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Natural elimination of upconversion YVO_4 : Yb, Er nanoparticles from a grape snail

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We report on a method for detection of upconversion nanoparticles (UNPs) in biological preparations by using confocal microscopy technique. Approbation of the method was carried out by monitoring the natural excretion of an injected colloidal solution of YVO4 : Yb, Er nanoparticles (0.2 ml, containing 15 mg ANP, dosage 600 mg/kg) with sizes from 10 nm to 700 nm into the snail. The experiments showed that the animal naturally eliminated the nanoparticles from the body within three days, while the excretion rate decreased over time. The threshold of sensitivity in the experiments was equal to one nanoparticle in 1 μl.

Keywords: upconversion, vanadate nanoparticles, rare earth ions, confocal microscopy, terrestrial snails.

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Introduction

Successful use of noninvasive fluorescent nanoprobes laid a reliable foundation for the development of a wide range of biological areas. There is no doubt that such methods will be the basic tools in the study of physiological intracellular activity principles in the nearest future. Various compounds are used as bioprobes: organic dyes, quantum dots, fluorescent proteins and luminescent transition metal complexes [1–6]. Generally, for their excitation, UV emission is used and extensively scattered and absorbed by biological tissues. This fact causes autofluorescence, photodestrction and cell heating which significantly limits potential applications. Low photostability of organic fluorochromes, undesired broad fluorescent protein emission spectrum, quantum dot flickering and toxicity and a set of other undesired factors [7–11].

To avoid any problems with the use of fluorescent nanosensors operated in the down-conversion scheme, we use an essentially other approach based on the upconversion. This approach uses upconversion nanoparticle (UNP) as nanosensors. These UNP are doped with rare-earth ions which have bright visible luminescence when excited by near IR region radiation (biological tissue transmission window). Undeniable advantages of such approach include UNP photostability, absence of stray autofluorescence [12–14], large exciting radiation penetration depth in tissues [15], very low local heating, extremely low phodestruction of biomolecules [16]. This shall be added with narrow luminescence lines of rare-earth ions and very low UNP toxicity in some cases [15,17–19]. These properties explain the growing interest in UNP as promising bionanoprobes in multiple biological tasks (biovisualization, bionanosensing, drug transport, theranostics, phototherapy, optogenetics, etc.) [20,21].

Yb^{3+} and Er^{3+} ions are the most popular dopants. It is due to the fact that Yb^{3+} ions have large absorption cross-section at 980 nm and are capable of efficiently transferring the energy to resonant levels of Er^{3+} ions. Since metastable ion levels take part in the energy transfer, this ensures high probability of two-quantum processes [22,23]. As matrix, we use YVO_4 nanocrystals with phonon energy 880 cm^{-1} which is much higher than phonon energy 350 cm^{-1} frequently used by matrix β-$NaYF_4$, [24,25]. Advantage of YVO_4 : Yb, Er nanoparticles relative to fluoride equivalents is in their low sensitivity to surface extinguisher in biological media. In addition, oxide nanoparticles are low toxic [26], which in general makes them attractive or the use in biological media. Out recent experiments for biovisualization on grapevine snails Helix lucorum have shown their potential for the use as fluorescent nanoprobes [27,28]. In should be noted that vinegrape snails are traditional test objects for nervous system research [29–31]. They are extensively used for the study of learning and memory mechanisms due to relatively simple nervous system with wide variety of behavioral reactions defined by complex interaction of inborn reflexes modulated by habituation, sensitization and associative learning processes. In [28], we performed comparative analysis of behavioral activity in animals with introduced UNP and control animals without UNP, and no deviations were found. It has been found that UNP (injected into snail body with dose up to 200 mg/kg of

