

DEJAN MARKOVIC<sup>1</sup>  
IVANA KARADZIC<sup>2</sup>  
VUKOMAN JOKANOVIC<sup>3</sup>  
ANA VUKOVIC<sup>1</sup>  
VESNA VUCIC<sup>2</sup>

<sup>1</sup>Department of Paediatric and Preventive Dentistry, Faculty of Dentistry, University of Belgrade, Belgrade, Serbia

<sup>2</sup>Centre of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade, Belgrade, Serbia

<sup>3</sup>Laboratory of Radiation Chemistry and Physics, Institute of Nuclear Sciences "Vinca", University of Belgrade, Belgrade, Serbia

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## BIOLOGICAL ASPECTS OF APPLICATION OF NANOMATERIALS IN TISSUE ENGINEERING\*

### Article Highlights

- Stem cells and scaffolds - an essential role in the production of new tissue by tissue engineering
- Nanotechnology - a field of high importance and rapid development
- Functional necessities of scaffolds - biocompatibility, biodegradability and mechanical properties
- The main challenge: transforming tissue engineering into regenerative engineering

### Abstract

*Millions of patients worldwide need surgery to repair or replace tissue that has been damaged through trauma or disease. To solve the problem of lost tissue, a major emphasis of tissue engineering (TE) is on tissue regeneration. Stem cells and highly porous biomaterials used as cell carriers (scaffolds) have an essential role in the production of new tissue by TE. The cellular component is important for the generation and establishment of the extracellular matrix, while a scaffold is necessary to determine the shape of the newly formed tissue and facilitate migration of cells into the desired location, as well as their growth and differentiation. This review describes the types, characteristics and classification of stem cells. Furthermore, it includes functional features of cell carriers - biocompatibility, biodegradability and mechanical properties of biomaterials used in developing state-of-the-art scaffolds for TE applications, as well as suitability for different tissues. Moreover, it explains the importance of nanotechnology and defines the challenges and the purpose of future research in this rapidly advancing field.*

*Keywords: tissue engineering, nanomaterials, scaffolds, stem cells, tissue regeneration.*

### Tissue engineering

Millions of patients worldwide need surgical procedures to repair or replace tissue that has been damaged through trauma or disease [1]. Today, conventional therapy addresses the problem of lost tissue by appropriate tissue replacement - tissue graft. The majority of defects can be healed using standard conservative or surgical methods. However, large defects occurring after tumor surgery, cysts or multiple frac-

tures require a more complex procedure of tissue repair [2,3].

With respect to lost tissue treatment, the main emphasis of tissue engineering is on tissue regeneration (TE) rather than tissue replacement [4-7]. Thus, stem cells and highly porous biomaterials used as scaffolds have an essential role in the production of new tissue by TE (Figure 1). The cellular component is important for the generation and establishment of extracellular matrix (ECM) in the new tissue, while a scaffold is necessary for providing mechanical stability and foundation for a new three-dimensional tissue structure [6,8,9].

Since it has been demonstrated that biological systems correspond better to nano- than micro-dimensional biomaterial structures, nanotechnology has become a field of high importance and rapid development [10-12]. Also, composition, size, morphology and geometry of nanostructured materials can be

Correspondence: I. Karadzic, Centre of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade, Tadeusa Kosciuska 1, P.O. Box 102, 11129 Belgrade, Serbia.

E-mail: [ivana.colak@gmail.com](mailto:ivana.colak@gmail.com)

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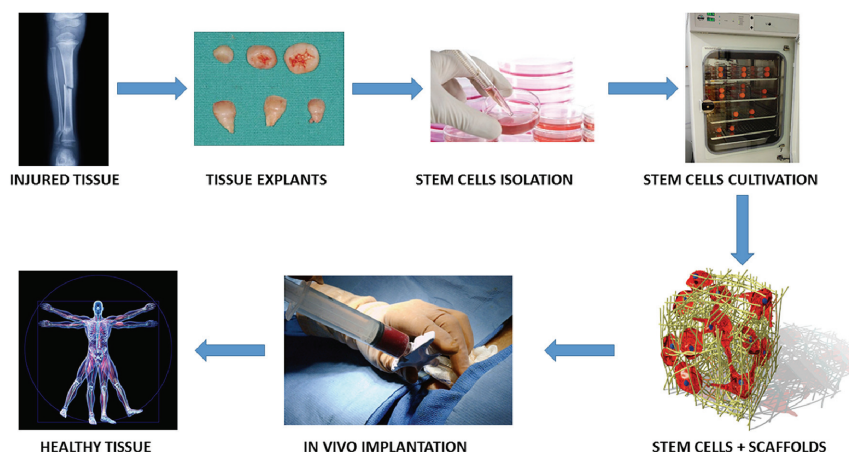


