Supplementary information

Use of folic acid nanosensors with excellent photostability for hybrid imaging

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Materials and methods

1 Materials

We purchased the following materials for use in the study: selenium (Se, 99.7%), tellurium (Te, 99.8%), oleylamine (OLA, 80%–90%), *N*-vinyl-2-pyrrolidone (VP, 99%), ethylene glycol dimethacrylate (EGDMA, 98%), *N*,*N*-dimethylformamide (DMF, 99%), dimethylsulfoxide (DMSO, 99%), folic acid (99%), tetramethylammonium hydroxide pentahydrate (TMAOH, 99%), and trimethylamine (TEA, 99%) from Acros Organics (Geel, Belgium); zinc acetate ($Zn(OAc)_2$, 99.99%), octadecene-1 (ODE, 90%), octadecylphosphonic acid (ODPA), octadecylamine (ODA, 99%), octanothiol (OT, 98.5%), zinc diethyl dithiocarbamate (ZDEC, 98%), zinc phthalocyanine (97%), trioctylphosphine (TOP, tech. grade, 90%), trioctylphosphine oxide (TPO, 99%), *N*-hydroxysuccinimide (NHS, 98%), magnesium phthalocyanine (90%), 4,4′-azobis(4-cyanovaleric acid) (ABCVA, 98%), 1,6-hexanedithiol (HT, 97%), and Superose 6 prep grade from Sigma-Aldrich (Burlington, USA); maleic anhydride (MAN, 99.0%), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 98%) from Fluka (Illinois, USA); sulfur (S, extra pure), oleic acid (OA, tech. grade 70%), and 1,3-dicyclohexylcarbodiimide (DCC, 98%) from Labtech (Moscow, Russia); cadmium oxide (CdO, 99.95%) from Alfa Aesar (Massachusetts, USA); tetrahydrofuran (THF, 99.9%) from Scharlau (Instanbul, Turkey); tris(hydroxymethyl) aminomethane (Tris, ultrapure) and polyvinylpyrrolidone K 30 (PVP) from MP Biomedicals (Solon, USA); agarose LE 2 from Helicon (Moscow, Russia).

2 Synthesis

The synthesis of nanocrystals was performed according to a method that we developed earlier (Dezhurov et al., 2017). Hydrophilization of QDs was conducted using 3-mercaptopropionic acid (MPA). The solution of QDs in toluene was centrifuged with 4× anhydrous ethanol (18000*g*, 15 min), and the precipitate was resuspended in chloroform. An aqueous solution of 100 mmol/L MPA (800 μL), the pH of which was brought up to 11.0 by adding tetramethylammonium hydroxide (TMAH), was added to the QD solution in chloroform and shaken vigorously for 4 h in the dark. The aqueous phase containing the QDs was separated, and the colorless organic phase was discarded. QDs were centrifuged (15000*g*, 15 min) to remove large aggregates, and the supernatant was dialyzed for 8 h repeatedly against 10 mmol/L phosphate buffer (pH 7.4) in a capped flask in the dark. We synthesized the copolymer of polyvinylpyrrolidone and acrylic acid (PTVP) according to the following procedure. Into a 250-mL three-neck flask, 15 mL (140 mmol) of VP, 5 g (51 mmol) of MAN in a mixture of 10 mL of acetic acid, and 15 mL of THF were placed. The MAN was dissolved by stirring and then 0.66 g (2.4 mmol) of ABCVA was added. The mixture was purged with argon and boiled for 15 min with stirring. Then 2 mL (10.6 mmol) of EGDMA and 0.3 g (1.2 mmol) of ABCVA dissolved in 20 mL of THF were

