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## **Cancer optical imaging using fluorescent nanoparticles**

Developing imaging technologies and molecular probes that allow cancer detection at its earlier stages, that would also provide predictive information on the chances of a given tumor to respond to a therapy, to monitor non-invasively and in real time how efficiently this therapy will reach the tumor as well as the early or long-term tumor's response is of course of major importance. Imaging methods are also expected to play major roles in the drug development process very early in the laboratory, during the translation phase from in vitro assays to preclinical systems, as well as to evaluate their ADME (pharmacokinetics and pharmacology for absorption, distribution, metabolism, and excretion). Finally, functional imaging will also bring some more details on the local and real time biological activity of the drug.

Anatomical imaging methods such as computed x-ray tomography (CT) and magnetic resonance imaging (MRI) represent the foundation of clinical imaging. These methods are complementary of other techniques, which are specially dedicated to the acquisition of molecular information including Positron Emission Tomography (PET), Single photon emission computed tomography (SPECT) and optical imaging. Except for PET and SPECT that absolutely require the use of radiotracers, the other methods can provide anatomical of physio-pathological information without the use of contrast agent, but adapted molecular probes will dramatically augment their performances. I will focus my presentation only on imaging systems that can be translated in the clinic.

Optical imaging is based on the detection of light passing through the tissues. Since living tissues are not transparent, the information that can be obtained is strongly depth-weighted and will depend on the thickness and optical properties of the tissues to be imaged. The tissues are less absorbing in a spectral window ranging from 650 nm to 900 nm and thus most of the optical applications are using this near-infrared (NIR) window.

In fluorescence, photons are absorbed by a fluorescent molecule, which emits light (fluoresces) at a longer wavelength in order to return from its excited state to its baseline level in a certain period of time. This emitted light can be captured with a cooled CCD camera and the quality of the detection will rely mainly on our capacity to filtrate the emitted photons from the illumination light. It is usually limited to more or less 1 cm depth. Time-resolved fluorescence can “go deeper” because it is possible to remove some background noise (less than 3 cm). It exploits differences in fluorescence lifetimes of the order of nanoseconds to monitor target fluorescence, and this technique has been used to report on the local environment of fluorophores, for example, local pH, refractive index, ion or oxygen concentration.

A suitable label should be excitable, without simultaneous excitation of autofluorescence of the tissues. It must be bright (high molar absorption coefficient and high fluorescence quantum yield) and have an adapted fluorescence lifetime and the largest Stokes shift. The Stokes shift determines the separation of excitation from emission. It is thus linked to the efficiency of signal collection but also determine the possible spectral cross-talk in two- or multi-fluorophore applications such as fluorescence resonance energy transfer (FRET) or spectral multiplexing. FRET is a major asset in fluorescence imaging. Indeed, introduction of a cleavable link between the FRET pair of fluorophores will allow us to measure enzymatic activities or physico-chemical variations. As well, this can allow us to follow the possible degradation of a macromolecule, including nanoparticles, labeled with a FRET-pair of fluorophores. Additional features include steric and size-related effects of the label, its absence of toxicity and the possibility to attach it covalently or not to the molecule/particle of interest and finally its solubility and stability in biological fluids.

Fluorophores can be separated in 2 categories: organics (Cyanines, Alexa Fluors, IRDyes, ICG, DiD, DiL...) and inorganics (Quantum dots (QDs), lanthanides).

An important number of fluorescent organic dyes have been developed for biological applications and especially microscopy (Fluoresceins, Rhodamines, cyanines...). Unfortunately, they are mostly emitting in the visible spectrum, which limits dramatically their use in vivo. NIR fluorophores are less diversified although their number is increasing. They usually have a low toxicity, and their solubility can be tuned chemically. On the bad side, NIR fluorophores usually suffer from low quantum yield efficiency. So far, the only NIR fluorophore that can be used in the clinic is Indocyanine Green (ICG), although ICG has major drawbacks as discussed thereafter.

Quantum dots (QDs) are fluorescent inorganic nanocrystals with size-tunable emission properties, which have been applied in vitro and in small animals for biomedical purposes including imaging, diagnostic, drug delivery or therapy [1]. In contrast to classic dyes, quantum dots can be used with a single excitation source independent of the emission profile allowing for efficient multiplexing. In addition, narrow emission spectra and increased resistance to photobleaching of fluorescent nanocrystals make of QDs very interesting macromolecules. However, they also have disadvantages including a complex surface chemistry, potential chemical toxicity and nanotoxicity, which prevent them from going to the clinic so far.

Luminescent lanthanide complexes such as europium ( $\text{Eu}^{3+}$ ) and terbium ( $\text{Tb}^{3+}$ ) complexes also have interesting luminescence properties like long luminescence lifetime and a Stoke's shift of  $>200$  nm. Their long-lived luminescence is especially suitable for time-resolved measurements [2]. Their use in vivo is still limited by their low absorbance, which imposes the presence of an "antenna" (a sensitizing chromophore covalently bound beside the lanthanide ion). Beside this "traditional fluorescence", it is also possible to use upconversion, a process where light of low energy is converted to higher energy light via multiphoton absorptions or energy transfer processes, but this application is highly depth-dependant and may not be efficient for deep imaging although recent studies are very promising in mice [3]. Since lanthanides have been used largely as MRI contrast agents (gadolinium), their toxicity is quite well documented.

