

Scaffold-free, Label-free, and Nozzle-free Magnetic Levitational Bioassembler for Rapid Formative Biofabrication of 3D Tissues and Organs

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Abstract: Scaffolding is the conceptual framework of conventional tissue engineering. Over the past decade, scaffold-free approaches as a potential alternative to classic scaffold-based methods have emerged, and scaffold-free magnetic levitational tissue engineering (magnetic force-based tissue engineering [Mag-TE]) is a type of this novel tissue engineering strategy. However, Mag-TE is often based on the use of potentially toxic magnetic nanoparticles. Scaffold-free and label-free magnetic levitational bioassembly do not employ magnetic nanoparticles and thus, the potential toxicity of magnetic nanoparticles can be avoided. In this short review, we describe the conceptual foundation of scaffold-free, label-free, and nozzle-free formative biofabrication using magnetic fields as “scaffolds.” The design and implementation of “Organ.Aut,” the first commercial magnetic levitational bioassembler, and the potential applications of magnetic bioassembler are discussed as well.

Keywords: Magnetic levitation, Bioassembler, Formative biofabrication, Tissue spheroids, Scaffold

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1 Introduction

The three-dimensional (3D) bioprinting could be defined as an automated, robotic, layer-by-layer additive biofabrication of 3D tissues, and organs

according to the digital model^[1-3]. 3D bioprinting promises to solve the shortage of human organs for transplantation, which is an urgent but still unsolved problem of clinical medicine. Today, there are three most common and well-established

types of 3D bioprinting technology: (i) Ink-jet bioprinting, (ii) extrusive bioprinting, and (iii) laser-based bioprinting such as laser-induced forward transfer (LIFT)^[4]. However, in recent reviews, especially in the market reports, new types of 3D bioprinting – magnetic and acoustic bioprinting were also mentioned^[5-8].

3D bioprinter is defined as a robotic device for additive (“layer-by-layer”) biofabrication of 3D tissue and organs in correspondence with digital models. Of note, a question is still under discussion – can magnetic and acoustic bioprinters be considered as real bioprinters, or must a new term “bioassemblers” be introduced to define the new type of equipment? There are still no commercially available magnetic or acoustic bioprinters. It is important to note that the development of scientific instrumentation is essential for technological progress and advances in the new research field. Thus, the focus of this paper is a presentation of a new custom-designed magnetic levitational bioassembler as a new type of device for rapid formative biofabrication of 3D tissue and organs. We also outline the conceptual framework for a new emerging biomedical field, which we call scaffold-free, label-free, and nozzle-free formative biofabrication. The scaffold-free and label-free magnetic levitational bioassembly are illustrative examples of emerging formative biofabrication. Finally, although many researchers^[5-8] and market analysts still prefer to use the more familiar term “magnetic bioprinter,” we hold a genuine belief that the name “magnetic levitational bioassembler” is more relevant to nozzle-free technology.

2 What does the “scaffold-free” approach mean?

Rapidly emerging scaffold-free tissue engineering is a potential alternative to traditional scaffold-based tissue engineering. There are several already published conceptual reviews on scaffold-free tissue engineering. The scaffold could be broadly defined as a temporal removable (sometimes biodegradable) support^[9,10]. In line

