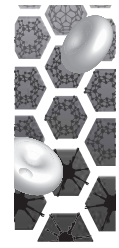
REVIEW



Optical molecular imaging agents for cancer diagnostics and therapeutics

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The National Cancer Institute has set the goal of eliminating suffering and death due to cancer by 2015. A key strategy to achieve this goal is to improve early detection and prevention using novel molecularly targeted cancer diagnostics and therapeutics. As we begin to better understand the cellular and molecular pathways of carcinogenesis, it is possible to identify and treat precursors to cancer before changes are detected at anatomical levels. Developing imaging techniques with the ability to detect molecular signatures will not only target these abnormalities for therapy at the earliest possible stages but will also prove useful in further unraveling the molecular origins of cancer. The ability to image noninvasively in real-time makes optical imaging well suited to early detection. Molecular characterization in combination with optical imaging provides a sensitive and specific method to detect and prevent the progression of precancerous lesions.

Molecular imaging

Improvements in biochemistry and molecular biology are rapidly uncovering the molecular mechanisms of disease. The role of human papillomavirus (HPV) in causing cervical cancer was discovered just 23 years ago and already today several prophylactic and therapeutic vaccines have been developed to combat this virus [1–3]. When a transformative pathway is triggered, as in an active HPV infection, a unique set of biomolecules undergo changes in expression levels that correlate to the transformation [4]. Charting these changes at various stages of the precancer to cancer sequence gives a detailed map of the carcinogenesis process and provides potential targets for diagnosis and therapy. Detection of these target molecules provides the opportunity to arrest neoplasia at its earliest stages and effectively prevent the subsequent progression to cancer and the costly and painful treatment associated with it. Prevention through early detection remains the best strategy to reduce both cancer incidence and mortality. Beyond early detection, visualization of the molecular features of carcinogenesis may also offer opportunities to guide and monitor the response to therapy.

Advances in the emergent fields of biophotonics and nanotechnology are enabling the ability to visualize molecular changes in tissue with subcellular spatial resolution. Optical imaging enables real-time, noninvasive visualization of biomarkers, affording multiple and frequent measurements, thereby reducing or avoiding the need for costly, time-consuming and often painful biopsy and subsequent histological analysis. Optical molecular imaging requires two vital components: a molecular-specific signal and an imaging system to detect this signal. While optical imaging devices have greatly advanced in recent years, this review focuses on recent advances in nano-engineering to develop novel optically active probes to enable imaging of disease-specific targets [5]. Optical probes that provide molecular-specific signal are being designed with a number of novel imaging agents including metal nanoparticles, composite nanoshells, quantum dots (Qdots) and smart fluorophores.

Targeting

Naturally occurring differences in the optical properties of normal and abnormal tissue have been exploited frequently in optical detection systems [6]. However, image contrast and diagnostic accuracy can be improved drastically by using targeted, optically active contrast agents to enhance the optical signal from disease-specific molecular markers. Regardless of the imaging agent employed, targeting of disease-specific markers is used to provide a meaningful signal for pathological diagnostics. Several factors need to be evaluated in choosing an appropriate target for detection. These include epitope availability, specificity to disease and the availability of a suitable probe molecule that binds to the candidate marker. The probe molecule itself should exhibit little to no toxicity, have high

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future medicine

affinity for the target and provide high contrast to background noise. High disease specificity is achieved by choosing markers that are expressed uniquely in pathological processes. Fortunately, the same targeting molecule used in studying the candidate biomarker often provides a suitable probe molecule. The targeting molecule used to characterize the molecular features of pathogenesis can be bioconjugated to imaging agents to provide good contrast agents. Monoclonal antibodies or antibody fragments, for example, which are used routinely in immunohistochemistry studies, can be used as probe molecules for contrast agents for *in vivo* imaging, in a manner analogous to traditional *in vitro* molecular biochemistry techniques [7]. In addition, advances in phage display-related technologies are facilitating the use of small peptide derivatives and aptamers as probe molecules for recognition and targeting. Phage display uses bacteriophages to produce and select synthetic proteins and aptamers that have similar target recognition qualities to those of natural antibodies. The phage is genetically engineered to display a protein of interest on its coat while retaining the encoding DNA within itself. Affinity purification for the target biomarker of interest is used to select a suitable phage displaying the probe molecule of interest from a vast library of random phages [8,9].

