenchymal stem cells (hMSCs) were cultured on these substrates for a period of 72 hours, and subsequent histological differentiation analysis revealed the osteoinductive potential of this substrate, with wider nano-grooves directly correlating with increased differentiation efficiency. The epigenetic state of these cells was further investigated by using a novel high-content image analysis technique that elucidates subtle patterns of posttranslational modifications by quantifiably analyzing their textures [77]. After employing dimension reduction algorithms in combination with k-means cluster analysis, it was found that the patterns of trimethylation on lysine residues 4 and 27 on histone 3 were very similar between those hMSCs cultured on the widest topographic units and pre-differentiated osteogenic hMSCs, thereby reinforcing the notion that key epigenetic mechanisms are largely responsible for the proper gene transcription regulation necessary for cellular differentiation. Further, the authors developed this system to detect various phenotypic changes in stem cells when cultivated on various nanoscale topographies [78] (Fig. 4). This system was termed as Epi-mark Descriptor Imaging of Cell Transitional States (EDICTS).

Aside from topographical cues, the mechanical properties of a cell's microenvironment heavily influence its behavior. It is evident that different cell phenotypes found throughout the mammalian body reside and thrive in microenvironments with varying tissue elasticity, from osteocytes and osteoblasts that maintain rigid bone tissue to neurons which function in much softer brain tissue [61, 79]. These facts inspired studies to investigate the quantifiable properties of substrates of varying matrix elasticity and correlate this with specific lineage specification patterns in stem cells [80]. However, a common aspect of most of these studies is the type of data that is acquired; a quantitative assessment of how cells respond to these different materials is usually made by probing a downstream reporter, whether it is protein/gene expression change, cell viability and proliferation, metabolomics and other downstream effects. All of these consequential downstream changes are ultimately orchestrated by specific instructions indicating which combinations of genes are activated or silenced spatially and temporally, which is a result of epigenetic mechanisms.

To address this gap, some studies have begun to investigate the epigenetic response of developing stem cells of varied stiffness. One particular study employed continuous modulus gradient polyethylene glycol dimethacrylate (PEGDM) hydrogels fabricated via the controlled deposition of varying PEGDM concentrations, which yielded substrates ranging in stiffness from 2.7 to 30 kPa. hMSCs were subsequently seeded onto these hydrogels, cultured for 72 hours, and their epigenetic state was probed via the aforementioned high content image analytical technique of H3K4me3 and H3K27me3 expression. Interestingly, K-means cluster analysis revealed that the most elastic area of the hydrogel (less than 10 kPa) yielded hMSCs with similar H3K4me3 and H3K27me3 patterns to those of pre-differentiated adipocytes. Long-term differentiation assessment after two weeks via histological staining revealed that there is indeed a

