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The effect of "Jelly" CdTe QD uptake on RAW264.7 monocytes: immune responses and cell fate study

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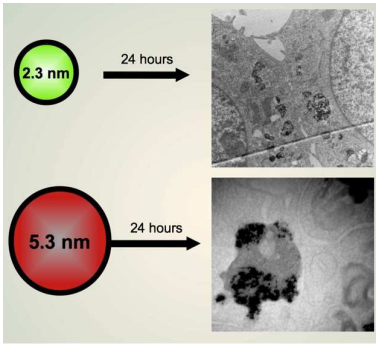
Graphical abstract



Abstract

Encapsulation of quantum dots (QDs) has become an essential factor for defying particle cytotoxicity while at the same time providing physical and chemical stability. Negatively charged cellular membranes have a great affinity to nanoparticles with surface molecules carrying positive charge, hence creating perfect conditions for fast and aggressive intracellular penetration. The preference of non-charged outer shells is topical in QDs design and various applications. In the current paper we develop gelatination as a prominent coating approach to create neutrally passivated QDs with improved biocompatibility. We have revealed the trends in particle's uptake, accumulation, intracellular localisation and retaining time as well as RAW264.7 monocyte cell fate and immune responses. Also the difference in particles endocytosis kinetics and dynamics has been shown to depend on QD core size. The intracellular QD content along with cell responses on population level were quantified by flow cytometry.

Key words: quantum dots, RAW264.7 cell, nanoparticles quantification, QDs uptake, nanotoxicity, TEM

Introduction

The quantum dots (QDs) are small semiconducting nanoparticles which are composed of a few hundred atoms; that leads to quantum confinement effects, high surface-to-volume ratio and consequently to their exceptional optical sensitivity and reactivity [1-4]. The exposure of a high number of core atoms to the surface of the quantum dot frequently results in leakage by ions from the particle core and associated free radical formation. Thus non-coated nanoparticles are not suitable for any biological applications due to their low compatibility to physiological media conditions and irregularities in optical parameters. Several strategies have been applied to reduce QD cytotoxicity including: incorporation in micelles and covering with polymers (TOPO, PEG), proteins (albumin), amino-acids and sulphur-containing compounds (TGA).

Gelatination has been explored as an effective approach to significantly increase particle biocompatibility without reducing its quantum yield and fluorescence intensity [5]. The surface of "Jelly" CdTe QDs has a mixture of functional groups (e.g. amino, carboxyl, mercapto-groups, etc) due to the nature of gelatin – it consists of fragmented peptides of dehydrolysed collagen, therefore it doesn't have a regular structure. As a result, gelatinated QDs can be linked to biomolecules (proteins, antibodies, oligonucleotides, drugs) by multiple paths [6,7].

Macrophages serve as antigen-presenting cells (APCs) expressing CD80 and CD86 receptors belonging to the B7 superfamily of the genes. These two bio-molecules (also known as B7-1 and B7-2) play an important role in T-cell activation by providing co-stimulatory signals. T-cell promotion requires either the presence of T-cell receptors (TCR) or the ligation of CD28 molecules. However binding of CD152 (or so-called CTLA-4, cytotoxic T-lymphocyte antigen-4) opposes T cells initiation. At first sight it appears that there is no difference between CD80 and CD86 molecules: they are complimentary to the same ligands, expressed by the same cell types and have the same functions. The distinct behaviours of these two proteins affect T-cell fate. CD86 has a higher dissociation/association ability and shorter activation time, and it preferably binds to CD28

ligands. CD80 has more affinity to CD152 receptors, but it is expressed on macrophages after CD86 triggering. It is intriguing that although a quicker CD86-CD28 interaction results in enhanced T-cell activation, the opposite pair, CD80-CD152, has a higher affinity, hence its amplified silencing effect [8].

