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Coupling Agents and Biomimetic Methods of Calcium Hydroxyapatites Design as Basic Elements of the Hierarchically Structured Bone Scaffold

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SUMMARY

In this paper, various modifications of agent coupling and biomimetic methods of synthesis and design of calcium-hydroxyapatites as basic elements of the hierarchically structured bone scaffold are described. These elements are created to have a very strictly defined geometry and phase composition, which can have essential importance for the morphology and geometry design of the bone scaffold thin inner walls, with strong influence to the bone cell activity and their balance during the processes of bone modeling and remodeling. Different kind of these methods for synthesis basic elements, e.g. calcium hydroxyapatite, as constitutive elements of the scaffold inner walls with their specific design from the aspects of the size and shape of their particles are the key factors in the processes of the tissue engineering and osteointegration of these materials used as building blocks of scaffold structure.

Keywords: agent coupling method; biomimetic method; calcium hydroxyapatite; bone tissue engineering

INTRODUCTION

The essential factors of bone tissue engineering are the biocompatible scaffolds suitable for osteogenic cell attachment, proliferation and differentiation, the osteogenic stem cells and the bio-environment which are suitable for long-term bone tissue formation and maintenance. These factors have the crucial importance for equilibrium between processes modeling (process bone formation by osteoblasts) and remodeling (process of the bone resorption by osteoclasts) providing coherent tissue structure.

The activity of the osteogenic steam cells and progenitor's cells, which are responsible for increasing osteoblasts concentration: cell division –proliferation, cell differentiation and cell death-apoptosis, mostly depends not only on the chemical and biological properties of calciumhydroxyapatites-CHA but also on its morphology, shape and size distribution, which are very important for the adequate structure of the scaffold inner walls. The chemical and biological properties have to be similar as possible to the corresponding properties of the biological CHA, which is the main constituent of bone.

Briefly, optimal material may have a poor crystallinity, chemical composition more or less far from stochiometry of CHA, due to cationic and anionic replacement of Ca^{2+} and PO_4^{3-} ions in CHA by corresponding other cations and anions as they are: Mg^{2+}, Sr^{2+}, Zn^{2+}, B^{3+}, SiO_4^{2-}, CO_3^{2-} etc. In agreement to this, for example, well-known Mg^{2+} ions are attendant in amount of cca 6 mol% in cartilage and bone during initial phase of osteogenesis-in young bone, while they significantly decrease in mature bone. From the other side, Mg^{2+} ions can stimulate nucleation of a high number of CHA nuclei, causing their further crystallization and inhibition of crystal growth, what has a crucial importance for better mechanical properties of bone. Also, the CO_3^{2-} ions present in CHA crystal cell, in so called carbonated CHA improve their chemical and biological activity.

If PO_4^{3-} ions are replaced inside of CHA crystal cell by CO_3^{2-}, those CHA which are prevailing in young bone are so called B-type of CHA, have higher degree of biological activity then CHA in which OH^- ions are replaced by CO_3^{2-} making so called A-type of CHA, prevailing in old bone. The typical crystallite size of natural bone is mostly in the range: length 30-50 nm, sometimes reaches 200 nm, width 15-30 nm and thickness 2-10 nm. These values are induced with shape and size of voids in the collagen fibers networks.

The next essential properties of bone scaffold are strong protein adhesion and osteoblast proliferation. From the numerous results given elsewhere [1-5], it is clear that for better activity of apatite and subsequent its better compatibility with biological apatite it is necessary to have corresponding shifted more or less chemical composition of synthesized CHA comparing to stoichiometric CHA, which can provide similar rate of the CHA biodegradation, e.g., rate of the release of corresponding ions in the crystal cell of CHA and their surface activity. The second important factor for bioactivity of CHA is corresponding modification of CHA surface structure to attain its better adhesion and proliferation by bone cells. That can be provided by the corresponding design and chemical composition of the CHA particles, which are used as a suitable building block of the final scaffold structure.

To show possible variety in the structure and properties of basic particles for scaffold tailoring, in this paper is shown a set of different modifications of agent coupling and biomimetic methods with its corresponding specificity, as a possible set of different approaches in the designing of the scaffold building blocks [6, 7, 8]. Beside these

methods described in this paper, in our laboratory, we used a set of others methods, also very important and promising as a methods described in this paper like: hydrothermal method and its modification by using surface active substances (SAS) with and without combination with mechanochemical method; reflux method with dominant partition of organic solvent with and without external physical field (RF field); ultrasonic spray pyrolysis method; double inverse micelle method and method of CHA synthesis assisted by enzyme. In the next papers, some of them will be presented.

METHODS OF CHA SYNTHESIS AND POWDER PROPERTIES

The first method which will be briefly described in this paper will be conditionally named method of coupling agents. The diverse class of coupling agents and polymers may be used as a cage matrix for CHA self-nucleation during its synthesis. Coupling agents, particularly SAS can cause specific chemical reactions occurring between polar groups of surface active substances through their electrostatic interactions with Ca^{2+} ions. These polar group acts as active sites, which have a prevailing influence to CHA nucleation and oriented crystal growth. By this way, the aggregation state of the polymer /surfactant coupling agents becomes the main factor for the control of CHA grain/crystallite size, shape, morphology and poly-dispersity.

Typical appearance of CHA powders synthesized using this method, in the mixture of polyvinyl alcohol (PVA) and ethylene vinyl acetate /versatate (EVA/EVV) coupling agents is shown in Figures 1a and 1b. Briefly, in this specific case, the negative charge of acetate and versatate ions (carboxyl polar groups) plays a role of the active sites for opposite charged Ca^{2+} ions, while inside of the PVA/SAS micelles nuclei formation and oriented growth of CHA is occurred by subsequent diffusion of PO_4^{3-} and Ca^{2+} ions inside of the micelle volume. Typical mechanism of forming these micelles which has a role of one kind of specific incubator for CHA self-nucleation is given in Figure 1c.

Beside the PVA in our experiments, we also used alginate, hydroxyl methyl celulose, modified starch, hydroxymethyl metacrylate as polymers-hydrogels; as SAS we used: eethylene vinyl acetate /versatate, polyoxyethylene octyl phenyl ether, polysorbate 80, polyoxyethylene - sorbitan monooleate, melaminesulphonate, cethyl three methyl ammonium bromide, sodium dodecyl benzene sulfonate and sodium dodecyl sulfonate.

