

Review Article

Nanotechnological Strategies for Biofabrication of Human Organs

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Nanotechnology is a rapidly emerging technology dealing with so-called nanomaterials which at least in one dimension have size smaller than 100 nm. One of the most potentially promising applications of nanotechnology is in the area of tissue engineering, including biofabrication of 3D human tissues and organs. This paper focused on demonstrating how nanomaterials with nanolevel size can contribute to development of 3D human tissues and organs which have macrolevel organization. Specific nanomaterials such as nanofibers and nanoparticles are discussed in the context of their application for biofabricating 3D human tissues and organs. Several examples of novel tissue and organ biofabrication technologies based on using novel nanomaterials are presented and their recent limitations are analyzed. A robotic device for fabrication of compliant composite electrospun vascular graft is described. The concept of self-assembling magnetic tissue spheroids as an intermediate structure between nano- and macrolevel organization and building blocks for biofabrication of complex 3D human tissues and organs is introduced. The design of in vivo robotic bioprinter based on this concept and magnetic levitation of tissue spheroids labeled with magnetic nanoparticles is presented. The challenges and future prospects of applying nanomaterials and nanotechnological strategies in organ biofabrication are outlined.

1. Introduction

Biofabrication may be defined as an application principle of engineering and information sciences for automated robotic bioassembly of living 3D human tissue and organs [1–3]. In a more narrow sense, biofabrication is basically a biomedical application of rapid prototyping technology or computer-aided additive fabrication. Although biofabrication is closely related to the more established field of tissue engineering and could even be considered as an integral part of this broader field, biofabrication focuses on and emphasizes using robotic automated engineering approaches in tissue bioassembly. In this context, the recent situation in tissue engineering and biofabrication could be compared to the situation of the

microelectronic industry before and after the introduction of automated robotic technologies for fabricating microchips and microprocessors. The transformation of the labor intensive and still predominantly manual field of tissue engineering into a robotic automated industry needs innovative and creative approaches. The history of technology development teaches us that one of the important principles that contribute to the emergence of a new technology is the creative application of knowledge from other disciplines outside the narrow domain of an existing technology. It is also a well-established fact that robotization and automation help to transform emerging promising technologies into economically feasible industries.

Nanotechnology is one of the most promising technologies of the XXI century. During last decade we witnessed the exponentially growing applications of nanotechnology in the area of tissue engineering [4–12]. Tissue engineering meetings and conferences now regularly include nanotechnology sessions. The number of nanotechnology-related papers in tissue engineering journals and tissue-engineering-related papers in nanotechnology journal has also dramatically increased especially during last decade. Finally, recent reviews on nanotechnology applications in tissue engineering are another confirmation of this trend [13–22]. However, it is not immediately obvious how nanomaterials with nanolevel organization could be used for biofabricating 3D tissue and organs which have macrolevel of organization. In other words, application of nanotechnology does not reduce existing size of human organs. However, the review of already existing and emerging approaches can provide interesting insights on probably the most important and nontrivial question on the interface of nanotechnology and tissue engineering: how employment of nanomaterials can enable biofabrication of human organs on macrolevel?

Thus, this paper is focused on demonstrating how recent advances in application of nanomaterials in tissue engineering can enable robotic and automated biofabrication of 3D human tissues and organs. The most impressive examples of emerging robotic tissue and organ biofabrication devices and related technologies will be presented. Finally, challenges and future prospects of application of nanomaterials in tissue engineering and nanotechnological strategies in organ biofabrication will be outlined.

2. Nanofibers

Over the last two decades, electrospinning has emerged as a relatively simple and scalable nanotechnological method for the generation of nanostructured scaffolds that closely mimic the dimensions of collagen fibrils of ECM. The general aspects of this technology have been reviewed extensively elsewhere [15, 23–25]. We will focus our attention here on the accomplishments and challenges in the utilization of electrospinning technology in the field of vascular tissue engineering.

A broad spectrum of synthetic polymers has been successfully employed in electrospinning. Moreover, electrospinning of natural proteins, such as collagen and elastin, either alone or in combinations, as well as in blends with synthetic polymers, has been reported [23, 26–29]. However, it is naive to believe that imitating the relative compositions of structural proteins, such as collagen and elastin, in natural vascular walls would lead to the development of vascular scaffolds with natural-like biomechanical properties. Indeed, the reported stability of vascular scaffolds created by electrospinning of only natural proteins is still far from desirable levels [27].

It has been demonstrated that electrospinning allows the control of the diameter of the spun fibers and produces nanofibers and scaffolds that imitate the nanostructure of natural ECMs, as confirmed by a more efficient vascular

cell attachment and spreading [27–31]. Although dense nanofiber meshworks provide excellent conditions for cell attachment and the spreading of endothelial cells on the luminal surface of the scaffold [30], they also preclude effective cell migration into the scaffold and thus impede smooth muscle cell migration and the sequential formation of muscular layers inside the vascular tissue engineered constructs.

