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Naphthalimides in fluorescent imaging of tumor hypoxia – An up-to-date review

Rashmi Kumari[a], Dhanya Sunil[a,*], Raghumani S. Ningthoujam[b]

[a] Department of Chemistry, Manipal Institute of Technology, Manipal Academy of Higher Education, Manipal 576 104, Karnataka, India
[b] Chemistry Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

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ABSTRACT

Hypoxia is a distinctive characteristic of advanced solid malignancies that results from a disparity between oxygen supply and its consumption. The degree of hypoxia is believed to have adverse prognostic significance. Therefore detecting cellular hypoxia can potentially offer insights into the grade of tumour as well as its evolution towards a progressive malignant phenotype, which clinically translates to greater metastatic potential and treatment resistance. Fluorescence imaging to visualize hypoxia in biological systems is a minimally-invasive method. Recently there are several reports on interdisciplinary research that aims at developing functional probes that can be efficiently used for non-invasive imaging of hypoxic tumours. Upregulated levels of nitroreductase (NTR) is detected in hypoxic solid malignancies, and this characteristic feature is increasingly utilized in the development of NTR-targeted fluorescent molecules to selectively sense hypoxia *in vivo*. The present review summarizes various reports published on the design concepts of nitro naphthalimide-based bio-reductive fluorescent sensors that can be applied noninvasively to image hypoxia in cancer.

1. Introduction

Hypoxia is a pathological phenomenon in aggressive malignant tumours characterised by low partial pressures in tissue oxygen (pO_2 values ≤ 2.5 mmHg) [1]. The mean oxygen level in these cancer cells is usually below 4.4%, but could reach 0% in focal areas [2,3]. This is essentially due to an imbalance between supply and demand in oxygen. Poor oxygen supply could be due to an inefficient vascular network or low oxygen saturation of haemoglobin. The subsequent hypoxia putatively alters tumour geometry and acquirement of an EMT (Epithelial-to-mesenchymal transition) phenotype resulting in an enhanced cancer cell mobility and ensuing metastasis to distant parts of the body. An enhanced oxygen demand is inherent to high cellular metabolic activity. Also, inadequate oxygen supply alters cancer cell metabolism and results in phenotypic diversity in tumours. The hypoxic tumours in turn limit the perfusion of therapeutic agents, thereby contributing to chemo-, bio-, thermo- and radio-therapy resistance by inducing cell quiescence [1,4]. The significant role that hypoxia owns in cancer progression and treatment is pictorially presented in Fig. 1. There has been surging studies on possible ways to lessen the mortality level amongst patients bearing hypoxic tumours [5]. Consequently, development of novel methods for hypoxia assessment is of high relevance in the detection and treatment of aggressive solid cancers [6,7].

2. Strategies for hypoxia imaging

Hypoxia is found to be existent in over 60% of solid tumours and can be either chronic or cyclical (acute or transient) [8]. In the majority of cases, conventional medical imaging techniques detect solid tumours *in vivo* only after they have considerably progressed. Imaging hypoxia could potentially offer an avenue to an earlier cancer diagnosis as this phenomenon starts evolving with tumour diameters of about 350 μm [9]. Several techniques, both invasive and non-invasive, have been exploited to measure hypoxia in tumours. Invasive approaches include immunohistochemical staining, oxygen electrodes, DNA (Deoxyribonucleic acid) strand breaks *etc.* Less-invasive techniques include polarographic needle electrodes, MRI (Magnetic resonance imaging), radionuclide imaging involving PET (Positron emission tomography), SPECT (Single-photon emission computed tomography), and optical imaging methods encompassing fluorescence and bioluminescence [10–15].

2.1. Imaging techniques for detecting tumour hypoxia

Various imaging approaches such as PET, MRI, photoacoustic and optical probes have been used for the sensitive and precise imaging of the hypoxic tumour microenvironment. Clinically available PET

* Corresponding author at: Department of Chemistry, Manipal Institute of Technology, Manipal, Karnataka, India.
E-mail address: dhanya.s@manipal.edu (D. Sunil).

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Nomenclature

List of acronyms

Acronyms	definition
ADEPT	antibody-directed enzyme prodrug therapy
ATP	adenosine triphosphate
AzoR	azoreductase
BOLD-MRI	blood oxygen level dependent- magnetic resonance imaging
CEST	chemical exchange saturation transfer
ctDNA	circulating tumour DNA
DIPEA	*N*,*N*-Diisopropylethylamine
DNA	deoxyribonucleic acid
EMT	epithelial-to-mesenchymal transition
FAD	flavin adenine dinucleotide
FMN	flavin-mononucleotide
FRET	fluorescence resonance energy transfer
GDEPT	gene directed enzyme prodrug therapy
HIF-1	hypoxia-Inducible Factor 1
ICT	intramolecular charge transfer
IPET	intra-molecular photo-induced electron transfer
MRI	magnetic resonance imaging
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of NAD
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form NADP
NIR	near infra-red
NTR	nitroreductase
PET	positron emission tomography
ROS	reactive oxygen species
SPECT	single-photon emission computed tomography
VDEPT	virus directed enzyme prodrug therapy

imaging relies on the detection of radioisotope-labelled markers that selectively accumulate in hypoxic regions, and have low detection limits [11,16–18]. BOLD (Blood oxygenation level dependent)-MRI is yet another clinically utilized imaging strategy that exhibits low detection limit, wherein the water relaxation behaviour of T_2^* is inspected by the concentration of deoxyhemoglobin in the bloodstream [19]. T_1-MRI and CEST (Chemical exchange saturation transfer)-MRI with high detection limits are still in their *in vitro* and *in vivo* laboratory level testing stage. T_1-MRI monitors the hypoxia-induced variations of relaxivity, and is centred on the altered number of inner-sphere water molecules or the electronic relaxation time. CEST imaging detects the hypoxia-induced CEST effect changes based on the varying proton lifetime τ_M or T_1 relaxation time [20–23]. Photoacoustics is an alternate modality used for hypoxia imaging with a moderate detection limit that

