Study of the impact of amino acids hydrochloride forms on the formation of biomimetic composites in the presence of nanocrystalline hydroxyapatite

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Abstract. The study of molecular composition of the biomimetic composites on the basis of nanocrystalline carbonate-substituted (C-HAP) and polar amino acids of L-lysine hydrochloride and L-arginine hydrochloride was performed in the work. Results of the study show that the change in acidity of the medium (pH≥11.2, ≥7.55 и ≤5) occurs under transformation of the conformational environment of L-lysine hydrochloride molecules with the formation of L-lysine structure as while increase of pH level as in the presence of C-HAP nanocrystals. At the same time composites basing on L-arginine keep their amorphous structure in the presence of synthesized C-HAP in the different media. The detected differences in the molecular environment of hydrochloride forms of amino acids during the interaction with nanocrystalline C-HAP prove to be significant for the development of technology of the integration between biocomposites and hard dental tissues.

1. Introduction
In the dental material science integration of the filling materials with enamel and dentin of the human teeth is an urgent and challenging problem that calls for: 1) development of new biomaterials; 2) determination of the type of interaction between the components comprising these materials and the inner organization of the composite; 3) identification of the conditions for the high-performance integration of biocomposite with the native tissues of the dental matrix [1,2]. Reproduction of the inorganic part of nanostructured hierarchical complex in the enamel and dentin of the human teeth within the biomimetic concept that can be achieved due to the introduction into the composites of carbonate-substituted hydroxyapatite (C-HAP) (Ca₁₀₋₁₃(PO₄)₆₋₇(CO₃)₃(OH)₂₋₃, 0.1<x<0.3) due to the correspondence of its chemical composition – inorganic part of the dental matrix [1,3,4]. In a number of works [3,5–8], concerned with the problem of restoration of the dental tissue a special attention is focused on the use of organic matrix together with C-HAP for the formation of the active biocompatible medium capable to fill the surface and bulk defects in the teeth. A special issue of the studies is the interaction of the components comprising biocomposites between each other as well as with the surface of the prepared dental tissues [7,9]. It should be noted that the interaction between the components of biomimetic composite at the nanolevel occurs due to the formation of the bonds between nanocrystalline non-stoichiometric hydroxyapatite with a great number of isomorphic
substitutions as well as with amino acids, proteins and collagen, and in case of hybrid systems – with the complexes of artificial resins and different polymers [3,8,10].

Being sub-units of the protein matrix in the teeth amino acids realize a lot of functions allowing HAP to form with the required configuration. A crucial aspect in the formation of the robust bond between the protein/amino acid matrix and the mineral (phosphate) complexes/compounds is not only the presence of the lateral bonds for amino acids as well as the free radicals on the surface of mineral complexes [5,10], but also some certain conditions (type of the solvent, temperature, pH value of the environment) for the behaviour of the integration process between biomimetic composite and the native tissue [11,12]. Note that in some part of the investigations a considerable effect of the extraneous ions on the process of interaction of hydroxyapatite with amino acids that were present in the enamel matrix was identified [13].

Therefore, the study of the changes in the molecular environment of carbonate-substituted hydroxyapatite and hydrochloride forms of the polar amino acids which are present within the enamel matrix was performed in order to specify the mechanisms of formation for biomimetic systems.

2. Materials and methods

2.1. Synthesis

Formation of biomimetic systems was made in the aqueous medium. At the first stage a homogeneous suspension was obtained which was comprised of nanocrystalline C-HAP reproducing the features of the enamel apatite [4,14]. At the second stage aqueous solutions of amino acids were obtained with the use of crystallized powders of L-lysine hydrochloride (LysHCl) and L-arginine hydrochloride (ArgHCl) in the concentrations corresponding to the content of L-lysine and L-arginine in the dental matrix. Before mixing solutions containing suspension of C-HAP and solutions of amino acids were subjected to ultrasound treatment for 5 minutes using homogenizer Q55 Sonica W=55W. In order to study the dependences of molecular environment for C-HAP on the charge state of amino acids the investigations were performed in the media with pH≥11.2, ≥7.55 and ≤5- above and below isoelectric points of L-lysine and L-arginine.