with this definition, it is easy to demonstrate that many so-called scaffold-free technologies are scaffold-based. For example, (i) based on the most cited paper by Gabor Forgacs’s group on scaffold-free tissue engineering, removable agarose hydrogel is not considered as a scaffold^[11]; (ii) the removable supporting metallic needles in the most popular and already automated and commercialized scaffold-free tissue engineering technology are not considered as temporal support or scaffold^[12]; and (iii) in the so-called scaffold-free bioprinting technology that is based on LIFT, fibrin hydrogel is not mentioned as a scaffold^[13]. The absence of a clear definition of scaffold-free technology leads to some semantic confusion when authors use some sort of materials as temporal and removable support, and claim their techniques as “scaffold-free.” Conceptually, if it is not a scaffold-free but still scaffold-based technology, then it automatically loses its desirable principal conceptual novelty. We are facing the situation when some researchers who try to show pseudo-novelty and attract attention are using certain semantic tricks. The logical question arises: Does any actual scaffold-free or material-free tissue engineering technology exist? For example, magnetic force-based tissue engineering (Mag-TE) could also be considered as a scaffold-free or material-free technology. However, in our opinion, nanoparticles be classified just as a new form of scaffold or at least temporal and removable supporting structure with both intracellular and extracellular localization. Thus, Mag-TE is not material-free and cannot be considered as scaffold-free technology.

3 Label-based magnetic tissue engineering

Mag-TE is a type of scaffold-free but label-based biofabrication that uses magnetic nanoparticles for enabling magnetic levitation. This technology was initially developed by Ito and Honda as an attempt to optimize cell sheet technology, which was initially developed by Prof. Okano’s group in Japan^[14]. The application of magnetic nanoparticles enables manipulation, handling, and rapid assembling of cell sheets into 3D tissue

constructs. There are already several published well-written reviews describing label-based Mag-TE^[15-17].

The main limitation of this promising technology is the employment of potentially toxic magnetic nanoparticles. There are two opposite opinions about the toxic effect of magnetic nanoparticles. One group of researchers, i.e., Claire Wilhelm's group, is trying to demonstrate that superparamagnetic iron oxide nanoparticle is practically not toxic and could be biodegraded to natural ferritin^[18]. Moreover, a type of magnetic nanoparticles-based contrast agent called "Feridex" has been used in clinical practice for the decades. From another point of view, there is a growing number of researchers who demonstrate the toxic effect of magnetic nanoparticles and claim that the accumulation of iron-based magnetic nanoparticles could lead to undesirable side effects such as liver and spleen hemosiderosis or the local accumulation of iron.

In a recently published review^[19], the authors defined three main challenges for tissue engineering based on removable physical fields (including Mag-TE): (i) Transition from academic-styled biofabrication of mini tissues to biofabrication of clinically relevant tissue fragments; (ii) the use of more advanced materials; and (iii) development of affordable instrumentation. The current paper is dealing with the third challenge – the production of bioassembler for magnetic levitational bioassembly^[18].

4 Concept of "scaffield" and "formative biofabrication"

There are three main ways of manufacturing: (i) Subtractive, (ii) additive, and (iii) formative manufacturing^[19]. During a long period from the early attempts until the end of the 20th century, subtractive manufacturing was the primary way of making things from wood, stones, and metals. At the end of the last century, additive manufacturing, initially known as the rapid prototyping technology, became a brilliant technology that formed the technological basis of the so-called third or fourth industrial revolution. Formative manufacturing

uses processes such as injection molding, die casting, pressing, and stamping to create materials into the desired shape. In traditional formative manufacturing, molds are applied to achieve the desired shape of used materials.

The term "formative biofabrication" was coined by Prof. Chua Chee Kai at the International Bioprinting Congress in Singapore to describe magnetic and acoustic levitational bioassembly using magnetic and acoustic fields as desirable shape forming scaffold (personal communication). Formative biofabrication comprises the technologies that uses magnetic, acoustic, electric, photonic, and other physical forces for levitational bioassembly of 3D tissue and organ constructs. A new word "scaffield" has been proposed to define magnetic and acoustic fields, which serve as some sort of temporal and removable support for levitating and assembling biological objects.

Thus, formative biofabrication is a scaffold-free and label-free levitational bioassembly of 3D tissues and organs that uses the combination of different physical fields as removable temporal support for levitating living building blocks which are capable of self-assembly. The device enabling magnetic levitational bioassembly of 3D tissue and organs should be called magnetic "bioassembler." Nowadays, there are no commercially available magnetic levitational bioassemblers. Here, we describe the first one of bioassemblers.