Why do we need fluorescent nanoparticles for clinical oncology?

Fluorescence imaging can be used for the characterization of new drugs or of new formulations of existing drugs especially at the preclinical level [4]. It would be preferred to radioactive labeling because fluorescence can be applied very early in the development of a drug, in vitro on cells, then in vivo in small animals and finally translated into the clinic with some limitations. Although it is not realistic to apply this method to small drugs due to the large size of the fluorophores, this can be done for a nanoparticle, assuming that the labeling should not profoundly modify the particle properties or that the fluorophore could be part of the final formulation. Nanoparticles that can be controllably loaded with large amounts of active compounds like Poly(DL-lactic-co-glycolic acid) (PLGA) loaded with vincristine [5], or mesoporous silica nanoparticles [6], lipid nanoemulsions or nanocapsules loaded with Paclitaxel ([7]; I Texier personal communication), block copolymer vesicles of poly(trimethylene carbonate)-b-poly(L-glutamic acid) (PTMC-b-PGA) loaded with doxorubicin [8] are very actively being developed at the preclinical level (review in [9]). Introducing the NIR fluorophores within the final therapeutic particle greatly speeds up the characterization of these objects in vitro and in vivo [10-12] but the interest and feasibility of producing fluorescent clinical grade particles is questionable.

Organic fluorescent dyes like indocyanine green (ICG) have been used in medical applications such as sentinel lymph nodes (SLN) detection and biopsy, optical-guided surgery in oncology or in blood flow measurements, in vascular surgery, retinal angiography in ophthalmology.... [13,14]. ICG can be injected intravenously in humans, but its solubility is not satisfying. It will immediately bind to plasma proteins and will be very rapidly eliminated with an half live of  $\pm 3$  min. Formulations of ICG in an emulsion [15] or in a liposome [16] will significantly augment its residence time in blood and provide a 2 to 3 fold increase in its absorbing capacity. In this example of application, the use of a clinical grade nanoparticle is interesting because it augments the solubility of the dyes, their intensity of fluorescence, their stability, their bio-availability and also their capacity to accumulate passively in different tissues like tumors using the enhanced permeability and retention (EPR) effect or by filtration in the lymph nodes. More sophisticated formulation can also be generated to use ICG in photothermal ablation of tumors, using antibodies-targeted colloidal materials [17].

Another way to combine fluorescence and therapy is indeed to generate theragnostic agents in which the absorbed energy from the excitation light can be used as well for diagnostic than for therapy. This is in particular the case for biodegradable or non-biodegradable particles that contains photosensitizers (PS) like porphyrins, phthalocyanines, and chlorine derivatives or inorganic particles like QDots for photodynamic therapy (PDT). PDT is a very well documented therapy currently used alone or in combination for the treatment of several types of pre-cancerous conditions (skin, Barrett's oesophagus) and cancers (biliary tract, brain, head and neck, lung, oesophagus, bladder and skin). PS are drugs that can transfer their absorbed energy to neighboring oxygen molecules leading to the production of toxic compounds (singlet oxygen and other Reactive Oxygen Species (ROS)) that may lead to cancer cell damage, tumor microvascular occlusion and host immune response. However, PS have a limited solubility in water and are forming inefficient aggregates. In addition, they should be specifically targeted to tumors in order to reduce their non-specific toxicity. For these reasons, PS have been encapsulated in nanoparticles thus augmenting their persistence in a monomeric form and a more pronounced tumor targeting due the EPR effect [18-20].

As mentioned earlier, pure optical imaging is limited in depth due to light scattering in tissues. Photoacoustic tomography (PAT) [21] is an emerging hybrid imaging modality with unprecedented high-resolution visualization (20-200  $\mu\text{m}$ ) of optical contrast several centimeters deep in tissues of small animals, primates and humans [22,23]. A short-pulsed laser beam illuminates the target. In function of the target's absorption coefficient, a portion of the light is absorbed and partially converted into heat, which generates a pressure rise propagated as an acoustic wave and detected by ultrasonic transducers. PAT can provide strong endogenous and exogenous optical absorption contrasts. It was initially used for real time tracking of fast hemodynamic changes, but recently provided interesting results with other tissues including bone and fat. Finally it can also be applied to the study of the biodistribution of diagnostic agents. NIR absorbing molecules can be used as contrast agents. Absorptive organic dyes, nanoparticles, reporter genes and fluorescence proteins have been successfully applied as PA contrast agents. In particular, gold nanostructures (gold nanospheres, gold nanoshells, gold nanorods, and gold nanocages) are extremely interesting objects for PAT imaging because their surface plasmon resonance peaks can be tuned from the visible to the near infrared region by controlling the shape and structure of the particle [21,24]. In addition, gold is known for its good biocompatibility, easily modifiable surface, absence of toxicity, capacity to encapsulate drugs and photothermal properties particularly interesting for inducing a laser- or X-Ray-mediated remote thermal ablation of the tumors.

Problems still to be addressed

As discussed before, nanoparticles are promising tools for luminescent imaging in oncology. However, beside possible nanotoxicity, the main problem still remaining concerns the active targeting of the nanoparticles to the tumor. The EPR effect is so far the only truly documented phenomenon allowing a certain but variable and unpredictable accumulation of the particles in the tumor microenvironment [25]. Introducing sophisticated targeting ligands may work in vitro and in preclinical experiments, but the true challenge is to do so only with the help of FDA approved molecules and methods.

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