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Sawitri Mardyani, Anupam Singhal, Wen Jiang, Warren C. W. Chan, "Interfacing peptides identified using phage-display screening with quantum dots for the design of nanoprobes," Proc. SPIE 5705, Nanobiophotonics and Biomedical Applications II, (28 April 2005); doi: 10.1117/12.601609

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Event: SPIE BiOS, 2005, San Jose, CA, United States

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Interfacing Peptides, Identified using Phage-Display Screening, with Quantum Dots for the Design of Nanoprobes

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ABSTRACT

The interface of targeting molecules that can recognize and identify specific biomolecules with highly luminescent semiconductor nanocrystals or quantum dots can lead to a novel and powerful new class of probes for studying biomolecules in real-time or for imaging and detecting diseases. We describe the rationale design of optical nanoprobes by using fluorescent semiconductor quantum dots with targeting molecules (TMs)-identified using phage display screening. Quantum dots are nanometer-sized particles with unique and tunable optical properties. They offer numerous optical advantages over traditional organic fluorophores in biological analysis and detection (e.g., photostability, continuous absorption profile).

Keywords: Nanoprobes, quantum dots, phage-display screening, targeting molecules, cell labeling, optical imaging.

INTRODUCTION

Over three centuries after the introduction of the light microscope, advances in optical microscopy continue to impact scientific research, particularly in the areas of biology and clinical health. The introduction of video cameras, photomultiplier tubes, and contrast agents has led to the development of a new set of optical microscopy tools and techniques that provides biological researchers with a 'window' through which to look at both the cellular and tissue levels of biological organisms with high resolution. The ultimate goal of biological researchers and health practitioners is to understand and elucidate the complex molecular interactions inside living cells and tissues.[2] Optical imaging techniques provide a useful tool to monitor these interactions. The high sensitivity of fluorescence microscopy makes it one of the most promising optical microscopy techniques for noninvasive biological imaging at the cellular and tissue levels. Conventional approaches to fluorescence microscopy employ fluorescent probes as contrast agents, which are delivered to cells or tissues and subsequently excited using electromagnetic radiation (e.g. ultraviolet radiation or visible light generated by lasers). By using an optical microscope to detect photons emitted from the excited fluorescent probes, high-resolution images of the cells and tissues surrounding these probes can be obtained. At present, most fluorescent probes are made from organic dye molecules (e.g. rhodamine 6G, fluorescein, Texas Red) conjugated to biomolecules (e.g. antibodies, peptides, DNA), which are used to target the probes to specific locations inside a cell or tissue [1-2]. Fluorescent probes constructed with this molecular architecture have been successfully employed in numerous biological applications, including the real-time imaging of live cell, gene expression profiling, cell sorting, and clinical diagnostics [1-3].

Despite the successful application of organic dyes in biological applications, several unfavourable optical properties of these dyes both limit the amount of useful information collected using these dyes as well as restrict their potential use in more diverse and sophisticated biological studies [1-4]. Firstly, the fluorescence of conventional organic dyes bleaches upon exposure to light in a process referred to as photobleaching. As a result, fluorescent probes composed of these dyes cannot

Nanobiophotonics and Biomedical Applications II, edited by Alexander N. Cartwright, Marek Osinski, Proceedings of SPIE Vol. 5705 (SPIE, Bellingham, WA, 2005) · 1605-7422/05/$15 · doi: 10.1117/12.601609

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be used to image dynamic cellular processes that take place on timescales larger than the rapid photobleaching rates of the dyes. Secondly, the narrow excitation (absorbance) spectra of organic dyes make it difficult to simultaneously excite multiple fluorescent probes with unique emission wavelengths. Thus, complex and expensive optical instrumentation consisting of multiple laser excitation sources each with a unique excitation wavelength are often required in applications where multiple organic dyes are used to simultaneously image and detect multiple cellular targets (i.e. "multiplexed" imaging and detection applications). For example, Roederer et al. require an eight-colour, three-laser system to conduct flow cytometry of ten parameters of cellular antigens [5]. Multiplexed imaging and detection of organic dyes is further complicated by their broad emission spectra with long tails at wavelengths red-shifted from the peak emission wavelength, which often result in overlapping emission spectra ("spectral cross-talk") between distinct dyes. In some situations, spectral cross-talk can be removed by using sophisticated filter systems to separate distinct detection channel [5].

