Recent Progress of Near-Infrared Fluorescence in vivo Bioimaging in the Second and Third Biological Window

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Abstract

Near-infrared (NIR) fluorescence bioimaging using above 1000 nm wavelength region is a promising analytical method in visualizing deep tissues. As compared to the short-wavelength ultraviolet (UV: < 400 nm) or visible (VIS: 400–700 nm) region, which results in extremely low absorption or scattering of biomolecules and water in the body, NIR light passes through the tissues. Various fluorescent probes that emit NIR emission in the second (1100–1400 nm) or third (1550–1800 nm) biological windows have been developed and used for NIR in vivo imaging. Single-walled carbon nanotubes (SWCNTs), quantum dots (QDs), rare-earth doped ceramic nanoparticles (RED-CNPs), and organic dye-based probes have been proposed by many researchers, and they are used to successfully visualize the bloodstream, organs, and disease-affected regions, such as cancer. NIR imaging in the second and third biological windows is an effective analytical method visualizing deep tissues.
Keywords
Bioimaging, near-infrared, second biological window, NIR-II, third biological window, NIR-III, nanoparticles, upconversion, QD, SWCNT,

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1. Introduction

In vivo imaging technique enables us to observe various phenomena occurring inside the body. Recently, near-infrared (NIR) fluorescence imaging has attracted attention, in particular, of biologists and medical scientists since NIR light (> 700 nm) can penetrate tissues.\textsuperscript{1-5} As compared to the short-wavelength ultraviolet (UV: < 400 nm) or visible (VIS: 400 - 700 nm) region, which results in low absorption or scattering of biomolecules and water in the body, NIR light passes through the tissues. Thus, the NIR wavelength region (700 - 1800 nm) is referred to as the “biological window” and used for bioanalysis.\textsuperscript{6-8}

In the past two decades, NIR imaging has mainly been carried out using the 700–900 nm (first biological window: NIR-I) region. Most NIR fluorescent materials emit NIR-I emission, observed by commonly used in vivo imaging systems that exhibit Si-based CCD cameras. However, more recently, NIR light in the 1000-1400 nm (second biological window: NIR-II) and 1550-1800 nm (third biological window: NIR-III) wavelength regions have been proposed for in vivo analysis as they penetrate living tissues more deeply than the commonly used NIR-I wavelength region, about 20-30 mm (Fig. 1).\textsuperscript{1-3, 9-19} Most NIR-I fluorescent probes require VIS excitation light, and thus, it is difficult to visualize the deeper tissues. Contrastingly, NIR-II or III fluorescent probes are excited by tissue-penetrable NIR-I light. The observation of NIR-II or III emission under NIR-I excitation is one of the most promising technological advances for deep tissue analysis (Fig. 2).

For NIR-II and III imaging, various fluorescent probes, such as single-walled carbon nanotubes (SWCNTs), quantum dots (QDs), rare-earth doped ceramic nanophosphors (RED-CNPs), or organic dyes, have been proposed by many researchers (Fig. 3). These nanophosphors possess each strong point and are used for in vivo imaging.
This review describes the recent progress in NIR-II and III in vivo fluorescence imaging techniques for analytical methods of the deeper tissues of the body.

2. Single Walled Carbon Nanotubes (SWCNTs)

The first study on NIR-II in vivo imaging was reported by Dai and his coauthors in 2009. In their study, SWCNTs were injected into mice to visualize vascular tissues. SWCNTs have recently been used as biomaterials with several functions, including drug delivery carriers and bioimaging agents. SWCNTs possess a hollow cylinder structure formed from rolled up graphite sheets. These SWCNTs possess absorption in the NIR region, thereby demonstrating tissue-penetrable NIR-II emission. Dai et al. reported several examples of in vivo imaging based on SWCNTs. Although SWCNTs show strong NIR-II emission, the hydrophobic surface character of SWCNTs is not suitable for biological applications as it causes difficulty during dispersion in an aqueous environment.

