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Using molecular beacons for cancer imaging and treatment

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1. ABSTRACT

Molecular beacons are essentially all probes that illuminate particular cellular target or cells with similar characteristics. In this review we focus on those molecular beacons that use near-infrared fluorescence imaging (NIRF-I) to identify the unique cellular and metabolic markers characteristic of cancer. They employ various delivery and activation pathways, selectively or specifically targeting proliferating and immortal cancer cells. These beacons can either be used in an imaging step separate from therapy or they can intimately connect these two steps into a single process. Matching cancer therapy to NIRF-I is photodynamic therapy (PDT) that uses the light-triggered phototoxic properties of some porphyrin-based dyes. Guided by beacon's restored fluorescence, the PDT laser could be focused on affected sites, killing the cancer cells using the enhanced photoactivity of the same beacon. Or vice versa—the restored fluorescence from the cleaved beacon could be used as an indication of the beacon's own therapeutic success, imaging the post-PDT apoptotic cells.

2. INTRODUCTION

Imaging cancer is a crucial step in the process of curing this complex disease. Using imaging for cancer diagnosis as well as post-treatment follow-up is the primary means of fighting cancer in present clinical applications. The future of clinical work is directed towards imaging of specific molecules involved in cancer development and proliferation, backed-up by the extensive research focused on the molecular basis of cancer that can help us to understand the disease better and fight it more efficiently (1).

Fast metabolism is an obvious characteristic of tumors that can be used in distinguishing the insatiable cancer from normal-growing tissues. An example of such an indicator is 2-deoxyglucose (2DG) that is used primarily in Positron-emission tomography (PET) (2), but it recently also found its place in fluorescence imaging (3, 4). Near-infrared fluorescence imaging (NIRF-I), which can take advantage of the wide variety of small fluorescent probes

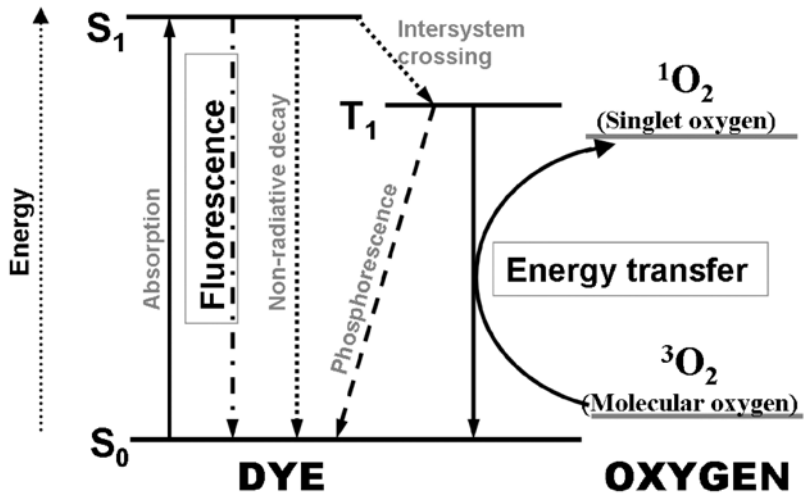

Figure 1. Jablonsky diagram modified to include the photosensitization processes during PDT. It shows the relaxation from an excited singlet state by fluorescence emission or by energy transfer to the molecular oxygen after intersystem crossing to the triplet state.

that can be conjugated to almost every molecule, together with sensitivity approaching the molecular level, is especially useful if specific molecules, rather than physical differences, are the target of imaging (5). To focus on cancer, varieties of molecules are unique to or overexpressed in cancer (6) and can serve as targets for molecular imaging. In order to image solely cancer cells, one must adopt strategies of selective delivery and activation that will favor the small volume of cancer cells over the rest of the body composed of variable tissues. This challenging goal can be achieved by using probes that employ cell-specific controlled activation: molecular beacons (MBs). Depending on the purpose, MBs may incorporate both a quencher that silences the beacons in normal tissues and thus reduces the background and toxicity, and a delivery vehicle that directs the MB to the affected cells and increases the beacon's efficacy. The basic design could be easily modified to redirect the beacon's specificity and also, if needed, to implement a therapy into the imaging core function as in so-called "photodynamic molecular beacons".

3. BASIC PRINCIPLES

3.1. Near-infrared fluorescence imaging (NIRF-I)

Although fluorescence imaging is a method widely used in physics, chemistry, and medicine for a respectable period of time, its *in vivo* application became more intriguing only recently, once shifted to the near-infrared (NIR) region. The NIR region (650-900 nm) is an ideal range of wavelengths for *in vivo* imaging where the influence of the main tissue absorbing components, oxy- and deoxyhemoglobin (λ_{max} ~560 nm) and water, is minimal. As a result, the NIR light can penetrate deeper into and out of tissues than visible light (7). The transition of fluorescence imaging from solution into tissues has its positives and negatives. The excitement of the possible new applications utilizing the molecular sensitivity of imaging biological processes is counterbalanced by the non-linear dependence (because of quenching) of the fluorescence signal on the abundance of the marker that is traced *in vivo*. The interpretation of the fluorescence signal is complicated by a large number of factors that one must keep in mind when choosing the right set up (7). Starting with the dye, its good water solubility is a prerequisite to any systemic application and detailed information about the absorption and emission maxima, and the extinction coefficient in water should be known, since the environment influences the dye's performance. Also, the dye's biodistribution is a function of its accumulation in tissues, clearance rate, and interaction with blood proteins and strongly influences the availability of the dye ready to interact with the target. Comparing the three main groups of dyes—quantum dots, small indocyanine dyes and fluorescent proteins—the difference in size and water solubility results in very different biodistribution, where quantum dots struggle because their size (3-20 nm) results in an unfortunate toxicity (8).

