The Interplay of manganese and nitrate in hydroxyapatite nanoparticles as revealed by pulsed EPR and DFT†

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The interplay of oppositely charged substitutions in the structure of hydroxyapatite (HAp) nanopowders is investigated on the atomic level by pulsed electron paramagnetic resonance (EPR) technique and ab initio density functional theory calculations. Benefits of EPR to determine Mn2+ ions in nano-HAp samples are demonstrated. A simple approach based on the measurements of electron spin relaxation times allowed observing the strong influence of fast-relaxing Mn2+ ions on the relaxation characteristics of the nitrate ions (NO3−/NO32−) incorporated in trace amounts. Based on the results of ab initio calculations, we show the propensity of Mn2+ and NO3−/NO32− to associate within the HAp crystal lattice. This could have a direct impact on the functional properties of the material especially to resorption and ion exchange. Furthermore, such an effect can increase a propensity of undesired impurities to incorporate into the doped nanocrystals.

1. Introduction

Hydroxyapatite (Ca10(PO4)6(OH)2; HAp) has been recognized as a promising biomaterial more than 30 years ago. Currently, it finds various applications: as a material for bone implant surgery,1–3 as a vehicle for bio-imaging and therapeutic delivery,4,5 and in chemical engineering.6–8 HAp can be quite easily synthesized in the form of nanocrystals doped by various ionic species, thereby obtaining desired functionality and biocompatibility of the material. Therefore, the mechanisms of uptake and release of substitutational ions attract a great interest from researchers. Among all the possible substitutions, divalent cationic additives (transition metals Zn2+, Co2+, Cu2+, Mn2+, alkaline earths and other metals of the II-nd group of the periodic table) are of particular importance.9 These dopants can either alternate physicochemical properties of the material and its surface (solubility and mechanical strength) or directly induce a biological response when released in the host environment via an ion exchange pathway.

There are numerous theoretical and experimental studies devoted to the thermodynamics of ion exchange and uptake with special attention on the functional characteristics of the material.10–12 However, the existing models usually do not consider a possible influence of the lattice defects (including those that are created by other additives) on the distribution of divalent ions within the crystal, their uptake and their release. Moreover, HAp always contains a certain level of intrinsic defects and impurities, which appears to be a relevant issue. However, one can expect that the propensity for defects or undesired impurities (which also can be potentially harmful) incorporated into the modified HAp might differ from the case of the pure HAp material. Therefore, it is quite important to be able to characterize and predict a mutual impact of different ionic substitutions within HAp crystals and to understand the underlying mechanisms.

Only few studies providing an atomic-level insight on this problem exist. The recent study by Matsunaga et al. contains a detailed discussion of a tendency of substitutional Zn2+ ion to associate with charge-neutral Ca2+ vacancy defect complex.13 Another related issue regarding the propensity of the segregation of Mg2+ ions (instead of uniform distribution) within the lattice. There are several publications describing the effects of HAp co-doping by Mg2+ and CO32−,14 Zn2+ and CO32−,15 Sr2+ and CO32−,16 Mg2+, Sr2+ and Mn2+17 on the functional characteristics of HAp. To the best of our knowledge, the issue is still poorly explored, especially for the case of the nanosized material.

The vast majority of the studies devoted to the doping of nanostructures are focused on semiconducting materials.19,20

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c5cp01986a
In general, doping of nanocrystals is widely appreciated to be a very challenging problem (for example, due to self-purification mechanisms). In this sense, for effective development and production of functional nanostructured materials, understanding the fundamental process of doping is mandatory. This objective is often addressed to and successfully treated by theoretical analysis. However, theoretical studies and calculations should be supported experimentally. It motivates the search for reliable analytical methods to characterize the novel nanostructures, including HAp-based materials. Electron paramagnetic/spin resonance (EPR/ESR) has been proven as a powerful analytical tool for apatite studies. In our recent studies, we exploited the capabilities (mainly the increased sensitivity and resolution) of modern commercial equipment along with techniques, such as pulsed (Fourier transformed, FT) EPR, relaxation measurements and pulsed electron-nuclear double resonance (ENDOR), to extend the boundaries of the EPR methods to characterize the nano-HAp containing species. We have shown that when supported with an appropriate computational approach, EPR spectroscopy can not only provide unique information about the chemical and electronic structure of a defect or an impurity in the crystal lattice but also shed light on the physical properties and composition of the crystalline environment.

