NANOTECHNOLOGIES

Interactions of Quantum Dots with Donor Blood Erythrocytes *In Vitro*

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The effects of quantum dots CdSe/ZnS-mercaptopropionic acid, (CdSe/CdZnS)ZnS-polyT, and CdSeCdSZnS/polyT/SiO_2-NH_2 on human erythrocytes were studied. The nanomaterials reduced significantly the erythrocyte sedimentation rate and modified the erythrocyte membrane resistance to induced (acid and hypo-osmotic) hemolysis. Evaluation of the erythrocyte morphology by atomic force microscopy in the control and after exposure to quantum dots showed significant differences in erythrocyte size and changes in their morphology as a result of exposure to the nanomaterials.

Key Words: *quantum dots; erythrocytes; atomic force microscopy*

Quantum dots (QD) are actively used for the creation of biosensor systems and in experimental studies *in vivo* [2,7,12]. The toxicity of nanocrystals for various cell types has been postulated not once [6,8,10]. Despite the fact that nanomaterials introduced into the body via any route are found in the blood [9], studies of QD effects on blood cells are still confined to the analysis of the toxic effects towards lymphocytes [15] and neutrophilic granulocytes [3]. We have previously determined LD_{50} for QD used in our studies for neutrophilic granulocytes: LD_{50}(QD-MPA)=0.025 mg/ml, LD_{50}(QD-polyT)=0.04 mg/ml, and LD_{50}(QD-NH_2)=16.5 mg/ml. However, studies of QD interactions with leukocytes and erythrocytes are essential for better understanding of their effects on blood cells.

Here we studied QD effects on the erythrocyte fraction of human blood.

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MATERIALS AND METHODS

Venous blood from male and female donors aged 20-40 years was collected in the morning and stabilized with 5% sodium citrate for evaluation of erythrocyte sedimentation rate (ESR) and with heparin (50 U/ml) for other studies.

Three QD types were used: CdSe/ZnS-mercaptopropionic acid (maximum emission at 620 nm) – QD-MPA; (CdSe/CdZnS)ZnS with a coating from polymeric chains consisting of polyacrylic acid residues modified with dihydrolipoic acid and PEG (maximum emission at 580 nm) – QD-polyT; and CdSeCdSZnS/polyT/SiO_2-NH_2 (maximum emission at 605 nm) – QD-NH_2, synthesized at Nanotech-Dubna Center. The spectra were recorded on an Ntegra Spectra (NT-MDT) after QD stimulation by solid-state laser (0.14 mW, 473 nm, 0.1 sec exposure). According to specification, QD size varied from 9 nm (minimum) to 30 nm (maximum); 90% QD were 11-20 nm in size. ζ-potential was measured on a Zetasizer Nano (Malvern Instruments Ltd.). The values were processed by Dis-

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persion Technology Software. Before the experiments, QD were stirred (10 min) on a Vortex (ELMI Ltd.), sonicated for 15 min (RELTEK) to make maximally homogenous suspension, and diluted to the working concentration. The final concentration of QD in all experimental series was 0.1 mg/ml.

Erythrocyte sedimentation rate was evaluated by the Panchenkov's method. QD (experiment) or an equivalent volume of saline (control) were added to citrate-stabilized blood (3:1) and incubated (30 min, 37°C). The blood was then transferred into Panchenkov's capillaries and ESR was evaluated after 60 min by the height of transparent plasma column. Medium pH was additionally evaluated at the beginning and end of the experiment (Metler Toledo).

The osmotic resistance of erythrocytes after incubation with QD (30 min, 37°C) was evaluated by the universal method modified by L. I. Idelson [1]. In brief, whole blood (20 ml per sample) was transferred into hypo-osmotic NaCl solutions (concentrations from 0.5 to 0.2%, at a 0.1% step). The blood without QD in hypo-osmotic solutions (control) or with QD (experiment) was incubated (30 min, 24°C), centrifugated (200*g*, 5 min), the supernatant was collected, and extinction coefficient was measured (λ=540 nm). Hemolysis percentage was calculated for each sample by the formula:

$$\frac{Ex}{E_1} \times 100\%,$$

where E_1 was extinction coefficient in the tube with 0.2% NaCl solution, E_x extinction coefficient of the test sample, and 100 is hemolysis percent in the tube with 0.2% NaCl.

