PNAS
www.pnas.org

Supplementary Information for

RADIOACTIVE (^{90}Y) UPCONVERSION NANOPARTICLES CONJUGATED WITH RECOMBINANT TARGETED TOXIN FOR SYNERGISTIC NANOTHERANOSTICS OF CANCER

Evgenii L. Guryev[a,1], Natalia O. Volodina[a,1], Natalia Y. Shilyagina[a], Sergey V. Gudkov[a,b,c], Irina V. Balalaeva[a,d], Arthur B. Volovetskiy[a], Alexander V. Lyubeshkin[e], Alexey V. Sen'[f], Sergey A. Ermilov[f], Vladimir A. Vodeneev[a], Rem V. Petrov[g], Andrei V. Zvyagin[a,d,g,h,2], Zhores I. Alferov[i,2], and Sergey M. Deyev[a,g]

[a]The Institute of Biology and Biomedicine, Lobachevsky State University of Nizhny Novgorod, 603950 Nizhny Novgorod, Russia;
[b]Moscow Regional Research and Clinical Institute, 129110 Moscow, Russia;
[c]A.M. Prokhorov General Physics Institute, Russian Academy of Sciences, 119991 Moscow, Russia;
[d]Center of Biomedical Engineering, Sechenov University, 119991 Moscow, Russia;
[e]Center of Crystallography and Photonics, Russian Academy of Sciences, 119333 Moscow, Russia;
[f]Scientific and Technical Center "Amplitude" Ltd., Zelenograd, 124460 Moscow, Russia;
[g]M. M. Shemyakin & Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117997 Moscow, Russia;
[h]Australian Research Council Centre of Excellence for Nanoscale Biophotonics, Macquarie University, Sydney, NSW 2109, Australia;
[i]Nanotechnology Research and Education Centre of Russian Academy of Sciences, St. Petersburg Academic University, 194021 St. Petersburg, Russia

[1] E.L.G. and N.O.V. contributed equally to this work.
[2] To whom correspondence may be addressed. E-mail addresses: andrei.zvyagin@.mq.edu.au (A. Zvyagin), zhores.alferov@ras.ru (Z. Alferov).

www.pnas.org/cgi/doi/10.1073/pnas.1809258115

This PDF file includes:

Supplementary text
Figs. S1 to S6
Table S1
References for SI reference citations

Supplementary Information Text

RESULTS

Characterization of the HER2 expression in cell lines. The expression level of the HER2 receptor in the cells of the cultures used in the study was tested by flow cytofluorometry after staining the cell suspension with a HER2-specific monoclonal antibody Herceptin conjugated with FITC fluorescent dye (Herceptin-FITC conjugates). The cells were removed from culture flasks with Versene solution, counted in Goryaev's chamber and centrifuged for 5 min at 200 g and room temperature. Then the cells were diluted with a solution of 3% skim milk in PBS to a concentration of 0.6×10^6 cells/ml and incubated at 4°C for 20 min. The resulting cell suspension in a volume of 500 μl, was supplemented with 0.5 μg of Herceptin-FITC conjugates or IgG-FITC conjugates (isotypic control) and incubated for 30 min on a rotator at 15 rpm. The cells were then washed with 500 μl of 3% milk in PBS, pelleted by centrifugation for 5 min at 200 g and washed again with 500 μl of 1% BSA in PBS. The cell pellet was resuspended in 300 μl of 1% BSA in PBS and analyzed by flow cytofluorometry using FACSCalibur (BD Biosciences). The obtained histograms (Figure S1) indicate nearly complete absence of HER2 receptor expression in CHO cells (the intensity of cell fluorescence after staining with Herceptin-FITC conjugates is at the level of isotype control) and its high expression (overexpression) in SK-BR-3 cells (the intensity of cell fluorescence after staining with Herceptin-FITC conjugates is several orders of magnitude higher than the fluorescence intensity of isotypic control).

In vitro DARPin-PE40 cytotoxicity assay. A dependence of the toxic effect of the targeted toxin DARPin-PE40 on the expression level of HER2 receptors was studied using several cell lines and reported by us in Ref [1] (Figure S2). In brief, the effect of DARPin-PE40, as well as DARPin and PE40, on the cell viability was assayed using the following cell types expressing variable amounts of HER2: SK-BR-3, HeLa and CHO cells characterized by overexpression, low-level expression and no expression of HER2, respectively. DARPin-PE40 was shown to potently inhibit the growth of HER2-positive cells SK-BR-3 and HeLa while affecting HER2-negative CHO cells only slightly (Figure S2). Moreover, cytotoxic efficacy of DARPin-PE40 strongly correlated with the level of HER2 expression: IC_{50} was 0.1 pM for SK-BR-3, which was about 400-fold lower than IC_{50} for HeLa cells. IC_{50} for CHO cells was about 6 orders of magnitude greater than that for SK-BR-3 cells.

