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Chapter

Hypotoxic Fluorescent Nanoparticles Delivery by Cell-Penetrating Peptides in Multiple Organisms: From Prokaryotes to Mammalians Cells

Betty Revon Liu, Yue-Wern Huang and Han-Jung Lee

Abstract

Nanotechnology is the study of materials in the nanoscale. By its nature, nanotechnology is interdisciplinary. Nanotechnology has made a significant stride in recent two decades in various industries. Numerous nanomaterials are devised for biomedical applications which include intracellular tracking and labeling, gene detection and hybridization, tumor or tissue targeting, pharmaceutical therapies, pathogenic inhibiting, and medical instrument coating for disinfections. High photostability and quantum yield of fluorescent nanoparticles are ideal for long-term monitoring of molecular events in living organisms. Here, we discuss delivery of three fluorescent nanoparticles in A549 cells, rotifers, Gram-negative bacteria, Gram-positive bacteria, and archaea. As these nanoparticles cannot enter cells, arginine-rich cell-penetrating peptides (CPPs) were used to enhance their internalization at the cellular or organismal level. The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay and sulforhodamine B (SRB) assay demonstrated that CPP complexed fluorescent nanoparticles did not produce lethal effect in all organisms tested. The discussion of these nanomaterials in this chapter intends to broaden our understanding of their biocompatibility in organisms of various hierarchical levels.

Keywords: fluorescent nanoparticles, cell-penetrating peptides, hypotoxicity, rotifer, prokaryotes

1. Introduction

Nanoscience and nanotechnology are fast growing multidisciplinary fields in the past two decades [1]. Nanomaterials are the foundation of devices and systems in various industries that revolutionize functionalities of end-user products [2]. Nanomaterials range from simple zero-dimensional structures such as nanodots [3], wire-like nanocomposites in one-dimension nanoscales [4], to two-dimensional nanosheets, and to three-dimensional structures [5, 6]. Furthermore, anisotropy and unique nano level physical and chemical properties can result in nanomaterials of the same elemental compositions having totally different functionalities [6, 7].

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Nanomaterials have contributed to various biomedical applications, including molecular labeling and tracking, DNA/RNA/proteins probing, tumor or tissue targeting, drug delivery and therapies, pathogenic intervention, and biomedical imaging [2, 8–10]. In general, nanomaterials are classified into four categories: carbon-based nanomaterials, metal and metal oxide related nanomaterials, organic-based nanopolymers, and composite nanomaterials with complicated structures [11]. Carbon-based nanomaterials such as fullerenes, carbon nanotubes (CNTs), and graphenes have been used as tissue scaffolds, biosensors, targeted drug delivery, and cosmetic additives [10, 12]. The studies and applications of metal- or metal oxide-nanocomposites are commonly found in toxicology, cancer therapies, and antimicrobial infections [13–15]. Organic-based nanomaterials such as liposomes, micelles, microemulsions, and dendrimers are mainly used in pharmaceuticals and drug delivery systems [16]. These organic nanopolymers can be combined with metal or carbon-based nanoparticles for controlled release of drug delivery and antitumor targeting [11]. There is an alternative classification of nanomaterials based upon their applications: drugs and medications, manufacturing and materials, the environment, electronics, energy harvest, and mechanical industries [17]. The first category can be further divided into cell specificity enhancement [18, 19], drugs, peptides, genes, vaccine delivery [16, 18, 20–25], diagnostics and imaging [2, 24, 26–28], anti-infectious agents or germicides [15, 21, 29], cancer therapy [24, 28–31], tissue/organ/tumor targeting [19, 24–26, 28, 31], scavengers for free radical or thrombosis [32, 33], DNA/RNA/PNA sensor and sequencing [34, 35], and angiogenesis inhibition [36, 37]. Toxicities and health implications become a dominant issue, even though most nanomaterials are hypotoxic for cell/tissue separation or identification, pharmaco-therapeutic molecules delivery, diagnostics, as well as imaging [2, 24–26, 28].

