

PREPARATION AND CHARACTERIZATION OF PASTES BASED ON CALCIUM PHOSPHATE POWDERS AND SODIUM HYALURONATE

Radost Ilieva¹, Ognyan Petrov², Elena Dyulgerova³

¹Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences, Acad. G. Bontchev Str., Bl.11, Sofia, Bulgaria

²Institute of Mineralogy and Crystallography, Bulgarian Academy of Sciences, Acad. G. Bontchev Str., Bl.107, 1113 Sofia, Bulgaria

³Dental Medicine Faculty, University of Medicine, 1 G. Sofiiski Str., 1431 Sofia, Bulgaria

Abstract

Series of pastes were prepared on the basis of mono- or bi-phased calcium phosphate powders and solutions of sodium hyaluronate and its modifications as a liquid phase. Three procedures for preparation of the powders were applied and as a result samples with different chemical, phase and morphological characteristics were obtained. The influence of the particle size and morphology of the phases as well as of the composition of the liquid phase and the solid to liquid ratio on the formation features and mechanical strength of the pastes were investigated. Their influence on the micro-environment surrounding by in vitro tests of dried molds in simulated body fluid and in distilled water were followed as well. The results showed that the materials are promising for practical use in medicine.

Key words: *paste-like materials, calcium phosphates, hydroxyapatite, β -tricalcium phosphate, sodium hyaluronate*

1. INTRODUCTION

Natural bone and teeth apatite crystals, which mediate and regulate load-bearing functions are bounded with macromolecular matrix. One of the strategies for preparation of biomaterials for bone engineering includes biodegradable paste or composite of monophase (hydroxyapatite (HA) or bi-phase (HA and β -tricalcium phosphate (β -TCP)) calcium phosphates with organic macromolecules. The ideal material for substitution of mineralized biological structures must be native in composition, promoting bone regeneration, and providing minimal inflammatory responses. In this context the idea for syntheses of "biological smart material" as paste-like material to be used in the medicine and dentistry is a hopeful task (Dan et al. 2009). The paste-like materials should possess good injectability, proper setting time convenient for surgery, excellent anti-washout ability and good mechanical strength (Alkhraisat et al. 2009). So far, some additives, such as citric acid, chitosan and sodium alginate, have been used to optimize the properties of such materials (Suzuki et al. 2014). It has been reported that addition of a certain amount of citric acid could improve the injectability of calcium phosphate but its anti-washout ability was still poor. On the other hand, some documents have confirmed that the anti-washout ability of calcium phosphate could be effectively improved by the addition of chitosan and sodium alginate (Qiao et al. 2013). However, these two biopolymers would inhibit the setting process. Furthermore, the addition of them would influence the calcium phosphate paste rheology and reduce the injectability.

Sodium hyaluronate (NaHA), a polysaccharide having a disaccharide repeating unit (D-glucuronic acid and N-acetyl-D-glucosamine), is a water soluble polymer known to be a bioactive biomaterial. Besides, NaHA has good viscoelastic and hydrating properties when it is in the dissolved form. In extracellular matrix, hyaluronate participates in a hydrated network and acts as an organizing core in the intercellular matrix, connecting complex intercellular aggregates (Suzuki et al. 2014, Qiuhong et al. 2012)

The aim of this study was to investigate the influence of the composition and morphology of calcium phosphate powders as well as of the composition of the liquid phase on the formation features and

mechanical strength of calcium phosphate pastes. For this purpose a solution of sodium hyaluronate and its modifications with citric acid and chitosan were used as a liquid phase. Three procedures were applied for the synthesis of calcium phosphate powders, providing modified reaction medium and including different techniques for sequent treatment of the precipitated precursors. The potential of pastes obtained to be a base for preparation of new "biological smart material" was checked by *in vitro* tests in simulated body fluid and in distilled water.

