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ORIGINAL RESEARCH PAPER

Targeting and sensing cancer cells with ZnO nanoprobes in vitro

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Abstract A new class of zinc oxide quantum dots (ZnO QDs) was investigated as nanoprobes for targeting cancer cells in vitro. ZnO nanoparticles were synthesized using conventional sol–gel method and encapsulated using trimethoxy aminopropyl silane. Transferrin, the ligand targeting the cancer cells, was conjugated to the ZnO QDs. In vitro imaging studies using MDA-MB-231 showed the biocompatible ZnO nanoprobe selectively binding to the cell surface receptor and internalizing through receptor-mediated endocytosis. Time-lapsed photobleaching studies indicate the ZnO QDs to be resistant to photobleaching, making them suitable for long term imaging purpose. Investigation of the ZnO nanoprobe as a platform for sensitive bioassays indicates that it can be used as an alternative fluoroprobe for cancer cell targeting and sensing applications.

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Introduction

Semi-conductor nanocrystals, also known as quantum dots (QDs), are emerging as an alternative fluoroprobe in the imaging and diagnosis of diseases like cancer (Warren et al. 1998; Yezhelyev et al. 2006). QDs possess an intense narrow fluorescence emission, a broad excitation spectrum and are resistant to photobleaching compared to small organic dyes making them suitable for biological applications (Chang et al. 2005; Dahan et al. 2003). Widespread applications of QDs include in vivo imaging studies and detection of multiple molecular targets simultaneously in small tumor samples (Dubertret et al. 2002; So et al. 2006). The commercially available quantum dots are based on Cadmium and selenium (CdSe) core shell passivated by zinc sulphide (ZnS). Despite their enormous potential the main drawback appears to be the toxicity of the cadmium-based QDs towards cells under UV illumination for extended periods of time. UV radiation acts by dissolving the semiconductor nanocrystals and releasing the toxic cadmium ions into the medium or body fluids, leading to investigations on the development of modified nanoprobes as tools for biological applications (Derfus et al. 2004; Gao et al. 2004).

A new class of ZnO based nanoprobe for in vitro imaging which could also serve as a sensitive bioassay for cancer diagnosis has been investigated. ZnO is a non-toxic versatile material having applications ranging from gas sensors, electroluminescent devices, solar cells, ultraviolet laser diodes, ointments, creams, and lotion and are highly biocompatible. ZnO has a wide band-gap of 3.37 eV and a large exciton binding energy making the exciton state stable at room temperature (Bang et al. 2006; Hossain et al. 2005). Further, ZnO is an environmentally friendly material making it useful for live cell imaging and early cancer detection. The ZnO bioconjugate of this study contains ZnO nano-crystal, a silane coating with amino functional group for ligand conjugation and transferrin as a tumor targeting ligand. The effect of the ZnO nanoprobes in cellular toxicity, non-specific binding, photobleaching, targeting of breast cancer cells and as a sensitive protease bioassay is reported herein.

Materials and methods

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. EDAC and transferrin were obtained from Merck. Cell culture medium, fetal bovine serum was from Invitrogen. Carboxyl-functionalized CdSe quantum dots (emission—515 nm) were procured from Evident Technologies.

Synthesis, surface modification and bioconjugation of ZnO nanoprobe

ZnO nanoparticles were prepared by precipitation from zinc acetate and NaOH (Bang et al. 2006). Briefly, 0.2 g NaOH in 10 ml ethanol was added into to 0.46 g zinc acetate in 30 ml ethanol with vigorous stirring in an ice bath, repeatedly washed with *n*-heptane and kept at 4°C for further characterization. The ZnO nanoparticles were then reacted with 3-amino propyl ethoxy silane resulting in the presence of free amino groups at the surface. For the carboxy nanoparticles, the amino ZnO particles were further reacted with diglycolic anhydride overnight with constant stirring at room temperature. Standard EDAC procedures were used for the conjugation of free carboxylic groups with the amino containing ligand transferrin.