In this chapter, we focus on fluorescent nanomaterials which are used as a probe for detection, biomedical imaging, and diagnosis. Their properties and applications in different species will be discussed; their toxicity to the test organisms will be evaluated. Mammalian cell lines, rotifers, Gram-negative bacteria, Gram-positive bacteria, and archaea are the choices of our topic. The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) and sulforhodamine B (SRB) assays are indicative of cytotoxicity.

1.1 Fluorescent nanoparticles

Bioimaging is increasingly popular and important because of its noninvasive, dynamic, and real-time properties. Fluorescent molecules play a pivotal role in optical imaging of life science research and biomedical applications. Fluorescent probes can be used to detect RNA and DNA; analyze proteins, hormones, and viral antigens; and identify organelles, specific proteins, tissues, or tumors via antibody conjugation [38]. However, traditional fluorescent probes made by organic compounds have a lot of room for improvement such as sensitivity, fluorescent intensity, water-solubility, and photo-bleaching [38]. Another critical issue is their asymmetric excitation spectrum with a long tail leading to significant overlapping with other fluorescent dyes [38]. Various fluorescent nanomaterials have high quantum yield and permanent fluorescence with the potential to 1 day replacing the traditional probes [39]. Advantages of fluorescent nanoparticles include, but not limited to, tunable in both sizes and compositions, ultra-bright with narrow spectrum of emission but broad spectrum of excitation, resistant to chemical degradation and photobleaching for long-term observation [40, 41]. Semiconductor nanoparticles such as CdSe/ZnS quantum dots (QDs) are the best example in bioimaging, diagnostics, and therapeutic molecules delivery [42]. Additional fluorescent nanomaterials have been developed to serve as bioimaging probes. We describe some popular fluorescent nanoparticles as follows.

1.1.1 Cadmium-based QDs

Cadmium-based QDs are the first colloidal semiconductor which started the new era of nanotechnology in bioimaging [43]. Extreme brightness and sharp peak of emission wavelength are their signature properties [40–43]. High stability in the biological environment makes them applicable for long-term bioimaging [40–43]. The versatile surface modifications of their outer shells greatly expand their functionalities and biocompatibilities [40–43].

1.1.2 Indium-based QDs

Indium phosphate/zinc sulfate (InP/ZnS) QDs which belongs to the III-V groups of semiconductors replace the toxic cadmium core as a new generation of nanoparticles in biomedical imaging [44]. InP/ZnS QDs modified with PEG-containing negative charges enable them to interact with cationic peptides [44]. PEG modification also increase stability in the aqueous environment. These new complexes entered cells via endocytosis without toxicity [44].

1.1.3 Graphene quantum dots (GQDs)

Graphene is a two-dimensional sheet structure with single-atom thickness. GQDs have attracted plenty of attention due to their low toxicity, water solubility, high stability, stable emission spectrum, better surface grafting, and high electrical and thermal conductivity [45]. There are a variety of synthesis methods for GQDs that can control the shape, size, and yield [45].

1.1.4 Carbon dots (CDs)

The fluorescent property of carbon dots were found accidentally [39]. CDs are low cytotoxic, eco-friendly, and highly biocompatible. These features allow them to overtake cadmium-cored QDs in bioimaging [39]. Safety studies have shown that they neither enter nucleus and damage chromosomes nor accumulate in mice bodies [39].

1.1.5 Zirconium porphyrinic metal-organic framework nanoparticles (ZrMOF)

Metal–organic frameworks (MOF) combined the inorganic and organic materials and the distinguished features are tunable pore size, high surface areas, and alterable internal surface properties. MOFs do not emit fluorescence, but can be loaded with organic dyes for biomedical sensing. Quenching and therefore became the target-induced sensor [46]. ZrMOF nanoparticles can be unquenched in the target site, thereby facilitating the effect of photodynamic cancer therapies [46].

1.1.6 Soft fluorescent nanomaterials

Soft fluorescent nanomaterials include dye-doped polymer, semiconducting polymer, organic-complex nanoparticles, micelles, and nanogels [47]. This group of fluorescent nanomaterials is a complicated convention. For example, semiconductor QDs are composed of V-III or VI-II groups of elements in their cores and shells, but they can also be functionized by organic materials via chemical linkage on the surface [41, 44]. Modified QDs are classified as soft fluorescent nanomaterials while FDA approved molecules are used and these complexes are fabricated under a mild condition [47].