Figure 1. Basic concept of tissue engineering.

controlled. Further, the surface of these materials can be modified in order to enhance biocompatibility, immune compatibility and/or cell adhesion [13].

This review describes the functional necessities and types of stem cells and biomaterials used in developing state-of-the-art scaffolds for tissue engineering applications. Furthermore, it defines the challenges and the purpose of future research in this fast advancing field.

### Stem cells and tissue engineering

Stem cells are unspecialized cells in the early stage of the development, which under normal conditions have the ability to differentiate into specialized mature cells and to divide in order to produce more stem cells [14–17]. Two functions define stem cells: unlimited self-renewal capacity, which makes them potentially immortal, and pluripotency [18]. Stem cells can be divided in several different ways, as shown in Table 1 [15,19–22].

Although they have less ability to differentiate, adult stem cells are far more applicable in regenerative medicine than embryonic stem cells, primarily because of being relatively easy to isolate, lack of oncogenic potential and no ethical constraints over their application [23–26].

### Architecture and nanotechnology of scaffolds

The ECM represents a biological 3D carrier for the cells and provides appropriate environment and architecture specific for each tissue [27]. Therefore, the key to a successful TE is proper design of cell carriers - scaffolds, which mimic the native ECM, combined with adequate stem cells. The role of these carriers is to determine the shape of the newly formed tissue and facilitate the migration of cells into the desired location, their growth and differentiation [28,29].

The key characteristic of every scaffold is that it must be biocompatible - to provide physical and mechanical functions and provoke a preferred response without causing any undesirable reactions in the host. Hence, the choice of material is a crucial point in tissue engineering [28,30]. It is desirable for the scaffold to disintegrate during the formation of new tissue, to allow the body's own cells, over time, to eventually replace the implanted material [31]. Therefore, the biodegradability of the scaffold is also considered very important in these processes.

Developing scaffolds with adequate mechanical properties is one of the greatest challenges in attempting to engineer bone or cartilage [32,33]. A balance must be achieved between the mechanical

Table 1. Stem cells classification according to different criteria

Criterion	Stem cells type	Properties
Cell potency	Totipotent stem cells	Potential to differentiate in any human cell even whole organism
	Pluripotent stem cells	Potential to differentiate in various tissue types but not whole organism
	Multipotent stem cells	Potential to differentiate in various cell types within tissue - progenitor cell
	Unipotent stem cells	Potential to differentiate in one cell type - precursor cell
Function	Normal stem cells	Not involved in pathologic process
	Cancer stem cells	Associated with most cancer disease
Sources	Embryonic stem cells	Derived from inner cell mass of blastocyst
	Adult stem cells	Derived from endoderm, mesoderm or ectoderm

properties and a sufficiently porous architecture in order to obtain the desired scaffold [34]. Adequate porosity allows cell migration and provides a suitable microenvironment for cell proliferation and differentiation, with adequate vascularization, flow of nutrients and oxygen and elimination of degradation products [35–37]. The porosity should be in the optimal range: small enough to ensure mechanical integrity and sufficiently large to provide optimal bioactivity. For this reason, the size of the pores should be less than 300 nm [29].

