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Features of ROS generation during hydrogen peroxide decomposition by nanoceria at different pH values

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Nanoceria (CeO_{2-x}) is well-known due to its superior ROS (reactive oxygen species) scavenging properties, but recent studies have revealed its ability to generate various types of ROS as well. Value of pH of nanoceria water solution is definitely one of the key parameters determining the type of redox activity of nanoceria (antioxidant vs. prooxidant), but generally both types of activities can co-exist at the same time, and the resulting effect of nanoceria-ROS interaction is determined by subtle interplay between pro- and antioxidant properties of these nanoparticles at specific pH value. In this paper three types of fluorescent sensors (DPPP, coumarin, and epinephrine) were used to study the processes of ROS scavenging/generation during hydrogen peroxide (HP) decomposition by nanoceria. Increase of pH of HP-nanoceria solutions from 4 to 10 is accompanied by suppression of the processes of generation of hydroxyl radicals ($^{\cdot}OH$), but, in turn, leads to advanced generation of superoxide anions (O_2^{-}) indicating two competing routes of HP decomposition prevailing at different pH values. Generally, prooxidant properties of nanoceria can be observed at low pH that can be useful for selective activation of apoptosis in cancer cells which pH value is less than for non-cancer ones.

Keywords: nanoceria, pH, hydrogen peroxide, hydroxyl radicals, superoxide anions.

Особливості генерації АФК при розкладанні перекису водню нанокристалами оксиду церію при різних значеннях pH. *В.В.Семінько, П.О.Максимчук, Г.В.Григорова, К.О.Губенко, С.Л.Єфімова*

Нанокристали оксиду церію (CeO_{2-x}) добре відомі завдяки своїй здатності до нейтралізації АФК (активних форм кисню), але нещодавні дослідження показали їх здатність також і до генерації різних типів АФК. Значення pH водного розчину оксиду церію, безумовно, є одним із ключових параметрів, що визначають тип редокс-активності оксиду церію (антиоксидантна або прооксидантна), але загалом обидва типи активності можуть співіснувати, і результат взаємодії оксиду церію з АФК визначається балансом між про- та антиоксидантними властивостями цих наночастинок при певному значенні pH. У роботі використано три типи флуоресцентних сенсорів (DPPP, кумарин та адреналін) для вивчення процесів знешкодження/утворення АФК під час розкладання оксидом церію перекису водню. Підвищення pH розчинів перекису водню з оксидом церію з 4 до 10 супроводжується зниженням концентрації гідроксил-радикалів ($^{\cdot}OH$), але, в свою чергу, приводить до зростання вмісту супероксид-аніонів (O_2^{-}), що вказує на два конкуруючих шляхи розкладання перекису водню, що переважають при різних значеннях pH. Як правило, прооксидантні властивості оксиду церію спостерігаються при низькому значенні pH, що може бути корисним для селективної активації апоптозу у ракових клітинах, значення pH яких менше, ніж у звичайних.

Нанокристаллы оксида церия (CeO_{2-x}) хорошо известны благодаря своей способности нейтрализовать АФК (активные формы кислорода), но недавние исследования показали их способность также и к генерации различных типов АФК. Значение pH водного раствора оксида церия, безусловно, является одним из ключевых параметров, определяющих тип редокс-активности нанокристаллов оксида церия (антиоксидантная или прооксидантная), но в целом оба типа активности могут сосуществовать, и результат взаимодействия оксида церия с АФК определяется балансом между про- и антиоксидантными свойствами этих наночастиц при определенном значении pH. В работе использованы три типа флуоресцентных сенсоров (DPPP, кумарин и адреналин) для изучения процессов нейтрализации/генерации АФК при разложении оксидом церия перекиси водорода. Повышение pH растворов перекиси водорода с оксидом церия с 4 до 10 сопровождается снижением концентрации гидроксил-радикалов ($^{\cdot}OH$), но, в свою очередь, приводит к росту содержания супероксид-анионов (O_2^-), что указывает на два конкурирующих механизма разложения перекиси водорода, преобладающих при различных значениях pH. Как правило, прооксидантные свойства оксида церия наблюдаются при низком значении pH, что может быть полезным для селективной активации апоптоза в раковых клетках, значение pH которых меньше, чем для обычных.