Preparation of protease sensing ZnO nanoprobes

Carboxylic ZnO QDs were activated by EDAC for 30 min followed by the addition of fibronectin (molar ratio of 1:50) and allowed to react for 2 h at room temperature. Fibronectin-labeled QDs are then reacted with QSY quencher (5 mg/ml) overnight and kept at 4°C for further assays.

Mammalian cell culture and cytotoxicity assessment

MDA-MB-231 (breast cancer cell line) was used. Cell viability was measured using the MTT reduction assay (Sathya et al. 2010). The culture supernatant was removed and supplemented with DMEM and ZnO nano-particles (0.1–100 μg/ml). Data represents the mean values of three independent experiments.

Labeling of cancer cell with ZnO bioconjugate

Cells were grown on sterile cover glass, the medium was aspirated, and incubated with 50 μl ZnO–transferrin bioconjugate for 1 and 6 h at 37°C in 5% CO_2. The medium was discarded and cells washed three times with 500 μl autoclaved PBS. Control experiments were carried out using CdSe–transferrin bioconjugates. Fluorescence imaging experiments were carried out using standard fluorescent microscope (×50 with N/A 1) under a 100 W mercury lamp. The ZnO was analysed at excitation 365 nm and emission 560 nm. Acquisition of fluorescence images was performed with CCD camera (JVC, USA) through image acquisition software Image Impact 4.0 (Matrix Vision GmbH, Germany).

Detection of activity of MMPs in cancer cells

The MDA-MB-231 cells were cultured and incubated with 20 ng PMA/ml overnight for the secretion of MMPs. The activity of MMPs were detected by the incubation of ZnO-fibronectin-QSY in the medium at different time periods. Fluorescence was monitored with an excitation of 365 nm and emission of 560 nm.