Once an appropriate probe molecule is identified, it is conjugated to the surface of an optically active imaging agent. Conjugation can either be direct or indirect via a linker segment, as shown in Figure 1. In choosing a linker, length and composition can play an important role in retaining the function and availability of the probe molecule. In addition to the probe molecules themselves, the imaging agents can be further functionalized with other moieties that can reduce nonspecific labeling or improve retention within the body. Polyethylene glycol (PEG), a hydrophilic biocompatible polymer, is often used in this capacity. Various peptides and polymers have also been proven effective in enhancing delivery and to direct passive or active targeting strategies. These include, but are not limited to, the nuclear localization peptides and various biological and synthetic polymers [10].

A number of optically active nanoparticles have been used to provide a strong source of optical signal in targeted contrast agents, including metal nanoparticles, nanoshells, Qdots and fluorophore/quencher pairs. Recent advances in each area are described in the following sections.

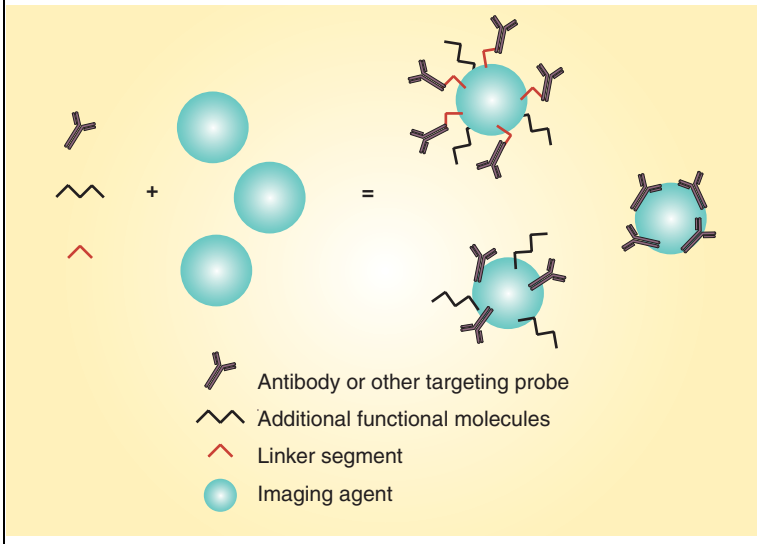
Metal nanoparticles

Probes based on metal nanoparticles utilize their unique interactions with light to provide optical contrast. Nanoparticles resonantly scatter light upon excitation of a conduction band of electrons on the surface of the nanoparticle, known as the surface plasmon [11,12]. The surface plasmon resonance depends on various intrinsic and environmental factors of the particles, including composition, size, shape, aggregation state and dielectric medium. Several properties of these plasmonic nanoparticles make them advantageous for imaging in biological systems. Since resonant scattering is elastic in nature, the signal benefits from photostability, allowing the particles to be imaged indefinitely. The sensitivity of the surface plasmon resonance to size and shape also provides optical tunability over a range of wavelengths. Colloidal gold nanoparticles are easily synthesized in aqueous solution by reducing $HAuCl_4$ with sodium citrate [13]. This reaction also affords size control of the monodisperse colloidal nanoparticle suspension. Gold nanoparticles are nontoxic and have been used in various other medical and biological applications. Since gold nanoparticles have long been used in electron microscopy, the strategies for conjugation to various probe molecules are well established [14,15].

Optical contrast can be further enhanced by exploiting the distance-dependent scattering properties of closely spaced assemblies of metal nanoparticles. As the mean particle–particle distance decreases, the scattering efficiency per particle increases and there is a marked red shift in the scattering spectra. Thus, the intensity and peak wavelength of the scattered signal is extremely sensitive to particle spacing [16]. This distance dependence has been exploited recently in developing a number of *in vitro* DNA assays, including monitoring hybridization kinetics [17], DNA probe assays with zeptomole detection limits [18] and oligonucleotide detection with single base-pair mismatch sensitivity [19].