Electrospinning allows the fabrication of a large variety of nanofibers and nanostructured scaffolds with special characteristics and functionalities (Figure 1). The obtained nanofibers vary in size, shape, and composition: they can be solid, composite, hollow, porous, decorated, helical, and branched. This diversity of possible electrospun nanofibers offers interesting opportunities for the enhancement of vascular-scaffold functionality. For example, the hollow nanofibers and nanoshells created by a coaxial extruder, as well as composite-coated or decorated nanofibers, could provide additional functionalities, including the capacity to release oxygen and to present growth factors and RGD peptides [32]. However, the full potential of electrospinning for the engineering of the full array of nanofibers with different functionalities remains to be explored.

Significant progress has already been made in controlling fiber orientation [33], which is an important step toward the rational design of biomimetic vascular scaffolds. However, in our opinion, controlling only the orientation of the nanofiber will probably not be sufficient. The recapitulation of the entire matrix architecture and the nonlinear biomechanical behavior of the natural vascular wall are equally crucial.

The most exciting breakthrough in electrospinning is the successful one-step rapid fabrication of a vascular scaffold with integrated living cells [34]. In these studies, previously reported methods for the encapsulation of living cells were combined with the electrospinning of nanofibers into one procedure. Further optimization of this electrospinning strategy might offer the greatest potential for rapid biofabrication of vascular-tissue constructs and might eventually eliminate the need for time-consuming and expensive bioreactor-based cell seeding and scaffold cellularization. Despite this impressive progress in vascular tissue engineering with the help of innovative electrospinning technologies, rapid cell integration into scaffolds and their optimal mechanical properties remain the main challenges.

Due to mimicking geometrical size and organization of natural extracellular matrix fibers, the electrospun matrices are ideal substrates for growing and implantation of cell monolayers and can even compete with popular cell sheet technology developed by Teruo Okano group in Japan [35]. Their main advantage is strong potential for functionalization and turning them into drug eluting matrices and scaffold. However, in case of 3D tissue engineering despite the existence of numerous publications and patents on using electrospinning in tissue engineering, we have paradoxical situation. Electrospun matrices are not permissive for cell invasion and seeding and their gross material properties are inferior. Scaffold which is not permissive for effective cell seeding and which has poor material properties is not

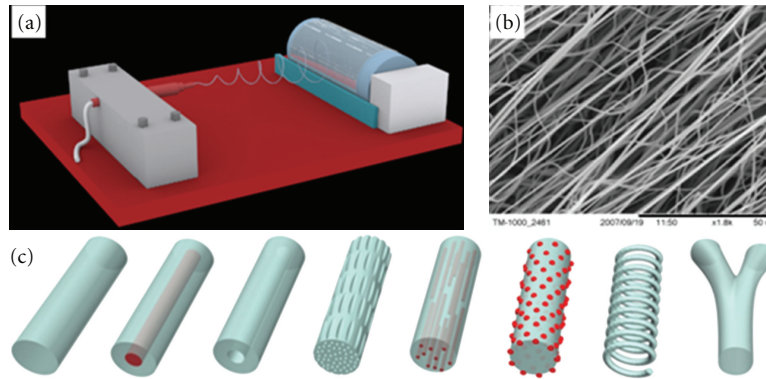


FIGURE 1: Nanofibers: electrospinning nanotechnology. (a) Scheme demonstrating principle of electrospinning technology and electrospinning apparatus; (b) Electrospun tissue engineered scaffold (scanning electron microscopy), (c) Scheme demonstrating different diverse classes of nanofibers that can be obtained by electrospinning. Shown are, from left to right, solid, core-shell, hollow, porous, composite, decorated, spiral, and branched nanofibers [3].

highly desirable scaffold in tissue engineering. These two still unsolved problems are main impediments on the way of successful clinical application of electrospun matrices from nanofibers.

In recent excellent review the first problem was very carefully addressed. It looks like it is possible to create large pore scaffold permissive for cell seeding using additional electrospinning sacrificial polymers or cryoelectrospinning [36].

We have recently developed an elegant solution for the second problem-creating electrospun scaffold with superior natural-like gross material properties using hybrid composite approach. In order to accomplish this, a special fabrication apparatus has been developed which includes a X-Y-Z robot with two nozzles allowing using two different polymers and a rotational collecting cylinder with periodically changeable diameter enabling formation of wavy nanofibers (Figure 2).

Development of an “out-of-shelf” compliant composite electrospun vascular graft will be important development in the application of nanofibers in vascular tissue engineering.

Combination of compliant composite electrospun scaffold with the use of coaxial electrospinning will allow creating also drug eluting vascular graft which in case of clinical success could be considered a very important development in the application of electrospinning in tissue engineering. Finally, another interesting breakthrough development is the combination of electrospinning with rapid prototyping, and developing hybrid composite technology, which allows to dramatic increasing of the bioprinted scaffold properties [37]. Thus, using nanofibers fabricated by the electrospinning method offers realistic opportunities to enhance tissue engineering.