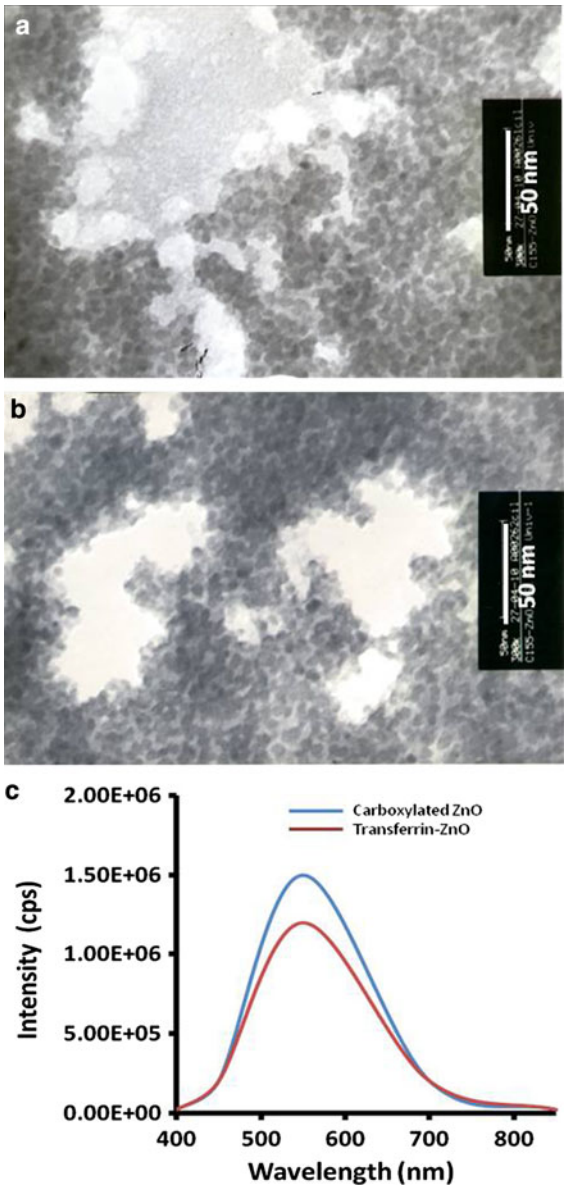

Fig. 1 TEM image of ZnO QDs dried on a carbon-formvar coated 200-mesh nickel grid. **a** ZnO QDs coated with 3-amino propyl ethoxy silane followed by diglycolic anhydride. **b** ZnO QDs conjugated with transferrin protein. **c** Photoluminescence spectrum of ZnO QDs and ZnO–transferrin nanoprobe under 365 nm excitation and emission at 550 nm. *Bar* 50 nm

Results and discussion

Characterization of ZnO nanoparticles

Transmission electron microscopy shows the ZnO QDs to be spherical with diameter ranging between

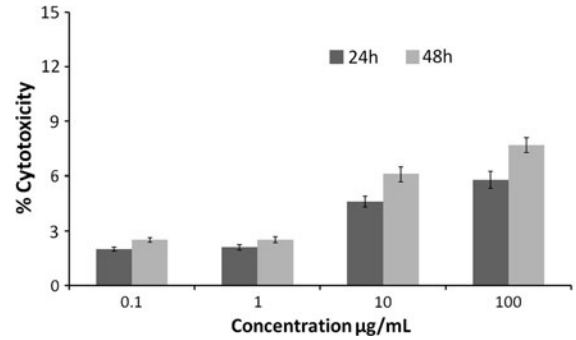

Fig. 2 Cytotoxic effect of ZnO QDs in vitro. Cytotoxic effect of ZnO QDs measured on MDA-MB-231 cells were treated with indicated doses of ZnO. After 24 and 48 h, cell death was estimated by MTT assay. Data shown reflect the mean ± SD of triplicates of three independent experiments

5 and 10 nm. Figure 1a, b shows the TEM images of unconjugated and transferrin conjugated ZnO QDs, respectively. Figure 1c shows the photo luminescence spectra of carboxylated ZnO and transferrin conjugated ZnO nanoparticles.

Assessment of cytotoxicity

Toxicity of CdSe QD depends on multiple factors such as surface oxidation and the bioactivity of the outer coating, resulting in release of cadmium and selenium ions to the system leading to toxicity (Hoshino et al. 2004). A 40% decrease in viability of HEK cells at 48 h was observed with 20 nM CdSe QDs (Ryman-Rasmussen et al. 2007). Cells exposed to 0.1–100 µg ZnO/ml showed no evidence of toxicity up to 48 h (Fig. 2) and our results indicate that even at 100 µg ZnO/ml the nanoparticles are biocompatible and are non-toxic in vitro.

Probing cancer cells with ZnO nanoprobes

Bioconjugated ZnO nanoprobes for in vitro cancer targeting and imaging were designed using drug delivery and targeting principles. For active cancer cell targeting, iron carrying protein transferrin, which is widely expressed in many cancer cells was selected as ligand to target breast cancer cells (Qian et al. 2002). The biocompatibility of ZnO QD–transferrin is demonstrated using fluorescence images acquired from cultured MDA-MB-231 breast cancer cells incubated with 1 and 6 h either with control ZnO or with transferrin conjugates at 37°C and washed