1. Introduction

Enzyme-like antioxidant action of nanoceria has been widely discussed since the first reports of its favorable effect on the lifespan of neurons [1], and protection of retinal cells from reactive oxygen intermediates [2], up to the most recent evidences on its role in management of COVID-19 [3]. Rather surprisingly, according to [4] nanoceria offers radioprotection to normal cells, but not to tumor ones. As pH of tumor cells is less than for normal cells (so-called Warburg effect [5]), the effect of pH on the type of redox activity of nanoceria (anti- or prooxidant) can be determinative for cytoprotective or cytotoxic biological action of these nanoparticles *in vivo*. The pH-dependent redox properties of nanoceria [6] were confirmed by combined studies of its antioxidant activity in water solutions and both normal (cardiomyocytes and dermal fibroblasts), and tumoral (lung and breast carcinoma) cell lines. At the same time, an alternative study on the different cell lines [7] has shown that nanoceria offers protection to tumoral and transformed cells as well. For now, it is clear that the size and shape of nanoparticles, degree of oxygen nonstoichiometry, and presence of surface ligands can change the type of redox activity of nanoceria blurring the effect of pH value [8]. Also pH decrease can favor elimination of some ROS, but provoke formation of other ones instead.

Decomposition of hydrogen peroxide (HP) molecules by nanoceria and nanoceria-based materials can be accompanied either by generation of hydroxyl radicals (Fenton-like reaction) [9], or by catalase-like activity (decomposition of HP to water and oxygen without ROS formation) [10]. Also HP decomposition can be accompanied by formation of superoxide radicals which are by far less reactive and harmful in comparison with hydroxyl radicals. Preventing Fenton-like reaction is a key task in obtaining effective and safe antioxidant materials for biological use [11]. At the same time, as nanoceria can scavenge both hydroxyl ($^{\cdot}OH$) and superoxide (O_2^-) radicals, the final effect of nanoceria interaction with HP should depend on the balance between its ROS generating and ROS scavenging properties. The rate of radical scavenging, in turn, is determined by the rate of recycling of surface active sites of nanoceria (Ce^{3+}/Ce^{4+} ions or $Ce^{3+}–Vo–Ce^{3+}$ complexes). The recovery rate of surface sites can be increased by variation of temperature and UV irradiation. According to some papers [6], the rate is strongly pH-dependent, and this dependence can be considered as one of the main factors changing the type of redox activity from antioxidant to prooxidant at low pH values.

In our study we analyze the formation of different ROS types (hydroxyl radicals, superoxide anions) during HP decomposition by nanoceria. We have revealed that subtle interplay between ROS generation and neutralization processes during HP decomposition is strongly pH-dependent and can change easily the overall redox properties of ceria nanoparticles from pro- to antioxidant ones at pH variation as well as during the process of HP decomposition.

2. Experimental

Aqueous solutions of ceria nanoparticles were obtained by the following method. $CeCl_3$ solution (100 ml, 2 mM) was mixed with 100 ml of hexamethylenetetramine

(HMTA) solution (4 mM) and stirred by means of magnetic stirrer for 3 h at room temperature. After that 1.8 ml NH_4OH and 0.6 ml of H_2O_2 were added into the solution. Then, the solution was put in round-bottom flask and refluxed for 5 h. As a result, transparent colorless solutions were obtained. The solution was evaporated in a rotary evaporator flask under vacuum at the bath temperature of 70°C to 30 ml. A solution of 2 M NaCl was added to the obtained solution until the resulting solution became turbid. Then the solid phase was precipitated by centrifugation. The precipitate was separated and solution of sodium chloride was added again. The procedure of precipitate cleaning was repeated three times. After the last stage of centrifugation, solution of sodium citrate with molar ratio $CeO_2/NaCt$ of 1:1 was added to the precipitate. Cerium (III) chloride and hexamethylenetetramine were mixed resulting in formation of ~ 2 nm CeO_{2-x} nanoparticles. CeO_{2-x} nanoparticles were stabilized by sodium citrate with molar ratio 1:1. The solutions were additionally dialyzed for 24 h against deionized water to remove the excess of ions and organics species. Dialysis membrane tubing with a molecular weight cutoff of "Cellu Sep H1" 3.5 KDa was used. All sols were transparent in transmitted light and passed through membrane filters with pores of 100 nm without loss. The pH values of nanoceria solutions were varied by addition of HCl (acidic pH) or NaOH (basic pH).

