Quantum Dots for Molecular Imaging and Cancer Medicine

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The past few decades have witnessed technical advances that have introduced cell biologists and physicians to a new, dynamic, subcellular world where genes and gene products can be visualized to interact in space and time and in health and disease. The accelerating field of molecular imaging has been critically dependent on indicator probes which show when and where genetically or biochemically defined molecules, signals or processes appear, interact and disappear, with high spatial and temporal resolution in living cells and whole organisms.

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For example, the use of radionuclide tracers combined with 3-dimensional (3-D) imaging systems such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) are now helping clinicians to characterize the molecular status of tumors deep within patients.

Other types of imaging probes rely on the bioluminescence and fluorescence of genetically encoded proteins (originally found in fireflies and jellyfish, respectively) or entirely synthetic fluorochromes, or a combination of both.

New powerful biological fluorescence microscopes provide the ability to study single molecules within single cells. Multiphoton confocal microscopy has been developed to allow for the capturing of high-resolution, 3-D images of living tissues that have been tagged with highly specific fluorophores.

Each of these indicator molecules is designed to maximize sensitivity and specificity and each approach has its own pros and cons.

While effective, the use of radioactive tracers is accompanied by limitations inherent to their complex fabrication, short half-life due to radioactive decay and issues related to patient/provider safety and radioactive waste disposal. Furthermore the radioactivity-based methods are limited by their inability to visualize simultaneous signals (of more than one marker) and the lack of detection sensitivity towards very small tumor burdens thus confining its use to macro-scale imaging. Typically, millions of tumor cells are needed to provide a suffi-

ciently large tumor signal-to-background ratio.

On the other hand, fluorescence microscopy is noninvasive and provides imaging with high sensitivity down to the nano-scale (10^{-9}) level, but conventional dye molecules impose stringent demands on the optical systems used to make such measurements. Moreover, fluorescent molecules suffer from major limitations that include: (i) poor photostability; they "bleach", or irreversibly cease to emit light, under repeated excitation and (ii) phototoxicity; dyes produce toxic radicals (highly reactive compounds) and photo-products upon repeated excitation and/or photo-destruction. This toxicity prevents long-term observation of labeled components in living cells.

Table 1. Glossary of Terms

Autofluorescence	The fluorescence from endogenous cell constituents such as NADH, riboflavin and flavin coenzymes, which can contribute to background levels during cell imaging.
Background	Nonspecific signal given off by the sample.
Bioluminescence	Production of light by living organisms from conversion of chemical to radiant energy.
Bioconjugate	Attachment of a marker molecule (fluorochrome or enzyme) to a specific binding molecule (e.g., peptide, antibody, or oligonucleotide) to form a probe.
Excited state	Condition of a fluorophore that has absorbed light energy; the energy is then released as fluorescence or heat.
Fluorescence	Light emitted during the rapid relaxation of fluorescent molecules following excitation by light absorption.
Fluorochrome, fluorophore	A molecule or chemical group responsible for fluorescence.
Isotopes	Different forms of the same element. Isotopes of an element have different numbers of neutrons in the nuclei of their atoms, but the same number of protons. Some isotopes, called radioisotopes, are unstable and emit radiation.
Multiphoton	Excitation of the fluorophores is achieved by two or more low-energy confocal microscopy: infrared photons instead of a single high-energy visible photon which results in deeper tissue penetration of the light and also less cellular damage.
Photon	A "particle" of light energy.
Positron emission	A technique to map the emission of particles called positrons from the tomography (PET): decay of radioactive elements injected into the body.
Probe	A molecular complex that specifically attaches to a target and indicates its location.
Radionuclide	Atom with an unstable nucleus. The radionuclide undergoes radioactive decay by emitting gamma rays and/or subatomic particles.
Single Photon Emission Computed Tomography	Image is formed by a computer compiling data as transmitted by single gamma photons emitted by radionuclides administered to the (SPECT): patient.

