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Monitoring of microwave emission of HRP system during the enzyme functioning



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ABSTRACT

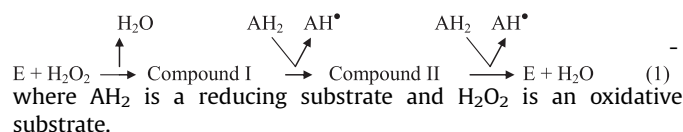
Monitoring of microwave emission from aqueous solution of horseradish peroxidase (HRP) in the process of the enzyme functioning was carried out. For the monitoring, a system containing HRP, luminol and H₂O₂ was employed. Microwave emission measurements were carried out in the 3.4–4.2 GHz frequency range using the active and passive modes (active-mode and passive-mode measurements). In the active mode, excitation of the solution in the pulsed electromagnetic field was accomplished. In the passive mode, no excitation was induced. It appears that the passive-mode measurements taken in the course of the peroxidase reaction in the enzyme system have shown a 0.5 °C increase of the microwave signal. Upon the active-mode measurements, taken in the same reaction conditions, the forced excitation of the solution has also led to the increase (by 2 °C) of the level of the microwave signal – i.e. to its 4-fold enhancement compared to the signal obtained in passive-mode measurements.

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1. Introduction

HRP pertains to heme-containing enzymes. The study of peroxidases is of great interest due to the fact that these enzymes are well represented in plant and animal tissues [1] and play an important functional role in the organism. Peroxidase catalyzes oxidation of a broad spectrum of organic and inorganic compounds by hydrogen peroxide [2]. Plant peroxidase takes part in the transformation of peroxides and compounds foreign to the organism. The interest in the plant peroxidase is also conditioned by the fact that currently ELISA methods using HRP-labeled chemiluminescent tags for signal amplification have found wide application in analytical biochemistry and diagnostics. ELISA-based analysis of sera from patients with autoimmune thyroid disease, when monoclonal antibodies against TPO conjugated with peroxidase are used, gives an example of such studies [3].

For both plant and animal peroxidases, including heme-containing human peroxidases TPO, LPO and MPO, the peroxidase reaction occurs in a similar way [4]. In general form this reaction can be written as [5]:



For HRP-based system peroxidase catalytic cycle is most studied. In practice, luminol, the artificial electron donor ABTS and other substrates are used as reducing substrates for peroxidase [6,7]. Reaction (1) is often used in clinical practice, in biosensors for environmental monitoring and in pharmaceutical therapy [7]. In this reaction luminol is often used to enhance the analysis sensitivity at the expense of chemiluminescence occurring upon the reaction of peroxidase-based catalysis. It is known that the maximum of the chemiluminescence spectrum in the HRP-luminol-H₂O₂ system corresponds to the wavelength ~425 nm [7].

In the present work, the possibility of monitoring the emission from the HRP-luminol-H₂O₂ system not in the visible, but in microwave frequency range, was examined. Measurements were carried out in two modes. Here, they were designated for convenience as the passive-mode and active-mode measurements. The passive mode consists in registration of extremely weak self-radiation of the observation object. In contrast to passive

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radiometry, in the active mode of measurements the object is probed with weak electromagnetic radiation with simultaneous registration of the microwave signal.

Earlier we have shown that microwave emission in the 3.4–4.2 GHz frequency range can be registered in CYP102 A1 containing monooxygenase system which catalyzes fatty acids hydroxylation [8,9]. This system is interesting in that it is a model of cytochrome P450-containing systems, as it contains the heme and flavin domains in one polypeptide chain [10]. That is why this system is often used as an object in enzymatic reaction studies [10–14] and was chosen in the present work to investigate the microwave emission. Besides, in our earlier study on the same it was shown by atomic force microscopy that the functioning of the enzyme is accompanied by fluctuations of the protein globule [12–16]. These fluctuations are able to cause disturbances of aqueous environment of the cytochrome CYP102 A1 molecules thereby generating the microwave emission from the whole solution. We have found that in the case of the heme-containing protein HRP, the fluctuations of the protein globule during its functioning also take place [17]. The amplitudes of height fluctuation of the protein globules of HRP and CYP102 A1 enzymes are equal (~ 0.8 nm). It was assumed therefore that the functioning of HRP-based system – similar to CYP102 A1-based system – is accompanied by microwave emission. The results of our present study demonstrate that the above-made assumption is correct.

