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Electroinduced Delivery of Hydrogel Nanoparticles in Colon 26 Cells, Visualized by Confocal Fluorescence System

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Abstract. *Background: Nano-scale drug delivery systems (nano-DDS) are under intense investigation. Nano-platforms are developed for specific administration of small molecules, drugs, genes, contrast agents [quantum dots (QDs)] both in vivo and in vitro. Electroporation is a biophysical phenomenon which consists of the application of external electrical pulses across the cell membrane. The aim of this study was to research electro-assisted Colon 26 cell line internalization of QDs and QD-loaded nano-hydrogels (polymersomes) visualized by confocal microscopy and their influence on cell viability. Materials and Methods: The experiments were performed on the Colon 26 cancer cell line, using a confocal fluorescent imaging system and cell viability test. Results: Electroporation facilitated the delivery of nanoparticles in vivo. We demonstrated increased voltage-dependent delivery of nanoparticles into cells after electrotreatment, without significant cell viability reduction. Conclusion: The delivery and retention of the polymersomes in vitro is a promising tool for future cancer treatment strategies and nanomedcine.*

The theranostic strategy has become an important element of cancer treatment as it helps to develop anticancer therapeutics based on imaging and therapy. In this concept of future cancer treatment, nano-platforms have been introduced as nanobiomaterial-based formulations. Nano-scale drug delivery systems (nano-DDS) are currently under active investigation in different fields of science (biomedical applications of pharmacology, bioengineering, medicine). Nano-platforms are being developed for specific administration of small molecules, drugs, genes, contrast agents and peptides both *in vivo* and *in vitro*. The selective disposition of nano-carriers into the target tissue is an essential issue in drug delivery. The achievement of a high local drug concentration, minimal drug leakage during transfer, biocompatibility and reduced toxicity are among other important characteristics of nano-DDS.

The preparation of nano-hydrogels combines the advantages of nano-hydrogel as a soft polymeric material and nano-particulate systems. Nano-hydrogels are a promising class of nanoparticles with adjustable properties, capable of encapsulating both hydrophilic and hydrophobic molecules (drugs or contrast agents).

Quantum dots (QDs) in the near-infrared spectra are of great interest for *in vivo* biological imaging, diagnostics and possible therapeutics due to separation from autofluorescence background and emission of light through thick tissues (1). Semiconductor QDs are recognized as advantageous imaging contrast agents for bright infrared emission over molecular fluorophores (2, 3). The unique optical properties of QDs can be used for optimization of the signal-to-background ratio, improving the sensitivity of fluorescence detection, and for increasing of the quality of fluorescent deep tissue imaging *in vivo*. In addition, QDs are extremely photostable, thus making them suitable for long-term observations or repeated measurements (4). Despite the great potential of QDs for *in vivo* studies, they possess some disadvantages: their relatively short circulation half-life in blood vascular system due to their rapid uptake and accumulation in the liver, and

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potential cytotoxicity due to the heavy metals constituting the QDs. Clearance from the body predominantly through the kidney is prerequisite for the approval for clinical use of any contrast agent. An appropriate organic coating of QDs can optimize their biodistribution, as well as clearance, and minimize their potential cytotoxicity (5).

Electroporation is a biophysical phenomenon which consists of the application of external short high-voltage electrical pulses across the plasma cell membrane, in order to increase its natural permeability. This phenomenon is associated with the creation of reversible pores as a result of the applied electrical field. Currently this technique is used for enhanced delivery of chemotherapeutic drugs in clinical skin cancer treatment (6).

Recently, we reported passive and electro-assisted delivery of QD-loaded nano-hydrogel particles to solid tumors visualized by fluorescence imaging and magnetic resonance imaging in colon cancer-grafted mice as a model (7-11).

In our previous studies, the QDs were used as a tool for tracing of pharmacodynamics and target-specific accumulation of nano-hydrogels using different imaging techniques. We considered nano-hydrogel particles to be very promising for *in vivo* applications. Investigating further on the celluIar level, we designed this study aiming to facilitate and document cellular uptake of QD-loaded nano-hydrogels into the colon cancer cell line Colon 26. We continued our studies in the field of nanotechnology by delivery, localization and visualization of QD-loaded nano-hydrogels.

