ISSN 0012-5008, Doklady Chemistry, 2011, Vol. 437, Part 2, pp. 103–106. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © O.S. Ivanova, T.O. Shekunova, V.K. Ivanov, A.B. Shcherbakov, A.L. Popov, G.A. Davydova, I.I. Selezneva, G.P. Kopitsa, Yu.D. Tret'yakov, 2011, published in Doklady Akademii Nauk, 2011, Vol. 437, No. 5, pp. 638–641.

CHEMISTRY

One-Stage Synthesis of Ceria Colloid Solutions for Biomedical Use

O. S. Ivanova[a], T. O. Shekunova[e], V. K. Ivanov[a], A. B. Shcherbakov[b], A. L. Popov[c], G. A. Davydova[c], I. I. Selezneva[c], G. P. Kopitsa[d], and Academician Yu. D. Tret'yakov[e]

Received November 23, 2010

DOI: 10.1134/S0012500811040070

Nanocrystalline ceria CeO_2 is a unique inorganic polyfunctional material with high biological activity [1]. It has been recently shown that ceria is a strong antioxidant; i.e., it can inactivate reactive oxygen species (ROS) [2]. This activity makes it possible to introduce ceria into X-ray and UV protection preparations (for example, it can be used in sun protection cosmetics) [3]. The ability of CeO_2 nanoparticles to scavenge ROS makes nanoceria a good candidate for prevention and treatment of disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases [4].

The biological activity of ceria directly depends on the size of nanoceria particles [5]. Such dependence is caused by the fact that the decrease in the size of ceria particles sharply enhances their oxygen nonstoichiometry, which is accompanied by an increase in the lattice parameter [6, 7].

By now, numerous methods have been developed for preparing CeO_2 nanopowders with controllable particle size and shape [8–11]. At the same time, biomedical applications of ceria-based preparations call for nanoceria in the form suitable for dosing, i.e., as aggregatively stable CeO_2 sols stabilized by biocompatible ligands [12, 13]. Therefore, in the present paper, we report a new method of synthesis of stable nontoxic colloid solutions of ultrasmall CeO_2 nanoparticles (~2 nm).

EXPERIMENTAL

To prepare initial solutions, $Ce(NO_3)_3 \cdot 6H_2O$ (reagent grade), citric acid (reagent grade), and aqueous ammonia (pure grade) were used. A CeO_2 sol was obtained by dissolving 0.24 g of citric acid in 25 mL of a 0.05 M aqueous cerium(III) nitrate solution. The resulting solution was rapidly poured under stirring into 100 mL of a 3 M ammonia solution and allowed to stand for 2 h.

The size and shape of nanoceria particles were determined by transmission electron microscopy on a Leo 912 AB Omega electron microscope (accelerating voltage, 100 kV). The optical absorption spectra were recorded on an Ocean Optics QE-65000 spectrophotometer in the wavelength range of 275–900 nm. The size of CeO_2 particles was measured by the dynamic light scattering method on a Malvern Zetasizer Nano ZS analyzer. Before measurements, the sol was diluted with distilled water.

The biocompatibility of CeO_2 sols was studied with the use of NCTC clone L929 fibroblasts from the collection of cell cultures of the Institute of Cell Biophysics, RAS (Pushchino). The cells were cultured in a DMEM/F12 (1 : 1) medium containing 5% of fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in humid atmosphere containing 5% CO_2. The cells were seeded onto cover slips and into the wells of a 96-well plate in a concentration of 20 000 cells/cm^2. Two hours later, the medium in the wells was replaced by nanoceria sols diluted in a culture medium with different nanoparticle content (from 10^{-3} to 10^{-8} M). The cells were incubated for 1, 2, and 3 days at 37°C in humid atmosphere containing 5% CO_2. Cells cultured in a medium with-

[a] *Kurnakov Institute of General and Inorganic Chemistry, Russian Academy of Sciences, Leninskii pr. 31, Moscow, 119991 Russia*

[b] *Zabolotny Institute of Microbiology and Virusology, National Academy of Sciences of Ukraine, ul. Zabolotnogo 154, Kiev, 03143 Ukraine*

[c] *Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, ul. Institutskaya 3, Pushchino, Moscow oblast, 142290 Russia*

[d] *Konstantinov Institute of Nuclear Physics, Russian Academy of Sciences, Orlova roshcha, Gatchina, Leningrad oblast, 188300 Russia*