3. Nanoparticles

There are at least several rapidly emerging nanotechnological strategies for using nanoparticles in tissue engineering [13–15, 38–40]. They are mostly based on employing magnetic nanoparticles. The emerging platform technology was

named as magnetic-forces-driven tissue engineering [41]. It is important to indicate that living cells can endocytose magnetic nanoparticles without strong toxic effect (Figure 3).

Moreover, at least one type of superparamagnetic iron oxide nanoparticles known as “Feridex” has been already approved for clinical use as a MRI contrast enhancing agent. First strategy is magnetic-forces-enhanced cell seeding of tissue-engineered scaffold. In certain extent this approach is similar to enhancing cell seeding using centrifugal forces. The principal difference is that driven force is not centrifugal forces but magnetic field forces that translocate cells labeled with magnetic nanoparticles. It has been demonstrated that using this technology dramatically accelerates and improves both speed and quality of cell seeding in porous tissue engineered biodegradable scaffolds [42–44]. One of the most interesting areas of application of magnetic-force-driven cell seeding is vascular tissue engineering. Several groups around the world independently developed rapid magnetic-force-based cell seeding technologies for vascular graft and scaffold [39, 40] summarized and illustrated on Figure 4.

The second strategy is based on using magnetic forces and so-called “nanoshuttle” for biofabrication and bioassembly of tissue spheroids [45]. However, in the reported approach the size of fabricated tissue spheroids is variable. In this context, the reported technology is still inferior as compared to other tissue spheroids biofabrication, but elegant simplicity of this method is attractive and it could be optimized. It is interesting that authors suggested to use their “nanoshuttle” method for magnetic-forces-based biofabrication histological structure of complex geometry [45]. Tissue spheroids fabricated from living cells labeled by magnetic nanoparticles have been used for patterning and assembly of 3D patterns by several groups [46, 47]. The way how magnetic force can be used for layer-by-layer fabrication of a 3D structure has been recently demonstrated by George Whiteside group from Harvard University [48]. Another group from the same University used hydrogel labeled with nanoparticles for successful assembly of a 3D living structure [49]. However, the most advanced published record in using

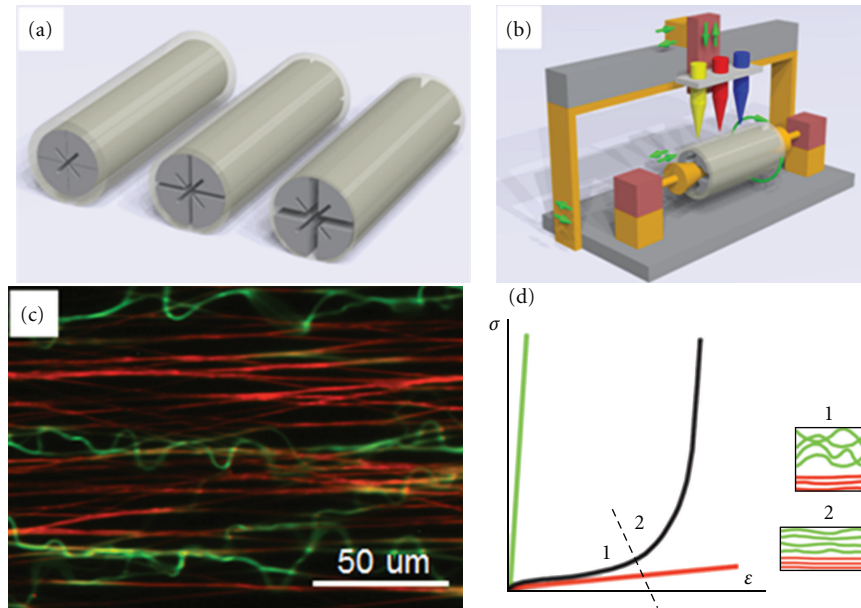


FIGURE 2: Robotic fabricator of compliant composite electrospun vascular graft. (a) Computer-aided design of rotational collecting cylinder with periodically changing external diameter; (b) Computer-aided design of robotic fabricator of compliant composite electrospun vascular graft (c) Confocal microscopy of compliant vascular graft fabricated from wavy (green) and nonwavy nanofibers (red); (d) Scheme explaining biomimetic mechanical behavior of compliant composite electrospun vascular graft.