Other factors, like light delivery differing for subsurface imaging and deep-tissue imaging and sensitive detection, influence the success of NIRF-I. In this review we focus on the issues of biodistribution that could be modulated by attachment of other molecules (Section 3.5.) and signal amplification by means of silencing the fluorescent probes in the absence of target and subsequent activation (Sections 3.2. and 3.3.). The connection of NIRF-I with specific activation and delivery is the necessary step towards effective imaging of cancer.

3.2. Fluorescence resonance energy transfer (FRET)

When a dye absorbs a photon, it can lose this excess energy by interaction with other molecules (e.g. solvent) or by intramolecular processes, particularly internal conversion (e.g. S_2 to S_1), intersystem crossing (e.g. S_1 to T_1), or radiative transitions (Figure 1). The

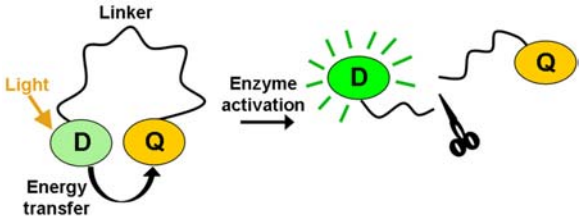

Figure 2. Molecular beacons activated by enzymatic cleavage.

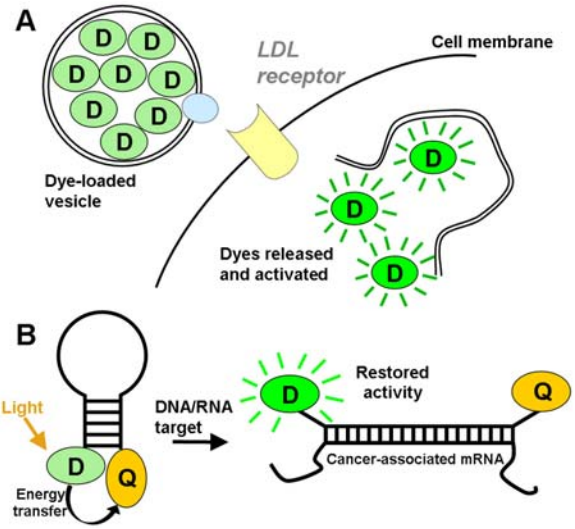

Figure 3. Molecular beacons activated by separation in space. A) Vesicles loaded with partially self-quenched dyes are selectively delivered into cancer cells (e.g. in the case of LDL, targeting the LDL receptors), releasing the dyes inside the target cells, B) DNA/RNA-based beacons have quenched photoactivity until they encounter the cancer cell-associated target mRNA.

detectable result of radiative transitions is fluorescence. In order to prevent this photon emission and therefore quench the fluorescence, the energy of the dye's excited singlet state should be transferred to a quencher. Depending on the spectral properties of the donor (dye) and the acceptor (quencher) and the distance between them, the acceptor can either absorb the already emitted photon (in the case of close overlap of their emission and absorption spectra respectively) or accept the energy of the excited donor nonradiatively (9). Out of the nonradiative energy transfers, the long-range dipole-dipole one is of particular interest. Unlike the short-range transfer occurring only if the dye and quencher are within their collision radii, long-range transfer can be highly efficient over longer distances (up to 100 Å) provided that the emission spectra of dye and absorption spectra of quencher have sufficient overlap. This physical process of nonradiative energy transfer is called fluorescence resonance energy transfer (FRET) and its inverse dependence on the sixth power of donor-acceptor distance is used as an estimation of molecular proximity (10). By measuring the changes in fluorescence intensity, this "spectroscopic ruler" provides information about the distance between dye and quencher to within 10-100 Å (11). This is used in a broad range of applications (12), such as protein-protein or protein-DNA interactions or studying a variety of conformational changes (13). The quencher can then dissipate the energy either again by radiative transition, but this time emitting a photon of a higher wavelength (in the case of dye-dye quenching) or by other means (in the case of so-called black hole quenchers), decreasing significantly the overall photon count. One of the simple applications of FRET is for quenched fluorescence probes, or molecular beacons. Using FRET to give "yes or no" information, these beacons report the presence of their target, signaling the intact or activated state in solution or in the presence of the target, respectively.

3.3. Protease- and mRNA-activated molecular beacons (MBs)

Molecular beacons (MBs) are designed to report specific events by restoring their fluorescence that is quenched in their native state. The way fluorescence is restored depends on the nature of the target. For example, if the target is an enzyme capable of cleaving chemical bonds, the fluorescence is restored when the linker between the dye and the quencher is physically cut (Figure 2). The linkers are therefore chosen from families of possible enzyme substrates—short peptides cleavable by endoproteases, oligonucleotides cleavable by DNAses or RNAses, or phospholipids cleavable by phospholipases.

On the other hand, the target could be a molecule that interacts strongly with a linker or a carrier, originally holding the dye and quencher in a close proximity, so that once these interact, the dye and quencher are forced apart and fluorescence is restored. An example of such a carrier is liposome-like formulations (e.g. low-density lipoprotein, LDL) loaded with self-quenched dyes released after interaction with membrane or membrane-associated receptor (e.g. LDL receptor) (Figure 3A). Out of the possible linkers capable of opening, the use of oligonucleotide linker, as a part of molecular beacons originally published by Tyagi and Kramer (14), is well-studied in a variety applications, reporting the presence of specific nucleic acid strand by monitoring the fluorescence increase in nucleic acid research and real time PCR (Figure 3B) (15). These beacons are composed of a dye and a quencher conjugated to opposing sites of a short nucleic acid strand. The ends (4-6 bases) are composed of bases complementary to each other, forming a stem that keeps the dye and quencher in close proximity in the native state. The middle part of this nucleic acid strand (the loop, containing 15-20 bases) is complementary to the nucleic acid strand that is to be reported/visualized. The FRET is disrupted upon the hybridization of the loop to a DNA or RNA target that outcompetes the stem's hydrogen bonds, or by denaturation of the MB's stem with temperature, pH or denaturing reagents (16). Special care needs to be taken when designing these beacons so that they will stay closed in experimental conditions but will open once the target is present, excluding non-complementary targets only with 1 mismatch by strength of hybridization (17).

