

1 Article

2 **In Vivo Assessment of Acute Toxicity and Immunotoxicity of**  
3 **Quantum Dots with Different Physicochemical Properties**4 Evgeniia Gerasimovich <sup>1,2</sup>, Svetlana Bozrova <sup>2</sup>, Maria Baryshnikova <sup>3</sup>, Zinaida Sokolova <sup>3</sup>, Pavel Samokhvalov <sup>2</sup>,  
5 Chris Guhrenz <sup>4</sup>, Nikolai Gaponik <sup>4</sup>, Alexander Karaulov <sup>5</sup>, Igor Nabiev <sup>1,2,5\*</sup>, Alyona Sukhanova <sup>1\*</sup>6 <sup>1</sup> Laboratoire de Recherche en Nanosciences, LRN-EA4682, Université de Reims Champagne-Ardenne, 51100  
7 Reims, France ; [igor.nabiev@univ-reims.fr](mailto:igor.nabiev@univ-reims.fr) (I.N.), [alyona.sukhanova@univ-reims.fr](mailto:alyona.sukhanova@univ-reims.fr) (A.S.)  
8 <sup>2</sup> National Research Nuclear University MEPhI (Moscow Engineering Physics Institute), Laboratory of  
9 Nano-Bioengineering, 115409 Moscow, Russian Federation; [svetaboz@yandex.ru](mailto:svetaboz@yandex.ru) (S.B.), [gerasi-](mailto:gerasi-)  
10 [movich.evg@gmail.com](mailto:movich.evg@gmail.com) (E.G.), [p.samokhvalov@gmail.com](mailto:p.samokhvalov@gmail.com) (P.S.)  
11 <sup>3</sup> Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences, Moscow, Russian Federa-  
12 tion; [ma\\_ba@mail.ru](mailto:ma_ba@mail.ru) (M.B.), [zasokolova@mail.ru](mailto:zasokolova@mail.ru) (Z.S.)  
13 <sup>4</sup> Physical Chemistry, Technische Universität Dresden, Zellescher Weg 19, 01069 Dresden, Germany; [niko-](mailto:niko-)  
14 [lai.gaponik@tu-dresden.de](mailto:lai.gaponik@tu-dresden.de) (N.G.)  
15 <sup>5</sup> Sechenov First Moscow State Medical University (Sechenov University), 119146 Moscow, Russian Federa-  
16 tion; [drkaraulov@mail.ru](mailto:drkaraulov@mail.ru) (A.K.)17  
18 **Citation:** Gerasimovich, E.;  
19 Bozrova, E.; Baryshnikova, M.;  
20 Sokolova, Z.; Samokhvalov, P.;  
21 Guhrenz, Ch.; Gaponik, N.;  
22 Karaulov, A.; Nabiev, I.; Sukhanova,  
23 A. In Vivo Assessment of Acute  
24 Toxicity and Immunotoxicity of  
25 Quantum Dots with Different  
26 Physicochemical Properties.  
27 *Nanomaterials* **2022**, *12*, x.  
28 <https://doi.org/10.3390/xxxxx>29 Academic Editor: Firstname  
30 Lastname,

31 Received: date

32 Accepted: date

33 Published: date

34 **Publisher's Note:** MDPI stays  
35 neutral with regard to jurisdictional  
36 claims in published maps and  
37 institutional affiliations.38 **Copyright:** © 2022 by the authors  
39 Submitted for possible open access  
40 publication under the terms and  
41 conditions of the Creative Commons  
42 Attribution (CC BY) license  
43 (<https://creativecommons.org/licenses/by/4.0/>).\* Correspondence: A.S., [alyona.sukhanova@univ-reims.fr](mailto:alyona.sukhanova@univ-reims.fr); I.N., [igor.nabiev@univ-reims.fr](mailto:igor.nabiev@univ-reims.fr)

**Abstract:** Semiconductor nanocrystals, or quantum dots, have received a great attention in the biomedical area because of their unique optical properties. However, their use in biomedical applications such as imaging, molecular tracking and drug delivery is limited due to safety concerns. The present study was conducted to assess acute toxicity of quantum dots with different chemical composition of the core and surface charge in mouse model. In addition, the immunotoxicity of CdSe/ZnS QDs was estimated. CdSe/ZnS, PbS/CdS/ZnS, CuInS<sub>2</sub>/ZnS QDs were synthesized and modified with PEG derivatives following standard procedure. After solubilization the hydrodynamic diameter and surface charge of QDs were characterized. Our results showed that the acute toxicity of QDs is determined by both their surface charge and the chemical composition of the core. Immunotoxicity of CdSe/ZnS-PEG QDs was evaluated at the dose of 25 mg/kg (LD<sub>10</sub>). Concentration of IL-12p70, TNF, IFN- $\gamma$ , MCP-1, IL-6, IL-10 was measured using cytometric bead array. The intravenous injection of CdSe/ZnS-PEG QDs was demonstrated to cause short-term elevation of the level of two pro-inflammatory cytokines.

**Keywords:** quantum dots; semiconductor nanocrystals; toxicity *in vivo*; immunotoxicity; cytokine profile

## 1. Introduction

Quantum dots (QDs) are semiconductor fluorescent nanocrystals with a size from 2 to 10 nm [1]. Their optical characteristics such as broad excitation and narrow emission spectra, high photostability and size-depending fluorescence peak spectral position make them a promising tool in the biomedical field [2]. Possible biomedical applications of QDs include their use in bioanalytical systems, cell and tissue imaging, drug delivery and tracking *in vivo* [3–5]. However, the potential toxicity of QDs is a serious limitation to their use in living organisms. In particular, the immunotoxicity of QDs is one of the actual topics because the immune system plays an important role in protecting the body from the foreign substances and preventing diseases.