1.2 Biocompatible enhancer for nanoparticle delivery

Most organic and inorganic nanomaterials including the nanoparticles of the six categories described above are either hydrophobic or water-insoluble, which means they are difficult to be delivered into cells or organisms. Lipid-derived or peptide-based modifications of shells on the surfaces of nanoparticles enhance the biocompatibility and increase the efficiency of cellular uptake [48, 49]. The routes of cellular deliveries in these nanoparticles functionized by lipid-derived or peptide-based linking chains are related to endocytosis [50, 51]. Antibody-functionalized nanoparticles are advantageous in targeting [51]; however, lysosomal degradation of the delivered drugs or bioactive molecules was a major concern. Several strategies have been developed to circumvent this issue. Among them are cell-penetrating peptides (CPPs) [52–54].

CPPs (a.k.a., PTDs), are proteins that possess the ability to penetrate cell membranes. In recent decades, they have attracted immense popularity in delivering genes, bioactive macromolecules, and drugs due to their effect intracellular translocation. The first CPPs were identified in the human immunodeficiency virus type I (HIV-1) transcriptional activator Tat which consists of 11-amino acids (YGRKKRRQRRR) [55, 56]. Later, various natural CPPs were found in many organisms and numerous synthetic CPPs were designed [57]. To date, there are more than 1700 CPPs in the databank [58]. CPPs can be classified into three categories: cationic, amphipathic, and hydrophobic [57]. Modifications such as cyclization, branches, D-form alteration, and non-primary amino-acid utilization have been performed to improve their transduction efficiency [59–62]. The mechanisms by which CPPs internalized have been vigorously studied. Numerous studies suggest that depending on the physicochemical properties and secondary structures of CPPs, energy-dependent endocytosis or direct membrane translocation is employed [52, 57]. Four major endocytoses are: clathrin-dependent, caveolae-dependent, clathrin and caveolae-independent, and macropinocytosis [57]. Entry of molecules via these four pathways is trapped in lysosomes and their bioactive and therapeutic characteristics would be lost in the low pH environment [63]. CPP-mediated direct membrane location is an option for delivering drugs and other bioactive molecules [52, 53, 64].

CPPs can interact with cargoes in a covalent, noncovalent, or covalent and noncovalent protein transduction (CNPT) manner. Cargoes are various including proteins, DNA, siRNA, and semiconductors QDs [44, 64–68]. CPP-mediated cellular uptake can be found from prokaryotic to eukaryotic organisms including mammalian cells, insect cells, aquatic microorganism, yeasts, plant tissue, mice dermis, Gram-negative and Gram-positive bacteria, and archaea [53, 65, 69–73]. Uptake mechanisms vary depending upon peptide sequences. IR9 consists of the INF7 fusion peptide and nona-arginine CPP. IR9 without taking any cargoes penetrated cell membranes via macropinocytosis. However, when IR9 was mixed with DNAs or QDs, classical endocytosis was utilized [74]. HR9 contains nona-arginine in the center, flanks with five histidines on either side, and caps with a cysteine in both ends. Out study showed that HR9-mediated cellular entry involves direct membrane translocation [53, 75].

PR9 which consists of nona-arginine and a penetration accelerating peptide sequence has been used to deliver ODs. The complexes entered cells by classical endocytosis [54]. Subsequently, the PR9/QD complexes escaped from lysosomes and entered nucleus [54]. The fluorescent quantum yield and complexes properties were unaltered indicating that CPP/QD complexes were suitable for long-term intracellular imaging and tracking [54, 77, 78].

In the following sections, we discuss biocompatibility of CPP-mediated delivery in various systems including mammalian cells, rotifers, Gram-negative bacteria, Gram-positive bacteria, and archaea.