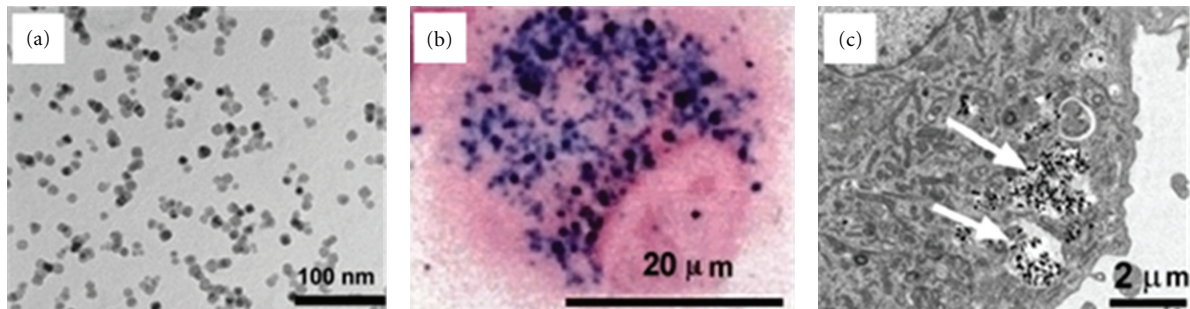


FIGURE 3: Nanoparticles. Labeling of cells with nanoparticles for magnetic-driven tissue engineering [3]: (a) Transmission electron micrograph of a typical population of metallic nanoparticles; (b) Light micrograph of human mesenchymal cells derived from bone marrow, which are capable of vascular lineage differentiation. The cells have been labeled with superparamagnetic iron oxide nanoparticles. Staining with Prussian Blue indicates the presence of nanoparticles inside of cells. (c) Transmission electron micrograph of human endothelial cells that were labeled with nanoparticles. White arrows indicate clusters of nanoparticles that had been endocytosed inside the cell.

nanoparticles for biofabrication of diverse human tissues belongs to members of Japanese group [41] who are pioneers of magnetic-force-driven tissue engineering. These authors, using magnetic forces driven tissue engineering, were able to fabricate functional epithelial, myocardial, skeletal muscle, skin, liver tissue and many other tissues. Finally, there is an emerging third approach which represents probably the most interesting strategy of using nanoparticles in tissue engineering. We are talking about certain attempts of using nanoparticles in bioprinting process [50]. Up to now, these attempts are still limited to incorporating nanoparticles in bioprintable hydrogels [50]. However, as it was proposed in our previous review publication using magnetic force and specially fabricated magnetic tissue spheroids could eventually lead to the development of a bioprinter based on

principles of magnetic levitation [15]. Another interesting approach is a fabrication janus-like tissue spheroids [51], which enables self-directed self-assembly of tissue spheroids in linear, circular, and branched structures (Figure 5).

4. Nanotechnology and Organ Printing

Organ printing is a biomedical variant of rapid prototyping technology or computer-aided robotic layer-by-layer additive biofabrication of 3D human tissues and organs using self-assembling tissue spheroids as building blocks [52–54]. The fundamental basic principle of organ bioprinting technology is the phenomenon of tissue fusion of closely placed tissue spheroids. The tissue fusion process is driven by physical forces such as surface tension and implies

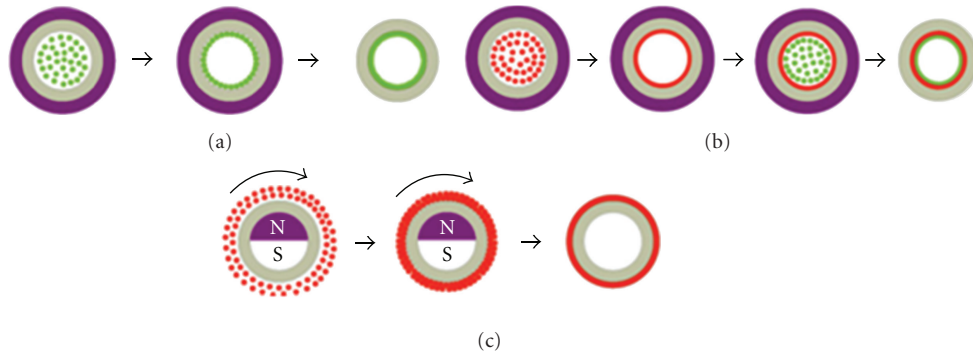


FIGURE 4: Magnetic-forces-driven vascular tissue engineering. The schematic illustrations shown above present three possible variations of magnetic-driven vascular tissue engineering, depending on the cells used and the placement of the vascular scaffold. In the first arrangement (a), the magnet (purple) is placed outside of the vascular scaffold and cells labeled with magnetic nanoparticles (green) are placed into the lumen. The magnet force causes the endothelial cells to adhere to and spread on the luminal surface of the vascular scaffold with the subsequent formation of a continuous endothelial monolayer. The second variation (b) is similar to the previous in that the magnet (purple) is placed outside of the scaffold (grey), but smooth muscle (red) and endothelial cells (green), both labeled with magnetic nanoparticles, are sequentially placed into the lumen for rapid adhesion and cellularization. In the third variation (c), the magnet (purple) is placed inside the vascular scaffold (grey) and smooth muscle cells labeled with magnetic nanoparticles (red) are placed outside of the scaffold. Rotation of the magnet enables the cell attachment with the subsequent formation of a concentric layer of smooth muscle cells on the external surface of the vascular scaffold. Endothelialization of the internal luminal surface can then be achieved as described in first variation.