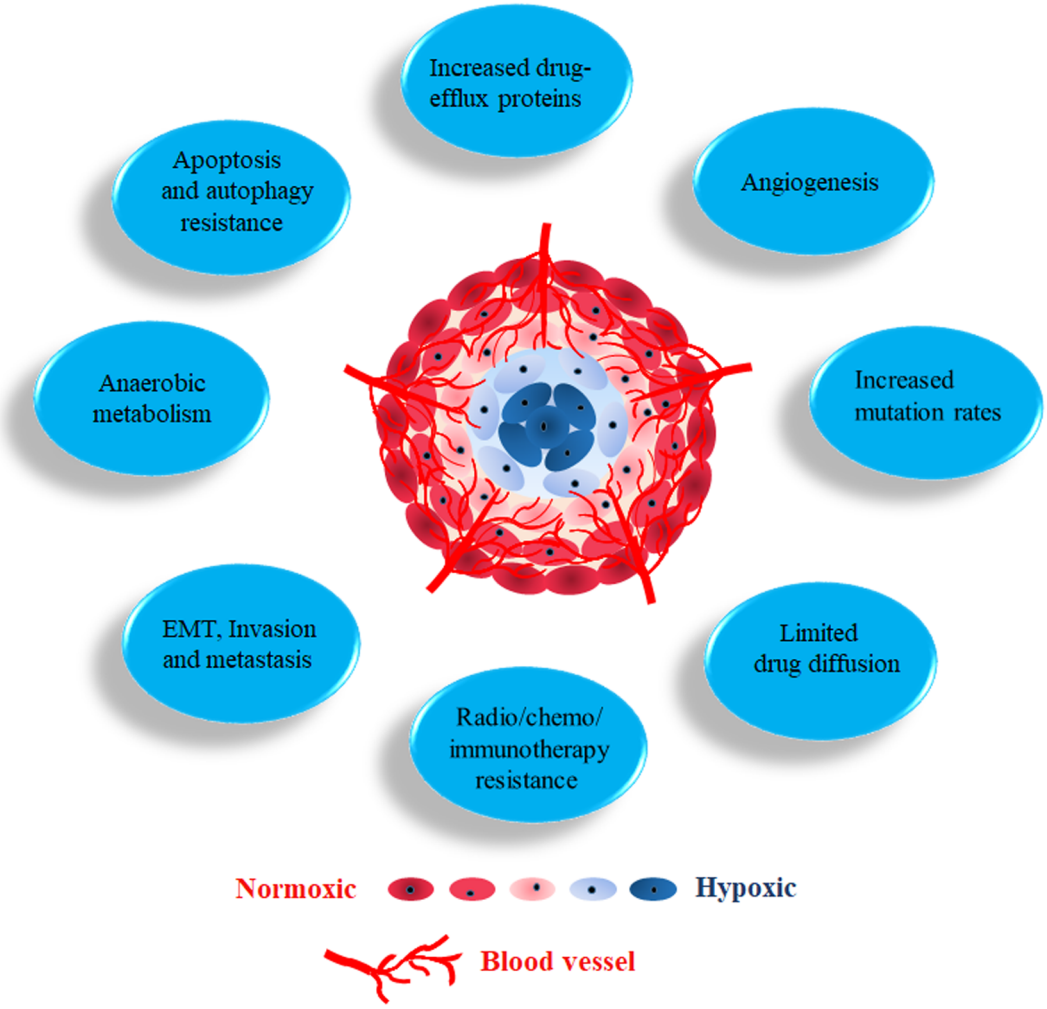


Fig. 1. Role of hypoxia in the progression of solid malignancies and anticancer therapy.

interprets the outputs of ultrasound signal by oxyhemoglobin and deoxyhemoglobin and is yet to progress to the clinical scenario [24,25]. Optical imaging can be satisfactorily achieved by analysing two intrinsic cellular parameters: redox levels and oxygen status. Thus either the alterations in the fluorescence intensities induced by changes in the intracellular redox environments or oxygen-triggered phosphorescence quenching of the exogenous sensors can be measured. The testing of optical imaging probes is still in the *in vitro* and *in vivo* phase and their hypoxia detection limit is extremely low [26–28].

2.2. Desired features of hypoxia imaging probes

The design of a good hypoxia imaging probe is critical. It should exhibit certain desired features; (i) require only a low dosage in order to minimise toxic effects, (ii) be highly selective to hypoxic tissues, (iii) form less metabolites, (iv) have long retention times in hypoxic cells, with rapid clearance from normal cells, (v) be less dependent on pharmacokinetic features that co-varies with hypoxia such as blood flow and pH and (vi) have simple and fast synthesis with high yield. The most widely used 'direct imaging' strategy for clinical detection of hypoxia is based on localizing the imaging probe within the less oxygenated regions and monitoring subsequent structural variations in the probe following interaction between the molecule and oxygen. Surrogates that are released due to the cascade of hypoxia triggered signalling pathways can function as targets for imaging hypoxia, and these represent potential "indirect imaging" approaches. Though the magnitude to which these pathways correlate with hypoxia has not been well-realized, it is evident that they have been intensely linked with clinical prognosis and hence continue to be important targets for imaging tumour hypoxia.

2.3. Fluorescence imaging of hypoxia

Fluorescence imaging is a highly favorable method for observing and monitoring bioactive species in living systems. It has received key attention in hypoxia detection, attributed to its low toxic profiles, sensitivity, selectivity, rapidity, good spatio-temporal resolution, high signal-to-background ratios, reversible responses, less expensive, simple operation and non-invasive real time monitoring in living systems [29–36]. The fluorescent probes reported till date for imaging tumour hypoxia are categorized based on: (i) the hypoxia associated biological macromolecules such as carbonic anhydrase and HIF-1 (Hypoxia-Inducible Factor 1) [37,38] and (ii) the distinct pathological hypoxic milieu, such as lesser partial pressure of oxygen and robust bio-reduction capability compared to normal cells [39–41]. Hence the hypoxia-responsive probes can exhibit variations in their fluorescence behaviors