In the quest for alternative labels, quantum dots (qdots) were introduced as a new class of multimodal contrasting agents for bioimaging (Bruchez et al., 1998; Chan and Nie, 1998). Fluorescent qdots are small nanocrystals (1-10 nm) made of inorganic semiconductor materials in which electronic excitations (electron-hole pairs, or excitons) are confined. Their small size imparts them with intriguing optical properties, very different from the properties of the bulk material on which they are based. They behave as single "atom-like" quantum entities and adopt new properties that depend directly on their size (Alivisatos, 1996). Perhaps their most useful size-dependent property is the tuning of the absorbance and emission with size, an effect known as quantum confinement, hence the name "quantum dots."

When these confined excitons excited with a beam of light, they will re-emit light (i.e., fluoresce) with a narrow and symmetric emission spectrum that is directly dependent upon the size of the nanocrystal. As a result, chemists can use the duration of the nanocrystal synthesis as a precision knob to produce qdot particles of a given size, which will absorb and emit light at a desired wavelength. As the size of the nanoparticle gets bigger, its emission shifts from the blue to the red end of the visible spectrum (Figure 1).

Other unique optical properties set the qdots apart from conventional dyes: (i) qdots are brighter (they emit many more photons per nanocrystal) which means that a very small number of qdots (even a single one!) are sufficient to produce a signal, (ii) they are more photostable (they live longer) allowing for the acquisition of images over long periods of time (minutes to hours) that are crisp and well contrasted, and (iii) they have a broader excitation spectrum. The latter means that a complex mixture of nanocrystals of different sizes can be excited by the same single wavelength light source and simultaneously detected and imaged in color (multiplexed detection). Thus, qdots enable users to multiplex many different biological signals in complex environments such as the living cell or the whole organism with an unprecedented kaleidoscope of colors.

Importantly, qdots can also be made to emit in the near-infrared (NIR) region of the spectrum, where autofluorescence is considerably reduced and where no good dyes exist.

In a recent study (Kim et al., 2004), NIR qdots (emitting at 850 nm) were used in sentinel lymph node mapping, a major procedure in cancer surgery, whereby the lymph node closest to the organ affected is monitored for the presence of roaming cancer cells. Following intradermal injection of the qdots in live mice and pigs, a surgeon could follow in real time the qdots homing to the nearby sentinel lymph node approximately 1 cm below the skin. Since qdots allow image guidance throughout the procedure virtually free of any background, the size of the incision that ensured the successful removal of the sentinel lymph node was reduced. The imaging of the sentinel lymph node in real time without the need of a traditional blue dye or radioactive tracer is a significant breakthrough that suggests that NIR imaging of qdots could possibly aid surgical procedures in humans.

Lastly, the high electronic density of qdots (they are made of heavy metals) makes them excellent contrasting agents for electron microscopy. Single receptors for the amino acid/neuromodulator glycine have been successfully tracked with the high-resolution spatial and temporal information afforded by single-molecule fluorescence microscopy and visualized with the very high-resolution structural information afforded by electron microscopy as they were diffusing in synaptic and extra-synaptic domains (Dahan et al., 2003). Thus, only qdots have the potential to provide images at all length scales, from the level of the whole body (macro-scale) down to the nanometer resolution using one type of

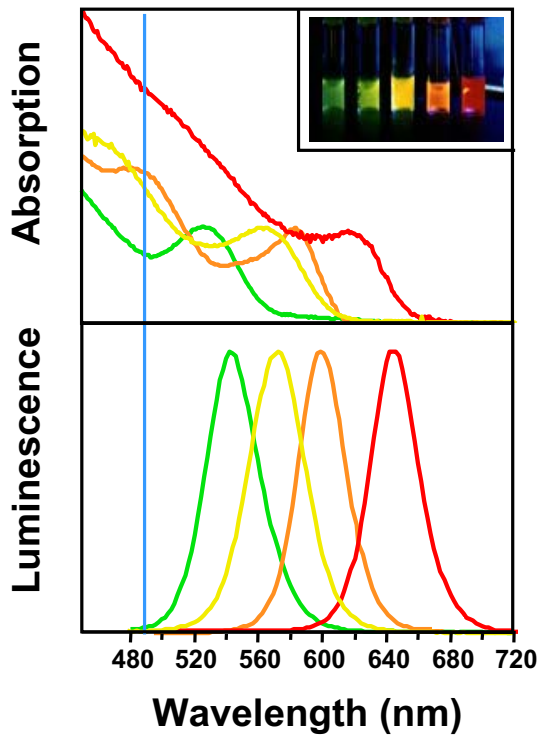

Figure 1. Absorption (upper curves) and emission (lower curves) spectra of four CdSe/ZnS qdot samples. The blue vertical line indicates the emission wavelength of the 488 nm line of an argon ion laser, which can be used to efficiently excite all four types of qdots simultaneously. Inset: a series of CdSe/ZnS dots in aqueous buffer, simultaneously illuminated by a laser (true-color).

probe.

