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Serbian Dental Journal, vol. 64, N° 1, 2017

INFORMATIVE ARTICLE
INFORMATIVNI RAD

DOI: 10.1515/sdj-2017-0004
UDC: 615.46; 616.71-089.843

Scaffold in bone tissue engineering

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SUMMARY

Treatment of bone tissue injuries and diseases is still a great challenge for surgeons, but also for researchers who work with materials. Today stem cells are commonly used in bone tissue engineering. However, advances in biocompatible materials design, especially biodegradable porous structure (scaffold) is gaining an important role in the treatment of diseased bone tissue. The basic advantage of these carriers is specifically designed scaffold with defined porosity and pore structure that is favourable for cells settlement. Scaffolds are most commonly used as ceramic brackets because they have excellent characteristics in biodegradation and bioactivity. The process of scaffold production is important because the appropriate technology must ensure control of liquids and reproducibility of scaffold production through standardized process.

The aim of this study was to present some of different procedures of scaffold production in bone tissue engineering and point out the advantages and disadvantages of these methods.

Keywords: scaffold; bone tissue engineering; the polymer matrix; scaffold prototype

INTRODUCTION

The treatments of trauma and bone tissue diseases, as well as reconstruction of bone defects represent a great challenge for orthopaedic surgeons and engineers [1]. The most common procedures in tissue engineering involve the use of stem cells or differentiated adults cells that are plated in a biodegradable scaffold and cultured in a bioreactor, before implanting in the defective area. Advances in design and functionalization of biocompatible materials and the progress of processing, allow development of biodegradable porous structure with well-designed architecture – scaffold for tissue engineering [1, 2].

Scaffold should be designed in a specific way, with appropriate porosity and biodegradability, and meet specific requirements for individual defects, such as their shape and size. From the technological aspect, design and production of biodegradable scaffold represent great challenge in skeletal tissue engineering, due to defined porosity and pore structure, that are suitable for settling cells and can be maintained for a long time [3].

Ceramic brackets can be used as scaffold with well degradation properties and bioactivity. With such mechanical properties, they are used during new bone forming as polymeric carriers, which hydrophilicity encapsules cells (similar to the natural extracellular matrix) [4]. Procedural processing techniques of conventional polymer materials are popular, tailored and extended to the installation of bioactive inorganic phase in a porous 3D polymer network.

In addition, implantation of bioactive molecules into biodegradable scaffold promotes bone regeneration with many positive effects [4, 5]. Big challenge in materials science and tissue engineering technology is control of accuracy and reproducibility of scaffold production through standardized process. Different techniques of scaffold producing, which include processing of various polymeric and composite materials and development of different microstructures are very current topics in research. Despite numerous techniques applied, each of them has certain disadvantages in the control of scaffold porosity, pore size and distribution, as well as the presence of residues of toxic solvents in the scaffold.

The aim of this study was to present some of different methodological procedures used in scaffold production in bone tissue engineering.

METHODS OF SCAFFOLD PRODUCTION

Scaffold producing process must ensure high level of control of their macro- and micro-structural properties. Depending on scaffold's material and strategies of tissue engineering, there are different methodologies and conditions of scaffold processing to optimize predetermined purpose. Correspondingly, the process procedure, in any of the specific cases should be selected not to change chemical and biocompatible properties of the material, and not to limit its clinical effects. In addition, scaffold should have interconnected pores and sufficiently high density of pores with proper morphology, size and distribution, and its quality should be highly reproducible.

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The method of polymer matrix

Among many methods of designing scaffold structure, one of the most commonly used methods is the polymer matrix (foam) that is used as a model system for designing ceramic scaffold structure. This method comprises applying suspension of ceramic powder through the matrix and, after drying and solidification of the suspension, burning of polymeric foam to provide porous ceramic with a porosity that depends on matrix [6, 7].

In our research, we used matrix of polyurethane foam as a model system for obtaining and formatting internal geometry of hydroxyapatite (HAP) scaffold [8, 9]. After the combustion process of the polymer phase and the ceramic phase sintering at 800 °C, the resulting structure of the scaffold is obtained (Figure 1).

The combination methods of polymer matrices and biomimicry

To functionalize the interior walls of scaffold obtained with polymeric matrix method, in our experiments, we used different types of polymers, such as poly (lactic-co-glycolic) acid (PLGA), hydroxyethyl cellulose, modified starch, alginate, etc. [10]. Subsequent biomimicry treatment in simulating body fluid allowed us to obtain active scaffold structure with specific structural design (Figure 2).

Beside these polymers, and other polymers, such as polycaprolactone, hydroxyethyl chitosan, biopolymers with RGD motifs, silk fibroin, as well as various growth factors may be involved in the functionalization of interior walls of scaffold. In our experiments we used materials with specific physical properties, such as super-paramagnetic materials-based ferro-fluid magnetite, maghemite, cobalt-ferrite, gadolinium oxide, etc. [10].

Gas-foam method with the effect of supercritical CO_2

The supercritical fluid of substance is obtained when the substance is exposed to an environment where the temperature and pressure are above the critical pressure and temperature for that substance. Under these conditions, liquid and gaseous material components become identical, and further compression of the fluid phase will not result in dissolution [11, 12, 13].

