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Biodistribution and stability of CdSe core quantum dots in mouse digestive tract following *per os* administration: Advantages of double polymer/silica coated nanocrystals

Y.F. Loginova [a], S.V. Dezhurov [b], V.V. Zherdeva [a], N.I. Kazachkina [a], M.S. Wakstein [b], A.P. Savitsky [a,*]

[a] *Laboratory of Physical Biochemistry, A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow 119071, Russian Federation*
[b] *Trial Center For Science and Technology "Nanotech-Dubna", Dubna, Moscow Region 141983, Russian Federation*

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ABSTRACT

CdSe-core, ZnS-capped semiconductor quantum dots (QDs) are of great potential for biomedical applications. However, applications in the gastrointestinal tract for *in vivo* imaging and therapeutic purposes are hampered by their sensitivity to acidic environments and potential toxicity. Here we report the use of coatings with a combination of polythiol ligands and silica shell (QDs PolyT–APS) to stabilize QDs fluorescence under acidic conditions. We demonstrated the stability of water-soluble QDs PolyT–APS both *in vitro*, in strong acidic solutions, and *in vivo*. The biodistribution, stability and photoluminescence properties of QDs in the gastrointestinal tract of mice after *per os* administration were assessed. We demonstrated that QDs coated with current traditional materials – mercapto compounds (QDs MPA) and pendant thiol group (QDs PolyT) – are not capable of protecting QDs from chemically induced degradation and surface modification. Polythiol ligands and silica shell quantum dots (QDs PolyT–APS) are suitable for biological and biomedical applications in the gastrointestinal tract.

1. Introduction

Quantum dots (QDs) are of great interest as fluorescent biomarkers for various biomedical applications. CdSe-core, ZnS-capped QDs are 1–10-nm semiconductor nanocrystals with unique optical and photophysical properties such as size- and composition-tunable emission, high brightness, large absorption cross sections, narrow emission bands, and high resistance to photobleaching [1–3]. Polymer-coated quantum dots could be used for many purposes *in vivo*, for example, as long-circulating vascular markers [4], for mapping the reticuloendothelial system [5], for lymph node mapping [6], for detection of cancer cells [7], for cell imaging and *in vivo* animal imaging [8]. Bioconjugated QDs are also being explored as targeted diagnostic cancer-imaging agents [9] and drug-delivery systems [10].

For biomedical applications QDs surfaces should be modified so that they became water-soluble, biocompatible, and maintain the optical properties of the organic-soluble QDs [11]. QDs could be functionalized using a variety of surface coating materials and synthesis strategies, such as silanization [12], encapsulation with block-copolymer micelles [13] or the ligand exchange method in which the surface ligands are exchanging with a thiol compounds such a mercapto-acetic acid [14]. The most common coatings include amphiphilic polymers [7], amphiphilic polymers conjugated with poly(ethylene glycol) [15], cysteines [16] and dihydrolipoic acid [17].

Generally, the coated QDs are stable under neutral or basic conditions. Recent study has shown that traditional surface coatings were ineffective in protecting QDs under strongly acidic conditions [18]. This is a major problem for QDs applications as agents for imaging or targeted delivering throughout the gastrointestinal tract (GI) because QDs pass firstly into the stomach with strongly acidic fluid (pH 1–2). Many of available QDs cannot be applied *per os*, because of their degradation in the GI [19]. The stability of nanoparticles is expected to affect potential toxicity of QDs core materials. After the organic ligands were detached, the nanocrystals became extremely toxic [20]. The development of acid-stable surface coatings is a main goal towards obtaining of nanoparticles that can effectively cross the gastrointestinal tract for *per os* administration.

The new approach for preparation of stable QDs is to combine the silica and amphiphilic polymer encapsulation techniques [21]. We reported here newly synthesized water-soluble CdSe/CdS/ZnS QDs with a previously undescribed combination of polythiol ligands and silica shell (QDs PolyT–APS). We have introduced an additional coating on the surface of QDs using silica precursor 3-aminopropyltriethoxysilane (APS) as an amino derivative. In the present study we demonstrated stability of QDs PolyT–APS,

* Corresponding author. Fax: +7 4959528725.
E-mail address: apsavitsky@inbi.ras.ru (A.P. Savitsky).