One of the qdots' limitations has long been the hydrophobic nature of the outer shell that has excluded them from being used in aqueous biological environments. A great deal of effort has therefore been concentrated on developing robust, versatile and biocompatible (non-toxic) surface chemistries to both solubilize and functionalize the nanocrystals for various biological applications (Michalet et al., 2005).

Any modifications of the qdots' outer surface has to fulfill the following requirements: (i) render the qdots water soluble and biocompatible; (ii) offer reactive groups on their surface for subsequent bioconjugation; (iii) maintain all of the qdots' good photophysical properties (photostability, wide excitation band, narrow emission, spectral range, brightness); (iv) ensure that the particles are monodispersed; and (v) add only a thin coat layer (that does not significantly increase the particles' diameter).

Towards this goal, we have developed a peptide-coating approach that "disguises" qdots into soluble protein-like entities. This was achieved by covering the qdots' surface with synthetic peptides inspired by naturally-occurring phytochelatin α-peptides that are involved in heavy metal detoxification processes in plants and yeasts (Pinaud et al., 2004).

These peptides comprise a metal-chelating and hydrophobic domain ensuring binding to the qdot surface, and a hydrophilic (water-loving) tail that provides solubilization and stability in buffers. A single binding domain of the peptide, containing cysteines (C) and hydrophobic unnatural amino acids such as 3-cyclohexylalanines (Cha), is responsible for surface recognition and their attachment to qdots, while the variable, more hydrophilic domain provides solubilization and functional groups. This surface chemistry is rather sim-