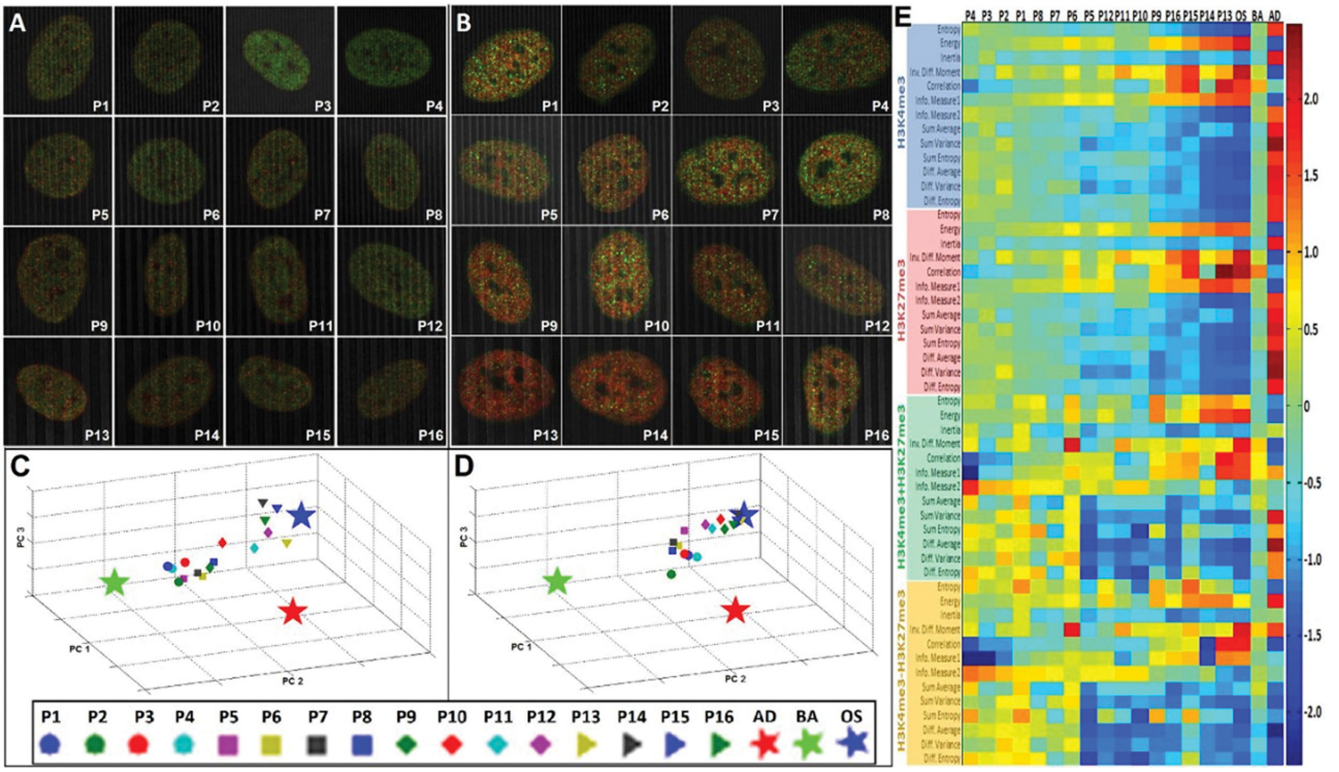


Fig. 4. Representative figure of Epi-mark Descriptor Imaging of Cell Transitional States (EDICTS) approach, which can differentiate the stem cell states depending on the nanotopography. H3K4K27me3 expression is responsive to varied topographic line/space patterns. (A and B) LSCM G-STED images of hMSCs cultured on PUA topographic line/space patterns labeled for H3K4me3 (green) and H3K27me3 (red) reveal a shift in balance from H3K4me3 to H3K27me3 as line/space widths widen that can be qualitatively observed after 2 weeks (B), but less apparent earlier after 72hours (A). (C and D) K-means cluster analysis reveals that centroids of hMSCs seeded on the widest line/space patterns (triangles) are closest in proximity to osteogenically differentiated hMSCs after 72hours (C) and migrate closer after 2 weeks (D), whereas centroids of hMSCs cultured on the narrowest line/space patterns (circles) are closest in proximity to undifferentiated hMSCs after 72 hours (C), but gradually migrate closer towards osteogenic after 2 weeks (D). (E) Heat-map of normalized texture descriptor values reveal increasing and decreasing trends that vary with increasing line/space width (left to right). The correlation and information of correlation 1 descriptors increase with line/space width, whereas the information of correlation 2 descriptor decreases, which is a trend not previously seen on hMSCs cultured on PEGDM hydrogels, indicating a subset of "material-specific" descriptors. (Line widths in nm - P1: 500 nm P2: 450 P3: 400 P4: 350 P5: 800 P6: 750 P7: 700 P8: 600 P9: 1250 P10: 1,200 P11: 1,000 P12: 900 P13: 2,000 P14: 1,800 P15: 1,600 P16: 1,500). Reproduced with permission from [78].

direct correlation between increasing elasticity and adipocytic differentiation (data not shown).

Although the scope of the epigenetic investigations was rather limited in these studies, they represent major milestones in a movement towards bridging the gap between the physical properties of a cell's microenvironment and how they influence the most upstream regulators of gene transcription. Such influence on the epigenomes by nanotopography and nanomaterials could be used directly to control/regulate the epigenome expression. As can be seen from the above examples, by changing the epigenetic state through nanotopography, the researchers were able to control the differentiation of stem cells; this could be utilized in the emerging biomaterials to stimulate regeneration. By controlling the epigenetic states, theoretically a powerful tissue response could be envisioned. It has now been understood that stem cells could even accumulate the epigenetic memories of their native state. Such stem cell epigenetic memories could be utilized for long-term fitness of tissues in a beneficial way [81]. Therefore, it is possible that by introducing well-planned nano features into a biomaterial, we could alter the epigenome and utilize it in favor of better outcomes. However, it is long way to study, understand and validate how to implement such systems *in vivo* in a reliable and consistent manner. Further investigations into how this epigenetic plasticity governs cellular behavior as a response to microenvironmental cues will pave the way for increased precision in the control over desired cellular responses, and thus will have a tremendous impact on regenerative medicine.

NANOFIBERS IN TISSUE ENGINEERING

Electrospun nanofiber has been the most highlighted in tissue engineering field over the past few decades. Rayleigh and Zeleny were the first researchers who observed electrospinning phenomena [82], and Reneker et al. confirmed the term "electrospinning" in the mid 1990s [83]. An electrospinning system is composed of four major apparatus (syringe pump, spinneret, high-voltage-supply and grounded collector) and several minor components (tub-

ing, syringe and wire). A syringe pump makes a steady flow of polymeric solution into a blunt-end spinneret. Following a high voltage supply impressing the spinneret, a Taylor cone is formed at the tip of spinneret by electrostatic pulling force. A liquid polymer jet heads to a grounded collector covered with aluminum foil for the final nanofiber collection. There are many operation parameters that can affect the characteristics of nanofiber: the concentration of polymeric solution, flow rate controlled by syringe pump, distance between spinneret and collector, and types of collector. These are easily controllable parameters in nanofiber fabrication processes [84].

Electrospun nanofiber has a great potential because it can be fabricated with specific characteristics. By choosing appropriate synthetic materials and the specific spinning techniques, the nanofiber system can obtain desirable mechanical properties suited for specific tissue engineering applications [85]. For example, poly(L-lactic-co-glycolide) (PLGA) nanofiber has larger stiffness compared to poly(caprolactone) (PCL) nanofiber [86]. Another remarkable nature of nanofiber is a high surface-area-to-volume ratio which enables electrospun nanofiber to contain relatively large amount of chemotactic agents. Therefore, Kenawy et al. could make poly(ethylene-co-vinyl acetate) (PEVA) and poly(lactic acid) (PLA) nanofiber by co-electrospinning and incorporate tetracycline into fabricated PEVA/PLA nanofiber. The main advantage of this strategy is a controllable release profile of target chemicals to the maximum [87] (Fig. 5(a)). Similarly, methylprednisolone, a steroid, loaded core-shell fibers have been synthesized by Liu et al., which reduced the inflammation in aortic dissection [88]. Researchers have also utilized coaxial electrospinning techniques to synthesize poly(methyl methacrylate) and silk fibroin mats which exhibited high tensile strength (around 45 kPa) that could be used as ECM mimics for tissue engineering applications [89] (Fig. 5(c)).