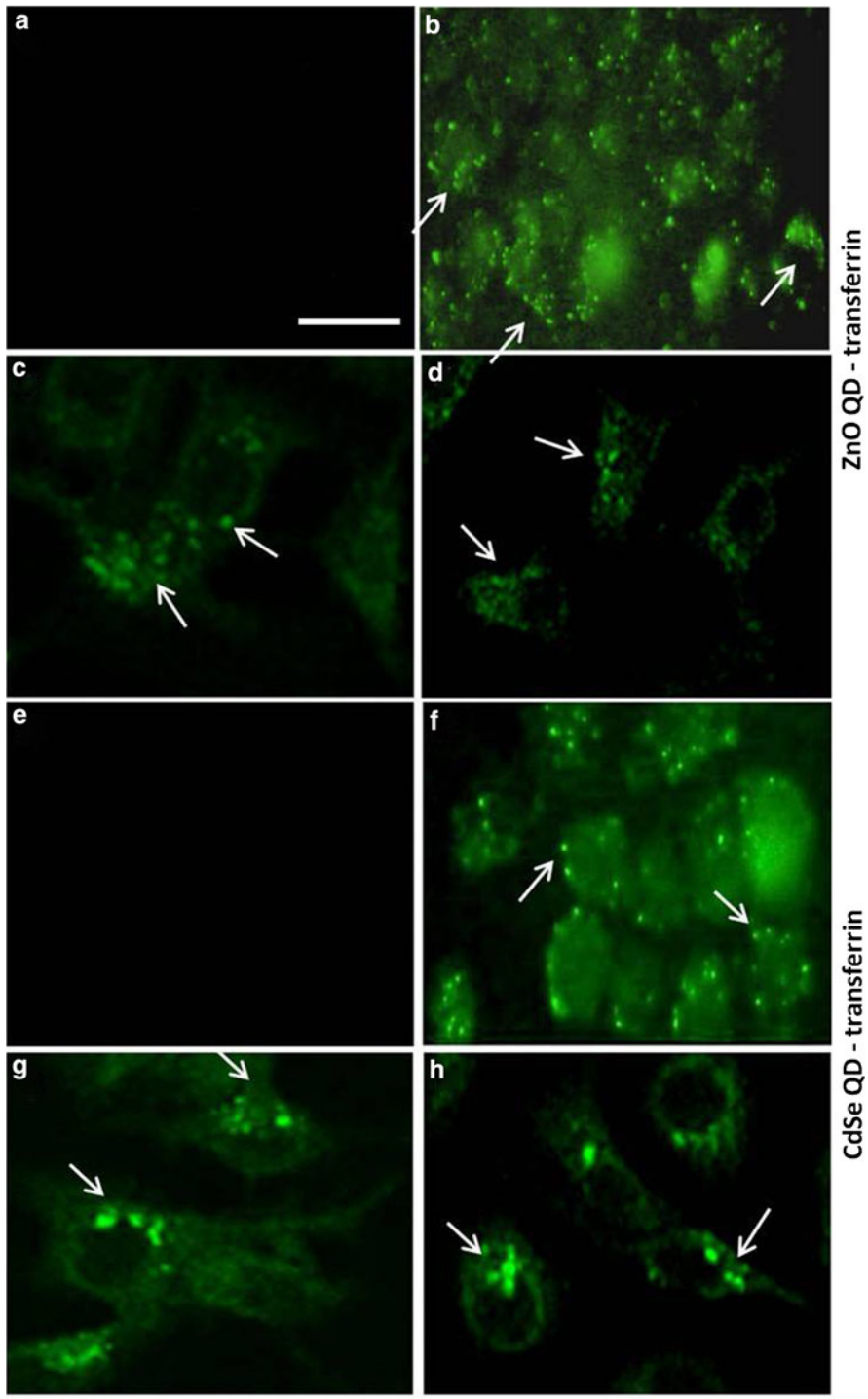

Fig. 3 Fluorescence images of cultured MDA-MB-231 cells incubated with ZnO–transferrin bioconjugates. **a** carboxylated ZnO QDs; **b** ZnO–transferrin bioconjugates recognize the membrane transferrin receptors; **c**, **d** after 6 h incubation ZnO–transferrin bioconjugates transported into the cell by receptor-mediated endocytosis, detected as aggregates; **e** carboxylated CdSe QDs; **f** CdSe–transferrin conjugate; **g**, **h** intracellular localization of CdSe–transferrin conjugates detected as aggregates after 6 h incubation. *Bar* 50 nm

repeatedly to eliminate excess ZnO. Fluorescence images revealed that in the absence of transferrin, no ZnO QDs were observed on the cells indicating that ZnO QDs are biologically neutral (Fig. 3a). The ZnO QDs conjugated with transferrin were able to recognize the transferrin receptor on the cell surface (Fig. 3b) and transport into the cell by receptor mediated endocytosis which are detected as clusters