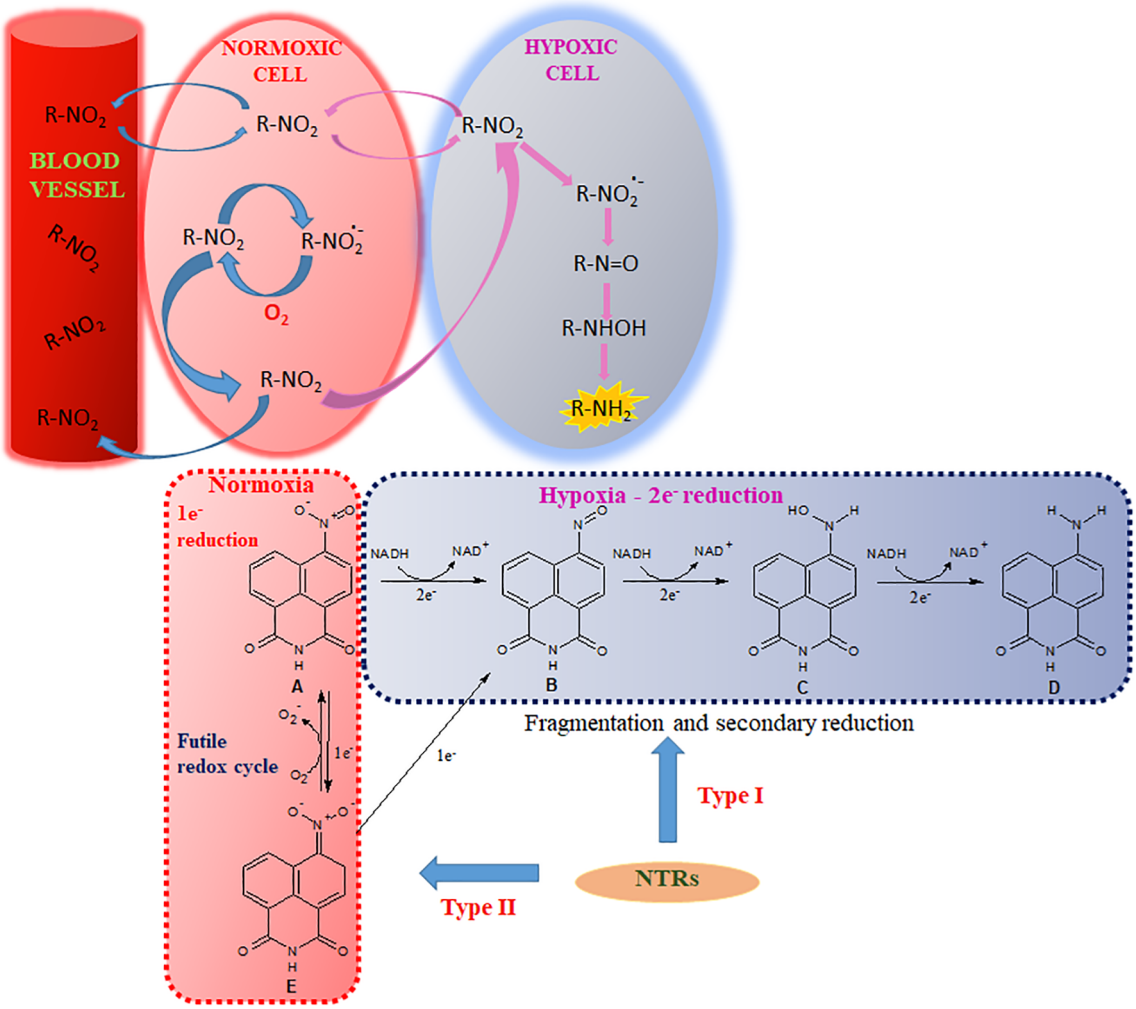


Fig. 2. The schematic representation of nitronaphthalimides in normoxic and hypoxic environments [65]. When diffused into the oxic cells, nitronaphthalimides are reduced to anionic radical species which are re-oxidised, whereas in hypoxic cells re-oxidation is prevented due to inadequate oxygen levels and the reduction process continues. The reduction mechanism of type I and type II NTRs on a nitronaphthalimide molecule [67] (A). Type I NTR transfers two electrons from NAD(P)H to generate the nitroso (B) and hydroxylamino (C) intermediates and lastly the amino group (D). Type II NTR transfers a single electron to the nitro moiety, to produce a nitro anion radical (A), which generates the superoxide anion in the presence of oxygen in a futile redox cycle, to regenerate the nitro entity.

due to either alteration in oxygen levels or interaction with target hypoxia markers in the less-oxygenated areas. These changes in fluorescence wavelengths or intensities can be monitored for hypoxia detection. Consequently oxygen-sensitive and bioreductive fluorescent probes have been constructed for both *in vitro* and *in vivo* imaging of cancer hypoxia. The oxygen-sensitive probe relies on the ability of oxygen to quench phosphorescence [42], while inadequate oxygen levels in hypoxic regions of tumour can considerably intensify the brightness of these probes, constructing them as good candidates for imaging applications [14,43,44]. Meanwhile, the bioreductive probes function based on the reduction of small molecules that contain nitro or azo groups in the hypoxic environment within tumours. These can also bring about alterations in the fluorescence emission in terms of intensity or emission wavelength [45–47]. The main focus of this review will be on the fluorescent probes that detect hypoxia through bioreduction mechanism.

The microenvironment in hypoxic cancers retains increased levels of redox, ROS (Reactive oxygen species), and acidosis (pH: 6.5–6.9) [48–50]. In achieving satisfactory fluorescence optical imaging of hypoxic regions, the intrinsic redox state and oxygen concentration play a crucial role [51]. The overexpression of intracellular reductases such as NTR (Nitroreductase), AzoR (Azoreductase) and DTD (DT-diaphorase) are observed [52–54] under hypoxic conditions. Generally the extent of hypoxia is related very closely to the native concentrations of these reductases [55–57] which can carry out one electron reduction. Various hypoxia sensitive bioreductive optical probes have been constructed relying on the principles of FRET (Fluorescence resonance energy transfer). The FRET donors and acceptors are connected through the hypoxia-sensitive nitro or azo- moieties and are cleaved through hypoxia-triggered reduction. The degree of hypoxia can be monitored by FRET on and off states correspondingly between hypoxic and normoxic situations.

3. Nitroreductases and their mechanism of action in hypoxic TME

As mentioned earlier, there are several endogenous and exogenous markers for monitoring the extent of hypoxia in the cancer microenvironment [15]. The catalysis of one electron reduction by intracellular reductases involving selective activation of specific functional compounds is a well-recognized pathophysiological feature in tumours exhibiting hypoxia [58]. One such electron reduction, mediated by NTR is a well-recognized and useful reaction for designing hypoxia targeted imaging probes. NTRs are a class of FMN (Flavin-mononucleotide)- or FAD (Flavin adenine dinucleotide)- reliant enzymes which are capable of metabolizing or catalysing the reduction of nitro-aromatic compounds to their respective hydroxylamines or amines, which in turn are often mutagenic or carcinogenic. This reduction is mediated by either FAD or FMN as prosthetic groups in the presence of reduced form of NAD (Nicotinamide adenine dinucleotide) or NADP (Nicotinamide adenine dinucleotide phosphate) as a reducing equivalent source [59,60]. NTR plays a central role in human health in various ways such as (i) mediating the toxic profiles of nitro-substituted compounds, (ii) bioremediation or bio-catalysis in biotechnological techniques, and (iii) supporting tumour chemotherapy, ablation of specific cells and resistance to antibiotics [61]. The clinical application of NTR is captivating owing to their capability to transform safe prodrugs such as CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide) into a potent DNA-crosslinking cytotoxic agent that kills cancer tissues. Besides, these enzymes are used in GDEPT (Gene directed enzyme prodrug therapy), VDEPT (Virus directed enzyme prodrug therapy), and ADEPT (Antibody-directed enzyme prodrug therapy) for possible application in drug screening and the treatment of certain types of malignant cells [62].

As NTR expression is directly related to the different degrees of hypoxia in aggressive solid tumours, the detection of NTR activities and hypoxia levels which can aid in tumour diagnosis and drug development is vitally important. Nitro-aromatics are well-recognized substrates for NTR and are readily metabolized under hypoxic environments in a stepwise reduction pathway in the presence of NADH (reduced NAD) as an electron donor [63–65]. In the nitro group, the polar bond between the nitrogen and more electronegative oxygen form partially positive and negative ends, and the positive end attracts the electrons, thereby undergoing reduction [66]. The NTR present abundantly in hypoxic tumours can catalyse the reduction of these nitro units through one- or two-electron transfers. Based on this capability, the NTRs are categorized into two groups as presented in Fig. 2; (i) Type I (oxygen-insensitive) NTRs that catalyse the stepwise transference of two electrons from NAD(P)H to the nitro group either in the presence or absence of oxygen, forming nitroso and hydroxylamine intermediates and finally primary amines [67], and (ii) Type II (oxygen-sensitive) NTRs that mediate one-electron reductions of the nitro moiety in the presence of oxygen, resulting in the formation of a nitro anion radical that further reacts with molecular oxygen, transferring one electron to oxygen producing a superoxide radical and regenerating the original nitro moiety. Thus, NTR performs the catalytic reduction of nitro-aromatics through two-electron transfers only in anaerobic conditions. Interestingly, the ubiquitous NTR catalyses the oxygen-dependent initial one-electron bio-reductive metabolism in biological systems. But in healthy tissues possessing normal oxygen levels this nitro-radical anion will be re-oxidised due to its highly reactive nature towards oxygen [68]. However under hypoxic conditions, the nitro-aromatics undergo a bio-reductive metabolism which results in subsequent sequential reduction of the one-electron reduction product to the nitroso ($2e^-$), hydroxylamine ($4e^-$) and finally amine ($6e^-$) derivatives as portrayed in Fig. 2. This chemical reactivity profile has been suitably exploited in the construction of hypoxia-triggered prodrugs. The nitro-aromatic entity masks the bioactive species, and forms an amine via reduction of the nitro group releasing the cytotoxic portion from the prodrug [69–71]. An analogous approach has been utilized for the fabrication of hypoxia-responsive fluorescent probes, for which a change in fluorescence occurs during the reduction process.

The nitro-aromatics have been utilized not only to develop bio-reductive prodrugs but also to fabricate fluorescent sensors for NTR and cancer hypoxia. Recently, advancements in nitro-aromatics-based fluorescent sensors to monitor NTR have received ample attention in the detection of hypoxia levels within a tumour using a selective 'switch' mechanism [72–77]. The transformation of the electron-withdrawing nitro unit to the electron-donating amino moiety results in a significant electronic change. This results in the breakage of the O (or N)-C bond, confirming the retrieval of the fluorescence signal that relies on an 'off-on' recognition approach. Interestingly, this reaction-based sensing strategy offers assurance of the highly selective nature of these probes towards NTR. The following section focuses on the comprehensive review of nitro derivatives of naphthalimides that have been explored for their potential use as molecular fluorescent probes for detection of solid malignancies through hypoxia imaging.