2. EXPERIMENTS

2.1. Synthesis of calcium phosphate powders

The calcium phosphate (CaP) powders used in this study were synthesized by conventional chemical procedures. All of them used as a start substances solutions of $\text{Ca}(\text{NO}_3)_2$ (1M) and $(\text{NH}_4)_2\text{HPO}_4$ (0.6M) (Merck, analytical grade) .

Procedure A (CaP1) - In this procedure the precipitation process occurred in the $\text{Ca}(\text{NO}_3)_2$ solution by slow dropping of the phosphate solution during continuous stirring and standard conditions. The pH of the system was kepted to be 11 (by NH_4OH). The precursor suspension was matured for 24 h at room temperature. The obtained precipitate was dried by heating at 90°C for 10 h, sintered at 1100°C for 1h and ground in agate planetary ball mill for 40 h.

Procedure B (CaP2) - In this procedure the precipitation process occurred at pH 8 and this was the only difference with the previous procedure. The other difference concerns the treatment of the obtained precipitate just before its drying. Solution of 2% $(\text{NH}_4)_2\text{SO}_4$ in an amount of 10% from the theoretical yield of calcium phosphate was added to the washed precipitate and stirred for 5 min. The obtained precipitate was dried by heating at 90°C for 10 h, sintered at 1100°C for 1h and ground in agate planetary ball mill for 20 h.

Procedure C (CaP3) - The differences in this procedure concern modification of the reaction medium as well as treatment of the precipitated precursor. Both reagent solutions were modified before their mixing. Tri-methyl ammonium bromide (STAB) was added to $(\text{NH}_4)_2\text{HPO}_4$ and polyethylene glycol (PEG) to $\text{Ca}(\text{NO}_3)_2$. The precipitation occurred at pH 8 (with addition of urea) during continuous stirring. After 24 h maturation the obtained precipitate was dried by lyophilisation and sintered at 900°C for 2h. Fine powders were obtained and grounding was not applied.

2.2. Preparing of mold samples

1% solution of sodium hyaluronate and its mixtures with 18% solution of citric acid and/or chitosan (low molecular weight) in different ratios were used as a liquid phase. The ratio calcium phosphate powder to liquid phase was varied from 0.2 to 3.3. The dense pastes were molded in rubber-molds with diameter of 10 mm and height of 5 mm and dried in air for 24 h.

2.3. Chemical analyses and characterization

Chemical analyses - The Ca^{2+} ions concentration was determined potentiometrically by EDTA complexometric titration in the presence of CuEDTA at pH 10, using a Cu ion-selective electrode and an Ag/AgCl reference electrode. The concentration of P-PO_4^{3-} ions was measured spectrophotometrically by NOVA 60 equipment, using Merck and Spectroquant[®] test kits.

Phase characterization - X-ray diffraction (Bruker D8 diffractometer, operating at 40 kV and 40 mA with $\text{CuK}\alpha$ radiation) was applied for phase registration of the CaP powders and the dried pastes.

Morphology studies – SEM images - The powder samples were sputter-coated with gold. Their morphology and microstructure were observed using scanning electron microscope JEOLJSM-5510 at 20 nA beam current and 20 kv accelerating voltage.

Formation characteristics - The initial and final setting times of the paste samples prepared in the rubber-molds were determined by the Vicat needle method (ASTM C191-9 1993). The mechanical strength of the final solid samples was visually checked.

Micro environmental behavior - *In vitro* testing of the air-dried solid samples in simulated body fluid (SBF) and in a distilled water was performed. Conventional simulated body fluid (SBF_c, pH 7.2-7.4) was prepared according to Kokubo (1990) Samples were air-dried for 24 h, taken out from the rubber-molds and were immersed in a solution at a solid to liquid ratio of 1:20. All experiments were conducted at room temperature under static conditions. The pH value of the liquid phases were measured permanently to check the solid samples influence.