44  
45

46 A number of studies concerning QDs toxicity clearly demonstrated that they can  
47 accumulate in various organs and tissues and cause pathological effects. The liver is  
48 shown to be one of the main organs for the accumulation of QDs [6]. In the study of  
49 hepatotoxicity of CdSe/ZnS QDs with carboxyl groups they were shown to cause  
50 hepatocytes damage and liver inflammation involving inflammasome activation and in-  
51 creased secretion of IL-1 $\beta$  cytokine [7]. Similarly, in the study of Cd/Se/Te-based QDs  
52 with ZnS shell the elevation of IL-6 and TNF- $\alpha$  level were shown, although no  
53 histopathological damage was observed [8]. The study of *in vivo* biodistribution of CdTe  
54 aqueous QDs stated the initial accumulation of QDs in liver and their further absorption  
55 in kidney during long-time blood circulation [9]. In addition, it was showed that  
56 Cd-containing QDs disrupted the redox balance of primary kidney cells, thus inducing  
57 their damage [10]. In conclusion, many evidences demonstrate that QDs can accumulate  
58 in major organs and cause pathological damage [11].

59 After the intravenous administration QDs were shown to accumulate in the spleen  
60 and thymus and maintain fluorescence ability for a long time after injection [12]. The  
61 results of immunotoxicity study of CdSe/ZnS QDs show that the majority of adminis-  
62 tered QDs are taken up by the immune organs (spleen and thymus). Lymphocytes from  
63 QD-treated mice exhibited lower viability and increase of the release of TNF- $\alpha$  and IL-6  
64 [13]. The study of the acute toxicity of CdSe/CdS-MPA QDs after repeated intraperitoneal  
65 injection demonstrated the accumulation of QDs in the liver and spleen and elevated  
66 level of IL-6 in these organs and plasma [14]. The study of intraperitoneal injection of  
67 PEG-InP/ZnS QDs in mice showed the increase of the percentage of neutrophils and IL-6  
68 levels in mouse PLF (peritoneal lavage fluid) and plasma, leading to the acute-phase in-  
69 flammation in mice [15]. Intravenous administration of CdTe QDs resulted in the altera-  
70 tion of the level of several pro-inflammatory cytokines. Specifically, IL-6 level was sig-  
71 nificantly increased starting at the dose of 0.4 mg/kg, while IL-12 (p70) and TNF- $\alpha$  levels  
72 were elevated starting from 5 mg/kg, but showed significant reduction at the highest  
73 doses [16]. In contrast, immunotoxicity study *in vivo* demonstrated little effect of  
74 CdInS<sub>2</sub>/ZnS QDs on immune organs and the level of pro-inflammatory cytokines [17].  
75 Another study revealed the dependence of immunotoxicity on molecules used for surface  
76 coating of QDs [18]. Summing up, the immunotoxicity of QDs can be affected by QD  
77 type, surface modifications and dose in a non-linear manner.

78 Assessment of cytokines concentration is one of the indicative methods for the  
79 immunotoxicity studies [19]. The cytokines are divided into two groups: the  
80 pro-inflammatory such as IL-6 [20], TNF [21], IL-12 (p70) [22], IFN- $\gamma$  [23] and an-  
81 ti-inflammatory such as IL-10 [22]. Monocyte chemoattractant protein 1 (MCP-1) is one of  
82 the key chemokines which regulates migration and infiltration of mono-  
83 cytes/macrophages during inflammatory response [24]. Thus, the alterations in cytokines  
84 and chemokines concentration can reflect the reaction of the immune system to the ad-  
85 ministration of potentially toxic substances.

86 Our study aimed to assess the acute toxicity *in vivo* of different types of QDs by  
87 calculating the LD<sub>50</sub> values for all types of QDs, estimating the physiological responses of  
88 laboratory animals on the injection of QD preparations, and toxic effects of QDs on dif-  
89 ferent organs. In addition, we used the most toxic CdSe/ZnS-PEG-OH QDs to investigate  
90 the immunotoxicity after intravenous administration in mice. For this purpose, six cyto-  
91 kines (IL-12p70, TNF, IFN- $\gamma$ , MCP-1, IL-6, IL-10) concentration was measured to assess  
92 the potential inflammation response after exposure to CdSe/ZnS-PEG-OH QDs.

## 93 2. Materials and Methods

94 Methanol, chloroform, sodium phosphate dibasic, sodium phosphate monobasic,  
95 sodium hydroxide, DL-cysteine hydrochloride hydrate were purchased from Sig-  
96 ma-Aldrich, US. PEG derivatives HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH, HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OCH<sub>2</sub>-COOH,  
97 HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-NH<sub>2</sub> were purchased from ProChimia Surfaces Sp, Poland.  
98

## 2.1 Quantum dots synthesis and solubilization

CdSe/ZnS (core/shell) quantum dots with a fluorescence maximum at 592 nm were synthesized as described earlier [25]. The synthesis of CuInS<sub>2</sub>/ZnS and PbS/CdS/ZnS QDs was carried out by the method described in [26] for CuInS<sub>2</sub>/ZnS QDs. After the synthesis quantum dots were transferred from organic phase to water solution by replacing hydrophobic surface ligands with polyethylene glycol derivatives following the procedure described earlier [25]. Briefly, quantum dots were dissolved in chloroform and precipitated with 10 mg/ml DL-cysteine solution in methanol. The excess of DL-cysteine was washed off with methanol. The precipitate was dried and then dissolved in a weak alkaline solution.