The dynamics of HP decomposition by nanoceria samples in aqueous solutions was measured using DPPP (diphenyl-1-pyrenylphosphine) peroxide sensor [12]. Being oxidized to phosphine oxide, DPPP becomes fluorescent with a wide band at 380 nm. During our experiment 980 µl portions of nanoceria-HP water solution ($C = 60$ mM) taken at different times after HP addition were pipetted to quarts cuvettes with subsequent addition of 20 µl of DPPP-DMSO solution (1 mM). The obtained solution was kept for 30 min for completing DPPP reaction with HP, and then a fluorescence spectrum was taken.

The processes of $^{\cdot}OH$ generation from HP in the presence of nanoceria was determined using standard coumarin test [13]. Intensity of 7-hydroxycoumarin (the product of interaction between coumarin molecule and hydroxyl radicals) fluorescence was measured during HP decomposition process. Concentration of coumarin in all samples was equal to 10^{-4} mol/l. The processes of O_2^- generation were studied for nanoceria-HP water solutions in the presence of epinephrine that is well-known superoxide sensor forming fluorescent form (adrenochrome) being oxidized by superoxide radical [14].

Concentrations of nanoceria in aqueous solutions were similar in all experiments and equal to 0.1 g/l.

3. Results and discussion

The ability of nanoceria to decompose HP was shown in the number of papers [9, 10]. The processes of HP decomposition by nanoceria can be both catalase-like (without formation of hydroxyl radicals) and Fenton-like (accompanied with formation of hydroxyl radicals). The HP decomposition according to Fenton-like reaction:

$$H_2O_2 + Ce^{3+} \rightarrow Ce^{4+} + OH^- + {\cdot}OH \qquad (1)$$

can be dangerous for living cells as it tends to produce hydroxyl radicals ($^{\cdot}OH$) which oxidative capability suppresses sufficiently the oxidative capability of HP. So, Fenton-like reaction can lead to peroxidation of lipid membranes and damage of different cell organelles by hydroxyl radicals. Another possible pathway of nanoceria-HP interaction includes formation of superoxide anions as an intermediate product:

$$H_2O_2 + Ce^{4+} \rightarrow Ce^{3+} + O_2^- + 2H^+. \qquad (2)$$

This pathway is less harmful to the cell, as superoxide anions can be easily utilized by nanoceria in SOD-like manner:

$$O_2^- + Ce^{4+} \rightarrow Ce^{3+} + O_2 \qquad (3)$$

and

$$O_2^- + Ce^{3+} + 2H^+ \rightarrow Ce^{4+} + H_2O_2. \qquad (4)$$

So, the types of ROS formed during HP decomposition by nanoceria determine the overall biological activity of these nanoparticles. One of the main factors controlling realization of specific ROS scavenging/generation reaction is the pH of nanoceria-HP water solution.

The influence of pH on the rate of HP decomposition by ceria nanoparticles was studied using DPPP sensor. In Fig. 1 the dynamics of the change of HP concentration during HP-nanoceria interaction is shown at pH = 4, 7.5 and 10. The dynamics consists of two well-defined stages corresponding to

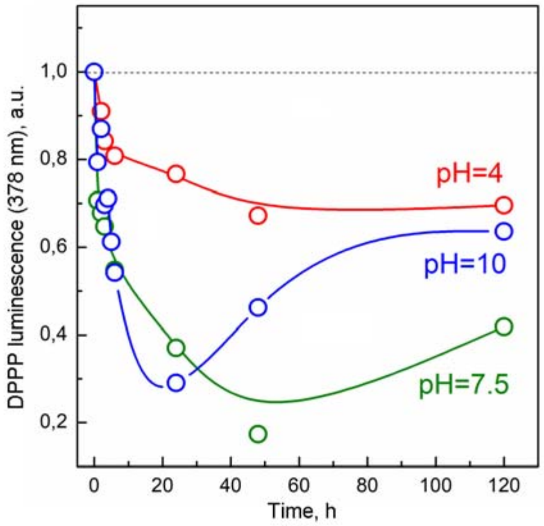

Fig. 1. Dynamics of HP decomposition by nanoceria at different pH values measured by DPPP luminescence intensity (λ = 378 nm) after HP addition.