The degree of acid hemolysis resultant from incubation with QD (30 min, 37°C) was evaluated as described previously [4]. In order to plot the erythrogram, reflecting the kinetics of induced (acid) hemolysis, the blood diluted 1000-fold was mixed (1:1) with 0.004 n HCl (hemolysis inductor), after which the extinction coefficient was directly measured (λ=670 nm). The measurements were carried out every 30 sec until two equal values were obtained, *i.e.* to the end of hemolysis (E_n). The percentage of erythrocytes destroyed during every 30 sec was calculated from the extinction values. The E_e-E_n difference was taken as 100%, where E_e was the starting point of hemolysis (first measurement) and E_n hemolysis completion (last measurement).

Erythrocyte morphology was studied by atomic force microscopy (AFM). Erythrocytes were isolated from the heparin-treated blood, washed 3 times in saline, and incubated with QD (9:1; experiment) in a final concentration of 0.1 mg/ml or with an equivalent volume of saline (control; 30 min, 37°C). Erythrocytes were transferred onto slides and fixed in glutaraldehyde (1.5%, 20 min, 24°C), washed 3 times, and scanned in a contact mode on a Solver Bio (NT-MDT). Scanning results were processed using SPMLab Analysis software (Topometrix). The NSG probes (NT-MDT) with the tip rounding radius of 10 nm, tip angle of 22°, and resonance frequency of about 42 kHz were used. The basic morphological parameters of cells were evaluated by the profile lateral section using SPMLab Analysis software.

The results were statistically processed by Origin 7.0 SRO and Statistica 6.1 software.

RESULTS

The ζ-potential values, measured by laser Doppler electrophoresis, were negative for all the studied QD: -18.2±0.8 mV for QD-MPA, -15.3±1.0 mV for QD-polyT, and -8.5±0.7 mV for QD-NH_2. The electrokinetic QD potential values indicated instability of the studied colloid systems and their liability to aggregation.

All QD types (final concentration 0.1 mg/ml) significantly reduced ESR from 7.1±1.9 in the control (n=16) to 4.9±1.9 with QD-MPA (t=2.78, n=11, p<0.05), 3.8±1.8 with QD-polyT (t=4.36, n=11, p<0.05), and 3.4±1.6 with QD-NH_2 (t=4.86, n=10, p<0.05). Several explanations of this effect could be offered: addition of an extra dispersed phase created a mechanical obstacle for erythrocyte sedimentation; the electrokinetic potential of QD similar to the membrane charge, prevented erythrocyte aggregation, thus reducing the integral ESR value; the cell membrane resistance changed under the effect of QD, which led to changes in the cell viability and, indirectly, in ESR; the erythrocyte morphology changed under the effect of QD; intricate complex interactions between QD, plasma proteins, and erythrocytes eventually inhibited ESR; changes in the medium pH in the presence of nanomaterials led to changes in the ESR. We excluded the last version, as only QD-MPA and QD-polyT caused significant changes in the pH values after 30-min exposure with QD (7.93 and 7.94, respectively). On the other hand, no pH changes in comparison with the control were detected for QD-NH_2, maximally inhibiting the ESR.

Hemolysis resistance of erythrocyte membranes after incubation with QD was modified by induced (hypo-osmotic and acid) hemolysis.