3.4. Photodynamic therapy (PDT)

When a dye absorbs a photon, it can lose this energy by emitting fluorescence (*via* radiative transition from its singlet excited state to the ground state, Section 3.1.) or by intersystem crossing to its long-lived triplet state. From there, it can relax either by transferring the energy to another triplet molecule or by emitting phosphorescence ($T_1 \rightarrow S_0$) (Figure 1). Many porphyrin-like molecules can both emit fluorescence and have a high population of triplet state when excited by light. In the presence of oxygen, this porphyrin can transfer the energy from its T_1 to the oxygen, generating singlet oxygen ($^1O_2$). This is a highly reactive molecule with a short lifetime that can cause destruction of cells by reacting with biomolecules like proteins (mainly with Cys, Met, Trp, Tyr, and His), lipid membranes (unsaturated fatty acids and cholesterol) (18, 19), DNA (mainly guanine), or carbohydrates. Photodynamic therapy (PDT) utilizes this light-triggered production of $^1O_2$ for selective destruction of cancer cells and vasculature. The dye, in this case termed a photosensitizer (PS), can be applied topically (e.g. 5-aminolevulinic acid metabolized inside the body to Protoporphyrin IX used for skin cancer) or systemically. Depending on the injection-treatment delay, PSs can mainly target the vasculature (if the light treatment follows the intravenous injection of, for example, Verteporfin, approved for destruction of abnormal vasculature in macular degeneration) or accumulate selectively in tumors over a span of 3-96 hours, depending on the type of tumor and PS.

The right therapy conditions and the therapy's outcome is the result of a complex interplay of all four components involved—type and dose of PS and its site of accumulation, light dose, type and possible PDT resistance of cells, and the tissue oxygenation. On the cellular level, the therapy can result in apoptosis, necrosis, or cell survival and the conditions leading to each will be discussed in Section 5.3. The final effect depends on both the mode of cell death (where necrosis can complicate healing by inflammatory reaction) and the ability to destroy all cancer cells. Although most PSs accumulate somewhat more in abnormal cells, the persisting accumulation in normal tissues resulting in phototoxicity and the inability to destroy all the cancer cells calls for better selectivity of PSs. The types and characteristics of PSs and the physical basis and clinical aspects of PDT are extensively reviewed elsewhere (20-22). The diverse ways of introducing selectivity to existing PSs are discussed in this review.

3.5. Delivery vehicles for NIRF-I probes and PSs

A target-specific delivery is core to the successful application of NIRF-I probes and high efficacy PSs. Unlike many PSs that can enter cells and are retained slightly more by cancer cells than by normal cells, NIRF-I dyes alone have almost no selectivity towards specific cellular targets—they gain specificity by conjugation with delivery vehicles. Therefore, most of the promising delivery vehicles are molecules that can both enter specific cells or can selectively interact with them, are informative with respect to a disease that makes them worth tracking, and have functions that are not impaired by conjugation to the dye. These vehicles could deliver small molecules inside cancer cells either nonspecifically (23) or specifically by targeting "cancer fingerprints" (6).

Examples of nonspecific delivery are oil dispersions (e.g. liposomes), or hydrophilic polymers (PEG) that increase the water solubility of encapsulated dyes/PSs and deliver them inside cells by passive diffusion or phagocytosis which is enhanced in cancer cells. Non-specific cell permeation could be achieved by attaching short peptide sequences like Tat (24), antenapedia (25), transportan (26) or poly-arginine (27), although this approach has the possible drawback of subsequent delivery to the nucleus, an unwanted site of accumulation for PSs (Section 5.3). The examples of delivery vehicles that take advantage of the increased metabolism of cancer cells are either proteins, for example serum albumin or a transferrin (involved in iron transportation, (28)), or small molecules, for example 2-deoxyglucose (2DG), targeting glucose transporter that is overexpressed in most cancer cells regardless of their type (4).

Each of these non-specific (but selective) delivery vehicles has an analogy in the class of specific delivery vehicles. An example of a physiological liposomal formulation targeting specific receptors by incorporating a protein into the membrane is low density lipoprotein (LDL, 22 nm) that targets LDL receptors (LDLR) overexpressed on certain types of cancer cells (29) to satisfy their hunger for cholesterol that is required for membrane building (30). These could accommodate multiple dyes or PSs that are released selectively in cancer cells (31, 32). Although this design ingeniously combines the specific delivery of more than one probe with a partial dye-dye quenching of the probes encapsulated inside, the clinical transition could be complicated. LDL particles are limited by low tumor selectivity since liver, adrenal, and reproductive organs all express high level of LDLR and by the narrow range of LDLR-positive tumor types. On the bright side, the *in vivo* application seems to look more promising when using smaller high-density lipoprotein (HDL, 7-12 nm) that has better permeability through vascular membranes and can be prepared using synthetic lipids and recombinant proteins.

The analogy to plasma proteins are monoclonal antibodies (Ab) (33) that are directed against specific antigens of malignant cells and are designed to selectively cure particular tumor types. Despite promising *in vitro* results, the *in vivo* application is complicated by the large size of the Abs, immunogenicity, low loading capacity as well as tumor heterogeneity.