The aim of this study was to research electro-assisted Colon 26 cell line internalization of QDs and QD-loaded nano-hydrogels visualized by confocal microscopy and their influence on cell viability.

Materials and Methods

Chemicals. QD^{705} (Qdots@705 ITK™ non-tagged quantum dots) were purchased from Invitrogen (Carlsbad, CA, USA). Ammonium persulfate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tetra-armed star PEG linker (PEG-4AC) was prepared as described in our previous report (11).

Nano-hydrogel. In order to prepare QD^{705}@Nanogel, 200 μI of PEG-4AC (100 mg/ml) were mixed with 100 μl of 2 M QD^{705}, 50 μl of 0.1 M APS, and 25 ml of 0.1 M TEMED in that order and then the mixture was stirred for 20 min. After the reaction, the mixture was filtered by Vivaspin 6-30 K (Sartorius, Goettingen, Germany) at 6000 rpm for 3 min, at 4˚C. The purification procedure was repeated three times. The size of nanoparticles was analyzed by dynamic light scattering (DLS). The average size of the nanoparticles was 174 nm. They were almost electroneutral and stable in serum even after 24 h incubation at 37˚C (11).

Cells. Cell line Colon 26 (CLS, Eppelheim, Germany), established *in vitro* from the Colon 26 tumor of female mice, was used as a model of colon carcinoma. The cell line was grown as monolayer (RPMI-1640 medium, supplemented with 2 mM L-glutamine, 10% fetal bovine serum) at 37˚C in an incubator with humid atmosphere and 5% CO_2. Cells were passaged two times weekly by trypsinization.

Electroporation. An electroporator Chemopulse IV (developed at the Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Science, Sofia, Bulgaria), generating bipolar pulses, was used in the experiments (6). The instrument is equipped with a wide-range voltage control in the limits of 100-2,200 V, simplified operations, locking against illegal manipulations and enhanced protection against electrical hazards. The electrotreatment was carved out using 16 bipolar, rectangular pulses, each of them 50+50 μs duration with 20 μs pause between both phases and a pause between bipolar pulses of 880 μs. In each experiment parallel stainless steel electrodes were used. The intra-electrode distance was 1 cm. In this study, an electric pulse with intensity of 200 V/cm; 500 V/cm and 1000 V/cm were applied.

Electroporation protocol. Colon 26 cells (1×10^5 cells per well) were seeded 24 h before electroporation. QDs or QD-loaded nano-hydrogels at different concentrations (10 nM and 20 nM) were added immediately before pulse delivery. For fluorescent microscopy experiments, the cells were cultivated on cover glasses.

After the electrical treatment, 990/980 μl (respectively) RPMI-1640, supplemented with 10% fetal calf serum was added to each sample. The controls were treated under the same conditions, but without electrical pulse application.

Cell viability assay. The viability of Colon 26 cells was determined by MTS test [MTS, Owen's reagent: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; (Promega, Madison, WI, USA)]. The MTS test was applied after treatment of cells with electrical pulses with or without QD705/nanosomes. To evaluate the statistical significance of the reduction in cell viability, a comparison between exposed and control probes was performed by Student's *t*-test. *p*-Values lower than 0.05 were considered statistically significant.

In vitro imaging. For the *in vitro* imaging, Colon 26 cells were grown on cover glasses. Twenty-four hours after treatment, cells were fixed by 4% paraformaldehyde. The cover glasses were then mounted onto microscope slides using mounting media. Imaging analysis was performed via laser scanning confocal microscopy (Leica DM 2500; Leica Microsystems, Wetzlar, Germany). The excitation wavelength was 405 nm, and the collected fluorescence channels were 690-720 nm.

Results and Discussion

From our previous *in vivo* studies (11), we know that the embedding of a hard material such as QDs in nano-hydrogels changes their physical properties (size, negative charge and shape) and affects their tumor penetration. Application of external electrical stimuli (electroporation) facilitated the delivery of the nano-hydrogels to the tumor tissue.