In our body, tendon and ligament tissues are the toughest tissues in order to tolerate extremely severe tensile stress acting on them. The difference between tendon and ligament tissue is a neighboring tissue. Tendons connect muscle to bone; ligaments connect bone to bone. The major component of these tissues is type I collagen with a few fibroblast populations [90]. For this reason, the self-regeneration capacity of tendon/ligament tissues is poor, and they need a strong scaffold to anchor either bone or muscle tissue well. Native tendon/ligament tissue is a hierarchical structure of collagen fibril, so it facilitates aligned nanofiber to resemble more. Yin et al. demonstrated that human tendon progenitor cells (hTSPCs) expressed a higher level of tendon specific genes when they were seeded on an aligned poly(L-lactic acid) (PLLA) nanofiber compared to the random oriented PLLA nanofiber. This group also confirmed that an implanted aligned PLLA nanofiber formed a collagen fibril structure similar to a native tendon tissue after six weeks of *in vivo* testing [91] (Fig. 5(b)). To increase mechanical strength, Kolluru et al. performed surface mineralization on PLGA nanofiber by immersing in the ten times simulated body fluid (10xSBF) solution [92]. To mimic the dynamic condition of tendon/ligament tissues in our body, a number of attempts to demonstrate this type of mechanical stimulation have been also investigated. For instance, Nerurkar et al. applied uniaxial tensile stress on the PCL aligned nanofiber with bovine mesenchymal stem cells (MSCs) seeded. In this study, the dynamic culture conditions significantly enhanced invasion of MSCs into the PCL nanofiber and reduced glycosaminoglycan content in the scaffold. At the same time, the collagen content and a percent of the uniformly distributed collagen fibrils were increased in the scaffold treated with dynamic tensile stress [93]. Electrospun nanofiber has the potential to achieve a high level of mechanical strength and structural similarity to native tendon/ligament tissues using the advanced fabrication technique. Therefore, a novel replacement based on nanofiber scaffold will be a breakthrough beyond the current limitations of clinical implant. Recently, Tang et al., achieved mechanical and degradation profiles similar to anterior cruciate ligaments by electrospinning a dual-phase, random phase and aligned phase fibers, in a single system. This system was able to support ligamentous tissue regeneration in rodent models [94].

There is no doubt that skin is considered to be the most self-renewing and self-repairing in that it never stops to shed and renew a numerous number of skin cells every hour until death. For the regeneration of skin, autologous skin tissues have been utilized as the 'Gold standard' in current clinical treatment. This method has no issue on immune rejection or biocompatibility but leads to loss of skin located at another region and painful surgical procedure. Consequently, scaffolds to replace autologous skin tissues are necessary to supplement skin loss. Among various types of scaffolds, an electrospun nanofiber is introduced to be an effective wound dressing because of its unique characteristics, such as easily acquirable thin layer and a high surface-area-to-volume ratio to load a large amount of chemicals and drugs by electrospinning process. Poly(ethylene glycol)-poly(DL-lactide) nanofiber with basic fibroblast growth factor (bFGF) by Yang et al. could confirm that bFGF-loaded nanofiber revealed significantly higher wound recovery rate and better regeneration of skin layer compared to bFGF-free nanofiber in the diabetic rat model [95]. Furthermore, Jin et al. investigated the potential of nanofiber as wound dressing in terms stem cell differentiation. They manufactured electrospun collagen/poly(l-lactic acid)-co-poly(3-caprolactone) (Coll/PLLCL) nanofiber with human bone marrow-derived mesenchymal stem cells (hBM-MSCs) seeded on the scaffold. This group demonstrated that the cells on Coll/PLLCL nanofiber have good proliferation rate, round keratinocyte morphology and expression of epithelial marker proteins compared to PLLCL nanofiber [96]. Like growth factors loaded scaffolds, drug-loaded nanofibers are also studied as the candidates for novel wound dressing. Motealleh et al. generated an electrospun nanofiber from poly(caprolactone)/polystylene (PCL/PS) blends and incorporated apigenin extracted from Chamomile flower. The PCL/PS nanofiber with chamomile extraction showed a higher efficiency in the wound closure and healing process compared to it without extraction [97]. Khoshbakht et al., utilized a tretinoin (all-trans retinoic acid) loaded nanofiber in a low cost manner, and showed a controllable release of tertinoin that could be potentially used as topical delivery device for treating acne [98]. Similarly, natural bioactive molecules have also been incorporated into electrospun cellulose acetate fibers. These fibers support the fibroblast survival and migration and also have anti-bacterial effects, thus serving as a potential candidate for wound healing applications [99]. Alginate, which is difficult to electrospun, has been com-