Small delivery vehicles analogous to 2DG and peptides have a better chance of *in vivo* success. One example of a small and easy-to-conjugate vehicle is folate, capable of delivering a variety of probes (34, 35) to cells overexpressing folate receptor (FR), mainly ovary, breast, colon, lung, nose, prostate, and brain cancer cells (36). Another example of small delivery vehicles are short peptides or peptidomimetics that either target receptors overexpressed in cancer like somatostatin receptor, bombesin (37), and $alpha_v beta_3$ integrin (RGD peptide)

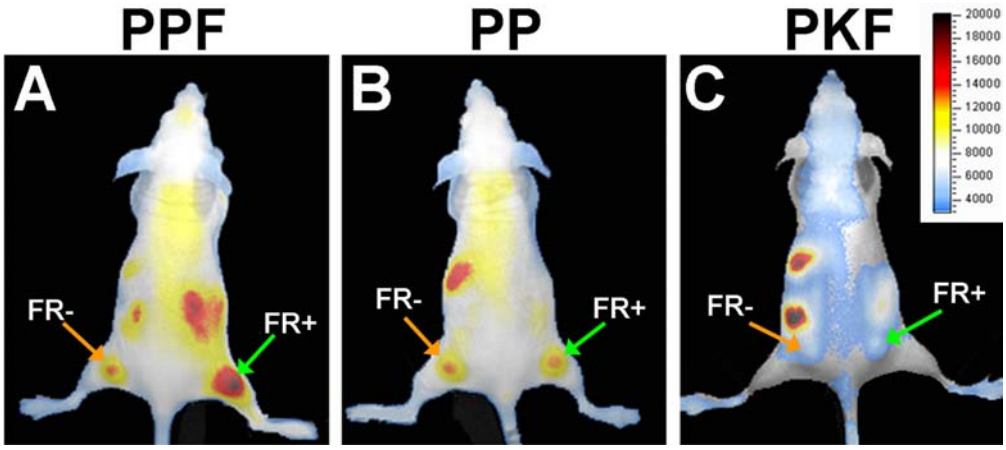

Figure 4. Peptide pharmacomodulation and folate-dictated redirection of the probe demonstrated using three probes, 6.5 hours after *iv* injection: A) Pyro-peptide-folate (PPF), accumulating more in folate receptor overexpressing tumor (FR+) than in FR negative (FR-) tumor and only slightly in kidney, B) Pyro-peptide (PP) accumulating significantly in both FR+ and FR- tumors but very little in other organs, and C) Pyro-Lys-folate (PKF) accumulating more in other organs (liver and spleen) than both FR+ and FR- tumors.

(38), or are selectively activated outside the cells, delivering the cargo only after this cell-specific activation. The latter one is based on "zipper" peptide beacons using a poly-cation (Arg) connected to the charge-neutralizing poly-anion through a protease-sensitive sequence. Only after cleaving this sequence, the poly-cation will deliver the attached molecule to the cells (39, 40).

4. MOLECULAR BEACONS FOR CANCER IMAGING

4.1. Unquenched beacons

A section on unquenched beacons could be very large, but for the purpose of this review, we only focus on those probes that contributed to the development of more efficient delivery and better tumor-to-tissue ratio, adopted by the quenched molecular beacons. One of the first unquenched probes for *in vitro* imaging of cancer is a dye conjugated to 2DG—an analogue of D-glucose delivered inside cancer cells with fast glucose metabolism (4, 41). When conjugated to a small non-NIR fluorophore (to give 2-NBDG, excitation, 475 nm; emission, 550 nm), 2DG delivers the probe preferentially to cancer cells *via* glucose transporters. Although a PS (Pyropheophorbide *a*) and a NIR dye (Cy 5.5) conjugated to 2DG also showed nice *in vitro* and *in vivo* accumulation in cancer cells and tumors respectively, it seems less likely that this delivery is due to the glucose transporter because of these dyes' larger size. The higher tumor to normal tissue ratio could be explained by the natural affinity of these dyes to tumor and exemplifies the likelihood of delivery pathway alteration when using small delivery vehicles and the need for introducing flexible linkers.

Linkers should be chosen at least partly according their length and hydrophobicity, with the rule of thumb that linkers that are too long or too hydrophobic should be avoided because of their potentially negative impact on the biodistribution. Recently, our group published a study using a short and hydrophilic linker between a fluorescent PS Pyropheophorbide *a* (Pyro) and folate, demonstrating the improvement of biodistribution of the Pyro-peptide-folate when compared to the probe replacing the 9-amino acid peptide with a single lysine in Pyro-Lys-folate (34). Pyro-peptide-folate has a better biodistribution, demonstrated by significantly lower accumulation in liver and spleen than its single amino acid analogue (Figure 4). Even when lacking folate, this probe still has an improved tumor to normal tissue ratio, supporting the role of the peptide as the main component that positively influences the probe's biodistribution. A similar beacon, using Cy 5.5 dye conjugated to a folate through a 2,2'-(ethylenedioxy)bis(ethylamine) linker synthesized with the focus on imaging, has shown a similar role for folate, with the ratio of signal from FR+ vs. FR- tumor 2.4 : 1 (compared with 2.5 : 1 for Pyro-peptide-folate). However, the unique role of peptide compared to the shorter and less hydrophilic linker used in this case cannot be elucidated since the full biodistribution was not evaluated.

As summarized in Section 3.5, many NIR dyes improve their biodistribution by attachment to short peptides targeting various receptors (42). As shown on the example of an RGD peptide, reported to improve the tumor-specific localization of the probe *via* targeting $alpha_v beta_3$ receptors by attachment of multiple peptides, these strategies are very popular and constantly improving. The literature dealing with *in vivo* fluorescence imaging of tumors that also covers quantum dots and green fluorescent protein imaging is summarized in a review from Ballou *et al.* (43).