The first part of this study was designed to compare the influence of the passive and electro-assisted delivery of the QD-loaded nano-hydrogels on cell viability using MTS test.

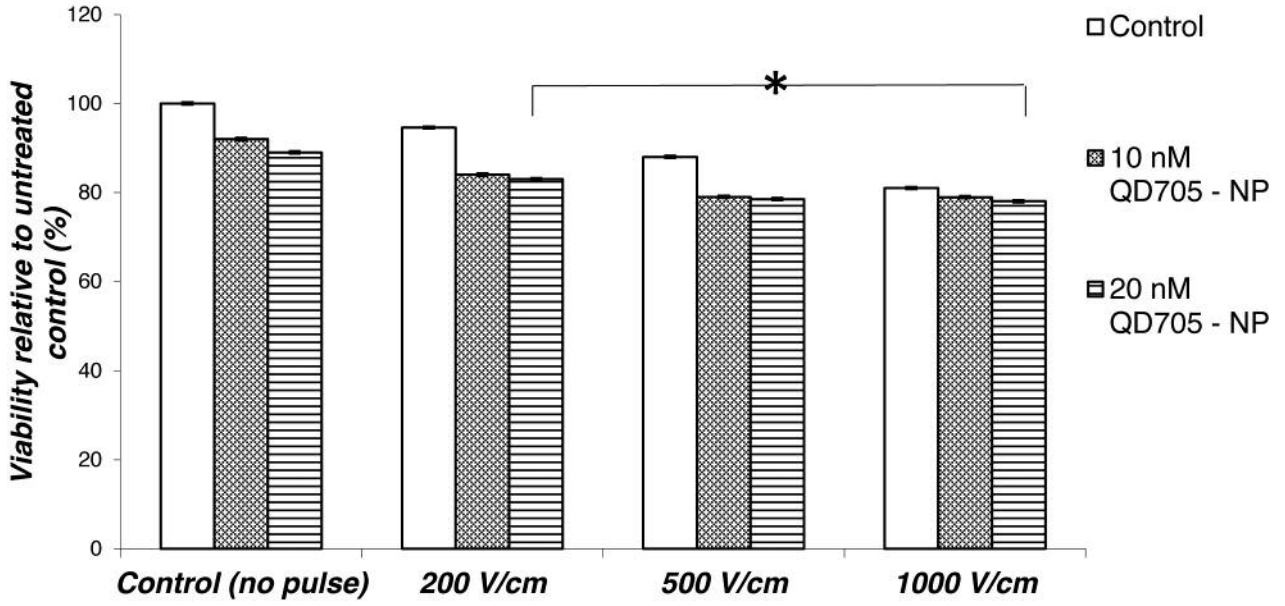

Figure 1. *Cell viability of Colon 26 cells 24 h after treatment with two different concentrations of QD[705]-loaded nanoparticles (NP) (10 nM and 20 nM) with and without electroporation at 200, 500, 1,000 V/cm (the data are averaged from three independent experiments). *Statistically significant ($p<0.05$) by Student's t-test.*

In order to assess theranostic possibilities of gel nanoparticles, firstly their uptake capacity into target cells (Colon 26), passively or under the influence of an electric field, should be determined; secondly whether the nanoparticles and QDs themselves have a cytostatic or cytotoxic effect on the cell line should be evaluated.

The results were obtained 24 h after treatment with and without electroporation (Figure 1). The data demonstrated that application of electrical pulses with different intensity (200 V/cm; 500 V/cm and 1,000 V/cm) affected the viability of Colon 26 cell line with at least 20% reduction at 1,000 V/cm. On the other hand, additional reduction (about 10%) was observed after treatment with nanosomes loaded with QDs at two selected concentrations (10 nM and 20 nM). The toxicity of QDs is associated with their physicochemical properties. It is known that the Invitrogen QD[705] have reduced cytotoxicity (12), which is why the reduction of cell viability after treatment with QD[705]-loaded nano-DDS is comparable with that after electrotreatment. The embedding of QDs into the nanosomes and their subsequent internalization into cells can be used as a marker for internalization of nanosomes without significant influence on cell viability.