In vitro UCNP-PMAO-DARPin cytotoxicity assay. The cytotoxicity of UCNP-PMAO-DARPin complexes against the SK-BR-3 cell lines (HER2 overexpression) and CHO (the absence of HER2 expression) characterized by over- and no expression of HER2

receptors, respectively, was tested (Fig. S3). We found negligible cytotoxic effects of the target complexes in the selected concentration range for SK-BR-3 and CHO cell cultures.

Investigating therapeutic effects of UCNP-R-T. To determine the tumor growth rate constant, the dependence of the natural logarithm of the relative tumor volume ($ln\ \alpha_v$) from time was analyzed (Figure S4B). The dependencies for different experimental groups were approximated by the function $\ln \alpha_{v(t)}=\ln \alpha_{v(0)}+kt$, where $\alpha_{v(t)}$ is the tumor volume in percent relative to the initial value ($\alpha_{v(0)}$), *k* is the tumor growth rate constant, *t* – the time of observation. The correlation coefficient R for all groups was not less than 0.9. It can be seen from Table S1, that intratumoral administration of UCNP-R-T at a dose of 15 μg/g results in a statistically significant decrease of the tumor growth rate constant: 0.09 $days^{-1}$ versus 0.13 $days^{-1}$ in the control and other experimental groups. UCNP-R-T administration at a dose of 10 μg/g leads to a reduction in the tumor growth rate constant to 0.11 $days^{-1}$.

The radioactivity of UCNP-R-T was calculated by using the tabulated decay rate of ^{90}Y and initial activity measured directly. In *in vivo* experiments, an initial dispersion of the tested formulation from the site of administration was also taken into account as 30% of the volume administered intratumorally.

To assess the systemic toxicity of UCNP-R-T, the dynamics of alteration of the animal body weight was studied. Intratumoral injection of UCNP-R-T at doses up to 15 μg/g demonstrated no significant reduction in the weight of animals compared to the control group (Figure S5). Thus, it can be assumed that UCNP-R-T does not exert pronounced general toxic effect on the experimental animals.

On Day 5, the administered formulation activity level fell below the therapeutically significant value (200 kBq), halting the tumor growth inhibition (Fig. 4). Since more than 80% of radioactive decay energy is released in the tumor node over 5 days, a further increase in the absorbed energy becomes small, and hence the absorbed dose calculated as a ratio of the integral of the released decay energy at the site to the current tumor mass value is diminished (Fig. S6).

DISCUSSION

UCNP-PMAO potential toxicity. A number of studies of the biodistribution of UCNP of different compositions and surface functionalizations have showed that they are capable of accumulating and retaining for long periods in the internal organs, predominantly, in the liver and spleen [2, 3]. The time of complete clearance of UCNP from the body varies from several days to months, depending on their size and surface characteristics [4]. Excretion of UCNP from the body typically occurs through the liver, bile duct and intestine; and through the kidneys in case of single-digit nanoparticles (diameter < 10 nm). Obviously, potential toxic effects depend on the retention of UCNP complexes in the critical and susceptible organs. A vast majority of reports consent on that UCNP complexes exert no cytotoxic effects and no toxic effects on the organs of

laboratory animals, or seldom, weak toxic effects [3, 5-7]. In addition the existing body of evidence, the authors have carried out a systematic study full-scale toxicity tests of PMAO-coated UCNPs (UCNP-PMAO) using murine laboratory animals. This study found no signs of acute and chronic toxicity, allergic and immunotoxic effects; no reprotoxic and mutagenic effects were found. The existing body of evidence suggest that UCNP-PMAO-s are safe to use as a universal platform for theranostics applications.

METHODS

Preparation of DARPin-PE40 protein and assembly of UCNP-R-T. For activation of carboxyl groups of PMAO, the UCNP-R/UCNP suspension was incubated in MES buffer (pH 6.0) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) cross-linkers (Sigma-Aldrich, USA) for 15 min, followed by the replacement of the buffer with PBS. A solution of immunotoxin DARPin-PE40 was added to the suspension, incubated for 2 h at 20°C and the resulting UCNP-R-T/UCNP-Ts were washed three times with PBS.

