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Review Article
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IN VIVO IMAGING TECHNIQUES FOR DETERMINATION OF THE FATE OF DRUG DELIVERY SYSTEMS

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ABSTRACT

The various bio imaging techniques provides a non invasive method to monitor the in vivo fate of the different pharmaceutical formulations post administration into the body. The different imaging techniques including gamma scintigraphy, near infra red fluorescent dyes helps to predict the pharmacokinetic behaviour of dosage form in subjects under investigation. The transit of dosage form through its intended site of delivery can be non-invasively imaged in vivo via the judicious introduction of an appropriate short lived gamma emitting radioisotope and non isotopic florescent dyes. Near-infrared (NIR) fluorescent probes offer advantages of high photon penetration, reduced light scattering and minimal autofluorescence from living tissues, rendering them valuable for noninvasive mapping of molecular events, assessment of therapeutic efficacy, and monitoring of disease progression in animal models. These studies provide an insight into the outcome of delivery systems, its integrity as well as enable the relationship between in vivo performance and resultant pharmacokinetic parameters. This review provides an overview of the recent development of the design and optical property of the different classes of NIR fluorescent nanoprobes as well gamma scintigraphic technique associated with in vivo imaging applications.

KEY WORDS

Bioimaging, Gamma Scintigraphy, Radiolablling, Fluroscent probes, NIR.

INTRODUCTION

The first medical imaging was realized in the late 1895 by Wilhelm Roentgen shortly after he discovered X-ray and applied to capturing the images of the bones of a hand on film. From the first anatomical charts to the most recent development of Magnetic Resonance Imaging (MRI) or Positron Emission Tomography (PET), setting into images what is normally unseen within living systems has been a major issue for the understanding of biological processes [1–3]. These imaging technologies differ predominantly in the following aspects: resolution, penetration depth, temporal resolution and energy expended for generation of the image. With the exception of MRI, which relies on magnetic properties of atom nuclei with half-integer spin, most of the imaging modalities rely on photons with wavelengths ranging from gamma emission to infrared. Any photon has specific interactions with tissues or bones that will define its uses and drawbacks. On one hand, high-energy photons such as gamma emission or X-rays can go through the tissues with low interactions, making them suitable for deep tissue or whole body imaging, while being potentially harmful because of the reactions they can trigger within cells. Their use is thus strictly restricted to dedicated areas and under the supervision of trained personnel. On the other hand, photons in the visible and

infrared wavelengths do not penetrate deeply within organic tissues, but they cause no or little damage to cells. Moreover, light sources and imaging apparatus are widespread, relatively cheap, their handling requires moderate levels of training and protection, and does not generate radioactive wastes.

Contrary to tissue auto-fluorescence recording, near-infrared/visible fluorescence is an optical imaging modality that relies on the injection of an exogenous probe that will emit light when excited with suitable wavelength. This method can be used from the macroscopic to the microscopic range, goes as further as sub-cellular resolution, and is highly sensitive. Targeted fluorescent probes can be designed to specifically mark and visualize different biological targets, which can be imaged with high contrast using the appropriate set of optical filters to minimize auto-fluorescence contribution of the surrounding tissues. Fluorescence is particularly suitable for *in vitro* and superficial *in vivo* imaging applications. However, fluorescence imaging using the near infrared range is also now routinely used for whole body imaging of small animals in preclinical studies [4–7], and the first human clinical trials have been performed in 2008/2009 for sentinel lymph node mapping in oncology by different groups [8–12]. The next challenge to address is to pursue the development of fluorescence imaging techniques in medical applications. Since the instrumentation tools now exist [13–18], the main blocking point to overcome for the expansion of clinical trials remains the availability of performing fluorescent tracers [19,20]. Nanotechnologies could facilitate their design by allowing modular and flexible constructions. This review will focus on the *in vivo* imaging and the coming challenges for their clinical translation.