At the next stage, DL-cysteine ligands were replaced with PEG derivatives with hydroxyl group. We used three types of PEG derivatives: HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH, HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OCH<sub>2</sub>-COOH, and HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-NH<sub>2</sub>. For the modification of CdSe/ZnS QDs mixtures of PEG derivatives were used at the following ratios: 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH/30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OCH<sub>2</sub>-COOH, 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH/30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-NH<sub>2</sub>, and 100% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH. The specified amounts of ligands were added to working mixtures with the corresponding pH values: 0.1 M sodium phosphate buffer solution (pH 7.2) if the HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH ligand was used, 0.1 M sodium phosphate buffer solution (pH 8.0) in the case of the mixture of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH / 30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OCH<sub>2</sub>-COOH, and 0.1 M sodium phosphate buffer solution (pH 6.6) in the case of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH/30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-NH<sub>2</sub>. After that, the mixtures were incubated at a temperature of 4°C overnight.

After the incubation, the samples were purified from unbound PEG derivatives using Amicon Ultra-15 centrifugal filter units with a 10 kDa cut-off (Millipore) by centrifugation following the adding 15 mL of 0.1 M sodium phosphate buffer (pH 7.2) in the case of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH, 0.1 M sodium phosphate buffer (pH 8.0) in the case of the mixture of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH 30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OCH<sub>2</sub>-COOH, and 0.1 M sodium phosphate buffer solution (pH 6.6) in the case of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH/30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-NH<sub>2</sub>. The centrifugation was performed three times at the room temperature at 4000 rpm for 10 min. Then, the obtained QDs preparations were additionally purified by gel exclusion chromatography on PD MiniTrap 25G columns (GE Healthcare) according to the manufacturer's protocol. For this purpose, 500 µL of a QD solution was applied onto a column preliminarily equilibrated with a 0.1 M sodium phosphate buffer solution with the corresponding pH. After that, 1 mL of the same buffer solution was used for elution. The fractions containing QDs were collected into a separate test tube. The purification procedure was performed twice. The resultant solution was sequentially filtered through Millex-GV 0.22 µm and Whatman Anotop 0.1 µm filter units. The solution of PEG derivatives with different functional groups (PEG filtrate solutions) were prepared by centrifugation of QDs solution using Amicon Ultra-4 centrifugal filter units with a 10 kDa cut-off (Millipore).

## 2.2 Quantum dots characterization

For the determination of QDs concentration at the final solution we used a weight method. The aliquot of 35 µL of the final preparation of the QDs solution was placed into a preliminarily weighted 0.5-mL low-bind test tube (Eppendorf) and then dried in the Concentrator Plus (Eppendorf, Germany) for 3 h at the temperature of 30°C. After that, the test tube was weighted again. The quantity of QDs contained in 35 µL of the original QD solution was calculated by subtracting the initial weight of the empty test tube from the final weight of the test tube containing the QD preparation after drying. The QD quantity per milliliter of solution was calculated to obtain the mass concentration. The hydrodynamic diameter and zeta potential of solubilized QD were measured by dynamic

151 laser scattering and laser Doppler electrophoresis, respectively, by using Zetasizer  
152 Nano-ZS device (Malvern Instrument Ltd., UK).

### 154 2.3. Estimation of acute cytotoxicity of QDs *in vivo*

155 The analysis of the acute *in vivo* toxicity of the QD preparations, including the esti-  
156 mation of their median lethal doses (LD<sub>50</sub>), was performed in experiments on female  
157 BALB/c mice obtained from the vivarium of the Blokhin Russian Cancer Research Center  
158 of the Ministry of Health of Russia. Standard animals weighing 18–22 g were used in the  
159 study. All the animals were healthy; they were kept in special roomy cages at room  
160 temperature (20–23°C), a relative humidity of 60–65%, natural illumination, and forced  
161 air supply, on a litter of wood shavings sterilized in a hot-air oven. The mice were fed on  
162 the standard commercial certified pelletized feed for rodents with a known expiry date.  
163 The mice had free round-the-clock access to pure drinking water.

164 The mice were divided into five groups (four mice each). CuInS<sub>2</sub>/ZnS, PbS/CdS/ZnS,  
165 and CdSe/ZnS QDs modified with the thiol-containing HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH ligand, as  
166 well as CdSe/ZnS QDs modified with the mixture of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH / 30% of  
167 HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OCH<sub>2</sub>-COOH, the mixture of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH / 30% of  
168 HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-NH<sub>2</sub>, or 100% HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH, were administered at doses from 100  
169 to 300 mg/kg as a single injection into the caudal vein. The substances studied were dis-  
170 solved in sterile 0.1 M sodium phosphate buffer solution (pH 7.2). Sterile 0.1 M sodium  
171 phosphate buffer solution (pH 7.2) and sterile filtered solutions of the corresponding  
172 modifying ligands in 0.1 M sodium phosphate buffer solution (pH 7.2) served as control  
173 solutions.

174 The day when the preparation was injected was taken to be day 0. In that day, the  
175 mice were watched for 6 h after the injection. After that, they were examined twice a day  
176 for 15 days. The number of the animals that died was the criterion for estimating the  
177 acute toxicity of the QDs. The toxicity was measured as the lethal dose (LD), i.e., the  
178 amount of QDs causing death of a specified percentage of the animals.

### 180 2.4. Assessment of CdSe/ZnS-PEG-OH QDs immunotoxicity

181 Eighty BALB/c mice were treated with 25 mg/kg CdSe/ZnS-PEG QDs. A solvent  
182 solution (0.05 M Na-phosphate buffer, pH 7.2) and PEG filtrate solution with the same  
183 concentration of PEG as in the CdSe/ZnS-PEG solution were used as negative controls. At  
184 the time points of 6, 24, 48 hours and 7 days intact animals was used as an additional  
185 negative control. All solutions were injected intravenously in the tail vein. Serum samples  
186 were collected at 6, 24, 48 hours, 7 and 30 days after injection. Serum from all mice in one  
187 group were pooled to obtain necessary amount of material and stored at -20°C until  
188 analysis.