5 The main dilemma of label-free magnetic levitational bioassembly

Label-free magnetic levitational bioassembly does not employ potentially toxic magnetic nanoparticles, which are often used in magnetic tissue engineering. Hence, label-free magnetic levitational bioassembly may avoid the problem of toxicity and associated undesirable potential side effect. However, the label-free magnetic levitational bioassembly uses a paramagnetic medium, for example, a medium that contains gadolinium salts. Gadolinium salts have already been approved for clinical imaging as a contrast agent in computer tomography by the U.S. Food and Drug Administration and other

national regulatory agencies. In the meantime, there is a growing body of evidence indicates that gadolinium can induce kidney fibrosis in human patients. Moreover, it has been shown that gadolinium blocks calcium channels and causes toxicological effects on cells *in vitro* and *in vivo*^[20-23]. The reduction of gadolinium salts concentration practically enables the complete elimination of undesirable toxic effects, but it could compromise the magnetic levitation. In other words, cells are alive, but they could not levitate. From another point of view, the increasing concentration of gadolinium salts not only enables magnetic levitation but also induces a significant cytotoxic effect.

Thus, cells can levitate, but they are dying in a toxic concentration of gadolinium. There are three theoretically possible, potential strategies to solve this dilemma: (i) The development of non-toxic gadolinium salts; (ii) the use of a high magnetic field to achieve a significant reduction of gadolinium salt concentration; and (iii) performing label-free magnetic levitational bioassembly under the condition of microgravity in space to decrease gadolinium salt concentration. We systematically tried to address and explore all three strategies. First, we failed to find non-toxic gadolinium or its adequate paramagnetic media alternative. Second, we were able to perform label-free magnetic levitational bioassembly in the high magnetic field with significant (more than 100 times) reduction of paramagnetic gadolinium salts^[24]. We also successfully performed a label-free magnetic levitational bioassembly at the microgravity conditions in space^[25].

6 Design and construction of bioassembler

“Organ.Aut” is a magnetic bioassembler that was created to study the possibility of formative 3D biofabrication of tissue structures using the method of self-assembly of living tissues and organs in microgravity (**Figure 1A and B**). The central part of the magnetic bioassembler is the inner case, with six pairs of magnets placed inside (**Figure 2B**). The shape of the case (**Figure 2A**) made it possible to minimize the overall dimensions of the device

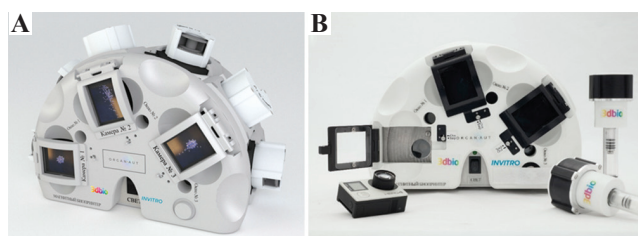


Figure 1. “Organ.Aut” is a magnetic levitational bioassembler. (A) Design of “Organ.Aut,” (B) the external view of “Organ.Aut.”

and conduct six experiments simultaneously. For the outer envelope, aluminum alloy D16-T was selected. Each pair of magnets (**Figure 2C**) was designed in such a way to form magnetic well in the center^[25].

The magnetic field was shielded to ensure the safe operation of the magnetic bioassembler on the International Space Station (ISS). Thus, individual screens were created (position 1, **Figure 2G**) from magnetically conductive stainless steel and connected to the inner case of the magnetic bioassembler. Furthermore, to ensure that various metal objects did not get into the magnetic bioprinter during its operation, unique covers with a diaphragm-based locking mechanism were added (**Figure 2E**). The material of these covers is magnetically conductive stainless steel.