Among the different imaging modalities, optical imaging, which owes its origin to single-cell *in vitro* studies, is attractive for small animal imaging because of its lower cost, portability, and potentially high spatial resolution with customizable fine-tuning. Despite being one of the most attractive techniques to provide noninvasive and nonionizing *in vivo* visualization [21], optical imaging is impeded by the tendency of living biological tissues to absorb and scatter photons and generate strong autofluorescence, which interferes with signal collection and processing[22]. In addition, living tissues also contain other major NIR absorbers, such as water, lipids oxyhemoglobin and deoxyhemoglobin [23] that prove challenging for optical imaging. To overcome these barriers, intense research has focused on developing highly sensitive and efficient fluorescent probes that function in the biologically transparent window of the first and second NIR region (NIR I, 650–950 nm, and NIR II, 1000–1350 nm)[24].

Information about the *in vivo* behaviour of dosage forms when obtained using radionuclide tagged with dosage forms is known as gamma scintigraphy. This radiolabelled method is a well established technique in medical practice for the past several decades. Moreover, instead of relying on pharmacokinetic data alone, it is better to combine it with the technique of gamma-scintigraphy or pharmacoscintigraphy to assess the performance of dosage form in man. The coupling of gamma scintigraphy with usual pharmacokinetic methods makes it possible to correlate the information obtained on the distribution of medicament with complete absorption profile. Radionuclide tagged with dosage forms can provide vital information regarding the extent, rate, site and mode of drug release and morphology of the drug delivery system during release in human under ethical norms. Conclusions can also be drawn about the performance of the formulation, including: the ability of a formulation to target a specific location; the rate of erosion in comparison with *in*

vitro dissolution data; the impact of absorption window on bioavailability.

Today, these modern imaging technologies coupled with newly developed imaging probes are widely used in monitoring disease progression, interrogating cellular and molecular events, evaluating safety and toxicology in drug discovery and development, and assessing therapeutic efficacy *in vivo* [25-33]

Gamma Scintigraphy Technique

Nuclear medicine images are primarily functional in nature, providing substantial information on physiology and more limited information on anatomy. The technique commonly termed as scintigraphy involves imaging the bio distribution of a radiopharmaceutical. Gamma camera images of the *in vivo* distribution of pharmaceutical formulation radiolabelled with a suitable gamma emitting radionucleotide, may be used to quantify the biodistribution of the formulation. No other technique can locate so precisely the site of disintegration of the formulation in the gastro intestinal tract. Scintigraphic technique also allows correlation between observed pharmacological effects and the precise site of delivery and thus facilitating drug targeting studies.

Radiolabelling by Gamma-Emitting Isotopes

Prior to imaging by this technique, the dosage form should be radiolabelled. Drug/formulations can be radiolabelled either by gamma emitting isotopes or by neutron activation technique. Emitted radiations are further captured by external detectors such as gamma cameras.

Radiolabelling of formulation involves utilization of a short-lived radioisotope that can spontaneously emit gamma radiation. The isotope is incorporated in the formulation as a salt (e.g., sodium pertechnetate) in normal saline. In this type of labelling, a reducing agent is used to reduce technetium, Tc (VII), into a lower valence state. The final solution is maintained in a buffered system, which is followed by incubation to enable labelling [34]. The most widely used reducing condition is the acidic stannous chloride or other stannous salts [35]. Various approaches for radiolabelling can be classified into whole dose radiolabelling, point radiolabelling and surrogate marker technique. Commonly used radionuclides are shown in table 1 along with their half-life [36].

Table 1: Commonly used radionucleotides

Radionucleotides	Approximate Half Life
81mKr (Krypton)	13 seconds
^{99}mTc (Technetium)	6.02 hours
^{111}In (Indium)	2.8 days
^{123}I (Iodine)	13 hours
^{131}I (Iodine)	8.05 days

Labelling by Neutron Activation Technology

This approach involves the incorporation of a stable isotope into the dosage form prior to its manufacture, followed by neutron irradiation of the intact dosage form (table 2). Neutron flux exposure is conducted for a very short time, generally 5-30 seconds. This exposure time has been shown to maintain the characteristics and integrity of the dosage form. Short period of time also prevents the degradation of drug under conditions of bombardment. Longer exposures may result in cross-linking of the polymers used in the dosage form. During this technique, thermal neutron irradiation converts the carefully selected stable isotopes (^{152}Sm or ^{170}Er) into radioactive gamma emitting isotopes (^{153}Sm or ^{171}Er) that can be detected by external imaging devices [37-40].