RAW264.7 murine macrophage-like cell line has been employed in a number of studies due to its quick doubling time, efficacy in internalizing, comparatively easy activation, good host quality for transfection, and expression of essential set of inflammatory proteins (IL-6, IL-10, TNF-a) and surface receptors (CD80, CD86). These adherent cells have monocyte morphology with the potential to be promoted to macrophages under certain conditions, for example being challenged by lipopolysaccharides (LPS); or being in the presence of mannose containing antigens or TLR. This cell line enables a broad use in *in vitro* biomaterial trials for investigating all kinds of cell-material interactions including: cell covering adhesion, cell growth, cell detachment, mitochondrial and proliferation activity, and immune and mitosis profiling [9,10]. Alternatively, the RAW264.7 cell line can be considered as a reasonable first approach for examining nanoparticle fate when injected in blood stream, following bio-imaging and final cleavage.

Materials and methods

QDs synthesis

CdTe QDs were synthesised according to a previously published procedure [11]. Briefly, Al_2Te_3 reacted with sulphuric acid to produce H_2Te gas which was bubbled through an aqueous solution of $CdCl_2$, thioglycolic acid (TGA) and 0.3g of gelatin, with pH buffered at 11. The molar ratio of Cd:Te:TGA was 1:0.25:1.4. The reaction mixture was then heated under reflux for 2 to 48 hours depending of the desired nanoparticle size. Narrow size distribution fractions were collected via size-selective precipitation using isopropanol.

UV-vis and PL spectra

Absorbance was examined on a Shimadzu UV-1601 spectrophotometer; distilled water was taken as a baseline. PL spectra were recorded on a Cary Eclipse spectrometer. All measurements were performed to characterize the optical properties of the nanoparticles obtained.

Cell culture

RAW 264.7 murine macrophages cell line was used in this study. Cells were cultured in Dulbecco's Modified Eagle Media (DMEM; Sigma), supplemented with 10% Foetal Bovine Serum (FBS; Sigma), 100 µg/mL of penicillin and 100 µg/mL of streptomycin. Macrophages were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Transmission Electron Microscopy (TEM)

Monocytes were seeded onto thermanox films (13 mm diameter) in 24 well-plate. The seeding density was 50,000 cells per well. The cells were cultured for 24 hours; after that, conditioned culture media was replaced by fresh media containing QDs in appropriate concentration. Cells were further incubated in presence of nanoparticles for next 12 or 24 hours as desired. Cells were fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium. Fixed samples were introduced to

ethanol gradient and embedded into low viscosity resin (TAAB, Berks, England). The obtained blocks were trimmed and proceeded to fine section cut. Samples were imaged at Hitachi H7000 transmission electron microscope.

ds-DNA Quantification

Quant-iT Pico Green dsDNA Assay Kit was used for a precise counting cell number in the probe. The cells were seeded in a 24-well plate to a density of $1x10^5$ cells per well, 24 hours prior to experiment. Different types of QDs (either TGA or TGA-gelatin-covered) within a range of concentrations (1-100 nM final concentration) were added to macrophages. After 24 hours of co-incubation, the cells were progressed to PicoGreen assay according to protocol.

Annexin V Apoptosis Assay

In this assay cells were seeded to a density of $2.5x10^5$ cells per well in 6 well-plates. After 24 hours of culture, appropriate amounts of QDs were added to each well. Control samples remained untreated. Cells were co-incubated with or without nanoparticles for 12 or 24 hours. Samples were harvested on the day of analysis. Briefly, the reduced media was removed and the cells were washed twice with phosphate buffered saline (PBS). Macrophages were harvested by pipetting in fresh media and then were placed in eppendorf tubes. Cells were washed twice with PBS immediately after harvesting, re-suspended in 500 µl buffer and stained with viability dye according to protocol. Afterwards cells were washed with serum-containing buffer. Finally, cells were prepared and stained with Annexin V Apoptosis Assay Kit (eBioscience) and directly proceeded to flow cytometry. All measurements were performed on BD FACS Canto A fitted with 2 lasers (blue, 488 nm; red, 633 nm) and 6 available colours. Unstained cells, single-stained samples, and cells treated with QDs only (without further staining) were used as quality controls.