[e] *Moscow State University, Moscow, 119992 Russia*

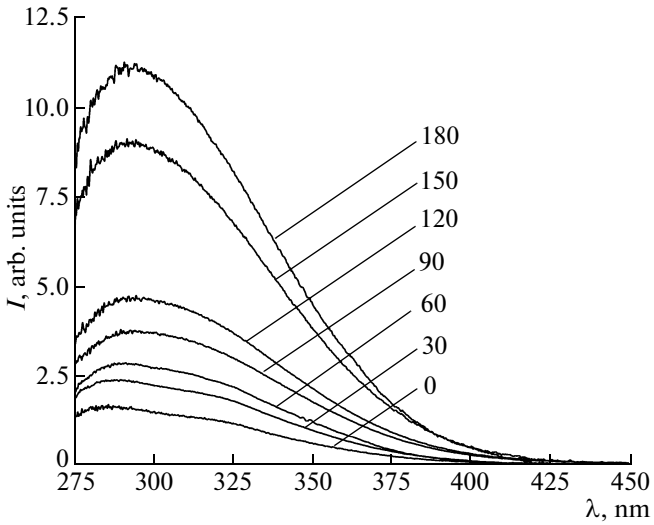

Fig. 1. UV-Vis absorption spectra of colloid solutions formed on mixing aqueous solutions of cerium(III) nitrate, citric acid, and ammonia. Synthesis durations (min) are given to the right of the spectra.

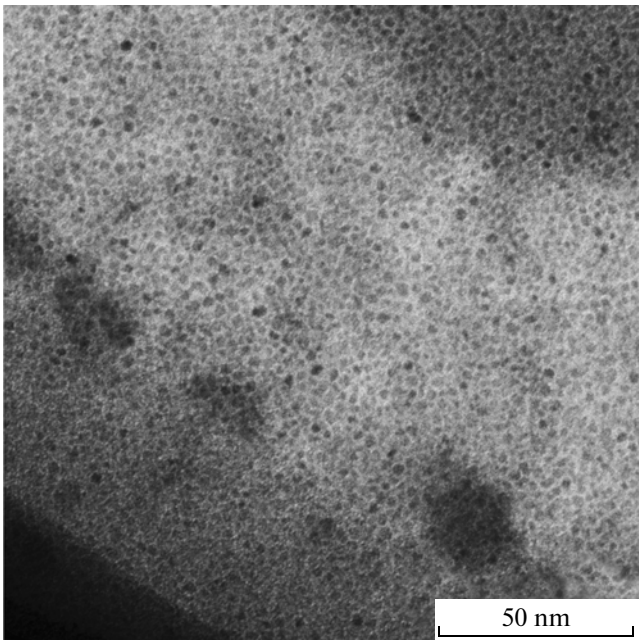

Fig. 2. SEM images of CeO_2 nanoparticles in citrate sol.

out CeO_2 nanoparticles served as the control. Cell viability was estimated by staining cells with SYTO 9 and propidium iodide fluorescent dyes.

After incubation was finished, the medium in the wells was replaced by a fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) and incubated for 3 h at 37°C in humid atmosphere containing 5% CO_2. Then, the liquid was removed, and the precipitate was dissolved in 100 μL of dimethyl sulfoxide. Color development was monitored by measuring the absorbance at 540 nm.

To prepare samples for scanning electron microscopy (SEM), after a 24-h cultivation period, the cells on the cover slips were washed with a 0.1 M phosphate buffer (pH 7.2) and fixed with a 2.5% solution of glutaraldehyde in the same buffer for 2 h at room temperature. After the samples were dehydrated in ethanol solutions (50–100%) at 4°C, ethanol was replaced with hexamethyldisilazane. The resulting preparations were studied on a Carl Zeiss NVision40 workstation at an accelerating voltage of 0.5 kV.

RESULTS AND DISCUSSION

Ceria forms immediately after mixing initial solutions of cerium(III) nitrate, citric acid, and ammonia, which is reflected by the appearance of an absorption band with an edge at ~400 nm in the UV-Vis spectrum (Fig. 1). The absorption intensity builds up as the reaction proceeds because of the increase in the concentration and size of CeO_2 nanoparticles in the sol. It is worth noting that the UV-Vis spectra recorded immediately after mixing the reagents also show the band at 285 nm, which evidently arises from intermediate cerium-containing compounds and vanishes as the reaction proceeds. Our data indicate that, under the conditions used, the formation of the CeO_2 phase was completed in 1.5–2 h, which is consistent with heat flow calorimetry data [14] The band gap (E_g) for the resulting sol is 3.53 eV, which noticeably exceeds the E_g value for macrocrystalline CeO_2 (~3.2 eV) and is evidence of small size of CeO_2 particles.