In this study, we show the capabilities of an approach based on the combination of the pulsed EPR methods and first-principles calculations on a specific case of simultaneous incorporation of oppositely charged ions in nanosized HAp. Namely, we study the interplay of substitutional Mn$^{2+}$ ions with nitrate NO$_3^-$/NO$_2^-$ anionic impurity.

We focus on manganese because it is one of the proposed additives to HAp to improve its osteoconductivity and to promote bone formation (see ref. 31 for the recent review on this issue). It is also known that for Mn in different types of crystals, electron paramagnetic resonance reveals spin interactions that are sensitive to the local environment of the impurity. The radiation-induced paramagnetic defect, NO$_2^-$, which can be easily formed under X-ray, ultraviolet, or some other type of irradiation of the samples grown by the wet precipitation technique, has well-established spectroscopic parameters and its structure is found to be similar to that of B-type carbonate CO$_3^{2-}$ impurity, which is a most abundant substitution in HAp and of great biological relevance. This motivates the use of NO$_3^-$/NO$_2^-$ as a model of anionic substitution introduced in trace amount to probe the expected effects.

Thus, we examine the impact of paramagnetic Mn$^{2+}$ ions on the spectral and relaxation characteristics of the NO$_3^-$/NO$_2^-$ radical and support the experimental results by density functional theory (DFT) calculations. Based on the obtained results, we discuss a possible role of anionic impurities present in HAp in the uptake and release of divalent metal ions during mineralization or ion exchange.

2. Materials and methods

2.1. Experiment

Mn$^{2+}$-containing HAp samples with the chemical formula Ca$_{10-0.005}$Mn$_{0.005}$(PO$_4$)$_6$(OH) (Mn-HAp) and with the average size of crystallites of about 30(10) nm were synthesized by the wet precipitation technique. Calcium nitrate monohydrate Ca(NO$_3$)$_2$·H$_2$O (235 g) was dissolved in 420 ml of 20% NH$_4$OH (solution A), whereas (NH$_4$)$_2$HPO$_4$ (72.2 g) was dissolved in 380 ml of deionized water (solution B). After (NH$_4$)$_2$HPO$_4$ was completely dissolved, 30 ml of 20% NH$_4$OH was added. Mn-containing reagent (MnSO$_4$·H$_2$O) was dissolved in 100 ml of deionized water and then added to solution A. Solution B was then added to the Mn-containing solution A and mixed by a magnetic stirrer for 24 h. After mixing, the precipitate was allowed to age for 48 h. Subsequently, the supernatant was decanted and then washed with 2.5 l of deionized water three times to remove excess of NH$_4$OH and NO$_3^-$. The washing procedure was repeated three times. After washing, the MnHA precipitate was separated from the liquid by filtering through a Buchner funnel. A part of the MnHA precipitate was heated at 900 °C for 3 h to remove residual NH$_4$NO$_3$ and improve sample crystallinity. The powders of nanosized HAp containing only nitrate impurity (hereinafter denoted as “pure HAp” or just HAp) were prepared by the same technique as described in ref. 34.

X-ray diffraction (XRD) analysis (D2 Phaser diffractometer, Bruker) shows that all the samples contain only one phase with the parameters of the unit cell typical for the bulk crystals of hydroxyapatite. Sizes of crystallites (30 nm) were extracted from X-ray diffraction line profile (002) using Scherrer- and Wilson-formulas from Williamson-Hall-plots according to the procedure of de Keijser, Langford, Mittemeijer.

The micromorphology of the powders was examined by scanning and transmission electron microscopy (JEM-2000FX II, JEOI, operated at 200 kV and FESEM LEO SUPRA 50VP, Carl Zeiss, 5 kV). It is consistent with the XRD outcomes and with the results of ref. 34: the nano-particles were grown mainly in the form of needles having an average length of 30(10) nm and cross-sectional size of less than 10 nm. Because the HAp cell volume is about 1 nm$^3$ it means that one 40 nm × 10 nm × 10 nm cuboid-shaped nano-particle contains about 4000 unit cells. This is obviously the upper bound for the studied nanostructures.

The chemical content of the powders was proved by EDS (INCA Energy+, Oxford Instruments, attached to LEO SUPRA 50 VP) and ICP-MS (ELAN – DRC II, Perkin Elmer). The formula Ca$_{10-0.005}$Mn$_{0.005}$(PO$_4$)$_6$(OH) defines the upper limit of 20 manganese ions per one nano-particle (assuming uniform distribution among the nano-particles).