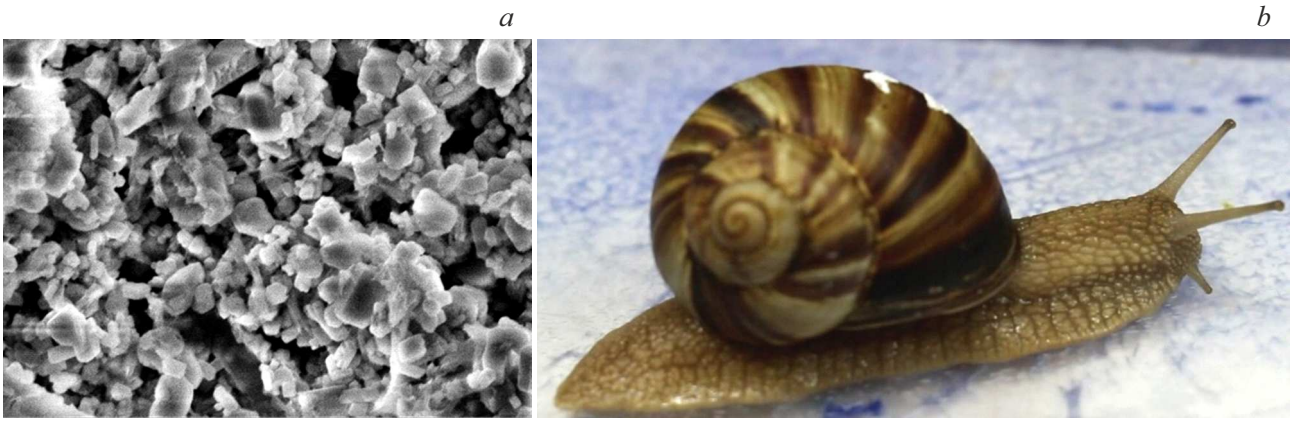

Figure 1. Image of YVO_4 : Yb, Er nanoparticles obtained by scanning election microscope (a). Helix lucorum grapevine snail photo (b).

animal weight) were virtually completely eliminated by the animals in natural way within 24 hours.

It should be understood that for further development of application strategies of YVO_4 : Yb, Er nanoparticles as fluorescent nanoprobes, information on their natural extraction rate is essential. For this purpose, we have developed the UNP control method in animal experiments. The sample preparation method proposed by us also makes it possible to detect UNP in drugs prepared from individual organs. This method was tested on grapevine snail Helix lucorum. As a result, we have found that despite the high dose (600 mg/kg) of introduced UNP, the snail was capable of full excretion within three days. Snail excretion samples for day 4 and further contained no UNP. No any deviation in behavioral reactions of snails after injection were observed. After 7 days, samples of individual snail organs were prepared. The experiments showed that these samples contained no UNP.

1. Experiment

UNP synthesis was performed in the same way described by us in [10] in accordance with the following procedure [32–34]. $Y(NO_3)_3$, $Er(NO_3)_3$, and $Yb(NO_3)_3$ ($c = 0.1$, 0.002 and 0.02 mol/l , respectively) aqueous solution was slowly added to Na_3VO_4 ($c = 0.1$ mol/l) aqueous solution with continuous stirring at room temperature. As a result, white sediment was formed which means that YVO_4 : Yb, Er nanoparticles are present; after this, the solution was dried to powder condition. Colloid silicone dioxide was prepared by heating tetraethoxysilane, ethanol and distilled water at pH = 1.25, $T = 60°C$ within 1 h. Then nanoparticles were coated with silicon dioxide and dispersed polymer (PE6800) (molar ratio V : Si : PE6800 = 1 : 5 : 0.05). After drying, mesoporous silicon network structure was formed containing nanoparticles. Calcination was carried out at 500°C within 1 h, and then nanopaticles were annealed within 10 min at 1000°C. After that, the silicon matrix was removed by means of three-hour treatment with fluoric acid with molar ratio HF : Si = 9 : 1. According to the electron microscopy data (Figure 1, a), the size of obtained UNP is within 10−700 nm.

The method was tested on a 25 g grapevine snail (Figure 1, b). Before the experiments, the animal was active for at least 2 weeks, received excessive food and was kept in wet atmosphere at room temperature (18−22°C). UNP aqueous colloid solutions were injected into the inner cavity of the animal through the sinoatrial node of the snail where no pain receptors are available. To prepare the UNP colloid solution, distilled water and UV dispersion procedure were used during 10 min directly before the injections. The colloid solution contained 15 mg UNP per 0.2 ml H_2O (600 mg/kg of animal weight).