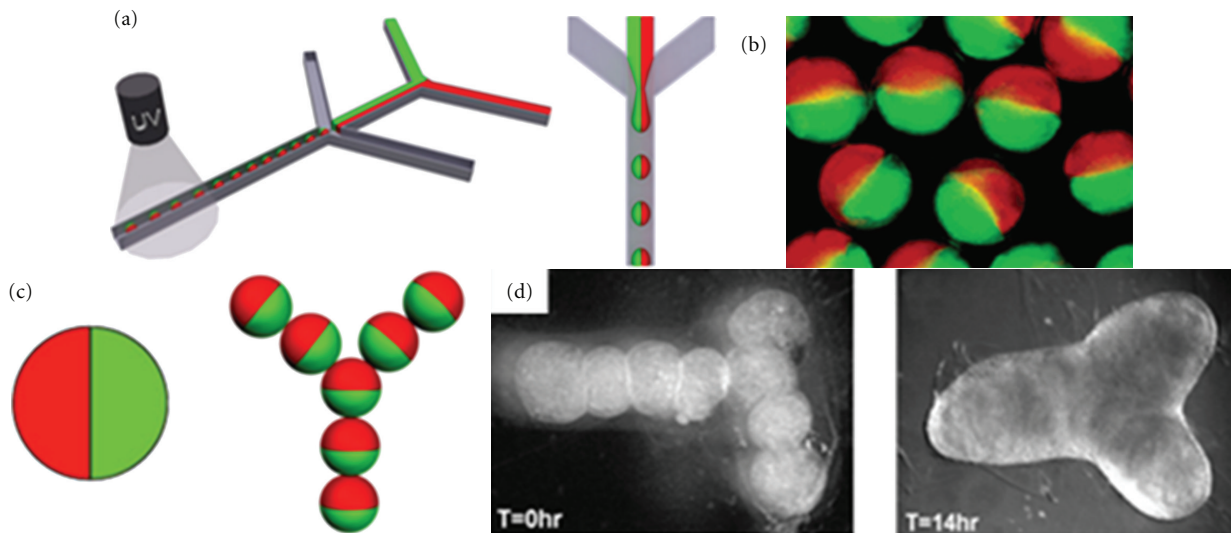


FIGURE 5: Fabrication of janus-like self-assembling tissue spheroids with magnetic nanoparticles. (a) Scheme demonstrating biofabrication of janus-like tissue spheroids using microfluidics [51]; (b) Janus-like spheroids fabricated by microfluidics devices [51]; (c) Scheme demonstrating magnetic-forces-driven self-directed self-assembly of closely placed janus-like magnetic tissue spheroids; (d) Branched structure formed as a result of fusion of closely placed tissue spheroids [52].

viscoelastic-plastic physical nature of tissue spheroids. However, when tissue spheroids under influence of inductive signal differentiate into so-called “osteospheres” or “chondrospheres,” then their material properties also change and their capacities for rapid tissue fusion are dramatically reduced. In order to escape this problem we have designed and developed interlockable miniscaffold or “lockyballs” which can interlock using specially designed “hook” and “lops” mechanism similar to famous Velcro and thus provide desirable material properties for 3D tissue constructs. Lockyballs also can contain engaged tissue spheroids which will still

have capacities for tissue fusion. The opposite is the situation when tissue spheroids are fusing too fast even before the actual bioprinting process. In order to prevent this situation, encapsulation in thin layer of sacrificial hydrogel is desirable. Thus, bioprinter can be loaded with three variant of tissue spheroids: (i) free tissue spheroids; (ii) tissue spheroids encapsulated in hydrogel; and (iii) tissue spheroids engaged into lockyballs or miniscaffold (Figure 6).

Now, if one wants to employ the magnetic levitation principle in the design of a robotic bioprinter for harvesting, translocating and delivery of tissue spheroids then

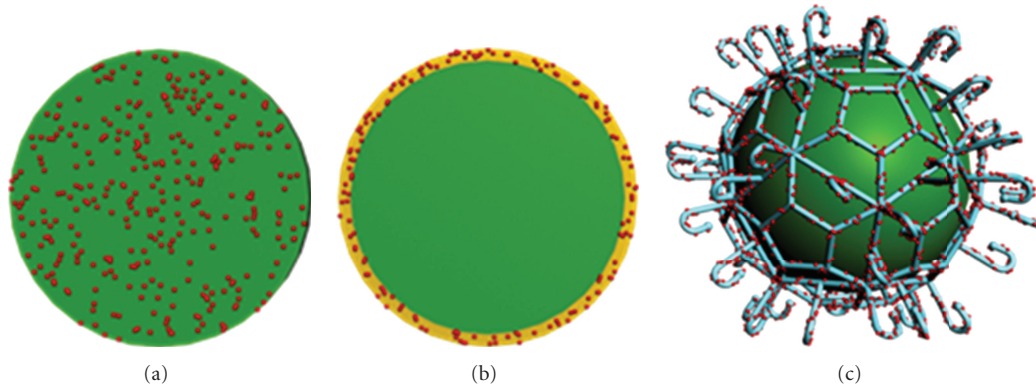


FIGURE 6: Enabling technologies for magnetic levitation of tissue spheroids. Three possible variants of modifications of tissue spheroids making them suitable for magnetic levitation: (a) biofabrication of tissue spheroid from cells labelled with magnetic nanoparticles; (b) encapsulation of tissue spheroid into hydrogel containing magnetic nanoparticles; (c) engaging tissue spheroid in magnetic microscaffolds.