QDs uptake and CD80/86 surface markers expression

Flow cytometry was used to detect the amount of internalized nanoparticles and to measure the expression of pro-inflammatory receptors caused by exposure to QDs. All measurements were performed on BD FACS Canto A. In this experiment cells were seeded into 6-well plates to a density of $2.5x10^5$ cells per well and left 24 hours to adhere. The next day, macrophages were loaded with red or green gelatin coated QDs within a range of concentrations (1-100 nM final concentration). After 12 hours of treatment (for the CD86 study) and 24 hours (for the CD80 study), the probes proceeded to the assay according to a standard protocol. Armenian hamster IgG and Rat IgG2a K were used as isotype controls for CD80 and CD86, respectively. All antibodies and isotype controls were purchased from BioLegend. The standard staining protocol recommended by manufacturer was employed. APC and FITC channels were used as references for signal detection. FlowJo software was used for interpretation of results.

Quantification of QDs

The amount of ingested QD nano-crystals was defined by FlowJo software. At least 10,000 events were recorded per tube. Consistent macrophage population was selected from light scatter graph, the level of fluorescence in FITC channel was evaluated from a histogram plot; the geometric mean value was used quantitatively as a statistical parameter. The percentage of population of interest was found from the overlay of two histograms of cells treated with QDs and untreated controls in the reference channel.

Statistical analysis

Two-tailed unpaired t-test has been used to evaluate statistical significance of the results. The experiments were compared to control group. The results were recognised as statistically significant if p-value is less than 0,05; they're marked with asterisk symbol (*) in the graphics. All p-values can be found in Table 1 Supplementary Information.

Results

Physico-chemical properties of QDs

As-obtained nanoparticles have been fully characterized. Table 1 shows main properties of QDs. Both batches have 29 nm Stokes shift and similar negative surface charge.

Sample name	Absorbtion, nm	Emission, nm	Core size, nm	Zeta-potential, mV	Standard deviation of zeta-potential
Green Gel	515	546	2.7	-61.7	2.1
Red Gel	600	629	3.7	-52.3	1.4

Table 1. Optical properties of green and red TGA-gelatin capped CdTe QDs.

ds-DNA quantification by PicoGreen

Only exposure to highest concentration (100 nM) had affected cell viability (Fig.1). The number of cells was reduced to 36-40% compared to untreated cells after 24 hours of co-incubation. It should be noticed, that the results of test reflect number of live cells in the sample on the day of acquisition, regardless nanoparticle internalising. Cell doubling time should be added to contributing factors. Exposure to low concentrations (1 and 10 nM) did not affect cell viability.