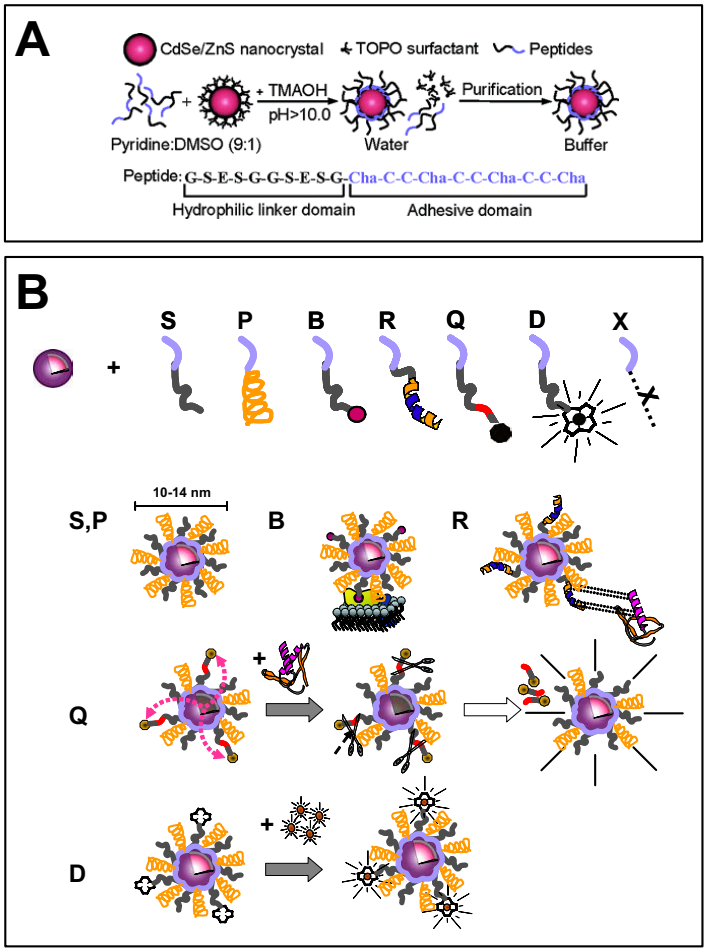

Figure 2. Nanocrystal peptide-coating approach. **A.** Schematic representation of the surface coating chemistry of CdSe/ZnS nanocrystals with phytochelatin-related -peptides. The peptide C-terminal adhesive domain binds to the surface of CdSe/ZnS nanocrystals after exchange with the trioctylphosphine oxide (TOPO) surfactant. A polar and negatively charged hydrophilic linker domain in the peptide sequence provides aqueous buffer solubility to the nanocrystals. TMAOH: Tetramethyl ammonium hydroxide; Cha: 3-cyclohexylalanine. **B.** Peptide toolkit. The light blue segment contains cysteines and hydrophobic amino acids ensuring binding to the nanocrystal (adhesive domain of Figure 2A) and is common to all peptides. S: solubilization sequence (hydrophilic linker domain of Figure 2A); P: PEG; B: biotin; R: recognition sequence; Q: quencher; D: DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) for radionuclide and nuclear spin label chelation; X: any unspecified peptide-encoded function. Qdots solubilization is obtained by a mixture of S and P. Qdots can be targeted with biotin (B), a peptide recognition sequences (R), or other chemical moieties. Qdots fluorescence can be turned on or off by attaching a quencher (Q) via a cleavable peptide link. In the presence of the appropriate enzyme, the quencher is separated from the qdot, restoring the photoluminescence and reporting on the enzyme activity. For simultaneous PET and fluorescence imaging, qdots can be rendered radioactive by chelation of radionuclides using DOTA (D).

ple and achieves both solubilization and bioconjugation in one single reaction step (Figure 2A).

Another advantage of the peptide exchange chemistry is the potential for combinatorial assembly of desired functions on the nanoparticle surface. We have demonstrated that this bioactivation approach is particularly suitable for targeting and detecting individual protein receptors in living cells (Pinaud et al., 2004).

The large number of potential surface attachment groups (covering the tens to hundreds of nm^2 of qdot's surface area) can be used to "graft" different functionalities to individual qdots, in order to produce multimodality probes (Figure 2B). For instance, additional contrast mechanisms like microPET imaging can easily be applied after adequate engineering of the peptide. Figure 3A shows that qdots having DOTA (a chelator used for radiolabeling) and 600-dalton Polyethylene Glycol (PEG) on their surface can be radiolabeled with ^{64}Cu (a positron-emitting isotope of copper with a 12.7-hour half-life). These qdots were injected (~ 80 µCi per animal) via tail-vein into nude mice under anesthesia and dynamically imaged in a small animal microPET scanner. Rapid and marked accumulation of qdots in the liver quickly follows their intravenous injection in normal adult nude mice. The liver is the single largest organ that traps the majority of the injected qdots. The uptake of qdots by Kupffer cells that are part of the reticulo-endothelial system in the liver is the likely reason for the high accumulation of radiolabeled qdots in the hepatic region, which could be minimized by using higher molecular weight PEG-peptide coating. Biopsies of the liver observed by optical microscopy confirm this accumulation of qdots within the liver (Figure 3B). A further step could involve electron microscope imaging of the precise localization of qdots in cells, illustrating the potential of qdots as probes at the macro-, micro-, and nano-scale.

A scenario could be envisioned in which cancer diagnosis and patient management could be greatly enhanced by imaging peptide-coated qdots at all scales, from the level of the whole body down to the nanometer resolution using a single probe and different imaging platforms such as microPET, two-photon light microscopy, single molecule localization and electron microscopy.

For example, patients at high risk for colorectal cancer would be intravenously injected with color-coded qdots coated with engineered antibodies specific for cancer-associated cell surface markers prior to colonoscopy. The colonoscopy could be performed using "next generation" endoscopes that will be able to excite and image the emission from the qdots. Alternatively, a fiber confocal-based catheter could be guided to the tumor site for real-time, in vivo "virtual optical biopsy." Where confirmation is needed, tissue removed in real biopsy could then be analyzed for the presence of cancerous cells using fluorescence microscopy (already stained with targeted qdots). This