It has been shown that in the HRP-luminol- H_2O_2 system the microwave emission in the 3.4–4.2 GHz frequency range is registered in passive-mode measurements, i.e. in the absence of external electromagnetic field. The influence of the pulsed electric field on the monitoring of microwave emission in the enzyme system (active-mode measurements) was also studied. The expected factor of the pulsed electric field influence was taken into account based on our data on the detection and concentrating of protein upon electrical stimulation [18,19]. As has been shown by us with cytochrome b5 as an example, the excitation of the medium containing this protein in the pulsed electric field with 1 ns front rise time leads to the increase in efficiency of protein detection on the chip surface [19]. In this study, the analogous conditions of measurements were used upon the monitoring of microwave emission from the HRP-luminol- H_2O_2 system to investigate the effect of the pulsed electric field on this system's emissivity.

The measurements were carried out using 10^{-6} M HRP solution. This concentration of the enzyme solution was chosen based on the fact that the concentration of this enzyme in horseradish root is at the same level, $C \sim 10^{-6}$ – 10^{-7} M [20].

2. Materials and methods

2.1. Reagents

Deionized ultrapure water was obtained using Millipore Simplicity UV system (Millipore, France). Hydrogen peroxide (H_2O_2) and luminol were purchased from Sigma (USA).

2.2. Protein

HRP-C (Sigma, USA) was used throughout. The specific activity of HRP claimed by the manufacturer is 1000 IU/mg. According to [21], HRP exhibits the maximum catalytic activity in the pH range 6.0–6.5. Test aqueous solutions of protein (10^{-6} M and 10^{-9} M) were prepared by dissolving calculated sample of the lyophilized protein in water and following consequent tenfold dilution with deionized ultrapure water.

2.3. Technique of microwave signal monitoring

2.3.1. Catalytic reaction in HRP-based system

The monitoring of microwave emission was carried out in aqueous solution containing reconstructed HRP-based enzyme system containing luminol. For this, 50 μ L of reducing substrate (10^{-3} M luminol solution) were added into the measuring cell containing 1150 μ L of HRP solution ($C = 10^{-6}$ M or 10^{-9} M); after that, 50 μ L of oxidizing substrate (10^{-4} M H_2O_2) were added into the cell. Catalytic reaction in the system occurred according to Eq. (1). Measurements were carried out at $T = 24$ °C for 50–60 min.

In control experiments, the following schemes of addition of the enzyme system's components were used in the above-described conditions:

- 1) only 50 μ L aliquots of water were added to the 10^{-6} M HRP solution;
- 2) 50 μ L aliquot of water and the 50 μ L aliquot of 10^{-3} M luminol solution were added to the 10^{-6} M HRP solution;
- 3) 50 μ L aliquot of water and the 50 μ L aliquot of 10^{-4} M H_2O_2 solution were added to the 10^{-6} M HRP solution.

2.3.2. Measurement of microwave emission from the solution

Broadband radiothermometer (microwave analyzer) RTM-01 RES, operating in the 3.4–4.2 GHz range, was used as a microwave emission detector. For the measurements, flagpole antenna of microwave analyzer was fully immersed into the sample solution in the measuring cell. The antenna was not removed from the cell throughout the experiment. The addition of solutions into the cuvette and stirring were carried out using automatic pipette.

The data on microwave emission measurements are presented using the brightness temperature (T_{SHF}) units, in which the radiothermometer is calibrated. The measurement accuracy was ± 0.1 °C. The measurement data are presented as the T_{SHF} vs time ($T_{SHF}(t)$) dependence.

