ISSN 1607-6729, Doklady Biochemistry and Biophysics, 2017, Vol. 477, pp. 394–397. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © E.V. Inzhevatkin, A.V. Baron, N.G. Maksimov, M.B. Volkova, A.P. Puzyr, V.S. Bondar, 2017, published in Doklady Akademii Nauk, 2017, Vol. 477, No. 6, pp. 738–741.

Detection of Nanodiamonds in Biological Samples by EPR Spectrometry

E. V. Inzhevatkin[a, b, *], A. V. Baron[b, c], N. G. Maksimov [b, d], M. B. Volkova[b, c], A. P. Puzyr[b], and V. S. Bondar[b]

Presented by Academician A.G. Degermendzhi May 5, 2017

Received August 1, 2017

Abstract—In model experiments in vitro, the applicability of the EPR spectrometry method for the detection of modified nanodiamonds (MNDs) in blood and homogenates of mouse organs has been established. A characteristic signal ($g = 2.003$, $\Delta H \approx 10$ G) is observed in the samples of biomaterials containing MNDs, the intensity of which increases linearly with the concentration of nanoparticles in the range of 1.6–200 μg MNDs per 1 mL of the sample. The EPR method in biomaterials reveals the presence of intrinsic paramagnetic centers, signals from which are superimposed on the signal from the MNDs. However, the intensity of these signals is small, which makes it possible to register the MNDs using EPR spectrometry with the necessary accuracy. The data obtained open up the prospects of using the EPR method for studies of the interorgan distribution, accumulation, and elimination of MNDs during their intravenous injection into experimental animals.

DOI: 10.1134/S1607672917060138

In recent years, the world has witnessed a marked activity in studying the applicability of nanoparticles of various physical and chemical nature for creating systems for targeted drug delivery [1, 2]. In particular, researchers show a great interest in using detonation nanodiamonds for these purposes [3–7]. It is predicted that the creation of tools for selective drug delivery to pathological foci will help to reduce the dose of therapeutics, improve their efficacy, and minimize the risks of side effects.

For researchers who study the applicability of detonation nanodiamonds in designing tools for targeted drug delivery, modified nanodiamonds (MNDs), which exhibit a high colloidal stability in various dispersion media (water and organic solvents) may be of interest [8, 9]. MND particles are obtained by treating commercially available detonation nanodiamonds produced in Russia and foreign countries with chemicals (in particular, with NaCl and EDTA) [8–10]. This reduces the amount of chemical impurities on the surface of nanodiamonds (primarily metal ions and organic compounds), which cause the agglomeration of nanoparticles. The MNDs obtained as a result of this treatment acquire a high sedimentation stability in aqueous and organic suspensions and can be used in a wide range of biomedical studies [11], for example, in designing targeted delivery systems for bioactive substances [3].

However, it should be emphasized that there is a number of issues in this field of research that are of fundamental importance and require a comprehensive study. One important aspect is the biodegradation of the carrier or its elimination from the body after the implementation of the targeted therapeutic function by the nanosystems in vivo. This necessitates the study of the pharmacokinetics and pharmacodynamics of nanoparticles introduced into the body as part of targeted delivery systems. The importance of studying these problems is determined by the possibility of adverse consequences at the accumulation of the carrier in organs and tissues. For these studies the EPR spectrometry method can be used, which allows the detection of nanoparticles with paramagnetic centers.