After injection, the animal was kept in terrarium with daily excretion sampling and sample preparation as follows. The snail excretion per day were thoroughly gathered from the terrarium walls and snail body. Then the gathered biomaterial was annealed at 500°C within 5 min , as a result grey ash was formed. Then it was dissolved in 0.06N hydrochloric acid and rinsed with water twice with microcentrifuging. At this stage, the sample constituted 2 ml colloid solution of unburnt and water insoluble sediment. To carry out optical experiments using a diluter, a drop of 1 μl solution was placed on a slide and spread over approx. 12.5 mm^2 and dried.

UNP luminescence was recorded using a confocal microscope with 980 nm diode laser excitation. Spatial resolution of the microscope was 1 μm. The use of single-photon detectors for radiation recording made it possible to reliably record luminescence from single UNP. The slide with the test sample was placed in front of the lens, then object scanning was carried out using a galvoscanner which moved the beam over the sample. As a result a pattern was obtained which constituted a scanned surface intensity gradient. To confirm that the glowing dots are the target UNP, spectrum of each glowing dot was recorded. It should be noted that the area of one scan on the confocal microscope was equal to 0.068 mm^2.

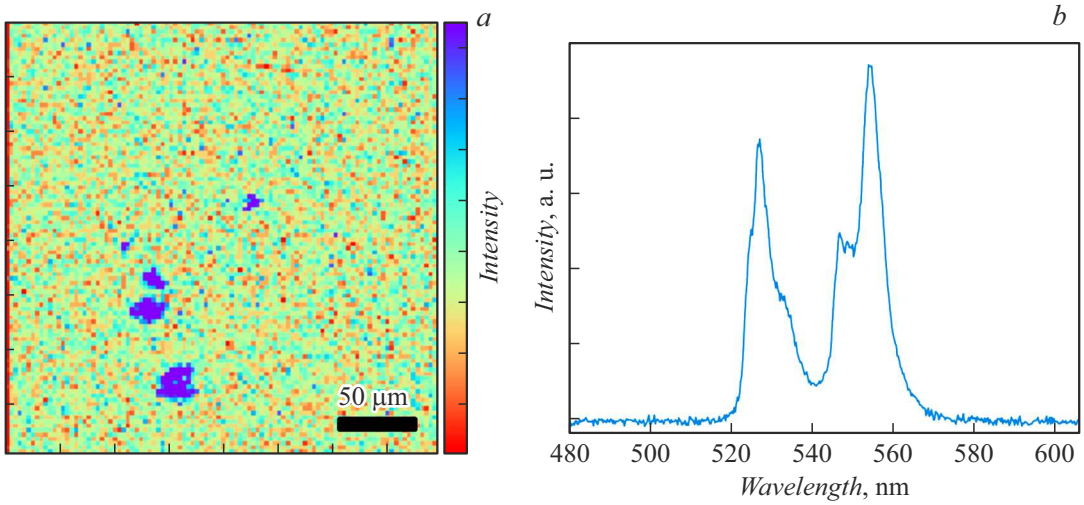

Figure 2. Typical scan of confocal microscope (a) made on snail excretion sample for 1 day after UNP injection. UNP luminescence spectrum confirming the UNP presence in five areas shown in blue (in online version) on the confocal microscope scan (b).