Over the last decade, a new class of inorganic fluorescent molecules, known as semiconductor nanocrystals (a.k.a. "quantum dots"), has generated much interest as a result of their potential applications in the design of novel biological probes [6-7]. Quantum dots are composed of atoms from groups I-VII, II-VI, or III-V of the periodic table and are defined as particles with physical dimensions smaller than the exciton Bohr radius. Because of this feature, quantum dots exhibit quantum confinement effects and thus, have unique optical and electronic properties not observed by larger crystallites [8-9]. Chan and Nie, Alivisatos and co-workers were the first groups to modify the surface chemistry of quantum dots to render them biocompatible and they demonstrated quantum dot applications in optical imaging [6-7]. Since the first two published papers, Wu et al., Dubertret et al., Goldman et al. demonstrated quantum dots for labeling EGF receptors on cancer cells and for long-term monitoring of cellular activity [10-12]. Also, Mitchell et al and Parak et al. showed that quantum dots could be used for genomic detection [13-14]. Furthermore, Akerman et al. used quantum dots for labeling tumor vasculatures in live animals [15] and Nie and coworkers linked quantum dots with a specific antibody (prostate-specific membrane antigen-specific monoclonal antibody) to image and detect prostate tumors in intact animals [16]. For biological applications, quantum dots have tremendous advantages over traditional organic-based dyes for optical labeling such as continuous absorption profiles, size and materials-tunable emission, 20 times brighter and 100 times more stable against photobleaching, and 2.5 times narrower spectral linewidths [4, 6-7]. In addition, the size of a quantum dot is similar to biological molecules (1 to 10 nm). The unique spectral properties and size renders quantum dots ideal for multiplexing biological imaging and analysis.

For most biomedical applications, quantum dots generally require targeting molecules to direct their biological activities. Recent advances in molecular bioengineering techniques have led to the development of novel screening approaches that can identify targeting molecules for specific cells, tissues, or organs [17]. The "marriage" of these two fields represents the development of novel powerful probes that enhance the biologist's toolbox for fundamental research and clinician's ability to more accurately diagnose a disease. In this manuscript, we describe the design and integration of semiconductor quantum dot, a fluorescent emitting nanostructure, with targeting molecules (identified using molecular engineering) to selectively label and detect immortalized cancer cells.

We selected the molecular bioengineering technique of phage-display screening to identify novel targets since this technique is easy to conduct as compared other screening approaches (such as the yeast hybridoma technology). Phage-display screening technology is a powerful combinatorial tool for discovering peptides that bind to specific or unknown biomolecular targets [17-19]. Using this technique, genes are inserted into bacterial viruses called phage, and expressed as peptides on the outer perimeter of the phage after replication in bacteria. These genes can be mutated or varied to create a diverse pool of phage that is screened against different populations of cancer cells. Until now, phage-display peptide libraries have been used to isolate and identify peptides that can map epitopes, bind to receptors on polystyrene surfaces, cells, tissues, and organs, and distinguish the motifs of protein-protein interactions [20-22].

EXPERIMENTAL APPROACH

Synthesis of quantum dots. The following procedure was adapted from ref. 23. In a glovebox, two sets of stock solutions were prepared: (A) $Cd(CH_3)_2$ and Se pellets were mixed in the solvent tri-n-ocytyphosphine (TOP) at a molar ratio of 1.4:1.0 and (B) $Zn(CH_3)_2$ and $(CH_3)_3SiSSi(CH_3)_3$ were mixed in the solvent TOP at a molar ratio of 1.0:1.0. 12 g of tri-n-octylphosphine oxide (TOPO) was measured out and placed into a three-neck flask, which was then heated, purified, and degassed at 200^{o}C under vacuum in a Schlenk line system. After 20 minutes, the temperature was raised to 360^{o}C and