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in strong acidic solutions both *in vitro* and *in vivo* in the digestive tract of mice after *per os* administration.

We compared QDs PolyT–APS with the surface capping method by mercapto compounds (QDs MPA) and pendant thiol group (QDs PolyT) *in vitro* and *in vivo*. The stability of QDs in previous studies was investigated only *in vitro* by simulation of gastric and intestinal fluids [22,23].

Biodistribution and accumulation of QDs in the tissues of the digestive system organs as well as in liver, pancreas and kidney, were monitored *in vivo* by fluorescence whole-body imaging and *ex vivo* by the local fluorescence spectroscopy of tissue samples.

2. Materials and methods

2.1. Synthesis and characterization of quantum dots

Tetramethylammonium hydroxide pentahydrate 97%, *N*,*N*′-diisopropylcarbodiimide (DIC) 99%, oxazine 170 perchlorate 95%, DL-α-lipoic acid (LA) 98%, tetrahydrofuran (THF) 99%, 0.45 μm PTFE membrane filters were purchased from Sigma–Aldrich Corporation. *N*-Hydroxysuccinimide (NHS) 97% were purchased from Fluka, 3-aminopropyltriethoxysilane (APS) 97% (Sigma,) methanol 99%, poly(acrylic acid) (PAA) Mw ~ 2 kDa 63 wt.% solution in water, rhodamine 6G 99% were purchased from Acros Organics. Chloroform, hexane, toluene, NaOH, KOH, Na_2HPO_4, phosphoric acid were chemically pure. PES syringe filters 0.22 μm were purchased from GE Healthcare.

CdSe/CdS/CdZnS/ZnS core/shell/shell nanocrystals were synthesized in accordance with an earlier developed protocol [24], using 3–4 monolayers of CdS, 3–4 monolayers of CdZnS and 4–5 monolayers of ZnS on the demanded emission wavelength.

2.1.1. Surface modification with 3-mercaptopropionic acid (MPA)

Modification by MPA was performed as described elsewhere [25].

2.1.2. Synthesis of PolyT (a poly(acrylate) backbone with pendant thiol groups and poly(ethylene glycol) chains)

DL-α-Lipoic acid (*O*,*O*′-bis aminopropylpolyethylenglycol) monoamide (LA-PEG-NH2) was synthesized according to previously reported procedures [26]. Modification of PAA with LA-PEG-NH2 was carried out using DIC and NHS according to previously reported procedures, with some modifications [27]. LA-PEG-NH2 was used in a ratio to PAA 1:4, respectively. Reduction of disulfide bonds of polymer was performed as described in [28]. Filtration through 0.45 μm PTFE membrane filter of obtained water–methanol solution of PolyT was performed as additional purification step.

2.1.3. Surface modification with PolyT

PolyT (0.10 g) was suspended in 5.0 ml of water and dissolved by adjusting pH to 9.0 with NaOH. Dispersion of 0.10 g of MPA capped QDs was added to solution of polymer dropwise. After 1 h of stirring, a small amount of aggregates was removed from colloid solution by centrifugation at 19,000*g* for 5 min. Then colloid solution was filtered through the 0.22 μm PES membrane filter.

2.1.4. Surface modification of PolyT with APS (combination of polythiol ligands and silica shell)

Dispersion of 0.10 g of QDs in 6.0 ml of $CHCl_3$ was added drop wise to a solution of 0.10 g of PolyT in 5 ml THF, 5 ml of methanol and 14 ml of $CHCl_3$. Mixture was stirred 1 h and evaporated under reduced pressure to volume 5 ml. Then dispersion was diluted with 10 ml of ethanol and same volume of THF. After that 0.30 ml of APS was added and suspension was stirred 1 h at room temperature. Water (0.10 ml) and 10 μl of 2 M KOH were added to mixture. After 3 days of stirring 5.0 ml of water was added to reaction. A resulting suspension was washed by hexan:diethyl ether (1:1) thrice. A residual organic solvent was distilled of under reduced pressure. A small amount of aggregates was removed from colloid solution by centrifugation at 19,000*g* for 1 min and filtration through the 0.45 μm PTFE membrane filter. The fluorescence quantum yield of the QDs was determined as described earlier [29] using oxazine 170 as a standard.