Two types of materials are currently used in TE: natural and synthetic [38]. The advantage of natural materials is biological recognition regarding cell adhesion and function. However, the downsides are uncontrolled mechanical properties and biodegradability, possible host immune reaction and the cost [39,40]. Various natural materials have been evaluated to date. Derivatives of ECM have been investigated for supporting cell growth. Proteins collagen and fibrin, as well as polysaccharides glycosaminoglycans, have all proved appropriate regarding cell compatibility, but some potential immunogenic issues still remain [35,41,42]. Hyaluronic acid, one of the most exploited glycosaminoglycans, in combination with glutaraldehyde or water soluble carbodiimide, is considered suitable for scaffold materials [43]. Polysaccharide chitin and chitosan based nanofibers have remarkable potential to be used as tissue engineering scaffolds, as well as drug delivery systems, wound dressing materials, antimicrobial agents and biosensors, due to their biocompatibility, biodegradability, antibacterial activity, low immunogenicity, wound healing capacity and cell binding capability [44]. Further, some scaffolds are tested for use in the delivery of small molecules (drugs) to specific tissues [45]. Finally, decellularized tissue extracts in which

the remaining cellular residues or ECM act as a scaffold, are another form of cell carriers undergoing investigation [46].

Synthetic materials, on the other hand, have the advantage of a commercial production, together with a control over mechanical properties, microstructure and degradation rate.

A commonly used synthetic material is polylactic acid (PLA). This is a polyester which degrades within the human body to form lactic acid, a chemical compound that plays a role in various biochemical processes and is easily removed from the body. The nanofibrous PLA mats incorporating carbon nanotubes and rectorite, fabricated using an electrospinning technique, have proven suitable for biomedical applications due to their increased thermal stability and low cytotoxicity [47]. Similar to PLA are polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA) and polycaprolactone (PCL), with degradation mechanisms similar to that of PLA but a different rate of degradation compared to PLA [48,49]. Other natural and synthetic materials have also been used in scaffold synthesis (Figure 2).

Due to their unique chemical, physical and biological functions, nano-sized particles/fillers, of both inorganic and organic origin, have been studied in detail. They differ in their structure, composition, design and application, and can be in the form of nanofibers, nanogels, etc, as presented in Figure 2. Nanocomposite hydrogels combine the advantages of nano-fillers and hydrogel matrices and thus may result in improved mechanical and biological properties and find their potential application in biomedicine as drug delivery matrices and scaffolds [50]. Recently, injectable scaffolds have received attention due to their potential for avoiding the invasive surgery normally required for tissue implantation. Natural

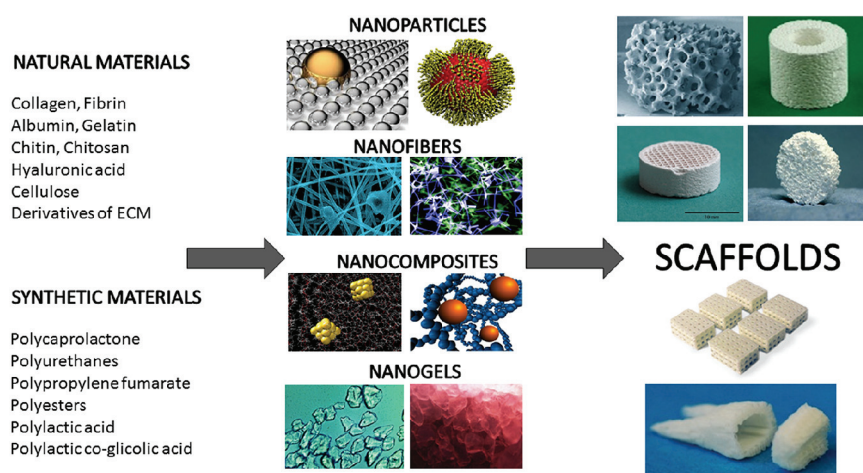


Figure 2. Synthesis, structure and design of different forms of nanomaterials used as scaffolds in tissue engineering.

polymers chitosan and alginate are used as coating materials to make positively and negatively charged PLGA nanoparticles, respectively. All the results demonstrate the potential use of the biodegradable colloidal gels as injectable scaffolds in tissue engineering and drug release [51].

