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Advances in contrast agents, reporters, and detection

Darryl J. Bornhop
Texas Tech University
Department of Chemistry and Biochemistry
Lubbock, TX 79409-1061

Christopher H. Contag
Stanford University School of Medicine
Department of Pediatrics
Stanford, CA 94305-5208

Kai Licha
Institut für Diagnostikforschung
GmbH an der Freien Universitaet
14050 Berlin, Germany

Catherine J. Murphy
Univ. of South Carolina/Columbia
Department of Chemistry
Columbia, SC 29208

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The use of exogenous probes to gain a deeper understanding of physiological and molecular processes *in vivo* through the acquisition of optical signals, particularly via enhanced contrast using molecular probes (physiologically transported, site-directed, or via reporter genes) has emerged with tremendous vigor in the past few years. One such area of expanded activity is in the area of early cancer detection, in great part because it is so critical to the clinical outcome in the treatment.[1–3] As an example, in colon cancer, which accounts for 15% of all U.S. cancer-related deaths, only 37% are found early enough for moderate treatment[1] and once these types of cancer reach metastatic activity the survival rate is only 7%. Oral and brain cancer represent other examples where a need exists for early detection or improved imaging during treatment. Each year about 31 000 Americans develop oral cancer. Squamous cell carcinoma (SCC) accounts for 95% of all malignant oral lesions with SCC having a survival rate of only 50%. Yet when this type of cancer is detected in its earliest stages, the survival rate becomes approximately 80%.[4] In cancers of the esophagus, the five-year survival rate is only listed at 5%. In contrast, if these cancers are detected when it is still contained in the mucosa the five-year survival rate becomes 90%.[5] For brain cancer the survival rate is abysmal—less than 2 years for younger patients and just weeks for those that are older—and is critically dependent on the imaging technique used during treatment.[6]

Current state-of-the-art detection techniques miss early stage disease. These detection protocols employ white light endoscopy or microscopy with gross visualization.[7,8] Yet the visual cues for determination of disease state are small, especially the discrimination between non-malignant and dysplastic and pre-malignant lesions. Visual assessment of early lesions within the colon and oral cavities depends on many factors, including the experience of the clinician and his/her ability to identify the suspect lesions at an early stage of development, and selection of the suspect site that is to be biopsied. Rex et al.[9] reported that patients undergoing back-to-back colonoscopies, performed by an experienced colonoscopist, have as many as 15–24% of their neoplastic polyps smaller than 1 cm overlooked. In addition, up to 6% of larger polyps would escape detection. Clearly there is a significant need for the enhancement of detection of diseased tissue at its earliest stages to improve the clinical outcome.

Alternative techniques to aid *in-vivo* diagnosis have recently been reported, including the use of techniques to detect changes in the *native* spectroscopic properties of tissue.[10–17] Of particular interest recently has been the use of autofluorescence[18] to quantify changes in pathology. Some evidence even exists that suggests tissue staging can be accomplished, allowing transformation from dysplasia to cancer to be detected. All tissue contains fluorophores that absorb light and subsequently emit light at a longer wavelength. Nicotinamide adenine dinucleotide (NAD[H]), flavins, collagen, and elastin are commonly known tissue fluorophores. It is currently believed that autofluorescence primarily detects changes in concentration or distribution of these components.[19,20] As normal tissue becomes dysplastic the concentration or distribution of these endongenous fluorophores changes, thus leading to a detectable change in the resulting fluorescent spectrum. These changes are wavelength dependent and correlate with changes in histology. While promising as a diagnostic tool, these signatures are generally not good candidates for the detection of *early* lesions. The inherent limitation of autofluorescence techniques for early detection is low S/N stemming from a relatively low signal (small changes in concentration of the solutes detected in early disease) and a large background (scattering, reflected

light, etc.) rendering the results of any quantitative measurements rather complicated.[21]

Because of the limitations associated with early detection by native spectroscopic technique and the desire to perform minimally invasive diagnosis, the use of contrast agents for diagnostic optical imaging has currently experienced an expanded level of attention.

One way to increase the effectiveness of autofluorescence is to make use of 5-aminolevulinic acid or ALA,[22] which is a precursor to the endogenous protoporphyrin IX. Protoporphyrin IX (PP IX) is a class of porphyrins that has been shown to over-accumulate in certain premalignant tissues and display somewhat attractive fluorescent properties, excitation in the blue region (ca. 450 nm) with emission in the red region (ca. 620 nm). The use of ALA has been primarily systemic with the goal of increasing the endogenous concentration of PP IX in order to give contrast for disease detection through the production of and subsequent detection of fluophore PP IX. The most successful uses of ALA have in some cases been the detection of dysplasia in Barrett's esophagus and colitis.[23]