### 189 2.5. Mouse cytokines concentration measurement

190 The concentration of six pro-inflammatory cytokines was measured using  
191 cytometric bead array kit (BD™ CBA Mouse Inflammation Kit, BD Biosciences,  
192 California, USA) according to manufacturer's instructions. This technique is based on six  
193 bead populations with distinct fluorescence intensities coated with antibodies specific to  
194 IL-12p70, TNF, IFN-γ, MCP-1, IL-6, IL-10. The cytokine capture beads and  
195 phycoerythrin-conjugated detection antibodies were incubated with serum samples or  
196 standards to form sandwich complexes. Then samples were measured on BD FACS  
197 Canto™ II cytometer (BD Biosciences, California, USA) and FCAP Array™ 3.0 software  
198 (BD Biosciences, California, USA) was used to calculate the concentration of cytokines  
199 using fluorescence intensity data. The standard curve range for each analyte was from 20  
200 to 5000 pg/ml.

### 3. Results

#### 3.1. QD synthesis, solubilization and characterization

In the presented study, QDs with various chemical composition and surface charge were used to investigate their toxic effects in mice model. After the synthesis of QDs in the organic phase, we modified their surface with thiol-containing PEG derivatives to obtain aqueous QD preparations. Using mixtures of PEG derivatives with different functional groups for the modification of CdSe/ZnS QDs, we obtained QD solutions with different surface charge. The surface charge and hydrodynamic diameter of QDs were determined using a Zetasizer Nano ZS instrument by the electrophoretic mobility method employing the Doppler effect and by the dynamic light scattering method, respectively. Table 1 shows the sizes and charges of the QDs used in the study.

**Table 1.** Size and surface charge of the quantum dots used in the study.

QD composition	Size, nm	ζ-potential, mV
PbS/CdS/ZnS-PEG-OH <sup>1</sup>	32.04±0.87	-10.60±2.92
CuInS <sub>2</sub> /ZnS-PEG-OH	16.08±0.51	-6.12±1.81
CdSe/ZnS-PEG-OH	16.74±0.28	-4.72±0.38
CdSe/ZnS-PEG-COOH <sup>2</sup>	15.37±0.14	-17.80±3.01
CdSe/ZnS-PEG-NH <sub>2</sub> <sup>3</sup>	22.77±0.36	6.43±1.12

<sup>1</sup>PEG-OH denotes 100% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH

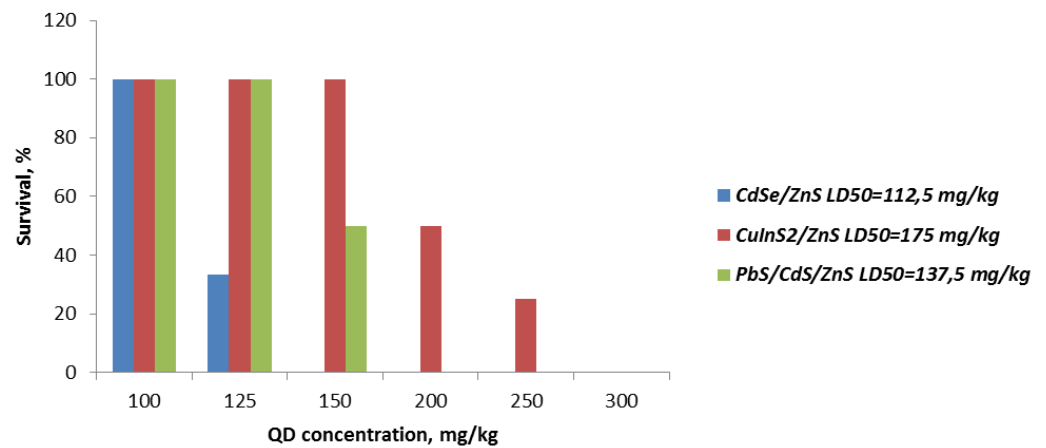
<sup>2</sup>PEG-COOH denotes a mixture of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH and 30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OCH<sub>2</sub>-COOH

<sup>3</sup>PEG-NH<sub>2</sub> denotes a mixture of 70% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-OH and 30% of HS-(CH<sub>2</sub>)<sub>11</sub>-EG<sub>6</sub>-NH<sub>2</sub>

#### 3.2. Acute *in vivo* cytotoxicity of quantum dots

The mice were injected with the QDs preparations into the caudal vein, after which we watched the animals carefully for 24 h. After that, the number of surviving animals was recorded.

We estimated the *in vivo* acute toxicity of QDs with different chemical compositions of the core (Figure 1) and with different surface charges (Figure 2). The data obtained in this experiment were used to calculate the LD<sub>50</sub> values for all types of QDs studied (Table 2). Our results showed that CuInS<sub>2</sub>/ZnS QDs had less toxic effect on mice than PbS/CdS/ZnS and CdSe/ZnS QDs, despite their surface charge were relatively similar due to the same PEG derivative used for their modification (Fig. 1). Furthermore, the obtained means of LD<sub>50</sub> for QDs with the same chemical composition of the core (CdSe/ZnS) but carried different surface charge varied considerably. CdSe/ZnS-PEG-OH QDS with low negative charge exhibited the highest toxic effect on mice, while the LD<sub>50</sub> of CdSe/ZnS-PEG-NH<sub>2</sub> QDS with low positive charge turned out to be approximately two times higher. CdSe/ZnS-PEG-COOH QDs were showed to cause the weakest toxic effect on mice.