4.2. Quenched beacons

There are two basic designs of beacons used for NIRF-I of cancer, depending on the type of quencher attached. The simpler one, where the donor and acceptor are the same molecules (dye-dye quenching), has the

advantage of a less complicated synthesis with the drawback of less efficiently quenched fluorescence. On the other hand, using an acceptor distinct from the donor (i.e. the quencher is chemically different from the dye) offers the advantage of careful design of the beacons, matching the dye with the most efficient quencher, but increases the complexity of the probe's synthesis. In this section, we focus on imaging enzymatic activity in the tumor environment that will manifest itself as an activation of beacons.

4.2.1. Beacons based on dye-dye quenching

One of the most widely applied series of probes, utilizing dye-dye quenching, was developed by Weissleder and colleagues. It uses methoxypolyethylen-glycol-derivatized (MPEG) poly-L-lysine as a backbone to which Cy 5.5 dyes are coupled either directly or through a short peptide sequence recognizable and cleavable by the target endoprotease. The polymer backbone serves as both delivery vehicle, accumulating in tumors by slow leakage across the permeable vasculature, and also as a site of attachment of Cy 5.5 dyes that are close enough to allow FRET (44). These dyes are released and the fluorescence restored upon cleavage by tumor-overexpressed proteases, later identified to be predominantly cathepsin B and H (45). By introducing an enzyme-specific peptide sequence between the lysine and Cy 5.5, the cleavage specificity could be directed towards other cancer-associated proteases like cathepsin D (46) and matrix metalloproteinase 2 (MMP-2) (47), or other proteases, for example caspase-1 redirecting the original role as a tumor-imaging probe towards apoptosis imaging (48). There are a few disadvantages that limit this approach. First, both cathepsins and MMP-2 are extracellular proteases, cleaving the probe outside the cancer cells. Therefore, without any additional intracellular delivery, the signal of the cleaved probes is more likely to quickly spread from the site of cleavage to the whole body (especially liver and spleen) and this leads to a decreasing signal-to-noise ratio with increasing time post-cleavage. Second, due to the tight assembling of the Cy 5.5 dyes to the bulky backbone, many less potent proteases will not efficiently cleave the Cy 5.5 from the backbone. Finally, there is an inverse relationship between coupling and cleaving efficiency, where the higher number of Cy 5.5 dyes means better quenching but worse cleavage from the backbone.

4.2.2. Beacons with fluorescence quenchers

Beacons composed of a linker that has a dye and a fluorescence quencher attached to opposite ends can be specifically activated either by cleavage with a particular enzyme overexpressed in cancer cells or by opening of the nucleic acid-based linker by hybridization to the target. Enzymatic cleavage generates a permanent fluorescence increase, diminished only by photobleaching and migration of the cleaved probe from the site of cleavage, whereas the opening of molecular beacons can be reversed.

Most examples of cleavable beacons whose fluorescence is diminished by the attachment of a distinct fluorescence quencher are from the family of peptide-based beacons, but other beacons, using phospholipid- or DNA-based linkers, are also emerging (49-51). There are over 500 proteases associated with cells. Naturally, the focus is drawn to imaging the *in vivo* activity only of those that are characteristic of, or overexpressed in, diseased cells. In cancer, these are the proteases involved mainly in assisting the cancer cells to alter the surrounding environment to better supply nutrients and oxygen and to expand (52). These are mainly from families involved in catabolism or tissue remodeling, like cathepsin D, B, and H (already mentioned in Section 4.2.1) (45, 46), matrix metalloproteinases (MMP), mainly MMP-2, MMP-7 (Section 6), and MMP-9 (39, 53-55); or others, like fibroblast activation protein (FAP), overexpressed by tumor stromal fibroblasts in tumor epithelium (56), and prostate-specific antigen (PSA).

But there are other possible enzymes that target biomolecules other than proteins. One of the examples is phospholipid-based beacons activated by phosphatidylcholine-specific phospholipase C (PC-PLC), involved in choline phospholipid metabolism altered in cancers (51, 57). Also, DNA-based MBs could also potentially serve as a sensitive fluorescence assay for detecting the presence of both specific sequence and enzyme (e.g. specific single-stranded DNA along with single strand-specific nucleases (49) or specific RNA along with ribonuclease H (58)).

The most widespread of the openable beacons are those derived from nucleic acids, widely used in nucleic acid research for detecting a specific DNA or RNA sequence by increased fluorescence (Figure 3B) (15, 59). The composition of these probes is similar: a dye and a quencher, but in this case conjugated to opposing sides of a short nucleic acid strand with specific architecture. It includes 4-6 nucleic acid bases at each end, complementary to each other so that they form a short stem keeping the dye and quencher in close proximity in the native state. The middle 15-20 bases (the loop) are complementary to the DNA or RNA target. The obvious target of these photodynamic molecular beacons are overexpressed or disease-unique mRNAs. The mRNAs overexpressed in cancers usually code for cancer-associated proteins like protein kinase c (responsible for signal transduction and implicated in cell growth and tumor development), Bcl-2 family (blocking the release of cytochrome C that would initiate apoptosis) or C-raf kinase (60). The pool of mRNAs that have a cancer-specific mutation (deletion or insertion) is growing with our understanding of the cancer genome and include for example the mutation in the BRAF gene that generates a unique mRNA in melanoma (61), or the deletion in the EGFRvIII gene (type III epidermal growth factor receptor) in breast cancer (62). These beacons are very specific and there are many possible targets, but the challenges of *in vitro* and *in vivo* applications are numerous. Although the rapid degradation of DNA probes in cells could be delayed by the use of phosphorothioate, 2'-O-Me-RNA nucleotide analogues (63), or peptide nucleic acids (64), the issues of accessible sequences on the target mRNA and *in vivo* delivery need to be resolved.