Inducing the aggregation of particles in the presence of a disease-specific target or interaction of multiple targets can provide highly sensitive contrast in reflectance imaging. Many cancer-related receptors exist as closely spaced hetero- or homo-dimers, providing opportunities to exploit this property of metal nanoparticles. Sokolov and colleagues have demonstrated this contrast using 20 nm gold nanoparticles targeted to epidermal growth factor receptor (EGFR), the overexpression of which is a hallmark of many epithelial

Figure 1. Targeting moieties can be conjugated to optical probes either directly or using various linkers in addition to other functional molecules.

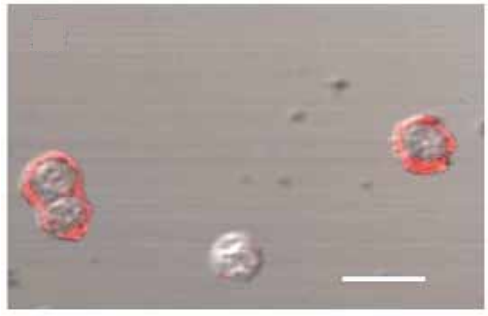


cancers. The nanoparticles are bioconjugated to anti-EGFR antibodies to image live cervical cancer cells as well as *ex vivo* cervical cancer tissue, as shown in Figures 2 & 3 [20–22].

Nanoshells

Nanoshells are composite nanoparticles consisting of a dielectric core and a thin metallic shell. Silica nanoparticles, which serve as the dielectric core, are nucleated with small gold colloid clusters and grown into a thin metallic shell by reducing $HAuCl_4$ on the surface in the presence of potassium carbonate and formaldehyde [23]. As in metal nanoparticles, the thin gold shell imparts significantly improved photostability. Nanoshells are novel in that their optical resonance can be tailored over a broad range from the near UV to the mid-infrared (IR) by varying the core:shell ratio and the overall size of the particles as shown in Figure 4. Optimizing nanoshells toward the near-IR (NIR), where hemoglobin and other biological chromophores have their lowest absorption, can be very useful in circumventing some of the limitations of depth penetration inherent in optical imaging. Tissue scattering and fluorescence is also minimal in the NIR wavelength regime, giving little to no background signal. Varying these parameters also influences the relative absorbance and scattering properties of these agents, which can modulate functionality. Backman and colleagues have recently described a theoretical model to achieve multicolor labeling using narrow controllable resonances [24]. Halas and West and colleagues are investigating the potential for nanoshell bioconjugates for use in molecular optical scattering-based reflectance imaging [25], the use of scattering nanoshells in optical coherence tomography (OCT) [26] and the use of absorbing nanoshells in NIR thermal therapy of tumors [27,28].

Figure 2. Laser scanning confocal reflectance overlaid on transmittance of epidermal growth factor receptor (EGFR)-positive cancer cells labeled with anti-EGFR gold nanoparticle conjugates.



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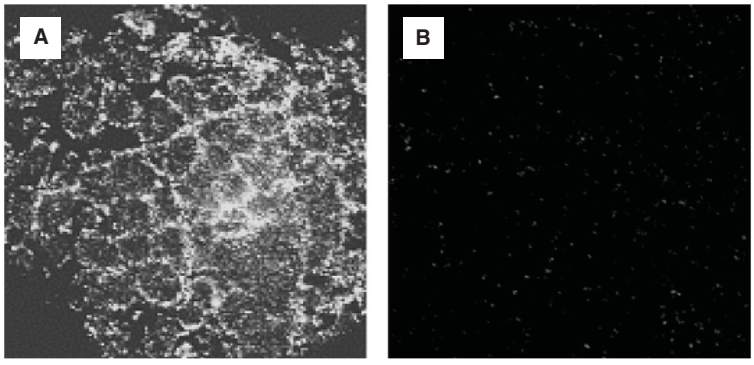
Scattering nanoshells exhibit a large optical cross-section, which is invaluable in high resolution reflectance microscopy and OCT. Nanoshells were conjugated to antibodies via a heterobifunctional linker and used to target HER2, a clinically significant marker of breast cancer, in SKBR3, a carcinoma cell line known to overexpress HER2. Darkfield microscopy showed significantly increased scattering intensity in targeted labeled HER2-positive cells over nonspecific nanoshells as well as in HER2-negative cell lines. Scattering nanoshells can also be tailored for *in vivo* imaging using OCT, an interferometric technique offering cross-sectional images of optical reflectivity [26].