2.1.5. QDs photoluminescence and stability of its suspension in 0.1 M HCl

Fifty microliters of aqueous 1 μM QDs dispersions were diluted by 3.0 ml of 0.1 M HCl and kept for 20 min at ambient temperature. Alternatively, the same procedures were followed, except that the HCl was replaced by 0.025 M phosphate buffer. All samples were characterized by fluorimetry and dynamic light scattering. Fluorescence measurements were carried out using spectrofluorometer Cary Eclipse (Varian). Absorption spectra were recorded using spectrophotometer Cary 100 (Varian). Hydrodynamic diameter of particles was measured by dynamic light scattering (DLS) using Nanotrac Ultra (Microtrac). Zeta potential of nanoparticles suspensions were measured by Zetasizer Nano Z (Malvern).

2.2. Animals

Female Nu/Nu and CD-1 mice were purchased from the breeder "Pushino" of M. Shemyakin and Yu. Ovchinnikov Institute of Bioorganic Chemistry of Russian Academy of Sciences (Russia). Mice are given food and water ad libitum and housed in a 12 h/12 h light/dark cycle. Solution of QDs (290 pmol QDs MPA, 200 pmol QDs PolyT and 200 pmol QDs PolyT–APS per animal) was administered in a volume of 0.1 ml into the stomach of mice ($n = 3$ for each time point, and each QDs type). Tissue specimens from the digestive system organs, liver, kidneys and spleen in 2 h, 4 h, 6 h and 24 h after injection of QDs were taken for analysis by the local fluorescence spectroscopy. QDs elimination by urine or feces was studied. Mice ($n = 3$) were kept in metabolic cages (Techniplast, Italy) for 48 h, urine and feces probes were collected daily and analyzed by the method of local fluorescence spectroscopy. All experimental procedures were approved by the local ethical committee for animal experiments. Animals were treated humanely with regard for alleviation of suffering.

2.3. Fluorescence imaging and local fluorescence spectroscopy

Whole body images were acquired by the imaging system iBox (UVP, USA) equipped a 16-bit monochrome cooled CCD camera (BioChemi HR Camera, UVP, USA) and a 150-W halogen quartz lamp (BioLite, UVP, USA). The *in vivo* fluorescence imaging was obtained using band-pass excitation (502–547 nm) and emission filters (605–680 nm for QDs MPA and QDs PolyT, and 665–720 nm for QDs PolyT–APS). Images were obtained at various time points after injection (over a 24 h period); image acquisition time was 25 s.

For *ex vivo* experiments organs were harvested and subjected to analysis by the method of local fluorescence spectroscopy using the fiber-optical multispectral fluorescence analysis system Spectr-Cluster (Cluster Ltd., Russia). The fluorescence was excited by irradiating with a DPPS laser (532 nm, 5 mW, Compact-export Co. Ltd., Russia). The probe was in contact with the tissue sample surface during the fluorescence emission measurements. Identification of the QDs was carried out by the presence of characteristic peaks in fluorescence spectra of tissue samples *ex vivo*.