The effects of QD-MPA and QD-NH_2 on erythrocyte membrane resistance were similar: addition of these nanomaterials at hypo-osmotic NaCl levels of 0.5, 0.4, and 0.2% reduced hemolysis, a paradoxical increase of erythrocyte membrane resistance was observed (Fig. 1). However, at NaCl concentration of 0.3% all QD types stimulated hemolysis. The effects of QD-polyT on erythrocyte membrane resistance

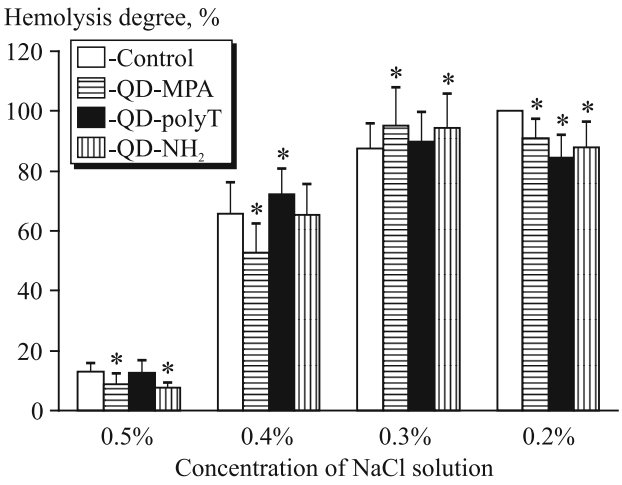

Fig. 1. Induced hypo-osmotic hemolysis of erythrocytes. Integral values of hemolysis degree at hypo-osmotic NaCl concentrations (0.5-0.2%) for cells not exposed to QD (control) and cells preincubated with QD (0.1 mg/ml, 30 min, 37°C).

were different: hemolysis values were at the level of control in the presence of hypo-osmotic NaCl concentrations of 0.5 and 0.3%, increased slightly at 0.4% NaCl concentration, and while decreased in the presence of 0.2% NaCl. Hence, erythrocyte hemolysis in hypo-osmotic medium was suppressed by two of the studied three QD types. As the duration of QD reactions with erythrocytes was limited by 30-min incubation, destruction of nanomaterials was hardly possible. A more likely explanation was the effect of QD coating on hemolysis degree, because QD coating directly interacted with the cells. Published data indicated that QD-MPA were one of the most toxic QD types, as they could be directly associated with the scavenger receptors on the cell surface [11]. On the other hand,

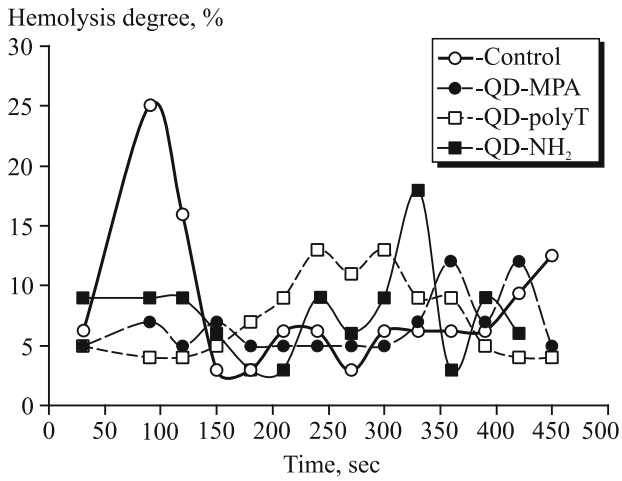

Fig. 2. Induced acid hemolysis of erythrocytes (typical erythrogram for a series of 24 experiments). Curves reflect the kinetics of induced hemolysis in control (no QD) and experiment (erythrocytes preincubated with QD, 0.1 mg/ml, 30 min, 37°C).

differences in the coating of the two QD types, QD-polyT and QD-NH_2, consisted in surface modification and addition of SiO_2-NH_2 group. However, even a slight modification of the coating surface could lead to significant modification of the QD biochemical and physiological effect. It was demonstrated [13] that slight modification of PEG with an active ligand significantly changed QD characteristics (including permeability). Presumably, addition of SiO_2-NH_2 group to the coating composition in our study enhanced QD interactions with the erythrocyte membranes and their effects on osmotic hemolysis.