Three cameras with special macro lenses were provided to visualize the biofabrication process. For this, Go Pro Hero 4 Silver (**Figure 2F**) having certificates for the use on ISS were used. Furthermore, a lighting unit which is powered by 6 A.A. batteries was provided for illumination during the process of video recording (**Figure 2D**).

A separate task was the development of special cuvettes for safe transportation of biological objects to the ISS, biological experiments, and fixing the fabricated constructs for further analysis (histological sections, etc.). Functionally, the cuvette is a system consisting of two V1 containers (the containers have the same volume but different contents) and the V2 biofabrication chamber. Between containers, there are valve assemblies that prevent fluid from moving from containers V1 to chamber V2 without pressing the pistons P (**Figure 3A**). The design of the cuvette was made of a transparent material

such as monolithic polycarbonate to provide airless filling of containers and the possibility of visual control of the biofabrication process. The inner parts of the cuvette are shown in **Figure 3B**. Since various fixatives, including paraformaldehyde, were used in the studies, the design of the cell requires three levels of biosafety. Accordingly, the layout of the cuvette that consists of three assembled cases was developed (**Figure 3C**). In addition to the three protection loops, the design of the cuvette includes a

locking mechanism that protects against accidental pressing of the piston systems (**Figure 3D**), which has a spring-loaded positioning mechanism in three positions (**Figure 3E**).

It should be noted that the use of the magnetic bioassembler “Organ.Aut” with cuvettes allows directed assembly of living biological constructs from tissue spheroids or various types of bacteria. Furthermore, it can be used to study the behavior of crystalline forms of chemical compounds

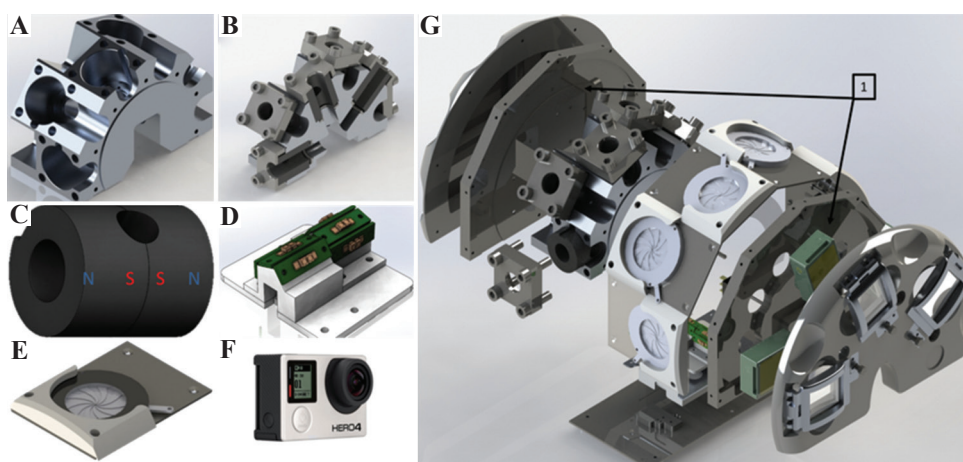


Figure 2. The basic design and construction of “Organ.Aut.” (A) The framework, (B) the framework with six pairs of cuvettes; (C) the assembly of two magnets; (D) the external view of illumination; (E) the external view of the cover shelf; (F) the external view of Go Pro Hero 4 Silver camera; (G) the blast-pattern of “Organ.Aut.”