[a] *International Scientific Center for Studies of Extreme States of Organism, Federal Research Center "Krasnoyarsk Scientific Center," Siberian Branch, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia*

[b] *Institute of Biophysics, Federal Research Center "Krasnoyarsk Scientific Center," Siberian Branch of the Russian Academy of Sciences, Krasnoyarsk, 660036 Russia*

[c] *Siberian Federal University, Krasnoyarsk, 660041 Russia*

[d] *Institute of Chemistry and Chemical Technology, Federal Research Center "Krasnoyarsk Scientific Center," Siberian Branch, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia*

** e-mail: inscience@ mail.ru*

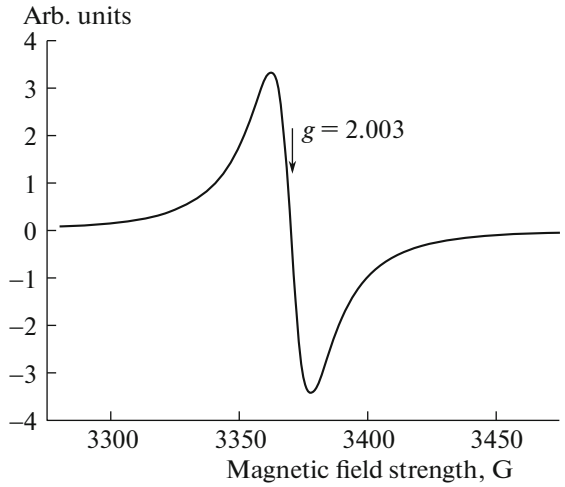

Fig. 1. Typical EPR signal recorded in samples of frozen MND hydrosols.

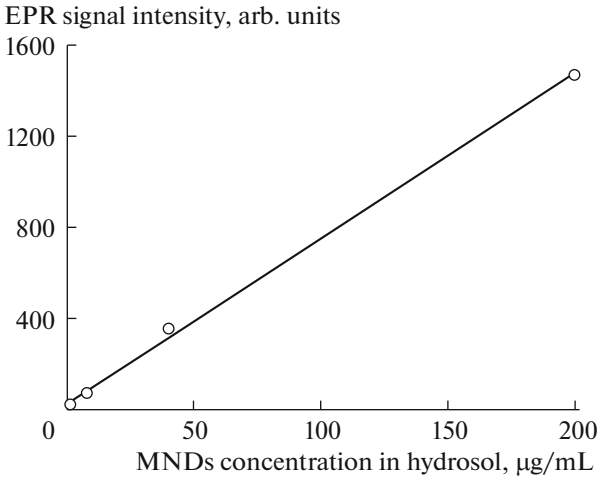

Fig. 2. Dependence of the EPR signal intensity on the MNDs concentration in hydrosol.

It is known that detonation nanodiamonds, including the MNDs, contain paramagnetic centers [12, 13].

In this work we studied the applicability of the EPR method for the determination of detonation nanodiamonds in biological materials in model experiments in vitro.

The study was performed with MNDs with an average size of nanoparticle clusters in hydrosols d_{50} = 70.6 nm (Zetasizer Nano ZS, Malvern Instruments Ltd., United Kingdom), which were obtained from the explosive nanodiamonds manufactured in Russian (OOO Real-Dzerzhinsk) by the previously developed method [8, 9]. These MNDs exhibit a high colloidal stability in aqueous suspensions and can be, therefore, used in biomedical studies [8, 11]. To perform the main experiments we first prepared the source MNDs hydrosol with a nanoparticles concentration of 1 mg/mL by adding deionized (DI) water (Milli-Q System, Millipore, United States) to an aliquot of MNDs powder. The source MNDs hydrosol was then used to prepare hydrosols with a nanoparticles concentration of 200, 40, and 8 μg/mL by serial dilutions with DI water.