The selective disposition of nanocarriers into the target tissue is an important issue in drug delivery. As we recently showed, QD-labeled nanosomes are capable to be introduced into the tumor tissue or to be used in lymph node mapping (10, 11).

We evaluated the efficiency of internalization of QDs and QD-containing nanoparticles by confocal microscope. The method of fluorescence microscopy has a greater sensitivity.

It is well known that short high-voltage electrical pulses cause transient and reversible pore formation. In our study, we used electroporation to enhance QD delivery through the formed pores.

The internalization efficiency of QDs alone is shown in Figure 2; with increasing applied voltage from 200 V/cm to 1,000 V/cm (Figure 2C-E), the intensity of the fluorescence increased significantly.

Cell internalization of QD-loaded nanoparticles is shown in Figure 3. Passive diffusion was found to be very low (Figure 3B), but the fluorescence strongly increased by applying of electroporation with intensity from 200 V/cm to 1,000 V/cm (Figure 3C-E). A reduction in the number of cells in the case of treatment of Colon 26 cancer cells with 1,000 V/cm in the presence of QD-loaded nanosomes is the reason for the decreasing intensity (Figure 3).

As shown in Figure 2B and Figure 3B, a low degree of passive diffusion of both QDs and QD-labeled nanosomes was apparent. In our study, we used electroporation to facilitate nanosome internalization into the Colon 26 cancer cell line.

We show convincing evidence of the low cytotoxicity of nanosomes applied at two different concentrations and elevated cellular internalization after application of electroporation both QDs and QD-labeled nanosomes.