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solution A was quickly injected into the reaction vessel. The solution initially appeared light yellow, indicating the successful nucleation of small quantum dots (< 1.5 nm diameter). The temperature was quickly lowered to 300°C. At this temperature, different sizes of quantum dots were prepared by the addition of excess precursor solution (solution A) in small aliquots to the reaction vessel at 300°C. When the desired quantum dot-size was obtained (measured by a spectrofluorimeter), solution B was injected into the reaction vessel to cap the quantum dots with a thin ZnS-layer. Finally, the temperature was lowered to 60°C, after which chloroform was added to the mixture to prevent TOPO re-solidification. Excess methanol was added to the reaction vessel to induce quantum dots to precipitate out of solution. The precipitated quantum dots were centrifuged (10,000 x rpm) for 10 minutes. The supernatant (containing un-reacted precursors) was discarded while the pellet was re-dissolved in chloroform. The remaining solution was generally optically clear. The remaining quantum dots were soluble in chloroform because of the hydrophobic stabilizing ligand TOPO on the surface of the quantum dots. Both the absorbance and fluorescence of the quantum dots were measured. The relative quantum yield of the quantum dots was determined by comparison of fluorescence intensity (or integrated peak area) of quantum dots with an organic dye molecule of a known quantum yield (e.g., rhodamine 6G in ethanol, quantum yield = 95%)..

Surface modification of quantum dots. TOPO coated quantum dots were rendered water-soluble by modifying the surface chemistry of the quantum dots A detailed procedure for the surface modification of quantum dots by the exchange of TOPO-molecule on the surface of the quantum dots with the more hydrophilic molecule mercaptoundecanoic acid (MUA) was recently described [24]. Briefly, MUA was measured out and melted in a three-neck flask. TOPO-coated quantum dots were then injected into the reaction vessel and stirred overnight. A small volume of dimethylsulfoxide (DMSO) was added to the reaction vessel to maintain optical clarity of the solution; the modification of the TOPO-quantum dots with MUA produced a more hydrophilic quantum dot that was insoluble in chloroform. Finally, the MUA-coated quantum dots were precipitated out of solution using hexane and re-dissolved in DMSO. The MUA on the surface of the quantum dots was cross-linked to prevent colloidal aggregation by using lysine in the presence of the organic catalyst dicyclohexylcarbodiimide. The final quantum dots were soluble in distilled water and available for conjugation to targeting molecules.

Using phage-display screening to identify targeting molecules. Phage display screening on whole cells was done using the New England Biolabs Ph.D.-7 Phage Display Peptide Library Kit (E8100S). Phage were selected to bind to HeLa cells using a selection/subtraction protocol adapted from Rasmussen et al. [25]. STO cells were used to subtract phage from the library. Before subtraction, cells were detached with a solution of Trypsin-ethylenediaminetetracetic acid (Trypsin-EDTA), washed two times in phosphate buffered saline (PBS), counted and re-suspended at 10^7 cells/ml in 10mM PBS containing 1% bovine serum albumin (BSA). 1.5 x 10^{11} phage plaque-forming units (pfu) were incubated with 10^7 STO cells for 1 hour at room temperature with slow shaking. The cells were pelleted in a centrifuge at 1500 rpm for 2 minutes. The supernatant was used to resuspend another 10^7 STO cells. This subtraction was repeated 3 times. The cells were then selected on 5 x 10^6 HeLa cells through incubation for 4 hours at 4C with slow shaking. The cells were washed 5 times with PBS containing 1% BSA and 0.1% TWEEN, changing centrifuge tubes between washes (centrifuge 1500rpm, 2 minutes). The bound phage were eluted by adding 100 ml 0.2M glycine-HCl (pH 2.2) for 10 minutes on ice. The cells were centrifuged for 2 minutes at 1500 rpm and the phage-containing supernatant collected by a pipette. The eluted phage were neutralized with 15_l of 1M Tris-HCl (pH 9.1). The phage were tittered and amplified using E-coli provided with the Peptide Library Kit and the entire subtraction process was repeated four times. After the fourth round, DNA from a single binding clone was sent to a sequencing lab (Hospital for Sick Children, Toronto, ON, Canada) for characterization.