3. RESULTS AND DISCUSSION

3.1. Calcium phosphate powders

The synthesis procedure and reaction conditions strongly influence the type of the precipitated phase and the morphology, size and specific surface area of the particles. In this study three procedures were used to synthesize the calcium phosphate powders. The Procedure A and Procedure B were analogous concerning the applied techniques but reactions medium pH were different - 11 for Procedure A and 8 for Procedure B. Thus, monophase HA was awaited in Procedure A and a bi-phase mixture of HA and β -TCP in Procedure B. Additionally, $(\text{NH}_4)_2\text{SO}_4$ solution was used in Procedure B in order to create better conditions for β -TCP formation. Procedure C was designed to obtain also a bi-phase mixture of HA and β -TCP. It was based on the recent approaches proposed as alternative route, using various additives and modification as the biomimetic template systems. In this strategy the nucleation centers are modulated by various macromolecules that act as a soft temporary template (Shanthi et al. 2010, Ye et al. 2008, Ye et al. 2010, Qiu et al. 2008). In this work as template macromolecules the surfactant tri-methyl ammonium bromide (STAB) (Shanthi et al. 2009, Yao et al. 2003, Liu et al. 2002) was used that was added to the solution of $(\text{NH}_4)_2\text{HPO}_4$. Surrounding the PO_4^{3-} ions with oriented water molecules it forms an outer diffuse layer in which the nucleation process starts. PEG was added to the solution of $\text{Ca}(\text{NO}_3)_2$ as a dispersing agent. It was dissolved in water and thus chelated Ca^{2+} ion, forming PEG-O-Ca-O-PEG (Liu et al 2002).

As a result of the applied synthesis three types of powders were prepared differing in chemical (Table 1) and phase (Fig.1) composition and as well in morphological characteristics (Fig.2).

The XRD study of the powder prepared at pH 11 confirmed that it is monophase hydroxyapatite (Fig.1a), while the powders prepared at pH 8 are bi-phase mixtures of HA and β -TCP. Different ratios between both phases were found for sample CaP2 (50% HA and 50% β -TCP) (Fig. 1b) and for sample CaP3 (70% HA and 30% β -TCP) (Fig. 1c). (The quantitative measurement was performed by external standard of whitlockite, using powder XRD data). These results are in accordance with the reported literature data on the influence of medium pH on the phase composition of calcium phosphate precipitates obtained by the wet method.

Table 1. Ca to P ratio in as-prepared CaP powders

Synthesis procedure and CaP powder	Ca/P
Procedure A, CaP1	1.6
Procedure B, CaP2	1.5
Procedure C, CaP3	1.55

The morphology of the obtained powders was characterised by SEM technique (Fig.2). The results confirmed the effect of high energy dry milling on the structural and crystalline state of sintered powders. As we have shown in our previous study (Ilieva et al. 2012), a progressive loss of the original crystalline morphology occurs. The particles are with irregular surfaces depending on the type of the solid phase. Flat agglomerates built from nanosized grains are observed in the case of monophase hydroxyapatite (Fig 2a). In the case of bi-phase powders, the agglomerates are composed of nanoscale round-edges grains (Fig.2b). Completely different are the particles constituting the

powder from Procedure C (Fig.3c) - well separated with round shape, a result from the presence of macromolecules soft template during the precipitation and sintering of the powders.

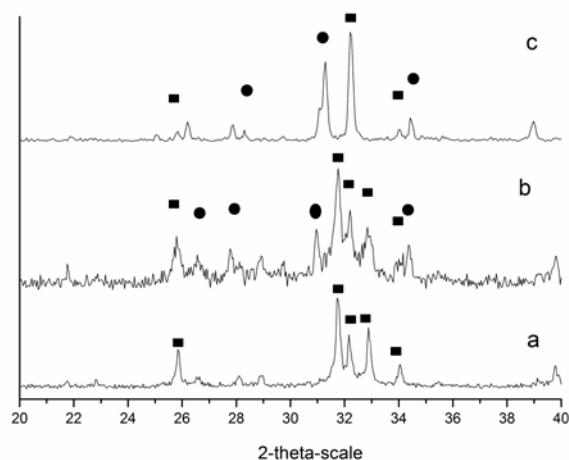


Fig. 1 XRD analysis of sintered powders: CaP1 (a) and CaP2 (b) after milling; CaP3 (c). ● – β -TCP, (■) – HA