One of the most common non-endogenous compounds that has been used clinically as a contrast agent is toluidine blue.[24] This compound has been used as a contrast agent for the detection of occult malignancies of the cervix;[25] has been found to provide some improvement for non-invasive detection of oral cancer;[26–28] and has also been employed in the detection of SCC of the upper aerodigestive tract.[29] While the exact mechanism of staining remains unclear, a study of the interaction of toludine blue with tissue using electron microscopy suggests the main factor governing selective uptake of this dye is the change in cellular membrane permeability.[30] It was noted in this report that both injured and malignant lesions exhibit a greater permeability to the dye than that of normal mucosa. Even though toluidine blue does show improvement of sensitivity over conventional white light imaging, it suffers from a lack of specificity.[26–28]

Another example of contrast enhancement agents or site-directed chemical agents that have seen recent success if the photodynamic therapy (PDT) class of markers.[31,32] While these types of markers have shown promise in a diagnostic setting, there are limitations. Long delays for accumulation in tumors, prolonged photosensitization of skin, and phototoxicity of tissues being imaged are some examples of these limitations.[33–37]

Among the many chemical approaches taken toward the design of efficient imaging probes, the cyanine dyes represent one of the most prominent classes of compounds. Synthesized for the first time at the beginning of the century, cyanine dyes have meanwhile been promoted to well-designed probes for numerous applications in bioanalytics and biomedicine. The particular advantage of cyanine dyes arises from their chemical structure. The chromophore's optical properties are in principal adjustable, thus making it possible to generate derivatives that occupy desired absorption and fluorescence ranges (extinction coefficients up to 250,000 L $mole^{-1}$ cm^{-1}) throughout the visible to near-infrared range. At the same time, chemical substitution appropriately introduced into the chromophore turns the compounds from dyes into reactive labels for biomolecules, living structures, and many other materials.

Such labels obviously serve not only as simple fluorescent emitters but can also report on molecular processes, such as target-ligand interactions, through molecular interactions that influence the signal output. Keywords are fluorescence resonance energy transfer (FRET), fluorescence quenching, or binding-dependent fluorescence enhancement. In this respect, it pays to relate the assessment of optical signals to the use of radioisotopes. The clear advantage of optical signals over radioactive decay is that optical signals can be specifically affected by the environment and are repeatedly inducible, and they are not subject to the unavoidable half-life decay of radiation.

Indocyanine green (ICG; caridogreen), a clinically approved NIR dye used for testing of hepatic function and fluorescence angiography in ophthalmology, has already been investigated as a potential contrast agent for the detection of tumors in both animal models[38,39] and humans.[40] For instance, the detection of breast tumors in humans by diffuse optical tomography using ICG as a contrast agent was shown to be feasible, correlating with the distribution of Gd-DTPA-enhanced as obtained by MRI.[40] ICG as well as structurally related hydrophilic derivatives of altered pharmacokinetics[41,42] represent the classical format of a contrast agent, with contrast-enhancing properties mainly relying upon a more distinct perfusion of tumor tissue compared to normal areas. Although not designed with any target-seeking moiety, the value of contrast-enhanced tumor detection was successfully demonstrated.

While the cyanine dyes mentioned herein are typical near-infrared agents of high molar absorbance and efficient, but short-lived fluorescence emission, we wish to mention the class of aromatic lanthanide chelates, which exhibit completely different, yet interesting optical properties. Again not displaying target-specific structural features, the use of a pyclen-based terbium chelate has recently shown promise in detecting chemically induced colon cancers in the Sprague Dawley rat.[43] This particular molecule, Tb-[N-(2-pyridylmethyl)-N′,N″, N‴-tris(methylenephosphonic acid butyl ester)-1,4,7,10 tetraazacyclododecane] or Tb-PCTMB, has excellent fluorescent properties, high specificity, and low toxicity.[43–46] Some of the spectroscopic properties of Tb-PCTMB that are advantageous to its use as fluorescent contrast enhancement marker include an extinction coefficient of $\approx$3000 L $mole^{-1}$ cm^{-1}, a high quantum efficiency of 0.51, an emission of extremely large Stokes' shift at 550 nm (where the human eye possesses almost its maximal sensitivity), and a relatively long fluorescent lifetime (2.2 ms).[44–46] With an emission signal that is spectrally removed from the background, inexpensive instrumentation can be used and thus low tissue doses administered. Sensitivity for this class of molecules is such that femtomole/pixel (picomolar) quantities have been quantified in intestinal tissue by endoscopy.[46] This high sensitive has allowed applications of millimolar solutions to be administered, very low light levels (TLC reader lamp) to be employed, and visual detection to be used for the detection of cancer tissues in the colon of the Sprague Dawley rat.[44] In this technique the bright green fluorescence from Tb-PCTMB used as an exogenous marker facilitated sensitivity as high as 94.7% for sites suspected to be dysplastic tissues.[43] Aiming at topical examinations, topical administration might be one advantage of this class of contrast agent.