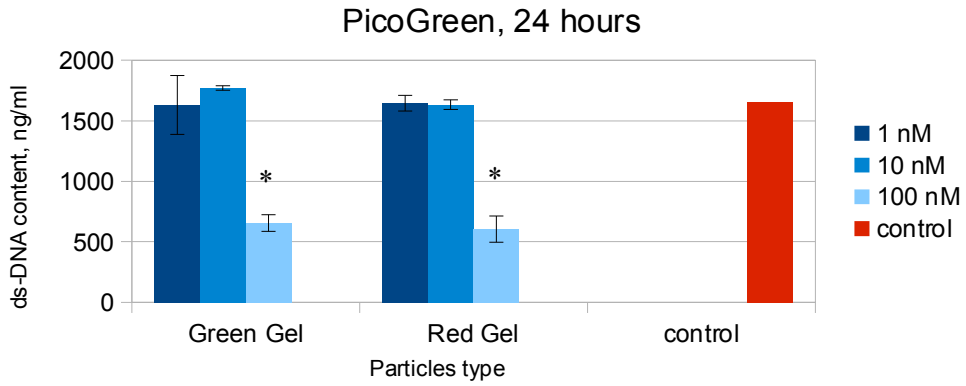
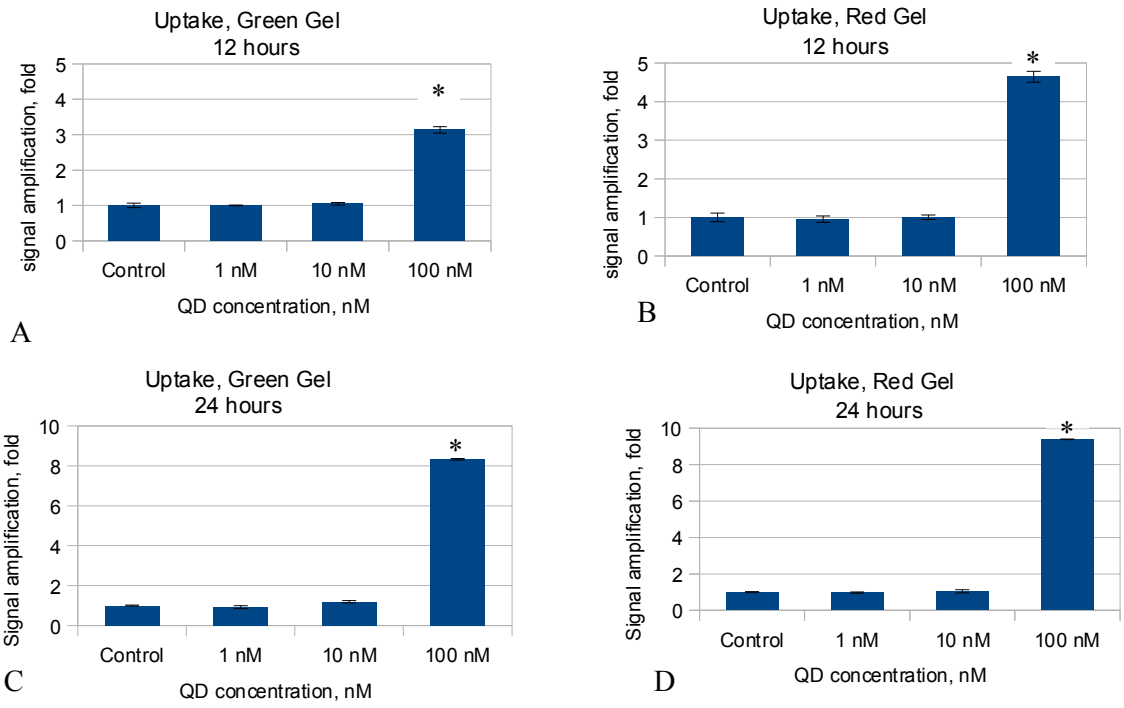


Fig. 1 ds-DNA content of RAW264.7 cells treated with red and green gelatinated QDs in various concentrations (1-100 nM) for 24 hours. Exposure to low concentrations (1 and 10 nM) did not affect cell viability. Only 100 nM dose reduced number of cells to 30%. All experiments were done in triplicate.

QD uptake evaluation by flow cytometry

Flow cytometry was employed as quick and simple acquisition method for nanoparticle ingestion. It allows to quantify intracellular amount of QDs on population level by measuring change in fluorescence and light scatter pattern in each individual cell. It's been shown that either red or green QDs are accumulating in monocytes over time (Fig. 2); the signal intensity in reference channels has doubled from 12 to 24 hours. The fluorescence response is higher for red QDs. As it was shown in PicoGreen assay, cell number has not been affected when treated with low doses. We speculate



that a threshold should be achieved to promote cellular reaction on introduced species [12,13].

Fig. 2 The uptake rate of green and red gelatinated QDs after 12 (A,B) or 24 (C,D) hours of co-culture with RAW264.7 cells. In both cases the overall dynamic remains same as observed at 12 hours – high response from 100 nM treated cells, the signal amplification is directly proportional to time of exposure. All experiments were done in triplicate.