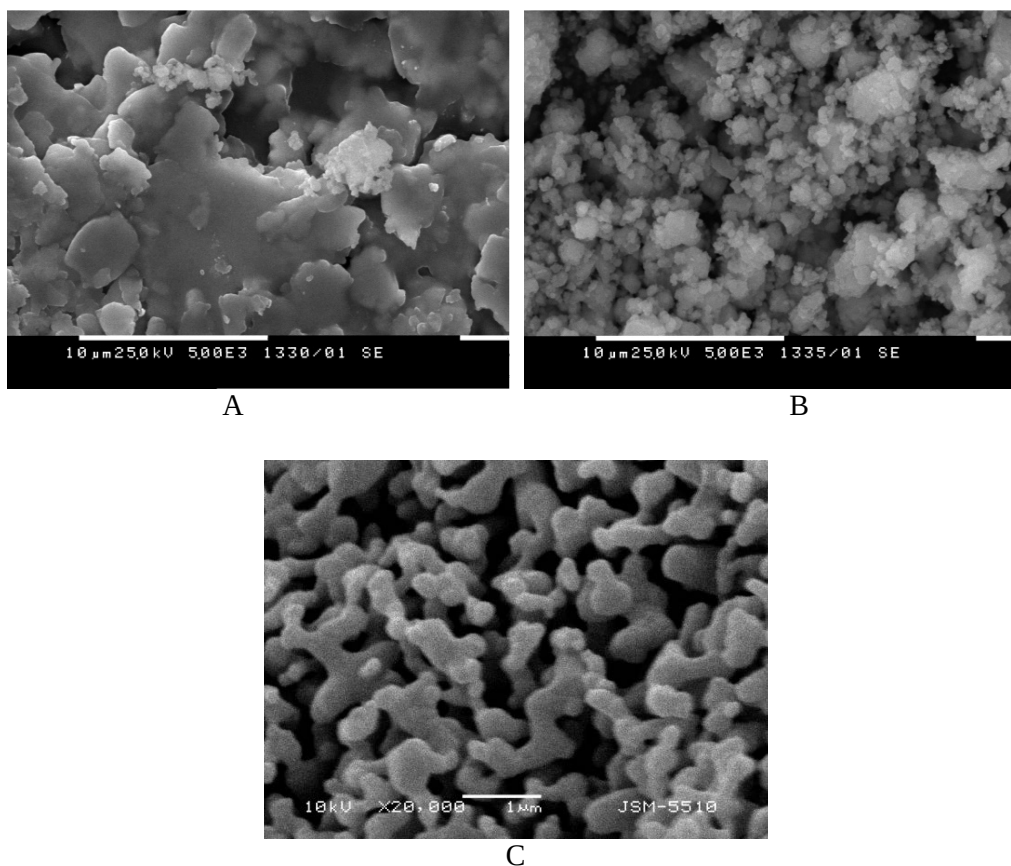


Fig. 2. SEM photos of: 40 h milled CaP1 (a); 20 h milled CaP2 (b) and CaP3 (c)

3.2. Mold Samples

A series of plastic paste-like materials was prepared on the basis of mono- and bi-phase calcium phosphate powders and 1% solution of sodium hyaluronate (NaHA), varying the solid to liquid ratio. Two additives, Chitosan (Ch) and 18% Citric acid (CA), were used also in the liquid phase. The formation features and the mechanical strength of the samples are presented in Table 2.