Additionally, CNTs have a “needle”-like conformation similar to asbestos. Therefore, there are growing concerns about their adverse effects on health regarding the use of CNT-related materials. Thus, surface modification of SWCNTs with biocompatible materials, such as biocompatible polymers, is required for in vivo applications. Most SWCNT-based biomaterials use poly(ethylene glycol) (PEG)-based derivatives as surface modification agents. For example, PEG possessing phospholipid structures are widely used for surface modification. The hydrophobic character of phospholipids is employed to immobilize PEG chains, which are coated on the SWCNT surface. The PEG-modified (PEGylated) SWCNTs usually display high biocompatibility. Furthermore, the blood circulation properties of SWCNTs are improved by PEGylation, due to the prevention of aggregation of the nanotubes and interaction with biomolecules.
However, although the surface-modified SWCNTs are an important candidate for NIR-II nanophosphors, they still present several challenges. The peculiar cylindrical shape of this material causes strange cellular uptake behavior, despite the PEG-modified surface. For example, PEGylated SWCNTs show peculiar cellular uptake speed, “delayed increase” uptake amount of nanotubes in the cells. This is not normal cellular uptake behavior of PEGylated nanoparticles. Unexpected cellular uptake behavior of fluorescent probe is difficult to perform successful fluorescence imaging. Therefore, improvements in the surface modification methods and cellular uptake studies are required for successful NIR-II in vivo fluorescence imaging based on SWCNTs.

3. Quantum Dots (QDs)

Semiconductor nanoparticles, QDs are one of the most famous fluorescent inorganic materials. They have been used as fluorescent probes for both in vitro and in vivo imaging. Currently, different colored fluorescence QDs with functional groups on nanoparticle surfaces are commercially available. The emission wavelength of QDs is tuned based on the particle size as they exhibit size-dependent emission spectra owing to their quantum size effect (Fig. 4). Thus, QD-based fluorescence imaging can be used for multicolor visualization under single-wavelength excitation. Additionally, QDs have several advantages as ideal fluorescence imaging probes. For example, the emission strength of QDs is quite strong and stable. Traditional fluorescent organic dyes exhibit photobleaching under strong excitation light and/or long-time observation due to the dissociation of molecules. In contrast, QDs show no photobleaching and can be used for long-term observation. The application of QDs for fluorescence bioimaging was first reported by Nie and Alivisatos in 1998, following which QD-based fluorescence
bioimaging rapidly accelerated.

Although QDs exhibit high performance as bioimaging probes, they are cytotoxic, as they are generally generated using heavy metals, such as lead (Pb) or cadmium (Cd). Thus, surface modification of QDs with biocompatible materials is required for bioimaging applications. Similar to its application in SWCNTs, PEG is employed as a surface modification polymer for QDs. The surface modification of QDs with PEG, improves their biocompatibility. Additionally, the dispersion stability of QDs and the resistivity against the non-specific adsorption of biomolecules are also improved by PEGylation. Therefore, most of the current QD studies use PEG as a surface modification agent.

For deep tissue imaging, NIR fluorescent QDs are widely reported. For example, Pb or indium (In) based QDs, such as InAs, PbSe, or PbS, have been proposed as NIR fluorescent probes. These QDs show NIR-II emission under NIR-I excitation and can be used for deep tissue in vivo imaging.

Lead chalcogenides, such as PbSe or PbS, are commonly used materials as NIR fluorescent QDs. The first report of PbS and PbSe QD-based NIR-II imaging was reported by Hyun et al. in 2007. This work reported in vitro NIR-II imaging of living cells. Thereafter, PbS or PbSe based on in vivo imaging of living animals, such as mice, has been reported by many researchers. Won et al. reported the application of PbSe QDs for NIR-II imaging of mice. Furthermore, in 2013, Jin et al. reported GSH-immobilized PbS for NIR-II imaging. The same group also reported various PbS-based probes for NIR-II imaging, such as PbS/CdS core-shell QDs and ligand protein-immobilized PbS QDs. In addition, further improvements in the emission properties of PbS QDs and the formation of a core-shell structure, which is one of the most important techniques, are required. For example, Dongling Ma et al. reported core-shell structures of PbS core-
based QDs, such as PbS/CdS/ZnS\textsuperscript{39} and PbS/CdS\textsuperscript{40} for NIR-II \textit{in vivo} imaging applications. Additionally, as an analytical method, observation techniques are also important for successful \textit{in vivo} imaging. For example, light-sheet microscopy has attracted much attention for 3-D image generation. Hongjie Dai et al. reported NIR-II light-sheet microscopy using PbS/CdS core-shell QDs for visualization of traumatic brain injury.\textsuperscript{41}