### Application of scaffolds in tissue engineering

Bone disease or bone defects such as osteosarcoma, osteoporosis, and bone fractures affect millions of individuals worldwide [1]. In order to solve the problem of lost bone tissue, bone tissue engineering emphasizes tissue regeneration rather than tissue replacement and is becoming a subject of growing interest. For over two decades, bioceramic material - hydroxyapatite (HAP) has been used as a substitute for bone as it has physical properties similar to the inorganic component of natural bone [52]. It is suitable material for hard tissue replacement due to its osteoconductivity, biocompatibility and slow resorption. On the other hand, the porosity of hydroxyapatite translates into poor mechanical properties [53]. In the attempt to compensate for these disadvantages, various polymers have been examined, however, none of these meet all of the requirements for the ideal cell carrier in bone TE [54]. This has led to the development of composite carriers consisting of both an inorganic and organic component where the inorganic particles are embedded into the surface of a polymer matrix [36,55]. Inorganic-organic composites are designed to mimic natural bone by combining the viscoelastic properties of polymers with the strength of the inorganic part of the composite, to create bioactive materials with improved mechanical properties and ability to degrade over time [56]. Also, the basic products of decomposition of hydroxyapatite and tricalcium-phosphate neutralize the acidity of the polymeric compounds. Regarding higher bioactivity, inorganic nanostructured components have received more interest than equivalent microstructured ones [57]. Nanocomposites based on hydroxyapatite-collagen are being particularly rapidly developed and showing promising results [58,59]. Recently, negatively charged inorganic hydroxyapatite nanoparticles (NPs) and positively charged organic PLGA NPs were assembled to form a cohesive colloidal gel which proved to be suitable as an injectable filling for the purpose of bone tissue regeneration [60]. Further, *in vivo* tests revealed that a similar colloidal gel, created by mixing PLGA nanoparticles of opposite charge, capable of controlled release, has shown good results as a filler for repair of cranial bone defects [61]. Use of a composite scaffold with high porosity (low mech-

anical properties) and fast degradation kinetics has led to the production of grafts that can be used in low load sites. In the middle of the last decade, a unique composite carrier consisting of a combination of biodegradable PLGA and bioresorbable calcium phosphate cement was created. This carrier is characterized by high porosity (81-91%), with macropores of 0.8-1.8 mm, and improved mechanical properties due to the polymer [62]. Another 3D scaffold suitable for bone regeneration, with porous design and mechanical properties similar to the trabecular bone is obtained by combining calcium phosphate ceramics (low crystalline CaP) fused in biodegradable PLGA microspheres [63].

Scaffolds are commonly applied in bone and cartilage tissue engineering, although notable results have been achieved in many other tissues, including skin, nerves, heart etc. However, different tissues have their own peculiarities. For instance, skin tissue engineering is complex for various reasons: greatly limited donor sites in patients with skin losses over 50-60%, transmission of infection (mostly human immunodeficiency virus or hepatitis related to allogeneic skin grafting), pain, and scarring at donor sites [64]. Several scaffolds have been investigated for applications in skin TE. In a rat model, regeneration of tissue with similar properties to the native dermis and significantly enhanced formation of blood capillaries has been achieved after a novel composite film of salmon DNA and collagen was implanted in a full-thickness skin wound in the dorsal region [65]. Further, alginate is also considered a promising agent in skin tissue engineering due to its ability to maintain a physiological, moist, microenvironment, reducing possibility for bacterial infection, and facilitating wound healing especially in deep-thickness wounds [66]. Polysaccharide chitin has been combined with other marine-derived composites and developed into a hydrogel wound dressing, providing good moist healing environment and encouraging capillary formation in a full-thickness skin wound in rat [67]. Composite nanofibrous cellulose acetate 3D mat coated with positively charged lysozyme and negatively charged layered silicate - rectorite, obtained by electrospraying, has shown promising results in pharmaceutical uses and antimicrobial wound dressing [68]. In addition, a nanofibrous scaffold made of PLGA-chitosan/polyvinyl alcohol, fabricated by electrospinning, is proving to be useful in skin tissue engineering [42]. Recently, novel nanofibrous mats have been developed, coated layer-by-layer with silk fibroin and lysozyme on a cellulose electrospun template via electrostatic interaction. These mats are promising tools in

dermal reconstruction due to their nontoxic, biodegradable, biocompatible, antibacterial and wound healing properties [69].