Table 2. Formation features and mechanical strength of the obtained pastes

Solid to liquid ratio	Liquid phase	Formation features		Mechanical strength of the dried molds
		Initial setting time, min	Final setting time, min	
CaP1 powders from Procedure 1				
3.3	NaHA	6	60	strong
2.8	NaHA	15	60	strong
2.0	NaHA	20	60	strong
1.4	NaHA	27	60	strong
0.4	NaHA:Ch = 4:1	30	60	strong
0.3	NaHA:Ch = 2:1	6	45	strong
0.2	NaHA:Ch = 1.3:1	6	30	strong
0.6	NaHA:CA = 2:1	15	60	strong
0.5	NaHA:CA = 1:1	3	10	strong
CaP2 powders from Procedure 2				
2.8	NaHA	22	45	strong
2.0	NaHA	21	60	strong
1.6	NaHA	30	<60	strong
1.25	NaHA	35	60	strong
0.6	NaHA:CA = 2:1	15	60	strong
0.5	NaHA:CA = 1:1	3	10	strong
CaP3 powders from Procedure 3				
2.8	NaHA	40	<60	cracky
2.0	NaHA	45	<60	cracky
1.6	NaHA	50	<60	strong
1.25	NaHA	55	<60	strong
0.5	NaHA:CA = 1:1	27	<60	strong
1.25	NaHA:CA:Ch=1:1:1.5	20	<60	strong

Note: NaHA - 1% Sodium hyaluronate, CA – 18% Citric acid, Ch – Chitosan

The results revealed that the finer the powder the higher is the amount of the liquid needed for preparation of plastic material that remains compact after drying (solid to liquid ratio of 3.3 for CaP1, 2.8 for CaP2 and 1.6 for CaP3). Samples composed of CaP1 powder display the shorter initial setting

time. The differences in the morphology of the surface grains of CaP2 and CaP3 lead to increasing of the initial setting times (Table 2). The modification of liquid phase with citric acid leads to defects on the grains surface and decreases the setting times for samples with CaP1 and CaP2 powders but do not influence significantly the samples with CaP3 powders. The formation of defects on the particle grains stimulates their ability to weld together through organic molecules of liquid phase. In the case of CaP particles with not defect surface the formation times increase.

3.3 *In vitro* testing of the dried molds in SBF and distilled water

The results from the *in vitro* testing of compact dried CaP moulds in SBF and in distilled water are presented in Figures 3-5.

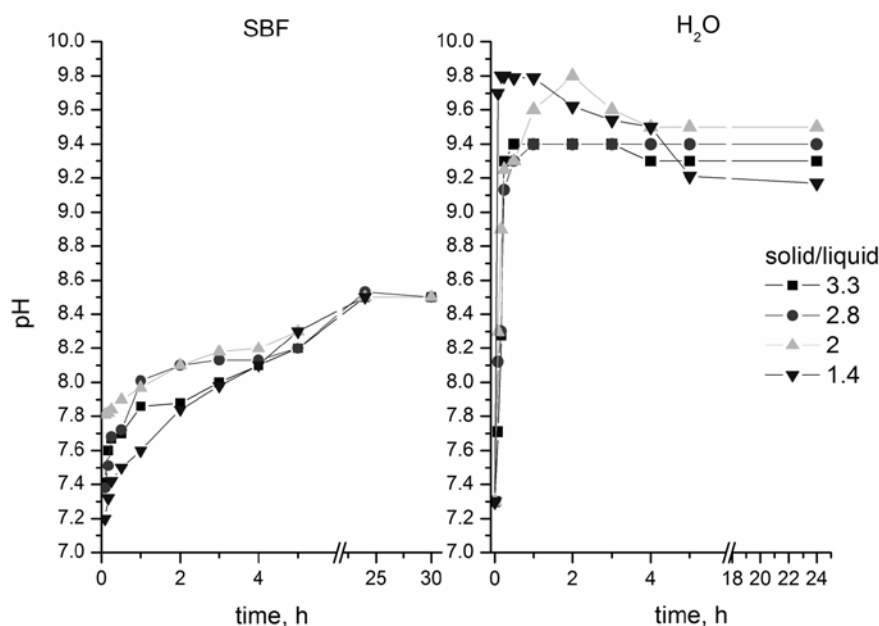


Fig.3. pH changes in SBF and in distilled water at *in vitro* testing of molds of CaP1 with sodium hyaluronate. - (SBF pH 7.2-7.4; water pH 7).

