Hydrophilic NaYF₄ nanocrystals doped with Eu³⁺: Effects of surface functionalization on glutamate and GABA transport in brain nerve terminals


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ABSTRACT

Neuroactive properties of NaYF₄ nanocrystals doped with Eu³⁺ were assessed based on the analysis of their effects on glutamate- and γ-aminobutyric acid (GABA) transport process in the nerve terminals isolated from rat brain. Two types of hydrophilic nanocrystals were examined in this work: (i) coated by polyethylene glycol (PEG) and (ii) with OH groups at the surface. The changes induced by nanocrystals in the nerve terminals’ neurotransmitters transport were compared to the experimental data obtained recently by our group using the same methods with carbon dots, detonation nanodiamonds, and an iron storage protein ferritin. Neuroactivity of nanocrystals can be considered lesser neurotoxic compared to the nanoparticles listed above. Based on the unique optical properties, these nanocrystals are good candidates for biosensing, in vitro and in vivo biomedical imaging, and after thorough preclinical testing for clinical use.

Keywords: lanthanide doped fluoride nanocrystals; glutamate; γ-aminobutyric acid; Na⁺-dependent uptake; membrane potential; brain nerve terminals

1 INTRODUCTION

Nanoparticles are envisioned to have great biotechnological and biomedical potential with a wide set of new applications. Many efforts in the research and development are focused on the design of the nanomaterials with multiple functions, particularly those that can be used in theranostics, a new branch of nanomedicine combining both diagnostic bio-imaging and disease treatment [1–3]. Physical and chemical properties of nanoparticles differ by the materials of origin, thus providing their unexpected behaviour and interaction with biological objects. It is clear that a detailed understanding of principles of influence of nanoparticles on the cell functioning is of value for further progress in nanobiotechnology and nanomedicine.

Nanoparticles can be used as bio-imaging contrast agents for magnetic resonance imaging, e.g. ferrum oxide [4], optical biomarkers for imaging, e.g. inorganic nanocrystals doped with rare Earth ions [5] and quantum dots [6]. Quantum dots, an optically active inorganic nanocrystals, composed of cadmium-selen and cadmium-sulfur have unique physical and chemical properties, and so are widely used in biomedical research as effective optical biomarkers. However, their use in daily medical practice clinics for diagnosis and treatment of different diseases is still uncertain doubtful mainly because of proven toxicity of Cd²⁺. We have demonstrated that exposure to Cd²⁺ can provoke development of neurotoxic consequences and neurological problems, and therefore it is tremendously dangerous to human health. The application of Cd²⁺ at a concentration of 200 µM to the rat brain nerve terminals caused a decrease in synaptic vesicles acidification and glutamate accumulation in digitonin-permeabilized preparations of the nerve terminals. Also, Cd²⁺ caused dissipation of the proton gradient of isolated rat brain synaptic vesicles. Therefore, Cd²⁺ provokes synaptic malfunction associated with a decrease in stimulated exocytosis due to incomplete filling of synaptic vesicles, and also an attenuation of Na⁺-dependent glutamate uptake [7–9]. Specific rare earth doped nanocrystals, a recent class of nanoparticles with fluorescent features, have great bioanalytical potential. Nanocrystals can overcome the difficulties of traditional fluorophores, for example organic dyes and fluorescent proteins, regarding spectral cross-talking, rapid photobleaching, blinking and limited brightness and signal intensity [10,11].

The aim of the study was to assess effects of surface functionalization of hydrophilic NaYF₄ nanocrystals doped with Eu³⁺ on glutamate and GABA transport in brain nerve terminals.

2 METHODS AND MATERIALS

2.1 Biological experiments

The effects of NaYF₄ nanocrystals doped with Eu³⁺ on glutamate- and γ-aminobutyric acid (GABA) transport process in the nerve terminals isolated from rat brain (synaptosomes) were investigated to clarify their neuroactive properties.

The assessment of neuroactivity was conducted at the neurochemical level, where an agent might alter the flow of

Animal experiments

Wistar rats (males, 100-120 g of body weight) were kept in animal facilities of the Palladin Institute of Biochemistry NAS of Ukraine, housed in a quiet, temperature-controlled room (22–23°C), and provided with water and dry food pellets ad libitum. Rats were decapitated before brain removing. All experimental procedures were conducted according to the Helsinki Declaration “Scientific Requirements and Research Protocols” and “Research Ethics Committees”. The Animal Care and Use Committee of the Palladin Institute of Biochemistry (Protocol from 19/09-2014) approved the experimental protocols.