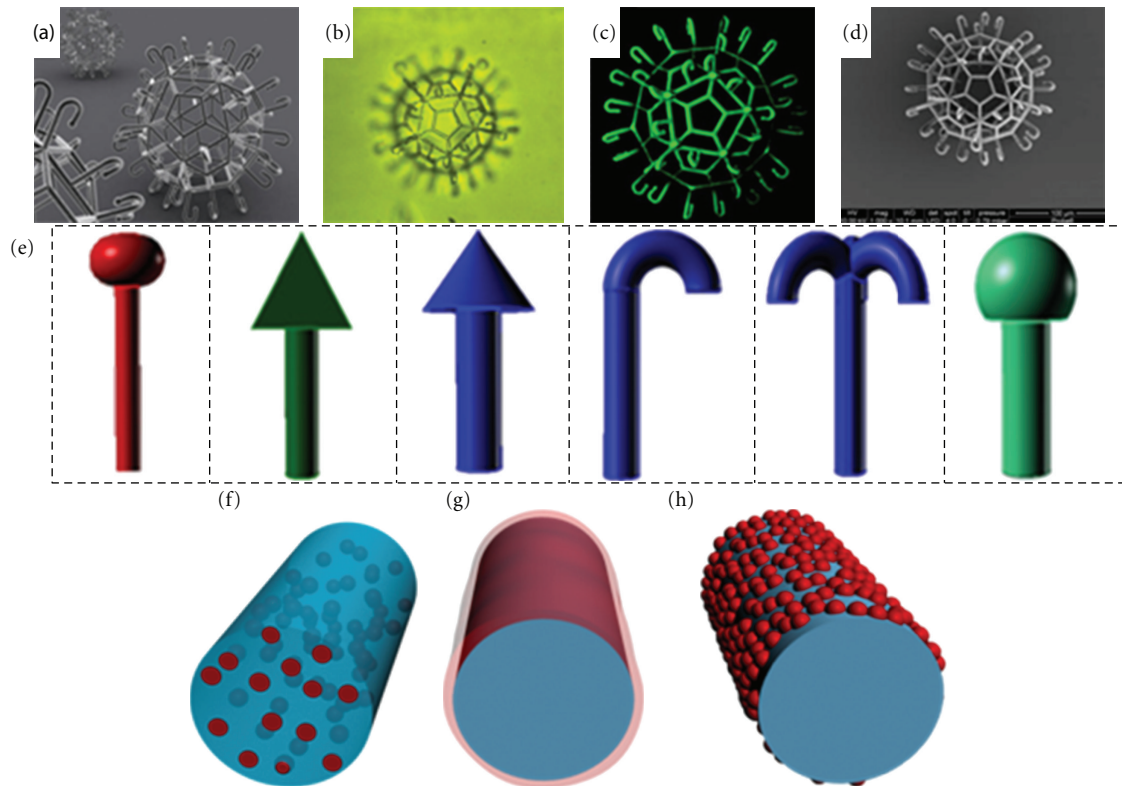


FIGURE 7: “Lockyballs” or interlockable microscaffolds for engaging living tissue spheroids. (a) Computer-aided design of lockyball; (b) Light microscopy of lockyball; (c) Confocal microscopy of lockyball; (d) Scanning electron microscopy of lockyball; (e) Different design variants of lockyball “hooks” which can be used as interlocking mechanism; (f) Section of lockyballs demonstrating magnetic nanoparticles inside; (g) Lockyball with magnetic nanosurface; (h) Section of lockyball demonstrating functionalization with nanoparticles on external surface.

nanotechnology can offer at least three basic approaches: (i) magnetic tissue spheroids could be biofabricated from cells labeled with magnetic nanoparticles; (ii) magnetic tissue spheroids can be fabricated from tissue spheroids encapsulated in hydrogel loaded with magnetic nanoparticles; and, finally, (iii) magnetic tissue spheroids can be fabricated by providing magnetic properties to miniscaffold or lockyballs engaging these tissue spheroids (Figure 7).

At least three different approaches may be adopted to obtain magnetic lockyballs. First, by placing magnetic nanoparticles inside a photosensitive polymer before two photon polymerization steps; second, by coating the lockyballs surface with a magnetic nanosurface; or third, by coating it with immobilized magnetic nanoparticles. All three approaches at least can make lockyballs and tissue spheroids engaged inside them suitable for magnetic levitation (Figure 8).