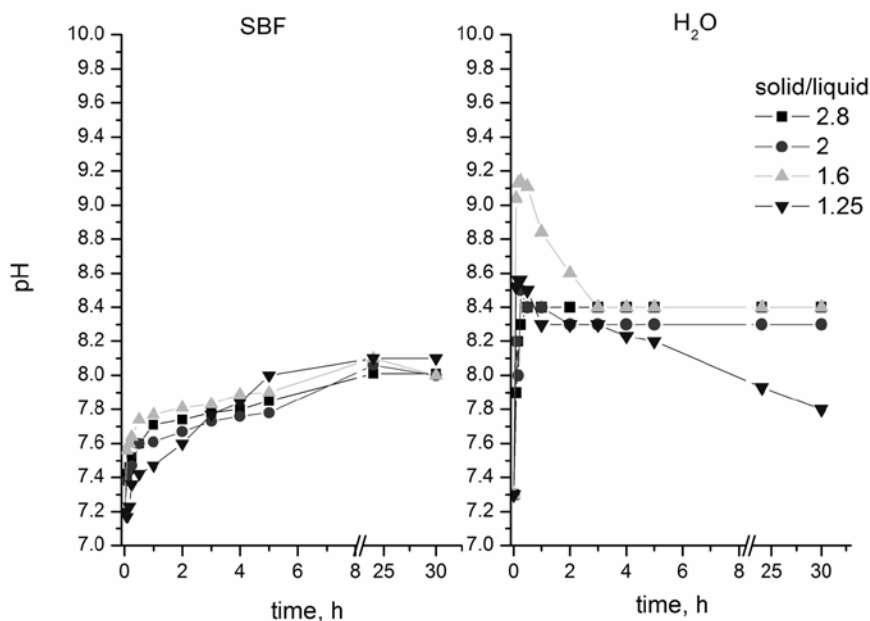


Fig.4. pH changes in SBF and distilled water at *in vitro* testing of molds of CaP2 with sodium hyaluronate. (SBF pH 7.2-7.4; water pH 7).

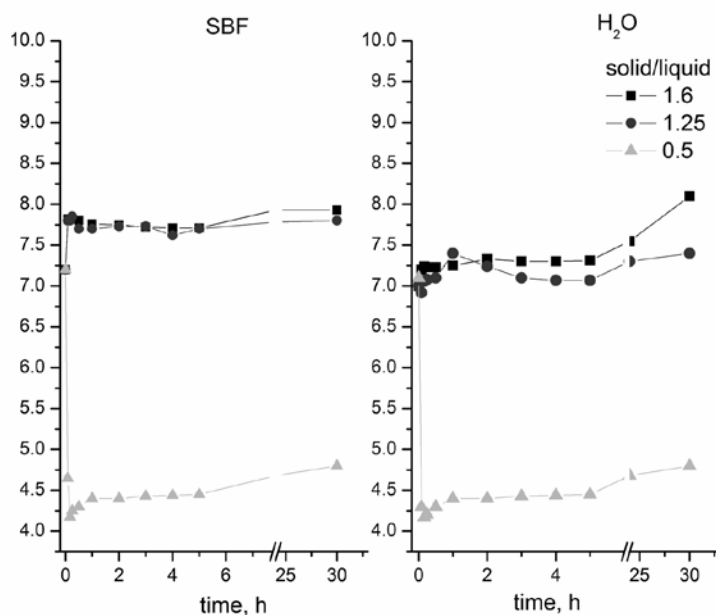


Fig.5. pH changes in SBF and distilled water at *in vitro* testing of molds of CaP3 with sodium hyaluronate (■; ●); with sodium hyaluronate and citric acid (1:1) (▲). (SBF pH 7.2-7.4; water pH 7).