Specimens of biomaterials were obtained from male ICR mice weighing 26–28 g. Before experiments the animals were euthanized under ether anesthesia and their blood was collected from the subclavian artery. Thereafter, the animals were sacrificed by cervical dislocation and their organs were removed using plastic and ceramic instruments to prevent contamination of biomaterials with metal particles. In the study we used the liver, spleen, kidneys, heart, brain, lungs, and hindleg muscles. The extracted organs were placed on ice, cleaned from connective and adipose tissues, and disintegrated in distilled water in a glass–glass homogenizer. MND hydrosols with different concentrations of nanoparticles were added to the obtained homogenates and blood samples in a ratio of 1 : 4 (200 μL of hydrosol per 800 μL of biomaterial sample). Thus, the final MNDs concentration in the samples was 200, 40, 8, and 1.6 μg/mL, respectively. The control samples (organ homogenates and blood) were supplemented with distilled water instead of MNDs suspension. All samples were thoroughly mixed, placed in plastic containers, and frozen in liquid nitrogen. Thereafter, the frozen samples were removed from the containers and placed at a liquid nitrogen temperature into a special holder, which was transferred into the cavity of an Elexsys E580 EPR spectrometer (Bruker Corp., United States), and the EPR spectra were recorded at a temperature of 85–90 K.

In preliminary experiments we have found that an important condition for effective use of the EPR method for detecting MNDs in biomaterial samples is their deep freezing at low temperatures and an increase in the test sample volume. For this purpose we have developed original plastic containers, which allowed us to freeze samples in liquid nitrogen, and a special holder, which was used to transfer the frozen samples from the containers at a liquid nitrogen temperature. In turn, the use of such a holder allowed us to fix test samples in the EPR spectrometer cavity without using ampoules and to increase the volume of test samples of biomaterials.

At the initial stage of the study we detected the characteristic signal (g = 2.003, $\Delta H \approx$ 10 G) in the EPR spectra of MNDs hydrosol samples (Fig. 1). These results are consistent with the results of our previous studies [13], in which a symmetrical EPR signal at g = 2.003, associated with the presence of paramagnetic centers, was recorded in MND samples with different cluster sizes. The magnitude of the EPR signal detected in MND hydrosols almost linearly depended

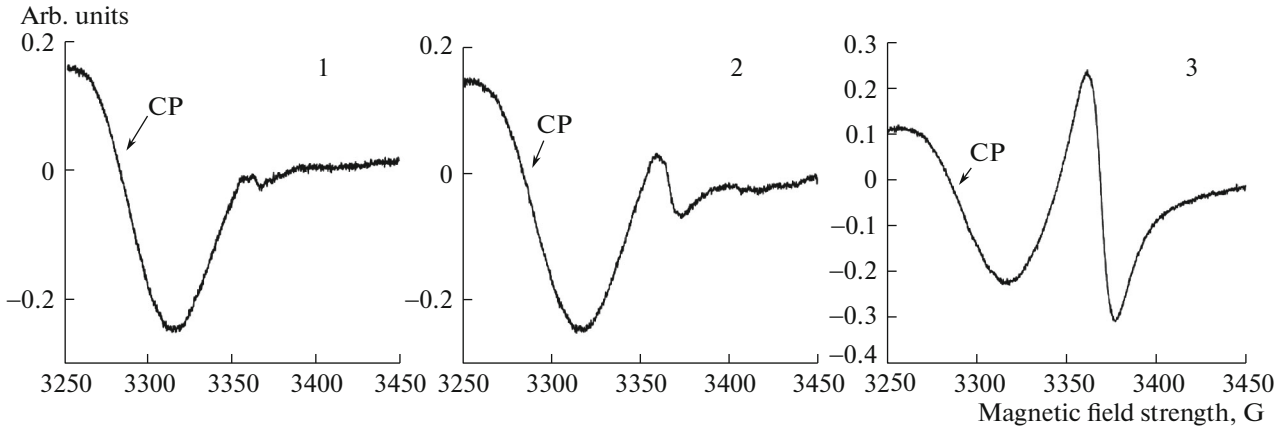

Fig. 3. EPR spectra in mouse blood samples: (1) control sample (without MNDs) and experimental samples with MNDs concentration of (2) 10 and and (3) 80 μg/mL, respectively. CP—part of the ceruloplasmin spectrum.