2.4. Active-mode measurements

The experimental scheme to measure the microwave emission is shown in Fig. 1 and is described in detail in [18]. In the present study this scheme was used with slight modification: the isolation of the HOPG electrode from the reaction medium was achieved by use of mica sheet. For this purpose, 0.25 mm-thick mica sheet was

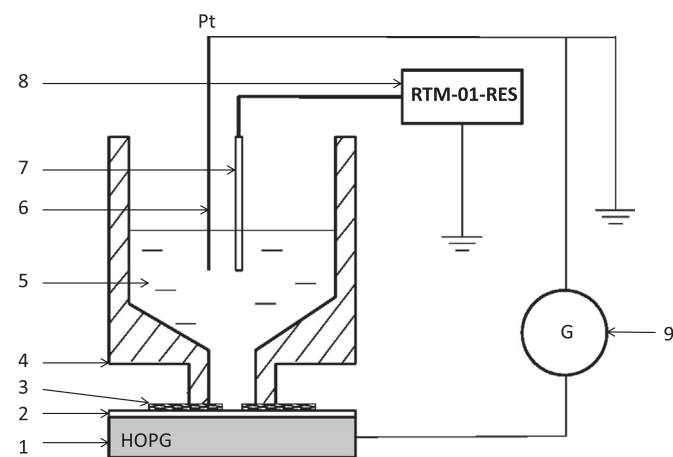


Fig. 1. Experimental scheme for the active-mode measurements of microwave emission from HRP solution. (1) – freshly cleaved HOPG; (2) – mica sheet; (3) – PTFE film; (4) – PTFE cell; (5) – HRP solution; (6) – Pt electrode; (7) – flagpole antenna; (8) – radiothermometer; (9) – pulse generator.

placed onto the freshly cleaved HOPG plate. Then, the mica sheet was pressed with the PTFE cell through PTFE film. So, the mica surface served as a bottom of the measuring cell.

In the *active-mode measurements*, the electrical excitation of the reaction solution was used. The following pulse parameters of the pulses applied to the HOPG plate were used: period 20 μ s, pulse width 10 μ s, amplitude 10 V. The voltage was maintained with a power supply — PG872 Pulse Generator (Resonance, Belarus).

In the *passive-mode measurements* the analogous experimental scheme was used, but no pulsed voltage was applied to HOPG.

3. Results

The monitoring of microwave emission was carried out in two series of experiments – in the active and passive modes of measurements. In each mode, control measurements by schemes (1)–(3) (see the “Materials and Methods” section) and working measurements in reconstructed HRP-based system (HRP-luminol- H_2O_2) were performed.

3.1. Passive-mode measurements

Results of measurements of microwave emission in passive mode are shown in Fig. 2 (A–C). As seen from Fig. 2(A), in control measurements carried out by schemes (1) and (2), upon addition of aliquot of water or luminol solution to aqueous HRP solution, the pulse-like changes in the microwave signal level by 0.1–0.3 $^{\circ}\text{C}$ were observed.

Based on the data obtained in control experiments, the baseline level of microwave noises the in inactive HRP-based system was determined: this level was found to be equal to 0.3 $^{\circ}\text{C}$.

Control measurements by scheme (3) (Fig. 2(B)) have shown that upon addition of an aliquot of water and an aliquot of H_2O_2 solution to the aqueous solution of HRP, a tendency in $T_{SHF}(t)$ dependence to the increase of the signal by $\Delta T_{SHF} \sim 0.2^{\circ}\text{C}$ is observed.

Measurements in reconstructed HRP-based system were carried out according to the technique described in the “Materials and Methods” section for enzyme solutions at two concentrations: 10^{-6} M and 10^{-9} M. The results of monitoring of microwave emission during catalytic reaction in passive-mode measurements are shown in Fig. 2(C).

As seen from this figure, addition of H_2O_2 to the solution containing the enzyme and luminol led to the increase of the signal by ΔT_{SHF} relative to the level of microwave emission from HRP-luminol solution. The elevated level of T_{SHF} remained unchanged during the entire observation time after the addition of H_2O_2 aliquot (~ 20 min). The increase in $T_{SHF}(t)$ signal indicates the generation of microwave emission from the solution during the peroxidase reaction. The measurements of microwave signal for the reconstructed system containing HRP at the lower concentration ($C = 10^{-9}$ M) have shown that upon addition of H_2O_2 to the enzyme-containing solution, no changes in T_{SHF} signal relative to the signal level in control experiments were observed (data not shown).