As it was shown previously, the exploited synthesis procedure leads to the incorporation of NO$_3^-$ ion into the HAp lattice (most probably substituting PO$_4^{3-}$ group). The stable NO$_3^{2-}$ paramagnetic center can be produced by X-ray irradiation. In the present study, the irradiation of the synthesized nanopowders was performed using URS-55 tube (U = 55 kV, I = 16 mA, W – anodic) at room temperature with the estimated dose of 10 kGy. Such high dose allows us to suggest that all possible NO$_3^-$ ions are ionized (cf. with our results in ref. 28–30).

Pulsed and continuous wave EPR measurements were done using combined X-band (9 GHz) and W-band (94 GHz) Bruker Elexsys 580/680 spectrometer equipped with the liquid helium
temperature controller. Electron spin echo (ESE) EPR spectra were obtained by means of field-swept two-pulse echo sequence π/2–τ–π with the pulse length of π pulse of 16 (X-band) or 36 ns (W-band), correspondingly, and time delay τ = 240 ns. For the phase-memory time measurements, τ was varied from 200 ns up to the desired value with the minimal possible step of 4 ns. The Inversion-Recovery pulse sequence π–T_{delay}–π/2–τ–π, where π pulse is defined above and T_{delay} was varied, for the spin–lattice relaxation times measurements was used. To separate the contributions from the paramagnetic centers with different electronic spins (S = 1/2 and S = 5/2), we changed the incident microwave power by switching the high power attenuation (HPA) between 7 dB and 16 dB.

2.2. DFT calculations

We employ the plane-wave pseudopotential approach with the Perdew–Burke–Ernzerhof version of the generalized gradient approximation of the exchange–correlation functional (GGA-PBE) and Vanderbilt ultrasoft pseudopotentials as implemented in the Quantum ESPRESSO package. The calculations were performed using an 88-atom HAp supercell (1 x 1 x 1 monoclinic cell; space group P2_1/b) with a 2 x 2 x 1 Monkhorst–Pack k-point mesh and energy cutoffs of 40 Ry for the smooth part of the electron wave functions and 320 Ry for the augmented electron density. The initial positional parameters for pure HAp were taken from ref. 40. Before computing the quantities of interest, the structures were allowed to fully relax. The convergence of the supercell size was analyzed by the test calculations using 176-atom HAp configurations (1 x 2 x 1 monoclinic cell). Since it is known that the GGA-PBE approach has limited accuracy in treatment of strongly localized d electrons, we employed DFT+U method that includes additional Coulomb repulsion for Mn^{2+} 3d shell. The Hubbard-type Coulomb repulsion U was set to 3 eV based on the linear response analysis performed in this study. Even though we found that use of both U = 0 and U = 3 eV leads to qualitatively similar trends in defect energetic, only the case of U = 3 eV is discussed in the study.

3. Results and discussion

3.1. ESE EPR of Mn^{2+} in the nanosized powders

Before the X-ray irradiation, pure HAp samples are EPR silent. For Mn-HAp samples, a broad line at 9 GHz (X-band) is observed (see the next Section 3.2), which is obviously due to the presence of manganese. Indeed, even in the perfect bulk crystals, the EPR spectra of Mn^{2+} are very complicated due to the fine structure of the electronic states (electronic spin S = 5/2), strong hyperfine interaction of electron spin with ^{55}Mn nuclear spin I = 5/2 and perceptible quadrupole moment of ^{55}Mn nuclei. Furthermore, in the nanosized samples, the EPR lines broaden causing spectral overlap.

At the same time, the spectrum is well resolved in the ESE W-band experiments (see Fig. 1). The six line pattern with the value of g-factor g = 2.001(1) and hyperfine splitting typical for ions embedded into the crystal structure of powder samples of A_{hf} \approx 9.1(1) mT clearly indicates the presence of manganese in 2+ charge state.

It can be noted that the quantitative determination of manganese in calcified materials, including bone and teeth, is still a challenge for the analytical tools because of the structural and chemical complexity of the investigated matrix. The benefits of the commercial W-band EPR instrument for the determination and quantification of manganese starting from ppm and sub-ppm levels in the apatite-like minerals was demonstrated in ref. 49. We show that EPR in the pulsed mode could be used for the non-destructive studies of the manganese-containing HAp species. In this sense, modern EPR spectroscopy, in our opinion, should be considered as an additional powerful method for the comprehensive analysis of the calcified tissues (materials). A location (position) of the introduced manganese even in the bulk samples is still debated. As for the natural apatites, two nonequivalent calcium positions Ca(1) and Ca(2) distinguishable in the HAp structure are proposed for the Mn^{2+} substitutions (see section DFT calculation for details). Experimentally, we washed the prepared samples in KOH and HCl solutions with various pH values, annealed the samples at different conditions and temperatures up to 850 °C and did not observe any changes in the Mn^{2+} EPR spectrum. This can serve as additional evidence that the Mn^{2+} ions we observe are embedded into the HAp lattice.