CD80/CD86 surface molecules expression

Monocytes are professional phagocytic cells aimed to ingest and destroy foreign bodies or trigger further signaling and consequent T cell activation. Change in CD80/86 surface markers expression evokes inflammatory pathway cascades and activation to macrophage. The markers levels were measured in respect to nanoparticle treatment. Fig. 3 depicts results of experiment. Both markers were significantly down-regulated when cells are introduced to 100 nM concentration regardless QD size (5.3 nm for red and 2.3 nm for green). Compared to control cultures, the levels of CD86 were elevated (20-40%) in case of red QDs ($p<0.005$) when treated with 1 and 10 nM.

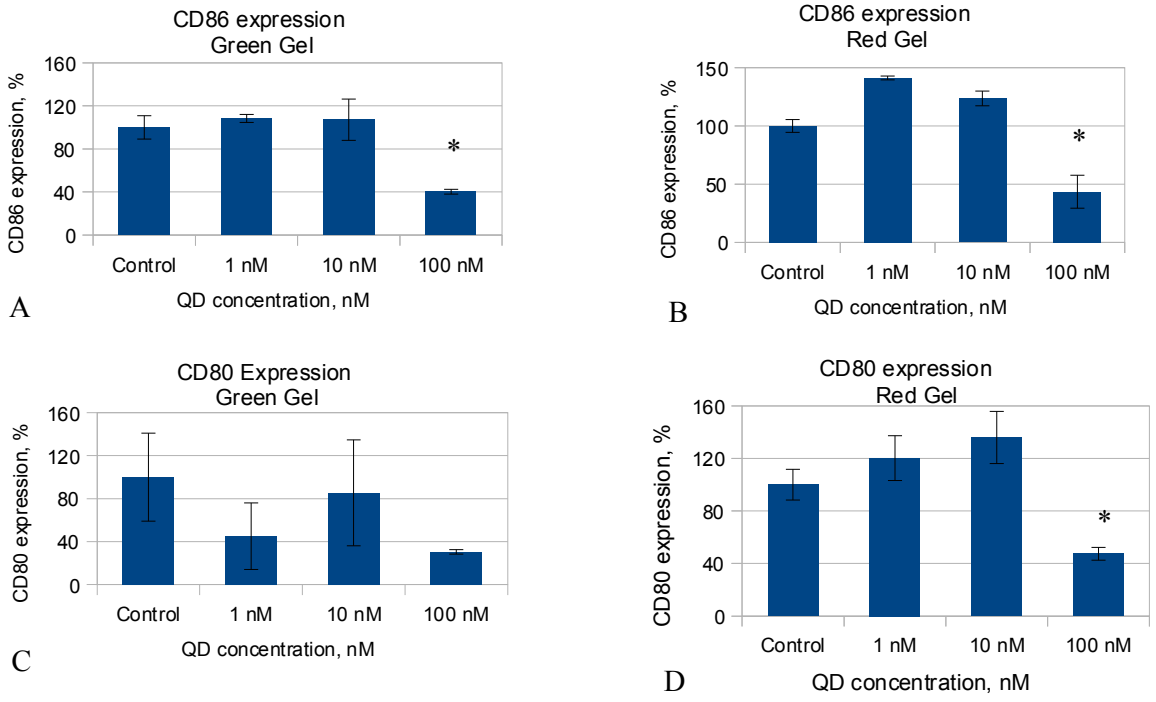


Fig. 3 The expression profile of pro-inflammatory surface molecules. **A,B:** CD86 expression profile for the monocytes treated with red and green gelatinated QDs for 12 hours. The marker undergoes drastic decrease when cells exposed to 100 nM dose due to high level of cell death and apoptotic response. In most cases, lower concentrations did not affect the surface markers profile. However, 1 and 10 nM of red gelatinated QDs have elevated CD86 expression; but down-regulation effect at 100 nM is more pronounced. **C,D:** CD80 expression profile for the monocytes treated with red and

green gelatinated QDs for 24 hours. Due to similar function, both CD80 and CD86 behave alike what confirms that expression "shutdown" is related to cell damage caused by high dose of QDs, without triggering inflammatory reaction as expected. All experiments were done in triplicate.