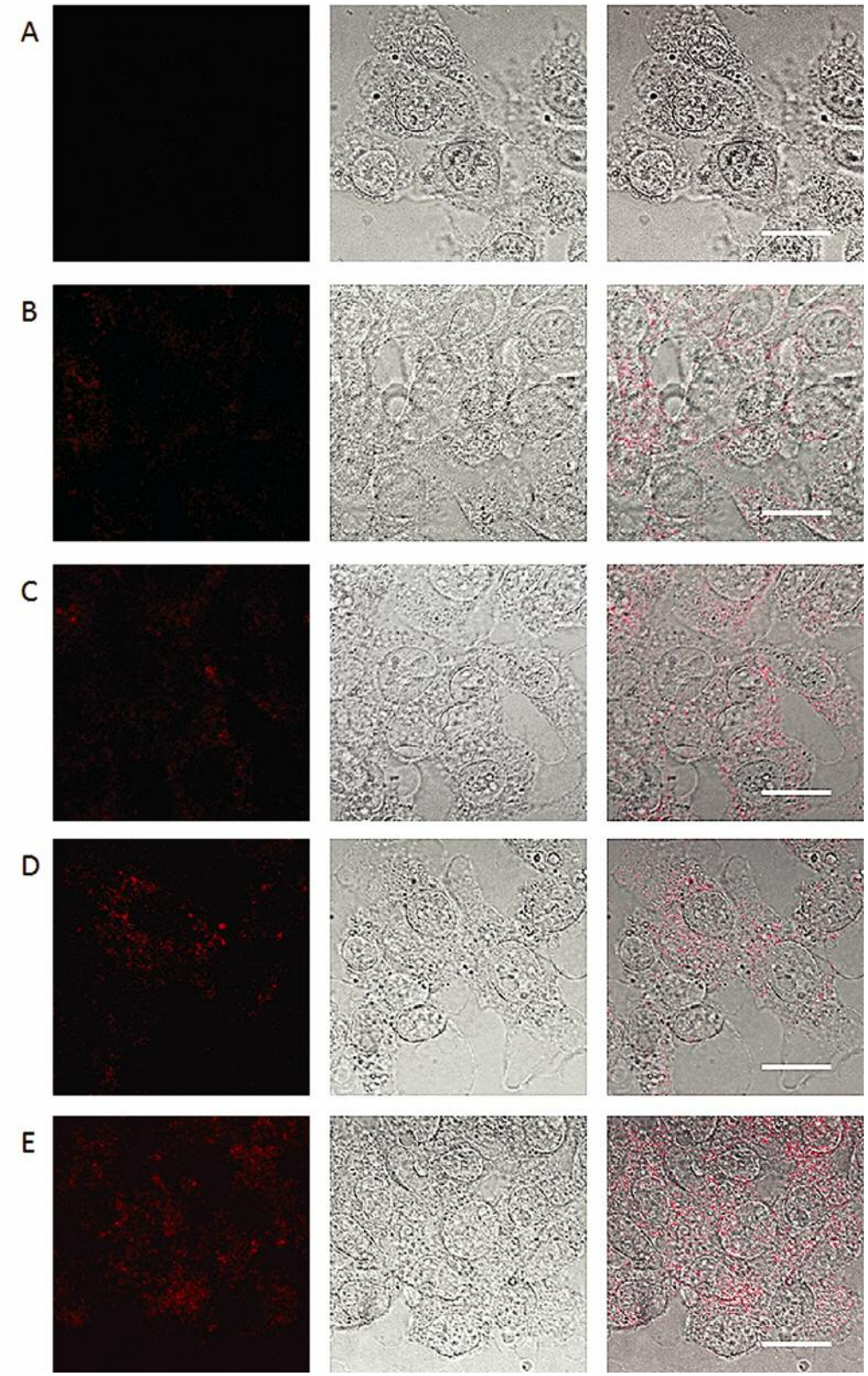

Figure 2. *Variation of efficiency of internalization of 10 nM QD^{705} in Colon 26 cancer cells with and without electroporation as evaluated by means of confocal microscopy. A: Control, untreated cells, B: cells + 10 μl QD^{705}, C: cells + 10 μl QD^{705} + 200 V/cm, D: cells + 10 μl QD^{705} + 500 V/cm, E: cells + 10 μl QD^{705} + 1,000 V/cm. Scale bar=100 μm.*