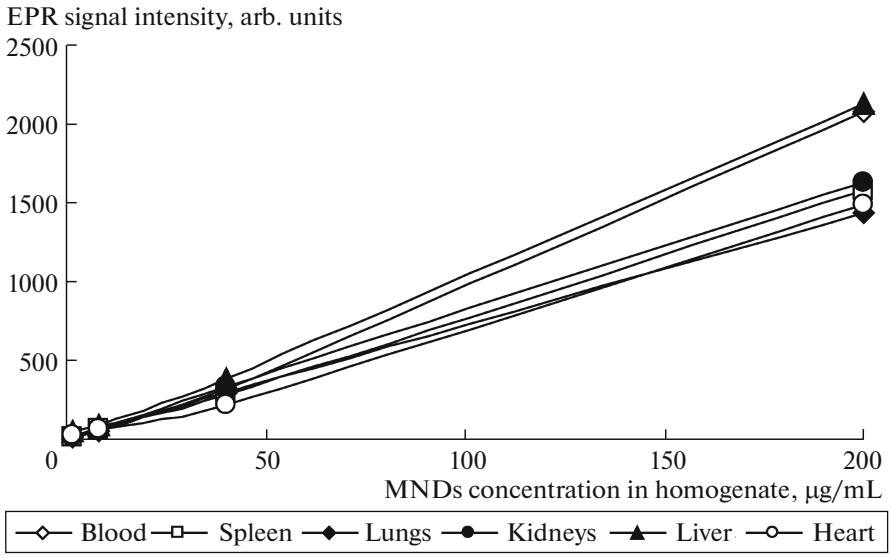

Fig. 4. EPR signal intensity depending on the MNDs concentration in samples of biomaterials.

on the concentration of nanoparticles in the range of 1.6–200 μg/mL (Fig. 2).

In the frozen samples of all studied biomaterials (blood and homogenates of animal organs) containing MNDs we also detected a symmetrical EPR signal ($g = 2.003$; $\Delta H \approx 10$ G). In all cases, the intensity of the EPR signals in the samples was directly proportional to the concentration of MNDs: greater signals were detected at higher concentrations of nanoparticles. An example of EPR signals detected in the blood samples of mice at different concentrations of MNDs is shown in Fig. 3.

EPR studies of the control samples of biological materials (containing no MNDs) showed the presence of intrinsic paramagnetic centers (radicals, heme-containing proteins, etc., Fig. 3) in them, the signals from which were superimposed on the signal from MNDs. However, at the sample volumes used in our study the values of EPR signals recorded from their intrinsic paramagnetic centers were usually small (Fig. 3). Nonetheless, for a greater accuracy of determination of MNDs in biomaterials we subtracted the EPR signal of the control sample (without MND) from the EPR signal of the experimental sample. This allowed us to detect MNDs in biomaterials with a greater accuracy.

The results of model experiments showed (Fig. 4) that in all test biomaterial samples the EPR signal value linearly increased with the concentration of MNDs (Fig. 4). At equal concentrations of nanoparticles higher EPR signals were detected in the test samples of blood and liver homogenates compared to other biomaterials studied. The causes of these differences remain unclear. We assume that they may be associated with an additional formation of intrinsic

paramagnetic centers in these biomaterials under the influence of MNDs. In particular, this can be observed when blood cells are destroyed. Earlier, in in vitro experiments we showed that nanodiamonds cause the destruction of red and white blood cells and activate the generation of reactive oxygen species (ROS) at the destruction of the white cells [14]. Therefore, the additional paramagnetic centers in the samples of blood and liver homogenates may form due to an increase in the free hemoglobin pool as a result of destruction of red blood cells and in the ROS pool as a result of destruction of the white-cell lineages. We believe that this version is true in both cases considered. It is commonly known that the liver is well supplied with blood and homogenates of this organ may contain a large number of blood corpuscles. Further studies are required to verify the above assumptions.

In general, our results suggest that the EPR spectrometry method can be used in studies in vivo for identification and quantitation of detonation nanodiamonds in organs and tissues of experimental animals after intravenous injection of nanoparticles.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 16-04-00999).