Nanoshells optimized to absorb light can be used to mediate the photothermal ablations of cancers. Photothermal ablation uses the nanoshells as photosensitizers to absorb light, which is converted to heat providing localized necrosis of tissue. The nanoparticle-assisted photothermal therapy (NAPT) has been demonstrated both in cancer cell lines where HER2–nanoshells effectively ablated human breast cancer cells (Figure 5) and in the live mice inoculated with murine colon carcinoma

Figure 3. Clinically (A) abnormal and (B) normal cervical biopsies topically labeled with anti-epidermal growth factor receptor gold nanoparticle conjugates.



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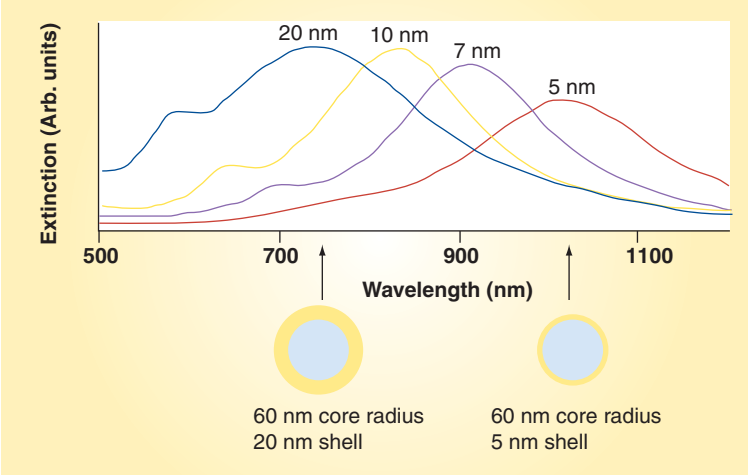
tumor cells. Nanoshells are delivered to the murine tumors via passive extravasation based on leaky vasculature often found around tumors. Within 10 days of photothermal treatment in the presence of nanoshells, the tumors were no longer visible. These mice remained healthy 90+ days post-treatment, whereas the tumors in the control/sham mice continued to grow unchecked. The control and sham mice were euthanized when the tumors exceeded 5% body weight on day 12 and 19, respectively. Nanoshells are also being designed to simultaneously provide both scattering and absorption properties at specific frequencies, which will enable a dual imaging/therapy approach [27,28].

Figure 4. The optical scattering of nanoshells varies as a function of shell thickness, with a constant 60 nm core radius.



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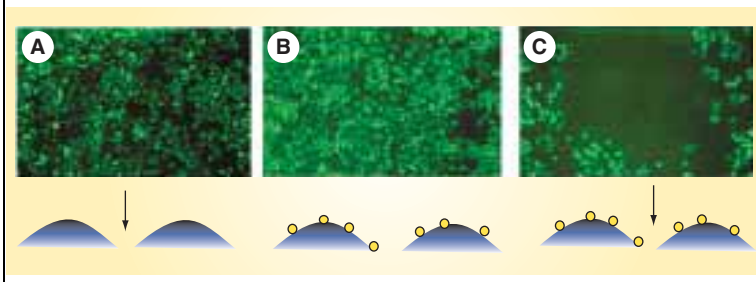
Quantum dots