Electron microscopy

Vesicles with trapped QDs are found within cytoplasm; cells maintain healthy morphology despite uptake. Fig. 4 shows obtained TEM images. No obvious hallmarks of apoptosis or necrosis were observed. The nucleus is smooth, chromatin is not condensed. The only difference between control and treated cells is increased number of endosomes. However, the number of cells in the sections is much smaller than analysed in any other assay.

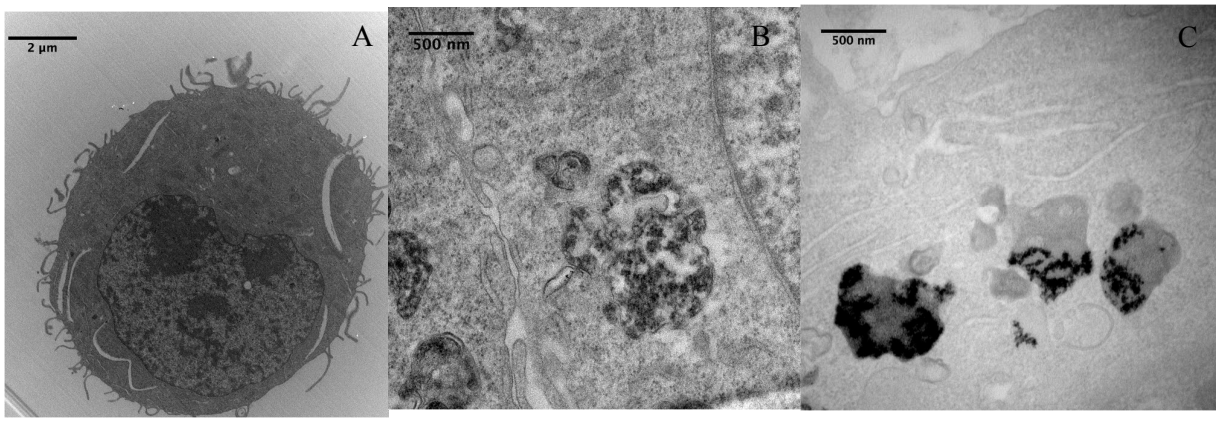


Fig. 4 TEM images of untreated monocytes (A), cells treated with 100 nM red (B) and green (C) gelatinated QDs. More images are provided in Supplementary Information.

Annexin V apoptosis test

The conditioned cell cultures were examined for prevalent cell fate. Annexin V detection kit was employed to distinguish live, apoptotic and necrotic stages. The exposure to 1 or 10 nM of nanoparticles did not show any deviations from control at any time points. The picture has drastically changed when monocytes received 100 nM QDs. Uptake rate did not change for green gelatin coated QDs. The intracellular content of red particles has increased twice from 12 to 24

hours and reached over 90% (Fig. 5 A-D). Then, this green or red positive subset was divided into 4 quadrants to quantify viable, early apoptotic, late apoptotic and necrotic cells according to annexin V/viability dye staining. (Fig. 5 E-H). Majority of cells treated with green QDs remained alive either at both control observations. Number of healthy cells also was constant. When cells were co-cultured with red nanoparticles, they mainly appeared to be dead by necrosis pathway.