The binding efficiency of the phage on STO and HeLa cells was characterized. 1.5 x 10^{11} pfu were incubated with 5 x 10^6 cells for 4 hours at 4C with slow shaking. Washing and elution was performed as described above and the eluted phage were tittered and eluted by the input number of plaque forming phage. When a phage with a suitable binding efficiency was found, its displayed peptide was synthesized at a peptide sequencing facility (Hospital for Sick Children, Toronto, ON, Canada).

Integration of targeting molecules with quantum dots. Targeting molecules were conjugated to quantum dots through a carbodiimide-mediated bioconjugation. Quantum dots, targeting molecules and carbodiimde (1– ethyl-3-(3-diaminopropyl carbodiimide hydrochloride) or EDC were mixed at a 1:25:1000 ratio and allowed to react for 2 hours with shaking and purified using sephadex columns.

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Cell incubation studies. Glass coverslips were placed in 60mm tissue culture dishes. 10^6 STO or HeLa cells were plated onto each dish and incubated overnight. 50 ml of quantum dots conjugated with targeting molecule were incubated with one dish of HeLa and one dish of STO for at least two hours. For controls, STO and HeLa were incubated with unconjugated quantum dots. After incubation, cells were washed five times with PBS and imaged at 10x using UV excitation (360 +/- 40 nm) and a long pass filter (520 nm).

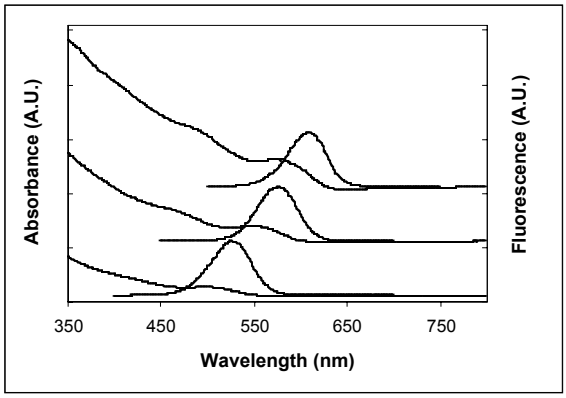

Figure 1. Absorbance and fluorescence spectra of three-different-sized ZnS-capped CdSe quantum dots. As can be observed, the absorbance spectra are continuous whereas the fluorescence spectra has minimum peak fronting or tailing. For ZnS-capped CdSe quantum dots, the fluorescence emission correspond to the size of the quantum dots. The emission wavelength is directly related to the size (longer emission wavelength = bigger quantum dot).

RESULTS and DISCUSION

Synthesis of quantum dots. We demonstrate the successful synthesis of different-emitting quantum dots by measuring their spectral properties (see figure 1). Once the Cadmium/Selenium precursor solutions are injected into the hot TOPO solvent, a rapid nucleation is observed through change in the color of the reaction vessel. Prior to injection, the solution appears relatively clear and colorless, while after nucleation, the color of the solution appears yellow. It has been postulated that nucleation occurs by the supersaturation of precursor atoms. At high temperatures, the size of the quantum dots can be dynamically altered to produce larger-sized quantum dots. High-temperature growth of quantum dots generally involves a process called Ostwald Ripening, while smaller quantum dots are broken down in order to support the growth of larger quantum dots. The color of the solution can indicate the growth of the quantum dots (e.g., darker colors indicate larger quantum dots). After initial injection of precursor solvents, the reaction vessel appears light yellow and as the quantum dots grow, they appear a light orange, then red, and finally a deep red. Figure 1 shows the absorbance and fluorescence spectra a series of visible-emitting ZnS-capped CdSe quantum dots.

It is important to place a thin ZnS-layer (~1 nm) on the surface of the core CdSe quantum dot. Our typical quantum yield values for core CdSe quantum dots is 5-10% but after coating with ZnS, the quantum yield improves to 30-50%. These values are obtained by comparison to the organic dye molecule rhodamine 6G. In addition, the ZnS-layer reduces cytotoxicity. Derfus and coworkers showed that the ZnS-layer is important for using quantum dots as probes for live cell labeling [26]. The ZnS-layer minimizes cellular toxicity (induced by cadmium poisoning) by preventing the core CdSe quantum dot from oxidizing. Finally, the ZnS improves the overall stability of CdSe core quantum dots against photobleaching (or loss of fluorescence signal). This renders quantum dots useful as a biological probe for long-term molecular or cell tracking.