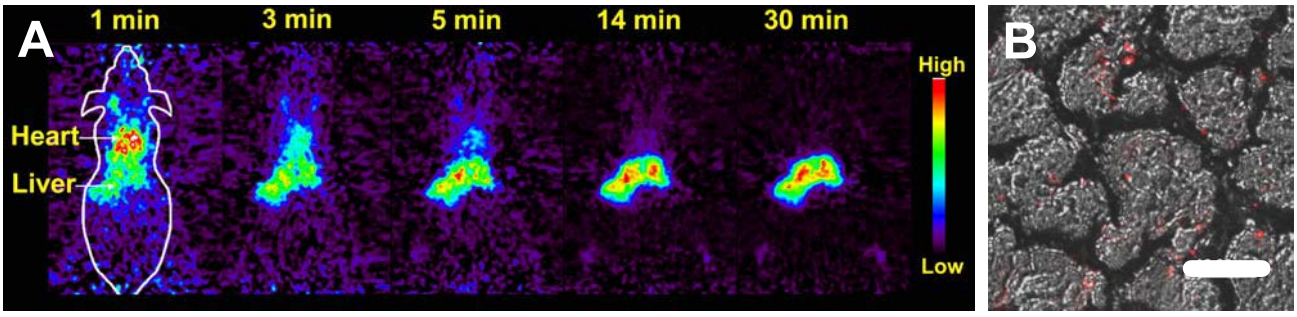

Figure 3. MicroPET of ^{64}Cu qdots. Qdots were injected via tail-vein into nude mice and imaged in a small animal scanner. **A.** Rapid and marked accumulation of qdots in the liver quickly follows their intravenous injection in normal adult nude mice. **B.** Overlay of a DIC image (Differential Interference Contrast is a popular method of contrast microscopy that utilizes dual-beam interference optics to yield images with a shadowy, three-dimensional relief effect) and a fluorescence image of liver sections showing the accumulation of qdots within cells.

would potentially allow earlier cancer detection by providing molecular specificity and sensitivity. In addition, this patient could also be imaged using clinical PET with radiolabeled qdots. This would allow for the determination of any other sites of cancer involvement and would also be useful for following up patients after initial treatment. Lastly, after diagnosis is made, targeted qdots could be irradiated by X-rays or infrared (IR) light to heat-up the tumor and trigger apoptosis (programmed cell death, Figure 4).

Peptide-coated qdots are undoubtedly opening up exciting new opportunities in cancer medicine given their unmatched potential as novel fluorescent, non-isotopic, intravascular probes for both diagnostic and therapeutic purposes.

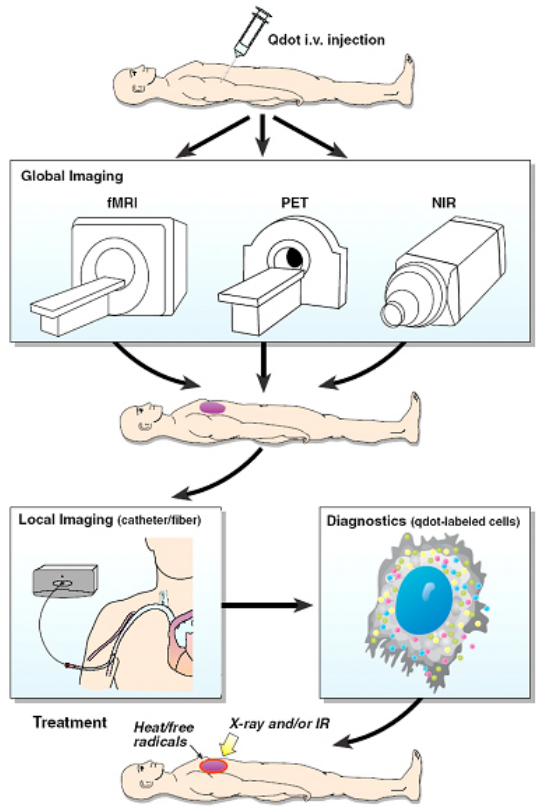

Figure 4. Envisioned future applications for qdots as cancer bioimaging and therapy agents. After injection, qdots homing to the tumor are visualized by PET/MRI/NIR. The tumor is diagnosed in real-time by a confocal catheter or by biopsy and fluorescence analysis. Treatment by specific X-ray or infrared (IR) light absorption by qdots or by free radical photogeneration can follow. Artwork by Lynne C. Olson, UCLA Photographic Services.

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