decrease and subsequent increase (for pH = 7.5 and 10) of HP concentration. This specific dynamics can be considered as a result of combination of catalase-like and SOD-like activities of nanoceria. Increase of pH value leads to sufficient increase of HP decomposition rate at the first stage of decomposition. Taking the density of nanoceria as 7.22 g/cm^3, the molar concentration of ceria nanoparticles, and so, the rate of HP decomposition by single nanoparticle was calculated. The average rates of HP decomposition at pH = 4, pH = 7.5, and pH = 10 (during first 24 hours) were equal to 1.9, 5.25, and 5.8 HP molecules/min per 1 ceria nanoparticle. The rate at pH = 7.5 (5.25 molecules/min) is slightly less than the one determined in our previous paper [15] by the change of HP absorption spectra (9.5 molecules/min). Interaction of HP molecules with Ce^{3+}–Vo–Ce^{3+} (or Ce^{4+}–Vo–Ce^{4+} in oxidized form) complexes that are the most abundant type of surface defects in nanoceria can provide HP decomposition to water and oxygen without formation of intermediate ROS [15–17]. At the same time, interaction with separate Ce^{3+}/Ce^{4+} ions should be accompanied with formation of ROS. So, during the process of HP decomposition besides oxygen formation, also superoxide anions (O_2^-) could be formed according to reaction (2). In [16] the SOD-like activity (i.e. the ability to turn superoxide anions to HP) of nanoceria was reported. So, on the second stage, SOD-like activity of nanoceria dominates leading to formation of additional HP, but its content is sufficiently less than initial HP concentration.

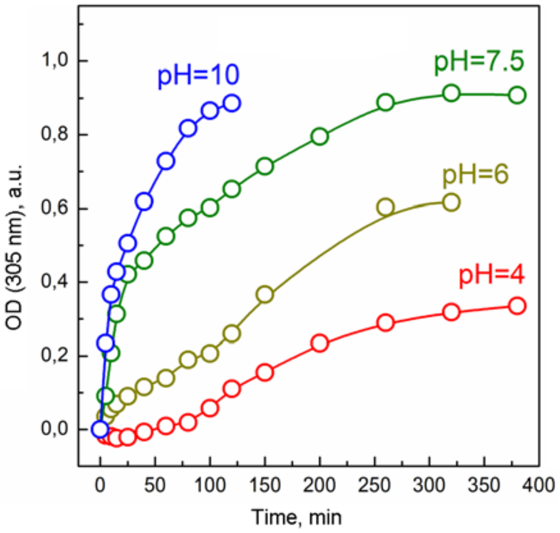

Fig. 2. Concentration of superoxide anions (O_2^-) in HP-nanoceria water solutions with different pH values determined by the change of adrenochrome absorption (λ = 305 nm).

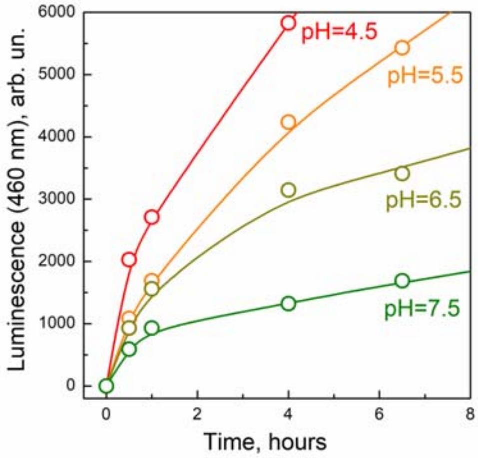

Fig. 3. Concentration of hydroxyl radicals ($^{\cdot}OH$) in HP-nanoceria water solutions with different pH values determined by the change of 7-hydroxycoumarin luminescence (λ = 460 nm).