Once synthesized, solvent precipitation is used to isolate the quantum dots from the precursor molecules. Since quantum dots are coated on the surface with hydrophobic molecule tri-n-octylphosphine oxide (TOPO), the quantum dots aggregate in the presence of a miscible polar solvent (e.g., methanol). The addition of methanol to TOPO-coated quantum dots dissolved in chloroform causes the quantum dots to precipitate out of solution upon centrifugation. The quantum dots can be subsequently isolated by disposal of the resulting supernatant and predisposing the precipitate in chloroform.

Modifying the surface of quantum dots to improve biocompatibility. TOPO-coated quantum dots are only soluble in non-polar solvents, such as chloroform, and cannot be interfaced or used for biological research. In order to alleviate this

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problem, we modified the surface of the quantum dots with bifunctional molecules – i.e., molecules that contain a thiol group (-SH) on one end and a carboxylic acid group (-COOH) on the other, or molecules containing a long-hydrophobic chain at one end and a –COOH at the other end. One such bifunctional molecule that we employ is mercaptoundecanoic acid. By mixing TOPO-coated quantum dots with a high concentration of mercaptoundecanoic acid, the mercapto-group binds to the surface of the quantum dot through a thiol-to-zinc or thiol-to-cadmium interaction. On the other end, the polar carboxylic acid functional group protrudes form the surface. Recently, we demonstrated the successful modification of the TOPO-coated quantum dots with mercaptoundecanoic acid [24]. We further showed the cross-linking of the mercaptoundecanoic acid on the surface of the quantum dots with the amino acid lysine. This rendered the quantum dots hydrophilic and these quantum dots can now be interfaced with biological systems. Figure 2 shows the change in solubility of the TOPO-coated quantum dots before and after surface modification with mercaptoundecanoic acid and subsequent cross-linking with lysine. In the vial, the bottom layer contains chloroform while the top layer contains distilled water. Only TOPO-coated quantum dots are soluble in the chloroform layer, while the mercaptoundecanoic acid-lysine cross-linked quantum dots are soluble in the distilled water layer. Furthermore, the carboxylic acid on the surface of the quantum dots permits conjugations to biomolecules (e.g., DNA, proteins, carbohydrates). This makes these quantum dots useful as biological probes.

Identification of targeting molecules and integration with quantum dots. The use of quantum dots for multiplexing applications may be important for elucidating and detecting the proteome or genome dynamics of cells. A library of optical nanoprobes may be useful for color-coding the molecular processes of cells, from the single cell to organ level. Quantum dots alone will not bind to cellular targets for imaging and detection. In order to target quantum dots to specific sites and produce quantum dot-target probes, a large corresponding library of recognition molecules or targeting molecules to specific proteins and nucleic acid sequences is needed. In effect, each uniquely emitting quantum dot represents a specific targeting molecule. In this section, we discuss the method of phage-display screening to identify targeting molecules and the integration of quantum dot with these targeting molecules to design an optical nanoprobe.

We examined the technique of phage-display screening to identify targeting molecules that were specific to one cell type. As a model, we panned the phage library against cell cultures of HeLa and STO cells. HeLa cells are an immortalized cervical cancer cells while STO cells are fibroblast cells. We used the combination of phage subtraction and selection to identify unique targeting molecules. In this technique, the phage that bind to STO cells are subtracted out. From the remaining library, the phage that bind to the HeLa cells are selected for expansion. In effect, the phage that will only bind preferentially to the HeLa cells as compared to the STO cells are selected. Using this screening approach, we identified the peptide HFYVSPW that specifically targets HeLa cells and not STO cells. Once identified, we synthesized the peptide sequence and conjugated the peptide to the surface of the lysine cross-linked mercaptoundecanoic acid quantum dots using