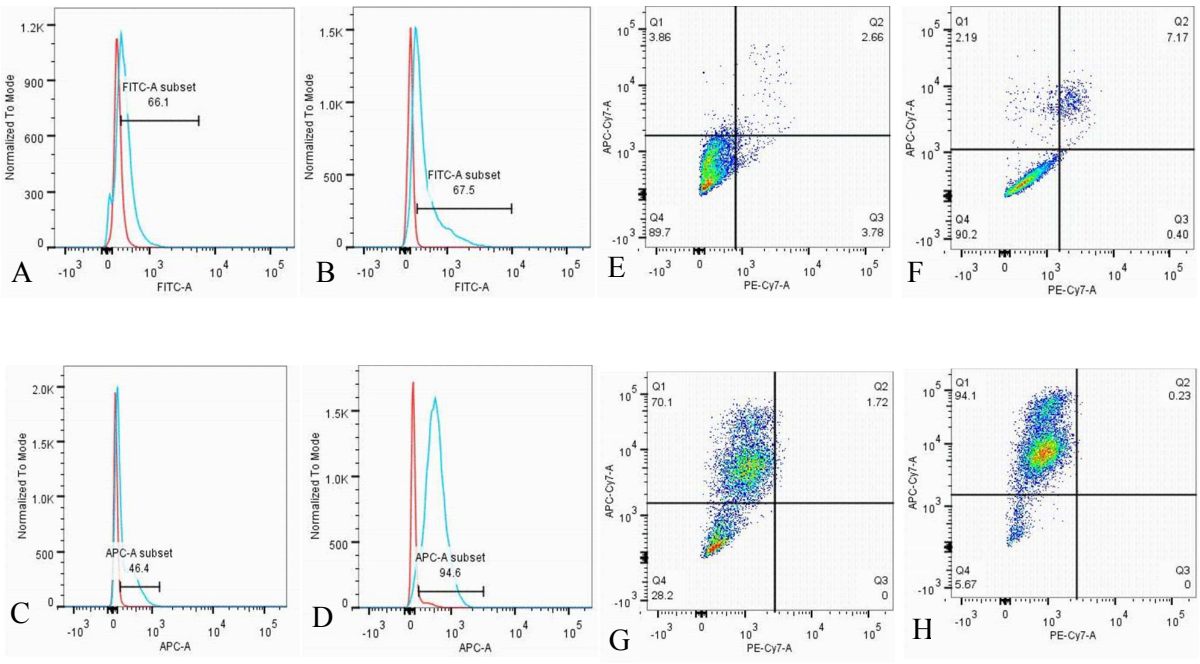


Fig. 5 Uptake rate of green (A,B) and red (C,D) gelatinated QDs incubated with RAW264.7 monocytes for 12 (A,C) or 24 (B,D) hours. Red line in histograms is untreated control, blue line is the experiment (cells exposed to QDs). **A-D:** X-axis is common logarithm of fluorescent intensity in reference green FITC (A,B) or red APC (C,D) channel. Y-axis is frequency of data distribution. **E-H:** observed apoptosis/necrosis profile after exposure to green (E,F) or red (G,H) gelatinated QDs for 12 (E,G) or 24 (F,H) hours. X-axis is common logarithm of fluorescence intensity in Annexin V corresponding channel, Y-axis is common logarithm of fluorescence intensity in viability dye reference channel. Cellular subsets: **Q1:** necrotic, **Q2:** late apoptotic, **Q3:** early apoptotic, **Q4:** live cells.

Discussion

In our work we used serum-containing culture media to emulate physiological media conditions. The formation of protein corona is one of the key events defying further cell-particle interaction and can't be neglected [14-17]. To reveal potential effects caused by long exposure to QDs, continuous cell culture is required, which is not feasible in serum-free environment. Wang [18] showed that ingestion pathway, rate and cytotoxicity are not the same once serum proteins are involved. Rapid intake of small amounts of QDs, by cells other than professional phagocytes, have been shown in number of studies [18-21]. Pulse co-incubation (typically up to few hours) with bare nanoparticles in solution was performed, with an excellent outcome in terms of high rate of targeting with no or very little cell death. This system is a good first approach for efficacy evaluation. However “real life” cell targeting and drug delivery have more complex routes than direct cargo-target contact.