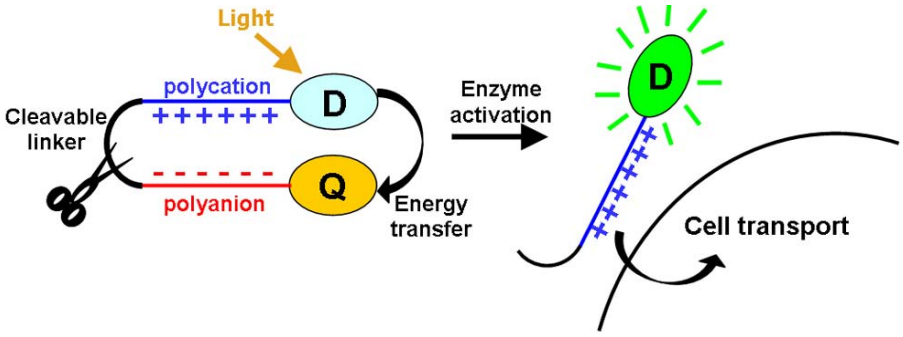

Figure 5. Activation and means of delivery of zipper beacons.

A combination of both openable and cleavable beacons that solves the problem of delivery are the zipper-like beacons (Figure 5, Section 3.5). They are composed of two strands—a poly-cation (arginines) and poly-anion (glutamic acids)—that form an electrostatic "zipper", bridged by a protease-cleavable linker. Poly-arginine is one of the cell-penetrating peptides, but this ability is effectively blocked in the intact probe by fusion with the negatively charged poly-anionic sequence. Therefore, a dye connected to the poly-cation is delivered inside the cells only once the peptide sequence is cleaved (39). These zipper beacons were shown to successfully identify prostate cancer cells with elevated expression of PSA (65) and tumors overexpressing MMP-2 and MMP-9 (39).

5. MOLECULAR BEACONS IMAGING RESPONSE TO THERAPY

5.1. Apoptosis vs. necrosis

There are two extreme modes of cell death—apoptosis and necrosis. Apoptosis, which is critical for the healthy development of a multicellular organism, is characterized by the organized energy-dependent fashion in which the damaged, unwanted, or otherwise compromised cells die. On the other hand, necrosis could be characterized as an uncontrolled, energy independent bursting of a cell that follows a sudden and severe physical damage and results in an inflammatory reaction. Although these two modes of cell death are in principle very different, it was shown in several cases that there is a gray zone in between these modes, having the characteristics of both apoptosis and necrosis (66).

The critical role of apoptosis in the everyday life of a multicellular organism became clear only recently, once its disregulation was linked with severe diseases (67). Cancer is one of the diseases whose master plan of circumventing the body's regulatory pathways includes the deactivation of apoptosis that would otherwise eliminate the rapidly proliferating cells (68). Therefore, imaging apoptosis—either its successful drug-related triggering or the lack of apoptotic response—is critical for understanding the process itself and its relatedness to successful cancer treatment.

5.2. Traditional imaging of apoptosis

Apoptosis imaging is complicated by the diverse features characterizing each stage combined with the non-harmonicity of the process (i.e. not all cells in apoptosis are simultaneously at the same stage of this process) (69). The cell starts its apoptosis with ATP-dependent digestion of its own biomolecules, leading to a slow shrinking and later to the formation of apoptotic bodies (membrane-encapsulated cell content, mainly organelles), while preserving membrane integrity. These morphological features can be used to identify the apoptotic cells in fixed specimens (70, 71). Currently, the focus is mainly drawn to the earlier stages, where the difference between apoptosis and necrosis is the most pronounced (72). The common methods use features of controlled cell degradation such as DNA laddering (e.g. TUNEL/Apoptag assay) (73), disruption of mitochondrial transmembrane potential, redistribution of phospholipids in the plasma membrane (e.g. Annexin V-FITC membrane binding) (74), or activation of caspases, the proteases of apoptosis (75). None of these features, except caspase activation, is unique to apoptosis though. DNA laddering and disruption of mitochondrial membrane are also associated with necrosis (76) and a recent study also revealed that Annexin V binds both apoptotic and necrotic cells (77). Therefore, when imaging apoptosis using these conventional methods, one must keep in mind that controls for necrosis should be included.

5.3. The mode of cell death as a response to PDT

Since the recognition of singlet oxygen as the major cytotoxic agent in 1976 (78), there has been great interest in the physics and chemistry of this reactive form of oxygen. Singlet oxygen has a very short lifetime (<200 ns *in vitro* and *in vivo*) (79), resulting in an average diffusion range of 20 nm from the site of generation. Therefore, the location of the PS in the cell is also the site of organelle damage. The damage on a cellular level can be either *via* direct destruction of malignant cells or changes in the tumor vasculature. The latter is the reason why PDT can also be used in treatment of macular degeneration and atherosclerosis (80). On the subcellular level, the targets—mainly cytoskeletal tubulins, lysosomes, mitochondria, plasma membrane, and nucleus—are very PS-dependent. Of these organelles, the damage caused to tubulins and lysosomes is either reversible or not very effective. In the case of lysosomes, this could be due to a PDT-associated destruction of lysosomal enzymes that would otherwise be involved in triggering the post-treatment apoptosis (81, 82). Although most PSs localize mainly in membranes and thus do not enter the nucleus, some damage to nuclear DNA has been reported (83). This is unwanted collateral damage