The formation of superoxide anions during HP decomposition by nanoceria was studied using the change of the absorption spectra of epinephrine after HP addition. Absorption band at 305 nm corresponds to adrenochrome absorption (adrenochrome is a product of epinephrine interaction with O_2^- anions). Without nanoceria no oxidation of epinephrine to adrenochrome was observed in epinephrine-HP solutions. Addition of nanoceria leads to fast HP decomposition according to reaction (2) accompanied with

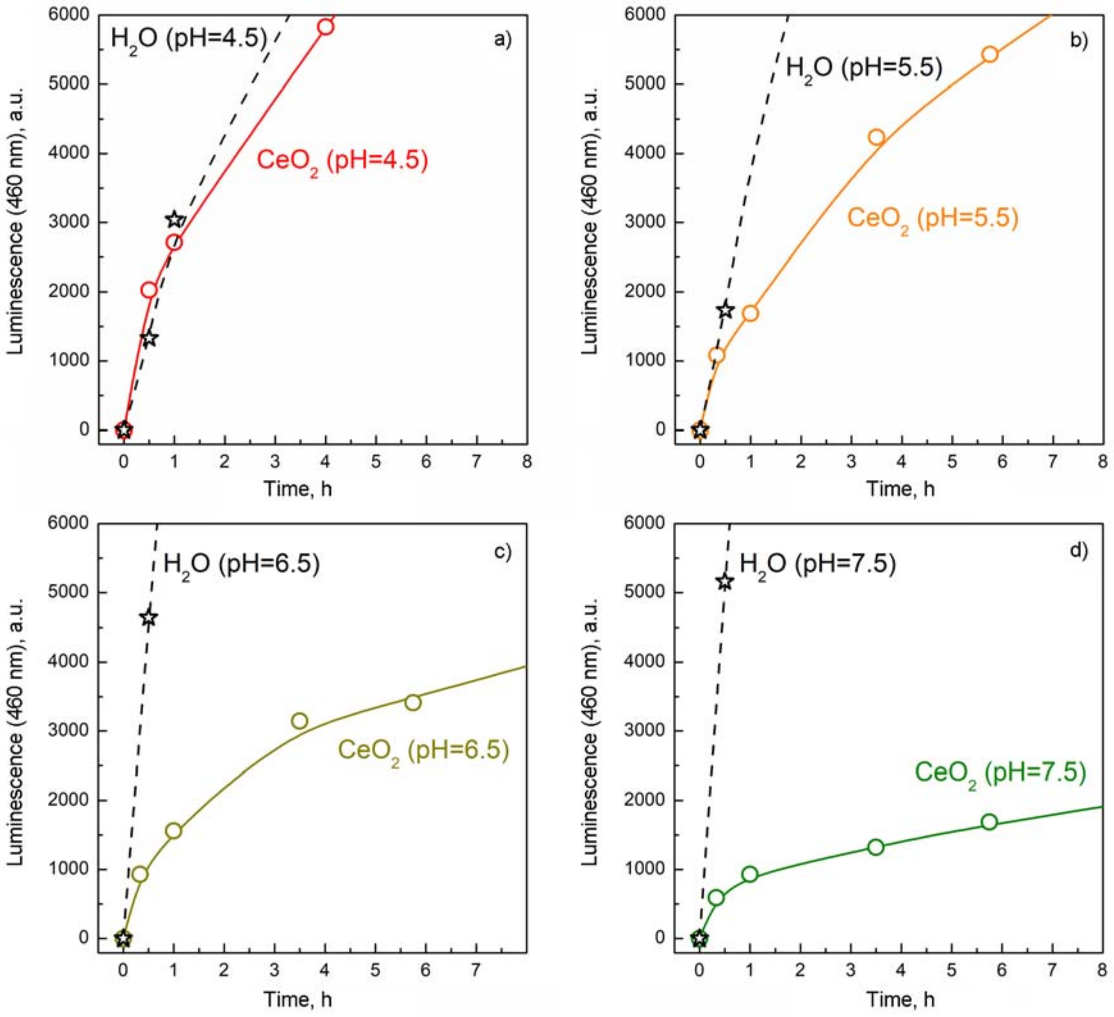

Fig. 4. Comparison of the content of hydroxyl radicals ($^{\cdot}$OH) in HP-water solutions with different pH values (a–d) with and without nanoceria determined by the change of 7-hydroxycoumarin luminescence (λ = 460 nm).

formation of superoxide anions (Fig. 2). The content of O_2^- anions formed at HP decomposition is pH-dependent being the highest for pH = 10.