Table 2: Commonly used radionucleotides used in neutron activated scintigraphy

Radionucleotide	Natural abundance (%)	Approximate Half Life	Stable Nuclide
^{139}Ba (Barrium)	71.7	83 minutes	138Ba (Barrium)
^{171}Er (Erbium)	14.9	7.5 hours	170Er (Erbium)
^{153}Sm (Samarium)	26.7	47 hours	151Sm (Samarium)

Gamma Camera

Gamma scintigraphy relies on the detection of radiations emitted from a radionuclide. In this technique, gamma camera is used to image gamma radiation emitted from radioisotopes Nuclear imaging is predominantly carried out with planar or SPECT (single photon emission computed tomography) cameras and by using radionuclides that emit gamma radiation with energies between 100 and 250 KeV. The single photon emitting radioisotopes such as ^{99}mTc and ^{111}In are widely used with these instruments. Gamma camera is composed of an array of photomultiplier tubes coupled to a sodium iodide crystal. The interaction of a gamma photon from the source with the crystal leads to the production of a flash and it is detected by photomultiplier. To ensure that radiation from the source is detected in straight line, a lead collimator is placed between the subject and the crystal. The camera provides two dimensional or planar images of the distribution of radioactivity in the subject. The planar image provides a good depiction of the position of the radiotracer. For this reason, radiolabelled drug delivery systems are best studied with planar camera [41]. If planar imaging cannot provide the required deposition details, then SPECT should be considered. SPECT is a technique for producing crosssectional images of radionuclide distribution in the body. This is achieved by imaging the organ at different angles (e.g. 64 or 128 images/ 180^0 or 360^0) using a rotating gamma-camera. The acquired raw data are then processed by high-speed computers [42].

Applications of Gamma Scintigraphy

The use of imaging techniques particularly gamma scintigraphy to follow the behavior of drug formulations has revolutionized our knowledge of absorption and distribution in drug delivery. Following are the few applications of Gamma Scintigraphy technique for determination of the fate of the dosage forms under study-

1. Pulmonary drug delivery
2. Oral drug delivery
a. Gastroretentive
b. Colon targeting
3. Ocular drug delivery
4. Rectal drug delivery
5. Site specific targeting
a. Brain targeting
b. Tumor targeting
c. Liver & spleen targeting

Further, formulations involving colloidal particles, liposomes, micro-emulsions, and dendrimers can also be effectively be evaluated. The major advantage of gamma scintigraphy is that it is free of animal scarification and can therefore be especially useful in exploring sources of inter-subject variation. This method can be applied to both experimental animals and humans alike. Hence, it is a versatile technique for the investigators who can use this method to generate real time pharmacokinetic and pharmacodynamic data during various stages of drug development.

Fluorescence imaging technique using NIR probes

Molecules that absorb in the near-infrared (NIR) region, 700–1000 nm, can be efficiently used to visualize and investigate *in vivo* molecular targets

because most tissues generate little NIR fluorescence [43, 44]. The most common organic NIR fluorophores are polymethines. Among them, pentamethine and heptamethine cyanines comprising benzoxazole, benzothaizole, indolyl, 2-quinoline or 4-quinoline have been found to be the most useful [45].

An emerging new class of probes for *in vivo* fluorescence imaging is semiconductor nanocrystals or quantum dots. Quantum dots (QDs) typically have a core/shell structure of 2–8 nm in diameter with size-dependent fluorescence emission. The unique optical properties of QDs for *in vivo* optical imaging include high absorbency, high quantum yield, narrow emission bands, large Stokes shifts, and high resistance to photobleaching. QDs that emit at several different wavelengths can be excited with a single wavelength, and thus are suitable for multiplex detection of multiple targets in a single experiment. Several recent reviews summarize the synthesis, bioconjugation chemistry, optical features and applications of
QDs for *in vivo* imaging [46, 47].