In contrast, in cardiac tissue engineering the aim is to produce tissue constructs that are thick and compact, contain physiological densities of metabolically active cardiac cells, and contract synchronously in response to electrical stimulation with sufficient force [70]. The most difficult requirements are probably related to the establishment of blood flow and to the integration and electromechanical coupling with the host tissue. Shinoka [71] presented the first results of tissue engineered heart valves implanted into the juvenile sheep model. The scaffolds used for these heart valves were created from biodegradable polymers which were seeded *in vitro* with autologous valve cells. Recently, additional studies were performed to examine tissue activity at the mitral site of the experimental model, allowing to assess the highest tissue stress that can be achieved by tissue engineered heart valves [72]. The next challenge is to insert these heart valves with minimally invasive technique without alteration of the tissue during the implantation. Finally, the field of the whole organ engineering has been expanding, in which the bio-artificial heart [73] could overcome the problem of organ deficiency for patients suffering from end-stage heart failure.

In the case of cartilage produced for the auricle and nose or for complex facial trauma, the emphasis is on maintaining the shape and accurate reproduction of the intended geometry [74]. Also, it is very important to imitate the mechanical characteristics found in the native cartilage and to control the mechanical stimulus for chondrogenesis and ECM production. Chen *et al.* [74] have shown that collagen in its natural form is a better surface modification material than gelatin for promoting cell adhesion, proliferation and secretion of ECM components. Transplantation of peptide hydrogels made of nanofiber scaffolds containing chondrocytes and growth factors, into cartilage defects in a bovine model, resulted in extensive synthesis of glycosaminoglycans and type-II collagen similar to the native cartilage [75]. *In vitro* experiments revealed that peptide scaffolds comprising growth factors are capable of inducing chondrogenic differentiation of human mesenchymal stem cells (MSCs). These scaffolds can promote substantial regeneration of the articular cartilage in full thickness chondral microfractured defects in the trochlea of adult rabbits in the presence of bone marrow MSCs [76].

In nerve tissue engineering, due to the complex system, involving neural cells, the microenvironment with a variety of cell receptors, the ECM and specific chemo-physical properties, electrospun guidance channels and hydrogels are considered to be the most promising types of scaffolds [77]. The soft nature of the nervous tissue potentially makes hydrogels the ideal material, considering also their biodegradability, flexibility and low inflammatory potential. Adequate matching between the mechanical properties of different materials and specific neural environments is crucial in achieving the correct morphology, neural growth and differentiation. It has been demonstrated that neurite extension in dorsal root ganglia cells conversely correlates with the mechanical stiffness of agarose gels [78]. Many natural and synthetic polymers have been investigated for use as neural scaffold materials. For example, a biodegradable glass material was used to repair the facial or median nerve in a sheep model [79] and carbon nanostructures, including nanotubes, nanofibers and graphene have been incorporated in some experimental neural prostheses and guides [80].

Various types of tissues and their stem cells that are commonly used and described for application in tissue engineering are shown in Figure 3.

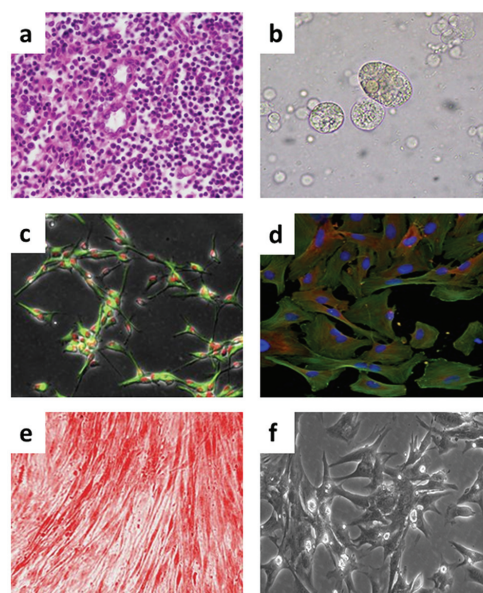


Figure 3. Various types of adult stem cells used in tissue engineering: (a) skin, (b) ligaments and cartilage, (c) brain, (d) muscle, (e) bone and (f) cardiovascular.