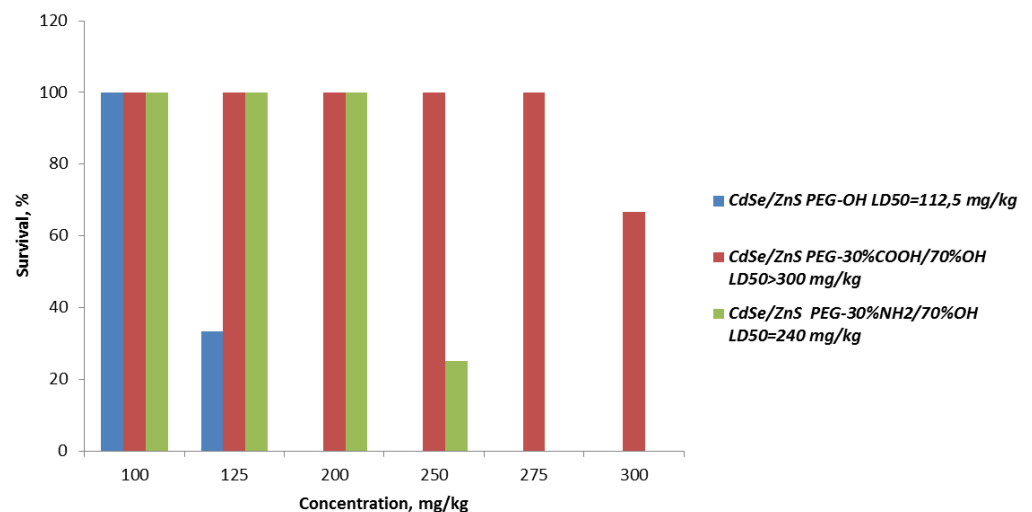


233

234

235

**Figure 1.** Survival rate of BALB/c mice as dependent on the quantum dot concentration and core composition.



236

237

238

**Figure 2.** Survival rate of BALB/c mice as dependent on the quantum dot concentration and charge.

**Table 2.** LD<sub>50</sub> values of different quantum dots for BALB/c mice.

QD type	LD <sub>50</sub> , mg/kg
CdSe/ZnS-PEG-OH	112.5
CuInS <sub>2</sub> /ZnS-PEG-OH	200
PbS/CdS/ZnS-PEG-OH	150
CdSe/ZnS-PEG-COOH	>300
CdSe/ZnS-PEG-NH <sub>2</sub>	240

239

240

241

242

243

244

245

246

### 3.2 Cytokine profile in mice after injection of QDs

In three experimental groups mice were treated with 0.1 M Na-phosphate buffer, PEG filtrate solution, and CdSe/ZnS-PEG solution at the dose of 25 mg/kg. The concentrations of six cytokines (IL-12p70, TNF, IFN- $\gamma$ , MCP-1, IL-10, IL-6) in mice serum determined by CBA assay are shown in Table 3. The data show that 24 hours after administration of CdSe/ZnS-PEG QDs the level of IL-12 was slightly elevated compared to control group and groups treated by buffer and PEG solutions, but starting from 48 hours the level of this cytokine was low in all groups of animals. The concentration of

another pro-inflammatory cytokine, TNF, is raised in group treated with CdSe/ZnS-PEG QDs after 48 hours after injection. In addition, the level of IL-6 was somewhat higher 6 hours after injection in the groups treated with buffer, PEG and CdSe/ZnS-PEG QDs solutions compared to intact animals, but after longer time periods it remained at the basic level. The level of anti-inflammatory cytokine, IL-10, was below the limit of detection in samples from all experimental groups. The level of MCP-1, pro-inflammatory chemokine, was elevated in groups treated with buffer and CdSe/ZnS-PEG QDs 6 hours after injection. However, 30 days after the start of the experiment the level of MCP-1 was twice higher in groups treated with PEG and CdSe/ZnS-PEG in comparison with group treated with buffer.

**Table 3.** Concentration of cytokines in mouse serum after the injection of CdSe/ZnS QDs.

Cytokine concentration, pg/ml	Time	control	Na-phosphate buffer	PEG solution	CdSe/ZnS-PEG
IL-12p70	6 h	10.26±2.75	10.36±7.06	7.38±7.81	0.00±0.00
	24 h	3.56±0.30	2.37±0.74	0.00±0.00	10.27±1.25
	48 h	1.50±0.50	0.00±0.00	0.00±0.00	1.85±0.00
	7 d	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	30 d	N/A	0.00±0.00	0.00±0.00	0.00±0.00
TNF	6 h	21.71±0.74	25.39±10.42	24.51±9.18	17.14±5.23
	24 h	24.69±4.47	18.19±6.72	26.09±0.99	24.87±1.24
	48 h	26.27±7.69	18.91±0.25	21.18±3.97	36.62±0.99
	7 d	5.25±2.29	4.72±0.90	4.66±0.33	5.70±0.66
	30 d	N/A	2.49±3.22	5.41±0.25	4.84±1.38
IFN- $\gamma$	6 h	2.68±0.57	3.12±0.51	3.55±1.34	2.84±0.34
	24 h	2.01±0.48	3.00±0.34	2.56±0.06	2.26±0.83
	48 h	3.59±1.4	2.84±0.57	2.88±0.40	2.05±0.54
	7 d	0.59±0.00	0.31±0.21	0.00±0.00	1.11±0.11
	30 d	N/A	0.67±0.06	0.81±0.00	0.42±0.13
MCP-1	6 h	32.06±5.21	79.47±12.2	49.05±5.71	77.72±5.59
	24 h	35.54±0.28	28.98±3.25	20.08±0.00	32.49±3.46
	48 h	20.62±7.36	26.03±1.51	24.73±2.76	21.88±1.26
	7 d	11.44±3.02	13.79±1.22	17.13±2.91	14.48±4.08
	30 d	N/A	7.51±3.52	13.68±0.16	12.7±0.93
IL-10	6 h	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	24 h	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	48 h	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	7 d	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	30 d	N/A	0.00±0.00	0.00±0.00	0.00±0.00
IL-6	6 h	1.54±0.13	6.20±2.83	3.68±0.74	8.56±1.85
	24 h	1.51±2.13	2.27±0.65	0.00±0.00	0.00±0.00
	48 h	0.00±0.00	0.00±0.00	0.00±0.00	1.33±0.43
	7 d	0.62±0.69	0.00±0.00	0.00±0.00	0.78±0.42
	30 d	N/A	0.33±0.47	0.00±0.00	0.00±0.00

#### 4. Discussion

Semiconductor nanocrystals, or quantum dots, have a great potential in the field of biomedicine, but their use is restricted by potential toxic effects on living organisms. The *in vivo* toxicity of CdSe/ZnS QDs has been reported in several studies. However, a more systematic evaluation of negative effects of QDs on living organisms is necessary for safe human use. One of the important aspects is an immunotoxicity as the immune system functions include protection from foreign substances.