CO_2 is most frequently used as the supercritical fluid, because it is low toxic, non-flammable, inexpensive, stable, and environmentally acceptable. It is used in foam-gas technique, where CO_2 under high pressure is used for scaffold processing (critical temperature of 31°C and pressure of 73.8 bar). In order to be properly processed by a mesoporous structure, a polymer disk is exposed to high pressure of (1-6 MPa) at room temperature at the beginning of the process, to allow saturation of the gas in the polymer and formation of a single phase between the gas and the polymer. Gas solubility in the polymer rapidly decreases with a decrease in pressure and CO_2 leads to nucleation and growth of gas bubbles, leading to the formation of pores larger than 500 mm. Macroporous foam poly-L-lactic acid (PLLA), polyglycolic acid (PGA) and poly-lactic-co-glycolic acid (PLGA) is obtained from such polymer discs inside which at high pressures of CO_2 an appropriate mixture of the polymer-gas is formed. Then comes a phase where gas molecules cluster, forming gas nuclei which diffuse, while inside of polymer discs macropores are left behind. Porosity and structure depend on the quantity of gas dissolved in the polymer, gas nucleation velocity and diffusion rate of gas molecules through the polymer (Figure 3) [12].

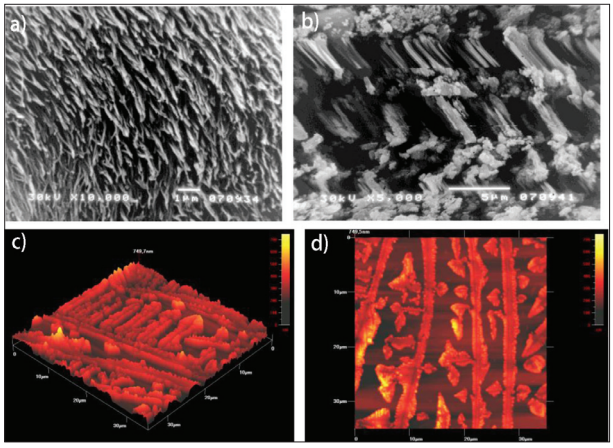

Figure 1. A typical layout structure HAP scaffold obtained by the polymer foam method: a) SEM recordings; b) AFM recordings
Slika 1. Tipičan izgled strukture HAP skafolda dobijenog metodom polimerne pene: a) SEM snimci; b) AFM snimci

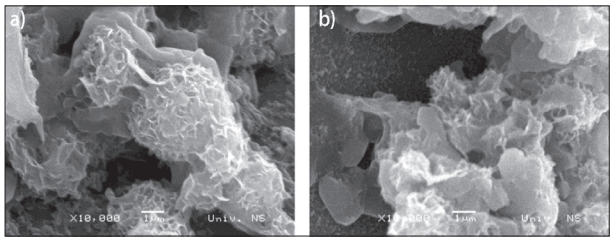

Figure 2. Scaffold obtained by combining the methods of polymer matrix, deposition of a thin polymeric film and biomimicry: a) a polymer film, PLGA; b) polymeric film hydroxy-ethyl cellulose
Slika 2. Skafoldi dobijeni kombinacijom metode polimerne matrice, depozicije tankog polimernog filma i biomimične metode: a) polimerni film, PLGA; b) polimerni film hidroksietilceluloze

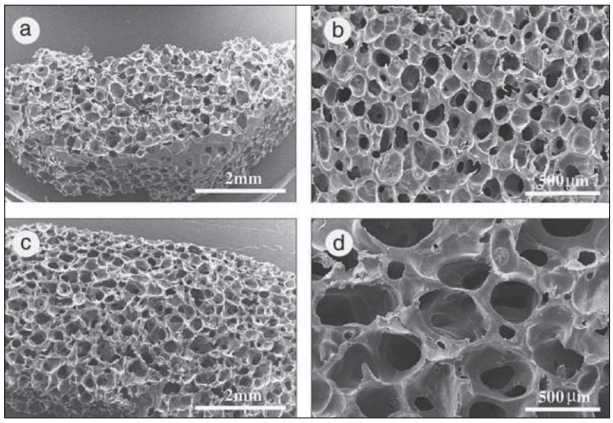

Figure 3. PLLA polymer after processing with supercritical CO2 at 240 bar, and 35°C for 12 minutes (a, b) and 60 min. (c, d) [12]
Slika 3. PLLA polimer posle procesiranja sa superkritičnim CO2 na 240 bari i 35°C u toku 12 minuta (a, b) i 60 minuta (c, d) [12]

Method of phase separation

During the phase separation, thermodynamic instability is established in a homogeneous multicomponent polymer solution, which under appropriate conditions indicates a tendency to split into more than one phase to reduce its total free energy. The polymer solution is separated into two phases, the phase rich in polymer and the phase poor in polymer. A polymer rich phase solidifies and the polymer poor phase is removed forming highly porous polymer network. Method of phase separation is known as thermal induced phase separation (TIPS). The basic principle of TIPS is to move polymer solution from its homogeneous single-phase region in the spinodal region of its binary system phase using rapid temperature change of the solution. This can be done by rapidly cooling polymer solution at the upper critical temperature or rapid heating at the lower critical temperature. When polymer solution is moved to the spinodal region, it becomes unstable and separates spontaneously into polymer-rich and solvent-rich region [14, 15, 16].

After thermodynamic separation of the homogenous solution of polymer-solvent, in the polymer rich and polymer-poor phase, the solution is usually exposed to another solvent or cooling below its binodal solubility. Then the solvent is removed by freeze drying, leaving the polymer in the form of foam. Morphology of the resulting scaffold is controlled with the phase transition that occurs during cooling, i.e. liquid-liquid phase separation, while in the solid-liquid phase anisotropic foam in the specific leaf form is obtained.

In general, micro and macrostructure of the polymer, obtained by the method of phase separation is possible to control with optimal selection of the process parameters such as polymer concentration, temperature and speed of separation. Incomplete solvent removal, especially in thicker constructs, results in reduced compatibility, and a change of the built-active factors. Although this method results in highly interconnected porous matrix, limited pore size obtained by this method causes serious problems related to the establishment of controlled macro- and microstructure scaffold (Figure 4) [17].