Qdots are fluorescent semiconductor nanocrystals that are smaller than the Bohr exciton radius, giving rise to quantum confinement effects. These 2–10 nm nanocrystals fluoresce upon the radiative recombination of an electron-hole pair (exciton) that is created with the absorption of a photon with energy above the semiconductor band gap energy. This absorption/recombination process offers favorable optical properties for Qdots. Absorption has as increased probability at higher energies (shorter wavelengths) and results in a broadband absorption spectrum. Recombination of the created exciton leads to emission of a photon in a narrow symmetric band defined by the size and composition of the Qdots. The broad absorption spectrum enables the excitation of multiple sizes of Qdots with a single light source while retaining the narrow spectral emission without overlap [29,30]. A thin layer of semiconductor material is often grown on top of the core to protect the nanocrystal surface from oxidation and other chemical reactions. The atomic-layer shell also covers surface defects that detract from radiative recombination and can increase the fluorescent quantum yield to more than 90% efficiency, comparable to fluorescent dyes. The protective shell also improves the photostability of the Qdot, which is a vast improvement over organic dyes. Other benefits of semiconductor Qdots over traditional fluorophores include long luminescent lifetimes, resistance to photobleaching and improved brightness owing to extinction coefficients that are an order of magnitude larger than most dyes. The relatively large extinction coefficient allows sensitivity limits down to one Qdot per target molecule, which can be utilized to perform nanometer resolution confocal microscopy [31].

Qdots have been imaged in a number of biologically and clinically relevant studies. Single Qdot trajectories have been followed laterally in cell membranes, targeting glycine receptors, as well as in internalization into phagokinetic human cancer cells [32,33]. Biotinlylated epidermal growth factor (EGF) has been used in conjunction with commercially available streptavidin Qdots to study receptor tyrosine kinase (RTK)-dependent signal transduction in various cancer cell lines [34]. Streptavidin Qdots have also been used to detect HER2 in SKBR2 breast cancer cells via biotinylated secondary human antibody and primary anti-HER2 antibody [35]. Åckerman and colleagues use peptides

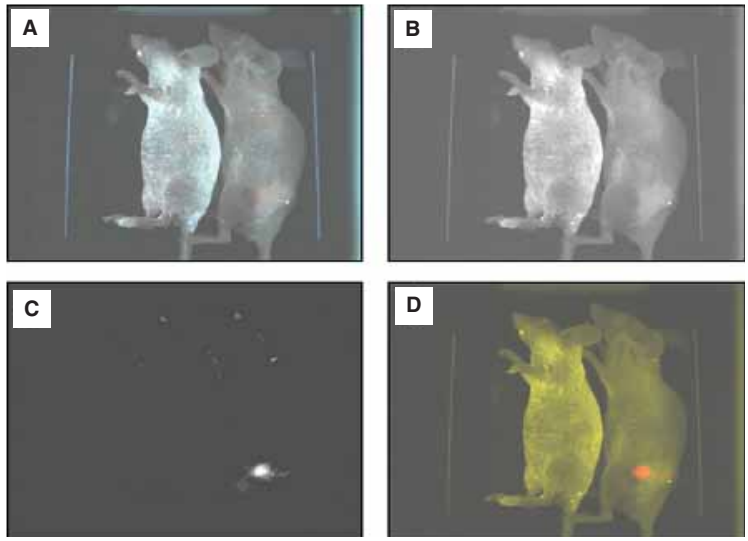
Figure 5. Nanoparticle-assisted photothermal ablation using calcein AM staining of cells to indicate viability.



(A) Cells without nanoshells exposed to laser light. **(B)** Cells incubated with nanoshells but not exposed to laser light. **(C)** Cells incubated with nanoshells after laser exposure are effectively ablated. Reprinted with permission from [26].