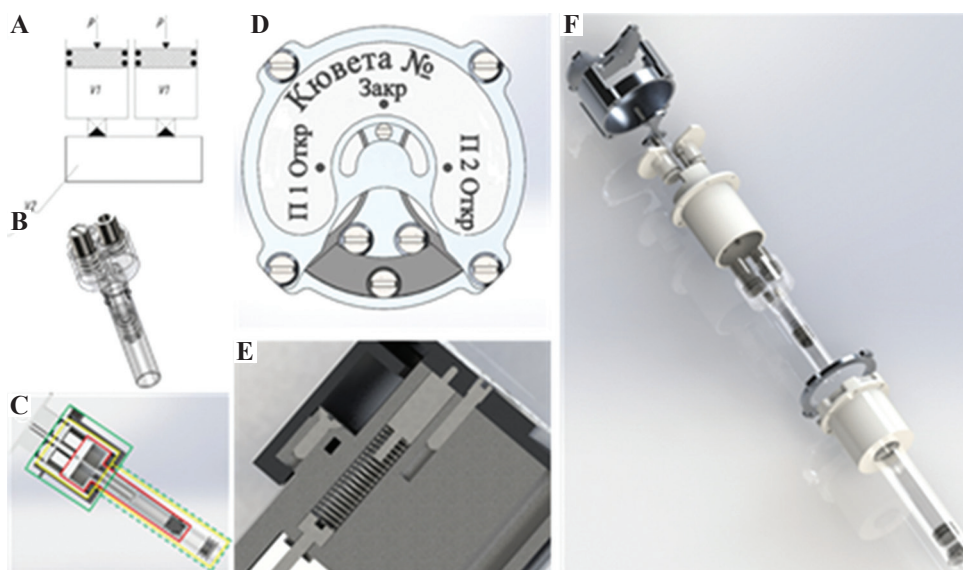


Figure 3. The design of cuvette. (A) The scheme of cuvette structure; (B) the inner parts of cuvette; (C) biosafety contours; (D) the protection from accidental hitting; (E) the fixation mechanism for protection from accidental hitting; (F) the assembly of the cuvette.

(inorganic components of the bone matrix, drugs, enzymes, hormones, etc.) placed in an inhomogeneous magnetic field with a high tension gradient. Thus, preliminary findings showed the unique capabilities of the magnetic fields used in the magnetic bioassembler “Organ.Aut” to induce faster recrystallization of synthetic calcium phosphates compared to traditional methods of inorganic synthesis, making it possible to obtain promising materials for bone grafting with high efficiency. Another area of research may be the assembly of 3D structures from bacteria in a magnetic field. Such conglomerates, otherwise called “bacterial biofilms,” are characterized by the ability to create or simulate volumetric living conditions under which bacterial cells exhibit different growth and synthetic activity compared to cultivation on flat surfaces.

7 Features and functionalities of magnetic bioassembler

Undeniably, our original magnetic levitational bioassembler has some unique advantages, which are outlined and emphasized in the following:

1. Magnetic bioassembler has a relatively small size, and it is not very heavy; therefore, it is compact, transportable, and space-saving.
2. Magnetic bioassembler is safe. It has three contours of defense for its chambers, and it has the necessary certification for use on the ISS.
3. Magnetic bioassembler has six chambers that allow multiple experiments to be run simultaneously; this may help with the generation of statistically sound experimental data.
4. Magnetic bioassembler has a video camera that assists experiment monitoring in real-time from long distance.
5. Magnetic bioassembler has a robust and durable design. This is evidenced by the sturdiness of the chambers as at least one from the six chambers survived the vigorous collisions as they fell with part of Russian spaceship from Space to the Earth. Now, the specimen is preserved in The Russian Museum of Space Research.
6. Magnetic bioassembler is very easy to operate. Russian cosmonauts have been trained on how to use bioassembler on the earth, and then they performed planned experiments in space without any technical problems.
7. Magnetic bioassembler could be optimized either by increasing its complexity (e.g., complete automation) or simplification (e.g., reduction of bioassembler size and number of its chambers).
8. Magnetic bioassembler has batteries. It is an autonomous device that does not need an external power supply.
9. Trained personnel are not required for the maintenance of magnetic bioassembler.
10. Magnetic bioassembler is commercially available, and it could serve as affordable instrumentation for performing magnetic levitational bioassembly in space.