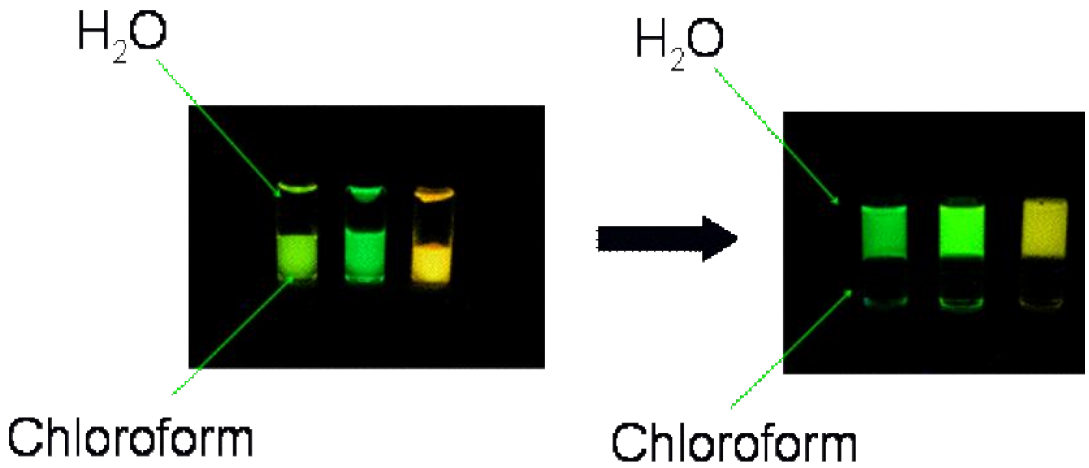

Figure 2. Change in solvent solubility of quantum dots after surface modification. Two solvent layers (bottom layer is chloroform and top layer is water) are distinguished by quantum dot fluorescence in this real-color photo. TOPO-coated quantum dots are soluble in the organic solvent. After surface modification with mercaptoundecanoic acid and cross-linked by lysine, the quantum dots are soluble in the aqueous layer. The fluorescence in the vials is observed by illumination with a handheld UV-lamp. In this figure, we show the surface modification of three-different sized ZnS-capped CdSe quantum dots. The water-soluble quantum dots appear monodisperse under epifluorescence illumination and transmission electron microscopy.

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EDC. During the synthesis of the peptide, we placed amino acid spacer of GGGS at the C-terminus. The C-terminus was then subsequently amidated. We placed a spacer between the peptide and the quantum dots surface. Otherwise, the binding peptide may adsorb onto the surface of the quantum dot and hence, the peptide may not bind to it's target. The final peptide has the sequence HFYVSPWGGGS.

The HFYVSPWGGGS-conjugated quantum dots were incubated in two sets of tissue culture dishes (one set containing HeLa cells and another set containing STO cells) at 37^{O}C for 2 hours. Afterward, the tissue culture dishes were cleaned to remove unbound peptide-quantum dot conjugates. Figure 3 shows fluorescence and DIC (differential interference contrast) images of the cells. As can be observed from figure 3, the quantum dots conjugated to HFYVSPWGGGS selectively labeled HeLa cells and not STO cells. We did observe some non-specific binding of quantum dots in the STO