to target intravenously injected Qdots to specific vascular markers and Ballou and colleagues have shown that coating Qdots with PEG improves circulating lifetimes and reduces accumulation in the liver and bone marrow [36,37]. Gao and colleagues have developed Qdots with triblock polymers to incorporate tumor-targeting ligands and PEG molecules in targeting human prostate cancers grafted in nude mice, as depicted in Figure 6. Using either subcutaneous injection or systemic injection, these Qdots accumulate at the tumors via passive-enhanced permeability at tumor sites and active targeting to cancer biomarkers [38]. Jain and colleagues have demonstrated the versatility of Qdots in studying various tumor pathophysiology models, including identifying tumor vessels from background vasculature, determining accessibility to the tumor site based on Qdot size and monitoring the recruitment of precursor cells to the tumor site [39]. Kim and colleagues have used near IR Qdots to guide real-time resection of sentinel lymph nodes. The Qdots are coated with polydentate phosphine coating to improve stability and injected intradermally into live mice and pigs, which then migrate into the lymph nodes within 3–4 min and allow complete mapping [40]. Near IR Qdots were also used successfully as angiographic contrast agents in mice at a depth of 1–2 mm through fat, skin and bone [41].

Figure 6. *In vivo* fluorescence of prostate-specific membrane antigen-targeted quantum dots in C4–2 human prostate tumor-bearing mice.



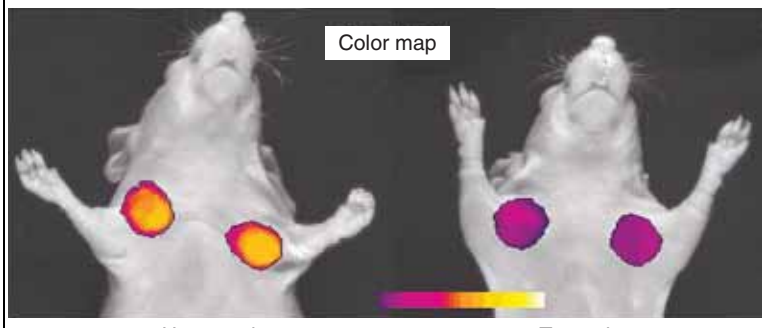
Orange fluorescence signals indicate a prostate tumor growing in a live mouse (right) as opposed to the healthy control mouse (left). **(A)** Original image; **(B)** unmixed autofluorescence image; **(C)** unmixed QD image; and **(D)** superimposed image. Reprinted with permission from Macmillan Publishers Ltd: *Nat. Biotechnol.* [38] © 2004.

Smart contrast agents

Fluorescent proteins and organic dyes have long been established in their ability to provide optical contrast. Their applications in biology and medicine have been described recently in a number of excellent reviews and will not be a focus here [42]. More recently, a number of smart contrast agents, which alter their signal intensity based on interactions with specific targets, have been developed. Activation of the signal is therefore tailored to the site of interest and can be based on changes in conformation, chemical structure or displacement. This provides two key benefits – low intrinsic background signal and inherent signal amplification dependent on target concentration. Because the smart agents give no signal intensity in their inactive or 'off' state, there is no need to account for signal that has not reached the target. When the site-specific smart contrast agents are activated or are in the 'on' state, the signal intensity will be a function of target concentration [43–45].

The first smart fluorophores, developed by Weissleder and Ntziachristos in 1999, transitioned from a quenched state to a fluorescent state upon proteolytic activation. They consist of NIR fluorophores attached to polylysine-PEG co-polymers that can be cleaved by the mouse tumor-associated lysosomal proteinase, cathepsin B [43]. A similar cathepsin B-targeting peptide linked to Cy5.5 was used to detect intestinal adenomas in a mouse model of adenomatous polyposis [46]. Various analogous smart detection systems have been expanded to other proteinase

Figure 7. *In vivo* near infrared fluorescence imaging of HT1080 tumor-bearing mice showing color-coded matrix metalloproteinase-2 activity overlaid on white-light images with and without treatment, demonstrating *in vivo* imaging of protease inhibition.



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settings, most notably cathepsin D, intracellular proteinases and matrix metalloproteinases (MMPs) [47–49]. Smart probes targeting cathepsin D successfully assayed well differentiated and undifferentiated mammary tumors in a mouse model of human breast cancer [47]. Intracellular proteinases, such as caspase-3, which are correlated with apoptosis, have been detected using caspase-3-cleavable recombinant luciferase fusion proteins [48]. Figure 7 shows MMP smart agents used to image the reduction of signal in HT1080 fibrosarcoma-grafted nude mice after treatment with MMP inhibitors, thus providing a novel approach for monitoring the effectiveness of anti-tumor therapies [49].