The size of the obtained rod shape particles was in diameter between 100 and 200 nm and in length between 0.5 and 2 μm, with ratio length: diameter 10:1. A very nicely divided particles and separated one from others, with very expressed regular order in the rows, placed in almost equal distances, one from other, in its surrounding, make these systems very interesting for modeling scaffold-bone cells investigations which are of the essential importance, in the aspect of bone cells activity and degree of their adaptation on the scaffold surface morphology. It is expected that these systems can be one kind of

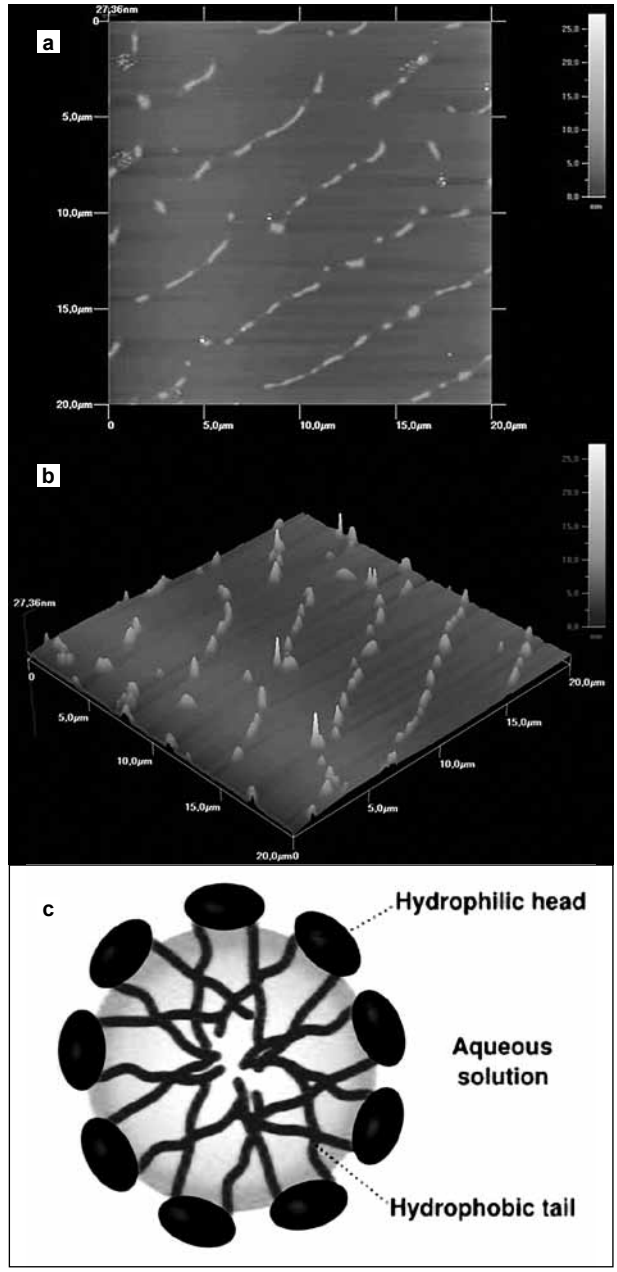

Figure 1. Typical appearance of CHA obtaine using coupling agents method on the example of PVA-EVA/EVV (a, b); Typical scheme of micelle in an aqueous solution (c).
Slika 1. Tipičan izgled čestica CHA dobijenih metodom udruženih agensa na primeru PVA-EVA/EVV (a, b); Tipična shema micele u vodenom rastvoru (c).

the very precisely tailored bridges supporter which can be easily joined to the biomimetically CHA growth inside the body, which is promoted by body fluid solution (SBF) consisted from the mixture of various ions, electrically charged proteins and amino acids.

Therefore, the next method of CHA synthesis shown in this paper was the biomimetic method, e.g. method CHA synthesis from supersaturated SBF solution. For such kind of investigations, the synthesis of silica thin films as substrate for further CHA synthesis, was firstly done, using thermal spray pyrolysis method. Very precisely tailored silica sol particles order of 6.7 nm with silica chains-length between 18-20 silica tetrahedrons were used for deposition on the surfaces of steel tape.

In the next step of CHA synthesis, the specimens of steel tape covered by silica were emerged in the slightly supersaturated SBF, which had been prepared by a partially modified recipe comparinf to a typical composition corresponding to the product solubility of the stochiometric CHA. Concentrations of corresponding ions were given as follows: $c(Cl^-)$=0.054 mol/L; $c(Na^+)$=0.0542 mol/L; $c(Ca^{2+})$=0.0025 mol/L; $c(PO_4^{3-})$=0.001 mol/L; $c(Mg^{2+})$=0.0003 mol/L; $c(NO_3^-)$=0.0006 mol/L and $c(K^+)$=0.0014 mol/L). Thin silica film on the surface of steel tape (thickness 22.5 μm), shown in the Figures 2a and 2b is consisted from clearly distinguishable rods which are very pronouncedly oriented along one specific direction.

For the mechanism of CHA self-nucleation, Si–OH groups are the most important, because they induce heterogeneous nucleation of CHA on the surface of silica thin films. Once CHA nuclei is formed, crystals of CHA grow spontaneously to form a layer of bone-like apatite, because SBF is supersaturated with respect to apatite. From the findings described above, it can be observed that the bone-like apatite deposition from SBF onto materials can be achieved by increase in the degree of supersaturation of SBF with respect to apatite and the existence of the functional groups, such as Si–OH group, which induce heterogeneous nucleation of apatite on the surface of materials.

The initial stage of the nucleation induced by Si–OH groups was described by Takadama et al. [1] and our recent investigations. As the given examination clearly shows, the process of CHA nucleation is induced by Si–OH groups in SBF and a silanol groups do not induce apatite nucleation directly but form amorphous calcium silicate compounds such as $\equiv SiOCa^+$ and $(\equiv SiO)_2Ca$ incorporating calcium ions. The amorphous calcium silicate compounds then incorporate phosphate ions to form CHA nuclei.