Similar results were obtained in studies of acid hemolysis. The kinetics of hemolytic reactions was evaluated on blood specimens from 7 donors (4 repeats for each experiment). A typical erythrogram is presented in Figure 2. Control erythrogram showed hemolysis maximum at ~25% by the 90th sec of the study (Fig. 2). All QD types suppressed acid hemolysis, which manifested in reduction of the hemolysis peaks (to 12% for QD-MPA, 13% for QD-polyT, and 18% for QD-NH_2) and in delayed onset of the hemolysis peak (to 360 sec for QD-MPA, to 240 sec for QD-polyT, and to 330 sec for QD-NH_2).

Hence, the results demonstrate minimum shifts in the degree of hypo-osmotic hemolysis and significant shifts in the erythrogram in acid hemolysis. These data are in line with a previous report [5] demonstrating negligible shifts in spontaneous hemolysis of erythrocytes under the effect of CdSe core QD.

ESR reduction could be caused by modification of cell morphology. The modifying effect of QD on erythrocyte morphology was studied by AFM (Fig. 3).

All control cells had the classical biconcave shape (Fig. 3, *a*). Cell size was measured by the lateral section of a normocyte (Fig. 3, *b*). The following parameters were measured and statistically processed: erythrocyte diameter, maximum cell height (in the tore zone) and minimum height (in the pellore zone; Table 1).

QD with different coating caused poikilocytosis and anisocytosis. Mainly stomatocytes and planocytes were found in smears after incubation with QD-MPA (Fig. 3, *c* and *d*, respectively), while cell incubation with QD-polyT and QD-NH_2 led to formation of mainly echinocytes, elliptocytes, and codocytes (Fig. 3, *e*, *f*). The maximum changes in the morphology and poikilocytosis were recorded for QD-MPA.

In a previous report [14], QD were conjugated with immune markers and tested as indicators of specific erythrocyte receptors [14]. These authors studied fixation of conjugated QD to the cell surface. However, their AFM scans clearly demonstrated alteration of erythrocytes. The erythrocyte morphology changed less than in our experiments, presumably due to protective effect of streptavidin conjugated with QD.