Method of sublimation drying

Sublimation drying (freeze drying) involves solvent removal (usually water), first with sublimation of frozen samples and then desorption of non frozen absorbed solvent under reduced pressure [18]. In the stage of freezing, polymer solution or dispersion is cooled below the temperature of solvent solidification. This leads to the formation of ice crystals and deployment of polymer molecules in the spaces between the ice crystals. In the second stage, the solvent is removed by applying pressure that is lower than the equilibrium vapour pressure of the frozen solvent. In the meantime, non-frozen water absorbed in the dried layer is desorbed. When frozen solvent is completely sublimated, the process continues with a slight heating of the sample until it is completely dried. Sublimation of ice crystals causes formation of highly porous sponge-like scaffold structure.

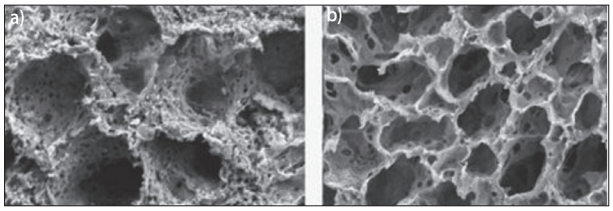

Figure 4. PLGA / nano-hydroxyapatite (95 : 5) scaffold prepared from a 10% (w/v) of a mixture of PLGA / dioxane / water with different solvent systems: a) water, 1,4-dioxane 85:15 and b) 1,4-dioxane water 87 : 13 [17]
Slika 4. PLGA/nanohidroksiapatit (95 : 5) skafold pripremljen iz 10% (w/v) smeše PLGA/ dioksan/ voda sa različitim sastavima rastvora: a) 1,4-dioksan – voda 85 : 15 i b) 1,4-dioksan – voda 87 : 13 [17]

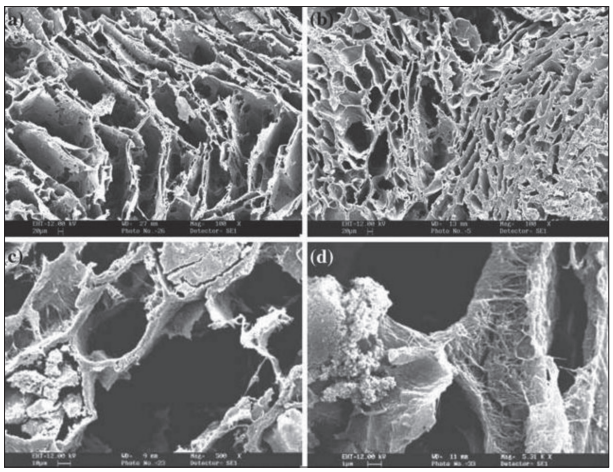

Figure 5. Scaffold obtained with sumblimation technique at least 10% (w/v) of emulsion: a) pure poly hydroxybutyrate-co-valerate (PHVB); b) 10% hydroxyapatite-HA: 90% PHVB; c and d) 20% HA: 90% PHVB [22]
Slika 5. Skafoldi dobijeni tehnikom sumblimacionog sušenja 10% (w/v) emulzije: a) čisti polihidroksibutirat-ko-valerat (PHVB); b) 10% hidroksiapatit-HA: 90% PHVB; c i d) 20% HA: 90% PHVB [22]

The final pore structure depends on conditions during the preparation of scaffold such as pH value, the speed of freezing and partial pressure. Rapid uncontrolled cooling leads to different nucleation and grow of ice crystals, and hence different morphological heterogeneity within the scaffold, caused by physical and temporal variables during the transfer of heat through the system. It has been shown that it is possible to obtain substantially uniformly porous scaffold at a constant cooling rate during the freezing process. Highly porous and interconnected structure with small pore size is achieved using the freeze-drying technique [18].

Sublimation method of drying the emulsion, as modified by the phase separation method is used in the production of aliphatic polyester scaffold. Scaffolds obtained by this method have porosity above 90%, while the mean pore size is between 15 and 35 microns, with the largest pores larger than 200 microns (Figure 5). Scaffold porosity primarily depends on the volume fraction of the disperse phase, polymer concentration and its molecular weight. Technique of sublimation drying of emulsion is used for the installation of proteins into the polymer scaffold. Sublimation drying of aqueous solutions of biopolymers such

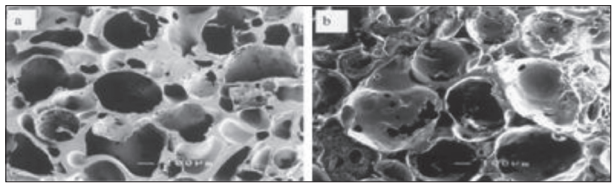

Figure 6. SEM: intersection of PLGA sponge obtained by the method of leaching (a-PLGA, b-hybrid PLGA-collagen) [17]
Slika 6. SEM: presek PLGA sunđera dobijenih metodom luženja (a-PLGA, b-hibrid PLGA-kolagen) [17]

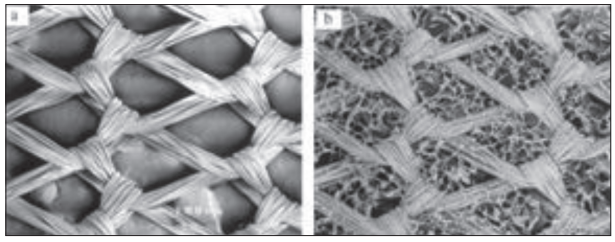

Figure 7. SEM: cross-section of the PLGA-collagen-hydroxy-apatite hybrid foam after 2 (a) and 4 (b) alternating cycles of dipping [24]
Slika 7. SEM: presek PLGA-kolagen-hidroksiapatit hibridnog sunđera posle dva (a) i četiri (b) alternirana ciklusa umakanja [24]