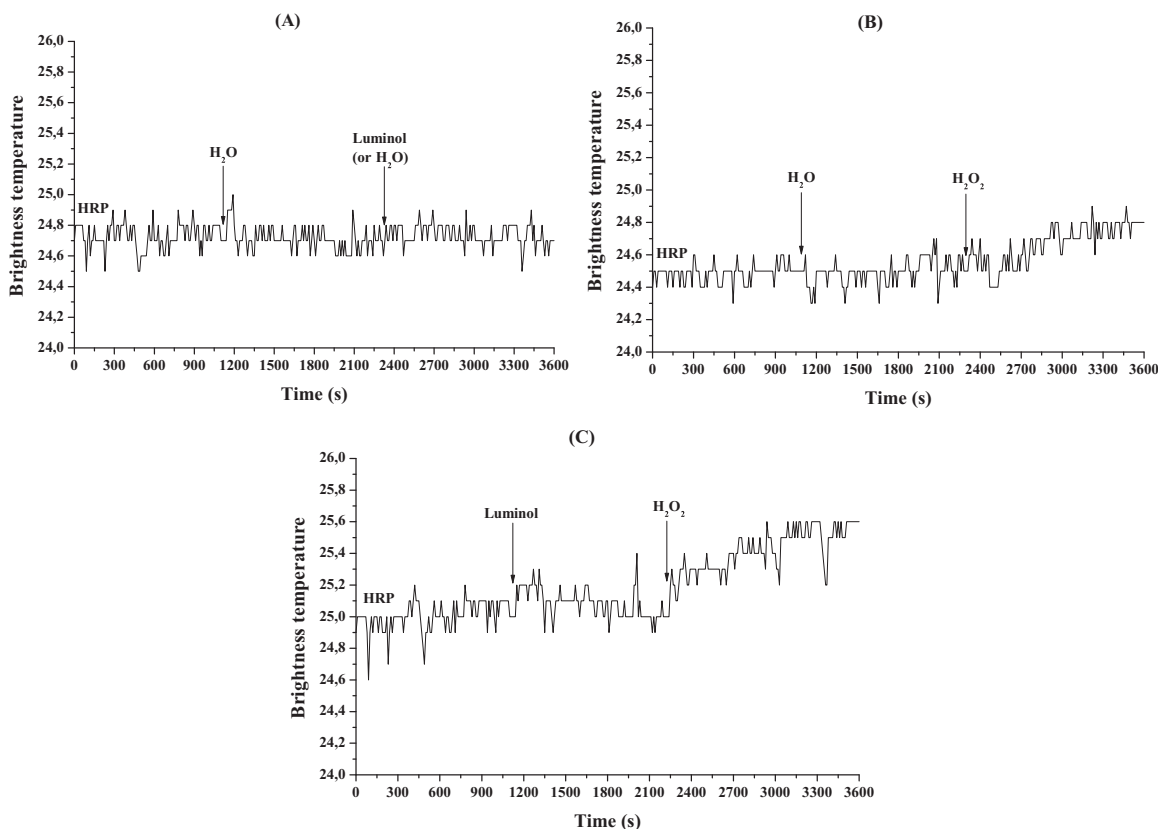


Fig. 2. Results of passive-mode measurements of microwave emission. (A) – Results of control experiments according to the schemes of enzyme system components addition (1) and (2) (see the “Materials and Methods” section); (B) – Results of control experiments according to the schemes of enzyme system components addition (3) (see the “Materials and Methods” section); (C) – Result of measurement in reconstructed HRP-based system. Experimental conditions: - components concentration: 1 μ M HRP; addition of 1 mM luminol; addition of 0.1 mM H_2O_2 ; - temperature of measurements 24 $^{\circ}\text{C}$; - absence of pulsed electric field; Arrows indicate the time points of addition of aliquots of enzyme system components solution or water into the reaction solution.