2. Cell viability in mammalian cells

In our studies, human bronchoalveolar carcinoma A549 cells were used as a model cell line to investigate CPP-mediated uptake of inorganic fluorescent nanoparticles which are CdSe/ZnS QD with green fluorescence, InP/ZnS emitted green fluorescence, and CdSe/ZnS QD with red fluorescence. Synthetic nona-arginine (named SR9) CPP were premixed with these three QDs respectively and incubated

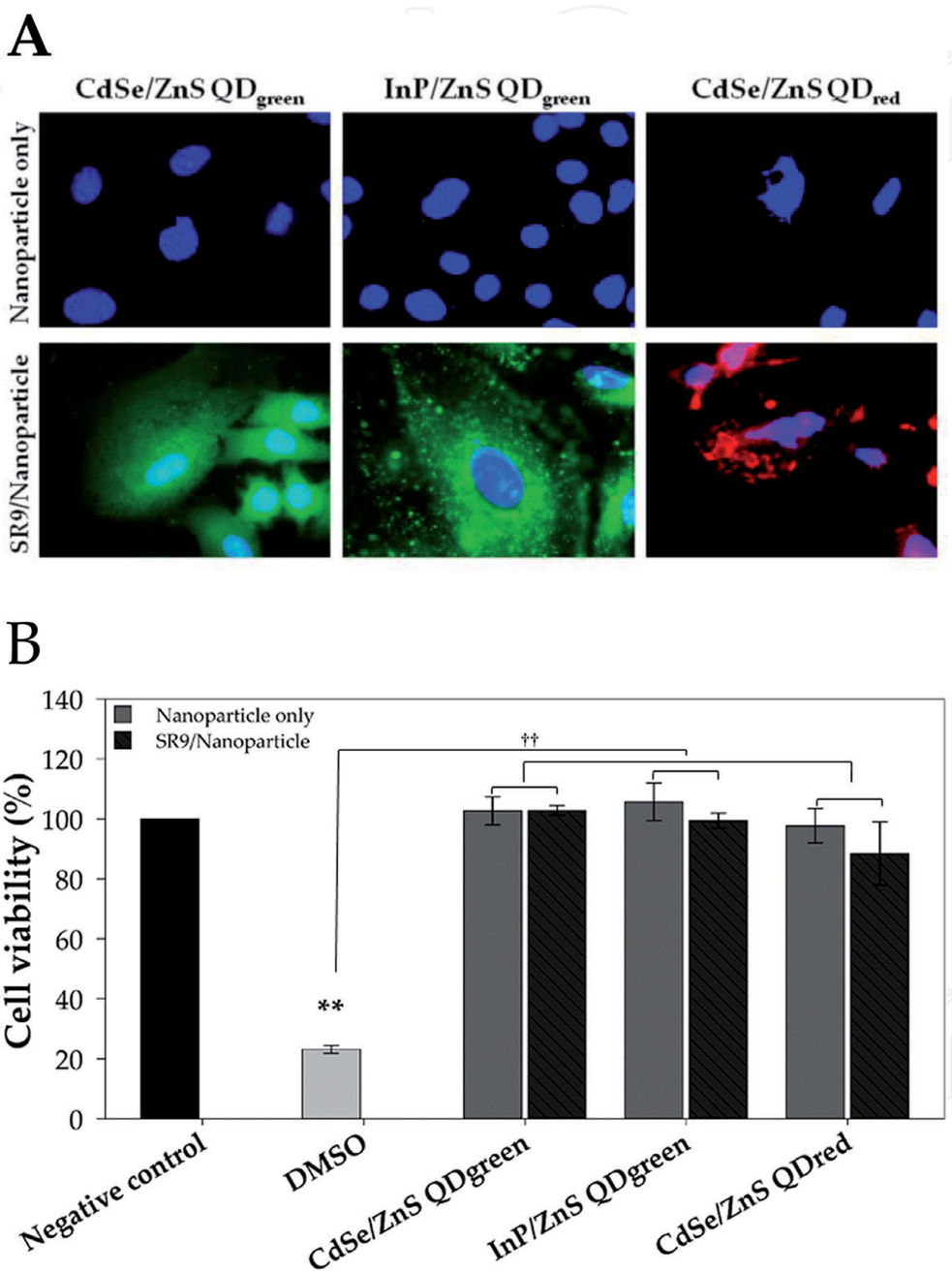


Figure 1.
*Semiconductor nanoparticles treatments in the mammalian A549 cell line. (A) Penetrations of various semiconductor nanoparticles and CPP/nanoparticles complexes in mammalian cells. A549 cells were treated with green fluorescent CdSe/ZnS QD, green fluorescent InP/ZnS QD, red fluorescent CdSe/ZnS QD, SR9/CdSe/ZnS QD_{green} complexes, SR9/InP/ZnS QD_{green} complexes, and SR9/CdSe/ZnS QD_{red} for 1 h at 37°C, respectively. Protein transductions were recorded using a BD pathway 435 system. Green and red fluorescence revealed the distribution of nanoparticles, and blue fluorescence indicated the nuclei. Images were taken at a magnification of 600×. (B) Cell viabilities in A549 cells treated with either nanoparticles alone or CPP/nanoparticles complexes. Cells were treated as previous description shown in (A) and the SRB assay was performed for cytotoxic analysis. Cells without any treatments and treated with 100% DMSO were served as the negative and positive groups, respectively. Histogram of cell viability was represented by mean ± SD from three independent experiments in each treatment group. Significant differences at $P < 0.01$ (**,††) are indicated [44, 53, 78].*

with A549 cells for 1 h followed by Hoechst 33342 nuclear staining. Internalizations of QDs and SR9/QD complexes were determined using confocal microscopy. Rare green fluorescence emitted from CdSe/ZnS QD_{green} and InP/ZnS QD_{green} as well as red fluorescence from CdSe/ZnS QD_{red} were observed while cells were treated with these semiconductors alone (**Figure 1A**). However, strong green and red fluorescence were observed in the groups of SR9-mediated QDs delivery, which meant SR9 facilitated the internalizations of nanoparticles (**Figure 1A**).