In the Figures 3a and 3b, the CHA film (after nucleation for 33 days on the surface of 22.5 μm thick SiO_2 film covering a surface of steel tape), is shown. The CHA film,shown in this figure, consisted of small 0.138-0.41 μm sized CHA particles and agglomerates, mostly between 1.66 and 2.77 μm, while the biggest of them were between 2.8 and 4.86 μm. The agglomerates were "flowers-shape-blow ball shape" with smaller branches of the smaller agglomerates and small elongated particles (range 200 nm in length, and 20-30 nm in diameter). The bottom darker layers were also visible as very dark spots. On the top of these layers were newly formed, brighter CHA layers.

In the Figures 4a and 4b, a three dimensional view of self-nucleated CHA is shown. The clearly distinguished layers of CHA, which are regularly ordered one to another from the bottom to the top of CHA thin film, are evident. They form the separated islands firstly, which are joined finally, due to capillary forces which cause the erosion of the islands edges, by induction of the corresponding fluid disturbances and instabilities inside of SBF.

The specific variety of this synthesis, more complex, is shown in the Figures 5a and 5b. This picture shows typical appearance of the self-nucleated CHA when used is not only SBF solution. In one case a mixture of SBF and EMEM-Eagles minimal essential medium which contain: amino acids and salts of $CaCl_2$, KCl, $MgSO_4$, NaCl and

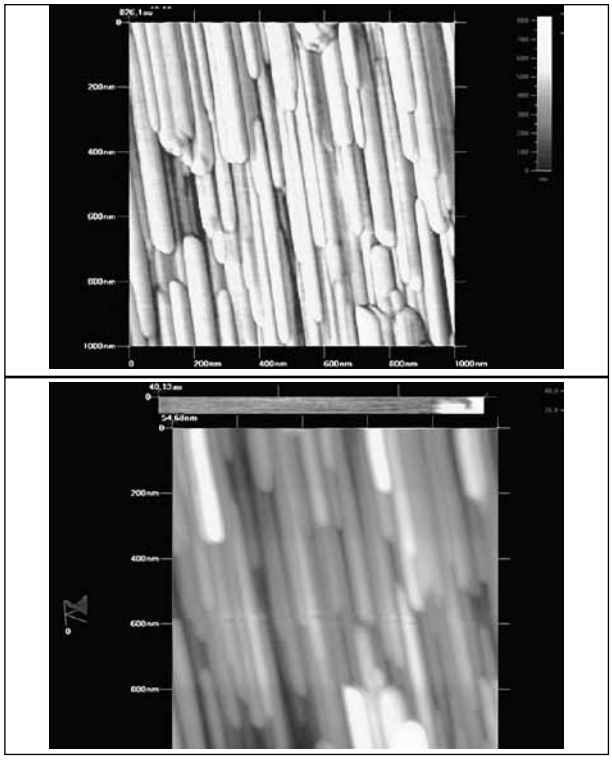

Figure 2. AFM: Thin silica film on the surface of steel tape
Slika 2. AFM: tanki silicijum-dioksidni film na površini čelične trake

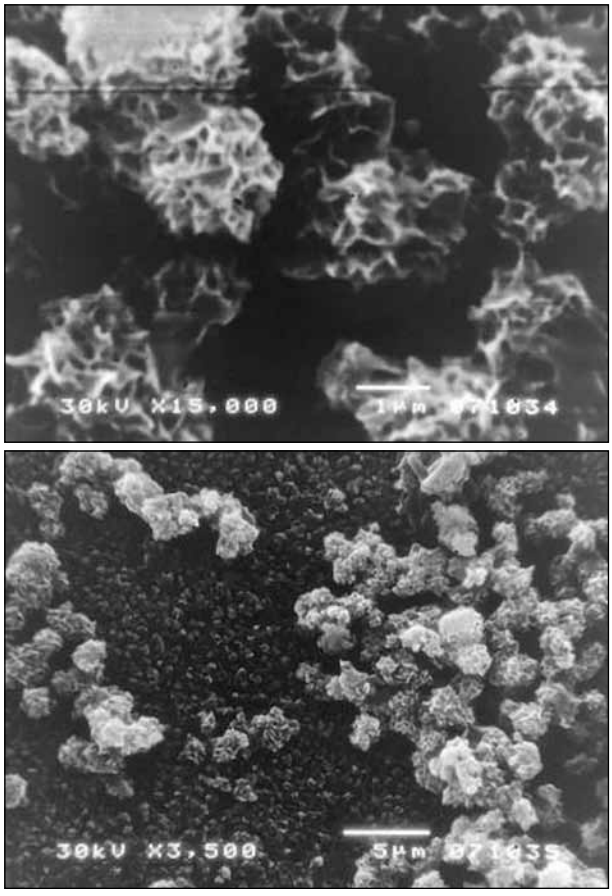

Figure 3. SEM: typical appearance of self-nucleated CHA, after 33 days nucleation in SBF on the surface of silica thin film covered steel tape.
Slika 3. SEM: tipičan izgled samonukleiranog filma CHA nakon 33 dana nukleacije SBF na površini tankoslojnog filma silicijum-dioksida nanetog na površinu čelične trake.

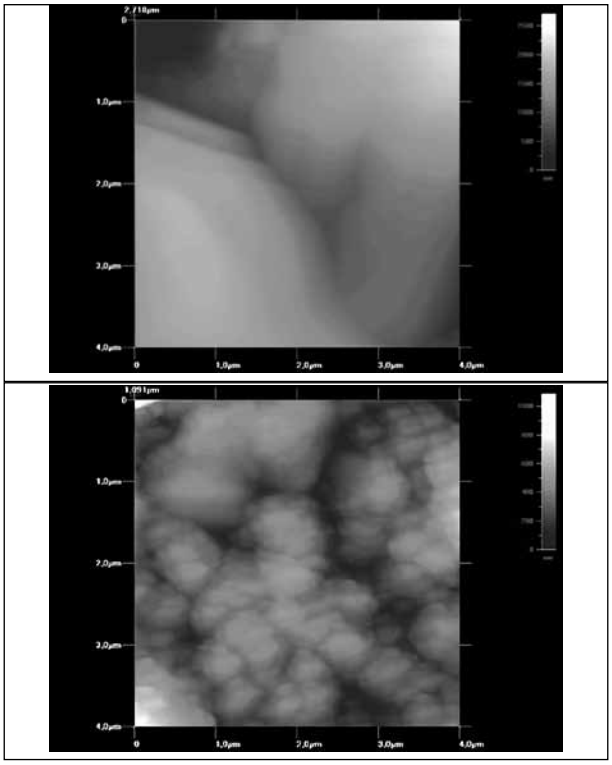

Figure 4. AFM: Typical three-dimensional appearance of self-nucleated CHA, after 33 days nucleation in SBF on the surface of silica thin film covered steel tape.
Slika 4. AFM: tipičan trodimenzionalni izgled slojeva tankog filma samonuleiranog CHA nakon 33 dana nukleacije SBF na površini tankoslojnog filma silicijum-dioksida nanetog na površinu čelične trake.