4. Naphthalimides as hypoxia sensitive biomarkers

1*H*-Benzo[*de*]isoquinoline-1,3-(2*H*)-diones or naphthalimides are one of the simplest polycyclic amides comprising of a flat, π-deficient aromatic system. Most of the molecules that incorporate a naphthalimide moiety are fluorescent and exhibit a broad range of biological properties such as antitumor [78–80], antitrypanosomal [81], antiviral [82], local anesthetic [83], analgesic [84], serotonin 5-HT3 and 5-HT4 receptor antagonist activities [85]. The DNA binding ability of naphthalimide derivatives were first reported by Braña and co-workers [86] and mitonafide and amonafide were approved for Phase II clinical trials. Moreover, naphthalimide derivatives offer non-biological applications as optical brighteners [87], chemosensors [88,89], fluorescent markers [90], liquid crystal additives [91], electro-optically sensitive functional materials for laser application [92] and lucifer dyes [93]. A

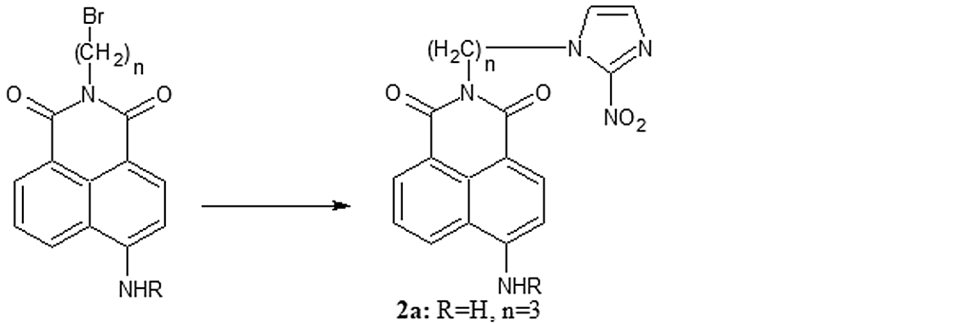
few diamino-1,8-naphthalimide derivatives have been developed as sensors for the detection of transition metal ions [94].

Naphthalimide being a perfect fluorophore owing to its good chemical stability, high fluorescence quantum yield, and multiple sites for chemical alteration has been one of the most widely used material in the fabrication of various fluorescent probes [95–98]. 1,8-Naphthalimides display excellent cell and tissue permeability and this has been extensively exploited in biological applications [99,100]. The incorporation of a nitro group in 1,8-naphthalimide unit leads to quenching or near quenching of fluorescence through energy transference and electron transference mechanism. Upon NTR-induced bio-reduction, the nitro group of nitronaphthalimide probe is converted to an amino group that stimulates a fluorescence off-on response. The formed reduction product (4-amino-1,8-naphthalimides) have extensive usage in biological sensing because of their inherent qualities such as large stokes' shifts, excellent photostability, and high quantum yields [88]. Its fluorescence mechanism functions via ICT (Intramolecular charge transfer) that involves the electron donation from the 4-amino moiety to the imide group. The replacement of the amino unit with a nitro group impedes ICT and thereby quenching the luminescence. However, fluorescence is turned on during the reduction of the nitro group to an amine [101]. Thus the pull-push transformation from electron-deficient $–NO_2$ to electron-rich $–NH_2$ would cause a spectroscopic modification within a highly conjugated system. This provides an effective and simple approach to construct an off-on fluorescent sensor.

Due to the pharmaceutically attractive structural features such as large planar aromatic chemical framework, good DNA-intercalating behaviour, and as a substrate with enormous synthetic possibilities, nitro group incorporated naphthalimides attracted the attention of various synthetic as well as medicinal chemists for its probable use in the detection of NTR/hypoxia. The hypoxia-specificity response of nitronaphthalimides stemmed from the hypoxia-dependency of the nitro moiety bio-reduction and the intensification in fluorescence from the metabolites of bio-reduction process. Several nitronaphthalimide derivatives which had remarkably low fluorescence because of the quenching influence by nitro groups were prepared. Hypoxia-induced reductive metabolism of nitro groups in these molecules occurs through the nitro radical-anion, recovering the fluorescence due to the formation of the corresponding amine metabolites. Thus their "futile metabolism" in normoxic cells can be exploited as a diagnostic tool that would evidently reflect the balance between NTR activity and oxygen status. Hence, hypoxia-sensitive nitro group bearing naphthalimide derivatives emerged as promising candidates in the construction of fluorescent sensors for imaging hypoxia. In this review, nitro-naphthalimides with varying chemical structures that have been developed as probes for imaging hypoxia in tumours with emphasis on their synthetic aspects are presented.

Hodgkiss et al. in 1991 prepared compounds **2a-f** bearing a fluorescent naphthalimide nucleus and a single side chain of 2-nitroimidazole ring system in their chemical structure which acted as hypoxic cell markers [102] (Fig. 3). The anion of 2-nitroimidazole (azomycin) was alkylated using *N*-(3-bromopropyl)- and *N*-(5-bromopentyl)-4-aminonaphthalimides (**1a** and **1c**) to give *N*-(nitroimidazolyl)-4-aminonaphthalimides **2a** and **2b** respectively. Further, the amino derivative **2a** was acetylated to achieve acetamido compound **2c** by considering the low levels of the fluorescent sensor that is expected to be available in the hypoxic tumours *in vivo*. Besides, **2a** was acylated with chloroacetyl chloride to prepare a more water soluble derivative **2d**. The successive nucleophilic displacement of chlorine atom in **2d** with *N,N*-bis(2-hydroxyethyl)-l,3-diaminopropane gave the trihydrochloride **2e,** which carried both hydroxyl and amine moieties in a side chain and retained an amide group as the 4-substituent on the naphthalimide core. Alkylation of 4-aminonaphthalimide with 1,9-dibromononane afforded **1d**, which on further reaction with azomycin generated **2f**. The fluorescence was quenched in the compounds due to the electron or energy transfer between the naphthalimide fluorophore and the electron-accepting nitroimidazole nucleus and hence they were non-fluorescent in normoxic cells. The bio-reduction of the nitro group to yield the respective fluorescent amines occurred only in the hypoxic cells. The large normoxic-hypoxic differences in metabolic drug binding in the case of all the target molecules was due to the electron-accepting nitroimidazole moiety present in them. The hypoxic-normoxic differential in fluorescence was sensitive to the fluorescent ring structure. However, the possible fluorescence quenching due to interaction between the naphthalimide core and the reduction products of the nitroimidazole unit was a prospective concern, though these reduction products were much less electron-accepting than the nitroimidazole. In addition, cellular fluorescence of bio-reductively derived metabolites was significantly influenced by the carbon chain length that connected the nitroimidazole unit to the naphthalimide. Compounds **2a** and **2b** showed a 5-fold or higher hypoxic-normoxic differential in the *in vitro* system. They were depleted easily due to high cellular uptake by diffusion through tumour tissues and hence could be used for *in vivo* application. Though the increase in the side imide chain length had little effect on the cellular uptake of compounds **2a**, **2b** and **2f**, the longer side arm on the 4-position of the naphthalimide unit of **2e** reduced the cellular uptake and interfered with its intercalation to DNA.