2.2. Methods

Investigations of the obtained composites were performed with the use of microscpecroscopy technique employing spectrometer RamMix 532 (EnSpectr), aligned with the microscope CX41 Olympus. The wavelength of the exciting radiation was of 532 nm and the spectra were registered in the range of 250-1750 cm⁻¹. IR spectra of the obtained composites were observed in the range of 4500-400 cm⁻¹ with Vertex-70 (Bruker) spectrometer employing the attenuated total reflectance attachment (ATR) PlatumunATR. Transmittance spectra were registered with a step of 2 cm⁻¹. Processing of the spectral data, normalization, background correction were performed with the use of Origin 9.0 program suite.

3. Results

3.1. Raman microspectroscopy

Figure 1 represents the spectra of the sample L-lysine hydrochloride after its staying in the medium with pH<5 (curve 1); composite C-HAP/LysHCl formed in the medium with pH<5 (curve 2); composite C-HAP/LysHCl formed in the medium with pH~7.8 (curve 3); composite C-HAP/LysHCl formed in the medium with pH>11.2 (curve 4) and the sample of L-lysine hydrochloride after its staying in the medium with pH>11.2 (curve 5).

While analyzing of Raman spectra it was found that under the same concentration of L-lysine hydrochloride during its dissolution and the following drying in the air crystalline modifications of the original components of L-lysine samples were formed [15]. Moreover, experimental data show that upon the dissolution of L-lysine hydrochloride with a specified concentration and the following drying of the sample in the air crystalline modifications of L-lysine are formed [15].
Upon dissolution of L-lysine hydrochloride in the media with pH>11 its recrystallization takes place with the formation of crystalline form that is more correspondent to the pure L-lysine.

It should be noted that introduction of nanocrystalline C-HAP into the solutions of L-lysine hydrochloride in the media with pH>7.8, results in the appearance of low-intensive vibrations in the Raman spectra (figure 1) within the range of 995, 1187, 1225 cm⁻¹, associated with NH₃⁺ group and redistribution of the modes intensities in the range of 1360 cm⁻¹, where vibrations of the group COO⁻ are observed [13]. From the analysis of Raman spectra (figure 1) for the samples obtained in the medium with pH<5, it follows that the synthesis is accompanied by the changes taking place as in the molecular environment of L-lysine hydrochloride in the presence of C-HAP. Conformational transformations of L-lysine hydrochloride are presented in the Raman spectrum as the changes in the intensity of vibrations correlated with the components of the carbon chain in amino acid.

Spectra of the sample of L-arginine hydrochloride after its staying in the medium with pH<5 (curve 1); composite of C-HAP/ArgHCl formed in the medium with pH~7.8 (curve 3); composite of C-HAP/ArgHCl formed in the medium with pH >11.2 (curve 4) and the sample of L-arginine hydrochloride after its staying in the medium with pH>11.2 (curve 5) are presented in figure 2. Unlike the samples of L-lysine hydrochloride, those of L-arginine hydrochloride keep their amorphous structure as in the neutral media as in the media with pH>11 and pH<5. Similar tendency for the samples of L-arginine is also observed in the presence of nanocrystalline C-HAP. The detailed analysis of Raman spectra for L-arginine revealed redistribution of the spectral lines attributed to guanidine complex, since its low-intensive vibrations in the Raman spectra can be observed in the range of 980 cm⁻¹ and 1395 cm⁻¹ (figure 2). An interest to this group of vibrations is caused by the fact that the interaction with C-HAP is realized due to the formation of the bond between the phosphate complexes of C-HAP and guanidine group of arginine as it was shown in [13]. However, it should be noted that upon comparison of the spectra presented in fig. 2 one can observe a stable ratio of intensities characteristic for the carbon and amide bonds that is possible related with the presence of chlorine atoms bonding to the guanidine complex of L-arginine.