The processes of formation of hydroxyl radicals at Fenton-like HP decomposition by nanoceria at different pH values were studied using coumarin as $^{\cdot}$OH sensor. The dynamics of fluorescence of 7-hydroxycoumarin at 460 nm was used to study the processes of $^{\cdot}$OH formation. Decrease of pH increases the content of hydroxyl radicals formed during HP decomposition (Fig. 3) in the presence of nanoceria. At the same time, the $^{\cdot}$OH scavenging by nanoceria can eliminate the large part of hydroxyl radicals formed during HP decomposition. We have noticed before [15] that for nanoceria at pH = 7 the content of hydroxyl radicals remains less than in control water-HP solution without nanoceria during whole process of HP decomposition. Two factors decreasing $^{\cdot}$OH generation in the presence of nanoceria were elucidated in [15]: first, the high content of surface Ce^{3+}–Vo–Ce^{3+} complexes able to decompose HP without formation of hydroxyl radicals, and, second, high $^{\cdot}$OH scavenging ability. The last can be also pH-dependent, so the final prooxidant or antioxidant effect of nanoceria should result from combined action of $^{\cdot}$OH generation and $^{\cdot}$OH scavenging processes at specific pH value.

In order to reveal the dominating type of redox-activity of nanoceria at different pH values, the change of $^{\cdot}$OH content in HP-water solutions with and without nanoceria were compared. It is known that generation of $^{\cdot}$OH radicals from HP is possible even without nanoceria due to presence of small quantities of uncontrolled impurities (dissolved ions etc.). The comparison between the samples with and without nanoceria show that despite $^{\cdot}OH$ generation ability, $^{\cdot}OH$ scavenging by nanoceria prevails at almost all pH values (Fig. 4, a–d). At the same time the difference between $^{\cdot}OH$ content formed in water solutions with and without nanoceria decreases at pH decrease from 7.5 to 4.5. So, at lower pH value protection against $^{\cdot}OH$ provided by nanoceria decreases, and at pH = 4.5 at the first stage of $^{\cdot}OH$ decomposition prooxidant properties of nanoceria dominate over antioxidant ones (Fig. 4a).

Monitoring the Ce^{3+}/Ce^{4+} ratio in nanoceria using luminescence techniques provided deeper insight into the role of nanoceria in the processes of generation and scavenging of specific types of ROS during HP decomposition at different pH values [18]. In [19] we have revealed that addition of HP to nanoceria water solution at pH = 7.5 leads to $Ce^{3+} \rightarrow Ce^{4+}$ oxidation followed by $Ce^{4+} \rightarrow Ce^{3+}$ recovery after decomposition of the most part of HP in the water solution. HP can act both as oxidant and reductant forming different ROS types ($^{\cdot}OH$ or O_2^-) during interaction with the active surface sites ($Ce^{3+} \rightarrow Ce^{4+}$ ions or Ce^{3+}–Vo–Ce^{3+} complexes) of nanoceria. In Fig. 5 the change of Ce^{3+} luminescence intensity after HP addition at different pH values is shown. At pH = 7.5 and pH = 6 addition of HP leads to fast $Ce^{3+} \rightarrow Ce^{4+}$ oxidation, at pH = 10 to fast $Ce^{4+} \rightarrow Ce^{3+}$ reduction, and at pH = 4 the Ce^{3+}/Ce^{4+} ratio remains the same as before HP addition. These results open the way to understand the whole mechanism of pH-dependent formation of $^{\cdot}OH$ or O_2^- during HP decomposition.