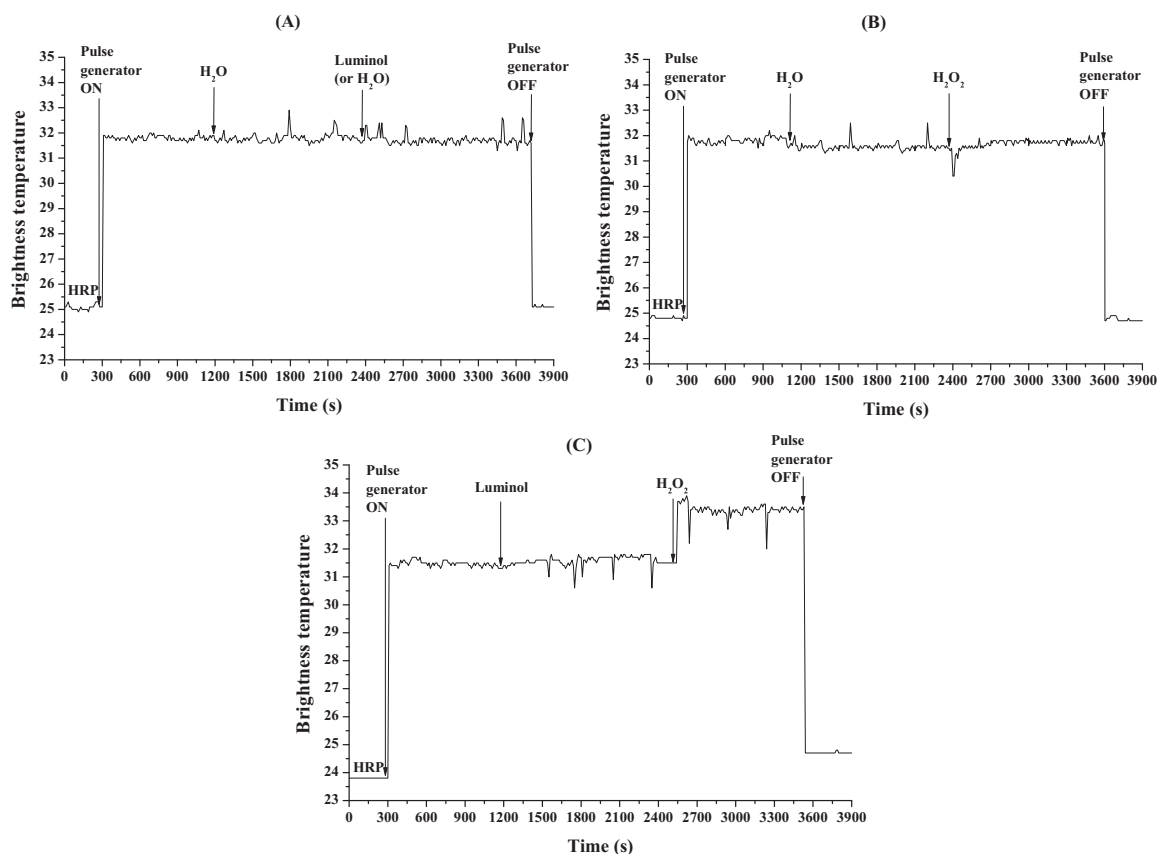


Fig. 3. Results of active-mode measurements of microwave emission. (A) – Results of control experiments according to the schemes of enzyme system components addition (1) and (2) (see the “Materials and Methods” section); (B) – Results of control experiments according to the schemes of enzyme system components addition (3) (see “Materials and Methods” section); (C) – Results of measurement in the reconstructed HRP-based system. Experimental conditions: - components concentration: 1 μ M HRP; addition of 1 mM luminol; addition of 0.1 mM H_2O_2 ; - temperature of measurements 24 $^{\circ}C$; - electric field pulse parameters: period 20 μ s, pulse width 10 μ s, amplitude 10 V. Arrows indicate the time points of addition of aliquots of enzyme system components solution or of water in the reaction solution.

3.2. Active-mode measurements

The results of control experiments of microwave emission in active mode are shown in Fig. 3(A–B). As seen from these figures, at the application of the pulsed electric field, the general increase in T_{SHF} of the solution by $(\Delta T_{SHF})_E \sim 7^{\circ}C$ is observed. Furthermore, upon addition of an aliquot of water or an aliquot of luminol solution (schemes of control experiments (1) and (2)), as well as after stirring, the minor fluctuations of the microwave signal (in the order of 0.3–0.8 $^{\circ}C$), i.e. somewhat more intensive ones than fluctuations registered in passive-mode measurements, were found to occur (Fig. 3A).

In control measurements by scheme (3), after the addition of an aliquot of H_2O_2 to the reaction solution, the level of microwave signal at first, decreases by $\sim 1.5^{\circ}C$. Then, after stirring, the increase in the signal by the same 2 $^{\circ}C$ was registered whereupon the tendency to a smooth increase in T_{SHF} by another 0.5 $^{\circ}C$ was apparent (Fig. 3B).