3.2. Results of IR-spectroscopy
IR-spectra of the samples of C-HAP/LysHCl composite formed in the medium with pH<5 (curve 1) in the medium with pH~7.8 (curve 2) and in the medium with pH>11.2 (curve 3) are presented in figure 3. It should be noted that in IR-spectra of C-HAP/L-lysine biocomposites there are vibrations associated either with C-HAP or with L-lysine. At the same time in the spectra of C-HAP/L-lysine a number of features are observed in a dependence of the medium and the charge state of L-lysine hydrochloride, respectively.
First, redistribution of the modes intensities for the vibrations of $\text{PO}_4^{3-} v_4$ (602, 561 cm$^{-1}$) and $\text{PO}_4^{3-} v_3$ (1091, 1024 cm$^{-1}$) was observed. Second, a decrease of intensity for the shoulder near 630 cm$^{-1}$ attributed to the vibrations of OH group in C-HAP can be seen (figure 3, curves 1,2,3). Simultaneously with the changes of C-HAP modes in IR-spectra the composites obtained in the acidic medium redistribution of intensity takes place as well as the shift of the bands attributed to the vibrations of the lateral chains of $\text{NH}_3^+$ and $\text{COO}^-$ L-lysine in the range of 1500-1576 cm$^{-1}$, 1148-1161 cm$^{-1}$ and 558-790 cm$^{-1}$, respectively (figure 3, curves 1,2,3).

The spectra of the samples of C-HAP/ArgHCl composite formed in the medium with pH<5 (curve 1), in the medium with pH=7.8 (curve 2) and in the medium with pH>11.2 (curve 3) are presented in figure 4. Analysis of IR spectra of the samples demonstrated that within the range of 1750-500 cm$^{-1}$ absorption bands attributed to the vibrations of $\text{PO}_4$ (1040 cm$^{-1}$, valence and deformational vibrations of the bonds $\text{P}=\text{O}$ and $\text{P}–\text{O}$) [8]. Note, that the vibration band at 1650 cm$^{-1}$ should be considered as the total vibration of $\text{C}=\text{O}$ and N-H bonds. The absorption band at 1665 cm$^{-1}$ should be attributed to the deformational vibrations of N-H bonds in amines and ions of $\text{NH}_3^+$ type. The detected bands in the range of 1449 and 1466 cm$^{-1}$ correspond to the deformational vibrations of C-H bonds in the structural fragments of -CH$_-$ and -CH$_2$ types, as well as to symmetrical deformational vibrations of N-H bonds in the ions of $\text{NH}_3^+$ type [13]. Let us focus on the changes in the position and intensity of the mode related with symmetrical deformational vibrations of the bonds in N-H group of $\text{NH}_3^+$ ion in the spectrum of L-arginine hydrochloride in the range near 1168 cm$^{-1}$ for the different pH levels. Intensity of this mode in the composites on the basis of L-arginine hydrochloride and C-HAP corresponds to the changes in the lateral bond of L-arginine in the presence of the foreign ions that is in accordance with the data presented in literature [13].

4. Discussion
The results of analysis for the molecular environment of L-lysine hydrochloride and L-arginine hydrochloride which are the main polar amino acids in the enamel matrix of the human tooth in the media with different values of pH support the data presented in literature on the change of the charge state in amino acids upon the increased or decreased acidity of the medium. In addition, one can observe the differences in conformational environment at formation of the bonds with nanocrystalline carbonate-substituted hydroxyapatite in the presence of hydrochloride form of amino acids.

The results of Raman spectroscopy show that during interaction of carbonate-substituted hydroxyapatite on the surface with L-lysine hydrochloride the latter one changes its charge state (figure 1). In the medium with pH<5 intensity of COO$^-$ vibrations and vibrations of CH in the main chain of
amino acid molecule increases that, in principle, differs from that the one observed for pure L-lysine [15]. An increase of the medium pH level resulted in a decrease of intensity for COO\(^{-}\) bands and an increase of low-intensive vibrations in the spectra within the range of 995, 1187,1225 cm\(^{-1}\) attributed to NH\(_3^+\) (figure 1). It means both the change of the charge state for amino acid and that of the main type of interaction with C-HAP which is due to its basic properties participates in reaction of neutralization with L-lysine in the cation form in the acidic medium.