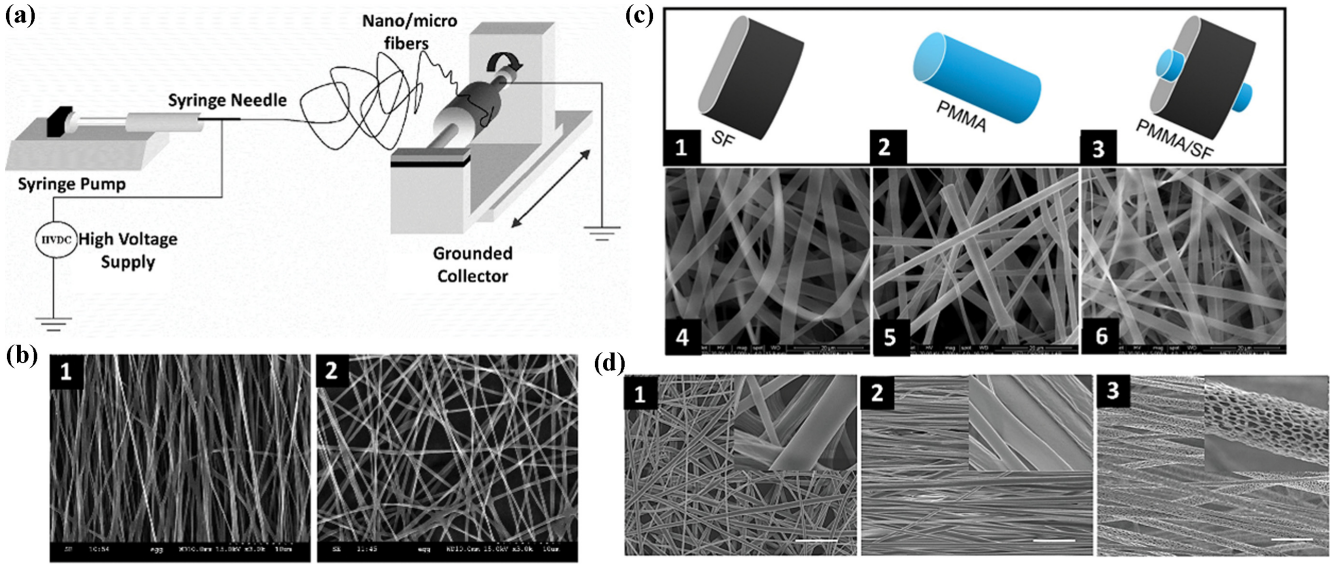


Fig. 5. Figure showing the electrospinning apparatus and various nano/micro fibers that could be achieved. (a) Schematic of the electrospinning apparatus consisting of the polymer solution loaded syringe, high voltage applied to the syringe needle and the collection of nano/micro fibers onto the grounded collector [87]. (b) Micrographs of the aligned and the random configuration of electrospun fibers [91]. (c) The development of flat electrospun with PMMA core and SF sheath in coaxial spinning [89]. (d) The development of nanoporous morphology on a electrospun fiber (D3) using vapor induced phase separation, compared to the non-porous fiber surface (D1-2) [105]. Reproduced with kind permission from 86, 90, 88 & 105.

bined with PVA, and a nanofiber scaffold was synthesized through electrospinning which was suitable for skin tissue engineering [100]. For skin tissue engineering, electrospun nanofiber has some benefits in mechanical property, combination with effective biomolecules, reasonably simple process and so on. These benefits will promote actual clinical application of nanofiber as wound healing and be step toward the bright future in the field of regenerative medicine.

The nervous system is a highly complex network, and electrical signals transfer from the central nervous system (CNS) to the peripheral nervous system (PNS) through their connections. If nerve injury occurs, nerve tissues require special clinical strategies to reconstruct rupture sites since they have a poor self-healing capacity. In the view of tissue engineering, specialized scaffolds incorporating nerve progenitor cells can be the most powerful candidate to connect between injured nerve tissues. Specifically, electrospun nanofiber with uniaxially aligned orientation induces one-direction axon guidance of nerve cells. For instance, Chew et al. fabricated an aligned poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP) nanofiber encapsulating human glial cell-derived neurotrophic factor (GDNF) and confirmed that aligned PCLEEP nanofibers were able to increase regeneration of peripheral nerves when they were compared to random oriented PCLEEP nanofibers in 15 mm nerve defect model [101]. Moreover, Xie et al. manufactured conductive core-sheath nanofiber with polypyrrole (PPy) coated on PCL and PLA nanofiber via *in situ* polymerization and reported that neurite was more elongated on aligned nanofiber than on random nanofiber. There was also further enhancement of length of neurite with electrical stimulation [102]. Electroactive materials have been utilized for synthesizing nanofibers through electrospinning [103]. These fibers can conduct biological electrical impulses when implanted in the tissues, thus enhancing the functional outcome of nerve regeneration. Researchers have also loaded vitamin-C into poly(caprolactone)/poly(glycerol sebacate) nanofibers for enhancing peripheral nerve regeneration [104]. Xu et al., were able to introduce nanoporous surface on poly(lactic acid)/collagen composite fibers [105] (Fig. 5(d)). These aligned fibers were able to enhance the proliferation and elongation of Schwann cell and the dorsal root ganglion neurites. Regardless of many technical barriers, a specialized nanofiber scaffold fully mimicking microenvironment of nerve tissue will give an opportunity to overcome tragedy from any unexpected severe accident.

When it comes to polymer scaffolds, electrospun nanofiber illustrates its conspicuous characteristics, such as high surface-area-to-volume ratio and adjustable scaffold parameters to be applicable in tissue engineering and regenerative medicine fields, which will gradually derive many remarkable experimental achievements. A high versatility of electrospun nanofiber can ease the modification to other scaffolds and soluble factors as well. Newer strategies such as in situ cross-linker electrospinning, near field direct writing melt electrospinning, and various other pre and post modifications methods will push forward the electrospinning for improving tissue regenerative medical devices [106-109]. Hence, it is assuredly expected that the prospect of clinical strategies based on electrospun nanofiber is as crystal-clear as knocking on the door to become common, and several types of nanofibers will bring us imminent improvement in tissue engineering and regenerative medicine fields in the future.