We have estimated the LD<sub>50</sub> values for different types of QDs, physiological responses of laboratory animals on the injection of QD preparations, and toxic effects of QDs on different organs. Such experiments could help to improve the safety testing standards for nanotechnological products based on nanocrystals.

The QD preparations were injected into the caudal vein of BALB/c mice, after which we carefully watched the mice for 24 h and then examined them twice a day for 15 days. We estimated the *in vivo* toxicity of QDs with different chemical compositions of the core (Fig. 1) and those with different surface charges (Fig. 2). The results were used to calculate the LD<sub>50</sub> values for all types of QDs studied (Table 2). Our data showed that the CdSe/ZnS-PEG-OH QDs with a low negative surface charge were the most toxic. The QDs with a high negative surface charge were the least toxic. It was also demonstrated that the QDs with CuInS<sub>2</sub> cores were less toxic than the QDs whose cores contained heavy metals.

In addition, our experiments on toxicity showed that intravenous injections of QDs with different chemical compositions provoked different physiological responses. The administration of CdSe/ZnS and PbS/ZnS QDs caused an increase in the respiration and heart rates, paresis of the legs, convulsive retching movements, and spasm of the caudal vein. After several hours, the surviving animals completely recovered. After the injection of CuInS<sub>2</sub>/ZnS QDs, the mice felt well for 5–10 min, after which leg paresis, rapid and shallow breathing and general paralysis were observed. These transitory disorders suggest the formation of metastable QD aggregations in the blood, which either were broken up due to opsonization and absorbed without affecting considerably the general state of health or, in more acute situations, led to death of the mice. There are published data that QDs may cause lung thrombosis [27], which explains the condition of the mice in our study. The mechanism of QD-induced thrombosis is unknown; however, it is assumed [27] that QDs are capable of triggering the clotting process in blood. A different physiological response to CuInS<sub>2</sub>/ZnS QDs suggests that the toxic effect of these QDs is underlain by a different mechanism, apparently determined by copper, which, unlike lead and cadmium, is not a heavy metal [28].

Our results clearly demonstrate that the QDs surface charge also influences their toxicity. Indeed, the QDs with a low negative surface charge have proved to be the most toxic, and those with a higher negative charge, the least toxic. The observed effect is most likely to be determined by the differences in the distribution and accumulation of QDs in the mouse body and the differences in the interactions with the components of biological fluids. For example, negatively charged QDs may rapidly accumulate in lymph nodes [29], and electrically neutral QDs are less prone to electrostatic interaction with proteins, which interferes with the formation of the "protein corona" and promotes their degradation [30].

After the most toxic type of QDs was revealed, we performed an additional experiment in order to assess the effect of these QDs on the immune system. For this purpose, we estimated the effect of CdSe/ZnS-PEG-OH QDs at the dose of 25 mg/kg, or LD<sub>10</sub>, on cytokine profile in mice. Buffer solution (0.1 M Na-phosphate, pH 7.2) and PEG-OH solution were used as controls in the study. The concentration of IL-12p70, TNF, IFN- $\gamma$ , MCP-1, IL-10, and IL-6 was determined using cytometric bead array kit (Table 3). The results demonstrated a slight increase in the production of two pro-inflammatory cytokines, IL-12 (p70) and TNF, 24 and 48 hours after injection, respectively. The level of MCP-1 was elevated in the groups treated with both buffer solution and CdSe/ZnS-PEG



312 QDs solution 6 hours after administration, but after 30 days it was relatively higher in  
313 groups treated with PEG solution and CdSe/ZnS-PEG QDs solution. Our results might  
314 evidence that injection of CdSe/ZnS-PEG QDs can cause short-term inflammatory reac-  
315 tion in mice. However, after 7 and 30 days after injection of QDs the level of the majority  
316 of pro-inflammatory cytokines was similar to that of control groups. Based on these re-  
317 sults, we can suggest that immune reaction to CdSe/ZnS QDs at the dose of 25 mg/kg is  
318 reversible, but this issue needs further investigation.  
319

320 **Author Contributions:** Conceptualization, A.S. and I.N.; methodology, M.B., Z.S., Ch.G., P.S.;  
321 formal analysis, N.G., A.K., and A.S.; investigation, P.S., S.B., E.G., M.B. and Z.S.; data curation,  
322 N.G., A.S., I.N. and A.K.; writing—original draft preparation, S.B., A.S.; writing—review and ed-  
323 iting, E.G., M.B., Z.S., N.G., A.K., and I.N.; supervision, I.N., A.S and N.G. All authors have read  
324 and agreed to the published version of the manuscript.

325 **Funding:** This work was supported by the Ministry of Science and Higher Education of the Russian  
326 Federation through the grant No. 075-15-2021-935 of the ERA.NET-RUS+ project NanoToBio in its  
327 part related to the synthesis of nanomaterials. This work was also supported by the French Na-  
328 tional Research Agency (ANR-20-CE19-009-02) and co-financed by the European Union via the  
329 European Regional Development Fund (FreeBioWave project) in its part related to the develop-  
330 ment of new surface chemistry approaches.