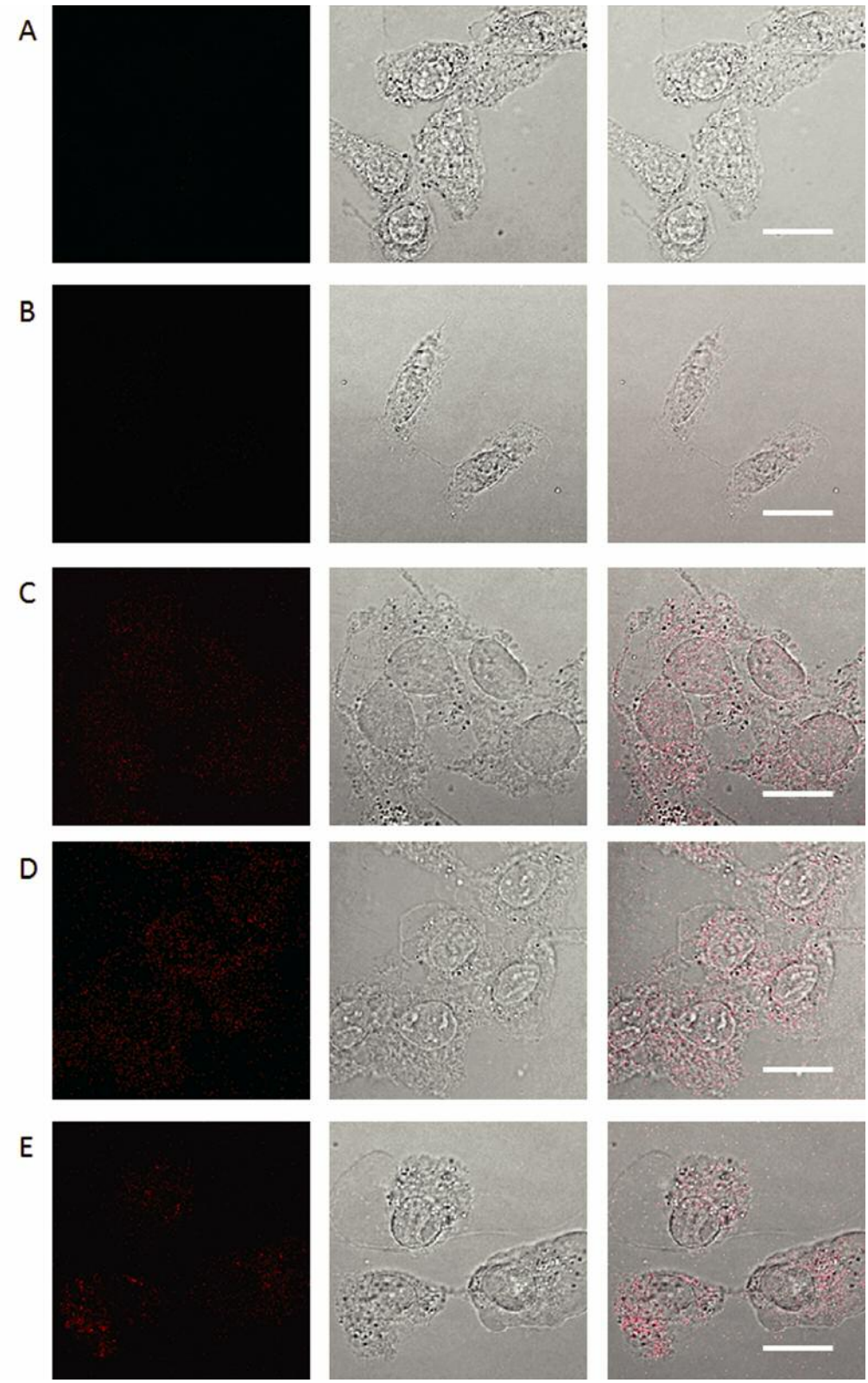

Figure 3. *Variation of efficiency of internalization of 10 nM QD^{705}-loaded nanoparticles (NP) in Colon 26 cancer cells with and without electroporation evaluated by means of confocal microscopy. A: Control, untreated cells, B: cells + 10 µl QD^{705}-NP, C: cells + 10 µl QD^{705}-NP + 200 V/cm, D: cells + 10 µl QD^{705}-NP + 500 V/cm, E: cells + 10 µl QD^{705}-NP + 1,000 V/cm. Scale bar=100 µm.*

One possible mechanism for passive cellular uptake of the nanosomes is endocytosis. In this case, surface charge and zeta potential of nanoparticles greatly influenced the internalization efficiency (13-15). Nanosomes used in the present study are of elongated shape and are almost electroneutral (11). This fact contributes to easier entry of the QD-loaded nanosomes into the target cells. The presence of specific receptors for mediation of penetration process, surface groups, charge, the functionalization layer *etc*., are also of great importance. Apart from the nanosomic properties, cell type, cell incubation media and temperature can influence the uptake process (12). The toxicity of QDs is associated with their physicochemical properties. A major cause for QD toxicity could be the induction of reactive oxygen species, which in most cases cause cellular changes leading to DNA damage. The embedding of the QDs in nanosomes prevents aggregation of QDs in the cytosol, reduces their cytotoxicity and makes internalization into cells more efficient (16).

With results presented in our study, we contribute to clarification of the process of uptake of nanoparticles into individual cells and to assessing the efficiency of electroporation for introducing perzistent (up to 24 h) nanoparticles into Colon 26 cancer cells.

The growing number of studies on nanosomes, including both *in vivo* and *in vitro* experiments, demonstrates that this technique is a new and valuable possibility in diagnostics and therapy. The applied electric field benefits internalization of the nanosomes without significant reduction in cell viability. We expect that the molecules other than QDs (anticancer drugs for example) can be incorporated and delivered into target cells by means of electroporation.

With our study, we directly demonstrated the delivery and retention for up to 24 h of the nanosomes, loaded with contrast agent QD^{705}, into the cancer cells. Electroporation is a promising biophysical approach for administration of therapeutic nanomaterials and could become a tool for future cancer treatment strategies.

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