Later Liu et al. extended the above study by synthesizing naphthalimides bearing two 2-nitroimidazole side arms for bio-reductive binding and as fluorescent markers for hypoxic cells. They considered



1a: R=H, n=3
1b: R=Ac, n=3
1c: R=H, n=5
1d: R=H, n=9

2a: R=H, n=3
2b: R=H, n=5
2c: R=Ac, n=3
2d: R=$COCH_2Cl$, n=3
2e: R=$COCH_2NH(CH_2)_2N(CH_2CH_2OH)_2.3HCl$, n=3
2f: R=H, n=9

Fig. 3. Fluorescent markers for hypoxic cells carrying a single side chain of 2-nitroimidazole ring system that undergo bio-reductive binding.

various important aspects such as: (i) the inclusion of two nitroimidazole moieties compared to one that could increase the bio-reductive unit and strongly retain the fluorescent reduction products inside the hypoxic tissues, (ii) modulation of the linker length connecting the two nitroimidazole units with naphthalimide that could avoid any possible quenching of fluorescence by either electron or energy transfer between the naphthalimide core and the reduction states of the electron-deficient nitroimidazole unit, (iii) introduction of the two longer connecters that might reduce the DNA intercalation affinity of the markers, as only the naphthalimide framework either without or with small substituents demonstrate strong DNA intercalation tendency, and (iv) incorporation of a hydrophilic ether moiety into the side chains that could enhance its water solubility. The naphthalimide derivatives **5a-d** and **6a-d** were prepared from 4-bromonaphthalic anhydride **3** and 1,4,5,8-naphthalic anhydride **4** respectively as depicted in Fig. 4. The hydrophilic ether moiety present in the side chains of **5c**, **5d**, **6c** and **6d** improved the *in vivo* transport of fluorescent hypoxic probes to the tumour cells. The introduction of 3-nitro-l,2,4-triazole in place of 2-nitroimidazole as in **5a**, **5c**, **6a** and **6c**, displayed very weak fluorescence intensities when incubated with hypoxic cells. Derivative **5d** displayed the maximum hypoxic–normoxic fluorescence differential (20 times) *in vitro* in 95D (human lung carcinoma) and CHO (Chinese Hamster ovary) cells due to improved water solubility and weaker fluorescence quenching by IPET (Intra-molecular photo-induced electron transfer) from the reduction products of the nitro imidazole entity to the naphthalimide core. The reduction product of **5b** displayed higher IPET efficiency due to the shorter side-chain length and resulted in more fluorescence quenching, thereby exhibiting a lower hypoxic–normoxic fluorescence differential than that of **5d**. The stronger electron deficient nature of 2-nitroimidazole compared to 3-nitro-l,2,4-triazole [103] and the higher electron-donating capability of the latter for bio-reductive product than that of the former resulted in the dissimilar fluorescence behavior amongst the two types of derivatives **5b**, **5d** and **5a**, **5c**. Hence, the strong IPET capacities to slake the luminescence for the reduction products of **5a** and **5c** compared to **5b** and **5d** balanced the fluorescence enhancement during bio-reduction, that ultimately caused little hypoxic–normoxic fluorescence difference in cells for **5a** and **5c** [104]. Among the naphthalimide derivatives **6a-d** that were incubated in 95D, CHO and V79 (Hamster Lung Fibroblast) cells, only **6d** in V79 cells exhibited 2.5 times hypoxic–normoxic fluorescence differential, attributed to the weak luminescence of the reductive product of **6**.

Zhou et al. in 2016 developed a simple and feasible lysosome-specific fluorescence off-on NTR probe through a single-step synthetic protocol by integrating a typical lysosome-targeting morpholine unit **8** into a 4-nitro-1,8-naphthalic anhydride **7** fluorochrome as a specific substrate for NTR. This was an attempt to image NTR and hypoxia in live cells and to gather better understanding of the action of lysosomal NTR under a hypoxic environment [101]. The detection mechanism was centred on the NTR-catalysed bio-reduction of the non-fluorescent probe **9** to highly intense fluorescent 4-amino-1,8-naphthalimide **10** product as presented in Fig. 5. The probe showed precise ability to target lysosome with a detection limit of 2.2 ng mL^{-1}. This highly sensitive and selective probe was used to image the variation in the levels of lysosomal NTR in live hypoxic cells. According to the study the rise in NTR levels in an acidic organelle like lysosomes might be lesser than that in the cytoplasm. The sensor demonstrated its real application in imaging the variations of lysosomal NTR in live A549 (human alveolar basal epithelial adenocarcinomic) cells under hypoxic conditions.

Wei and his team in 2017 synthesized an off-on fluorescent probe consisting of a naphthalimide fluorophore as a signalling unit decorated with 4-nitrobenzyl chloroformate as an exogenous hypoxia marker to detect NTR [105]. To a mixture of 2-butyl-6-hydroxy-1*H*-benzo[*de*] isoquinoline-1,3(2*H*)-dione **14** and triethylamine, a solution of 4-nitrobenzyl chloroformate in anhydrous dichloromethane was added to afford the probe 2-butyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl(4-nitrobenzyl)carbonate **15**. The detection mechanism involved the NTR-mediated reduction of 4-nitrobenzyl chloroformate moiety with NADH as electron source in the biological system releasing cyclohexa-2,5-diene-1,4-diimine and 2-butyl-6-hydroxy-1*H*-phenalene-1,3(2*H*)-dione **16**. A stronger electron donor that remained at the 6-position of naphthalimide during the cleavage of the diester bond triggered an intra-molecular charge transfer process leading to

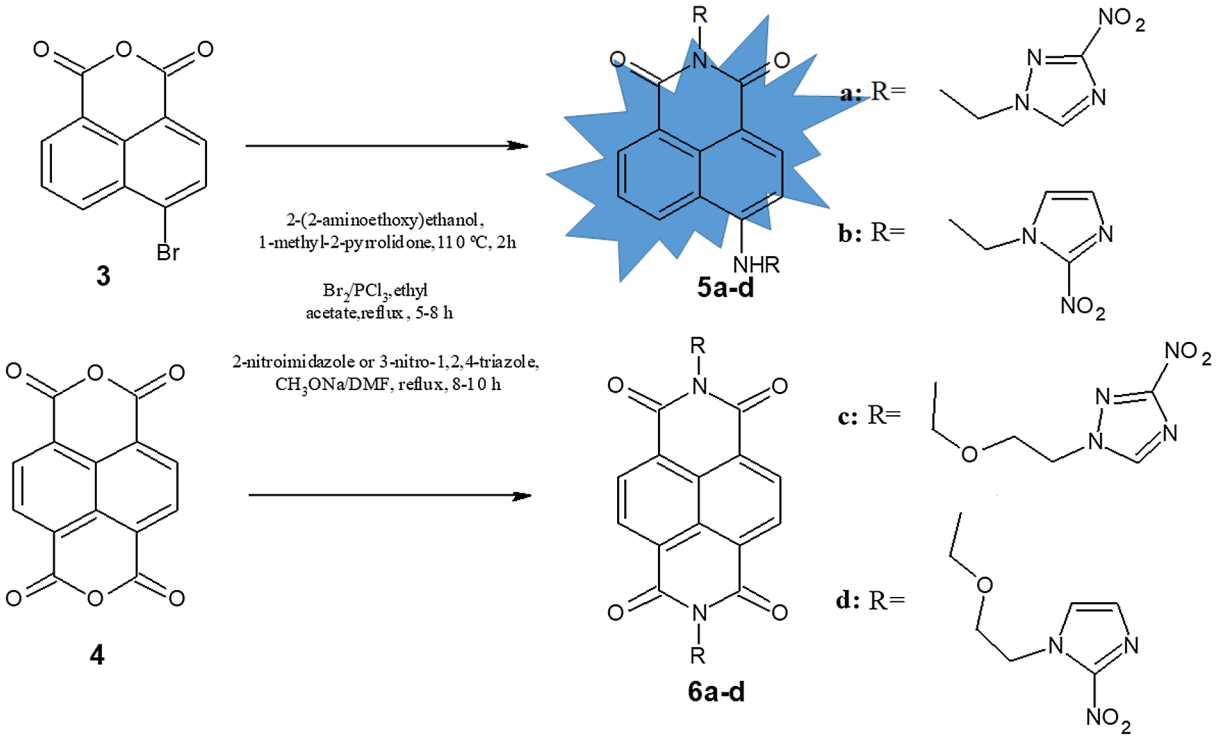


Fig. 4. Fluorescent markers for hypoxia carrying two 2-nitroimidazole side arms for bio-reductive binding.