Characterization of UCNP-R-T. The conversion coefficient was determined using an integrating sphere setup [8]. The upconversion coefficient (η_{uc}) was calculated as a ratio of the emitted photoluminescence power to the absorbed power:

$$\eta_{uc}=P_{em}/P_{abs}, \quad \textbf{[1]}$$

To obtain transmission electron microscope (TEM) images, samples of UCNP-R and UCNP-R-T aqueous suspensions were sonicated and applied to copper grids with diameter of 3.05 mm coated with a polymer film, which were then dried at room temperature. Images were acquired with a TEM LEO-912 AB OMEGA (Carl Zeiss, Germany).

Specificity of UCNP-R-T binding to the cell surface. Human mammary adenocarcinoma (SK-BR-3) and Chinese hamster ovary (CHO) cells were cultured in McCoy's medium with 10% fetal bovine serum (HyClone, USA) and 2 mM L-glutamine (PanEco, Russia) at 37°C in an atmosphere with 5% CO_2. Cells were plated at a concentration of 2.5×10^5 cells/mL on coverslips treated with 0.01% polylysine solution (Sigma-Aldrich, USA), placed in 6-well plate (Corning, USA) and cultured for 24 h. A colloidal solution of UCNP-R-T and UCNP-R was added to the medium at a concentration of 1 μg/mL and incubated for 1 h at 4°C to prevent endocytosis. Cells were washed three times with PBS and fixed with 4% formaldehyde for 30 min at 24°C in the dark. After cell three times washing with PBS and once with deionized water, the coverslips were dried and placed in UltraCrus Mounting Medium (Santa Crus Biotechnology, USA).

The binding of UCNP-R-T and UCNP-R to the cell surface was investigated by a fluorescence laser scanning confocal microscopy using a LSM 710 system (Carl Zeiss, Germany). The excitation of UCNP photoluminescence was carried out at a wavelength of 980 nm, and the photoluminescence signal was recorded in the spectral range of 769-849 nm.

In vitro UCNP-R-T cytotoxicity assay. Cells at a concentration of $2x10^3$ cells/mL were placed in 96-well plate (Corning, USA) and cultured for 12 h. Medium in the wells was replaced with growth medium containing UCNP-R-T/UCNP-T/UCNP-R/UCNP, followed by 96-h incubation. The cytotoxicity was assessed by MTT test [9]. MTT reagent (Alfa Aesar, UK) was added to the growth medium to a final concentration of 0.5 mg/mL, followed by 4-h incubation. The medium was removed and MTT-formazan crystals were dissolved in 200 μL of DMSO (PanEco, Russia). The optical density of the well contents was measured using a Synergy MX (BioTek, USA) plate photometer at a wavelength of 570 nm. The cell viability was assessed using a ratio of the optical density of the formazan solution in each well to the optical density in the control.

Experiments using laboratory animals. Balb/c nude athymic mice (female, 17-19 g, age 5-7 weeks) were housed under standard vivarium conditions. SK-BR-3 cells were injected subcutaneously into the left thigh at a rate of 5 million per animal to obtain experimental xenograft tumour models. Following 16 days after tumour grafting, when the tumour nodule reached 400 mm^3 in volume, animals were divided into 4 groups of 5 individuals: groups 1 and 2 received UCNP-R-T, as a single dose of 10 μg/g and 15 μg/g, respectively, and groups 3 and 4 (control) received a single intratumoural injection of PBS.