Labeling Mechanism of Fluorescent Probes

Optical imaging has increasingly been used for dynamic noninvasive imaging of biological events in mouse models. Due to the lack of NIR fluorescence contrast generated by most tissues in the biological transparency window, exogenous fluorescent probes have to be administered for *in vivo* studies in order to visualize living tissues in its native physiological state. Ideally, the fluorescent probes to be administered should be biologically stable in the *in vivo* environment, and accumulate and produce imaging contrast at the target site. Fluorescent probes are classified according to their mechanism of contrast generation, collectively as non-specific, targeting and activatable.

Non-specific probes simply have differential distribution and are used to assess physiological processes such as changes in blood volume, permeability and perfusion in angiogenesis. Typically, they achieve only low target-to-background signals due to the non-binding circulating probes producing significant background fluorescence within the compartment.

Tissue- or cell-specific contrast is created by coupling a targeting moiety to a fluorescent robe that binds specifically to a receptor thus generating the readout signal. These probes can report more detailed information about the biological events than non-specific probes. Targeting probes can achieve high target-to-background signals provided the targeting moiety has a high affinity for the receptor and any unbound probes are thoroughly removed from the system, thereby reducing the background fluorescence.

Activatable probes comprise of donor-acceptor fluorophores that are coupled to each other in close proximity to maintain a quenched state. The fluorescence emission is activated by enzyme-mediated cleavage that releases the fluorescent probes. Activatable probes can attain high target-to-background signals as these probes in their native injected state are relatively undetectable.

Non-Specific Organic-Dye Probes

The choice of a suitable dye for *in vivo* imaging depends on many factors. The most important consideration would be the molar extinction coefficient and quantum yield of the dye in the NIR region [48, 49]. Of all the dyes, cyanine makes up the majority of commercial fluorescent probes for *in vivo* applications. Cyanine is a synthetic dye family of the polymethine group with a conjugated chain of odd number of carbon atoms linked between two nitrogen centers [50]. Among this class of compounds, carbocyanine dyes with indolic groups are readily available commercially and have also been synthesized in a large variety of analogues. The structure of carbocyanine is

formed by reaction of two indolic isomers (either identical or different) linked on each end of a C1, C3 or C5 methane. The absorption and fluorescence emission wavelength is determined both by the chain length of the methane and the side chains attached to the indolic groups. Carbocyanine dyes emits in the visible to NIR range. These dyes generally exhibit high molar extinction coefficients but only have moderate fluorescence quantum yields up to 30%. A prominent representative of the NIR cyanine dye is Indocyanine green (ICG). ICG was first synthesized in the fifties [51] and is the only clinically approved dye available commercially. It has been used in optical imaging of changes in blood-brain barrier permeability after thrombus formation in a mouse model of cerebral venous thrombosis [52] and liver perfusion in mouse [53]. Clinically, ICG has been used in clinical retinal angiography [54], hepatic function testing [55] and imaging of human brain [56, 57]. Since ICG binds tightly to plasma proteins and becomes confined to the vascular system, they are widely used for intraoperative assessment of vascular flow in cardiovascular surgery [58–60]. Because of its amphophilicity and a shortage of functional groups available for conjugation, ICG could only function as a non-specific probe for *in vivo* imaging.

To improve the pharmacokinetics of *in vivo* contrast agents, a possible approach is to reduce the conjugate molecular size while still preserving the targeting affinity of the labeled dyes. Neri *et al.* demonstrated this idea by conjugating antibody single chain fragments selected from phage display libraries against an angiogenesis-associated oncofetal fibronectin isoform to cyanine dyes and applied to imaging of angiogenesis in a variety of animal models [61, 62]. Alternative strategy is also shown in the work by Achilefu *et al.* and Licha *et al.* highlighting the successful application of receptor-specific peptide-dye conjugates for fluorescent imaging of tumors [63–65]. As a whole, fluorescent dyes can be readily functionalized to achieve targeting.

Targeting Organic-Dye Probes

Covalent attachment of targeting moieties such as antibodies, antibody fragments, proteins and peptides to fluorescent dyes can significantly improve the target-to-background signal. The strategy of conjugating antibodies to cyanine dyes were first demonstrated by Folli *et al.* and Ballou *et al.* [66, 67] and applied to fluorescence imaging of tumor in mice. Antibodies, however, have found limited utility due to their unfavorable pharmacokinetics. The typical circulating half-life of antibodies is much shorter than the time required to access a small tumor with reasonable accumulation, thus rendering effective targeting rare using antibodies [68].