8 Magnetic levitational bioassembly in microgravity in space

To perform the magnetic levitational bioassembly experiments under the condition of microgravity in space at The Russian Orbital Segment, three main steps have to be followed: (i) Delivering bioassembler and tissue spheroids in the thermo-reversible hydrogel to the space station; (ii) performing the actual magnetic levitational bioassembly experiments at the ISS; and (iii) fixing biofabricated 3D tissue constructs in space and delivering fixed specimen to the earth for sequential histological analysis (**Figure 4**).

The logistics of space experiments involving living cells and tissue are hugely complex and challenging. Getting official permission to perform operations on the ISS needs tremendous efforts and an unbelievable amount of paperwork. The certification of magnetic bioassembler is also not an easy task. Finally, the researchers are also running the risk of unable to perform the experiments due to unexpected technical problems with a spaceship. Despite the apparent limitations and restrictions associated with conducting space research, we were still able to get exciting results. We developed original cuvette with three levels

of defense or three contours of biosafety. We founded commercial thermo-reversible hydrogel, which allowed safe delivery of viable tissue spheroids without preliminary undesirable tissue fusion. Magnetic levitational bioassembly of 3D tissue constructs from chondrospheres (tissue spheroids prepared from a primary culture of human chondrocytes) using original magnetic levitational bioassembler have been successfully performed (**Figure 5**). After a short incubation, the bioassembled 3D tissue constructs were fixed in formalin and delivered to the earth for subsequent histological investigation. The correspondent article has been accepted for publication^[25].

9 Challenges and future perspectives: Biomaterials are forever

The main challenge in the development of magnetic levitational bioassembly as well as in the broader field – formative biofabrication is the potential employment of biomaterials. On the one hand, formative biofabrication is positioned as a

truly scaffold-free technology. On the other hand, it is becoming increasingly apparent that without biomaterials, this field could not evolve. How could these obvious contradictions be logically solved? How can one still use biomaterials and, at the same time, call magnetic biofabrication technology scaffold-free? At first, we must remember that any bioprinting or biofabrication technology has three main steps: (i) Pre-processing or design digital model and fabrication of building blocks; (ii) processing or actual biofabrication or bioassembly of 3D tissue and organs construct from building blocks; and (iii) post-processing. Second, to be precise, scaffold-free formative biofabrication refers to the second step or actual bioassembly, in which magnetic levitational bioassembly is indeed scaffold-free. However, using scaffold-free levitational bioassembly at the second step does not necessarily eliminate the need for using biomaterials for fabrication of building blocks at the first step and for accelerated tissue maturation at the third, post-processing step. Thus,