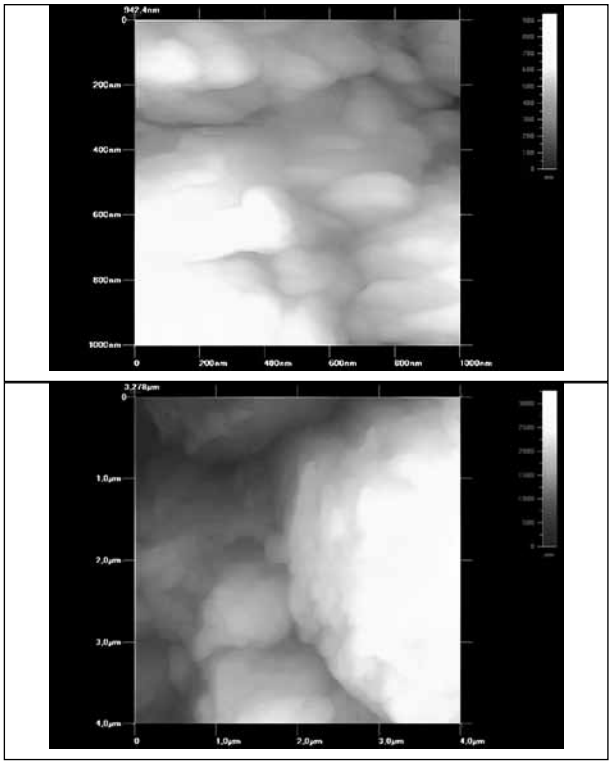

Figure 6. AFM: Typical three-dimensional appearance of self-nucleated CHA, after 33 days nucleation in SBF on the surface of silica thin film covered steel tape.
Slika 6. AFM: tipičan trodimenzionalni izgled samonukleiranog CHA nakon 33 dana nukleacije SBF na površini troslojnog filma silicijum-dioksida nanetog na površinu čelične trake.

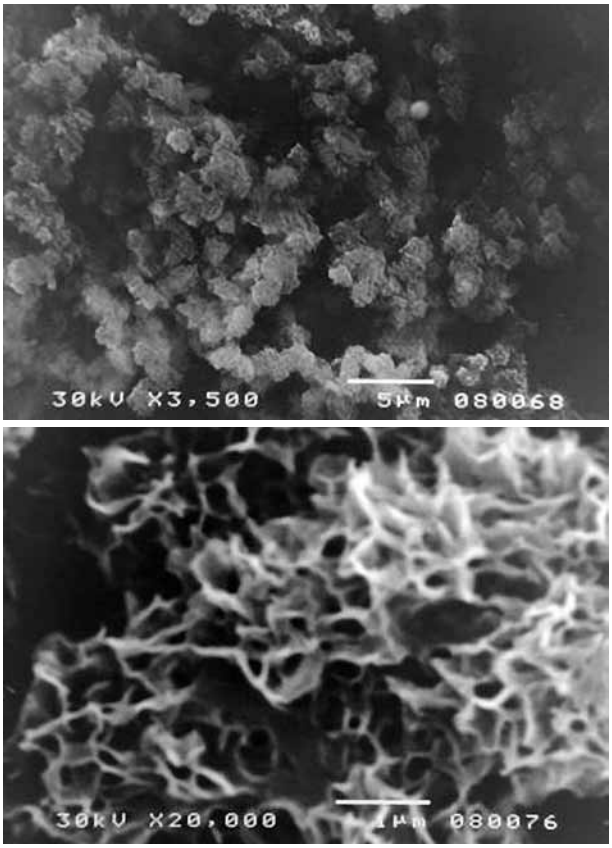

Figure 5. SEM: Typical appearance of self-nucleated CHA after 33 days nucleation in mixture solution of supersaturated SBF and EMEM.
Slika 5. SEM: tipičan izgled samonukleiranog CHA nakon 33 dana u mešovitom rastvoru prezasićenog SBF i EMEM.

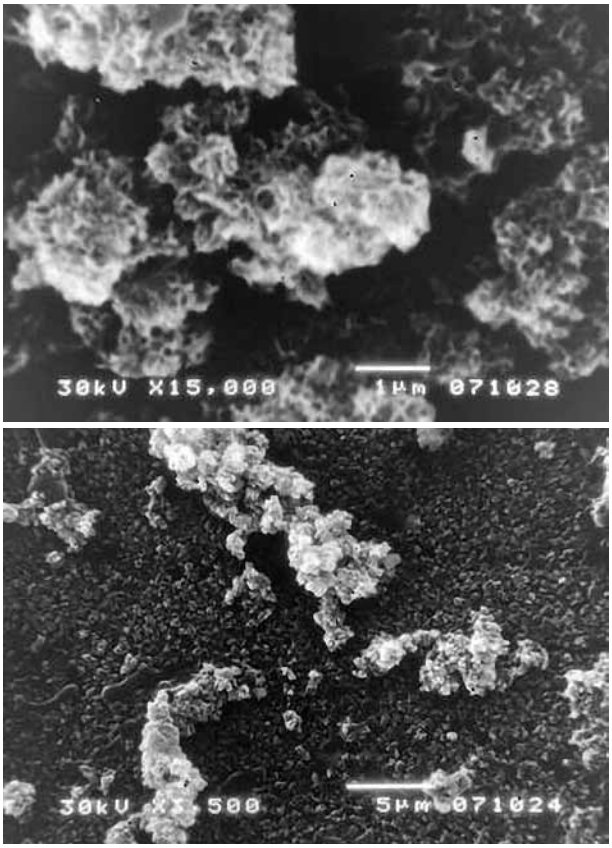

Figure 7. SEM: Typical appearance of self-nucleated CHA, after 33 days nucleation in mixture solution of supersaturated SBF and FCS.
Slika 7. SEM: tipičan izgled samonukleiranog CHA nakon 33 dana nukleacije u mešovitom rastvoru prezasićenog SBF i FCS.