MAGNETIC NANOPARTICLES IN TISSUE ENGINEERING

The assembly of tissues that resemble the characteristics of those

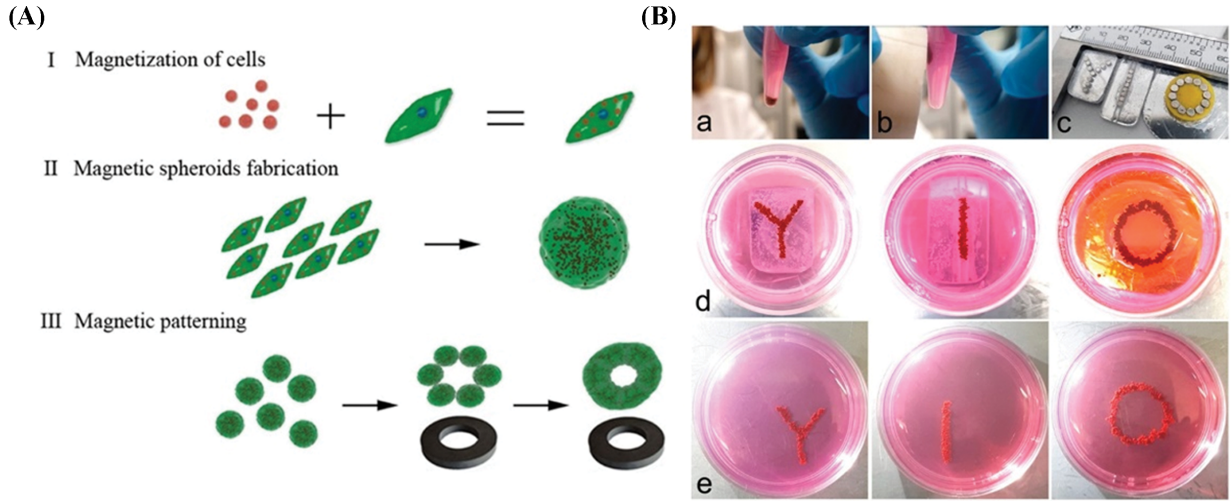


Fig. 6. Magnetic nanoparticle based spheroid patterning. (A) Schematic showing the method to fabricate the magnetic spheroids. (B) Patterning of tissue spheroids labeled with 12 magnetic microcapsules per cell with an applied magnetic field. (a) Aggregation of magnetic spheroids at the bottom of the tube due to the gravity in the absence of a magnetic field; (b) attraction of magnetic spheroids to a magnet; (c) Photograph of neodymium magnets of different shapes; (d) Photographs of magnetic-directed patterning of spheroids; (e) Photographs of tissue constructs formed from magnetic spheroids during 24 h. Reproduced with permission from [128].

found *in vivo* remains a challenge in tissue engineering. Tissues with high fidelity to native tissues could be used for drug discovery and diagnosis, among other possibilities [110]. However, the bulk of knowledge in cell culture comes from 2D cultures, which in many cases are not successful in recreating the complex tissues found in the body [111-113]. To assess these problems, many approaches have been tried. Current approaches rely on intrinsic adhesive properties of the cells or on the engineering of cellular interactions. The first one requires the cell surface to be chemically modified, which may lead to alternation in stem cell behavior, since the interactions in which the extracellular matrix is altered and important signaling pathways for cell differentiation may be affected. The second approach consists in entrapping cells within biomaterials that provide support for cell growth, such as hydrogels, which can then be assembled by engineering the chemical and physical properties of the biomaterial building blocks [114,115]. With this technology, the spatial assembly of multi-cellular modules in composite structure can be obtained. However, the interaction of cells within different modules is limited, and properties of hydrogels may influence the stem cell phenotype [116-118].

The above two approaches make 3D assembly possible, but they require *a priori* design of a series of parameters, which makes it impossible to use as a general technique that can be applied for all kinds of cells without the need for previous cell modification or specific materials design. A straightforward technique for assembly of 3D cell culture remains an unmet need [119,120]. Recently use of magnetic particles as a means for the assembly has been established [121-126].

The idea behind magnetic micro/nanoparticles embedded spheroids lies in the physical entrapment of iron magMP on the outer region of mouse embryonic stem cells (EScs) spheroids and their manipulation using an external magnetic field [119]. Since there is no uptake of the magMP due to its big size, stem cell differentiation is not affected. Liu et al. describe the various methods through which magnetic nanoparticles can be used to synthesize cell spheroids [127]. With this technique, the position and, therefore, the shear stress for the stem cell can be controlled and a rational process can be applied to obtain the desired lineage differentiation.

Iron oxide magnetic nanoparticles can be utilized to produce patterning of cells and tissues *in vitro*. When the cells are loaded with magnetic nanoparticles, under an external magnetic field, such as ring, bar, or magnetics arranged in any shape, the cells can align with the magnetic field and form organoids of the required shape [128] (Fig. 6). It has also become possible to obtain cellular fibers by manipulating and aligning the magnetic nanoparticle loaded cells remotely. Using such a technique researchers were able to achieve cellular alignment and muscle tissue differentiation on a chip, with a resolution of 10-100 μm [129]. Similarly, Byun et al. were able to magnetically control the magnetic nanoparticle coated fibers and stem cell aggregates to form into spheroids and further differentiate into osteo-chondral lineages. This method avoids incorporation of magnetic nanoparticles into the cells, thereby preventing toxicity of the system [130].