Activatable Organic-Dye Probes

The concept of activatable NIR probes was introduced by Weissleder *et al.* for *in vivo* imaging of enzyme activity [69]. Enzyme-activatable probes contain either two identical or different fluorophores linked in close proximity to each other by a specific peptide linker. The fluorescence of the probes is essentially undetectable in the quenched state. After enzymatic cleavage, the fluorophores are separated to restore the fluorescence emission. A large number of activatable probes have been documented, among them the enzyme activation of NIR targeting dyes is mediated mainly by tumor associated proteases, such as cathepsins, caspases and matrix metalloproteinases [70–75]. The ability of activatable fluorescent probes to detect gene expression is particularly useful as a means to diagnose malignant molecular process in the early disease state [76].

Nanomaterial-Based Fluorescent Probes

Despite the long history of *in vivo* optical imaging, organic dyes have suffered from small Stokes shifts, poor photostability, high plasma protein

binding rate, aggregation and background fluorescence in aqueous medium [77]. Attention has been increasingly channeled toward nanotechnology to search for new class of more effective probes. Many nanomaterials are already widely used as catalysts, and energy storage and electronic devices [78–82]. Their application to the biomedical arena has attracted a similarly enthusiastic following. Interest in the nanomaterials arises from the fact that at nanoscale the properties of materials can be very different from their bulk counterparts. Firstly, nanomaterials when compared to the same mass of material existing in a larger form have a much higher surface area. As such, nanomaterials are more chemically reactive because of the high surface energy. In addition, at nanoscale range, quantum effects begin to dominate the behavior of matter, affecting the optical, electrical and magnetic behavior of materials [83, 84].

Lately, the integration of nanotechnology with medicine has found many novel applications of nanomaterials. Nanoparticles can be engineered to overcome biological barriers for effective and targeted delivery of drugs, genes, and contrast agents [85]. Active moieties such as proteins, peptides and nucleic acids can easily be conjugated to the surface of the nanoparticles for use as non-specific, targeting or activatable nanoprobes. The following sections are devoted to the recent development of some particulate NIR fluorescent nanoprobes for potential *in vivo* imaging.

CONCLUSION

From the foregoing discussion it is evident that the bioimaging of Pharmaceutical dosage form for determination of the *in vivo* efficacy past administration in the body can be done effectively by using the above mentioned techniques. Pharmacoscintigraphic technique is an elegant approach to gain insight of the actual *in vivo* distribution pattern of dosage form along with the assessment of pharmacokinetic information. Conventional methods for evaluating these formulations *in vivo* are laborious and time-consuming. The data obtained from scintigrams, although qualitative, are quite reliable in predicting the extent to which a formulation has been targeted to its destined site. As a tool to examine drug delivery to the lung and to the eye, scintigraphy is the method of choice and needs to be exploited to the maximum for its potentials in the evaluation of new molecular entities, drug delivery systems/formulations and in therapeutic drug monitoring. The potential of NIR fluorescent nanoprobes for *in vivo* application is unlimited. In particular the versatile, nanomaterials-based NIR fluorescent probes offer ample opportunity to optimize their optical and targeting properties. Among all nanomaterial-based fluorescent probes, Si QDs is the only system that can be directly loaded with drugs and biodegraded into silicic acid that can be excreted efficiently through renal clearance. Like other nanomaterial-based fluorescent probes, improvement of their quantum efficiency, however, will still require significant effort. Robust and environmentally friendly synthetic routes that can produce these nanomaterials-based NIR nanoprobes with uniform size and colloidal stability represent other significant challenges. The widespread application of NIR fluorescent nanoprobes awaits demonstration of their long term biocompatibility, *in vivo* targeting efficacy as well as parallel development of advanced optical instrumentation for deep-tissue imaging capability. Nevertheless, their role in advancing the field of nanomedicine will undoubtedly be essential.

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