Our electron-nuclear double resonance (ENDOR) measurements in the W-band reveal the splittings due to the interaction with the neighboring ^{1}H and ^{31}P nuclei with I = 1/2. The detailed analysis of the similar ENDOR spectra for the Pb^{3+} and NO_{2}− paramagnetic impurities in HAp samples allowed us to precisely define their location in the HAp cell. The simple simulations of the experimental ENDOR spectra in the case of Mn^{2+} in HAp nanoparticles match both the calcium positions, and therefore, the ENDOR spectra (shown as Fig. S1 in ESI†) are not discussed in this study. For the DFT calculations, we consider models in which the Mn^{2+} ions occupy either Ca(1) or Ca(2) sites.
3.2. EPR and relaxation time measurements in the irradiated samples

In the EPR spectra of the irradiated samples, the spectrum of NO$_3^{2-}$ paramagnetic centers is the dominant one. Fig. 2A and B present the spectra of NO$_3^{2-}$ obtained in the HAp and Mn-HAp after X-ray irradiation. The observed three line pattern is a typical powder spectrum due to the hyperfine interaction (described by the hyperfine constant of axial symmetry $A$) between the electronic spin with $S = 1/2$ and one nuclear spin with $I = 1$ as a case for $^{14}$N. Detailed interpretation of the NO$_3^{2-}$ spectrum in (nano)-HAp samples is given in ref. 29, 30, and 33. The signal of Mn$^{2+}$ ions in the EPR spectrum of the irradiated Mn-HAp is manifested as a broad Gaussian line due to unresolved fine structure. No changes in EPR spectra with temperature within the investigated temperature range were noticed.

No significant difference in EPR spectrum of NO$_3^{2-}$ is observed with Mn-codoping. However, the relaxation characteristics (spin–lattice/longitudinal relaxation time $T_1$ and phase-memory/spin–spin/transverse relaxation time $T_2$) of the NO$_3^{2-}$ paramagnetic center change drastically. This is illustrated in Fig. 2C for $T_2$ relaxation time ($T_2^*$ decay and $T_{1e}$ recovery curves could be described by the mono-exponential functions) obtained in the magnetic field corresponding to the maximal ESE signal [marked as $B_2$ on Fig. 2B]. It can be seen that the phase memory time of NO$_3^{2-}$ in Mn-HAp dramatically shortens (up to 30 times) tending to the value of $T_2^*$ of Mn$^{2+}$ below 80 K. The modulation of the electron spin echo due to electron-nuclear couplings (ESEEM) was observed only at $T < 15$ K. Its depth is estimated to be less than 10% of the ESE amplitude and therefore, ESEEM does not considerably influence the presented results.

The measured temperature dependencies of the spin–lattice relaxation rates $T_1$ are quite complicated due to the contributions of different mechanisms and their detailed analysis is beyond the scope of this study. The effect of the Mn-codoping can be illustrated by the data for $T = 50$ K in X-band: $T_{1e}$(HAp, 343.5 mT) = 2500(30) μs; $T_{1e}$(Mn-HAp, 343.5 mT) = 280(5) μs; and $T_{1e}$(Mn-HAp, 326 mT) = 10(1) μs. Within the studied temperature range $T_1 > 8T_2^*$, one can exclude the influence of $T_1$ relaxation mechanisms on $T_2^*$ for the data analysis.

The values of the spin–spin and spin–lattice relaxation times especially at low temperatures (at room temperatures they can be too short to measure and track the influence of the neighboring spins) can be sensitive indicators of spin–spin interactions in distance regimes, wherein the lineshape changes in conventional EPR are very small to detect. The stronger the dipolar interaction, the more effectively the relaxation of the fast relaxing spin is communicated to the more slowly-relaxing spin. In the limit of strong interaction, the relaxation time for the slowly-relaxing spin becomes equal to that for the rapidly-relaxing spin. Strong influence of Mn$^{2+}$ on the relaxation of NO$_3^{2-}$ indicates that the distances between them are less than 1–2 nm if we account only single inter-ionic interaction.