3. Results and discussion

3.1. Characterization of QDs and chemical stability study

Recent research has shown that the surface coating chemistry has a dramatic effect on the chemical stability and biocompatibility of QDs [30]. In this research, three types of water-soluble QDs modified by various functional ligands were prepared (see Fig. S1 in the Supplemental material). In the case of QDs MPA (λem 630 nm, $d \sim 8$–11 nm), the original hydrophobic TOPO (trioctylphosphine oxide) surfactant was replaced by the 3-mercaptopropionic acid ligands (MPA) to achieve good solubility in aqueous solution and to provide specific functionality (Suppl. Fig. 1A). QDs PolyT (λem 626 nm, $d \sim 15$–16 nm) have a poly(acrylate) backbone with pendant thiol groups and poly(ethylene glycol) chains (Suppl. Fig. 1B). QDs coated with polydentate ligands are the most promising in terms of stability and functionality. The presence of several coordination centers can improve the stability of particles suspension and increases their biocompatibility [28].

Stability of fluorescence signal *in vivo* can significantly decrease under the influence of the medium of a living organism, in particular in the acidic medium of the stomach, endosomes, etc. It was shown that a combination of a silica shell with polymer coating yielded stable particles. This combination can protect QDs fluorescence signal even when treated with acidic solutions pH 1.0 [21]. The mechanism of protection by the silica–amphiphilic polymer coating is not entirely understood at this time and deserves further systematic studies. We have introduced an additional coating on the surface of QDs PolyT using silica precursor 3-aminopropyltriethoxysilane (APS) as an amino derivative. Since the carboxyl groups PolyT-coatings have a negative charge, we hypothesized that positively charged amino groups of APS are mainly located in close proximity to the polymer in solution due to electrostatic interactions. As a result, QDs PolyT were functionalized to obtain water-soluble QDs with a combination silica–amphiphilic polymer coating (PolyT–APS) similar to described in [31]. The average size of the QDs PolyT–APS with emission maximum at about 680 nm was estimated to be ~33–36 nm (Suppl. Fig. 1C).

We have demonstrated the change of zeta potential on the surface of QDs from negative (–COO^- groups) for PolyT to positive (–$NH3^+$ groups) for PolyT–APS (Fig. 1). The obtained data indicate that the use of PolyT–APS-coating causes significant increase in zeta potential, especially in range of pH 2–8, where the corresponding values of zeta potential for other types of QDs are lower (PolyT), or reflect the degradation of QDs due to the ligand dissociation from the surface (MPA), which leads to the complete disappearance of fluorescence and rapid aggregation of particles. Importantly, the isoelectric point of QDs PolyT–APS is above pH 9.0, which determines their use *in vivo*, because pH values found in human body fall in the range between pH 1 and 8.5 [32].

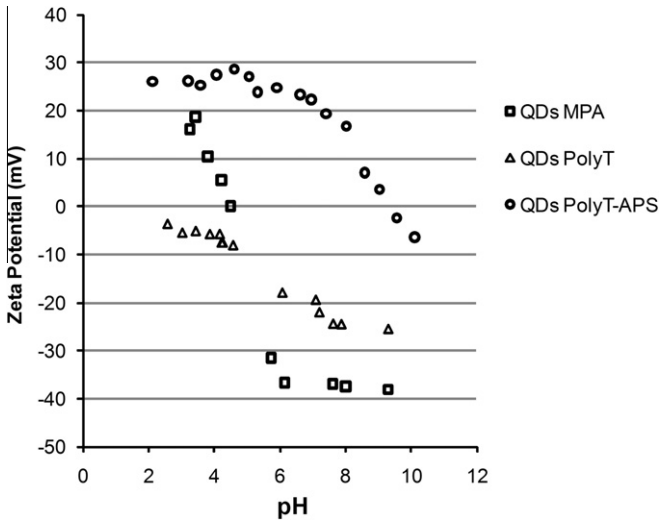

Fig. 1. Zeta potential of QDs modified by different functional ligands at various pH values. The measure of zeta potential was performed using a Zetasizer Nano Z instrument (Malvern). pH values of QDs solutions were tuned with 0.01 M HCl (pH 2–10). The concentrations of QDs were 0.1 μM.

Hydrodynamic diameter (HD) and dispersity of QDs are important properties for their application for *in vivo* fluorescence imaging [33]. HD analyses of all QDs at pH 1.0 and 7.0 were performed by dynamic light scattering (DLS). HD increase was observed for the QDs PolyT–APS while HD dispersion increase was observed in the case of QDs PolyT (Fig. 2).