The measurements in the reconstructed HRP-based system in the presence of electric field were carried out according to the technique described in the “Materials and Methods” section for 10^{-6} M enzyme solution. The results obtained are presented in Fig. 3(C). As seen from this figure, during the peroxidase reaction in the system containing enzyme and luminol, the addition of H_2O_2 aliquot caused the increase in the level of microwave emission by 2 $^{\circ}C$ (Fig. 3(C)) relative to the signal level before H_2O_2 addition. Thus, in the presence of electric field, upon active-mode measurements the signal was four times greater than that registered in passive mode (Fig. 2(C)). Such enhancement of the signal

corresponds to the amplification factor $K \approx 400\%$. The duration of microwave emission from solution after H_2O_2 addition was about 20 min

4. Discussion

As was mentioned in the “Introduction” section, the activity of the HRP-luminol- H_2O_2 system was fairly well studied (for example, [4,7,22]). The functioning of this enzyme system is accompanied by chemiluminescence in the visible range. The results presented in this study indicate that during the functioning of HRP-based enzyme system the generation of emission is also observed in the microwave frequency range also takes place. Microwave emission was detected during the peroxidase reaction in reconstructable HRP-luminol- H_2O_2 at $T=24^{\circ}C$. As mentioned in the “Introduction” section, it was reported by us earlier that the functioning of each HRP and CYP102 A1 is accompanied by the fluctuation of the protein globule with approximately the same amplitude (in the order of 0.08 nm) [17]. Also, we have demonstrated the occurrence of microwave emission in CYP102 A1-based system during the enzyme functioning [8,9]. Emergence of microwave emission in the enzyme system can possibly be explained by the following factors [9]:

- (1) the generation of emission, corresponding to rotational transitions of molecules and ions excited during catalysis (OH^{\cdot} , H_2O and H_2O_2); the frequency of these transitions is observed in the GHz range [23];

- (2) the generation of emission caused by the change in the ratio between para- and ortho-isomers of H₂O; the possibility of such transitions between para- and ortho-isomers of H₂O was mentioned in range [24];
- (3) the emergence of emission caused by the increased mobility of the protein globule during the catalytic reaction.

Factors (2) and (3) directly influence the water structure, which, as is known, is not a homogeneous medium, but consists of nano-sized clusters [25,26]. Besides, these factors influence the structure of the ice-like hydration shell of enzyme in solution: its existence at 20 °C is discussed in [27]. In the present study the measurements were carried out at 24 °C, i.e. at the temperature near the phase transition of water, associated with surface tension [28]. Therefore, water condition factors significantly influence the level of the microwave emission signal in the aqueous enzyme system.

It should be noted that the data obtained in passive-mode measurements coincide for two enzyme systems – the HRP-based one (present study) and the CYP102 A1-based one [9]. Thus, the microwave emission is only generated in the presence of all components of the enzyme system. In control experiments, the level of signal change upon addition of system components or upon stirring does not exceed the noise level. The cause of emission emerging in the HRP-luminol-H₂O₂ system is possibly the same as in the CYP102 A1-based system.

Investigation of the influence of the pulsed electric field has shown that in its presence the level of microwave emission within the catalytic cycle (according to equation (1)) increases up to 2 °C, which is four times greater than the increase occurring without electrical stimulation. At that, microwave emission – as measured in both the active and passive modes – was observed within ~20 min. Manifestation of the amplification effect of microwave emission – as registered by active-mode measurements – is possibly due to the fact that probing the reaction medium by microwave emission is accompanied by additional excitation and change in the state of water molecules' clusters in solution of enzymes occurring in their different proportions close to the phase transition, while the subsequent reduction of this excitation manifests itself in the form of microwave emission. Also, the effect of enhancement of microwave emission may be connected with the radio enlightenment of aqueous medium due to changes in the absorption spectrum of changing water clusters.

5. Conclusion

Microwave emission from the solution of functioning HRP and containing luminol in the 3.4–4.2 GHz range has been registered. In the passive-mode measurements, microwave emission with corresponding increase in T_{SHF} by 0.5 °C was observed. The influence of the pulsed electric field on the microwave emission from the HRP solution has been investigated. It has been shown that the increase in the level of microwave emission from the solution in the presence of pulsed electric field was much greater, up to 2 °C.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.05.003>.

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