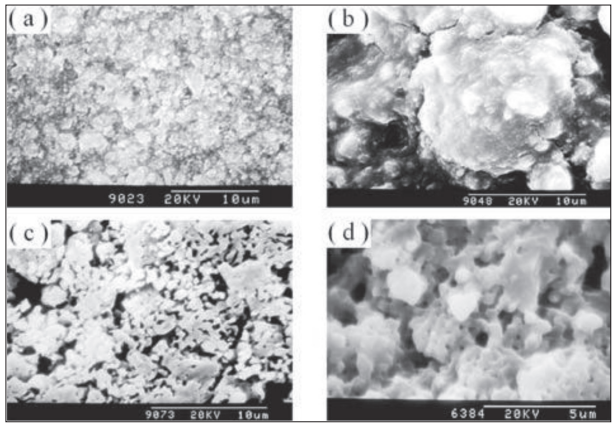

Figure 8. SEM: sintered coatings obtained with EPD in various dispersion media at different temperatures and vacuum sintering: a) ethanol, 800°C; b) glycol, 800°C; c) ethanol, 1000°C; d) glycol, 1000°C [26]
Slika 8. SEM: sinterovane prevlake dobijene sa EPD u raznim disperzionim medijumima pri različitim temperaturama vakumskog sinterovanja: a) etanol, 800°C; b) glikol, 800°C; c) etanol, 1000°C; d) glikol, 1000°C [26]

as collagen is also used in obtaining well-defined porous matrices (well-defined pore size and orientation) [19-22].

The method of casting / leaching of porogen

Casting solvent allows preparing porous structure with normal porosity but of limited thickness [17, 23]. A method of casting / leaching of porogen involves casting of the mixture of polymer and porogen into a mold, drying the mixture, followed by leaching of porogens with water and finally obtaining the pores. Salts are most commonly used porogens. Pore structure is most easily controllable with porogens contribution. Highly porous PLLA membrane with controlled porosity, the ratio of area:volume, and crystallinity are prepared by casting a dispersion of crystalline salts in the organic solution of PLLA, and then exposure to water. NaCl, Na-tartrate and Na-citrate with different particle sizes are used as materials for pore forming by leaching. Properties of obtained foam depend exclusively on fractions of salt and size of its particles, and not on the type of polymer solvent used. Therefore, it is used for the preparation of porous three-dimensional scaffold suitable for tissue engineering (Figure 6). With this technique it is possible to obtain highly porous scaffold structure with a porosity of over 93% and an average diameter of pores up to 500 microns.

The main advantage of this method is easy production of scaffold, without need for any expensive special equipment. This technique allows obtaining wide range of pore size and porosity that is easily controlled. The disadvantage of this method is the necessity to remove solvent particles inside the polymer. Casting method combined with leaching can be used to create a thin membrane or 3D samples of small thickness (up to 2 mm). One of the main disadvantages of this method is porogen particle agglomeration during the process of obtaining scaffold, causing uneven distribution of scaffold pores. Another drawback is linked to the use of organic solvents. They must be removed in order to avoid possible damage of seeded cells and proteins or other active molecules that are built into it [23].

Combined method of sublimation drying and casting porogen

Hybrid sponge of synthetic polymer, collagen and hydroxyapatite is suitable for the deposition of hydroxyapatite on its surface, because it mimics the structure of collagen and hydroxyapatite of the natural bone (Figure 7) [24]. Deposition of hydroxyapatite takes place through the process of alternating immersion PLGA-collagen sponge in aqueous $CaCl_2$ and Na_2HPO_4. PLGA-collagen sponge is first immersed in a solution of $CaCl_2$ in vacuum at 37°C for 12h, so that the space within the sponge is soaked with the best possible solution. After removing from $CaCl_2$ solution, the sponge is centrifuged to remove excess solution of Na_2HPO_4. This process of alternating immersion and centrifugation defines a cycle of depositing hydroxyapatite within the foam structure of PLGA-collagen scaffold. The deposition process is usually performed in a series of repeated cycles.

Electrophoretic deposition method

The method of electrophoretic deposition (EPD) is one of the most effective techniques in assembling fine particles of hydroxyapatite. It is very simple and it is possible to obtain very complex forms of particles. The equipment for performing EPD is cheap. At first, this method was mainly used for the deposition of hydroxyapatite coatings on metal or ceramics, while the deposition of sufficiently thick layers of apatite of adequate porosity is still great challenge [25]. Therefore, attention is on obtaining stable dispersions of hydroxyapatite, with high values of zeta potential and an appropriate concentration, that would in suitably selected conditions of depositing be able to give appropriate scaffold structure (Figure 8) [26].

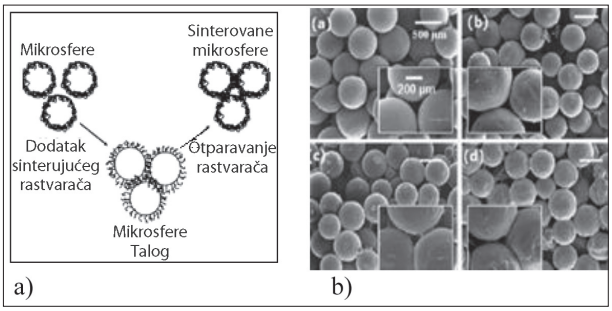

Figure 9. a) Schematic representation of sintering microspheres; b) scaffold morphology obtained by sintering microspheres [27]
Slika 9. a) Šematski prikaz sinterovanja mikrosfera; b) Morfologija skafolda dobijenog sinterovanjem mikrosfera [27]