From the comparison of the integrated intensities of cw and ESE detected EPR spectra of Mn$^{2+}$ and NO$_3^{2-}$ between each other and with the external reference (aqueous solution of CuHis complex), we estimated the relative number of ions studied (cf. with the inset in Fig. 2B), which was found as $N$(Mn$^{2+}$)$/N$(NO$_3^{2-}$) $\approx$ 25(5). It implies (assuming the homogeneous distribution of NO$_3^{2-}$) that not more than one NO$_3^{2-}$ radical can be found in one nano-particle in the investigated...
species, whereas the number of Mn\(^{2+}\) ions can reach the value of about 20 (see Section 2.1). Along with the fact that all the observed T\(_2^+\) decay and T\(_1\) recovery curves are the monoeponential functions, it may be assumed that the Mn\(^{2+}\) ions are not randomly distributed in the vicinity of NO\(_3^-\)/NO\(_2^-\)/NO\(_4\) within nanocrystal. To address this issue, we performed first-principles calculations presented in the next section.

Probably due to the fast relaxation mechanisms and sufficiently large number of Mn\(^{2+}\) ions around the nitrate radical, we could not observe the influence of Mn\(^{2+}\) ions on NO\(_3^-\)/NO\(_2^-\)/NO\(_4\) in the attempted ESEEM, ENDOR or electron-electron double resonance (ELDOR, DEER) measurements using our EPR technique and by applying the standard pulse schemes. It makes it tricky to estimate the interatomic distances and ion distribution directly from the experiment.

3.3. DFT calculations

First, we analyzed the thermodynamic stability and the crystallographic surroundings of Mn\(^{3+}\) in pure HAp (without addition of nitrate impurity). Basically, we calculated total energy of the 88-atom HAp supercell with a single Mn\(^{2+}\) ion incorporated at the various Ca atomic sites. Though the real concentration of Mn\(^{2+}\) ions in the investigated species is much lower, we believe that this model properly describes an isolated substitution. From a symmetry standpoint, there are two nonequivalent types of Ca(1) site and three types of Ca(2) site in the crystal structure of HAp.\(^{3,9,15,46}\) We tested all of these atomic positions. In each case, the incorporation of Mn\(^{2+}\) leads to appreciable structural deformations associated with the shortening of Mn–O distances compared to Ca–O bonds. The analysis of the calculated energies suggests that Ca(2) sites are energetically more favorable for Mn\(^{2+}\) substitution compared to Ca(1) positions. The energy between the Ca(1) substitutional sites and the most stable Ca(2) position is found to be about 0.13 eV, whereas for the less stable Ca(2), this value is 0.06 eV. Similar results were reported in ref. 14 for Mg\(^{2+}\) substitution. The last one has an ionic radius close to that of Mn\(^{2+}\).

For the nitrate-containing HAp, we used a model of nitrate defect described in detail in ref. 29 and 30. We assume that the impurity substitutes PO\(_4\)\(^{3-}\) group with charge balancing maintained via removal of the adjacent Ca ion (Fig. 3). The position of Ca vacancy is chosen to obtain the lowest ground-state energy of the HAp supercell in the presence of NO\(_3^-\) (without Mn\(^{3+}\)) only and is fixed throughout the simulations.

To investigate the propensity of NO\(_3^-\) and Mn\(^{2+}\) substitutions to associate within a supercell, we calculate the binding energy of the pair:

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E_b = E_{\text{tot}}(\text{NO}_3/\text{Mn}) + E_{\text{tot}}(\text{HAp}) - E_{\text{tot}}(\text{NO}_3) - E_{\text{tot}}(\text{Mn}),
\]

where \(E_{\text{tot}}(\text{NO}_3)\), \(E_{\text{tot}}(\text{Mn})\) and \(E_{\text{tot}}(\text{NO}_3/\text{Mn})\) are the total energies of the HAp supercells containing NO\(_3^-\) ion, Mn\(^{2+}\) ion and both NO\(_3^-\) and Mn\(^{2+}\), respectively; \(E_{\text{tot}}(\text{HAp})\) is the total energy of the same supercell without impurities.