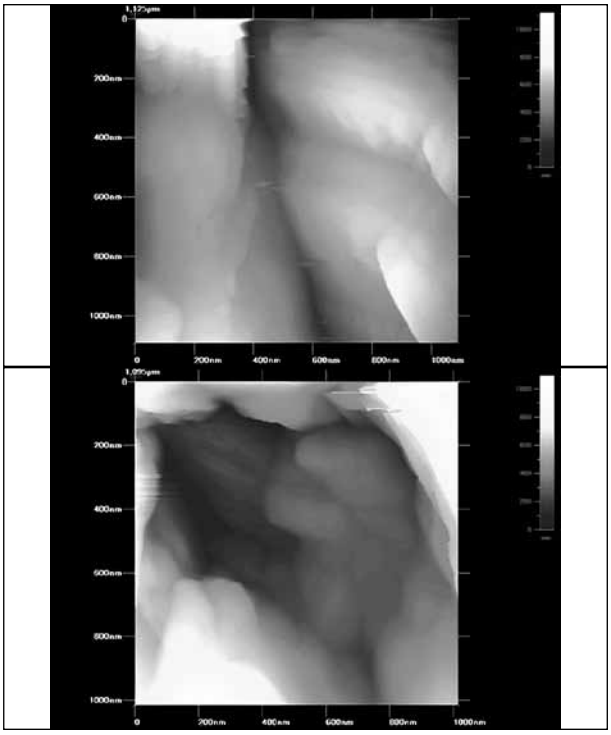

Figure 8. AFM: Typical appearance of self-nucleated CHA, after 33 days nucleation in mixture solution of supersaturated SBF and EMEM.
Slika 8. AFM: tipičan trodimenzionalni izgled samonukleiranog CHA nakon 33 dana nukleacije u mešovitom rastvoru prezasićenog SBF i EMEM.

$NaHPO_4$ in the amount overhead the balance value given by Kokubo was used.

The appearance of the nucleated CHA shown in Figures 5a, 5b, 6a and 6b very similar to the previous, corresponding to these, when SBF is used only. These pictures show very pronounced branches of CHA, which are very strongly networked, with ratio length: diameter =10:1.

Similar synthesis is made by using mixture of the SBF and fetal bovine serum (FBS), which contains dominantly globular bovine serum albumin as protein, very important for surviving, growth and division of bone cells (Figures 7a, 7b, 8a and 8a).

Basically, experiments with EMEM and FCS were initiated following idea about the mechanism of bone formation and proliferation inside of collagen fibers in live bone tissue. As it is recently known, polymer induced liquid precursor (PILP) plays a dominant role in the bone mineralization [5]. PILP is responsible for initial nucleation of CHA within hollow zones of collagenous tissue, in which voids PILP can be drown with capillary forces. The mechanism of new bone formation given by Gower can be briefly explained as follows: the negative charge of polyanionic proteins (phosphoproteins: ostecaclin, phosphoryn and bone sialoprotein) refered as "acidic proteins" and amino acids: asparatic acid, acts as active sites for opposite electrically charged Ca^{2+} ions, causing its further attraction of PO_4^{3-} ions into polyanionic proteins micelles, sited inside of collagen fibril voids. Formation of CHA occurs by diffusion of Ca^{2+} and PO_4^{3-} ions into those voids and so obtained CHA initially was in the form of a hydrated amorphous structure, while in the further phase of the ions rearrangement releases excess of bound water transforming its amorphous structure into crystal structure.

Similar to this process, process of nucleation and crystallization of CHA in EMEM and FBS is occurred. In both cases of these biological serums, the additional role of amino acids and proteins are significantly included in the mechanism of apatite formation in the mixed solution of SBF and EMEM (amino acids) and FBS-bovine albumin.

These facts influence significantly the kinetic of CHA crystal growth, showing that presence of amino acids (in the case of EMEM) and electrically charged proteins (bovine albumin in the case of FCS) significantly increase the rate of CHA crystal growth (Table 1). The rate of CHA thin film self-nucleation is more than two times accelerated when it is used EMEM or FCS, in the mixture with supersaturated SBF, in comparison with supersaturated SBF, used only. On the other side, the shape and morphology of obtained powders, is similar in all these cases.

CONCLUSION

The diverse modifications of the agents coupling and biomimetic methods are described in this paper. The main mechanism, which follows such kind of synthesis are described. The specificity of obtained particles as potential basic elements of the scaffold thin walls is shown from the aspect of their average size, shape, size distribution and ratio length: diameter. The role of their geometry design and phase non homogeneities in the bone tissue engineering processes responsible for equilibrium between rate of modeling by osteoblasts and rate of remodeling by osteoclasts is pointed in this paper. The numerous different methods are also very promising and important

Table 1. Thicknesses of the self-nucleated CHA thin films on the surfaces of SiO_2 thin film
Tabela 1. Debljina samonukleiranog CHA tankog filma na površini SiO_2 tankog filma

Sample Uzorak	Time of the CHA nucleation (days) Vreme nukleacije CHA (dani)	Mass of the CHA Masa CHA	Specific surfaces Specifične površine	Volume of pores Zapremina pora	CHA film thickness Debljina filma CHA	SiO_2 film depth SiO_2 dubina filma
Supersaturated SBF Prezasićeni SBF	33	1.2 mg	94 m^2/g	0.116 cm^3/g	4.2 μm	22.5 μm
Supersaturated SBF-EMEM Prezasićeni SBF-EMEM	14	1.3 mg	112 m^2/g	0.097 cm^3/g	4.8 μm	
Supersaturated SBF-FCS Prezasićeni SBF-FCS	14	1.1 mg	102 m^2/g	0.107 cm^3/g	4.1 μm	

methods used in our laboratory investigations for synthesis of CHA powders as building blocks for construction of scaffold thin inner walls will be probably published in some next issues of this journal.