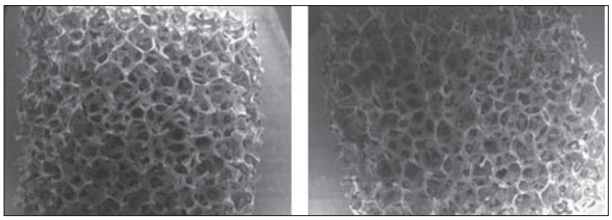

Figure 10. SEM a) Sintered compact on the surface obtained by dipping (18% paste) and b) combined method of dipping and spraying (18% paste, 6% of a sprayed suspension for 30 minutes) [30]
Slika 10. SEM a) Sinterovani kompakt na površini dobijen umakanjem (18% pasta) i b) kombinovanim postupkom umakanja i sprejovanja (18% pasta, 6% suspenzija za sprejovanje, 30 minuta vreme sprejovanja) [30]

Method of sintering microspheres

Non-toxic, neutral degradation products of amino acid esters (polyphosphazene) are ideal candidates for the use in orthopedic applications *in vivo.* Esters of amino acids (substituted poly polyphosphazenes) are used often due to good mechanical properties as scaffold is exposed to heavy loading. The most interesting are leucine, valine, and ethyl ester phenylaniline. Among these esters, ethyl ester phenyl amine shows the highest glass transition temperature (41.6°C). Therefore, it is used as a composite, in the mixture with hydroxyapatite whose particle size is in 100 nm order [27, 28, 29]. Composite first forms geometry in the form of microspheres, which is then sintered in a 3-dimensional porous scaffold, using so-called dynamic sintering of the solvent, as schematically presented in Figure 9.

Composite microspheres obtained by this method have pressure modulus of 46-81 MPa and mean pore size between 86 and 145 micrometers. 3D polyphosphazene-HA composite possesses good adhesion of osteoblasts, enables their optimal proliferation, and expression of alkaline phosphatase, what makes it suitable for use in the reconstruction of bone tissue.

Electrospray method

Electrospray method is typical replication method. It is used to obtain a scaffold without internal voids and limited number of micro cracks. In this method, ceramic suspension is pumped between the needle and the outer electrode while electric field breaks liquid into the little mono-disperse droplets [30]. Little particles of hydroxyapatite that are deposited this way provide good attachment for cells (Figure 10). It has been shown that foam coating with ceramics is better than foam obtained by the traditional method of soaking [30].

NOTE

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project no. 172026).

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Received: 09.01.2017 • Accepted: 01.03.2017

Skafoldi u inženjerstvu koštanog tkiva

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KRATAK SADRŽAJ

Terapija i lečenje brojnih povreda i oboljenja koštanog tkiva je još uvek veliki izazov za hirurge, ali i za one istraživače koji se bave materijalima. Na polju inženjerstva koštanog tkiva danas se najčešće koriste matične ćelije. Međutim, napredak u dizajniranju biokompatibilnih materijala, a posebno biodegradibilnih poroznih struktura (skafolda) sve više dobija vrlo značajnu ulogu u lečenju obolelih koštanih tkiva. Specifično dizajnirani skafoldi sa definisanom poroznošću i strukturom pora koja je povoljna za naseljavanje ćelija osnovna je prednost ovih nosača. Skafoldi se najčešće koriste kao keramički nosači jer imaju izvanredne osobine vezane za biodegradaciju i jako izraženu bioaktivnost. Postupak izrade skafolda je vrlo važan jer se odgovarajućom tehnologijom mora obezbediti kontrola tečnosti i reproduktivnost izrade skafolda kroz standardizaciju procesa.

Cilj ovog rada je bio da se predstave različiti metodološki postupci izrade skafolda u inženjerstvu koštanog tkiva i ukaže na određene prednosti i nedostatke tih metoda.

Ključne reči: skafold; inženjerstvo koštanog tkiva; polimerne matrice; prototip skafolda

UVOD

Lečenje trauma i oboljenja koštanog tkiva, kao i rekonstrukcija koštanih defekata predstavljaju veliki izazov kako za ortopedske hirurge tako i za inženjere [1]. Najčešći pristupi na polju inženjerstva tkiva uključuju korišćenje matičnih ili diferenciranih ćelija odraslih, koje se zasejavaju u biodegradibilne skafolde i kultivišu u biorekatorima, pre implantiranja na defektno mesto. Napredak u dizajnu i funkcionalizaciji biokompatibilnih materijala, kao i napredak tehnika procesiranja omogućavaju razvoj biodegradibilnih poroznih struktura sa dobro dizajniranom arhitekturom, skafolda za tkivno inženjerstvo [1, 2].

Skafold treba da bude dizajniran na specifičan način, tako da poseduje odgovarajuću poroznost i biodegradibilnost i da ispunjava specifične zahteve vezane za individualne defekte, kao što je njihov oblik i veličina. Sa tehnološke tačke gledišta, veliki izazov kod skeletnog tkivnog inženjerstva je dizajniranje i izrada biodegradibilnog skafolda, koji ima definisanu poroznost i strukturu pora, koja je pogodna za naseljavanje ćelija i koja može da se održi u dovoljno dugom vremenu [3].

Kao skafoldi mogu da se koriste keramički nosači sa dobro prilagođenim osobinama degradacije i bioaktivnosti. Sa takvim mehaničkim osobinama oni služe kao potpora tokom stvaranja novog koštanog tkiva ili kao polimerni nosači zbog svoje hidrofilnosti pogoduju inkapsulaciji ćelija (slično prirodnom ekstracelularnom matriksu) [4]. Veoma su popularne procesne tehnike obrade konvencionalnih polimernih materijala, prilagođene i proširene na ugradnju neorganskih bioaktivnih faza u poroznu 3D polimernu mrežu.