Recent advances in magnetic nanoparticles have enabled their application in cell levitation [121,122,131-133]. This technique is based on the usage of a hydrogel consisting of phage derived particles targeting the av integrins, gold nanoparticles, and magnetic iron oxide (MIO) nanoparticles. The hydrogel and human glioblastoma or murine neural stem cells were cultured and hydrogels were allowed to up-taken by the cells. After cellular up-take of hydrogels, an external magnetic field was used to levitate the mixture to the air-medium interface. Between 72-192 hrs, the cells assembled in a spheroid shape [122]. It was observed that the cell growth in this 3D fashion was much high than in 2D culture. Chan et al. described the enhanced stemness features and the differentiation potential of pulp-derived mesenchymal stem cell when cultured under 3-D magnetic levitation [131]. By using the magnetic levitation method, Tseng and colleagues created a three-dimensional bronchiole structure

[123]. The bronchiole is formed by four different types of cell layers: epithelial cells (EpiCs), smooth muscle cells (SMCs), pulmonary fibroblast (PFs), and pulmonary endothelial cells (PECs). This coculture was achieved by using a magnetic pen to sequentially prick up the levitated mono cellular cultures. The ECM production by the bronchiole coculture was highly organized and allowed the cells to have a close *in vivo* like behavior. In fact, mucus production was higher in the coculture than in monotype 3D and 2D EpiC cultures, which indicates that cell-cell interaction and adequate ECM plays an important role in maintaining cell phenotype. Several researchers have also shown the utilization of magnetic nanoparticles in bone tissue regeneration [134-136]. It has also been found that mesenchymal stem cells can be triggered with the use of magnetic nanoparticles and static magnetic field to secrete exosomal miR-1260a, thereby leading to better osteogenesis and angiogenesis, thus enhancing tissue regeneration [137].

The use of magnetic particles for the assembly of three-dimensional structures demonstrates a great potential for the formation of complex tissues. This potential is due to the generality of the methods, which have no need to chemically alter the cell or to develop cell-specific materials [138]. Also, the easy usage of this technique compared to others allows us to think in different ways of generating tissues. Although magnetic nanoparticles are interesting and have many advantages in the field of regenerative medicine, certain issues have to be addressed before utilizing them in full scale. Some of the notable issues include biocompatibility, immunological reactions, accumulation, biodegradability and clearance of magnetic nanoparticles and their degradation products. Since most magnetic nanoparticles are metallic core based, it could be understood that they are not easily degradable. The biocompatibility can be overcome by utilizing various strategies, such as polymeric coating, providing a shell of compatible materials such a hydroxyapatite and choosing the appropriate magnetic core. For example, Fe_3O_4 or Fe_2O_3 core will more biocompatible when compared to nickel or cobalt core, due to reduced oxidation [139,140]. Magnetic nanoparticles could be encountered by the macrophages when placed *in vivo*, thereby leading to immunological responses. This issue is being overcome by altering the surface charges on the magnetic nanoparticles, providing surface functionalization and also by surface neutralization by attaching PEG molecules or acetylation [141]. Another important issue is the biodegradation and clearance of the magnetic nanoparticles. If the magnetic nanoparticles are not cleared efficiently, this could lead to acute as well as chronic accumulation in various organs. In due course this would lead to toxicity of the specific organ or in multiple organs. The efficient breakdown and/or clearance, avoiding the accumulation could be achieved by providing the surface with functional groups or coating with lipids that can enhance the macrophagic uptake for degradation or by enhancing renal clearance. However, these issues still remain a great challenge, so that the magnetic nanoparticles could be used very effectively for tissue regeneration therapies.

NANOGELS FOR TISSUE REGENERATION

Hydrogels are widely used as tissue scaffold materials due to their highly swollen network structure, ability to encapsulate cells and bioactive molecules, and efficient mass transfer [2]. Researchers have successfully applied various hydrogels, which were chemically and physically modified, for different conditions. Nanogels are a specially designed class of hydrogels in the sub-micron range, which can be used for controlled release of various chemotactic factors, exosomes, drugs and even other nanoparticles [142,143]. Similar to the regular hydrogels, nanogels are usually synthesized through cross-linking chemistries and click-chemistries [144]. Fujioka et al. developed a cholesteryl group and acryloyl group bearing pullulan based nanogels for effective delivery of various growth factors such as BMP2 and FGF-18. This nanogel system has been shown to improve bone regeneration *in vivo* [145,146]. Hyaluronic acid based nanostructured hydrogels loaded with chitosan nanogels have been utilized for wound tissue healing. This nanogel loaded hydrogel also contained amoxicillin, thereby exhibiting anti-bacterial effect [147]. Bioderived nanogels are also being developed for tissue engineering applications. Kongprayoon et al. synthesized PLA-Sericin based nanogels and incorporated them into electrospun nanofibers which exhibited good cytocompatibility [148] (Fig. 7). Microfluidics has been utilized to synthesize alginate based nanogels which were loaded with TGF-B3. These nanogels elicited a controlled release of the growth factor and were able to enhance the chondrogenic differentiation of mesenchymal stem cells [149]. Several advances are being made in the chemistry of nanogels, such as stimuli responsiveness, nanogels-tectonic material, which has proven to be a promising drug/protein delivery system for varied applications [150,151]. Oxidoreductase radical polymerization reactions are also being employed to fabricate a new genre of nanogels for tissue engineering applications [152]. Kader et al. developed an interesting nanogel/nanoparticle system, in which VEGF as well as BMP2 were conjugated to the nanogels. These nanogels were then further loaded into patterned microchannel system, in which localized and on-demand delivery of BMP2 and VEGF were achieved, enhancing both osteogenesis as well as angiogenesis [153]. Fardous et al. utilized a gel in oil emulsion system to prepare heparin-immobilized gelatin nanogels and loaded them with bFGF. The nanogels showed efficient transdermal delivery of bFGF and induced angiogenesis *in vivo* [154]. Mizuta et al. integrated the nanogels with the ability to be navigated magnetically, thereby developing magnetic nanogels. These magnetic nanogels were able to be loaded with exosomes; they could be magnetically guided into adipose derived stem cells and able to induce neuron-like differentiation [155]. Kwon et al. were able to successfully deliver oncomodulin proteins using ε-poly(L-lysine) nanogels that showed remarkable spinal cord regeneration [156]. Pushing the boundaries further, very miniscule nanogels such as attoliter protein nanogels are being exploited for intracellular delivery [157]. With the expanding need for precise and targeted delivery of molecules and genes, for efficient programming of cells towards enhanced regeneration, and newer chemical synthesis routes, researchers can envision that nanogels will prove to be an efficient content in the field of nanotechnology with respect to tissue engineering and regenerative medicine.