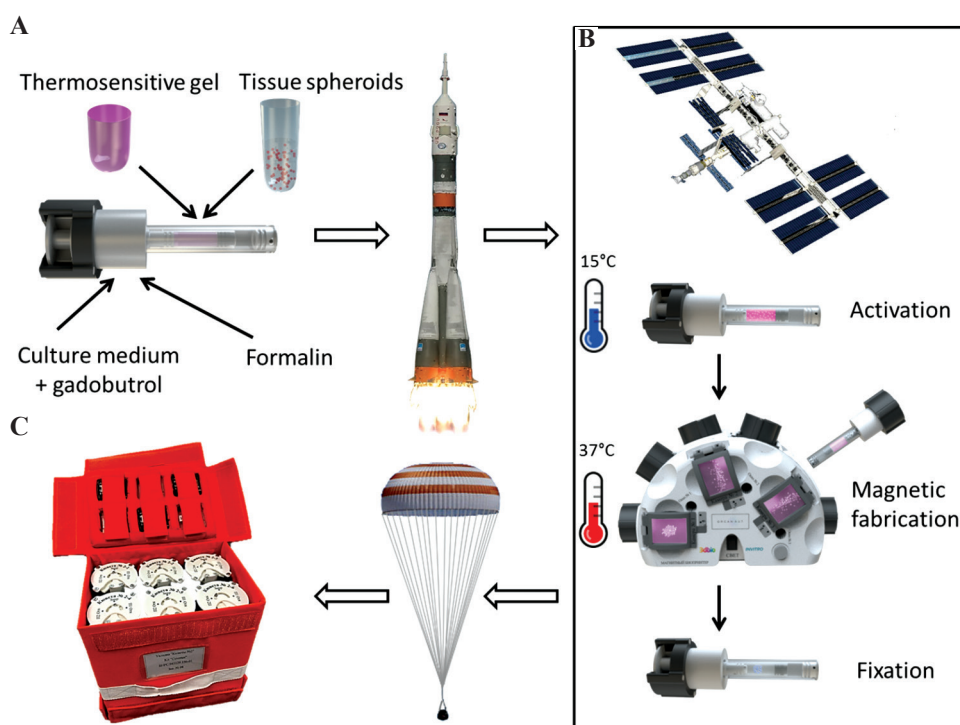


Figure 4. Schematic diagram of magnetic levitational bioassembly experiments at the Russian Orbital Segment (with permission from Science Advances)^[25]. (A) The filling of the cuvettes and their delivery to International Space Station (ISS); (B) the scheme of experiment on ISS; (C) the transportation of the cuvettes from ISS to earth.

such an approach not only solves the contradiction but also opens new avenues for chemists and material scientists to contribute to the development of formative biofabrication technology. The new types of synthetic and natural hydrogels could be used before bioassembly for sizing, shaping, and guided differentiation of organoids as potential new building blocks for scaffold-free formative biofabrication. Moreover, biomaterials and the special, thin fusogenic coating could be used for remote-controlled enhancements of tissue fusion process after initial bioassembly of 3D tissue and organ constructs. Finally, biomaterials could be used for directed tissue differentiation

and accelerated tissue maturation during post-processing. Hence, biomaterials are forever!

The development of magnetic levitational bioassembly is associated with another big challenge – toxicity of gadolinium salt. One of the potential ways to solve this problem is to develop novel bioassembler based on superconductive magnets. High magnetic fields generated by superconductive magnets reduce the concentration of gadolinium salts. To decrease power consumption, the superconductive magnets could be cooled by liquid nitrogen, which is a more affordable alternative compared to the expensive water perfusion-based cooling system.

The second potential strategy which could be employed for reducing concentration of gadolinium salts is the development of hybrid levitational bioassembly technology which is a combination of different physical forces and field. The hybrid magneto-acoustic bioassembler with built-in perfusion bioreactor allows switching from initial magnetic levitation to acoustic supporting levitation and complete removal of paramagnetic gadolinium salts through the nutrient flow.

The third perspective approach is the development of laser magneto-acoustic bioassembler. We already demonstrated that laser could rotate, elongate, and split 3D tissue

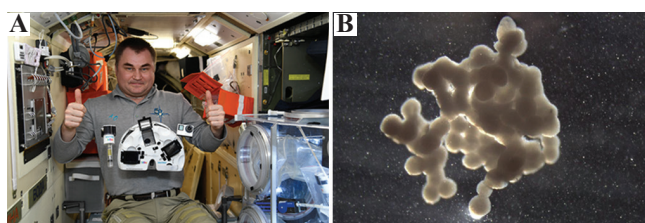


Figure 5. Space experiments. (A) “Organ. Aut” bioassembler at The Russian Orbital Segment, operated by Russian cosmonaut Alexey Ovchinin; (B) 3D tissue construct assembled with magnetic levitational from chondrospheres under microgravity in space on the International Space Station.