The behavior of the molds prepared with CaP1 (Fig.3) and with CaP2 (Fig.4) powders is completely different in SBF and in water, whereas with CaP3 (Fig.5) powder is similar in both media and is the most stable. pH of the SBF gradually increased during 24h up to 8 for CaP2 and up to 8.4 for CaP1. pH of the water drastically increased up to 8.6-9.2 for CaP2 and up to 9.2-9.4 for CaP1 in the first 2h

and after that slightly reduced and the systems reached equilibrium at values higher than the physiological pH (7.2-7.4). The highest registered pH was for CaP1 in water. This result probably is due to a partial dissolution of HA that could be stimulated by the defects of grains surface as a result of the high energy milling. The buffer capacity of SBF, due to its buffer systems $\text{HCO}_3^-/\text{CO}_2$ and $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$, keeps the pH. For the molds with CaP3 powder pH increased up to 7.8 and remained unchanged in SBF, but slightly increased after 24th h in water. The presence of citric acid decreased pH up to 4.2 of both SBF and distilled water. All molds with CaP2 powder remain compact after 30h treatment in SBF or distilled water while the others were destroyed.

4. CONCLUSIONS

Series of paste-like materials were prepared on the basis of mono- or bi-phase calcium phosphate powders and sodium hyaluronate. Three procedures for preparation of CaP powders were applied and as a results samples with different chemical, phase and morphological characteristics were obtained. The influence of the latest as well as of the composition of the liquid phase and the solid to liquid ratio on the formation features and mechanical strength of the materials were investigated. It was found that high energy milling stimulates the binding capacity of the hydroxyapatite but not of β -TCP. In the cases of sodium hyaluronate solution the initial setting time was more than 20 min and the final about 1h. The included citric acid significantly reduced the settings time (3 min initial setting time) but decrease the pH of the SBF and distilled water below 4. Other samples increased pH.

It is of interest to underline that this study was inspired by the idea to prepare smart material for endodontics as root canal sealers, which is bio-inductive and promotes regeneration processes in apical region (Gusiyska 2014, Gusiyska 2014a, Gusiyska 2012, Gusiyska & Ilieva 2015) This is a new challenge in 21st century endodontics studies related with the clinical application of new materials that can adopt to ever-changing microenvironment of the canal system (Glickman & Koch 2000) Having in mind this, we are look hopefully for future investigation and results in this new disputable fields and to assist dentists in providing more predictable and reliable endodontic treatments.

ACKNOWLEDGEMENTS

This work was financially supported by the Bulgarian Ministry of Education and Science under Project DFNI T02-5/2014

REFERENCES

- Alkhraisat, M, Rueda, C, Marino FT, Torres, J, Jerez, LB, Glureck, U & Cabarcos, E 2009, 'The effect of hyaluronic acid on brushite cement cohesion', *Acta Biomater.*, vol. 5, pp. 3150-3156.
- ASTM C191-9 1993, 'Standard test method for time of setting of hydraulic cement paste by Vicat needle' In: *Annual book of ASTM standards*, vol. 04. 01: Cement, Lime, Gypsum, American Society for Testing and Materials: Philadelphia, USA, pp. 158-160
- Dan, K, Dongxiao, L, Xiangdony, Z, Lei, Z, Hongsong, F & Xingdoug, Z 2009, 'Addition of sodium hyaluronate and the effect on performace of the injectable calcium phosphate cement', *J. Mater Sci:Mater. Med*, vol. 20, pp.1595-1602.
- Glickman, G & Koch, A 2000, '21st Century endodontics' *JADA*, vol. 131, pp.39S-46S.
- Gusiyska, A & Ilieva, R 2015, 'Nanosize Biphasic Calcium Phosphate used for Treatment of Periapical Lesions', *IJCR*, vol. 7, no.1, pp. 11564-11567.
- Gusiyska, A 2012, 'Orthograde treatment of chronic apical periodontitis - biological approaches', *PhD Thesis*, Medical University, FDM-Sofia, pp. 254.