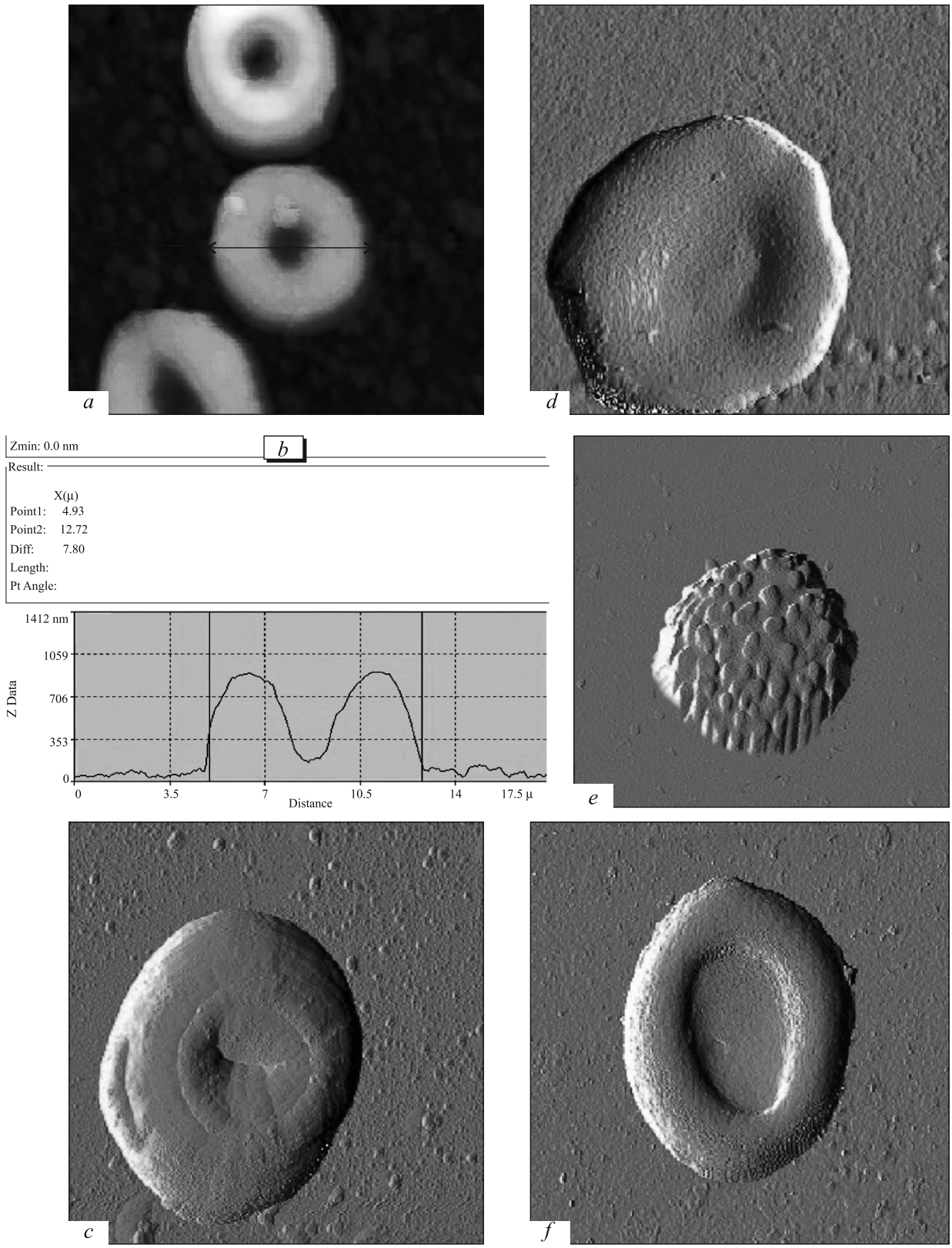

Fig. 3. AFM scans of erythrocytes. *a*) intact erythrocyte morphology: normocyte (discocyte); *b*) lateral section of an erythrocyte: algorithm of cell diameter measurement with consideration for convolution effect is shown; *c*) stomatocyte: the most incident erythrocyte alteration under the effect of QD-MPA; *d*) planocyte: rather incident alteration of erythrocytes under the effect of QD-MPA; *e*) echinocyte: minimum changes in the morphology under the effect of QD-polyT, though several echinocytes and codocytes were found; *f*) elliptocyte: typical alteration of erythrocytes under the effect of QD-NH_2; this QD type is characterized by emergence of stomatocytes and echinocytes as well.

TABLE 1. Erythrocyte Morphology without QD (Control) and with 0.1 mg/ml QD (37°C, 30 min)

Experiment conditions	Erythrocyte diameter, μ	Maximum height, μ	
		tore zone	pellore zone
Control	7.70±0.72 (*n*=23)	0.89±0.11 (*n*=23)	0.21±0.09 (*n*=23)
QD-MPA	11.38±0.98* (t=-14.72; *n*=23)	1.57±0.20* (t=-12.45; *n*=23)	0.91±0.37* (t=-9.41; *n*=23)
QD-polyT	7.10±0.14* (t=2.47; *n*=47)	1.60±0.28* (t=-11.35; *n*=47)	0.28±0.14* (t=-2.02; *n*=47)
QD-NH_2	6.37±0.83* (t=6.55; *n*=45)	1.10±0.18* (t=-4.89; *n*=45)	0.33±0.19* (t=-2.76; *n*=45)

Note. *$p<0.05$ in comparison with the control. *n*: number of experiments.

Hence, ESR reduction was a standard reaction to addition of all QD types to the blood. Maximum inhibition of ESR was observed in response to QD-NH_2. QD caused shifts in the erythrograms, the minimum shifts were recorded in response to QD-polyT. Kinetic criteria (acid hemolysis) were more sensitive for evaluation of the modifying effect of QD on erythrocyte membrane resistance than the integral parameters (hypo-osmotic hemolysis). Poikilocytosis and anisocytosis were the common structural and morphological reaction of erythrocytes to QD.

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