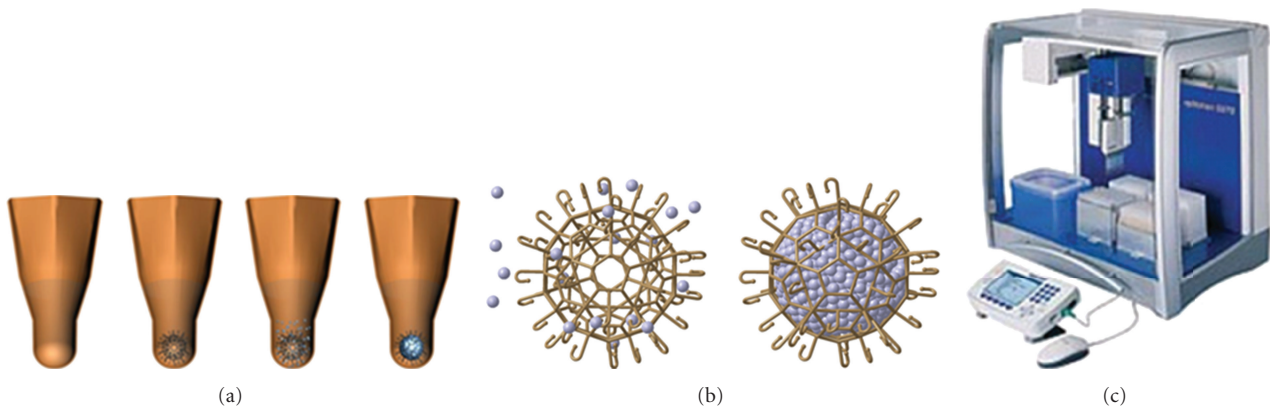


FIGURE 8: Robotic tissue spheroids biofabricator. (a) Scheme demonstrating sequential steps of encasing tissue spheroids in micro scaffold (lockyballs) in molded agarose hydrogel; (b) Scheme demonstrating cell seeding and tissue spheroid formation in lockyballs; (c) Robotic dispenser (Eppendorf, Germany).