To understand toxicity of these CPP-QDs complexes, the SRB assay was conducted for viability analysis. A549 cells were treated with the materials for 24 h and then stained with SRB. Cells without any treatment or with 100% DMSO served as a negative control or a positive control, respectively. Cell viability of CdSe/ZnS QD_{green}, InP/ZnS QD_{green}, CdSe/ZnS QD_{red}, and SR9-modified CdSe/ZnS QD_{green}, InP/ZnS QD_{green}, and CdSe/ZnS QD_{red} complexes did not differ from the negative control (**Figure 1B**). Collectively, semiconductor fluorescent nanoparticles and their CPP-modified complexes did not reduce cell viability.

3. Survival rate in rotifers

Rotifers are non-arthropoda, metazoan aquatic invertebrates with a completed digestive systems. They form the basis of the microzooplankton community in the plankton food web and link the energy flow to higher organisms. Recently, a growing number of studies considers rotifers as an indicator of marine pollution and toxicity of plastic nanoparticles [79–81], as well as a model species for pharmaceutical and toxicological studies [80, 82]. To test the toxicity and uptake efficiency of fluorescent nanoparticles, *Brachionus calyciflorus* were treated with CdSe/ZnS QD_{red} and IR9-FITC mixed CdSe/ZnS QD_{red} complexes respectively (**Figure 2**). Low red fluorescent intensity was detected in rotifers which meant CdSe/ZnS QD_{red} were difficult to enter rotifers without any help. Contrarily, CdSe/ZnS QD_{red} enter rotifers easily by forming complexes with IR9-FITC (**Figure 2A**).

To investigate potential cytotoxicity of CPP-associated quantum dots on rotifers, the MTT assay was performed (**Figure 1B**). *Brachionus calyciflorus* were treated with CdSe/ZnS QD_{red} alone, IR9-FITC alone, and IR9-FITC/QD_{red} complexes for 24 h. Rotifers without treatment served as a negative control, while rotifers treated with 100% DMSO as a positive control. Hypotoxicity was observed in the QD_{red}, IR9-FITC, and IR9-FITC/QD_{red} complexes groups (**Figure 2B**). In contrast, DMSO significantly reduced the survival of rotifers (**Figure 2B**). Collectively, CPP-mediated cellular entry of quantum dots resulted in relatively harmless in rotifers.