2.2 Isolation of nerve terminals (synaptosomes) from rat brain

The cerebral hemispheres were rapidly removed and the synaptosomes were prepared by differential and Ficoll-400 density gradient centrifugation of the homogenate according to [12,13].

2.3 Measurements of L-[14C]glutamate uptake by synaptosomes

The uptake of L-[14C]glutamate by synaptosomes was measured as follows. The synaptosomal suspensions (125 µl; of the suspension, 0.2 mg of protein/ml) were pre-incubated in the standard salt solution at 37°C for 8 min, then nanocrystals (0.5-7.5 mg/ml) were added to the synaptosomal suspensions and incubated for 10 min. The uptake was initiated by the addition of 10 µM L-glutamate supplemented with 420 nM L-[14C]glutamate (0.1 µCi/ml), and incubated at 37°C during different time intervals (1, 2, 10 min), and then rapidly sedimented using a microcentrifuge (20 s at 10,000 g). The L-[14C]glutamate uptake was determined as a decrease in radioactivity in aliquots of the supernatants (100 µl) and an increase in radioactivity of the pellets (SDS-treated) measured by liquid scintillation counting with ACS scintillation cocktail (1.5 ml).

2.4 Measurements of [3H]GABA uptake by synaptosomes

The synaptosomes were diluted in the standard salt solution containing GABA transaminase inhibitor aminooxyacetic acid at a concentration of 100 µM to minimize formation of GABA metabolites. Concentration of protein in the synaptosomal samples was 200 µg/ml. The samples were preincubated at 37°C for 8 min, then nanocrystals (0.5-7.5 mg/ml) were added to the synaptosomal suspension and incubated for 10 min. Uptake was initiated by the addition of GABA and [3H]GABA (1µM and 50 nM-0.1µCi/ml, respectively). The GABA uptake was terminated in different time intervals (1, 5, 10 min) by filtering aliquots through a Whatman GF/C filters. After twice washing with 5 ml the standard salt solution, filters were dried, then were suspended in Organic Counting Scintillant and counted in a Delta 300 (Tracor Analytic, USA) scintillation counter [14]. Non-specific binding of [3H]GABA was evaluated in cooling samples sedimented immediately after the addition of radiolabeled GABA. Each measurement was performed in triplicate.

2.5 Assessment of the ambient level of L-[14C]glutamate in the preparations of synaptosomes

The synaptosomes were diluted in the standard saline solution to reach the concentration of 2 mg of protein/ml, and after pre-incubation at 37°C for 10 min they were loaded with L-[14C]glutamate (1 nmol/mg of protein, 238 mCi/mmol) in oxygenated standard saline solution at 37°C for 10 min. After loading, the suspensions were washed with 10 volumes of ice-cold standard saline solution; the pellets were re-suspended in the solution to a final concentration of 1 mg protein/ml and immediately used for release experiments. The synaptosomal suspensions (125 µl; 0.5 mg of protein/ml) were pre-incubated for 10 min, then the nanocrystals (0.5-7.5 mg/ml) were added at 37°C and incubated for different time intervals (0 min and 6 min), and then rapidly sedimented using a microcentrifuge (20 s at 10,000 g). The release was measured in the aliquots of the supernatants (100 µl) and the pellets by liquid scintillation counting with scintillation cocktail ACS (1.5 ml). The results were expressed as a percentage of total amount of radiolabeled neurotransmitter incorporated.

2.6 Assessment of the ambient level of [3H]GABA in the preparations of synaptosomes

The synaptosomes were diluted in standard saline solution to 2 mg of protein/ml and after pre-incubation for 10 min at 37°C were loaded with [3H]GABA (50 nM, 4.7 µCi/ml) in the oxygenated standard saline solution for 10 min. Aminooxyacetic acid at a concentration of 100 µM was present throughout all experiments of [3H]GABA loading and release. After loading, the suspensions were washed with 10 volumes of ice-cold oxygenated standard saline solution. The pellets were re-suspended in the standard saline solution to obtain protein concentration of 1 mg of protein/ml. The synaptosomes (120 µl of the suspension) were preincubated for 10 min with the nanocrystals (0.5-7.5 mg/ml) at 37°C, and then rapidly sedimented using a microcentrifuge (10,000×g, 20 s). [3H]GABA radioactivity was measured in the aliquots of supernatants (90 µl) by liquid scintillation counting with...
scintillation cocktail ACS (1.5 ml) and expressed as percentage of a total $[^3]$H[GABA accumulated.