The comparison of Raman spectra of L-arginine (figure 2), its alkaline, neutral and acidic solutions with the addition of C-HAP shows the change of the modes intensity for guanidine group and the corresponding vibration of C-N1-H\(_2\) L-arginine in the range of 980 cm\(^{-1}\), as well as the absence of the changes for vibrations in the range of 1150-1350 cm\(^{-1}\), attributed to NH\(_2^+\) and also known from the literature. As it was shown in [13] such behavior of arginine molecules can be connected with the presence of chlorine atoms in the original form of arginine that are bound with NH\(_3^+\) groups. Due to its defect nature HAP adsorbs on its surface molecular complexes of L-arginine hydrochloride but without formation of the chemical bond as it happened in case of the pure arginine [13,16].

The results of IR-spectroscopy are in agreement with the literature data on the change of the charge state of L-lysine hydrochloride and L-arginine hydrochloride in the media with different alkalinity (figure 3,4). This is supported by redistribution of the modes intensity attributed to the lateral, i.e. amide and carboxyl groups of amino acid [3,13,15]. The change in the ratio of intensity for OH modes of hydroxyapatite and phosphorus-oxygen tetrahedrons at the corresponding change of the ratio for the intensities of modes attributed to the lateral bonds of amino acid means that interaction between C-HAP and L-lysine proceeds by formation of the chemical bonds based on the mechanism of the charge compensation between C-HAP and L-lysine (figure 3). Formation of the bond between the group of PO\(_4^3-\) in C-HAP and NH\(_2^+\) of L-lysine mainly occurs in acidic medium while substitution of OH group in hydroxyapatite by carboxyl COO\(^{-}\) group of L-lysine proceeds in the alkaline medium [8,10,13].

In the IR-spectra of the samples of L-arginine with different pH values of the medium a slight redistributions of the modes intensity are observed associated with the vibrations of amide groups (figure 4). It mainly indicates the changes of conformation for the molecules of arginine in the media with different acidity. Introduction of C-HAP into the matrix of L-arginine results in the change of intensity for the transmission bands in the range of 1090, 962 cm\(^{-1}\), associated with the vibrations of phosphorus-oxygen tetrahedrons in hydroxyapatite [6]. Thus, redistribution of the modes intensities is observed; these modes are related to the vibrations of amide groups in L-arginine in case of increased and decreased alkalinity. It means that the interaction between C-HAP and L-arginine is realized with the help of amide groups.

5. Conclusion

The change of the medium acidity at the stage of formation of biomemimetic composites results in the transformation of conformational environment for the molecules of L-lysine hydrochloride with the formation of the structure of pure L-lysine; it is probably connected with the release of chlorine ions as upon an increase of pH solution as with the presence of C-HAP nanocrystals. Since the samples of L-arginine keep amorphous structure as in the neutral media as in the media with pH>11 and pH<5. Similar tendency for the samples of L-arginine is observed in the presence of C-HAP.

Using IR-spectroscopy it was found that under formation of the composite containing L-lysine hydrochloride redistribution of intensity takes place for the vibrating modes corresponding to the lateral bonds of L-lysine and OH-groups of hydroxyapatite. According to the data of Raman microspectroscopy, interaction of L-arginine amino acid with C-HAP is realized on the basis of molecular orientation mechanism with a participation of NH\(_2^+\) group. A low activity of this mode in the spectra as well as amorphous stable structure of L-arginine means stabilization of L-arginine in the presence of chlorine atoms and accounts for a weak interaction with C-HAP.

The differences in molecular environment of amino acids under interaction of their hydrochloride forms with nanocrystalline C-HAP are significantly important for the development of technique applied for the integration of biomimetic composites with the native hard dental tissues.
Acknowledgments
The study was performed under support of RFBR (grant № 18-29-11008 mk) and grant of the President of Russian Federation №MK-419.2019.2.