4. Hypotoxicity shown in prokaryotic organisms

Microorganisms are regarded as a vital member in the ecosystem as they play an important role in the natural recycling, elements and energy transforming, and environmental balancing of living materials [83, 84]. Disruption of microorganisms cause reduction of microbial diversity and obliquely influence our natural world [85]. Prokaryotic organisms are major microorganisms; the prokaryotic domain include bacteria and archaea [70, 86, 87]. Here, *Arthrobacter ilicis* D50–1 (Gram-positive bacteria), *Escherichia coli* DH5α (Gram-negative bacteria), and *Thermus aquaticus* (archaea) were studied for protein transduction and cytotoxicity. They were treated with Cd-core green semiconductor nanoparticles; hardly any green fluorescence detected (**Figure 3A**). Bright green fluorescence was observed in the SR9-mediated uptake of CdSe/ZnS QD_{green} in all three organisms (**Figure 3B**).

Toxicological studies of nanomaterials on prokaryotic organisms are important. Bactericidal nanomaterials can affect nonpathogenic bacteria leading to imbalance of a microbiome community and, to the greatest extent, ecological disasters [88]. The toxicity of CdSe/ZnS QD_{green} and SR9-modified QD_{green} complexes were studied using archaea, Gram-positive bacteria, and Gram-negative bacteria (**Figure 3B**). Organisms were treated for 1 h at room temperature. The MTT assay showed no reduction of viability