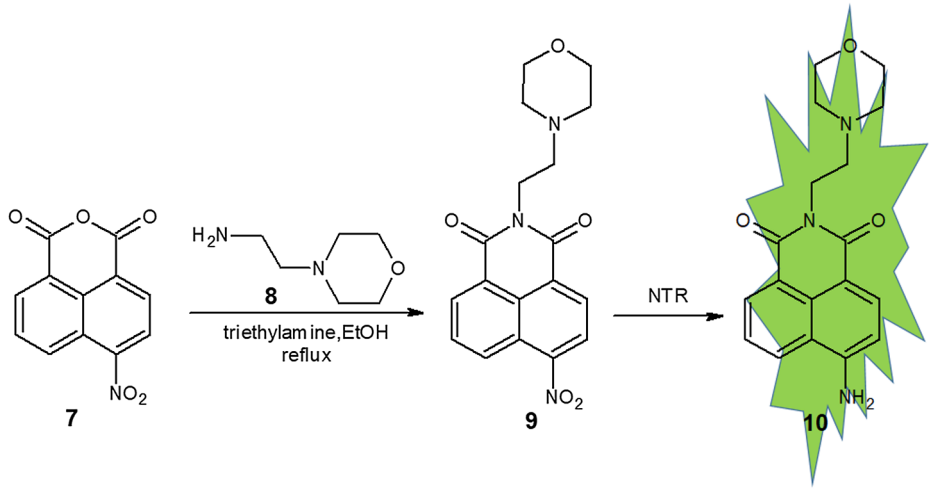


Fig. 5. Synthesis and bio-reductive mechanism of a lysosome-targeting off-on fluorescent sensor for NTR and hypoxia imaging in live cells.

enhanced fluorescence as depicted in Fig. 6. The fluorescence intensity of highly specific and biocompatible NTR-probe **15** showed a good linear increase within the NTR concentration range of 0.1–0.3 μg/mL. The sensor could efficiently distinguish normoxic (non-fluorescent) from hypoxic (strong green fluorescence) U87 (glioblastoma) cells. The probe could detect endogenous NTR and hence monitor the extent of cellular hypoxia in tumour cells.

Fang et al. in 2018 developed a fluorescence probe comprising of a 1,8-naphthalimide/rhodamine hybrid molecular structure [106]. The design of the sensor molecule was based on various aspects such as (i) choice of fluorochromic naphthalimide and rhodamine with excellent spectroscopic features and non-overlapping fluorescence emissions [107–110] (ii) diethylenetriamine and nitro groups as two different recognition units specifically for ATP (Adenosine triphosphate) and NTR respectively [101,110] (iii) easy synthesis of the probe with high purity and good yield. Diethylenetriamine on reaction with rhodamine B **19** in methanol gave **20**, which on subsequent treatment with nitro naphthalic anhydride **18**, obtained by the oxidation of nitro acenaphthene **17** afforded the naphthalimide derivative **21** as presented in Fig. 7. The probe **21** exhibited different fluorescence responses with ATP, NTR, and ATP/NTR. The cellular imaging using probe **21** revealed ATP to be a hypoxia-sensitive entity for the first time. Moreover, intracellular ATP and NTR demonstrated a contrasting changing trend in behaviour under hypoxia environment, *i.e.,* as ATP levels decreased, the NTR levels showed an exponential increase. This behaviour was effectively utilized to indicate the hypoxia status in living cells. The fluorescence quenching behavior of the nitro moiety of 1,8-naphthalimide and the generation of the spirocyclic rhodamine species rendered the sensor **21** with an inherent weak fluorescence. Conversely, the reduced product **24** obtained by addition of NTR to the sensor solution significantly recovered the fluorescence of 1,8-naphthalimide. This improved luminescence is attributed to the pull–push transformation from the electron-deficient nitro to the electron-rich amino group. The addition of ATP yielded the highly fluorescent species **22** due to the ATP-

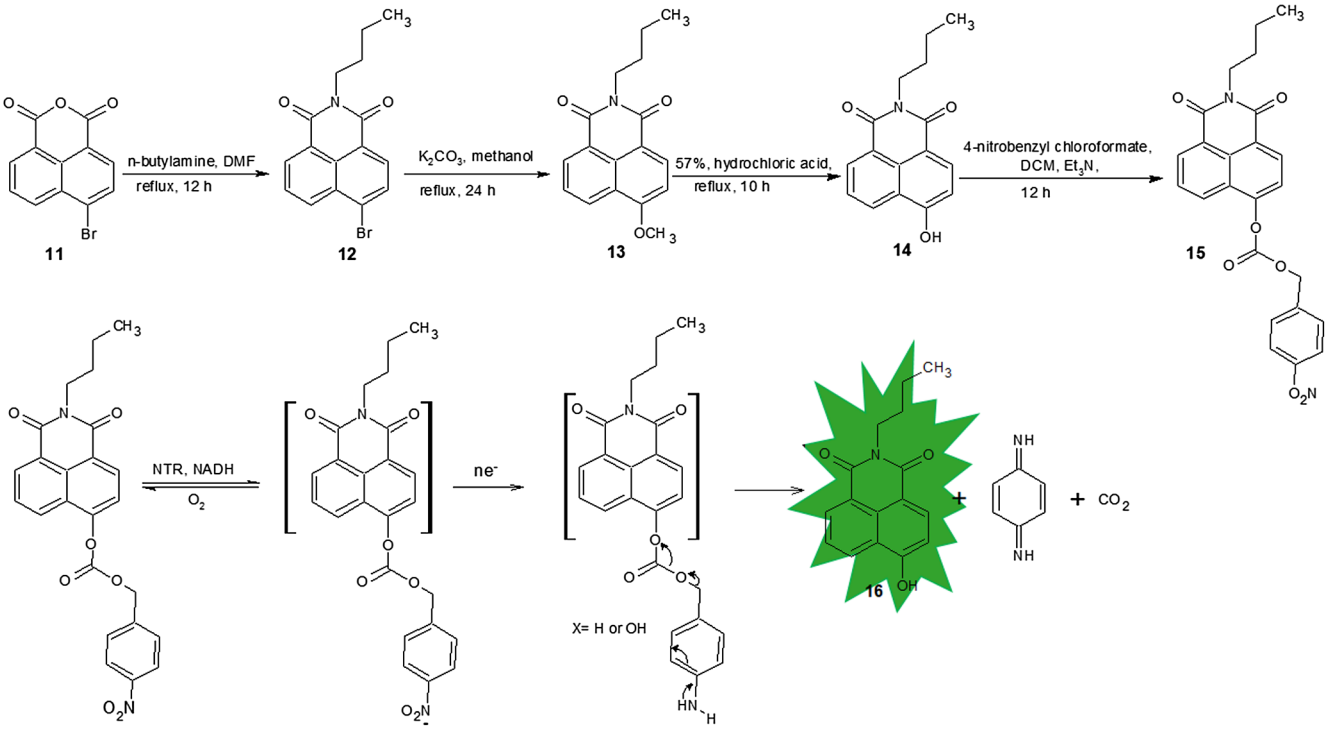


Fig. 6. Synthesis and plausible fluorescence off-on mechanism of the NTR Probe for hypoxia imaging in tumour cell.