- Gusiyska, A 2014, 'Periapical resorptive processes in chronic apical periodontitis: an overview and discussion of the literature', *J. of IMAB*, vol. 20, no.5, pp. 601-605.
- Gusiyska, A 2014a, 'In vivo analysis of some key characteristics of the apical zone in teeth with chronic apical periodontitis', *IJSR*, vol.3, no.12, pp. 2390-2393.
- Ilieva, R, Dyulgerova, E, Petrov, O, Aleksandrova, R & Titorenkova, R 2012, 'Effects of high energy dry milling on biphasic calcium phosphates', *Advances in applied ceramics*, vol. 112, no.4, pp. 219-226.
- Kokubo, T 1990, 'Surface chemistry of bioactive glass-ceramics', *J Non-Cryst Solids*, vol. 120, no. 1-3, pp. 138-151.
- Liu, Y, Wang, W, Zhan, Y, Zheng, C, Wang, G 2002, 'A simple route to hydroxyapatitenanofibers', *Mater. Lett.*, vol. 56, pp. 496-501.
- Qiaoa P, Wang J, Xie Q, Lia , Dongc L & Xu T 2013, 'Injectable calcium phosphate–alginate–chitosan microencapsulated MC3T3-E1 cell paste for bone tissue engineering in vivo', *Materials Science and Engineering: C*, vol. 33, no 8, pp 4633–4639
- Qiu, C, Xiao, X, Liu, R & She, H 2008, 'Biomimetic synthesis of spherical nanohydroxyapatite with polyvinylpyrrolidone as template', *Mater. Sci. Technol.*, vol. 24, pp. 612-617.
- Qiu hong L, Ming L, Peizhi . & Shicheng, W 2012, 'In vitro synthesis of bioactive hydroxyapatite using sodium hyaluronate as a template', *J. Mater. Chem*, vol. 22, pp. 20257-20264.
- Shanthi, P, Ashok, M, Balasubramanian, T, Riyasdeen, A & Akbarsha, M 2009, 'Synthesis and characterization of nano-hydroxyapatite at ambient temperature using cationic surfactant', *Mater. Lett.*, vol. 63, pp. 2123-2125.
- Shanthi, P, Mangalaraja, R, Uthirakumar, A, Velmathi, S, Balasubramanian, T & Ashok, M 2010, 'Synthesis and characterization of porous shell-like nanohydroxyapatite using Cetrimide as template', *J. Colloid Interface Sci.*, vol. 350, pp. 39-43.
- Suzuki, K, Anada, T, Miyazaki, T, Miyatake, N, Honda, Y, Kishimoto, K, Hosaka, M, Imaizumi, H, Itoi, E & Suzuki, O 2014, 'Effect of addition of hyaluronic acids on the osteoconductivity and biodegradability of synthetic octacalcium phosphate', *Acta. Biomater.*, vol. 10, pp. 531-543.
- Yao, J, Tjandra, W, Chen, YZ, Tam, KC, Ma, J & Soh, B 2003, 'Hydroxyapatite nanostructure material derived using cationic surfactant as a template', *J. Mater. Chem.*, vol. 13, pp. 3053-3057.
- Ye, F, Guo, H & Zhang, H 2008, 'Biomimetic synthesis of oriented hydroxyapatite mediated by nonionic surfactants', *Nanotechnology*, vol. 19, pp. 245605-245607.
- Ye, F, Guo, H, Zhang, H & He, X 2010, 'Polymeric micelle-templated synthesis of hydroxyapatite hollow nanoparticles for a drug delivery system', *Acta Biomater.*, vol. 6, pp. 2212-2218.