For the study of external factors influenced on the fluorescent properties of QDs we have compared the relative fluorescence intensity of QDs solutions before (pH 7.0) and after their exposure to an acidic medium (pH 1.0). The fluorescence intensity of QDs PolyT–APS increased at pH 1.0 for 20%, whereas the fluorescence intensity of QDs PolyT decreased more than 50%. Consequently, silica shell on the surface of QDs PolyT–APS prevents fluorescence quenching of QDs.

3.2. The stability of QDs in the digestive tract of mice

The effect of various functional ligand modification on the biodistribution, stability and photoluminescence properties of QDs in the digestive tract of Nu/Nu or CD-1 mice after *per os* administration was assessed using noninvasive fluorescence imaging system iBox (UVP, USA). Images were obtained for experimental and control animal groups at various time points after injection (up to 24 h). The distribution pattern of all types of QDs in experimental animal group was similar. A detectable fluorescent signal of QDs was found only in organs of the digestive system. Immediately after administration fluorescence of all QDs was easily seen in the stomach (Fig. 3). Signals of all QDs were registered in the duodenum 20 min after administration. By 2 h after administration, fluorescence of QDs having MPA coating decreased significantly, whereas QDs PolyT and QDs PolyT–APS remained visible in the small intestine for at least 4 h. Background fluorescence from intestinal contents was very low; the other vital organs (liver, pancreas and kidney) have no visible autofluorescence. Images obtained 24 h after administration did not differ significantly from control images.

Compared with the traditional surface capping method, the new class of stable QDs demonstrates high resistance to harsh chemical treatment including strong acidic conditions of the stomach. We observed the strongest fluorescence signal of QDs PolyT–APS for a long period after administration (up to 4 h) using fluorescence imaging. This finding is promising for using polymer/silica coated QDs for the study processes in the GI *in vivo*.

Well detectable fluorescence maxima of all QDs at the corresponding wavelengths were revealed in the spectra of some parts of mice digestive system after *per os* administration (data not shown). The distribution and accumulation of QDs MPA, QDs PolyT and QDs PolyT–APS in the digestive system of mice after *per os* administration were studied also by analysis of the fluorescence of organs *ex vivo* using a laser spectrometer Spectr-Cluster with excitation wavelength 532 nm. Fluorescence spectra of specimens were measured in 2 h, 4 h, 6 h and 24 h after administration of QDs. All obtained spectra were exactly corresponded to that obtained for QDs in buffer. No traces of any QDs were detected in liver, pancreas and spleen. The presence of QDs MPA was detected in the stomach in 2 h after *per os* administration. QDs MPA were no longer present after 4 h neither in the stomach nor in any parts