CONCLUSION AND FUTURE PERSPECTIVES

Nanotechnology for tissue engineering and regenerative medi-

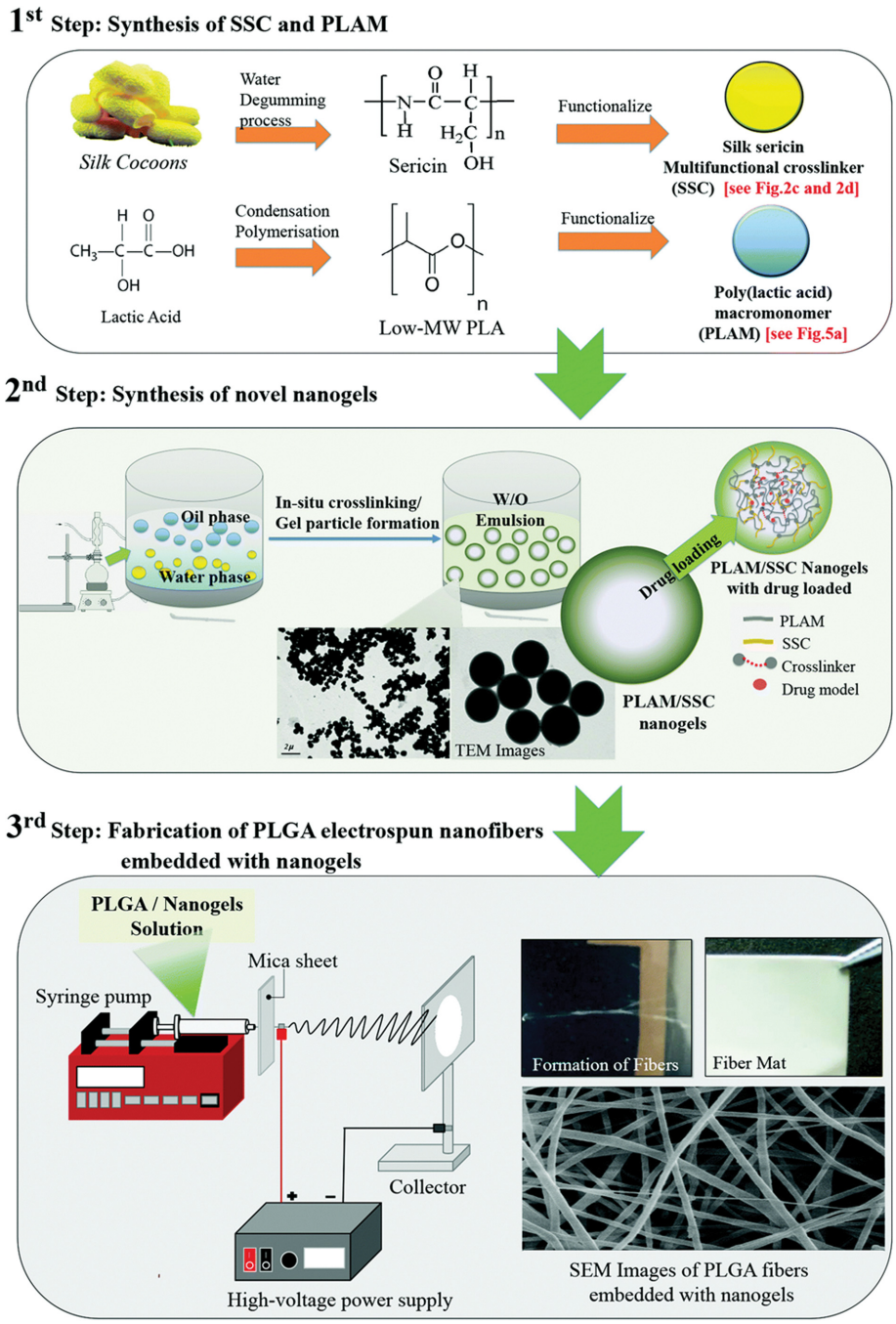


Fig. 7. Nanogel infused nanofiber mat. Schematic showing the synthesis of novel nanogels and further electrospinning of nanogels infused polymer. Reproduced with permission from [148].