REFERENCES

1. Jin-Wook, Y., Nishit, D., and Samir, M., Adaptive micro and nanoparticles: temporal control over carrier properties of facilitate drug delivery, *Adv. Drug Deliv. Rev.*, 2011, vol. 63, pp. 1247–1256.
2. Morachis, J.M., Mahmoud, E.A., and Almutairi, A., Physical and chemical strategies for therapeutic delivery by using polymeric nanoparticles, *Pharmacol. Rev.*, 2012, vol. 64, pp. 505–519.
3. Purtov, K.V., Petunin, A.I., Burov, A.E., Puzyr, A.P., and Bondar, V.S., Nanodiamonds as a carriers for address delivery of biologically active substances, *Nanoscale Res. Lett.*, 2010, vol. 5, pp. 631–636.
4. El-Say, Kh.M., Nanodiamond as a drug delivery system: applications and prospective, *J. Appl. Pharm. Sci.*, 2011, vol. 1, pp. 29–39.
5. Zhu, Y., Li, J., Zhang, Y., Yang, X., Chen, N., Sun, Y., Zhao, Y., Fan, C., and Huang, Q., The biocompatibility of nanodiamonds and their application in drug delivery systems, *Teranostics*, 2012, vol. 2, pp. 302–312.
6. Zhang, X., Wang, A.Q., Liu, M., Hui, J., Yang, B., Tao, L., and Wei, Y., Surfactant-dispersed nanodiamond: biocompatibility evaluation and drug delivery applications, *Toxicol. Res.*, 2013, vol. 2, pp. 335–342.
7. Kaur, P. and Badea, I., Nanodiamonds as novel nanomaterials for biomedical applications: drug delivery and imaging, *Int. J. Nanomed.*, 2013, vol. 8, pp. 203–220.
8. Bondar, V.S. and Puzyr, A.P., Nanodiamonds for biological investigations, *Phys. Solid State*, 2004, vol. 46, no. 4, pp. 716–719.
9. Puzyr, A.P. and Bondar, V.S., A method for obtaining nanodiamonds of detonation with an increased colloidal stability, RF Patent no. 2252192, *Byul. Izobret.*, 2005, no. 14.
10. Puzyr, A.P., Burov, A.E., and Bondar, V.S., Modification and comparative study of commercial nanodiamonds, *Full. Nanotub. Carb. Nanostruct.*, 2015, vol. 23, pp. 93–97.
11. Puzyr, A.P., Baron, A.V., Purtov, K.V., Bortnikov, E.V., Skobelev, N.N., Mogilnaya, O.A., and Bondar, V.S., Nanodiamonds with novel properties: a biological study, *Diam. Relat. Mater*, 2007, vol. 16, pp. 2124–2128.
12. Solmatova, A.A., Il'in, I.V., Shakhov, F.M., Kidalov, S.V., Vul', A.Ya., Yavkin, B.V., Mamin, G.V., Orlinskii, S.B., and Baranov, P.G., Electron paramagnetic resonance detection of the giant concentration of nitrogen vacancy defects in sintered detonation nanodiamonds, *JETP Lett.*, 2010, vol. 92, pp. 102–106.
13. Puzyr, A.P., Bondar, V.S., Bukayemsky, A.A., Selyutin, G.E., and Kargin, V.F., Physical and chemical properties of modified nanodiamonds, *NATO Sci. Ser. II. Math. Phys. Chem.*, 2005, vol. 192, pp. 261–270.
14. Puzyr, A.P., Neshumayev, D.A., Tarskikh, S.V., Makarskaya, G.V., Dolmatov, V.Yu., and Bondar, V.S., Destruction of human blood cells in interaction with detonation nanodiamonds in experiments in vitro, *Diam. Relat. Mater.*, 2004, vol. 13, pp. 2020–2023.

Translated by M. Batrukova