In a step to further improve the performance of contrast agents, target-specific conjugates have been used that introduce molecular specificity into the diagnostic process. Since many cancers over-express certain receptors, increased uptake for the corresponding ligand can act as a vehicle of active transport. The results is enhanced accumulation or association of this ligand with or in a certain type of cell, potentially providing high detection specificity. Conjugation of a fluorophore to these ligands has been used to target cancerous tissues or cells, thus facilitating improved imaging by fluorescence.[47,48] It is appropriate to mention the commercially available CyDye™ series consisting of Cy3, Cy3.5, Cy5, Cy5.5, and Cy7, which have been subjected to such approaches and have demonstrated to be fluorescent markers in *in vivo* optical imaging.[49] More examples of conjugates are those consisting of cyanine dyes and peptide ligands targeted for upregulated heptahelical receptors.[50,51] For example, animal tumors that over-express the somatostatin (sst2) receptor could be imaged using highly receptor-specific indocyanine green (ICG)-based peptide conjugates. In another approach dye-labeled single-chain fragment antibodies with a high affinity for angiogenesis-specific extracellular matrix proteins were used.[52] Finally, site-directed contrast enhanced imaging utilizing folate as a specific vehicle for diagnostic agents to target several different kinds of cancer cells that are known to upregulate a receptor for this complex was reported.[47]

An interesting alternate line of 'conjugated' markers are the protease-activated probes containing quenched fluorescent molecules that are cleaved off by tumor-specific proteases.[53] These compounds are attractive because the signal is suppressed until they come in contact with tissues exhibiting enhanced protease activity. The result is a distinct amplification of signal and an inherent S/N. It should be remembered, as R.K. Jain has taught us, there is an inherent resistance to transport for high molecular weight or bulky markers, which is often the case for conjugated complexes or those that depend on active delivery mechanisms.

One of most exciting developments in recent years is the use of inorganic nanoparticles as dyes and reporters in biological applications. These semiconductor nanoparticles or quantum dots fluoresce in the visible, with narrow bandwidths and tunable emission wavelengths that are size-dependent, and can be surface-conjugated with a variety of groups. Thus, these nanoparticles may function as dyes for a broad spectrum of biological and bioanalytical applications.[54–56] The synthetic preparation of these probes has progressed in the last few years, so that by choosing the appropriate reaction conditions, more or less any desired particle size (1–100 nm) is available in almost monodispersity. It has been shown that chemical surface modification is possible, and the fluorescence may be tuned by different adsorbates, thus functioning as sensors for physiological analytes, proteins, or DNA/RNA's.[57,58] Metallic nanoparticles can serve as reporters in biological applications based on induced aggregation to give visible color changes,[59,60] permit surface-enhanced Raman spectroscopy of anlaytes adsorbed to their surface,[61] or are applicable in surface plasmon resonance spectroscopy.[62] Recent SPIE symposia (Conference 3924 in 2000 and Conference 4258 in 2001) focused on nanoparticles. For this novel class of compounds numerous exciting applications are at the horizon.

An optical imaging approach of a fundamental different kind has recently emerged: the use of reporter genes with externally detectable optical signatures. This novel approach to contrast enhancement has aided in furthering our understanding of molecular and cellular events *in vivo*. Since light is transmitted through mammalian tissues, at a low level, optical signatures conferred on cells by expression of reporter genes, such as luciferases and fluorescent proteins from a variety of organisms, can be detected externally. Both of these types of reporter genes, which include a large number of variants with different physical properties and emission maxima, have been used to label tumor cells and monitor tumor cell growth and regression in responses to various therapies in living experimental animals.[63,64] Luciferase reporter genes have also been used to assess and follow whole body gene expression patterns[65,66] and to monitor the progression of infectious disease[67] or the efficacy of chemo- or immunotherapeutic treatment *in vivo*.[68] Detection of luciferase expressing cells *in vivo* is an extremely sensitive method with signals over background being apparent from as few as 100–1000 tumor cells in mice and can be accomplished with bench top scanning systems[69] that are relatively inexpensive and easy to use.

These tools that employ optical reporter genes for molecular and cellular imaging have been applied to several models of human cancer making it possible to follow tumor cell growth from a time when the lesion is comprised of very small numbers of cells to the point of extensive disease. Thus, therapies that are designed to treat minimal disease states can be evaluated, as well as those that target late stage disease where the tumor burden is significantly greater.[68,70]

In vivo imaging through the use of optical reporter genes is sensitive and quantitative, permits real time spatiotemporal analyses of the dynamics of neoplastic cell growth, and facilities rapid optimization of effective treatment regimens. The accessibility and versatility of using reporter genes for optical measurements *in vivo* provides investigators in a variety of disciplines with the ability to follow biological processes *in vivo* without requiring expensive instrumentation and dedicated imaging facilities. These methods have the potential to revolutionize *in vivo* biological investigation and could provide the basis for human imaging strategies that have broad clinical utility.

In summary, we believe that the combination of biological principles, quantitative imaging techniques, and optical probes (reporters) will lead to improved tools to monitor molecular level tissue transformation and disease-associated processes and to improve clinical outcomes. On the horizon is an ever-expanding need for contrast agents, particularly those that are ''smart'' because there is an inherent S/N limitation when using direct spectroscopic methods for the quantification solute concentration changes indicative of those present at the cellular level. While our enthusiasm about realizing *molecular biopsy* is high it still remains to be seen whether these agents will have the low toxicity, long-term stability, spectroscopic properties, and specificity necessary to be used as human clinical tools.

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