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Metoda udruženih agensa i biomimetska metoda dizajna čestica kalcijum-hidroksiapatita kao osnovnih elemenata u strukturiranju koštanih skafolda

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KRATAK SADRŽAJ
U radu su opisane različite modifikacije metoda udruženih agensa i biomimetske metode sinteze i strukturnog dizajna prahova kalcijum-hidroksiapatita, kao osnovnih elemenata u hijerarhijskom strukturiranju koštanih skafolda. Ti elementi su kreirani tako da poseduju dobro definisanu geometriju i fazni sastav, koji su od presudnog značaja za morfološki izgled i dizajn unutrašnjih tankih zidova koštanih skafolda, što ima veliki uticaj na aktivnost ćelija kosti i njihovu ravnotežu tokom procesa modeliranja i remodeliranja nove kosti. Posebno su analizirani različiti načini sinteze čestica kalcijum-hidroksiapatita, kao osnovnih konstitutivnih elemenata unutrašnjih zidova skafolda, s njihovim specifičnim dizajnom s aspekta veličine i oblika dobijenih tokom sinteze čestica. Oni predstavljaju ključne elemente u procesu integracije koštanog tkiva.

Ključne reči: metoda udruženih agensa; biomimetska metoda; kalcijum-hidroksiapatit; inženjering koštanog tkiva

UVOD

Glavni faktori inženjeringa koštanih tkiva su: biokompatibilni skafold pogodan za kačenje osteogenetskih ćelija, njihovu proliferaciju i diferencijaciju, osteogene matične ćelije i biosredina koja je pogodna za dugotrajno formiranje koštanog tkiva i njegovo održavanje. Ovi faktori imaju ključni značaj za ravnotežu između procesa modeliranja (procesa formiranja koštanog tkiva pomoću osteoblasta) i procesa remodeliranja (procesa resorpcije koštanog tkiva pomoću osteoklasta), obezbeđujući koherentnu strukturu koštanog tkiva.

Aktivnost osteogenih matičnih ćelija i progenitorskih ćelija, koje su odgovorne za povećanje koncentracije osteoblasta, ćelijsku deobu i proliferaciju, ćelijsku diferencijaciju i ćelijsku smrt apoptozom, zavisi najčešće od hemijskog sastava i bioloških osobina kalcijum-hidroksiapatita (CHA), kao i od njegovih morfoloških osobina, oblika i raspodele veličine čestica, što je veoma bitno za odgovarajuću strukturu unutrašnjih zidova skafolda. Hemijske i biološke osobine sintetizovanih CHA treba da budu što je moguće sličnije odgovarajućim osobinama biološkog CHA, glavnog sastojka prirodne kosti.

Optimalni CHA treba da ima slabu kristalnost, hemijski sastav u većoj ili manjoj meri pomeren u odnosu na hemijski sastav stehiometrijskog CHA, što je uslovljeno odgovarajućim katjonskim i anjonskim izmenama jona Ca^{2+} i PO_4^{3-} u CHA s odgovarajućim katjonima i anjonima, kao što su: Mg^{2+}, Sr^{2+}, Zn^{2+}, B^{3+}, SiO_4^{2-}, CO_3^{2-} itd. U skladu s tim, kod bioloških apatita – na primer, Mg^{2+} – jona ima oko 6 mol% tokom početne faze osteogeneze u mladoj kosti, dok se njihov sadržaj značajno smanjuje kod stare kosti. Pored toga, joni Mg^{2+} stimulišu nukleaciju velikog broja CHA nukleusa, utičući na njihovu dalju kristalizaciju i inhibiciju njihovog rasta, što je od presudne važnosti za bolje mehaničke osobine kosti. Takođe, joni CO_3^{2-}, koji se nalaze u CHA kristalnoj ćeliji, kod tzv. karbonatnih CHA poboljšavaju njihovu hemijsku i biološku aktivnost. Ako se joni PO_4^{3-} zamene u kristalnoj ćeliji CHA sa jonima CO_3^2, tada ovakav CHA (koji preovladava u mlađim kostima i poznat je kao CHA tipa B) ima veću biološku aktivnost nego CHA, u kojem su joni OH^- zamenjeni sa CO_3^{2-} (tzv. A tip CHA, koji preovladava kod starijih kosti). Uz to, tipična veličina kristalita kod prirodne kosti je dužine 30-50 nm (ponekad dostiže i 200 nm), širine 15-30 nm i debljine 2-10 nm. Ove vrednosti su uslovljene oblikom i veličinom šupljina unutar mreže kolagenskih vlakana.

Sledeće važne osobine koštanih skafolda su snažna proteinska adhezija i proliferacija osteoblasta. Na osnovu brojnih objavljenih rezultata [1-5], jasno je da je za bolju aktivnost CHA i njegovu bolju kompatibilnost sa biološkim apatitom neophodno imati manje ili više izmenjen hemijski sastav sintetizovanog CHA u odnosu na stehiometrijski CHA. Time se obezbeđuje slična brzina biodegradacije CHA, tj. brzina otpuštanja odgovarajućih jonskih vrsta iz kristalne ćelije CHA unutar bioloških medija i njegova bolja površinska aktivnost. Drugi važan faktor bioaktivnosti CHA je odgovarajuća modifikacija površinske strukture CHA, da bi se njome omogućile bolja adhezivnost i proliferacija koštanih ćelija. To se obezbeđuje odgovarajućim strukturnim dizajnom i hemijskim sastavom čestica CHA, koje se koriste kao odgovarajući gradivni elementi finalne strukture skafolda.

Da bi se pokazale promene u strukturi i osobinama osnovnih čestica radi dizajniranja strukture skafolda, u ovome radu prikazan je set različitih metoda udruženih agensa i biomimetskih metoda s odgovarajućim specifičnostima, kao mogućih prilaza u dizajniranju geometrije gradivnih elemenata skafolda [6, 7, 8]. Pored ovih metoda, u našoj laboratorijskoj praksi se primenjuju i mnoge druge, vrlo značajne i obećavajuće metode, kao što su: hidrotermalna metoda i njena modifikacija sa sudelovanjem površinski aktivnih supstanci (SAS) u kombinaciji sa mehanohemijskom metodom ili bez nje; metoda refluksa sa dominantnim udelom organskog rastvarača uz učešće spoljašnjih fizičkih polja (radiofrekventno polje) ili bez njih; ultrazvučna sprej-piroliza, metoda dvostruke inverzne micele i metoda CHA sinteze pomoću enzima. U nekom od sledećih radova neke od ovih metoda biće posebno predstavljene.