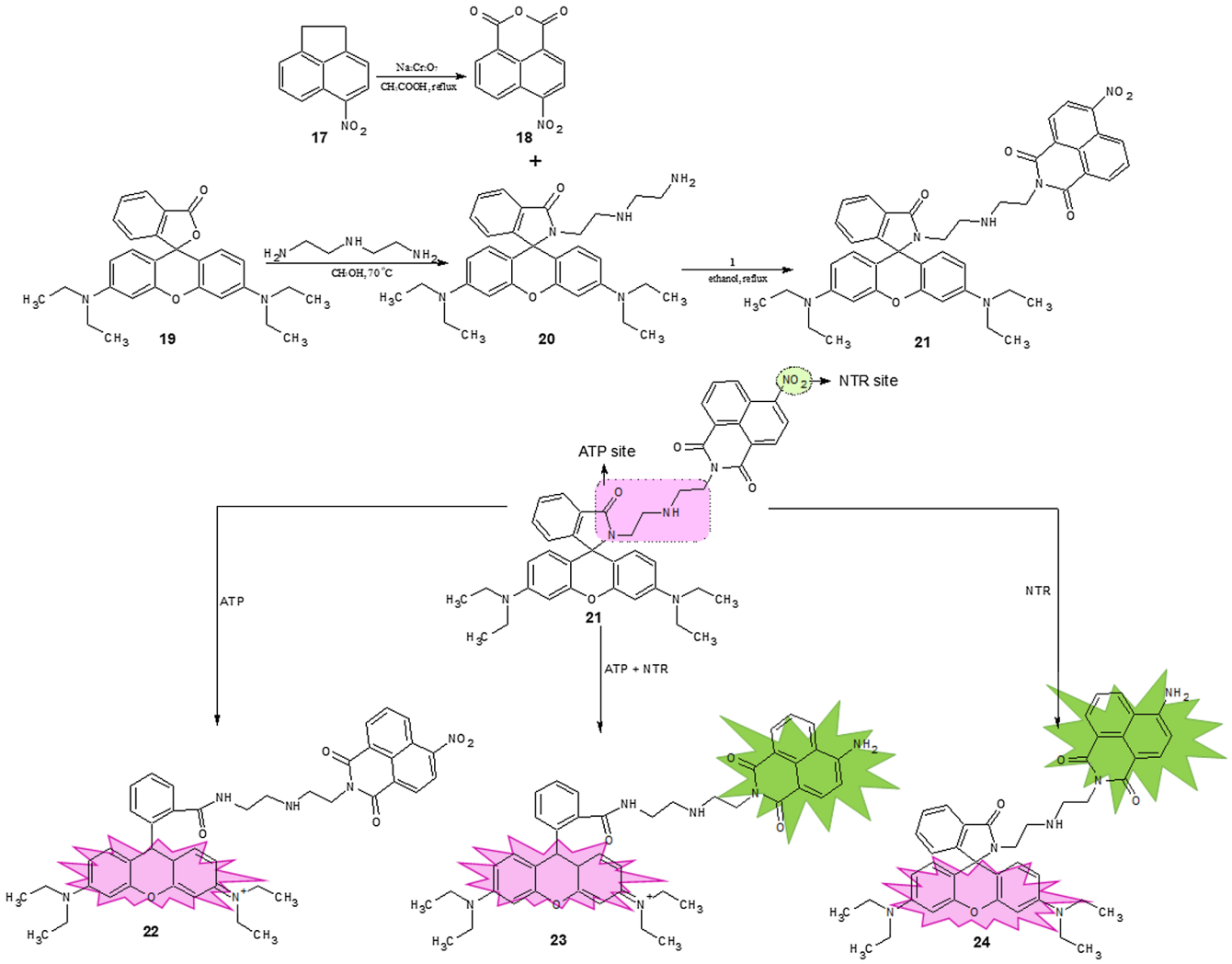


Fig. 7. Synthetic procedure of a dual-function fluorescent probe for monitoring the hypoxia levels in living cells via imaging of hypoxia sensitive NTR and ATP species.

induced spirolactam ring opening reaction of rhodamine, caused by the specific $\pi - \pi$ stacking interaction between the adenine unit of ATP and rhodamine. The pH changes (within 5–10) had negligible effect on the fluorescence of probe **21**, but its solution with ATP exhibited a maximum fluorescence intensity in the pH range of 7.0–7.4. Hence probe **21** could be used to detect ATP levels in hypoxic cancers that typically holds an intracellular pH values of 7.0–7.2. Thus the probe was able to sense the two hypoxia-sensitive ATP and NTR species within live HeLa (cervical cancer) cells.

Yang et al. in 2018 prepared nitronaphthalimide based three hypoxia sensing probes **25**, **28** and **32** [111] as shown in Fig. 8. The reaction between nitronaphthalic anhydride **18** and butylamine afforded *N*-butyl-4-nitro-1,8-naphthalimide **25**, which on further nitration gave *N*-butyl-4,5-dinitro-1,8-naphthalimide **26**. This nitro derivative was further treated with butylamine to give *N*-butyl-4-butylamino-5-nitro-1,8-naphthalimide **27**. The naphthalimide probe **28** was prepared by the reaction between **18** and aspartic acid in DMF, in presence of DIPEA (*N*,*N*-diisopropylethylamine). The reaction between 4-sulfo-1,8-naphthalimide potassium salt **29** and butylamine afforded *N*-butyl-4-sulfo-1,8-naphthalimide potassium salt **30**, which on further nitration and subsequent reaction with butylamine yielded *N*-butyl-4-butylamino-6-nitro-1,8-naphthalimide **32**. Although **25** was readily reduced within the cells, the much more oxidising situation outside the cells made it harder to achieve the reduction of probe, and hence had an appropriately high reduction potential that matched well with the extracellular redox milieu. Though the modification of **25** with a negatively-charged aspartic acid unit gave the analogue **28** having a comparable reduction potential, the cellular uptake was poor in DLD-1 (colorectal adenocarcinoma) cells. In spite of the structural similarity with cytotoxic **25**, derivative **28** was non-toxic to the cells even after prolonged incubation, consistent with its reduced cellular uptake. But the extracellular probe **28** selectively stained the hypoxic areas in 3D tumour spheroids. However, the less readily-reduced **32** was highly selective to hypoxic cells over normoxic ones and distinguished differential hypoxia between the two stem cell types.