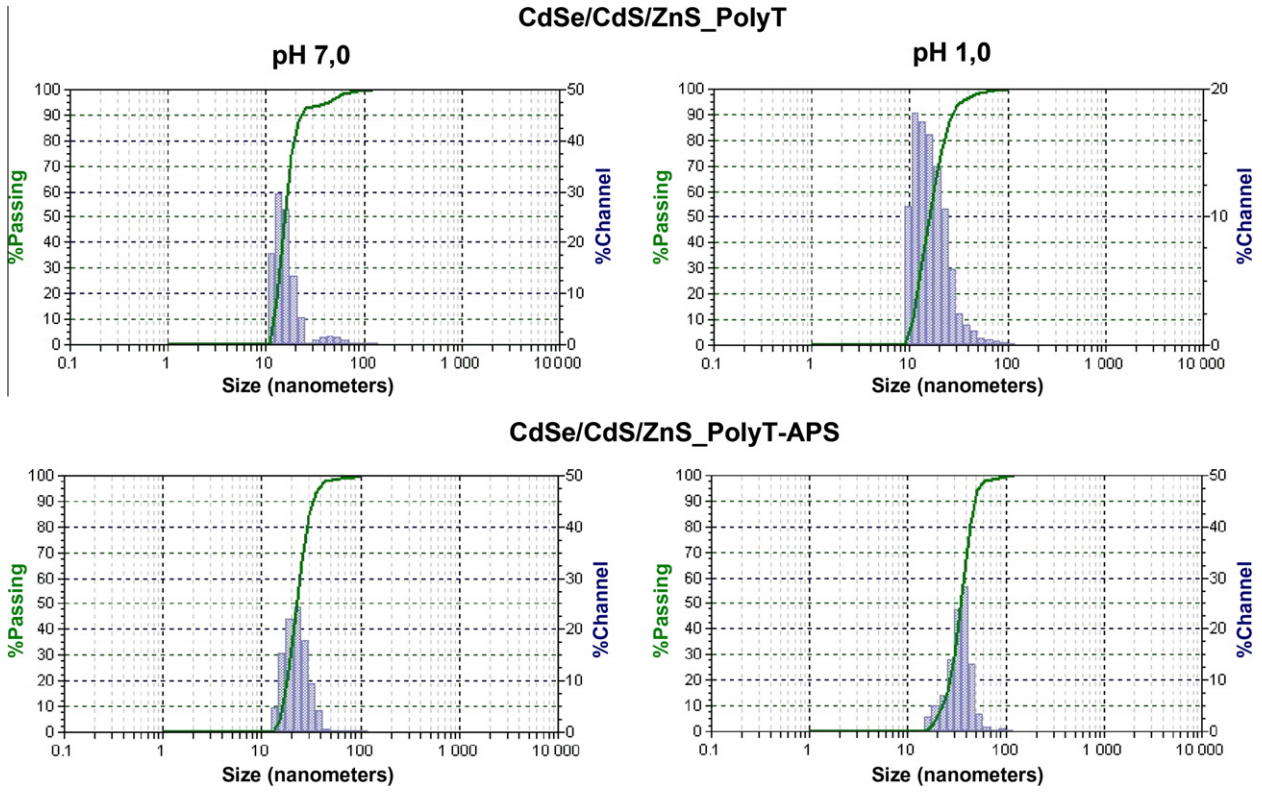

Fig. 2. Hydrodynamic diameter (HD) distribution of QDs PolyT (up) and QDs PolyT–APS (down) at pH 7.0 in deionized water and pH 1.0 in 0.1 M HCl, measured by DLS.