According to SEM (Fig. 2), the sol consists of weakly aggregated CeO_2 particles of nearly isotropic shape 1.5–2.5 nm in size. Dynamic light scattering data indicate that the hydrodynamic diameter of CeO_2 particles is 2.3 nm. This is consistent with the SEM data and is evidence that the presence of a monomolecular (or submonomolecular) layer of citrate ions has no noticeable effect on the particle size. During long-term storage (for more than a month), sols gradually lose their stability because of formation of nanoparticle agglomerates 100–2000 nm in size.

The CeO_2 sols contain a considerable amount of impurity ions (including nitrate ions) and have a strong alkaline reaction; hence, they are toxic. To remove impurities, the sols were acidified to pH ~ 2 with a hydrochloric acid solution, and the resulting CeO_2 precipitate was carefully washed and then pep-

Effect of CeO_2 nanoparticles on cell viability according to the MTT test data

Concentration of CeO_2 nanoparticles, M	Absorbance of MTT solutions at different durations of cell cultivation (days)		
	1	2	3
0	0.59 ± 0.1	1.02 ± 0.03	1.66 ± 0.13
10^{-11}	0.58 ± 0.09	1.03 ± 0.03	1.65 ± 0.10
10^{-10}	0.56 ± 0.06	0.98 ± 0.05	1.67 ± 0.17
10^{-9}	0.51 ± 0.14	1.16 ± 0.11	1.68 ± 0.10
10^{-8}	0.64 ± 0.07	1.09 ± 0.15	1.59 ± 0.02
10^{-7}	0.58 ± 0.12	0.97 ± 0.04	1.87 ± 0.12
10^{-6}	0.51 ± 0.08	0.99 ± 0.03	1.46 ± 0.14
10^{-5}	0.63 ± 0.10	0.91 ± 0.01	1.68 ± 0.08
10^{-4}	0.56 ± 0.09	0.86 ± 0.04	1.62 ± 0.25
10^{-3}	0.55 ± 0.04	0.85 ± 0.01	1.40 ± 0.16

tized by gradually adding a dilute ammonia solution to pH ~ 7.

The results of the MTT test (table) are evidence that no reliable decrease in the cell viability occurs in a wide range of CeO_2 concentrations (from 10^{-6} to 10^{-11} M). This testifies to the nontoxicity of citrate CeO_2 sols in such concentrations. At higher concentrations, the cell viability is reduced.

At the same time, in none of the samples with respect to the control did counting the ratio between dead and living cells colored with fluorescent dyes reveal a reliable increase in the content of unviable cells. Inasmuch as the rate of MTT transformation into formazan reflects the overall dehydrogenase activity of the cells and is modulated by the activity of conjugated enzyme systems, for example, the respiratory electron transfer chain, we may state that the decrease in cell viability at high CeO_2 concentrations (10^{-3}–10^{-6} M) is likely caused by the decrease in the respiratory activity of the cells rather than by their death.

The SEM data (Fig. 3) demonstrate that the introduction of CeO_2 nanoparticles (in a concentration of 10^{-3} M) into the NCTC L929 cell structure has no adverse effect on the morphologic characteristics of the cells. The cells in the controls and in the samples treated with CeO_2 have an oval cell nucleus typical of active fibroblasts and an abundant rough endoplasmic reticulum with microspikes, protrusions, lamellipodia, and filopodia. The presence of cells with spreading of the leading edge type is evidence of cell migration activity. The presence of rounded cell with the abundant rough endoplasmic reticulum and the absence of cells with a smooth or damaged endoplasmic reticulum are evidence of the proliferative activity of the cells and the lack of cell death.

Thus, we suggested a new-one-stage method of synthesis of cerium dioxide sols stabilized by ammonium citrate and carried out their complex physicochemical attestation. Our findings show that the sols have no cytotoxic effect of the cells and do not change their morphology; however, at concentrations above 10^{-5} M, the sols suppress the metabolic activity of the cells.