331 **Data Availability Statement:** The data that support the findings of this study are available from  
332 the corresponding authors, A.S. or I.N., upon reasonable request.

333 **Acknowledgments:** A.S. and I.N. acknowledge the support of the French Ministry of Higher Ed-  
334 ucation, Research and Innovation and the University of Reims Champagne-Ardenne. We thank  
335 Vladimir Ushakov for proofreading the manuscript.

336 **Conflicts of Interest:** The authors declare that the research was conducted in the absence of any  
337 commercial or financial relationships that could be construed as a potential conflict of interest.

## References

- 338  
339 1. Medintz, I.L.; Uyeda, H.T.; Goldman, E.R.; Mattoussi, H. Quantum Dot Bioconjugates for Imaging, Labelling and Sensing.  
340 *Nat. Mater.* **2005**, *4*, 435–446, doi:10.1038/nmat1390.
- 341 2. Matea, C.T.; Mocan, T.; Tabaran, F.; Pop, T.; Mosteanu, O.; Puia, C.; Iancu, C.; Mocan, L. Quantum Dots in Imaging, Drug  
342 Delivery and Sensor Applications. *Int. J. Nanomedicine* **2017**, *12*, 5421–5431, doi:10.2147/IJN.S138624.
- 343 3. Bilan, R.; Nabiev, I.; Sukhanova, A. Quantum Dot-Based Nanotools for Bioimaging, Diagnostics, and Drug Delivery.  
344 *ChemBioChem* **2016**, *17*, 2103–2114, doi:10.1002/cbic.201600357.
- 345 4. Brazhnik, K.; Sokolova, Z.; Baryshnikova, M.; Bilan, R.; Efimov, A.; Nabiev, I.; Sukhanova, A. Quantum Dot-Based  
346 Lab-on-a-Bead System for Multiplexed Detection of Free and Total Prostate-Specific Antigens in Clinical Human Serum  
347 Samples. *Nanomedicine Nanotechnology, Biol. Med.* **2015**, *11*, 1065–1075, doi:10.1016/j.nano.2015.03.003.
- 348 5. Wagner, A.M.; Knipe, J.M.; Orive, G.; Peppas, N.A. Quantum Dots in Biomedical Applications. *Acta Biomaterialia* **2019**, *94*,  
349 44–63, doi:10.1016/j.actbio.2019.05.022.Quantum.
- 350 6. Lu, J.; Tang, M.; Zhang, T. Review of Toxicological Effect of Quantum Dots on the Liver. *J. Appl. Toxicol.* **2019**, *39*, 72–86,  
351 doi:10.1002/jat.3660.
- 352 7. Lu, Y.; Xu, S.; Chen, H.; He, M.; Deng, Y.; Cao, Z.; Pi, H.; Chen, C.; Li, M.; Ma, Q.; et al. CdSe/ZnS Quantum Dots Induce  
353 Hepatocyte Pyroptosis and Liver Inflammation via NLRP3 Inflammasome Activation. *Biomaterials* **2016**, *90*, 27–39,  
354 doi:10.1016/j.biomaterials.2016.03.003.
- 355 8. Lin, C.H.; Yang, M.H.; Chang, L.W.; Yang, C.S.; Chang, H.; Chang, W.H.; Tsai, M.H.; Wang, C.J.; Lin, P. Cd/Se/Te-Based  
356 Quantum Dot 705 Modulated Redox Homeostasis with Hepatotoxicity in Mice. *Nanotoxicology* **2011**, *5*, 650–663,  
357 doi:10.3109/17435390.2010.539712.
- 358 9. Su, Y.; Peng, F.; Jiang, Z.; Zhong, Y.; Lu, Y.; Jiang, X.; Huang, Q.; Fan, C.; Lee, S.T.; He, Y. In Vivo Distribution,  
359 Pharmacokinetics, and Toxicity of Aqueous Synthesized Cadmium-Containing Quantum Dots. *Biomaterials* **2011**, *32*,  
360 5855–5862, doi:10.1016/j.biomaterials.2011.04.063.
- 361 10. Zhao, L.; Zong, W.; Zhang, H.; Liu, R. Kidney Toxicity and Response of Selenium Containing Protein-Glutathione  
362 Peroxidase (Gpx3) to CdTe QDs on Different Levels. *Toxicol. Sci.* **2019**, *168*, 201–208, doi:10.1093/toxsci/kfy297.
- 363 11. Liang, Y.; Zhang, T.; Tang, M. Toxicity of Quantum Dots on Target Organs and Immune System. *J. Appl. Toxicol.* **2021**, 1–24,  
364 doi:10.1002/jat.4180.
- 365 12. Fitzpatrick, J.A.J.; Andreko, S.K.; Ernst, L.A.; Waggoner, A.S.; Ballou, B.; Bruchez, M.P. Long Term Persistence and Spectral  
366 Blue Shifting of Quantum Dots in Vivo. *Nano Lett.* **2009**, *9*, 2736–2741, doi:10.1021/nl901534q.Long.
- 367 13. Wang, X.; Tian, J.; Yong, K.T.; Zhu, X.; Lin, M.C.M.; Jiang, W.; Li, J.; Huang, Q.; Lin, G. Immunotoxicity Assessment of  
368 CdSe/ZnS Quantum Dots in Macrophages, Lymphocytes and BALB/c Mice. *J. Nanobiotechnology* **2016**, *14*, 1–12,  
369 doi:10.1186/s12951-016-0162-4.
- 370 14. Haque, M.M.; Im, H.Y.; Seo, J.E.; Hasan, M.; Woo, K.; Kwon, O.S. Acute Toxicity and Tissue Distribution of  
371 CdSe/CdS-MPA Quantum Dots after Repeated Intraperitoneal Injection to Mice. *J. Appl. Toxicol.* **2013**, *33*, 940–950,  
372 doi:10.1002/jat.2775.
- 373 15. Chen, S.; Chen, Y.; Chen, Y.; Yao, Z. InP/ZnS Quantum Dots Cause Inflammatory Response in Macrophages through  
374 Endoplasmic Reticulum Stress and Oxidative Stress. *Int. J. Nanomedicine* **2019**, *14*, 9577–9586, doi:10.2147/IJN.S218748.
- 375 16. Nguyen, K.C.; Zhang, Y.; Todd, J.; Kittle, K.; Patry, D.; Caldwell, D.; Lalande, M.; Smith, S.; Parks, D.; Navarro, M.; et al.  
376 Biodistribution and Systemic Effects in Mice Following Intravenous Administration of Cadmium Telluride Quantum Dot  
377 Nanoparticles. *Chem. Res. Toxicol.* **2019**, *32*, 1491–1503, doi:10.1021/acs.chemrestox.8b00397.
- 378 17. Chen, T.; Li, L.; Lin, X.; Yang, Z.; Zou, W.; Chen, Y.; Xu, J.; Liu, D.; Wang, X.; Lin, G. In Vitro and in Vivo Immunotoxicity of  
379 PEGylated Cd-Free CuInS<sub>2</sub>/ZnS Quantum Dots. *Nanotoxicology* **2020**, *14*, 372–387, doi:10.1080/17435390.2019.1708495.