since chromosome aberrations and mutations may produce even more complications. The damage to mitochondria and plasma membrane is more important and plays a role in the "death decision" between apoptosis and necrosis (84). The response to PDT depends on all three components involved—the type of PS, oxygen level, and light dose. Generally, using high light doses, targeting tissues with low levels of oxygen, and using a PS that localizes in plasma membrane (mostly hydrophobic PSs) leads to necrosis while low light doses to well oxygenated tissue using a PS that localizes near the mitochondria leads to apoptosis (21). Most tumor tissues are heterogeneous, different tumor cell lines respond differently to the same treatment conditions, and many of the PSs accumulate near more than one organelle. As a consequence, most of the responses to PDT are a combination of necrosis, apoptosis, and cell survival with an unknown percentage of each. Although attempts were made in assessing the PDT outcome by combining the information from pre- and post-PDT fluorescence images of PS, deoxy- and oxyhemoglobin (85), specifically visualizing apoptosis after the PDT would help to identify the best combination of all actors involved more clearly.

5.4. Imaging apoptosis *in situ* and as a cellular response to PDT

Imaging apoptosis *in situ* or *in vivo*, especially following any therapy, is a very challenging goal. As outlined in Section 5.2, the obstacles lie in these facts: 1) there are many cells in different phases of apoptosis at any given time and they have several characteristics observed by each available method, 2) there is no clear dividing line between apoptosis and necrosis, 3) for successful *in situ* apoptosis detection, the target must be exposed on the surface of cells (as in the case of detection with Annexin V) or the probe should be capable of penetrating cells. Caspases—enzymes involved in the initiation and propagation of apoptosis—are an ideal target for molecular beacons. Caspase-1 was successfully visualized in cells treated with traditional apoptosis-inducing methods (e.g. Staurosporine), using Cy 5.5 dyes coupled through a caspase-1-cleavable peptide sequence to a MPEG poly-L-lysine (see Section 4.2.1.). This probe was also shown to image cells that were infected to express caspase-1 and subcutaneously implanted into mouse (48).

Although caspase-1 is part of the apoptotic machinery, it is also involved in the inflammatory processes and since it is an initiator caspase it may not be a clear indicator of cell death that is "beyond repair". Activation of executioner caspases, namely caspase-7 and caspase-3, is apoptosis-specific event, usually indicating the point of no-return (75). These proteases were targeted using a small peptide beacon with an Alexa Fluor 647 NIR dye and a QSY fluorescence quencher attached to opposing ends of a DEVD peptide sequence (86). Cellular delivery was achieved by conjugation of the nucleus-permeating Tat peptide and the construct identified apoptotic cells by the restored dye's fluorescence.

To visualize the apoptotic response immediately after the therapy, it is convenient to combine both the imaging and therapeutic role into one probe by exchanging a simple dye for a fluorescent PS. One such construct (PDT agent with a built-in apoptosis sensor, PDT-BIAS) is composed of PS Pyropheophorbide *a* (Pyro), a Chlorin with NIR emission that was also shown to penetrate cells and accumulate near mitochondria as a sensitive site for apoptosis-triggering damage; a GDEVDGSGK peptide sequence cleavable by executioner caspases (caspase-3); and a black hole quencher 3 (BHQ-3), used for an effective nonradiative quenching of Pyro's fluorescence. This construct enters cancer cells, triggers apoptosis by light-induced damage to mitochondria, and reports this apoptosis by caspase-3-dependent cleavage of the peptide sequence, resulting in fluorescence restoration. This probe was also capable of reporting apoptosis using traditional apoptosis-inducing methods like Staurosporin treatment (87). The attachment of a folate moiety as a cancer cell-specific delivery vehicle, allowed PDT-BIAS to distinguish FR+ tumor from FR- tumor and other organs and monitor the post-PDT apoptosis also *in vivo* (88). The increase of fluorescence following the PDT was correlated with *ex vivo* Apoptag staining as the traditional apoptosis-detecting method.

6. MOLECULAR BEACONS FOR IMAGE-GUIDED THERAPY

6.1. Image-guided therapy

There are many approaches that deal with improving current therapies to make them better targeted and less invasive. Combining imaging and therapy so that the affected sites are first identified by imaging and subsequently treated, was already shown to enhance both the accuracy and effectiveness of the treatment (89). Molecular beacons can take this approach a step further. They can combine imaging and therapy into one probe, capable of both identifying diseased cells with overexpressed molecular target (like proteases, mRNAs or various receptors) and directing the therapy to these sites using the activated beacon as a drug. In this case, the NIRF-I and PDT form a successful couple, where NIRF-I serves as a non-invasive method of imaging and PDT serves as a targeted therapy with an inherent level of targeting in the form of laser-directing.

6.2. Singlet oxygen quenching

When a PS absorbs a photon, it can release the energy by fluorescence, non-radiative processes or by intersystem crossing to its triplet state from where it can transfer this energy to any molecular (triplet) oxygen that is present, generating reactive singlet oxygen (Figure 1). Therefore, the production of $^1O_2$ can be inhibited in three main ways: 1) by scavenging the already generated $^1O_2$, 2) by quenching the *triplet* state of the PS, and 3) by quenching the *singlet* state of the PS (this will ultimately also reduce the population of triplet state and therefore indirectly lower the $^1O_2$ production). The molecules capable of all three ways of quenching are few, with carotenoids described as the best matching counterpart to PSs. On the other hand, any efficient fluorescence quencher could potentially also reduce the $^1O_2$ production by simply quenching the singlet state preceding the $^1O_2$-forming triplet state. When designing a beacon, both the character

of the quencher and the combination of distance and spatial stability of the dye-quencher pair will dictate the final quenching ability. Unlike the quenching of the short-lived singlet state, triplet state quenching and $^1O_2$ scavenging does not require a very intimate and locked position of PS and quencher, since the lifetimes of triplet state and $^1O_2$ are more long-lived than thermal fluctuations. The spatial relationship of the PS and quencher may not be clear in advance, so for any given application, both types of quenchers should be considered.