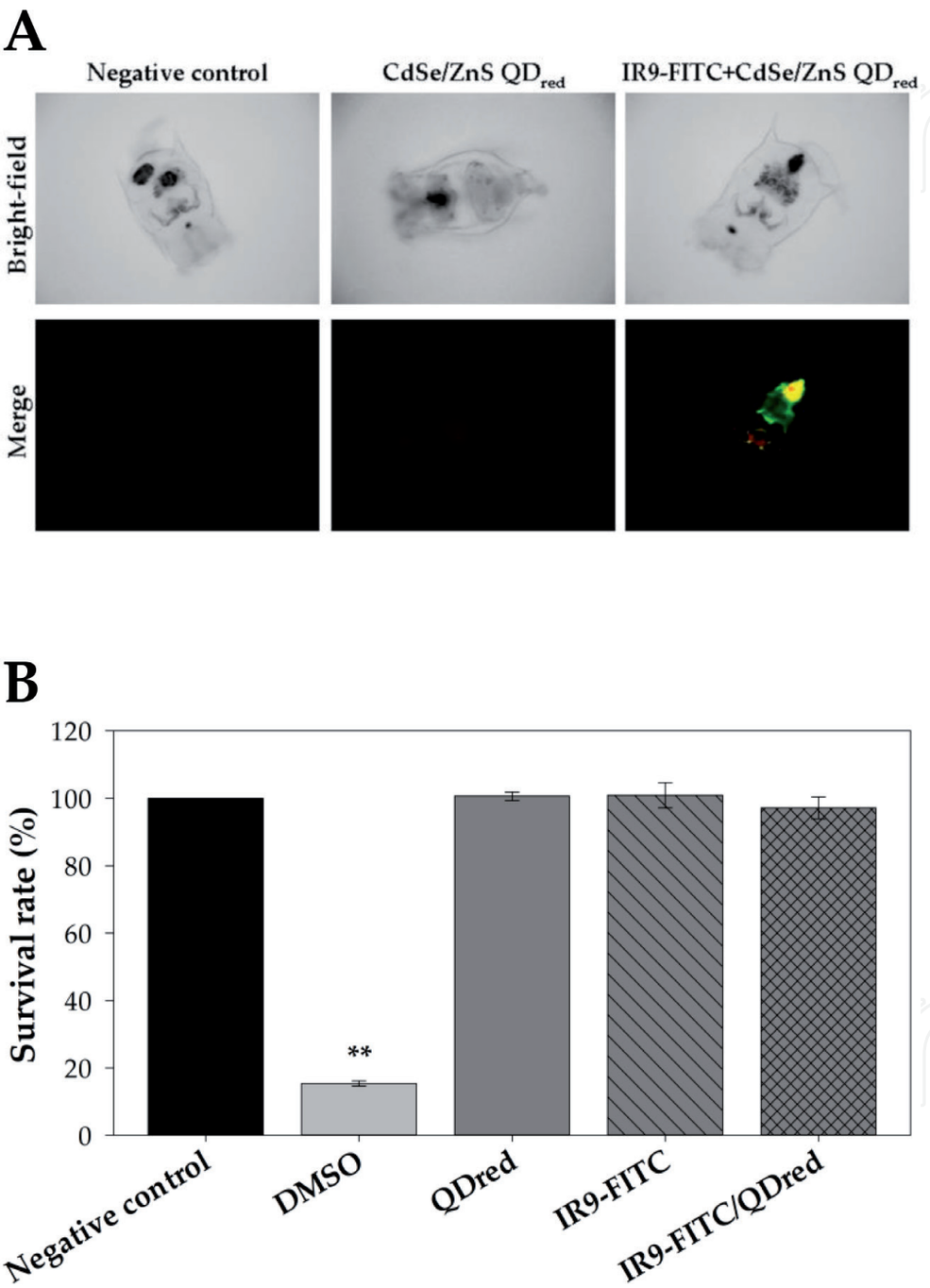


Figure 2.
Semiconductor nanoparticles treatments in the rotifer (Brachionus calyciflorus). (A) Protein transduction in rotifer treated with semiconductor nanoparticles CdSe/ZnS QD_{red} and IR9-FITC carpeted QD_{red} complexes. Rotifers were treated with QD_{red} alone or IR9-FITC/QD_{red} complexes for 1 h at 25–28°C. Green fluorescence referred to IR9-FITC and red fluorescence indicated $QD_{red.}$ Merged fluorescent images and bright-field images were recorded at a magnification of 200× using a BD pathway 435 system. (B) Histogram of rotifer survival rate. Rotifers were treated with CdSe/ZnS QD_{red} alone, IR9-FITC alone, and IR9-FITC/QD_{red} complexes, respectively and the survival rate was analyzed by MTT assay. Rotifers treated with water and 100% DMSO were served as negative and positive controls, respectively. Each treatment group was compared with the negative control. Significant differences at $P < 0.01$ (**) were indicated. Data were presented as mean ± standard deviation from three independent experiments in each treatment groups [74].