We compared the values of \(E_b\) for different arrangements of NO\(_3^-\)/Mn\(^{2+}\) pair. The selected configurations are shown in Fig. 3. Negative sign of \(E_b\) suggests the high probability of substitutions to occupy the corresponding adjacent positions in HAp lattice.\(^{53}\) The largest values of about \(-0.3\) eV were obtained for Mn\(^{2+}\) occupying the Ca(1) sites closest to nitrate (see Fig. 3; Mn\(_1\) site; the distance between the manganese and nitrogen centers is \(r_{\text{N-Mn}} = 0.34\) nm). Regarding the case of Mn\(^{2+}\) at Ca(2) positions neighboring the NO\(_3^-\) ion, this also gives the negative values of \(E_b\) (around \(-0.15\) eV; Mn\(_2\) site in Fig. 3, \(r_{\text{N-Mn}} = 0.63\) nm). At the same time, the binding energy of about \(-0.05\) eV obtained for the structures with Mn\(^{2+}\) located relatively far from NO\(_3^-\) within the same supercell indicates a somewhat lower energetic gain of the association (Mn\(_{\text{III}}\) and Mn\(_{\text{IV}}\) sites in Fig. 3, \(r_{\text{N-Mn}} \approx 0.85\) nm).

The results indicate thermodynamic preference for the simultaneous substitution of oppositely charged Mn\(^{3+}\) and NO\(_3^-\) ions into HAp lattice, i.e., the impurities are more stable in the case of simultaneous incorporation. Since the isolated Mn\(^{2+}\) and NO\(_3^-\) defects are both charge neutral and the spin density of Mn\(^{2+}\) is strongly localized, no contribution of coulombic or magnetic interaction is expected to be involved in the observed effect. Therefore, we suppose that the underlying mechanism is related only to structural differences, as it was previously suggested in ref. 13 for substitutional Zn\(^{2+}\) and Ca-vacancy complex in HAp. In other words, incorporation of the pair compensates the local lattice distortions induced separately by both substitutions, and thereby lowers the total energy of the nanocrystal.

In addition, the revealed presence of energetically preferable manganese sites in the vicinity of the anionic impurity suggests that certain content of nitrate in HAp can lead to non-uniform distribution of Mn\(^{2+}\) ions within the nanocrystal. In turn, this can alternate the pathways of Mn\(^{2+}\) ions release into the host.

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**Fig. 3** Optimized geometry of HAp containing NO\(_3^-\) defect with schematic of the selected positions of Mn\(^{3+}\) considered in the study. The values of binding energy calculated for the displayed Mn\(^{3+}\) sites are shown in parentheses (Mn\(_1\) and Mn\(_2\) represent the most stable configurations among Ca(1) and Ca(2) positions, respectively). Location of Ca\(^{2+}\) vacancy (VCa) is marked by a dashed circle. Ca(1), Ca(2), P, O, H and N atoms are shown as green, blue, purple, red, white and green spheres, respectively.
media and the mechanisms of surface resorption, thereby influencing osteoconductivity of the material. On the other hand, due to the energetic preference of co-substitution, the lattice of manganese doped HAp can become more labile for the incorporation of undesired impurities (which in turn can have a cytotoxic effect) as compared to that of pure crystal.

In principle, one can also expect a similar effect to take place in the case of another pair of substitutions (e.g. carbonate with some transition metal) instead of nitrate and manganese. To support this idea further experimental and computational analysis should be carried out.

4. Conclusions

Quite a simple approach based on pulsed EPR is shown to investigate the simultaneous doping of HAp nanoparticles by manganese and nitrate. To the best of our knowledge, the study provides the first direct experimental evidence of interplay between oppositely charged ionic additives in nanosized HAp supported by first-principles calculations.

The main outcome of the study can be summarized as follows:

1. Measurements of electron spin relaxation times allowed observing simultaneous incorporation of manganese and nitrate substitutions into the HAp nanoparticle. Due to the high sensitivity, EPR spectroscopy could be applicable for further research with the lower levels of impurities and smaller sizes of nanocrystals.

2. The performed ab initio analysis clearly indicates that it needs less energy to incorporate both manganese and nitrate into the HAp lattice simultaneously than to insert the separate impurities. Thereby, it supports the feasibility of HAp co-doping.

3. The results of the EPR measurements and calculations suggest preferably non-uniform arrangement of Mn$^{2+}$ ions within HAp nanocrystal in presence of nitrate impurity. In turn, this effect can have a direct impact on the functional properties of the material (such as ion exchange with environment and surface resorption).

Acknowledgements

This study was funded by the subsidy allocated to the Kazan Federal University for the state assignment in the sphere of scientific activities. M.G. acknowledges the subsidy of the Russian Government to support the Program of Competitive Growth of Kazan Federal University among World’s Leading Academic Centers. E.K and V.P. acknowledge partial support from Lomonosov Moscow State University Program of Development and Russian Foundation for Basic Research.

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