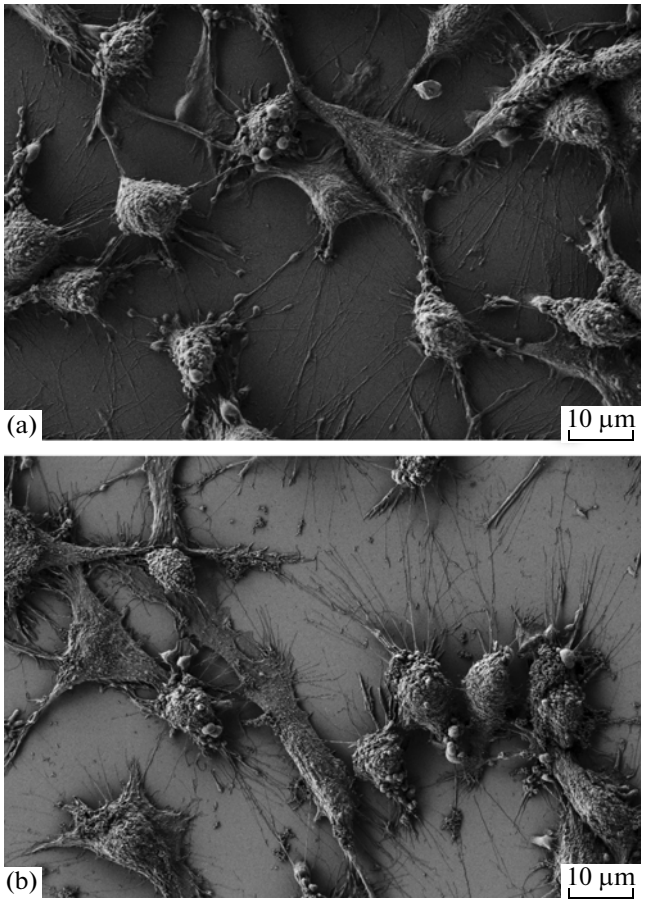

Fig. 3. SEM images of NCTC L929 cells: (a) the control and (b) after treatment with an aqueous CeO_2 sol.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education and Science of the Russian Federation (State contract no. P-2440), the Russian Foundation for Basic Research (project no. 11–03–00783), and the Presidium of the RAS (program no. P-21).

REFERENCES

1. Ivanov, V.K., Shcherbakov, A.B., and Usatenko, A.V., *Russ. Chem. Rev.,* 2009, vol. 78, pp. 855–871.
2. Heckert, E.G., Karakoti, A.S., Seal, S., and Self, W.T., *Biomaterials*, 2008, vol. 29, pp. 2705–2709.

3. Zholobak, N.M., Ivanov, V.K., Shcherbakov, A.B., et al., *J. Photochem. Photobiol. B*, 2011, vol. 102, pp. 32–38.
4. Chen, J., Patil, S., Seal, S., and McGinnis, J.F., *Nat. Nanotechnol.*, 2006, vol. 1, pp. 142–150.
5. Ivanov, V.K., Fedotov, G.N., Nikulina, M.V., et al., *Dokl. Chem.*, 2008, vol. 420, part 2, pp. 141–143 [*Dokl. Akad. Nauk*, 2008, vol. 420, no. 5, pp. 628–631].
6. Zhang, F., Chan, S.-W., Spanier, J.E., et al., *Appl. Phys. Lett.,* 2002, vol. 80, pp. 127–129.
7. Baranchikov, A.E., Polezhaeva, O.S., Ivanov, V.K., and Tretyakov, Yu.D., *CrystEngCom.,* 2010, vol. 12, pp. 3531–3533.
8. Polezhaeva, O.S., Yaroshinskaya, N.V., and Ivanov, V.K., *Neorg. Mater.*, 2008, vol. 44, pp. 57–63.
9. Ivanov, V.K. and Polezhaeva, O.S., *Zh. Neorg. Khim.*, 2009, vol. 54, pp. 1602–1604.
10. Ivanov, V.K., Polezhaeva, O.S., Baranchikov, A.E., and Shcherbakov, A.B., *Neorg. Mater.*, 2010, vol. 46, pp. 49–53.
11. Bumajdad, A., Eastoe, J., and Mathew, A., *Adv. Coll. Int. Sci.,* 2009, vol. 147/148, pp. 56–66.
12. Ivanov, V.K., Polezhaeva, O.S., Shaporev, A.S., et al., *Zh. Neorg. Khim.,* 2010, vol. 55, pp. 368–373.
13. Perez, J.M., Asati, A., Nath, S., and Kaittanis, C., *Small*, 2008, vol. 4, pp. 552–556.
14. Ivanov, V.K., Polezhaeva, O.S., Sharikov, F.Yu., and Tret'yakov, Yu.D., *Dokl. Chem.*, 2006, vol. 411, part 2, pp. 223–225 [*Dokl. Akad. Nauk*, 2006, vol. 411, no. 4, pp. 485–487].