- 380 18. Dai, T.; Li, N.; Liu, L.; Liu, Q.; Zhang, Y. AMP-Conjugated Quantum Dots: Low Immunotoxicity Both In Vitro and In Vivo.  
381 *Nanoscale Res. Lett.* **2015**, *10*, 1–9, doi:10.1186/s11671-015-1100-3.
- 382 19. Corsini, E.; House, R. V. Evaluating Cytokines in Immunotoxicity Testing. *Methods Mol. Biol.* **2018**, *1803*, 297–314,  
383 doi:10.1007/978-1-4939-8549-4\_18.
- 384 20. Tanaka, T.; Narazaki, M.; Kishimoto, T. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harb. Perspect. Biol.* **2014**,  
385 *6*, 1–16.
- 386 21. Zelová, H.; Hošek, J. TNF- $\alpha$  Signalling and Inflammation: Interactions between Old Acquaintances. *Inflamm. Res.* **2013**, *62*,  
387 641–651, doi:10.1007/s00011-013-0633-0.
- 388 22. Vignali, D.A.; Kuchroo, V.K. IL-12 Family Cytokines: Immunological Playmakers. *Nat. Immunol.* **2012**, *13*, 722–728,  
389 doi:10.1038/ni.2366.IL-12.
- 390 23. Schroder, K.; Hertzog, P.J.; Ravasi, T.; Hume, D.A. Interferon- $\gamma$ : An Overview of Signals, Mechanisms and Functions. *J.*  
391 *Leukoc. Biol.* **2004**, *75*, 163–189, doi:10.1189/jlb.0603252.
- 392 24. Deshmane, S.L.; Kremlev, S.; Amini, S.; Sawaya, B.E. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *J. Interf.*  
393 *Cytokine Res.* **2009**, *29*, 313–325, doi:10.1089/jir.2008.0027.
- 394 25. Nabiev, I.; Sukhanova, A.; Even-Desrumeaux, K.; Chames, P.; Baty, D.; Artemyev, M.; Oleinikov, V.; Nabiev, I. Engineering  
395 of Ultra-Small Diagnostic Nanoprobes through Oriented Conjugation of Single-Domain Antibodies and Quantum Dots.  
396 *Protoc. Exch.* **2012**, doi:10.1038/protex.2012.042.
- 397 26. Vokhmintcev, K. V; Linkov, P.A.; Samokhvalov, P.S.; Nabiev, I.R. Two-Stage ZnS Shell Coating on the CuInS<sub>2</sub> Quantum  
398 Dots for Their Effective Solubilization. *Ken Energy* **2018**, *3*, 535, doi:10.18502/ken.v3i2.1862.
- 399 27. Geys, J.; Nemmar, A.; Verbeken, E.; Smolders, E.; Ratoi, M.; Hoylaerts, M.F.; Nemery, B.; Hoet, P.H.M. Acute Toxicity and  
400 Prothrombotic Effects of Quantum Dots: Impact of Surface Charge. *Environ. Health Perspect.* **2008**, *116*, 1607–1613,  
401 doi:10.1289/ehp.11566.
- 402 28. Pons, T.; Pic, E.; Lequeux, N.; Cassette, E.; Bezdetsnaya, L.; Guillemin, F.; Marchal, F.; Dubertret, B. Cadmium-Free  
403 CuInS<sub>2</sub>/ZnS Quantum Dots for Sentinel Lymph Node Imaging with Reduced Toxicity. *ACS Nano* **2010**, *4*, 2531–2538,  
404 doi:10.1021/nn901421v.
- 405 29. Robe, A.; Pic, E.; Lassalle, H.P.; Bezdetsnaya, L.; Guillemin, F.; Marchal, F. Quantum Dots in Axillary Lymph Node Mapping:  
406 Biodistribution Study in Healthy Mice. *BMC Cancer* **2008**, *8*, 1–9, doi:10.1186/1471-2407-8-111.
- 407 30. Liu, W.; Choi, H.S.; Zimmer, J.P.; Tanaka, E.; Frangioni, J. V; Bawendi, M.G. Compact Cystein-Coated CdSe(ZnCdS) QDs  
408 for in Vivo Applications. *J. Am. Chem. Soc.* **2008**, *129*, 14530–14531, doi:10.1021/ja073790m.Compact.
- 409