Although Pb-based QDs show strong NIR-II emission and are used for \textit{in vivo} imaging, Pb cytotoxicity is a serious issue. To address this concern the use of various low-toxicity QDs, such as silver (Ag)-based QDs, have also been proposed. Hongjie Dai and his co-workers presented Ag\textsubscript{2}S QDs for NIR-II imaging of mice tumors.\textsuperscript{42} Yan Zhang et al. also reported the long-term \textit{in vivo} biodistribution of PEGylated Ag\textsubscript{2}S QDs.\textsuperscript{43} Li et al. reported real-time imaging of bloodstream and angiogenesis using Ag\textsubscript{2}S QDs.\textsuperscript{44} Additionally, owing to their excellent low toxicity, Ag-based QDs are commonly used for long-term tracking of cells.\textsuperscript{45-48} For example, Qiangbin Wang and his co-workers reported NIR-II \textit{in vivo} tracking of transplanted mesenchymal stem cells (MSCs) using Ag\textsubscript{2}S QDs.\textsuperscript{45} Thus, Ag\textsubscript{2}S is a promising candidate for labeling marker in regenerative medicine.

More recently, indium (In)-based NIR-II fluorescent QDs have been proposed, and they have been used as QD probes with high safety. Kameyama et al. reported rod-shaped AgInTe\textsubscript{2} QDs for NIR-II imaging.\textsuperscript{49} Synthesized AgInTe\textsubscript{2} QDs were incorporated into the liposome and used as NIR-II fluorescent probes. Moreover, Bawendi et al. proposed InAs/CdSe/CdS tri-layered QDs for NIR-II imaging.\textsuperscript{50} These “core-shell-shell” QDs visualized the blood vessels of living mice.

Although the NIR-II fluorescent QDs are powerful tools for deep tissue \textit{in vivo} imaging, most contain highly toxic elements, thereby making their medical use challenging. To address this, QD formation using low-toxicity elements and surface
modification with biocompatible polymers can enable the construction of high-performance *in vivo* imaging probes.

4. Rare-earth doped ceramic nanoparticles (RED-CNPs)

Rare-earth ion-doped ceramics are well-known fluorescent materials and used as optical components in industries.\textsuperscript{51-53} For example, 1550 nm emission of erbium (Er\textsuperscript{3+}) under 980 nm excitation in silicate glass fibers is used for optical communications.

In the past two decades, biological applications of RED-CNPs have been widely reported. Most of these studies mainly used the VIS emission of RED-CNPs under NIR excitation.\textsuperscript{54-56} RED-CNPs show short-wavelength VIS emission under longer-wavelength NIR excitation, owing to the stepwise excitation process of trivalent rare-earth ions. This phenomenon is called “upconversion,” and thus, this material is also well-known as “upconversion nanoparticles.” The VIS emission of RED-CNPs can be detected using a Si CCD camera. Therefore, upconversion fluorescence imaging is performed using normal fluorescence microscopy with a NIR laser excitation source. Although the VIS upconversion emission of RED-CNPs is easy to observe, the penetration depth of VIS emission is low, making visualization of the deep interior of the body difficult.

Instead, the observation of NIR-II or III emission of RED-CNPs is proposed for *in vivo* imaging applications.\textsuperscript{57-59} Under NIR excitation, such as NIR-I laser irradiation, RED-CNPs show VIS upconversion emission and simultaneous NIR-II or III emission. For *in vivo* imaging, observation of NIR-II or III emission is preferential compared with VIS emission due to the low scattering and absorption of biomolecules and water in the body. The first study of NIR-III *in vivo* imaging based on RED-CNP was reported by Kamimura et al., in 2011.\textsuperscript{60} In their work, Er\textsuperscript{3+}- and ytterbium (Yb\textsuperscript{3+})-doped yttrium oxide
(Y$_2$O$_3$) was synthesized, and the surface of the nanoparticles was modified with a PEG-based block copolymer. The obtained PEGylated nanoparticles showed strong NIR-III emission (1550 nm) under NIR-I excitation (980 nm) and visualized the spleen of a living mouse.

Following this, RED-CNP-based NIR-II or III imaging became widely reported.$^{61-68}$ Similar to other fluorescent nanomaterials, such as SWCNTs and QDs, surface modification of RED-CNPs is also quite important. In most of the current RED-CNP-based imaging studies, rare-earth ion-doped NaYF$_4$ or NaGdF$_4$ are used as fluorescent probes due to their strong emission intensities. However, due to the synthesis method of these nanoparticles, their surfaces are capped with hydrophobic capping agents. These nanoparticles are mainly synthesized using the thermal decomposition method with oleic acid as a capping agent for crystal growth. Thus, the surface of the obtained nanoparticles is capped with oleic acid, and their dispersion is difficult in an aqueous environment. To solve this problem, surface modification with hydrophilic polymers is required for successful biological applications. Most of the current reports of NaYF$_4$- or NaGdF$_4$-based imaging use PEG surface modifications. Ligand exchange reactions with PEG possessing anchor structures are widely used because of their weak adsorption of the COOH group of oleic acid on the nanoparticle surface.$^{69}$