As can be seen from Fig. 2 and Fig. 3, the pathway of HP decomposition by nanoceria (according to reaction (1) or reaction (2)) can be controlled by pH. Increasing the content of OH^- ions (pH = 10) one can stimulate the decomposition according to reaction (2) as this reaction leads to increase of H^+ content compensating initial OH^- excess. This reaction should be accompanied by formation of superoxide anions and reduction of Ce^{4+} ions to Ce^{3+} ones that agrees with increase of O_2^- content and Ce^{3+} luminescence intensity in Fig. 2 and Fig. 5, respectively. At slightly acidic pH values (pH = 6) excess of H^+ ions, in turn, provides preferable HP decomposition according to reaction (1) leading to formation of OH^- anions. Reaction (1) involves formation of $^{\cdot}OH$ radicals and oxidation of Ce^{3+} ions to Ce^{4+} ones explaining the higher content of $^{\cdot}OH$ radicals for lower pH values and Ce^{3+} intensity decrease seen in Fig. 3 and Fig. 5, respectively. For pH = 4 the concentration of $^{\cdot}OH$ is high, but the $Ce^{4+} \leftrightarrow Ce^{3+}$ reversibility is disrupted by very high H^+ content [6], so the Ce^{3+}/Ce^{4+} ratio (and, so, Ce^{3+} luminescence intensity) remains almost constant during HP decomposition.

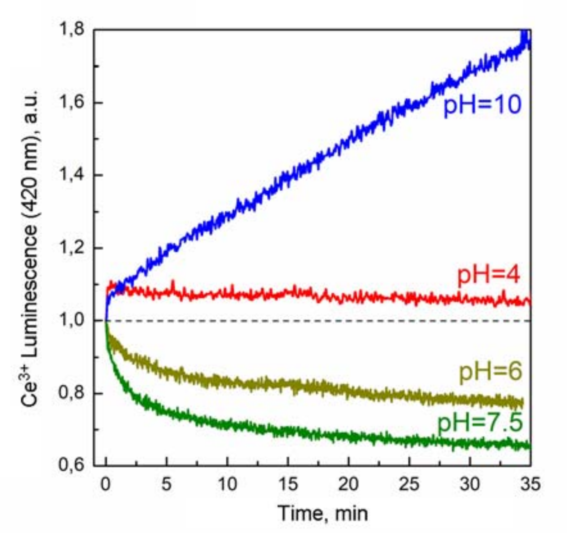

Fig. 5. Dynamics of Ce^{3+} luminescence intensity after HP addition for nanoceria water solutions with different pH values.

The resulting dynamics of HP decomposition (Fig. 1) by nanoceria at specific pH value is a result of its catalase- and SOD-like activity along with its O_2^- generation ability. The higher is pH value, the higher is the content of O_2^- formed at HP decomposition. At some O_2^- concentration in water solution, the SOD-like activity of nanoceria (reactions (3), (4)) starts to prevail leading to formation of additional H_2O_2 seen in Fig. 1 by increase of DPPP fluorescence intensity. As O_2^- content at pH = 10 is the highest, at this pH the strongest increase of H_2O_2 content is observed. On the contrary, for pH = 4 no formation of additional HP was observed, as HP decomposition at this pH occurs mainly by reaction (1) and is not accompanied by superoxide formation. In this way, changing the pH of nanoceria-HP water solution changes not only the rate, but also the mechanism of HP decomposition.

Conclusions

As the rate, so the specific mechanisms of HP decomposition by nanoceria depend on the value of pH of HP-nanoceria water solutions. The higher is pH value, the higher is the rate of HP decomposition by nanoceria. Two different pathways of HP decomposition were revealed: first one with formation of superoxide anions and prevailing at high pH values, and second one with formation of hydroxyl radicals dominating at low pH values. High $^{\cdot}OH$ scavenging capability of nanoceria leads to almost complete elimination of hydroxyl radicals formed at HP decomposition at neutral and slightly acidic pH. At the same type, prooxidant activity of nanoceria was observed at pH = 4.5 which can be related to suppression of Ce^{3+}/Ce^{4+} redox cycling leading to low-effective $^{\cdot}OH$ scavenging activity.

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