### 2.7 Measurement of synaptosomal plasma membrane potential ($E_m$)

Membrane potential was measured using rhodamine 6G, a potentiometric fluorescent dye according [12].

### 2.8 Measurements of synaptic vesicle acidification in the synaptosomes

Acridine orange, a pH-sensitive fluorescent dye, is known to be selectively accumulated by the acid compartments of synaptosomes (synaptic vesicles) [7]. Monitoring of synaptic vesicle acidification was conducted according [7].

### 2.9 Statistical analysis

The results were expressed as mean ± S.E.M. of $n$ independent experiments. The differences between two groups were compared by two-tailed Student's $t$-test. The differences were considered significant, when $P \leq 0.05$.

### 3 RESULTS AND DISCUSSION

Two types of hydrophilic nanocrystals were examined in this study, the first one is the nanocrystals coated by polyethylene glycol (PEG) and the second one – the nanocrystals with OH groups at the surface. It was found that NaYF$_4$:Eu-PEG and NaYF$_4$:Eu-OH within the concentration range of 0.5-3.5 mg/ml and 0.5-1.5 mg/ml, respectively, did not influence Na$^+$-dependent transporter-dependent L-$[^{14}]$glutamate and $[^3]$H[GABA uptake and the ambient level of the neurotransmitters in the synaptosomes.

An increase in NaYF$_4$:Eu-PEG and NaYF$_4$:Eu-OH concentrations up to 7.5 mg/ml and 3.5 mg/ml, respectively, led to: (1) attenuation of the initial velocity of uptake of L-$[^{14}]$glutamate and $[^3]$H[GABA; (2) elevation of ambient L-$[^{14}]$glutamate and $[^3]$H[GABA in the suspension of nerve terminals. NaYF$_4$:Eu-PEG and NaYF$_4$:Eu-OH at concentrations of 7.5 mg/ml and 3.5 mg/ml, respectively, did not influence acidification of synaptic vesicles that was shown with pH-sensitive fluorescent dye acridine orange (Fig.1), however decreased the potential of the plasma membrane of synaptosomes measured with potential-sensitive dye rhodamine 6G (Fig.2).

The changes in transport of the neurotransmitters in the synaptosomes were compared among nanocrystal, carbon dots, detonation nanodiamonds, and an iron storage protein ferritin using the same methods. The neuroactive properties of these nanoparticles were registered at concentrations of 0.08 mg/ml, 0.5 mg/ml, and 0.08 mg/ml, respectively [15,16], and the results showed that the nanocrystals can be considered less neurotoxic.

**Fig.1** Acidification of the synaptosomes in the presence of NaYF$_4$:Eu-PEG. The synaptosomes were loaded with acridine orange (5 µM); when the steady level of the dye fluorescence had been reached, NaYF$_4$:Eu-PEG at a concentration of 7.5 mg/ml (marked by arrow) were applied to the synaptosomes. Trace represents three experiments performed with different preparations.

**Fig.2** The membrane potential of the nerve terminals after the addition of NaYF$_4$:Eu-PEG. An increase in the fluorescence signal of rhodamine 6G in response to application of NaYF$_4$:Eu-PEG (7.5 mg/ml). The suspension of the synaptosomes was equilibrated with potential-sensitive dye rhodamine 6G (0.5 µM); when the steady level of the dye fluorescence had been reached, NaYF$_4$:Eu-PEG were applied to the synaptosomes. Trace represents three experiments performed with different preparations.
4 CONCLUSIONS

Based on the unique optical properties, the nanocrystals NaYF₄:Eu-PEG and NaYF₄:Eu-OH are good candidates for biosensing, \textit{in vitro} and \textit{in vivo} biomedical imaging, and after thorough preclinical testing for clinical use.

Importantly, for \textit{in vivo} bioimaging applications, precise control and monitoring of the concentrations of the nanocrystals and their usage at the concentrations below neuroactive ones should be implemented.

In turn, neuroactive properties of high concentrations of the nanocrystals shown in this study could be used in the treatment of neurodegenerative diseases and in neurotheranostics.

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