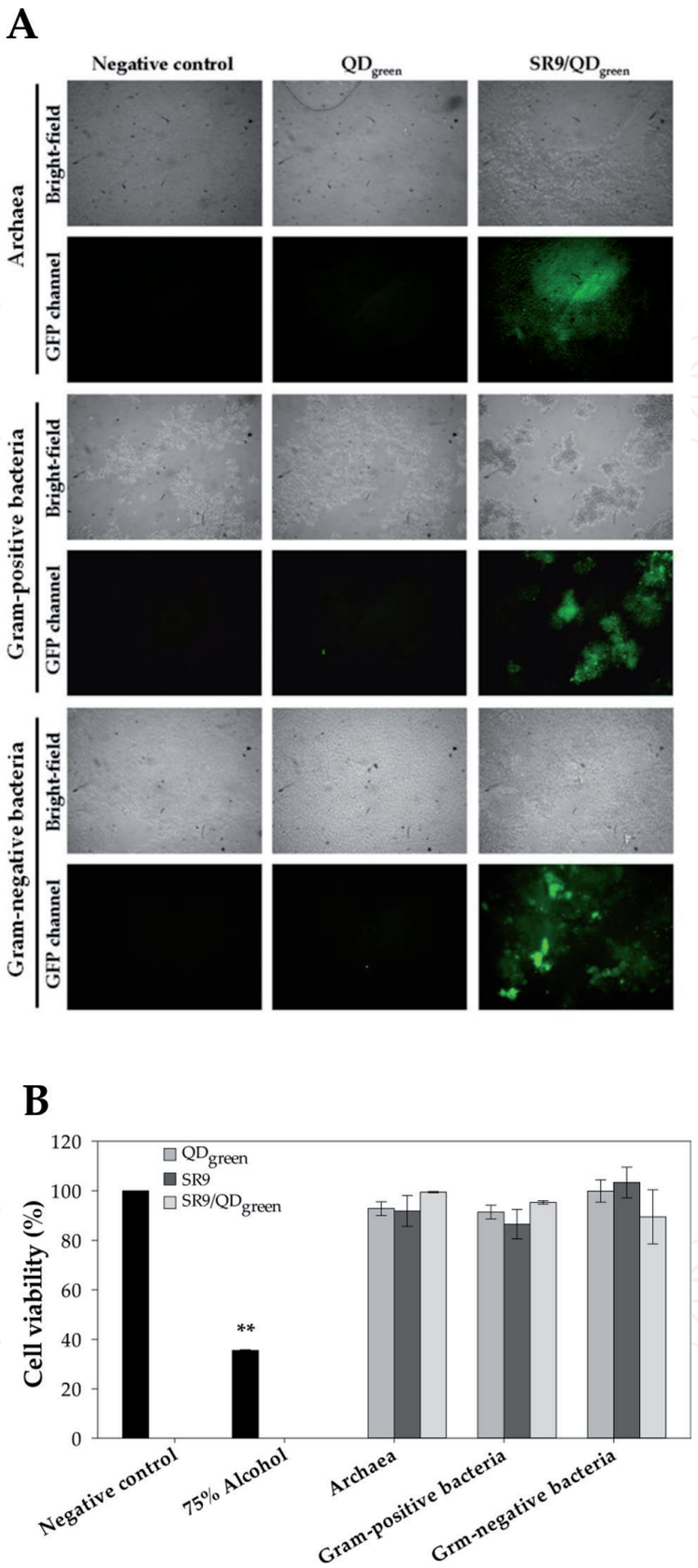


Figure 3.
Treatments of CdSe cored QD with green fluorescence in three types of prokaryocytes. (A) Fluorescent microscopy of Thermus aquaticus (archaea), Arthrobacter ilicis D50-1 (Gram-positive bacteria), and Escherichia coli DH5α (Gram-negative bacteria) treated with QD_{green} alone or $SR9/QD_{green}$ complexes. Three prokaryocytes were incubated with QD_{green} alone or $SR9/QD_{green}$ complexes for 1 h at room temperature. Protein transductions were detected in GFP channel and cell morphologies were observed in bright-field with an AE31 fluorescent microscope. (B) Cytotoxicity of nanoparticles in archaea, Gram-positive bacteria, and Gram-negative bacteria. Archaea, D50–1, and DH5α were treated with QD_{green}, SR9, and $SR9/QD_{green}$ complexes. Cells treated with their specific media and 75% alcohol as negative control and positive control, respectively. Significant differences from negative control at $P < 0.01$ (**) were indicated. Data were presented as mean ± standard deviation from three independent experiments [89].

in the groups of QD_{green}, SR9, and SR9/QD_{green} complexes (**Figure 3B**). This result indicated that Cd-core nanoparticles did not cause lethal effect to prokaryotic organisms. We reasonably ratiocinated that fluorescent nanoparticles applied in bioimaging and biotechnologies might not provoke natural imbalance and environmental problems.

5. Conclusion

We discussed applications and safety issues of various fluorescent nanoparticles. The cellular entry of particles of interest can be facilitated by CPPs. The particles did not produce lethal effects in mammalian cells, rotifers, archaea, Gram-positive bacteria, and Gram-negative bacteria. The outcome from assessing nanoparticle safety in mammalian cells suggests their potential medical applications. Hypotoxicity in rotifers and prokaryotes infers their environmental safety and eco-friendliness. In summary, these fluorescent nanoparticles and their CPP-modified complexes can be potent tools in various biological, environmental, and medical applications in the future.

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