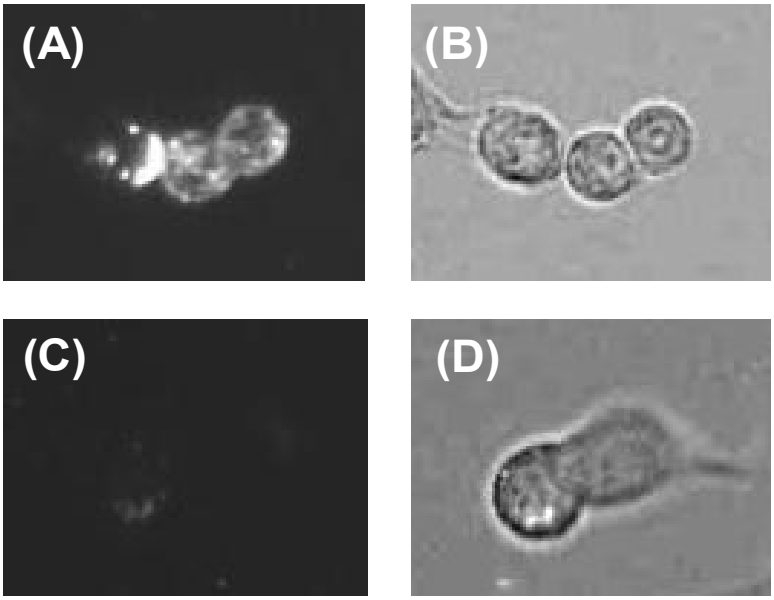

Figure 3. Staining of HeLa and STO cells by HFYVSPWGGG-conjugated quantum dots. The peptide-quantum dot conjugates labeled HeLa cells (A) much more than STO cells (B). Differential interference contrast images (B and D) are shown beside their corresponding fluorescent images (A and C). The peptide was identified using phage-display screening. Images were obtained using an Olympus IX epifluorescence microscope: 10x objective, excitation wavelength of 360 +/- 40 nm, long pass emission filter of 520 nm. These cells are ~ 10 mm. Of note, there are two cells in figure D; one of the two cells are not in focus.

cells. This clearly shows the use of phage-display screening to identify targeting molecules and the integration of these targeting molecules with quantum dots.

One of the interesting aspects of phage-display screening is that the target does not need to be known or identified. In this example, we discovered by the peptide but we do not currently know its target. Even though we do not know the target, figure 4 shows the ability of phage-display screening to selectively isolate a targeting molecule that can home to HeLa cells and not to STO cells.

An interesting note is that not all of the HeLa cells were labeled with quantum dots. In fact, we estimate that only 15-20% of the cells were labeled as compared to STO cells (<1%). Our speculated reason is that this targeting molecule may bind to a receptor of HeLa cells when it is going through a specific cellular process. At this stage, we do not know what

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receptor or process. However, this experiment shows that phage-display screening may be a useful technique for identifying targets for a subpopulation of cells (e.g., cell that may undergo apoptosis versus proliferation). The strength of phage-display screening is the ability to identify targeting molecules to specific receptors on/in cells without the need to identify the receptor. Researchers liken this technique to finding a needle in a haystack. With a large library of uniquely emitting quantum dots, phage-display screening will be an important tool to identify a library of targeting molecules. The merging of these two technologies will provide a large set of biological probes to fluorescently study or detect molecular and cellular processes in a multicolor or multiplexed fashion.

CONCLUSIONS

We showed the integration of two fields of research to build nanoprobes for bioimaging and detection. Visible-emitting ZnS-capped CdSe quantum dots were synthesized using an organometallic technique. They were altered by changing their surface chemistry or infused into microbeads to enhance their biocompatibility. The technique of phage-display screening was used to identify targeting molecules. We screened for a peptide (HFYVSPW) that bound to HeLa cells and not STO cells. We conjugated the peptide HFYVSPW with a GGGS spacer onto the surface of the quantum dots using EDC. We showed the labeling of only HeLa cells and not STO cells with HFYVSPW-conjugated quantum dots. These experiments demonstrated the feasibility of integrating targeting molecules (identified using phage-display screening) with quantum dots to design novel nanoprobes. The fusion of these two technologies may lead to the creation of a large library of nanoprobes for multiplex imaging in cells, tissues, organs, and animals.

ACKNOWLEDGEMENTS

W.C.W. Chan would like to acknowledge Canadian Institute of Health Research (Regenerative Medicine and Nanomedicine NET grant), Natural Science and Engineering Council (NSERC, Discovery Grant), Canadian Foundation for Innovation (New Opportunity), and Ontario Innovation Trust (New Opportunity) for financial support. S. M. would like to acknowledge NSERC and CFUW (Canadian Federation of University Women), A.R. would like to acknowledge NSERC for summer fellowship, W. J. would like to acknowledge Ontario Graduate Scholarship. Finally, W. C. W. Chan would acknowledge organizers of the Interanational Symposium of Environmental Nanotechnology for the invited presentation. We acknowledge the Zandstra lab at the University of Toronto for kind donation of cells.

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