The special feature of RED-CNP-based NIR-II or III imaging is temperature measurement applications (Fig. 5).$^{70-81}$ It is well-known that most fluorescent materials, such as organic dyes or QDs, show temperature-dependent changes in fluorescence properties, such as intensity, peak, and lifetime. Based on the temperature dependence of these properties, the temperature of the local nano- or micro-region of the living specimen can be measured by using fluorescence. This imaging technique is called “nanothermometry.” In particular, temperature measurement based on fluorescence
intensity changes is the easiest way to measure the temperature of cells in the body. However, most fluorescence intensity-based nanothermometry techniques have concentration-dependent challenges. Fluorescent probes diffuse intracellularly, causing fluorescence intensity changes. Additionally, most current fluorescence nanothermometers use VIS emission, making it difficult to measure temperature within the body.

To address this issue, temperature-dependent emission lines of RED-CNPs in NIR-II and III are attractive candidates for temperature measurement in deep tissues. As described previously, NIR-II or III emission of RED-CNPs under NIR-I excitation can be applied for deep tissue imaging. The fluorescence intensity of various trivalent rare-earth ions in the NIR-II or III region is different between rare-earth species. For example, holmium (Ho\(^{3+}\)) does not show temperature dependency, whereas Er\(^{3+}\) shows strong temperature dependence. Using these fluorescence characteristics, ratiometric nanothermometers can be designed. NaYF\(_4\) nanoparticles co-doped with Ho\(^{3+}\) and Er\(^{3+}\) showed a temperature non-dependent 1150 nm emission of Ho\(^{3+}\) in the NIR-II region and a temperature-dependent 1550 nm emission in the NIR-III region. Based on these two emission lines in the NIR-II and III regions, the temperature of the local region can be detected, besides the concentration problem of the probes.\(^6\)

Compared with other NIR-II and III fluorescent materials, the emission intensity of RED-CNPs is weak. However, this material has low toxicity compared with SWCNTs or QDs. Additionally, this nanophosphor has attractive fluorescence properties, such as ratiometric temperature imaging in the NIR-II and III regions. Thus, it is an important probe for NIR bioanalysis.
5. Organic Dyes

Organic dyes are the traditional fluorescent probes used for bioimaging. Although organic dyes have several weaknesses, such as low photostability, they are still major probes because of their low cost and functionality with various biomolecules through chemical bonding. Additionally, the small molecular size of organic dyes is also advantageous for safe imaging. These probes are removed from the body via renal clearance. Therefore, fluorescent dyes are promising candidates for clinical applications.

In the NIR wavelength region, various organic dyes are used for \textit{in vivo} imaging. For example, in the NIR-I window region, the USA Food and Drug Administration (FDA) approved dye, indocyanine green (ICG), is used as a fluorescence marker during clinical surgery.\textsuperscript{82, 83} Furthermore, in the NIR-II window region, commercially available NIR-II fluorescent dyes such as IR-26 or IR-1061 can be used as fluorescent probes (Fig. 6). However, due to their hydrophobic chemical structure, most NIR-II fluorescent dyes are difficult to use in aqueous environments. Therefore, the conjugation of these dyes with hydrophilic polymers has been proposed. The first report of fluorescent dyes based NIR-II \textit{in vivo} imaging was reported by Prof. H. Dai et al., in 2013.\textsuperscript{84} In their work, IR-1061 dye was conjugated with poly(acrylic acid) and phospholipid-PEG. The prepared dye-loaded nanoparticles were used for NIR-II imaging of mice under 808 nm excitation. Subsequently, several studies have reported on IR-1061 dye-based NIR-II imaging.\textsuperscript{85-87} For example, Kamimura et al. reported on biodegradable polymer micelles possessing IR-1061 dye in the core of micelles for NIR-II imaging.\textsuperscript{85} The polymer micelle is formed from PEG possessing a biodegradable segment. The dye-loaded micelles showed strong NIR-II emission and biodegradability. Thus, this IR-1061 dye-loaded polymer micelle can be used as a safety NIR-II imaging agent.