Conclusions

Optically active, molecular-specific contrast agents provide a new tool to enable noninvasive visualization of disease-specific molecular changes with subcellular spatial resolution. The combined use of contrast agents and optical imaging systems has the potential to improve the early detection of disease and to further study of the molecular mechanisms of disease. As probes become more sophisticated and new biomarkers are identified, dramatic improvements in early detection are expected.

Future perspectives

Molecular optical probes have proven their utility in both the clinical and laboratory setting. As improvements are made and more novel imaging agents developed, optical imaging will be invaluable in cancer diagnostics and therapy. The immediate clinical use of optical probes will require better active delivery strategies as well as further advances in incorporating smart targeting tactics. Various peptide- and polymer-based delivery vehicles are being developed and borrowed from the relatively well established field of drug delivery.

Probes that incorporate two imaging modalities are also being investigated and can be expected to be further developed. These multifunctional probes overcome the limitations of any one modality and provide the benefits of two. Recently, Weissleder employed a multimodal magnetic/optical probe consisting of an iron oxide/NIR fluorophore composite for the guided *in vivo* resection of brain tumors using both magnetic resonance and optical imaging [50]. Similar bifunctional gadolinium rhodamine dextran (GRID) particles have been used to trace stem cell transplants in the brain for neuroimaging [51].

As existing optical probes make their way through animal and clinical trials in the next 5–10 years, it will not be uncommon to see routine diagnostic assays that incorporate such probes.

Executive summary

Molecular markers

- The molecular mechanisms of disease are being discovered rapidly.
- A unique set of biomolecules undergo changes of expression during carcinogenesis and can be used to detect and potentially arrest the disease process.

Optical imaging

- Optical imaging is preferred for early detection because it allows non-invasive visualization, thus affording multiple measurements.
- Optical probes enable imaging of disease-specific biomarkers at the nanometer scale.

Executive summary

Targeting

- Targeting provides a means to obtain a meaningful diagnostic signal by providing high disease specificity.
- Factors involved in choosing a relevant target include epitope availability, specificity to disease and availability of a suitable probe.
- Monoclonal antibodies, antibody fragments, peptides and aptamers are all suitable targeting agents.

Metal nanoparticles

- Metal nanoparticles provide optical contrast by exploiting the distance-dependent properties of surface plasmon resonance.
- Sokolov and colleagues have illustrated this contrast in live cells and cervical biopsies using gold nanoparticles targeted for the detection of epidermal growth factor receptor, a hallmark of many epithelial cancers.

Nanoshells

- The relative optical absorbance and scattering of nanoshells, comprised of a metal shell and dielectric core, can be tailored over a broad range from the near UV to the mid-infrared (IR) by varying the core:shell ratio and the overall size of the particles.
- Scattering nanoshells show significantly increased scattering efficiency in targeting labeled cancer cell lines.
- Nanoparticle-assisted photothermal therapy has also been achieved in live tumor-bearing mice.
- Dual imaging/therapy is possible with nanoshells that simultaneously exhibit scattering and absorption at specific frequencies.

Quantum dots

- Quantum dots (Qdots) are 2–10 nm semiconductor nanocrystals that fluoresce upon radiative recombination of an exciton.
- Qdots offer many advantages over traditional organic fluorophores, including a broad absorption and narrow emission spectra (which allows multicolor labeling), resistance to photobleaching, improved brightness and long luminescent lifetimes.
- Qdots have been used successfully to target human prostate cancers grafted in mice as well as in guiding the real-time *in vivo* resection of sentinel lymph nodes.

Smart fluorophores

- Smart contrast agents alter their signal intensity based on interactions with specific targets.
- This enables low intrinsic background and signal amplification that is dependent on target concentration.
- Smart fluorophores have been shown to image various proteases, namely tumor-associated lysosomal proteinases, intracellular proteinases and matrix metalloproteinases.

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