added via syringe. After 10 min of boiling, the reaction was terminated by adding HT (15 mL, 102 mmol) into 20 mL of THF. After that, heating stopped and the solution was cooled to room temperature. About three volumes of diethyl ether were added to precipitate PTVP. The polymer was separated by centrifugation at 10000*g* and dissolved in DMF. The dissolution/deposition cycle was repeated twice to obtain a white powder. Next, we synthesized folic acid-conjugated polyethylenimine-modified PEGylated nanographene (PPG-FA). In a flask 3.2 g (6.7 mmol) folic acid was dissolved in 25 mL DMSO. After dissolution, 1.54 g (2 eq.) NHS and 2.76 g (2 eq.) DCC were added. The reaction mixture was stirred continuously for 16 h, after which the precipitate was filtered off. Then 1.88 mL (2 eq.) of TEA and PPG-NH_2 (33.5 mmol, 5 eq.) in DMSO were added to the reaction mixture. Afterward, PPG-FA was precipitated with a 1:1 (volume ratio) mixture of THF and diethyl ether (Et_2O). The yellow precipitate was centrifuged and washed with a mixture of THF and Et_2O before being centrifuged again. In the same way, the precipitate was washed twice in Et_2O, after which it was collected and dried under vacuum. The weight of the dried PPG-FA precipitate was 3.43 g. Next, 1 g of dry PPG-FA precipitate was dissolved in the solution of 5 mL DMFA+10 mL H_2O and mixed with 3 g of PTVP dissolved in 25 mL DMFA. Then PTVP-PPG-FA was synthesized. Dry PPG-FA precipitate (1 g) was dissolved in the solution of 5 mL DMFA+10 mL H_2O and mixed with 3 g of PTVP dissolved in 25 mL of DMFA. Then we stirred the mixture for 1 h at 30 °C, after which we diluted it with 40 mL water and removed the aggregates by centrifugation at 10 000*g* for 5 min. The polymer from the supernatant was precipitated with transforming growth factor (TGF) and separated by 5-min centrifugation at 10 000*g*. The residue was dried in a vacuum at room temperature. The copolymer was estimated by means of Fourier transform infrared (FTIR) spectra, using a Shimadzu-1800S spectrometer (Shimadzu, Kyoto, Japan) on a CaF_2 plate in the 800–4000 cm^{-1} range. Elemental C/H/N/S analysis was performed with a Carlo Erba CE1106 elemental analyzer (Carlo Erba, Val de Reuil, France). We obtained the values for C, H, N, and S are 52.17%, 6.61%, 7.56%, and 1.57%, respectively. Standard deviation (SD) is ±0.5% error in absolute units. Synthesis of the conjugate was performed by adding 0.75 g of PTVP-PPG-FA to 10 mL of water with a dispersion of 0.5 g QD-MPA in 7 mL of water. After stirring, the mixture was centrifuged, and aggregated particles were separated. The supernatant was treated with TGF until the dispersion became turbid. The precipitate was separated and dispersed in 10 mL of water. Purification was performed using Superose 6.

Blocking of –COOH groups was performed by activation of 5 mL of QDs (about 20 nmol) in the presence of 500 eq. NHS and 1000 eq. EDC. Then 10 000 eq. of diethanolamine was added. It was then incubated for 1 h under stirring. The purification was performed with Superose 6.

3 Transmission electron microscopy (TEM)

QD dispersions were deposited on formvar/carbon-coated copper mesh (300 mesh, TedPella, Inc., Redding, USA). Microphotographs were obtained over a wide magnification range (50–1500 thousand times) using a JEOL JEM 2100 microscope operating at 200 keV (1 ev=1.602×10^{-19} J), equipped with a LaB6 filament and an Olympus Quemesa 11-megapixel CDD camera (Olympus, Tokyo, Japan). The obtained high-resolution TEM images were processed using ImageJ software (Schindelin et al., 2012; Rueden et al., 2017).

4 Determination of QD quantum yield

Determination of the quantum yield of the obtained particles was carried out following the recommendations of the manufacturer of the HORIBA FluoroLog spectrofluorimeter (HORIBA, Kyoto, Japan). Zinc phthalocyanine in a 1% (0.01 g/mL) solution of pyridine in toluene (λ=680 nm, QDs 30%) was used as a fluorescence standard.