Recently, novel NIR-II fluorescent dyes have been designed and synthesized
by many researchers. Dai et al. reported a synthetic 970-Da organic molecule dye with a donor-acceptor-donor (D-A-D) charge structure (CH1055).\textsuperscript{88} The synthesized CH1055 dye was conjugated with PEG (CH1055-PEG) and used for NIR-II imaging. Moreover, the CH1055 dye also forms a complex with bovine serum for the brightness NIR-II probes.\textsuperscript{89} Polymethine derivatives are also used for NIR-II imaging. For example, E. M. Sletten et al. reported flavylium polymethine based dye (Flav7) for NIR-II imaging.\textsuperscript{90} Additionally, another type of polymethine derivative, FD-1080, has also been reported by Zhang et al.\textsuperscript{91} Furthermore, they also reported a similar pentamethine cyanine dye, BTC-1070, for NIR-II imaging.\textsuperscript{92}

In addition, fluorescent polymer materials have recently been proposed as fluorescent probes for \textit{in vivo} bioimaging. Compared with low-molecular-weight dyes, fluorescent polymers have several advantages, such as increased sensitivity, robustness, and functionality. Furthermore, polymer chemistry techniques achieve functionalization of these polymer-based fluorescent probes, such as pH, temperature, and photo-responsibility. In 2014, H. Dai et al. first reported that the NIR-II fluorescent polymer probe (pDA), a biocompatible polymer with a D-A structure, was synthesized and used as an imaging probe (Fig. 7).\textsuperscript{93} Additionally, another type of NIR-II fluorescent polymer, PDFT1032, has been reported as an imaging guide agent during tumor surgery.\textsuperscript{94}

Compared with inorganic nanophosphors, organic dyes have several advantages, specifically high biocompatibility and renal clearance from the body. It is anticipated that the clinical application of NIR-II imaging will be performed using these organic materials in the near future.
6. Conclusions

NIR imaging using the second (NIR-II) and third (NIR-III) biological windows have recently become an important analytical technique for visualizing deep tissues. NIR-II and NIR-III imaging can visualize the deep part of the body due to low absorption or scattering of biomolecules and water. Various types of nanophosphors demonstrating fluorescence in these wavelength regions have been developed and applied for \textit{in vivo} imaging studies. This bioanalytical method may discover unknown biological phenomena in deep tissues. Furthermore, this imaging method has recently been applied in clinical trials and may be employed to save patient lives in the future. Therefore, though further research is required, the use of this analytical method remains crucial in understanding fundamental biology and developing novel medical diagnostic tools.
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Figure Captions

Fig. 1  NIR-I, II, and III biological windows in NIR region. Absorption or scattering of biomolecules and water in the body is quite low in the NIR region.

Fig. 2  NIR-II vascular imaging of living mice by using NIR-II fluorescent dye loaded polymer micelles. The fluorescent probes were injected into mouse via tail vein and NIR-II emission was observed 1 min after injection. (Excitation wavelength: 980 nm, Emission wavelength: 1100 nm.)

Fig. 3  Typical NIR-II and III fluorescent probes. (a) Single-walled carbon nanotubes (SWCNTs), (b) QDs, (c) rare-earth doped ceramic nanoparticles (RED-CNPs), and (d) organic dyes.

Fig. 4  Emission wavelength of QDs. QDs consist of various elements are used as fluorescent probes of bioimaging.

Fig. 5  Schematic illustration of RED-CNPs based nanothermometry. Based on the temperature sensitivity of rare-earth ions, RED-CNPs show temperature dependent NIR-II or NIR-III emissions.

Fig. 6  Chemical structure of typical NIR-II fluorescent organic dyes. (a) IR-26, (b) IR-1061, (c) CH-1055, (d) Flav7, and (d) FD-1080.

Fig. 7  Chemical structure of pDA polymer. This polymer is biocompatible, and it possess a donor-acceptor (D-A) structure.
Fig. 1

![Diagram showing wavelength (nm) and tissue depth with skin and tissue layers, indicating poor, fair, good, and excellent conditions with specific depths for UV, VIS, and NIR regions.](image-url)
Fig. 3

(a) Single Walled Carbon Nanotubes (SWCNTs)
- Diameter: < 400 nm
- Thickness: < 2 nm

(b) QDs
- PbS, Ag2S, etc.
- Diameter: 5 - 10 nm

(c) Rare-Earth Doped Ceramic Nanoparticles (RED-CNP) 
- NaYF₄, NaGdF₄, etc.
- Diameter: 10 - 100 nm

(d) Organic Dyes
- Mw < 1,000 g/mol
Fig. 4
Fig. 6

(a) IR-26

(b) IR-1061

(c) CH-1055

(d) Flav7

(e) FD-1080
Graphical Index

Ex: NIR-I  Em: NIR-II and III

Deep Tissue *in vivo* Imaging