References
[1] Dorozhkin S V 2017 Hydroxyapatite and Other Calcium Orthophosphates: Bioceramics, Coatings and Dental Applications [Hardcover] (Nova Science Publishers, Inc New York)
[2] Goloshchapov D, Kashkarov V M, Ippolitov Yu A, Ippolitov I Yu, Vongsvivut J and Seredin P V 2019 Synchrotron IR-microspectroscopy-based visualization of molecular and chemical interactions between dental cement, biomimetic composite and native dental tissue Bull. Russ. State Med. Univ. 71–8
[3] Comeau P and Willett T 2018 Impact of Side Chain Polarity on Non-Stoichiometric Nano-Hydroxyapatite Surface Functionalization with Amino Acids Sci. Rep. 8 12700
[4] Goloshchapov D L, Lenshin A S, Savchenko D V and Seredin P V 2019 Importance of defect nanocrystalline calcium hydroxyapatite characteristics for developing the dental biomimetic composites Results Phys. 13 102158
[5] Tavafoghi Jahromi M and Cerruti M 2015 Amino Acid/Ion Aggregate Formation and Their Role in Hydroxyapatite Precipitation Cryst. Growth Des. 15 1096–104
[6] Seredin P V, Goloshchapov D L, Prutskij T and Ippolitov Yu A 2017 Fabrication and characterisation of composites materials similar optically and in composition to native dental tissues Results Phys. 7 1086–94
[7] Wu X, Zhao X, Li Y, Yang T, Yan X and Wang K 2015 In situ synthesis carbonated hydroxyapatite layers on enamel slices with acidic amino acids by a novel two-step method Mater. Sci. Eng. C Mater. Biol. Appl. 54 150–7
[8] Jahromi M T, Yao G and Cerruti M 2013 The importance of amino acid interactions in the crystallization of hydroxyapatite J. R. Soc. Interface 10 1–14
[9] Lagazzo A, Barberis F, Carbone C, Ramis G and Finocchio E 2017 Molecular level interactions in brushite-aminoacids composites Mater. Sci. Eng. C Mater. Biol. Appl. 70 721–7
[10] El Rhilassi A, Mourabet M, Bennani-Ziatni M, El Hamri R and Taitai A 2016 Interaction of some essential amino acids with synthesized poorly crystalline hydroxyapatite J. Saudi Chem. Soc. 20 Supplement 1 S632–40
[11] Gómez-Morales J, Delgado-López J M, Iafisco M, Hernández-Hernández A and Prat M 2011 Amino Acidic Control of Calcium Phosphate Precipitation by Using the Vapor Diffusion Method in Microdroplets Cryst. Growth Des. 11 4802–9
[12] Rimola A, Corno M, Garza J and Ugliengo P 2012 Ab initio modelling of protein–biomaterial interactions: influence of amino acid polar side chains on adsorption at hydroxyapatite surfaces Phil Trans R Soc A 370 1478–98
[13] Hernández B, Pflüger F, Derbel N, De Coninck J and Ghomi M 2010 Vibrational Analysis of Amino Acids and Short Peptides in Hydrated Media. VI. Amino Acids with Positively Charged Side Chains: L-Lysine and L-Arginine J. Phys. Chem. B 114 1077–88
[14] Kashkarov V M, Goloshchapov D L, Rumyantseva A N, Seredin P V, Domashevkaya E P, Spivakova I A and Shumilovich B R 2011 X-ray diffraction and IR spectroscopy investigation of synthesized and biogenic nanocrystalline hydroxyapatite J. Surf. Investig. X-Ray Synchrotron Neutron Tech. 5 1162–7
[15] Pazderka T and Kopecký V 2017 Drop coating deposition Raman spectroscopy of proteinogenic amino acids compared with their solution and crystalline state Spectrochim. Acta. A. Mol. Biomol. Spectrosc. 185 207–16
[16] Aliaga A E, Garrido C, Leyton P, Diaz F. G, Gomez-Jeria J S, Aguayo T, Clavijo E, Campos-Vallette M M and Sanchez-Cortes S 2010 SERS and theoretical studies of arginine Spectrochim. Acta. A. Mol. Biomol. Spectrosc. 76 458–63