5 Measurement of QD photodegradation kinetics

To remove excess ligands, the dispersions of QDs and their conjugates were precipitated twice before the experiment. Quartz 1-cm cuvettes (2.5 mL) with 0.1 μmol/L nanoparticle dispersions were irradiated with a laser (λ=440–450 nm, 6 W, 5 cm source distance). Fluorescence spectra were recorded at certain time intervals

(0, 2, 5, 10, 30, 60, 90, 120, and 180 min). The corresponding values of intensity at fluorescence peak maximum were plotted against the total irradiation time of the samples.

6 Flow cytometry analysis

We performed flow cytofluorometry on cell lines MCF-7 (ATCC HTB-22), B16-F10 (ATCC CRL-6475), HEK-293 (ATCC CRL-1573), and CHO using a FACScan (Becton Dickinson, New Jersey, USA).

7 In vitro cytotoxicity assays

The toxicity of QDs was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test. We applied the maximum possible molarity range, as determined by the initial molarity of the QD preparation. In the experiment, the CHO and HT29 cells were cultured in an incubator at 37 °C and in an atmosphere of 5% CO_2 on Dulbecco's modified Eagle's medium (DMEM) with 0.294 mg/mL L-glutamine supplemented with 10% (mass fraction) bovine fetal serum, 0.1 mg/mL streptomycin, and 100 U/mL penicillin. We counted the cells using the Goryaev chamber (Minimed LLC, Moscow, Russia).

A quantity of 1×10^4 cells were seeded on 96-well culture plates. After 24 h, the cells were added to the test substances at concentrations of 0.0001, 0.001, 0.01, and 0.1 μmol/L. Further incubation was performed for 48 h under standard conditions, after which the medium was withdrawn and fresh medium was added with 0.5 mg/mL MTT. After 2 h of incubation under standard conditions, cell lysis was conducted using DMSO. The resulting staining was quantified on a spectrophotometer at 600 nm. Half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 5.02 software (GraphPad Software Inc., San Diego, USA).

8 In vivo optical imaging

In vivo optical imaging was conducted using a Bruker In-Vivo FX PRO (Bruker, Coventry, UK). The research was carried out on nude and C57BL/6 mice. We created melanoma tumours by subcutaneous inoculation of B16F10 melanoma cells (ATCC CRL-6475) into female C57BL/6 mice at a concentration of 2×10^6 cells; the cells were obtained by culturing in 10% fetal bovine serum medium, 100 U/mL penicillin, and 100 μg/mL streptomycin.

9 Ex vivo optical imaging

Model preparation: isolated, chilled pork stomachs with the mucosa were turned inside out. The mucosa was washed with purified water. The areas to be examined were then treated with 6% (mass fraction) acetic acid to remove mucus, after which the mucosa was washed again with water. The stomachs were placed in an opaque container for optical radiation of the material.

We injected the samples into 1-mL syringes and applied them on the surface of the gastric mucosa (or injected them submucosally) in an amount of 10–20 μL. The dispersion concentration was 0.6 and 3.0 μmol/L. Then we inserted the end of the endoscope into each prepared container with a stomach. Photo and video recordings were made in white- and blue-light 440-nm illumination modes, which initiated autofluorescence. We used a K. Storz video camera control unit (TELECAM SL II, illuminator unit D-light C) and a 300-W xenon lamp with white and blue illumination. The ambient temperature was (−28±2) °C.

10 Statistical analysis

All experiments were carried out in triplicate unless otherwise indicated. The results are expressed as the means of three independent experiments. All data were expressed as the mean±standard error of the mean (SEM). Statistical analysis was performed using a two-tailed Student's *t*-test with GraphPad Prism 5.02 (GraphPad Software Inc, USA).

References

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