Pored toga, ugradnja bioaktivnih molekula u biodegradibilne skafolde pospešuje regeneraciju kosti sa mnogim pozitivnim efektima [4, 5]. Veliki izazov u nauci o materijalima i tehnologiji na području tkivnog inženjerstva je kontrola tačnosti i reproduktivnosti izrade skafolda kroz standardizaciju procesa. Razne tehnike izrade skafolda, koje uključuju procesiranje različitih polimernih i kompozitnih materijala i razvoj različitih mikrostruktura danas su veoma aktuelne teme istraživanja. Ipak, još uvek i pored brojnih tehnika koje se primenjuju, svaka od njih poseduje određene nedostatke sa stanovišta kontrole poroznosti skafolda, veličine pora i distribucije, kao i prisustva ostataka toksičnih rastvarača u skafoldu.

Cilj ovog rada je bio da se predstave različiti metodološki postupci izrade skafolda u inženjerstvu koštanog tkiva.

METODE IZRADE SKAFOLDA

Proces izrade skafolda mora obezbediti visok nivo kontrole njihovih makro i mikrostrukturnih osobina. Zavisno od materijala od kog se skafold izrađuje i strategije inženjerstva tkiva razrađene su i različite metodologije i uslovi procesiranja skafolda radi optimizacije za unapred određenu namenu. U saglasnosti sa tim, procesne procedure, u svakom od konkretnih slučajeva, treba da budu odabrane tako da ne menjaju hemijske i biokompatibilne osobine materijala, da ne bi tako ograničili efekte njegove kliničke primene. Pored toga, skafold treba da da ima međusobno povezane pore i dovoljno visoku gustinu pora sa pravilnom morfologijom, veličinom i distribucijom i da uz to njegov kvalitet bude visokoreproduktivan.

Metoda polimerne matrice

Među brojnim metodama dizajniranja strukture skafolda jedna od najčešće korišćenih je metoda polimerne matrice (pene), koja se koristi kao model sistem pri oblikovanju keramičke strukture skafolda. Postupak se sastoji u nanošenju suspenzije praha keramičkog sistema preko matrice i, nakon sušenja i očvršćavanja suspenzije, spaljivanja polimerne pene pri čemu se dobija porozna keramička strukrura čija je poroznost dirigovana matricom koja je korišćena [6, 7].

U našim istraživanjima smo koristili matricu od poliuretanske pene kao model sistem za dobijanje i oblikovanje unutrašnje geometrije hidroksiapatitnog (HAP) skafolda [8, 9]. Nakon procesa izgaranja polimerne faze i sinterovanja keramičke faze na 800°C dobijena je struktura skafolda data na slici 1.

Kombinacija metode polimerne matrice i biomimične metode

Za funkcionalizaciju unutrašnjih zidova skafolda dobijenih metodom polimerne matrice u našim eksperimentima korišćene su različite vrste polimera, kao što su: poli(mlečna-ko-glikolna) kiselina (PLGA), hidroksietil celuloza, modifikovani skrob, alginat i dr. [10]. Naknadnim biomimičnim tretmanom u simulirajućem telesnom fluidu dobijene su visokoaktivne strukture skafolda vrlo specifičnog strukturnog dizajna (Slika 2).

Pored ovih polimera, i niz drugih polimera, kao što su: polikaprolakton, hidroksietilmetakrilat, hitozan, biopolimeri sa RGD motivima, fibroin svile, kao i različiti faktori rasta mogu biti uključeni u funkcionalizaciju unutrašnjih zidova skafolda. U našim eksperimentima korišćeni su i materijali koji pokazuju specifična fizička svojstva, kao što su superparamagnetni materijali – ferofluidi na bazi magnetita, maghemita, kobaltferita, gadolinijum-oksida itd. [10].

Metoda gas–pena uz dejstvo superkritičnog CO_2

Superkritični fluid date supstance dobija se kad se ta supstanca izloži dejstvu okoline u kojoj su pritisak i temperatura iznad kritičnog pritiska i kritične temperature za datu supstancu. Pod ovim uslovima tečne i gasne komponente materijala postaju identične i dalja kompresija ove fluidne faze neće imati za rezultat rastapanje [11, 12, 13].

CO_2 se najčešće koristi u svojstvu superkritičnog fluida, jer je slabo toksičan, nezapaljiv i jeftin, stabilan i prihvatljiv za okolinu. Koristi se kao kod tehnike pena–gas, gde se za procesiranje skafolda koristi CO_2 pod visokim pritiskom (kritična temperatura od 31°C i pritisak 73,8 bara). Da bi se pravilno procesirala mezoporozna struktura, polimerni disk se u polaznoj fazi procesa izlaže visokom pritisku, od 1 do 6 MPa na sobnoj temperaturi, kako bi se omogućilo zasićenje gasa u polimeru i formiranje jedinstvene faze između polimera i gasa. Rasvorljivost gasa u polimeru opada brzo pa sa smanjenjem pritiska CO_2 dolazi do nukleacije i rasta gasnih mehurova, koji dovode do formiranja pora većih od 500 μm. Makroporozne pene poli-L-mlečne kiseline (PLLA), poliglikolne kiseline (PGA) i poli-mlečne-ko-glikolne kiseline (PLGA) dobijaju se iz takvih polimernih diskova unutar kojih pri visokim pritiscima CO_2 nastaje odgovarajuća smeša polimer–gas. Potom sledi faza gde se gasni molekuli klasteruju, formirajući gasne nukleuse, koji potom difunduju, a unutar diskova polimera zaostaju makropore. Poroznost i struktura pora zavise od količine gasa rastvorenog u polimeru, brzine gasne nukleacije i brzine difuzije molekula gasa kroz polimer (Slika 3) [12].