#### Examples of nanostructured scaffolds applied in bone TE

One of the most frequently used polymers for composite scaffolds today is PLGA, primarily due to

its proven biocompatibility and a variable degradation rate that can be regulated by modifying the proportions of its constituent polymers, PLA and PGA [2,5,81,82]. Moreover, the reversibility of a colloidal gel composed of oppositely charged PLGA nanoparticles makes it excellent material for molding, extrusion or injection of tissue engineering scaffolds [83]. There are, also, newly developed materials based on PLGA/HAP composites that are interesting for TE because of their high biocompatibility and ability to mimic natural bone. These materials have become a promising tool in load-bearing bone TE, and might provide optimal cell differentiation and mineralization of the bone tissue. Cells seeded on such materials easily adhere, especially on hydroxyapatite surface, which indicates good cell proliferation and integration of the bone implants [84].

In order to obtain better characteristics of scaffolds, new materials and new scaffold producing techniques are currently in development. Appropriate morphology of scaffold walls can be attained by using nanodesign hydroxyapatite particles inside a biomimetic medium, where they self-assemble on a polymer/ceramic scaffold structure [38]. It is an improvement on design of scaffold obtained using polymeric foam template based in polyurethane. This biomimetic method has shown that it stimulates the growth of “bone-like” structures on scaffold surfaces [85]. In this way, nanodesigned biomimetic apatite is very similar to biological apatite and very suitable for cell growth and proliferation [8].

A composite scaffold PLGA/HAP registered under the name ALBO-OSS has shown good cell adhesion of cells grown in control medium. Cells were spherical with clearly visible pseudopodia or cytoplasmic extensions (Figure 4a). After 7 days of culture in osteogenic medium, polygonal cells with very elongated cytoplasmic extensions were detected. The org-

anic fibrous-like structures have also been observed (Figure 4b). These structures and cell morphology are typical for stem cells undergoing osteogenic differentiation and indicate the beginning of the ECM formation. After 21 days, ECM dominated in the SEM micrographs covering the scaffold pores (Figure 4c). This highly developed ECM network demonstrated extensive differentiation and good biocompatibility between cells and materials, which is essential for use in tissue engineering [36].

Comparing the nano- and microstructured scaffolds, it was demonstrated by a difference in the optical density obtained by MTT testing and ALP activity that the larger surface area of the nanostructured scaffold allows better adhesion and provides more space for the differentiation of mesenchymal cells than in microstructured scaffolds [86].

Comparing the adhesion and quantity of human osteoblasts cultured on Bio Oss and synthetic bone substitute - Nano Bone for 7 days by SEM analysis, a significantly higher number of cells with cytoplasmic extensions was observed in the presence of Nano Bone [87].

SEM analysis showed similar results after studying cell morphology and adhesion of mesenchymal cells grown for 7 days on PLLA and on nano- and micro-HAP/PLLA composite scaffolds. The cells were spherical and their number was much higher in the presence of nano HAP/PLLA composite scaffold, indicating a superior biocompatibility of that material [88]. Hence, there are many scaffolds that exhibit good biocompatibility and could replace materials, such as golden standard Bio OSS, currently used in bone tissue engineering.

### Challenges and future research

The main requirement for a successful application of the scaffold is a high control level of their