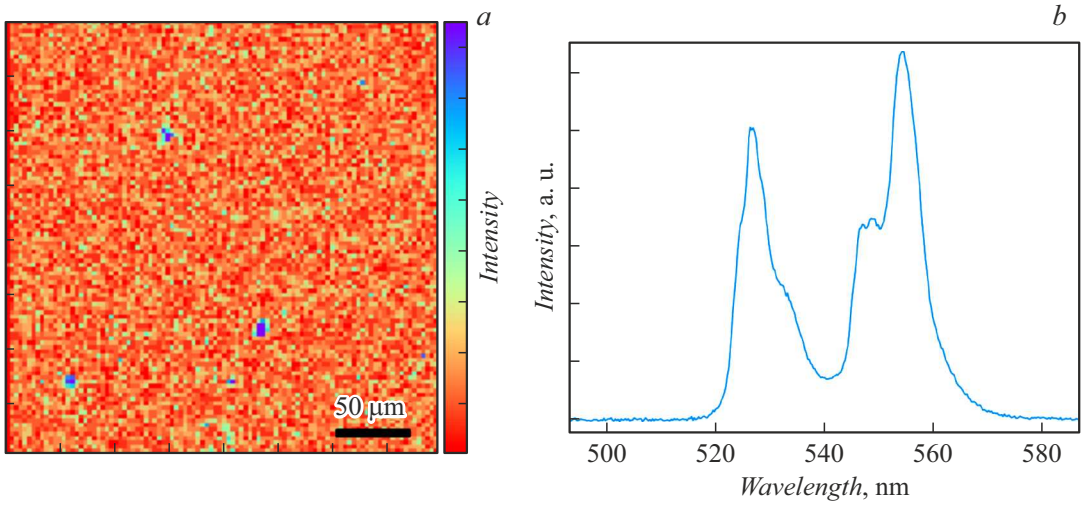

Figure 3. Typical scan of confocal microscope (a) made on snail excretion sample for 2 days after UNP injection. Luminescence spectrum confirming the UNP presence in four areas shown in blue (in online version) on the confocal microscope scan (b).

2. Results

Typical scan of a sample prepared from the snail excretion for the first day after injection is shown in Figure 2, a. This scan shows the areas with intensive luminescence highlighted in blue (in online version). Their luminescence spectrum shown in Figure 2, b is a typical spectrum of Er^{3+} ion emission into UNP which indicates the presence of UNP in these areas. The other scans made in random areas on the sample contain three to seven areas with bright luminescence. In general, this result shows that UNP is present in natural excretion of the animal for the 1st day.

Figure 3, a shows one of the scans of the snail excretion sample for day 2. There are three intensive luminescence areas whose spectrum (Figure 3, b) indicates that UNP is present. Other scans of this sample contain up to five areas with UNP. However, they are characterized by significant reduction of luminescent area sizes compared with the previous sample.

In the snail excretion sample for day 3, only a single luminescent area was detected and is shown in Figure 4, a. Its spectrum (Figure 4, b) confirms that the sample contains UNP. Other scans do not contain any UNP signal. The area has relatively large sizes comparable with the sizes of the largest areas on the first sample (Figure 2).

We have also prepared excretion samples for days 4–7. The spectroscopic tests found no luminescent signal from UNP. This indicates the absence or extremely low content of UNP beyond the sensitivity of the method used.

We used the biomaterial sample preparation method to find UNP in the preparations from the snail body fragments (liver, lungs, heart, nervous system, hemolymph, body, le)