METODE SINTEZE KALCIJUM-HIDROKSIAPATITA I OSOBINE DOBIJENIH PRAHOVA

Prva metoda koja će ukratko biti predstavljena u ovom radu mogla bi se uslovno nazvati metodom udruženih agensa. Različite vrste udruženih agensa, koje čine smese SAS i polimera, mogu se koristiti kao matrice kaveza (micela) u kojima dolazi do samonukleacije CHA tokom njihove sinteze. Udruženi agensi, posebno SAS, mogu izazvati različite specifične reakcije između polarnih grupa SAS i CHA kroz njihovu elektrostatičku interakciju sa jonima Ca^{2+}. Ove polarne grupe deluju kao aktivna mesta, koja imaju presudan uticaj na nukleaciju CHA i orijentisani rast kristala. Na taj način stanje agregacije udruženih agensa polimera i surfaktanta postaje glavni faktor kontrole veličine zrna CHA, njihovog oblika, morfologije i polidisperznosti.

Tipičan izgled praha CHA sintetizovanog pomoću ove metode, uz korišćenje smese polivinil-alkohola (PVA) i etilenvinil-acetata ili etilenvinil-versatata (EVA/EVV) kao udruženih agensa, prikazan je na slikama 1a i 1b. Najkraće, u ovom specifičnom slučaju negativno naelektrisanje acetatnih i versatatnih jona (karboksilnih polarnih grupa) igra ulogu aktivnih mesta za suprotno naelektrisane jone Ca^{2+}, dok unutar PVA/SAS micela dolazi do orijentisanog rasta CHA, koji se dešava kroz naizmeničnu difuziju jona PO_4^{3-} i Ca^{2+}. Tipičan mehanizam stvaranja ovakvih micela, koje imaju ulogu specifičnih inkubatora u kojima se odvija proces samonukleacije CHA, prikazan je na slici 1c.

Pored PVA, u ovim eksperimentima su kao polimeri korišćeni hidrogelovi alginata, hidroksimetilceluloze, modifikovanog skroba, hidroksimetilmetakrilata, a kao površinski SAS, EVA/EVV, polioksietilen-oktilfenil etar, polisorbat 80, polioksietilen-sorbitan monooleat, melaminsulfonat, acetiltrimetil amonijum-bromid, natrijum-dodecil benzen-sulfonat i natrijum-dodecil sulfonat.

Veličine dobijenih čestica štapićastog oblika bile su prečnika 100-200 nm i dužine 0,5-2 μm (odnos 10:1). Dobro međusobno razdvojene čestice sa veoma izraženim pravilnim rasporedom u redove, koji su udaljeni jedan od drugog skoro podjednako, čine ove sisteme izuzetno zanimljivim za modeliranje interakcija između skafolda i ćelija kosti, što je suštinski važno za istraživanje aktivnosti koštanih ćelija i njihovog prilagođavanja na morfološke osobine površine skafolda.

Može se očekivati da ovakvi sistemi odigraju ulogu precizno dizajniranih oslonaca "mostnih" konstrukcija, koje se potom lako međusobno mogu spojiti koristeći biomimetsku metodu daljeg rasta CHA, koja je potpuno stimulisana dejstvom telesnih tečnosti (SBF) koje se sastoje od smese različitih jona, naelektrisanih proteina i aminokiselina.

Biomimetska metoda je metoda sinteze CHA iz prezasićenih SBF rastvora. Za takvu vrstu istraživanja bila je neophodna prethodna sinteza tankog silicijum-dioksidnog filma pomoću metode termalne sprej-pirolize. Ovde se koristi dobro određen rastvor silicijum-dioksida sa lancima dužine 18-20 silikatetraedara, koji se deponuju na površinu čelične trake, da bi se tek nakon toga na površini tako formiranog silicijum-dioksida, sa mestimično ugrađenim OH^- grupama (na svakih 9-10 tetraedara jednom OH^- grupom, koja funkcionalizuje površinu tankog silicijum-dioksidnog filma), deponovao biomimetski CHA.

Čelična traka koja je na taj način prekrivena tankim silicijum-dioksidnim filmom uranja se potom u slabo prezasićeni rastvor SBF, koji je prethodno pripremljen po delimično izmenjenoj recepturi u odnosu na tipični sastav SBF dat prema recepturi Kokuba (*Kokubo*) i saradnika [1]. Koncentracije odgovarajućih jona u našoj recepturi bile su sledeće: $c(Cl^-)=0{,}054$ mol/l; $c(Na^+)=0{,}0542$ mol/l; $c(Ca^{2+})=0{,}0025$ mol/l; $c(PO_4^{3-})=0{,}001$ mol/l; $c(Mg^{2+})=0{,}0003$ mol/l; $c(NO_3^-)=0{,}0006$ mol/l i $c(K^+)=0{,}0014$ mol/l. Tanki silicijum-dioksidni film na površini čelične trake (film debljine 22,5 μm), prikazan na slikama 2a i 2b, sastoji se od štapića silicijum-dioksida, koji su izrazito orijentisani u određenom pravcu.

Za sam mehanizam samonukleacije CHA od presudnog su značaja Si-OH grupe, koje se nalaze na krajevima silicijum-dioksidnih lanaca, koje indukuju heterogenu nukleaciju CHA na površini tankog silicijum-dioksidnog filma. Jednom kad se nukleusi formiraju na mestima OH^- grupa, kristali CHA rastu spontano stvarajući slojeve apatita slične kostima, zbog toga što je koncentraciju tipičnih jona za CHA u SBF iznad granice zasićenja (posmatrano u odnosu na proizvod rastvorljivosti stehiometrijskog CHA). Na osnovu navedenih činjenica može se zaključiti da na depoziciju apatita sličnih kostima utiču rast, prezasićenost u odnosu na proizvod rastvorljivosti apatita i postojanje funkcionalnih grupa, kao što je Si-OH grupa, koje indukuju heterogenu nukleaciju apatita na površini materijala.