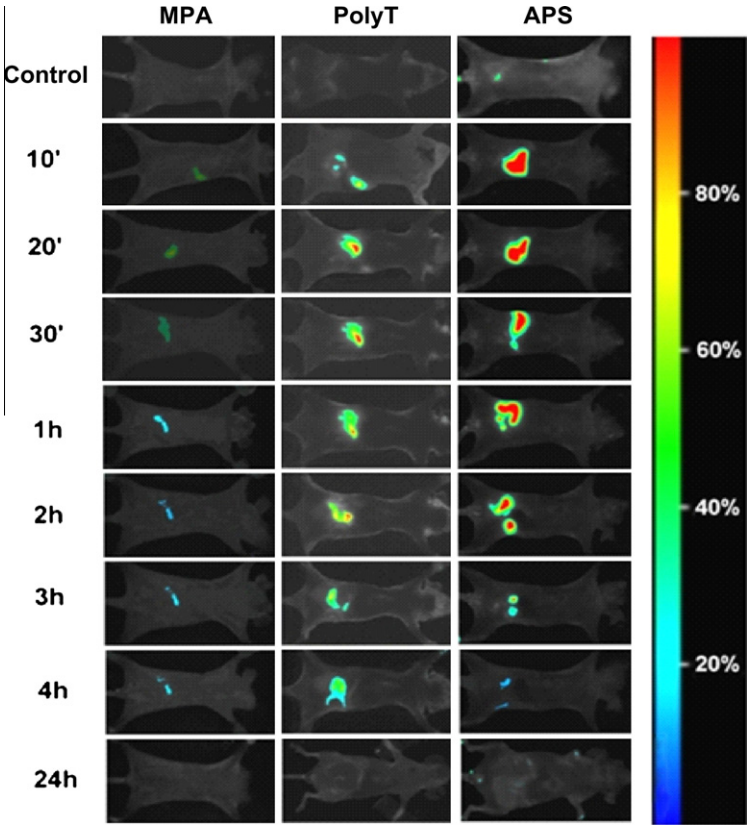

Fig. 3. Pseudocolor fluorescent images of mice at various time points after *per os* administration of QDs MPA, QDs PolyT and QDs PolyT–APS (red areas correspond to the maximal signal intensity on pseudoscale). The optical fluorescence *in vivo* imaging was obtained on iBox system (UVP, USA) using correspondent band-pass excitation (502–547 nm) and emission filters (570–640 nm or 670–720 nm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of intestine. A fluorescence maximum of QDs PolyT was observed in the spectra of the stomach wall and intestine measured over a 4 h period. QDs PolyT–APS were more stable than other types of QDs and the presence of QDs was clearly seen in spectra of stomach and intestine measured in 6 h after administration. For control measurements tissue specimens were taken from the same kind of organs of mice which were kept in the same conditions as experimental mice but not administered QDs.

The gradual decrease of fluorescence signals of QDs in tissues and organs of experimental animals over time could be first of all due to the clearance of the particles from the body or secondly due to the loss of their fluorescence signal caused by degradation in the digestive system. QDs could have a tendency to the aggregation and irreversible change in their chemical coating caused the action of gastric juice and enzymes. The proteolytic activity is highest in the stomach and duodenum. Probably, fluorescence of QDs is quenched in acidic solutions. The second hypothesis is in agreement with previously presented on *in vitro* degradation of QDs at acid pH. Suspensions of CdSe/ZnS quantum dots functionalized with a 3-mercapto-1-propanol and 3-mercaptopropionic acid via the thiol moiety were destabilized below pH 4 [34]. Apparently, the ligands are removed from the surface due to protonation of the thiol moiety in solution at a relatively low pH range, between 2 and 7 depending on the size. This will destroy the nanocrystal–ligand complexes.

The durability of QDs in the digestive system was assessed for the first time in an assay *in vitro* where exposure of CdSe/ZnS QDs functionalized by PEG_{350}–OCH_3 and PEG_{5000}–OCH_3 showed degradation in simulated gastric and intestinal fluids, loss of PEG ligand and subsequent aggregation/agglomeration of the particles. Moreover, erosion of the ZnS shell resulted in the release of Cd^{2+} [22].

At the oral administration hydrophobic CdSe/ZnS QDs were degraded in the digestive system of animals [35]. The authors observed a significant short-wavelength shift of the QDs band in photoluminescence spectra of the esophagus and stomach, and hypothesized that QDs undergo chemical and enzymatic degradation in the organs of the digestive system. Moreover, hydrophobic CdSe/ZnS QDs did not eliminate from the organism neither with urine nor feces. For hydrophilic QDs we demonstrated the appearance of detectable amounts of all QDs in the daily feces probes. No traces of any QDs were detected in urine probes.

The fluorescence of both QDs MPA (Fig. 4A) and QDs PolyT (Fig. 4B) measured in feces probes was very low, that strongly complicated the identification, and was detected only in rare spectra. In contrast, the well detectable fluorescent spectra of eliminated with feces QDs PolyT–APS possessed highest intensity similar to that of the original solution (Fig. 4C). It can be expected that additional silica layer protects QDs from aggregation and irreversible degradation in the highly acidic medium.

Thus, the QDs PolyT–APS was clearly seen in the spectra of intestine measured until 6 h following *per os* administration, and then the clearance of these QDs was started.