Metoda fazne separacije

Tokom fazne separacije termodinamička nestabilnost se uspostavlja u homogenom višekomponentnom polimernom rastvoru, koji pod odgovarajućim uslovima pokazuje težnju da se razdvoji u više od jedne faze kako bi se smanjila njegova ukupna slobodna energija. Polimerni rastvor se razdvaja na dve faze – fazu koja je bogata polimerom i fazu koja je siromašna polimerom. Polimerom bogata faza očvršćava, a polimerom siromašna faza se uklanja ostavljajući za sobom visoko poroznu polimernu mrežu. Metoda fazne separacije je poznata pod nazivom termički indukovana fazna separacija (TIPS). Osnovni princip TIPS-a je da se pomeri polimerni rastvor iz svoga homogenog jednofaznog regiona u spinodalni region njegovog binarnog faznog sistema brzom promenom temperature rastvora. To se može izvesti brzim hlađenjem polimernog rastvora na njegovoj gornjoj kritičnoj temperaturi ili brzim zagrevanjem na donjoj kritičnoj temperaturi. Kada se polimerni rastvor pomeri u spinodalni region, on postaje nestabilan i razdvaja se spontano u polimerom bogati i rastvaračem bogati region [14, 15, 16].

Nakon termodinamičkog razdvajanja homogenog rastvora polimer–rastvarač u polimerom bogatu i polimerom siromašnu fazu, rastvor se najčešće izlaže drugom rastvaraču ili hlađenju ispod tačke njegove binodalne rastvorljivosti. Rastvarač se potom uklanja sumblimacionim sušenjem (freeze drying), ostavljajući polimer u formi pene. Morfologija tako dobijenog skafolda kontroliše se faznim prelazom koji se dešava tokom hlađenja, tj. tečno-tečne fazne separacije, dok unutar faze čvrsto–tečnost nastaje anizotropna pena specifične lisnate forme.

Uopšteno govoreći, kontrolu mikro i makrostrukture skafolda datog polimera dobijenu metodom fazne separacije moguće je kontrolisati optimalnim izborom procesnih parametara kao što su koncentracija polimera, temperatura i brzina separacije. Nekompletno uklanjanje rastvarača, posebno kod debljih konstrukata, rezultuje smanjenoj kompatibilnosti i mogućoj promeni ugrađenih aktivnih faktora. Iako su pomoću ove metode dobijene visoko međusobno povezane porozne matrice, ograničena veličina pora koja se dobija korišćenjem takve metode uslovljava ozbiljne probleme vezane za uspostavljanje kontrolisane makro i mikrostrukture skafolda (Slika 4) [17].

Metoda sublimacionog sušenja

Sublimaciono sušenje (freeze drying) uključuje uklanjanje rastvarača (najčešće vode) prvo sumblimacijom zamrznutih uzoraka, a potom desorpcijom nezaleđenog apsorbovanog rastvarača pod smanjenim pritiskom [18]. U stadijumu zamrzavanja polimerni rastvor ili disperzija se hlade ispod temperature na kojoj rastvarač prelazi u čvrsto stanje, usled čega dolazi do formiranja kristala leda i razmeštanja polimernih molekula u prostore između kristala leda. U drugoj fazi rastvarač se uklanja primenom pritiska koji je niži od ravnotežnog pritiska pare zamrznutog rastvarača. U međuvremenu nezaleđena apsorbovana voda u sušenom sloju se desorbuje. Kada je zamrznuti rastvarač kompletno sumblimovan, proces se nastavlja dalje uz blago zagrevanje uzorka sve dok ne bude kompletno osušen. Sumblimacija kristala leda uzrokuje formiranje visoko porozne sunđeraste strukture skafolda.

Konačna struktura pora zavisi od procesnih uslova tokom izrade skafolda kao što su pH, brzina zamrzavanja i parcijalni pritisak. Brzo, nekontrolisano hlađenje vodi neuniformnoj nukleaciji i rastu kristala leda, a samim tim i različitim morfološkim heterogenostima unutar skafolda, koje su uslovljene prostornim i vremenskim promenljivima tokom transfera toplote kroz sistem. Pokazano je da je moguće dobiti znatno uniformnije porozne skafolde pri konstantnoj brzini hlađenja tokom procesa zamrzavanja. Tehnikom sušenja zamrzavanjem dobijaju se visoko porozne i međusobno povezane strukture sa malim veličinama pora [18].

Metoda sumblimacionog sušenja emulzije, kao modifikovana metoda fazne separacije, koristi se u proizvodnji alifatičkih

poliesterskih skafolda. Skafoldi dobijeni ovom metodom imaju poroznost iznad 90%, dok je srednja veličina pora između 15 i 35 μm, sa najvećim porama većim i od 200 μm (Slika 5). Poroznost skafolda prvenstveno zavisi od zapreminske frakcije dispergovane faze, koncentracije polimera i njegove molekulske mase. Tehnika sumblimacionog sušenja emulzije koristi se i za ugradnju proteina u polimerni skafold. Sumblimaciono sušenje vodenih rastvora biopolimera, kao što je kolagen, koristi se i kod dobijanja dobro definisanih poroznih matrica (dobro definisane veličine i orijentacije pora) [19–22].