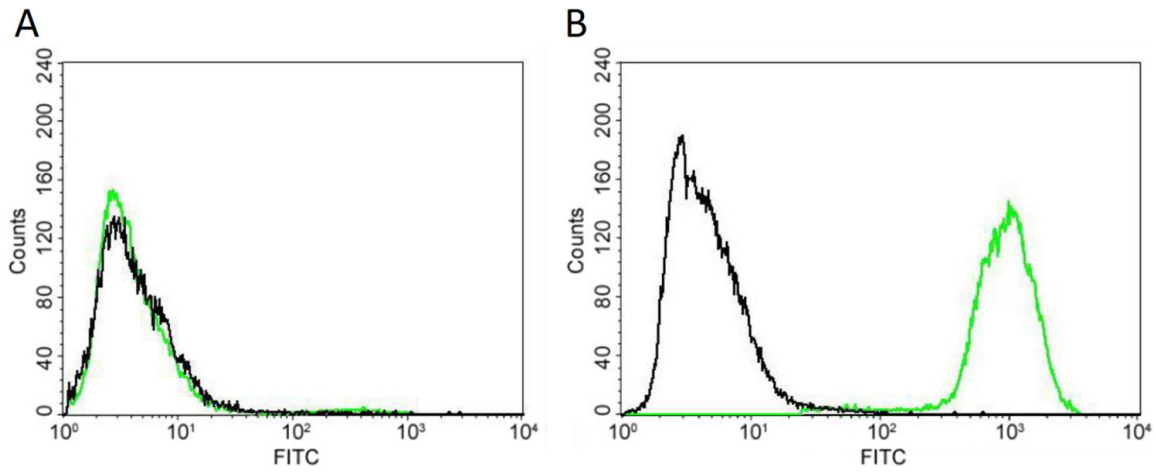

Fig. S1. The distribution of CHO (A) and SK-BR-3 (B) cells by the expression level of the HER2 receptor. Green line – cells incubated with HER2-specific Herceptin-FITC conjugates, black line – cells incubated with IgG-FITC conjugates (isotypic control).

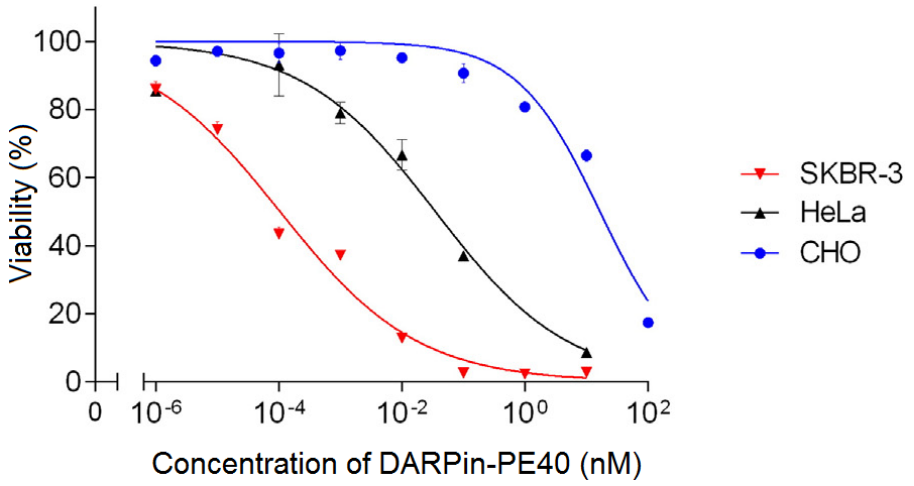

Fig. S2. In vitro analysis of the DARPin-PE40 cytotoxicity (MTT assay). Relative viability of the HER2-positive cells, SK-BR-3 (red line and inverted triangles), HeLa (black line and triangles) and HER2-negative cells, CHO (blue line and circles) after a 72-h treatment with DARPin-PE40 (adapted from E. Sokolova et al. / Journal of Controlled Release 233 (2016) 48–56)

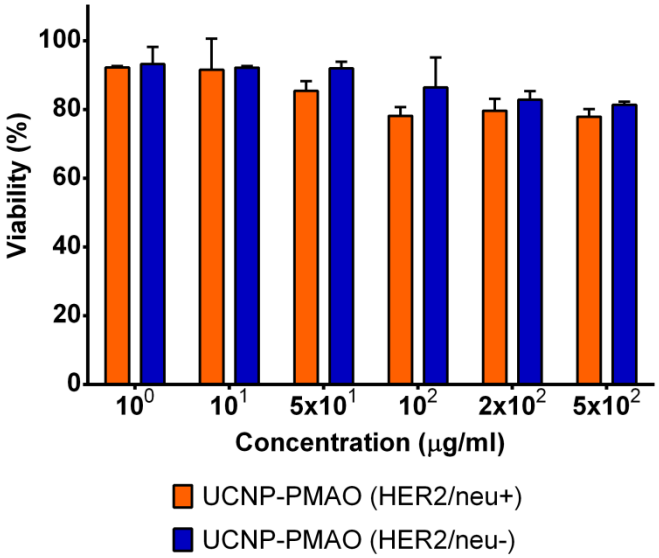

Fig. S3 A histogram of the viability of HER2+ and HER2- cells versus concentration of incubated UCNP-PMAO-DARPin complexes. HER2+ denote SK-BR-3 cells overexpressing HER2 receptors. HER2- denote CHO cells deprived of HER2 receptors.