cine has seen many milestones in the past few decades. Starting from simple nanoparticles, which had efficient functions compared to macro counterpart, we are in a period where we have a whole library of different nanoparticles of various shapes, sizes, formulations and configurations. As of today, one can select the required nanoscale system, such as bare nanoparticle, liposome, dendrimer, composite nanoparticles, drug loaded particles, nanofibers, nanogels and so forth, which suits their need in tissue engineering and regenerative medicine. Researchers and clinicians could utilize various products from nanotechnology for enhanced regenerative therapies in patients. For example, nanofiber based bandages could be used along with growth factors of regeneration enhancing molecules in efficiently treating the various wounds. Such nanotechnology based bandages could release the growth factors and help in skin regeneration without scarring. Various nanogel formulations can be incorporated into such bandages or skin patches and tuned for a controlled transdermal system, thereby enhancing the overall regenerative outcomes. Drug loaded nanofibers could be hierarchically arranged to form suture materials, which could improve the wound healing after surgery. Similar nanofiber arrangements

Table 1. Various nanotechnologies available for tissue engineering and regenerative medicine

S. No	Type of nanotechnology	Intended use	Experimental level	Reference
1	Magnetic nanochains	Enhancing osteogenesis	*In vitro*	[15]
2	Nanofilm caging system	Islet transplantation	*In Vivo*	[29]
3	Nanoparticles with bioactive peptides	Enhancing neovascularization	*In vivo*	[37]
4	Single walled carbon nanotube	Controlling stem cell lineage commitment	*In vitro*	[38]
5	Nanopatterned scaffold surface	Enhanced osteogenic differentiation	*In vitro*	[41]
6	Nanopatterned titanium implant	Enhanced bone formation	*In vivo*	[43]
7	Surface nano topography	Cellular alignment and organization	*In vitro*	[50]
8	Cellulose nanocrystal coating	Control cellular attachment and morphology	*In vitro*	[51]
9	PLA Nanofibers	Neural regeneration	*In vitro*	[52]
10	Hyaluronic acid-Silk fibroin nanofibers	Urothelium regeneration	*In vivo*	[54]
11	Antibiotic loaded Nanofiber mat	Wound closure and angiogenesis	*In vivo*	[56]
12	PLLA nanogrooved nanofiber	Enhancing cellular infiltration	*in vitro*	[57]
13	Nanogrooved microdiscs	Hierarchical bone-like construction	*in vitro*	[58]
14	Nanofiber covering for stent	Prevention of aortic inflammation	*In vivo*	[88]
15	PLGA nanofibers	Tendon to bone scaffolds	*In vitro*	[92]
16	Collagen/poly(l-lactic acid)-co-poly(3-caprolactone) nanofibers	Skin tissue engineering	*In vitro*	[96]
17	Tretinoin loaded nanofibers	Topical delivery	*In vitro*	[98]
18	Alginate -PVA nanofiber	Skin tissue engineering	*In vitro*	[100]
19	Poly(lactic acid)/Collagen fibers with nanoporous surface	Nerve tissue engineering	*In vitro*	[105]
20	Iron oxide nanoparticles	Magnetic patterning of tissue spheroids	*In vitro*	[128]
21	Iron oxide nanoparticles	Magnetic micro engineering and alignment of spheroids into fibers	*In vitro*	[129]
22	Magnetic nanoparticle coated fibers	Magnetic control of stem cell spheroid	*In vitro*	[130]
23	Magnetic nanoparticle	3-D culture under magnetic levitation	*In vitro*	[131]
24	Magnetic nanoparticle	Spheroid culture under magnetic levitation	*In vitro*	[132]
25	Superparamagnetic iron oxide nanoparticle	Enhancing bone regeneration	*In vivo*	[134]
26	Graphene oxide loaded magnetic nanoparticle	Bone regeneration and tumour treatment	*in vitro*	[135]
27	Cobalt ferrite magnetic nanoparticle loaded hydrogel	Bone regeneration	*In vitro*	[136]
28	Iron oxide nanoparticles	Static magnetic field triggered exosome release for enhanced osteogenesis	*In vitro*	[137]
29	Cholesterol-bearing pullulan nanogel	Osteogenic growth factor delivery	*in vivo*	[146]
30	Chitosan nanogels	Enhancing wound healing	*In vivo*	[147]
31	PLA/Silk sericin nanogels	Drug delivery & tissue engineering	*In vitro*	[148]
32	Alginate nanogels	Chondrogenic growth factor release	*In vitro*	[149]
33	Plasmin Cleavable nanogels	Osteogenic & angiogenic growth factor delivery	*In vitro*	[153]
34	Magnetic nanogel	Magnetic guidance and exosome delivery	*In vitro*	[155]
35	Epsilon-poly(L-lysine)-nanogel	Oncomodulin delivery for axonal regeneration	*In vitro*	[156]
36	Protein nanogels	Drug delivery	*In vitro*	[157]

could also be used for tendon regenerations. It could also be expected that nano-ceramic particles will be utilized to make 3-D printed patient specific custom implants for enhancing bone regeneration. Furthermore, nanoscale features could be added to the existing materials, such as metal implants for better osteointegration. By loading neuronal growth factors or molecules for enhancing neural regeneration into nanocarriers, it is possible to achieve functional spinal cord repairs. Some of the recent advancements in the nanotechnology for tissue engineering and regenerative medicine have been summarized in Table 1. With the advent of technology, such as lithography, selective etching, laser sintering and micro-casting, we can produce nano-scale features on almost any surface, including metals. Such an ability to introduce nano-scale features on biomaterials has allowed us to understand the biomechanics of a cell and its components, which has opened up a whole new field of mechanosignalling, which helps us to improve

tissue regeneration through physical cues. With the evolution of newer, greener and cost effective chemical routes for large scale synthesis of nanosystems, future researchers can achieve better tissue regeneration in an efficient manner.

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CONFLICT OF INTEREST

There are no conflicts to declare.

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