Metoda livenja / luženja porogena

Livenje rastvarača omogućava pripremu poroznih struktura sa normalnom poroznošću, ali sa ograničenom debljinom [17, 23]. Metoda livenja / luženja porogena uključuje u sebe livenje smeše polimera i porogena u kalup, sušenje smeše, potom luženje porogena sa vodom, da bi se napokon dobile pore. Kao porogeni najčešće se koriste soli. Strukturu pora je najlakše kontrolisati udelom porogena. Visoko porozne PLLA membrane kontrolisane poroznosti, kao i odnosa površina : zapremina, te kristaliničnosti pripremaju se livenjem disperzije kristalne soli u organski rastvor PLLA, da bi nakon toga so bila izlužena vodom. NaCl, Na-tartarat i Na-citrat raznih veličina čestica koriste se kao materijali za stvaranje pora luženjem. Osobine dobijenih pena zavise isključivo od frakcije soli i veličine njenih čestica, a ne zavise od vrste izabranog polimernog rastvarača. Zbog toga se ona koristi se za pripremu poroznih trodimenzionalnih skafolda pogodnih za inženjerstvo tkiva (Slika 6). Ovom tehnikom moguće je dobiti visoko porozne strukture skafolda sa poroznošću iznad 93% i srednjim prečnikom pora do 500 μm.

Glavna prednost ove metode je laka izrada skafolda, bez neophodnosti za nekim skupim specijalnim uređajima. Takva tehnika dopušta da se realizuju pore u širokom opsegu veličine i da se poroznost i veličina pora mogu kontrolisati. Nedostatak ove metode vezan je prvenstveno za neophodnost uklanjanja rastvornih čestica unutar polimera. Metoda livenja kombinovana sa luženjem može da se iskoristi za dobijanje tankih membrana ili 3D uzoraka male debljine (do 2 mm). Jedan od bitnih nedostataka metode je aglomeracija čestica porogena tokom procesa dobijanja skafolda, zbog čega pore skafolda imaju neujednačenu raspodelu. Drugi nedostatak je vezan za korišćenje organskih rastvarača. Oni moraju biti uklonjeni da bi se izbegla moguća oštećenja zasejanih ćelija i proteina ili drugih aktivnih molekula koji su u njega ugrađeni [23].

Kombinovana metoda sublimaciong sušenja i livenja porogena

Hibridni sunđer sintetičkog polimera, kolagena i hidroksiapatita pogodan je za deponovanje hidroksiapatitnih čestica na površini istog, jer oponaša strukturu kolagena i hidroksiapatita u prirodnoj kosti (Slika 7) [24]. Depozicija hidroksiapatita odvija se kroz proces alternirane imerzije PLGA-kolagenskog sunđera u vodenom rastvoru $CaCl_2$ i Na_2HPO_4. PLGA-kolagenski sunđer prvo se uranja u rastvor $CaCl_2$ pod vakuumom, na 37°C tokom 12 h, da bi se prostor unutar sunđera što bolje natopio rastvorom. Posle izvlačenja iz $CaCl_2$ rastvora sunđer se centrifugira da bi se odstranio višak Na_2HPO_4 rastvora. Ovaj proces alternirane imerzije i centrifugiranja definiše jedan ciklus deponovanja hidroksipatita unutar sunđeraste strukture PLGA-kolagen skafolda. Proces deponovanja najčešće se izvodi u nizu ponovljenih ciklusa.

Metoda elektroforetske depozicije

Metoda elektroforetske depozicije (EPD) jedna je od najefikasnijih tehnika asembliranja hidroksiapatitnih finih čestica. Vrlo je jednostavna i njome je moguće dobiti vrlo kompleksne forme čestica. Aparatura za izvođenje EPD je vrlo jeftina. U prvo vreme ova metoda je pretežno korišćena za deponovanje hidroksiapatitnih prevlaka na metal ili keramiku, dok je deponovanje (dovoljno debelih slojeva) apatita odgovarajuće poroznosti još uvek veliki izazov [25]. Zbog toga se velika pažnja posvećuje dobijanju hidroksiapatitnih stabilnih disperzija, sa visokim vrednostima zeta potencijala i odgovarajućom koncentracijom, koja bi u pogodno odabranim uslovima deponovanja mogla dati odgovarajuću strukturu skafolda (Slika 8) [26].

Metoda sinterovanje mikrosfera

Netoksični, neutralni degradacioni produkti estara amino kiselina (polifosfazena) idealni su kandidati za ortopedske primene *in vivo*. Estri aminokiselina (supstituisani polifosfofazeni) koriste se najčešće zbog svojih dobrih mehaničkih osobina kao skafoldi za tkiva koja trpe opterećenje. Među njima najzanimljiviji su leucin, valin i etil-estar fenilanilina. Među svim ovim estrima, etil-estar fenilamina pokazuje najvišu temperaturu staklastog prelaza (41,6°C). Zbog toga se koristi kao kompozit u smeši sa hidroksiapatitom, čija je veličina čestica reda 100 nm [27, 28, 29]. Kompozit prvo formira geometriju u formi mikrosfera, koje se potom sinteruju u 3-dimenzionalni porozni skafold, pomoću tzv. dinamičkog sinterovanja rastvarača, što je je šematski predstavljeno na slici 9.

Mikrosfere kompozita dobijene ovom metodom imaju modul pritiska 46–81 MPa i srednju veličinu pora između 86 i 145 μm. 3D polifosfazen-HA kompozit poseduje izuzetno dobru adhezivnost osteoblasta i omogućuje njihovu optimalnu proliferaciju i ekspresiju alkalne fosfataze, zbog čega je veoma pogodan za primenu u rekonstrukciji koštanih tkiva.

Elektrosprej metoda

Elektrosprej metoda je tipična replikaciona metoda. Koristi se da bi se dobio skafold bez unutrašnjih šupljina i da bi se sasvim limitirao broj mikropukotina. Kod ove metode keramička suspenzija se pumpa između igle i spoljašnje elektrode, pri čemu se zbog dejstva odgovarajućeg električnog polja tečnost kida u fine monodisperzne kapljice [30].

Fine čestice hidroksiapatita koje se deponuju na ovaj način obezbeđuju dobro kačenje ćelija (Slika 10). Pokazalo se da su dobijene prevlake pene sa keramikom kvalitetnije nego pene dobijene tradicionalnom metodom umakanja [30].