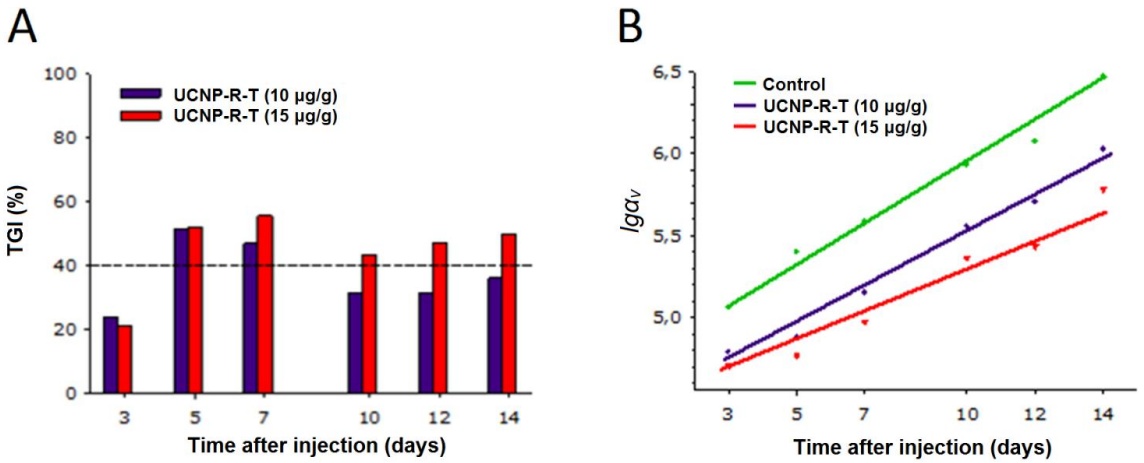

Fig. S4. The therapeutic effect of UCNP-R-T. A – Temporal changes in tumor growth inhibition factor in mice receiving UCNP-R-T at a single dose of 10 μg/g and 15 μg/g; B – Dynamics of growth of the experimental tumor in mice which received or did not receive a single injection of UCNP-R-T at doses of 10 μg/g and 15 μg/g. The ordinate axis is represented in semilogarithmic coordinates. α_v – the change in the initial tumor volume in percent.

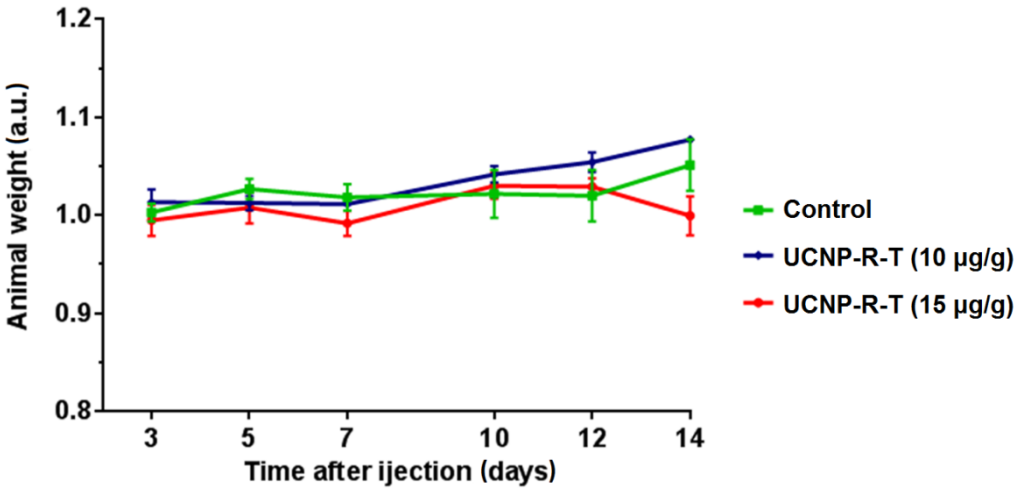

Fig. S5. The dynamics of animal weight in the control (control) and experimental groups (UCNP-R-T at doses 10 and 15 µg/g intratumorally). The weight of animals is indicated in relative units, with the weight on the day of injection taken as a unit. Error bars are represented by the SEM.