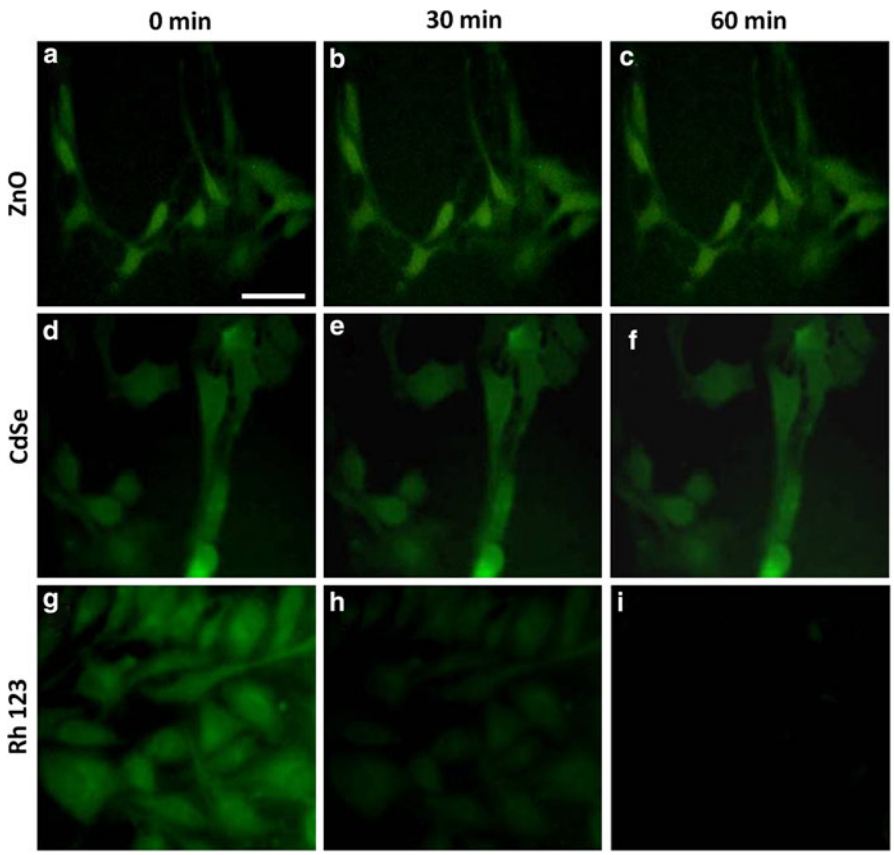

Fig. 4 Comparison of ZnO nanoparticles, CdSe QDs and Rhodamine 123 for their resistance to photobleaching. MDA-MB-231 cells were grown up to 60–80% confluency and fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton X-100. 1 μg ZnO QD/ml, CdSe QD and Rhodamine 123 (Rh 123) in PBS were added to the cells and incubated for 15 min at room temperature and were rinsed with PBS three times and observed under microscopy. **a–c** Consecutive images of ZnO QDs; **d–f** consecutive images of CdSe QDs; **g–i** consecutive images of Rh 123 were recorded. *Bar* 50 nm

or aggregates (Fig. 3c, d). Control experiments (Fig. 3e–h) carried out using commercially available carboxylated CdSe quantum dots conjugated with transferrin indicate the ZnO based QDs to be comparable with existing QDs. These results establish the biocompatibility ZnO–transferrin conjugates in retaining the receptor binding activity with specificity similar to the cadmium based quantum dots.