The drastic difference in uptake kinetics pattern is exemplified in Fig. 5. For green-emitting nanoparticles the uptake rate hasn't changed from 12 to 24 hours (66 and 67% respectively), or number of viable cells (93 and 90%). The histogram peak shifts to the right (higher fluorescence), proving QD accumulation over time. As it was described by Aberg [22], in continuous exposure to nanoparticles the internalising is heterogeneous process and depends on phase of cell cycle. According to their study, intracellular amount of nanoparticles can be ranked as G2/M> S>G1 [22,23]. Apparently, in long cultures (longer than one cell division cycle) with neutral nanoparticles two processes are competing: accumulation and export. In the case of toxic species a third parameter is contributing, namely, cell death and subsequent nanoparticle release to media. According to that, the diagram tail (Fig. 5B) is the signal from cells accumulated QDs (dividing cells) and the rest of histogram represents average response from cells in S/G1 phase. Small amount of cells goes through apoptotic (5.2 and 7.2% at 12 and 24 hours) or necrotic pathway (1.7 and 2.2% respectively).

The uptake heterogeneity concept is in striking agreement with results of experiment involved red-

emitting nanoparticles. If we assume, that only in one phase cells phagocyte QDs most, then after first 12 hours (full cell cycle) it has largest amount of nanoparticles. Taking that cytotoxicity is cumulative parameter, thus this subset is likely to die. In next 12 hours necrotic cells are eliminated from the system. The remaining cells are proceeding through proliferation cycle again and accumulate more QDs. It results in strong fluorescence intensity peak shift to the right and increase in number of dead cells.

QD size contributes to the uptake dynamics. Chitrani and Chan [24, 25] have found preferred QD size for efficient ingestion. This has been explained by the dependence of the wrapping time on the diameter of primary vesicle when loaded with nanoparticles. According to the study, optimal diameter for spherical particles is 50 nm. This result was confirmed by Osaki [26]. Nevertheless, when the core size of QDs does not exceed 10 nm, the protein corona increases hydrodynamic size up to hundreds nanometers. Similarly, Wang [14] and Jiang [27] suggested that if only large clusters of nanoparticles are formed locally, ingestion might occur. Apart from that, the mechanism and parameters defying uptake are still under discussion. It has been agreed that uptake is an energy dependent process for particles with core size 5 nm and above; smaller dots can be transported passively [18]. Red-emitting QDs enter the cells via clathrin-mediated route [21,27,28], however, Zhang and Monteiro-Riviere [20] have found caveolae/lipid raft as endocytosis mechanism via G protein receptor pathway and low-density lipoprotein (LDL)/scavenger receptor. Also there's no solidarity in the questions whether surface coating/charge [20,21] influence the uptake or makes no difference [28,29]. And is it more important than hydrodynamic size or not [30]. The observed contradictions may be related to different cell types used in the experiments.

Conclusion

In the current study we investigated behaviour of gelatin coated QDs in serum-containing conditions and their interaction with the cells in continuous cultures. Following earlier research,

suggesting distinct pattern rather than in protein-free media [18], it was confirmed elevation of toxic dose (100 nM over 10 nM in previous studies) and different cellular responses to exposed dots in various sizes (2.7 and 3.7 nm) for a time greater than the cell cycle. Our results suggest heterogeneity in the pace of uptake depends on the cell cycle phase. Unlike pulse treatment, where QDs were co-incubated for short time and particles captured regardless cell cycle phase, in our study it's one of the contributing parameters in endocytosis kinetics.

Only 100 nM concentration is considered to affect cell function. Surprisingly, surface marker expression levels have dropped down to less than 50% from control. Either green or red QDs drastically decrease cell number at 100 nM concentration. In case of red QDs massive cell death via necrosis was observed; this occurred with twice the uptake rate at the 24 hours acquisition point (from 50 to almost 100%). Overall, both QD types tend towards an intracellular occupancy and have a longer retention time when compared with less passivated particles. The ingested nanoparticles form conglomerates and are trapped into endosomes, clearly observable in cytoplasm (Fig. 4).

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