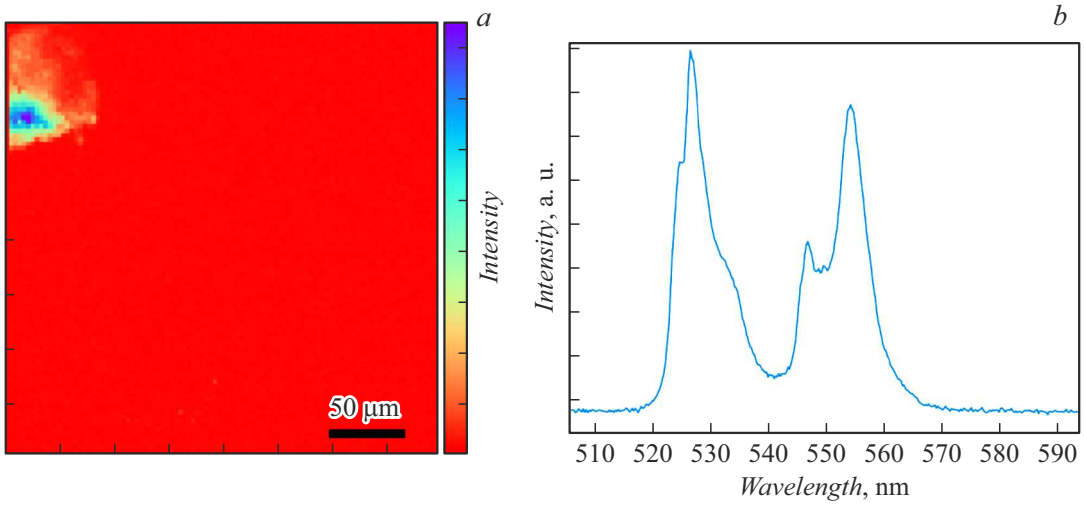

Figure 4. Typical confocal microscopes can (*a*) made on snail excretion sample for 3 days after UNP injection containing a single luminescent area. Luminescence spectrum confirming the UNP presence in the single area shown in blue (in online version) on the confocal microscope scan (*b*).

which we prepared 7 days after the injection. The study found no UNP signals in these samples. This indicates that there is no UNP in the animal body or low UNP concentration beyond the method sensitivity.

3. Discussion

The proposed method for sample preparation from biomass enables reliable detection of contained UNP. This method was successfully tested on the grapevine snail which received UNP aqueous solution injection (aqueous colloid solution, 0.2 ml, containing 15 mg UNP, dosing 600 mg/kg). The data obtained from monitoring of natural UNP excretion from the snail body indicates that the animal was able to get rid of foreign oxide nanoparticles within three days. We were not able to detect any luminescent signal in the samples prepared from the snail excretion for day 4 and further up to day 7. UNP were also found in the samples prepared from individual body fragments.

Here, it is important to make some sensitivity assessments for the method used. The used confocal spectroscopy equipment reliably detects luminescence of a single UNP. This means that the test samples where no luminescent signal was detected contain no UNP. Nevertheless, the method sensitivity is significantly restricted by the sample preparation technique which includes 1 μl solution application on the slide and drying. Accordingly, the method accuracy shall not exceed one 1 μl nanoparticle. This is probable the kind of accuracy limit which was achieved in the sample on experiment day 3 when a single luminescent area was detected. It should be noted that the absence of luminescent signal in the sample indicates a concentration lower than one 1 μl UNP.

Correction assessment of the absolute amount of UNP in the sample is the next method restriction. This is associated with the difficulties of UNP determination by the total luminescent signal. To implement this opportunity, a method shall be developed involving additional experiments, e.g. using electronic microscopy. Nevertheless, some observations can be made from the confocal microscope scan analysis at this stage. Though we cannot obtain absolute data on the amount of UNP in the sample, we can speak with confidence about a downward trend of the amount of UNP eliminated from the body over time within three first days. Since all samples were made using a single method, comparison of relative concentrations of luminescent areas containing UNP using confocal microscope scans is correct and reflects the general trend. It is also interesting to focus on luminescent area shapes in scans. It should be remembered that the sizes of injected UNP are 10–700 nm. We suppose that bright shapeless luminescent areas on the scan (Figure 2) may present agglomerates of the smallest UNP eliminated by the animal on day 1. Individual small luminescent spots in Figure 3 likely represent larger UNP which are eliminated from the body more slowly. And, finally the single luminescent area in Figure 4 may be one large UNP whose excretion was performed by the most complicated way. It should be noted that these discussions are qualitative and need additional experimental check.

Conclusion

Using the confocal microscopy equipment to ensure reliable detecting of upconversion luminescence of a single UNP, we have developed a method to determine natural excretion rate of oxide UNP from the animal. The method accuracy is limited primarily by the sample preparation technique and did not exceed one UNP in 1 μl of solution in our experiments. A grapevine snail was used to test the method. The experiments have shown that the animal

after 600 mg/kg UNP colloid solution injection eliminated by natural way UNP with sizes from 10 to 700 nm during 3 days, with the number of UNP in samples reduced over time. It has been also shown that after 7 days no UNP are present in the snail body or their concentration is beyond the method sensitivity limits. It should be noted that the proposed method may be used on any other animals. Experiments with a single snail are not sufficient for comprehensive assessment of UNP toxicity. Nevertheless, the represented results indicate the emerging opportunities of using UNP as minimally invasive luminescent nanoprobes in a wide range of biological tasks.

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Conflict of interest

The authors declare that they have no conflict of interest.

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