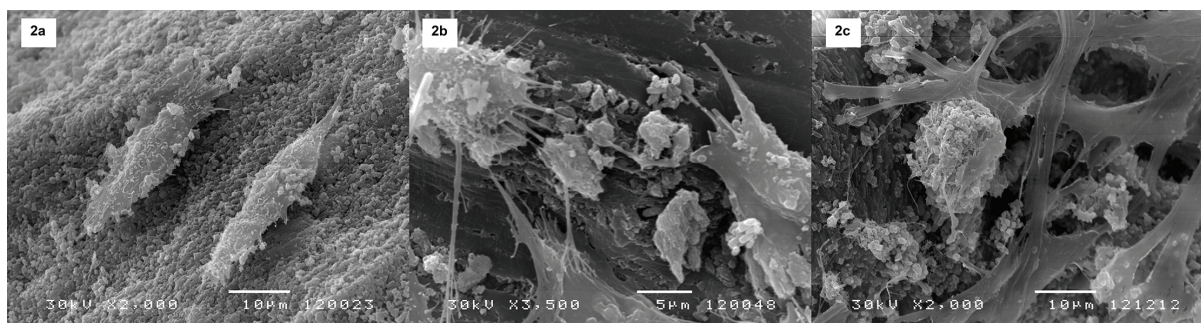


Figure 4. Scanning electron microscopy of dental pulp stem cells from deciduous teeth on Albo-Oss (porous hydroxyapatite + PLGA composite) scaffold: a) Spherical cells with cytoplasmic extensions indicate good cell adhesion after 7 days in control medium; (b) polygonal cells with elongated cytoplasmic extensions pseudopodia indicate very good cell adhesion; fibrous-like organic structures point toward beginning of ECM production after 7 days in osteogenic medium; (c) ECM was dominant in the SEM micrographs, covering the scaffold and the pores after 21 days in osteogenic medium.

micro- and macrostructural properties during production process. Mechanical properties of today's composite scaffolds still do not fully satisfy the properties of natural bone nor succeed in reaching their anisotropy [89].

Though a wide-range of strategies have been employed to produce the ideal scaffold that possesses the optimum dimensions, porosity, topography and mechanical properties, the clinical success of such constructs remains elusive [90].

The rapid development of techniques and fabrication tools that have arisen in the recent years have signified new beginnings in the field of TE. However, there are still obstacles in achieving the success of the *in vitro* experiments in an *in vivo* system. Moreover, the complexity of multiple tissues that form a functional organ poses a real challenge to tissue engineers. Further developments are awaited in reaching the goal of creating a completely functional organ, thus truly transforming tissue engineering into regenerative engineering.

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DEJAN MARKOVIĆ<sup>1</sup>  
IVANA KARADŽIĆ<sup>2</sup>  
VUKOMAN JOKANOVIĆ<sup>3</sup>  
ANA VUKOVIĆ<sup>1</sup>  
VESNA VUČIĆ<sup>2</sup>

<sup>1</sup>Klinika za dečju i preventivnu stomatologiju, Stomatološki fakultet, Univerzitet u Beogradu, Dr Subotića 11, 11000 Beograd, Srbija

<sup>2</sup>Centar izuzetnih vrednosti za ishranu i metabolizam, Institut za medicinska istraživanja, Univerzitet u Beogradu, Tadeuša Koščuška 1, 11000 Beograd, Srbija

<sup>3</sup>Laboratorija za radijacionu hemiju i fiziku, Institut za nuklearne nauke "Vinča", University of Belgrade, Mike Petrovića Alasa 12-14, 11000 Beograd, Srbija

NAUČNI RAD

## BIOLOŠKI ASPEKTI PRIMENE NANOMATERIJALA U TKIVNOM INŽENJERSTVU

*Milioni pacijenata širom sveta imaju potrebu za hirurškim procedurama radi reparacije ili nadoknade oštećenog tkiva nakon traume ili oboljenja. U cilju rešavanja problema izgubljenog tkiva, tkivno inženjerstvo glavni akcent stavlja na tkivnu regeneraciju a ne na zamenu tkiva, zbog čega postaje predmet sve većeg interesovanja. Matične ćelije i ćelijski nosači - visoko porozni biomaterijali, tzv. skafoldi, imaju esencijalnu ulogu u stvaranju novog tkiva putem tkivnog inženjerstva. Ćelijska komponenta je neophodna zbog stvaranja i uspostavljanja ekstracelularnog matriksa, dok je skafold zadužen za da odredi oblik novostvorenog tkiva i olakša migraciju ćelija na željeno mesto, njihov rast i diferencijaciju. Ovaj pregledni rad opisuje vrste, karakteristike i klasifikaciju matičnih ćelija. Osim toga, uključuje i neophodne funkcionalne osobine ćelijskih nosača - biokompatibilnost, biorazgradljivost i mehanička svojstva biomaterijala u primeni tkivnog inženjerstva. Takođe, objašnjava razlog interesovanja za praktičnu primenu nanomaterijala i nanotehnologije i definiše izazove i značaj daljih istraživanja u ovoj oblasti.*

*Ključne reči: tkivno inženjerstvo, nanomaterijali, ćelijski nosači (skafoldi), matične ćelije, regeneracija tkiva.*