Početna faza nukleacije indukovane Si-OH grupama opisana je u radovima Takadame (*Takadama*) i saradnika [1], kao i u našim prethodnim istraživanjima. U svim tim istraživanjima ispitivan je proces nukleacije CHA indukovan Si-OH grupama u SBF. Pokazano je da silanol grupe ne indukuju nukleaciju apatita direktno, nego prethodno formiraju amorfna kalcijum-silikatna jedinjenja, kao što su $\equiv SiOCa^+$ i $(\equiv SiO)_2Ca$ inkorporacijom jona kalcijuma. Amorfni kalcijum-silikati potom inkorporiraju u sebe fosfatne jone, stvarajući nukleuse CHA.

Na slikama 3a i 3b prikazan je film CHA posle nukleacije tokom 33 dana na površini SiO_2 filma debelog 22,5 μm koji je nanet na površinu čelične trake. Kao što se može videti, CHA film se sastoji od malih čestica veličine 0,138-0,41 μm i aglomerata CHA, najčešće veličine 1,66-2,77 μm, dok su najveći aglomerati obično 2,8-4,86 μm. Aglomerati imaju izgled koji podseća na bele kugle cvetova maslačka sa manjim grančicama, koje čine manji aglomerati i male izdužene čestice (dužine 200 nm i prečnika 20-30 nm). Donji tamni slojevi su takođe jasno vidljivi, kao i veoma tamni delovi koji se nalaze ispod njih. Na površini ovih slojeva jasno se uočavaju i novoformirani, svetliji slojevi CHA.

Trodimenzionalni izgled čestica samonukleiranog CHA prikazan je na AFM snimcima na slikama 4a i 4b. Jasno se uočavaju slojevi CHA koji rastu sloj po sloj, nižući se jedan na drugi, stvarajući prvo ostrvca, koja se potom međusobno spajaju u monolitne strukture procesima sekundarne nukleacije, procesima erozije površine ostrvaca usled dinamičkih nestabilnosti u kanalima između ostrvaca, izazvanih dejstvom kapilarnih sila unutar datog SBF.

Specifična varijacija ove vrste sinteze, ali malo složenija, prikazana je na slikama 5a i 5b, koja pokazuje tipičan izgled samonukleiranog CHA kada se umesto samog SBF u jednoj od varijacija koristi smesa SBF i EMEM (engl. *Eagles minimal essential medium*), koji sadrži soli $CaCl_2$, KCl, $MgSO_4$, NaCl i $NaHPO_4$ u koncentraciji koja prevazilazi proizvod rastvorljivosti stehiometrijskog CHA.

Na slikama 5a, 5b, 6a i 6b dat je prikaz samonukleiranog CHA u smesi prezasićenog SBF i EMEM, čiji je izgled vrlo sličan izgledu CHA kada je korišćen samo prezasićeni SBF. Slike

pokazuju vrlo naglašene grane CHA, koje su međusobno umrežene, sa odnosom dužine i prečnika čestica od 10:1.

Slična sinteza je izvedena korišćenjem smese SBF i fetalnog goveđeg seruma (engl. *fetal bovine serum – FBS*), koji sadrži dominantno globularni goveđi serumski albumin kao protein, koji je veoma važan za preživljavanje, rast i deobu ćelija kosti (Slike 7a, 7b, 8a i 8b).

U osnovi eksperimenata sa EMEM i FCS nalazi se početna ideja koja je izvedena iz mehanizma formiranja prave kosti i njene proliferacije u kolagenskim vlaknima u živom koštanom tkivu. Kao što je već rečeno, polimer-indukovani tečni prekursor (PILP) igra dominantnu ulogu u mineralizaciji kosti [5]. PILP je odgovoran za početnu nukleaciju CHA u zonama kolagenskih tkiva, u čije šupljine se uvlači pomoću kapilarnih sila. Mehanizam stvaranja nove kosti koji je opisao Gauer (*Gower*) može se ukratko objasniti na sledeći način: negativno naelektrisanje polianjonskih proteina (fosfoproteina, osteocaklina, fosforina i koštanog sialoproteina), tzv. kiselih proteina i aminokiselina (aspartinske kiseline) deluje kao aktivno mesto za suprotno naelektrisane jone Ca^{2+}, izazivajući potom privlačenje jona PO_4^{3-} u polianjonske proteinske micele, smeštene u šupljinama kolagenskih vlakana [5]. Formiranje CHA odvija se kroz difuziju jona Ca^{2+} i PO_4^{3-} u ove šupljine. Tako dobijeni CHA je u početku u obliku hidratne amorfne strukture, koja tokom dalje jonske preraspodele u daljim fazama procesa oslobađa višak vezane vode, pretvarajući svoju amorfnu strukturu u strukturu kristala.

Slično tome odvija se i proces nukleacije i CHA kristalizacije u EMEM i FBS. U oba slučaja ovih bioloških seruma posebnu ulogu u formiranju CHA imaju aminokiseline u mešovitom sistemu SBF-EMEM i proteini u sistemu SBF-FCS. Obe vrste ovih seruma značajno utiču na kinetiku rasta kristala CHA, pokazujući da prisustvo aminokiselina (u slučaju EMEM) i naelektrisanih proteina (goveđi albumin u slučaju FCS) značajno ubrzavaju njihov rast (Tabela 1). Brzina samonukleacije tankog CHA filma je približno dvostruko veća kad se koriste EMEM ili FCS u smesi sa prezasićenim SBF, nego kad se koristi sam SBF. Sa druge strane, oblik, morfološki izgled i veličina čestica dobijenog praha su slični u svim slučajevima.

ZAKLJUČAK

U radu su opisane različite varijante metode udruženih agensa i biomimetske metode. Opisani su ukratko njihovi glavni mehanizmi sinteze CHA. Specifičnosti dobijenih čestica, kao potencijalnih osnovnih elemenata tankih zidova skafolda, prikazane su sa stanovišta srednje veličine čestica, njihovog oblika, distribucije i odnosa dužine i prečnika. Posebno je naglašena uloga geometrije dobijenih čestica i njihovih faznih nehomogenosti na procese stvaranja koštanih tkiva. Brojne druge značajne metode koje se koriste u našoj laboratoriji za sintezu prahova CHA biće opisane u narednim radovima.