In summary, the new strategy for preparation of QDs stable in the digestive tract is to combine the silica and amphiphilic polymer encapsulation techniques. We demonstrated that our newly synthesized QDs PolyT–APS with a double polymer/silica coating exhibited a maximal stability both *in vitro* after their exposure to a strong acidic solution (pH 1.0) and *in vivo* in the digestive tract of mice after *per os* administration. The presence of silica shell on the surface of QDs PolyT–APS prevents their degradation and fluorescence quenching. We compared the chemical stability of our QDs PolyT–APS with QDs coated with current traditional materials. Nanoparticle coating technologies based on 3-mercaptopropionic acid or pendant thiol groups have tend to irreversible degradation under the strong acidic conditions.

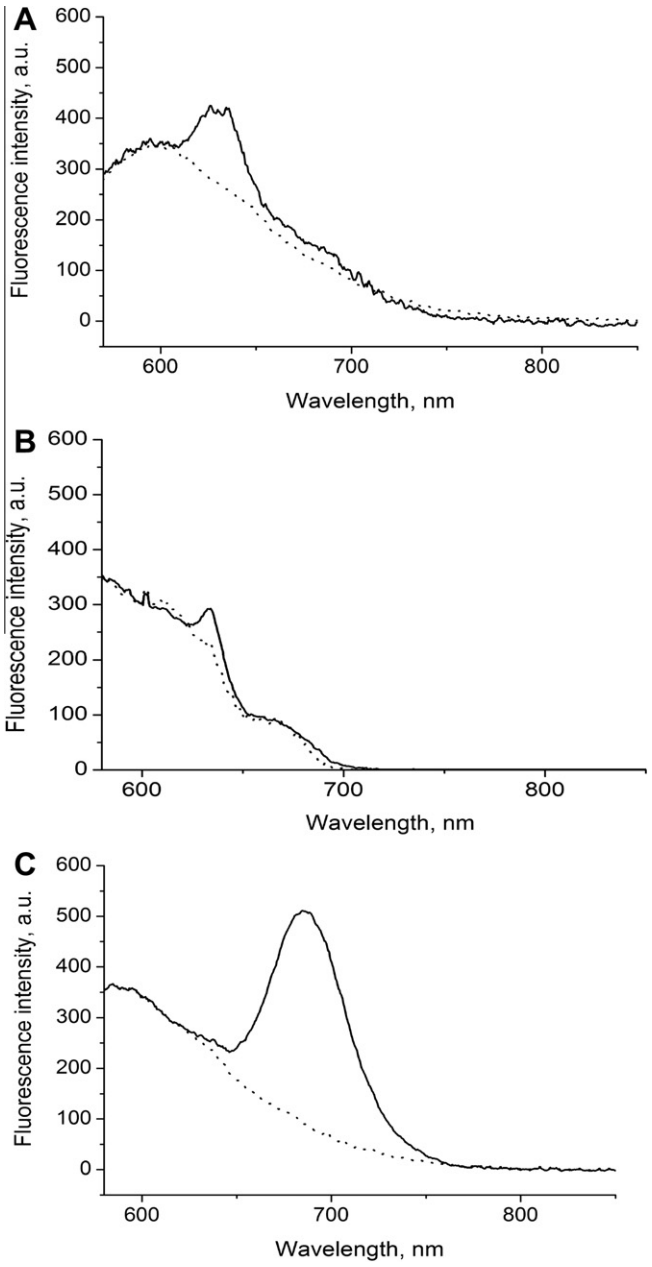

Fig. 4. Fluorescence spectra of feces probes 24 h after *per os* administration of (A) QDs MPA, (B) QDs PolyT and (C) QDs PolyT–APS: dashed curve – feces control, solid curve – feces after administration of QDs. Mice were kept in metabolic cages; feces probes were collected daily and analyzed by the method of local fluorescence spectroscopy. Excitation wavelength was 532 nm.

These coating technologies are not capable of protecting QDs from chemical-induced surface modification. Obtained results demonstrate the possibility of using of QDs PolyT–APS for the study *in vivo* processes in digestive system due to their high resistance to harsh chemical treatment including strong acidic conditions of the stomach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.123.

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