6.3. Quenched molecular beacons used in fluorescence-guided PDT

One of the first pre-beacons utilizing carotenoid as a singlet oxygen quencher are carotenoporphyrins, covalently connecting a carotenoid analogue to the porphyrin PS (meso-tetraphenyl porphyrins (90, 91), pheophorbide (92) or hematoporphyrin (93)). This type of beacon was designed to mimic the role of carotenoids in photoprotection and their antenna function in photosynthetic systems, and was utilized as a diagnostic agent for tumors, taking advantage of its low toxicity. However, both the lack of selective separation and partial fluorescence quenching makes this design somewhat limited.

The first carotenoid-containing beacon utilizing both $^1O_2$ quenching and activation was designed to replace the enzymatically-resistant linker with a peptide linker, cleavable by a specific protease (in this case caspase-3 as a model). Despite the linker's flexibility, the $^1O_2$ production was effectively inhibited 8-fold and selectively restored upon enzymatic cleavage in solution, although only 4-fold owing to the ability of free carotenoid to scavenge and $^1O_2$ that was already produced (94). These solution data were also confirmed *in vitro*, showing that carotenoid conjugated through this short peptide linker to a Pyro PS could protect the cells that do not express the target protease from the harmful effects of $^1O_2$. A similar set-up was used for the c-raf-1 mRNA-targeting beacons that replaced the protease-cleavable linker with an mRNA-openable linker (2'-OMe-RNA). These beacons have better singlet oxygen quenching (up to 15-fold) and also show very good fluorescence quenching (13-fold) and are promising probes since Pyro can, in this case, serve not only as a PS and dye, but can also deliver the beacon inside cells.

The purpose of attaching a fluorescence quencher is mainly to quench the singlet state of the PS with $^1O_2$ quenching being more a side product of this process. The difference lies in the fact that a fluorescence quencher, unlike carotenoids, does not have the capacity to quench the long-lived triplet state or scavenge $^1O_2$ so its $^1O_2$ quenching capabilities are more fragile and dictated by the requirements for good fluorescence quenching (95).

There are two beacon designs that use the previously explored architectures outlined in Sections 4.2.1. and 5.4. One uses Pyro PS conjugated to the MMP-7-cleavable GPLGLARK peptide sequence, quenched by BHQ-3 that is attached to the opposite end of this sequence. This hydrophobic sequence keeps the Pyro and BHQ-3 tightly together and results in an 18-fold $^1O_2$ quenching and 15-fold fluorescence quenching. The specific activation of this photodynamic molecular beacon in MMP-7 overexpressing (MMP-7+) cells was monitored by the restored fluorescence and also corresponds to the restored $^1O_2$ production, demonstrated by preferential death of the MMP-7+ cells when compared to the cells lacking MMP-7. Image-guidance was also shown *in vivo*, where directing the laser to the site identified by the restored fluorescence resulted in tumor ablation and disappearance, monitored up to 30 days post-PDT. Although this is a very efficient and versatile design, the specificity of the delivery should be improved for better signal-to-noise ratio (96).

Another design used for controlled production of $^1O_2$ uses Chlorin-e_6 conjugated to an MPEG poly-L-lysine backbone (dye-dye quenching), reporting the presence of tumor-associated cathepsin B. This probe was used to image and treat the cathepsin B-overexpressing HT 1080 tumor, showing a slower tumor growth of the probe- and light-treated tumor compared to the control tumors. The limitation of this approach lies in the inverse relationship of $^1O_2$ quenching and activation efficiency since a higher degree of PS substitution leads to both higher $^1O_2$ quenching efficiency and lower $^1O_2$ activation efficiency because of decreased availability of the cathepsin B recognition sites (97).

7. PERSPECTIVE

We have summarized the various flavors of molecular beacons used in cancer imaging and therapy, focusing on NIRF-I and PDT. These probes are inherently flexible and tunable for various purposes, ranging from illuminating the apoptotic response to therapy to precise therapy guidance and controlled production of reactive $^1O_2$. Protease activation connected with the improvement of delivery and biodistribution, as in the case of the zipper beacons (39) and peptide pharmacomodulation (34), is very promising for future clinical applications. Although they have a longer way to go, mRNA-activatable beacons are attractive probes for future studies, especially considering our increasing knowledge about the human and disease genomes.

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Abbreviations: 2DG: 2-deoxyglucose, 2-NBDG: 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose, Ab: antibody, BHQ-3: black hole quencher 3, FAP: Fibroblast activation protein, FR: folate receptor, FRET: Fluorescence resonance energy transfer, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, LDLR: Low-density lipoprotein receptor, MB: molecular beacon, MMP: Matrix metalloproteinase, MPEG: methoxypolyethylen-glycol, NIR: Near-infrared, NIRF-I: Near-infrared fluorescence imaging, PC-PLC: phosphatidylcholine-specific phopsholipase C, PDT: Photodynamic therapy, PDT-BIAS: Photodynamic therapy agent with built-in apoptosis sensor, PET: Positron-emission tomography, PS: photosensitizer, PSA: prostate-specific antigen.

Key Words: Near-Infrared Fluorescence Imaging, Fluorescence resonance energy transfer, Apoptosis Imaging, Image-Guided Therapy, Photodynamic Therapy, Molecular Beacon, Singlet Oxygen, Quenching, Delivery, Review

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