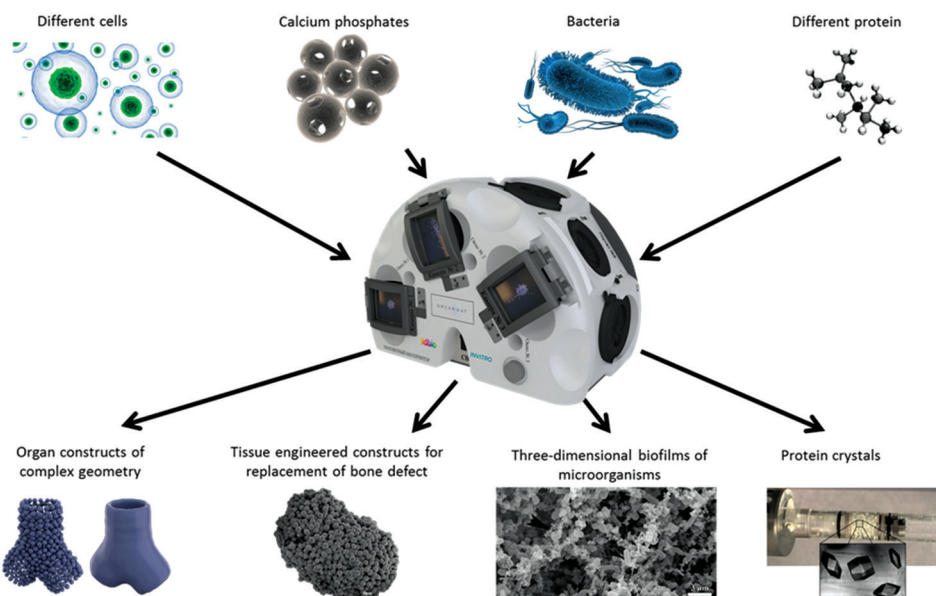


Figure 6. Potential applications of magnetic levitational bioassembler.

constructs. A laser could also be useful for the rapid fusion of tissue spheroids coated by a thin layer of photo-sensitive hydrogel after their magnetic levitation bioassembly in 3D tissue construct. The laser can be used for shaping more complex 3D tissue construct either by optogenetic apoptosis or laser-induced hyperthermia. A combination of magneto-acoustic levitational bioassembler with a laser could potentially improve the functionalities of bioassembler and enhances its capacities to form 3D tissue and organ constructs with complex geometry and structure. Incorporation of additional imaging facilities such as micro-computed tomography inside the bioassembler is also desirable, although it will significantly increase the cost of the instrument. Hence, there is also an unmet need for affordable bioassemblers.

10 Conclusion

The conceptual framework of scaffold-free, label-free, and nozzle-free magnetic levitational bioassembly has been described. Label-free magnetic levitational bioassembly is probably the most relevant example of the rapidly emerging, scaffold-free tissue engineering technology. The principal difference between label-free magnetic levitational bioassembly and the more traditional scaffold-based and label-based (using nanoparticles) magnetic tissue engineering are discussed. New terminology, including formative biofabrication, scaffold, and bioassembler, which represents the integral part of the novel conceptual framework, is also introduced.

Design, construction features, unique functionalities, and potential areas of applications of original magnetic levitational bioassembler have been described and illustrated. The most interesting example of using magnetic bioassembler for magnetic levitational bioassembly on the ISS under the condition of microgravity in space has been presented (**Figure 6**).

The challenges and perspectives of using magnetic bioassembler have been discussed. It is logical to assume that a combination of magnetic and acoustic levitation, as well as a combination of magnetic and acoustic levitation with laser or

photonic technology, will help develop a more functional magnetic bioassembler.

It is becoming evident that the development of affordable commercial bioassembler capable of rapid magnetic levitational bioassembly of clinically relevant tissue-engineered constructs in a non-toxic environment will become one of the main challenges.

As presented in this paper, original magnetic levitational bioassembler is the first commercial instrumentation specially designed for advancing scaffold-free tissue engineering and its clinical translation. While many researchers refer to their bioprinters as magnetic 3D bioprinters, we firmly believe that these instruments should be known as the “magnetic bioassembler for formative biofabrication.”

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Conflicts of interest

The authors declare no conflict of interest.

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