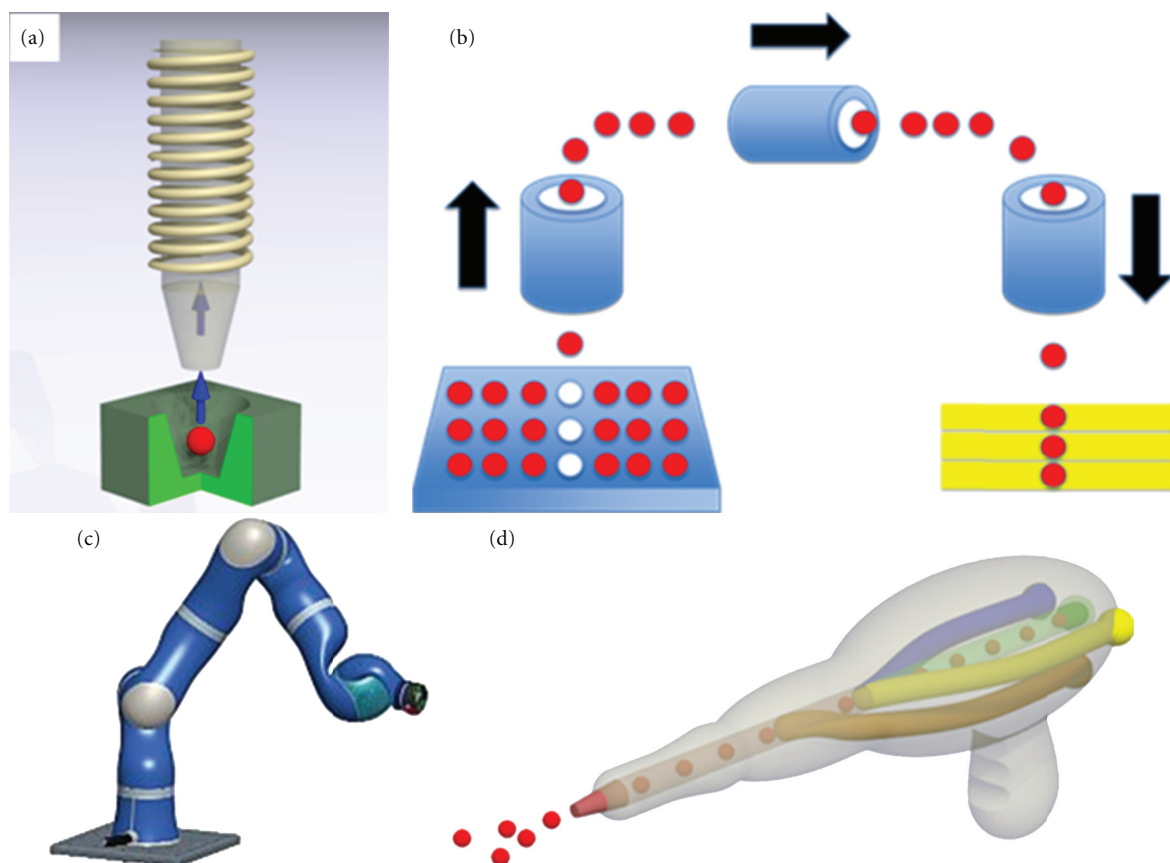


FIGURE 9: Design elements of a clinical robotic bioprinter. (a) Tissue spheroids harvester based on magnetic levitation of tissue spheroids; (b) Principal scheme of clinical robotic bioprinter demonstrating how tissue spheroids can be harvested from multiwells, translocated and dispensed in living tissue using magnetic levitation; (c) Elegant robotic hand developed by group of robotics and mechanotronics at German Aerospace Institute (http://www.dlr.de/rm/en/desktopdefault.aspx/tabid-3803/6175_read-8961/) which can be employed as an essential component in robotic clinical bioprinter for automated nozzle positioning; (d) Computer-aided design of nozzle of clinical robotic bioprinter containing several channels: two channels for fibrinogen and thrombin, one channel for tissue spheroids and additional channel for pressurized air for enabling fibrin hydrogel spraying.

The conceptual design of an *in vivo* robotic bioprinter based on the principle of magnetic levitation is presented on Figure 9. Magnetic forces driven *in vivo* bioprinter must have a magnetic harvesting device (magnetic solenoid) controlled by an X-Y-Z robot, a magnetic translocating device, and a magnetic dispensing nozzle controlled manually or by a robotic hand (Figure 9). The nozzle will include two channels for fibrinogen and thrombin, one or more channels for dispensing tissue spheroids (or different type of tissue spheroids) and one or more channels providing pressurized air which will enable spraying fibrin hydrogel. The potential areas of clinical application of such *in vivo* robotic bioprinter based on magnetic levitation are bioprinting of skin, cartilage and bone. The nanotechnological strategies described above could also be employed for designing more sophisticated bioprinters for bioprinting more complex 3D human tissues and organs such as kidney.

5. Conclusion and Future Perspectives

The research on the interface and integration of nanotechnology and tissue engineering is not just a highly desirable dream or science fiction anymore, but rather a well-established reality or already ongoing and rapidly expanding globally funded project. Nanotechnology found in tissue engineering field one of its most attractive, promising and also socially sounded applications. From another side, tissue engineers, by embracing nanotechnology, dramatically increased their research arsenal, broadening their “toolkit,” and were able to develop several novel impressive bioengineering platform technologies by applying nanomaterials. Thus, it is safe to predict that mutually beneficial collaboration of nanotechnologists and tissue engineers will only continue to grow. Moreover, with time the difference between these two professions will become increasingly semantic.

However, researches on the interface between nanotechnology and tissue engineering are still facing some serious challenges.

The first and most obvious challenge is toxicology of nanomaterials [55]. Although there are several solid researches which established principal safety of certain nanomaterials, the growing public concern about potential toxicity of nanomaterials must be systematically addressed. Thus, biocompatibility of nanomaterials for tissue engineering must be an integral part of the design principles as early as on the stage of project ideation.

The second challenge is functionalization of nanomaterials. Recent advance in designing drug eluting nanofibers using coaxial electrospinning is an excellent example of desirable progress in this direction. Functionalization of nanoparticles and carbon nanotubes is also under way.

The third challenge is design, synthesis, and fabrication of novel nanomaterials with biomimetic properties. This trend is already obvious in the fabrication of nanofibers using electrospinning technology. A similar approach is highly desirable for other forms of nanomaterials employed in tissue engineering. Development of composite nanomaterials is one of the most promising approaches in this direction.

The fourth challenge is standardization of nanomaterials. The standardization on certain stage of development of any emerging technology is a necessary step on the way to industrial product certification. Standardization is especially important in a biomedical application which needs regulatory agencies approval of new products. Finally, certain degree of standardization will enable comparing research results.

The fifth and last (we believe the most important) challenge is a seamless integration of nanomaterials into macrolevel biofabrication technologies. The introduction of nanomaterials will not reduce existing human organs size. Although we already have demonstrated some impressive examples of such integration, it is only the beginning and even more exciting new technology platforms will emerge on the interface between nanotechnology and biofabrication.

In the long term, perspective functional biomimetic nanomaterials can enable regenerative medicine (healing from inside) and thus reduce need for tissue engineering *ex vivo*. Drug eluting electrospun vascular graft is one possible example of this trend. Magnetic-forces-driven minimally-invasive technologies for targeted delivery of bone tissue constructing nanomaterials and stem cells *in vivo* in myocardial infarct area are other interesting examples. Finally, the *in vivo* robotic bioprinter described in this review could transform and reinvent surgical robotics.

Authors' Contribution

J. V. L. Silva and V. Mironov, both senior authors, made equal contribution to this paper.

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