Comparative analysis of photobleaching of ZnO, CdSe QDs and Rhodamine

An important feature of QDs is their resistance to photobleaching, making them brighter probes under extended time imaging conditions. Currently available QDs are highly photostable but release toxic ions over time. Organic fluorophores or fluorescent proteins are restricted by photo bleaching (Dubertret et al. 2002). Investigation of the photobleaching properties of ZnO QDs were compared with CdSe and an organic dye Rhodamine 123. Figure 4a–c demonstrates the photo stability of ZnO QDs which is comparable to the CdSe QD (Fig. 4d–f) and more stable than the organic dye Rhodamine 123 (Fig. 4g–i). After 60 min of constant UV exposure, the fluorescence intensity of ZnO QDs remained unchanged comparable with the widely reported CdSe QDs, whereas Rhodamine itself showed time-dependent photo bleaching characteristic of most of the organic dyes. These results indicate that ZnO QDs are highly resistant to photo-bleaching and could serve as an alternate to the existing cadmium based QDs in monitoring time-lapse in vitro as well as in vivo imaging studies.

Sensing of MMPs secretion by cancer Cells

MDA-MB-231 cells secrete high levels of MMP-2 and MMP-9. The efficacy of ZnO-fibronectin-QSY as a nanoprobe for the detection of cells secreting high levels of MMPs and as a sensitive bioassay was investigated. The Photoluminescence spectra of ZnO nanosensor is shown in Fig. 5a. The cells were grown and PMA was used to induce the secretion of MMP. The cell culture medium from PMA induced cells

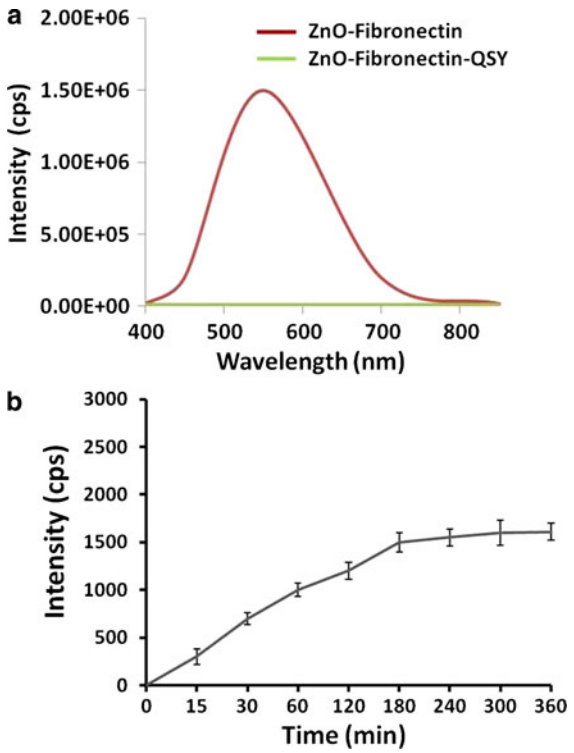

Fig. 5 Photophysical properties and protease sensing activity of ZnO nanoprobe. **a** Photoluminescence spectrum of protease sensing ZnO nanoprobe. **b** MMPs secreted by cells detected by protease sensing ZnO probes. Activity expressed as fold intensity of fluorescence

were collected and incubated with ZnO-fibronectin-QSY QDs for different time periods. The activity of MMP was detected in PMA stimulated cell culture medium within 1 h incubation in the cell culture medium which appeared to stabilize at 4 h (Fig. 5b) demonstrating the suitability of ZnO for sensitive bioassays.

Conclusion

ZnO QDs can be used for cancer targeting and sensitive bioassays. ZnO based nanoprobes exhibit no toxicity, are biocompatible and are resistant to photobleaching thus serving as a potent platform for sensitive bioassays.

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