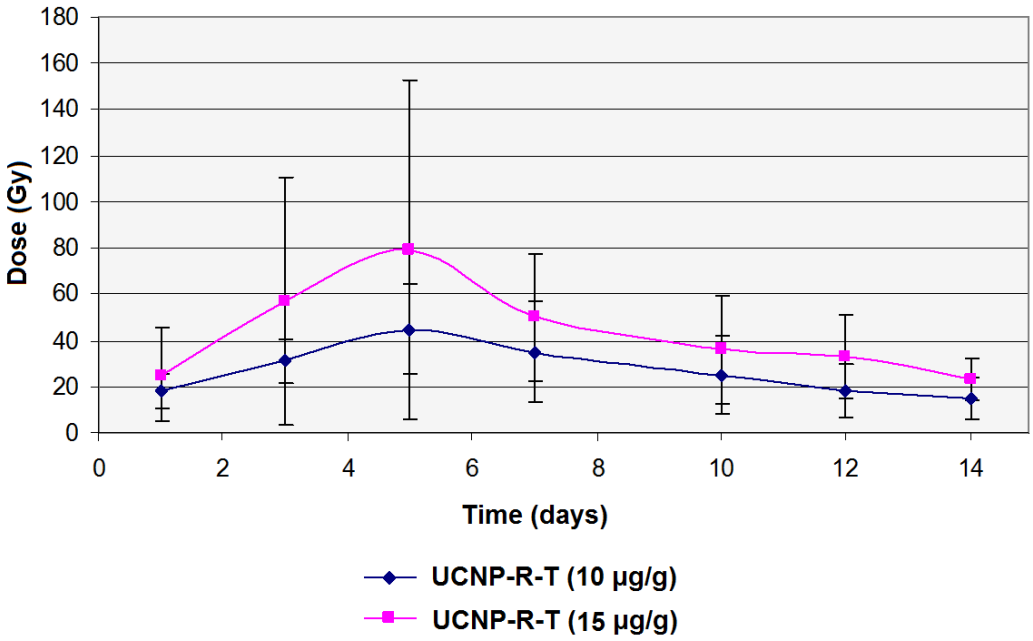

Fig. S6. The calculated dose burden on the tumor.

Table S1. Tumor growth rate constants

Group	k, days^{-1}	R	p
Control	0.13 ±0.01	0.95	0.0001
UCNP-R-T (10 mg/g)	0.11 ±0.01	0.95	0.0002
UCNP-R-T (10 mg/g)	0.09 ±0.01*	0.92	0.0006

* $p<0.05$, comparison to the control according to t-test with Bonferroni correction

References

1. Sokolova E, Proshkina G, Kutova O, Shilova O, Ryabova A, Schulga A, Stremovskiy O, Zdobnova T, Balalaeva I, Deyev S (2016) Recombinant targeted toxin based on HER2-specific DARPin possesses a strong selective cytotoxic effect in vitro and a potent antitumor activity in vivo. *J of Controlled Release* 233:48–56.
2. Cheng L, Yang K, Shao M, Lu X, Liu Z (2011) In vivo pharmacokinetics, long-term biodistribution and toxicology study of functionalized upconversion nanoparticles in mice. *Nanomedicine* 6(8):1327–1340.
3. Xiong L, Yang T, Yang Y, Xu C, Li F (2010) Long-term in vivo biodistribution imaging and toxicity of polyacrylic acid-coated upconversion nanophosphors. *Biomaterials* 31:7078e7085.
4. Sun Y, Feng W, Yang P, Huang C, Li F (2015) The biosafety of lanthanide upconversion nanomaterials. *Chem Soc Rev* 44:1509–1525.
5. Yu X F, Sun Z, Li M, Xiang Y, Wang Q Q, Tang F, Wu Y, Cao Z, Li W (2010) Neurotoxin-conjugated upconversion nanoprobes for direct visualization of tumors under near-infrared irradiation. *Biomaterials* 31(33):8724–8731.
6. Jalil R A, Zhang Y (2008) Biocompatibility of silica coated NaYF4 upconversion fluorescent nanocrystals. *Biomaterials* 29(30):4122–4128.
7. Kumar R, Nyk M, Ohulchanskyy T Y, Flask C A, Prasad P N (2009) Combined Optical and MR Bioimaging Using Rare Earth Ion Doped NaYF4 Nanocrystals. *Adv Funct Mater* 19:853–859.
8. Grebenik EA, et al. (2014) Specific Visualization of Tumour Cells Using Upconversion Nanophosphors. *Acta naturae* 6(4):48-53.
9. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth* 65(1-2):55-63.