A selective ratiometric fluorescent sensor **36** based on an ICT mechanism was designed by Cui et al. in 2011 for the detection of hypoxic microenvironment by linking pro-drug *p*-nitrobenzyl moiety and a signalling unit **39** via a carbamate group as presented in Fig. 9. The reaction between 4-amino-1,8-naphthalic anhydride **33** and 2-(2-aminoethoxy)ethanol formed 4-amino-*N*-(2-(2-hydroxyethoxy)ethyl)naphthalimide **34**, which on reaction with acetic anhydride in anhydrous pyridine yielded 4-amino-*N*-(2-(2-acetoxyethoxy)ethyl)naphthalimide **35**. The chemical reaction of **35** with DIPEA and triphosgene followed by addition of 4-nitrobenzylacohol in toluene afforded probe **36**. The presence of electron-withdrawing carbamate group in **36** weakened the ICT effect and thereby resulted in a blue shift in the fluorescent emission wavelength. The selective reduction of probe **36** by NTR released the amino derivative **39** and restored the fluorescence emission allowing it to be applied in *in vitro* hypoxia imaging in A549 cell line. The

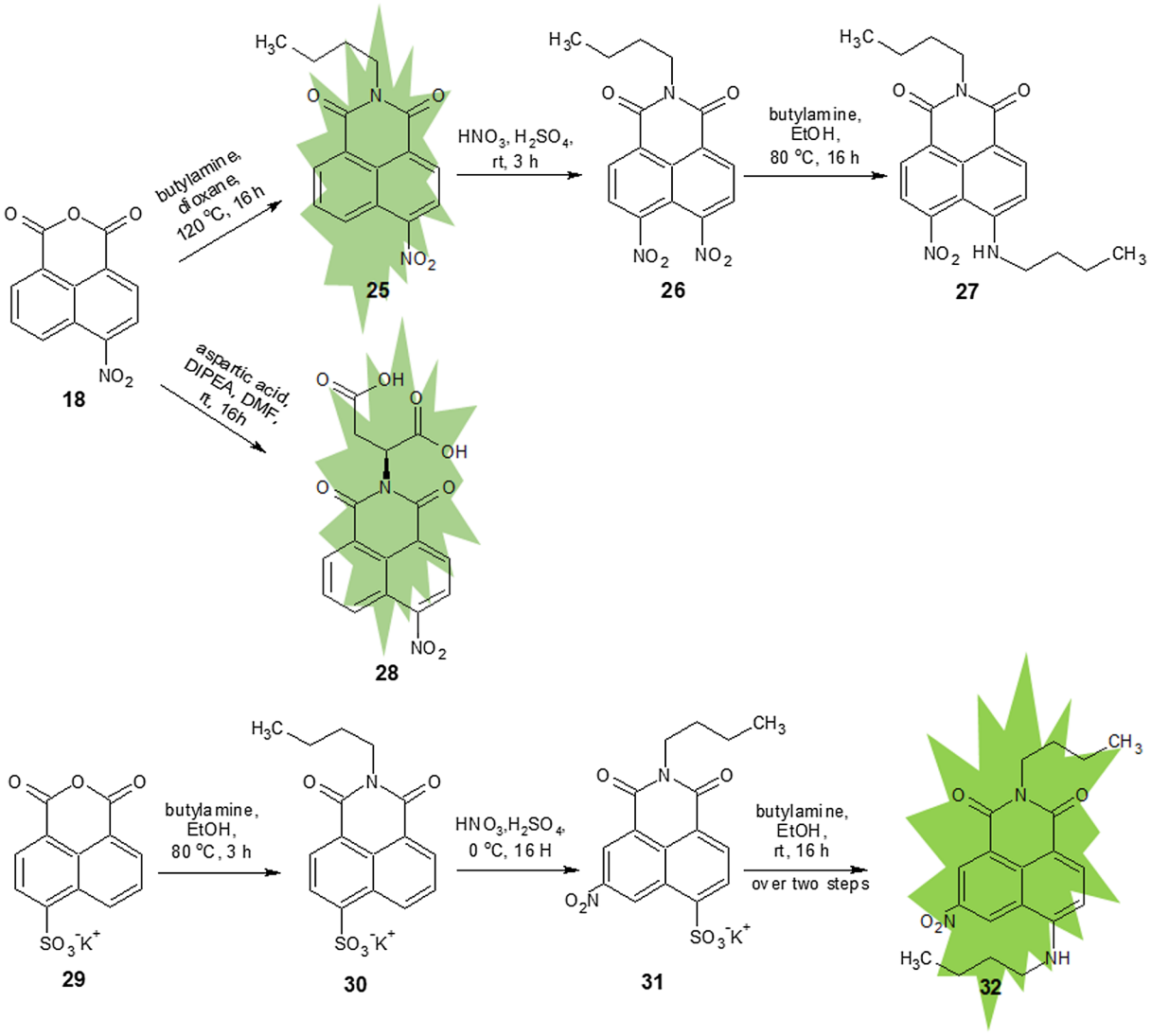


Fig. 8. Synthetic scheme for tailor-made 1,8-naphthalimide derivatives for hypoxia imaging.

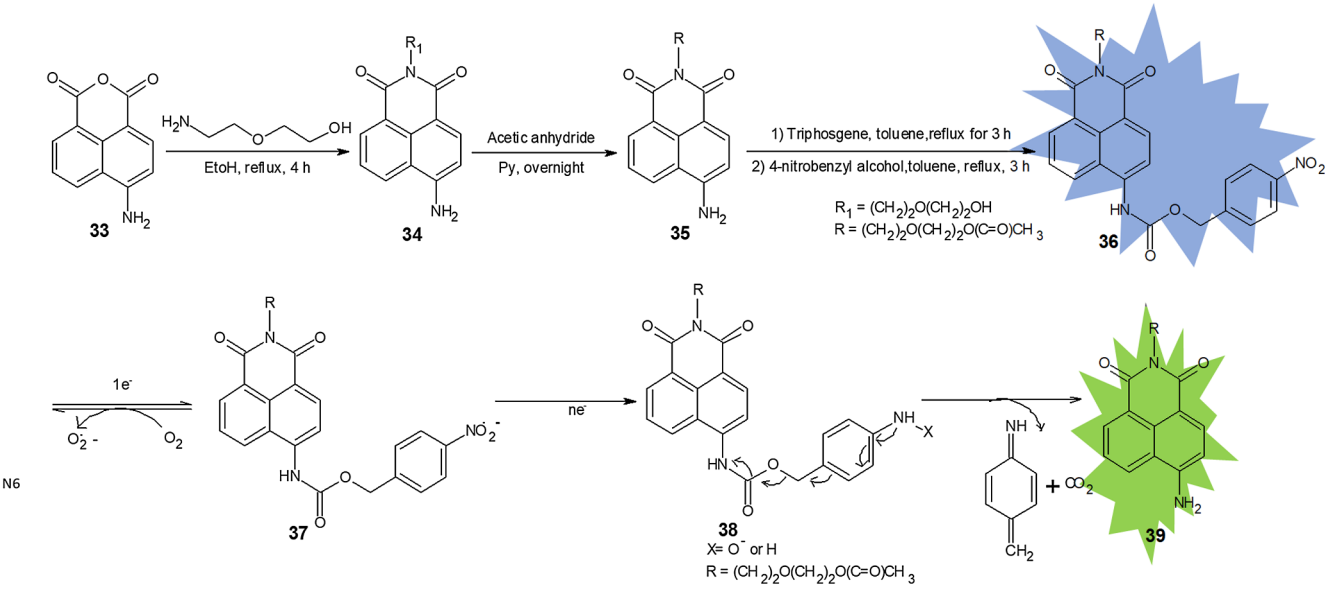


Fig. 9. Synthetic route of a pro-drug-derived ratiometric fluorescent probe for imaging hypoxia.

hypoxia-responsive probe **36** displayed no cytotoxicity, and could be used in tumour diagnosis via hypoxia imaging [112].

Following the failure of Mitonafide **40** and Amonafide **41** in phase II clinical trials, Yin et al. prepared tertiary amine *N*-oxides of naphthalimides as prospective anticancer agents against hypoxic solid tumours [113]. The reaction of 4-bromo-1,8-naphthalic anhydride **48** and *o*-nitrophenol with catalytic amount of sodium hydroxide and copper gave **49**, which on further treatment with iron powder in glacial acetic acid afforded **50** (Fig. 10). The reaction of sodium nitrite and hydrochloric acid with **50** at 0–5 °C, and subsequent treatment with copper sulphate

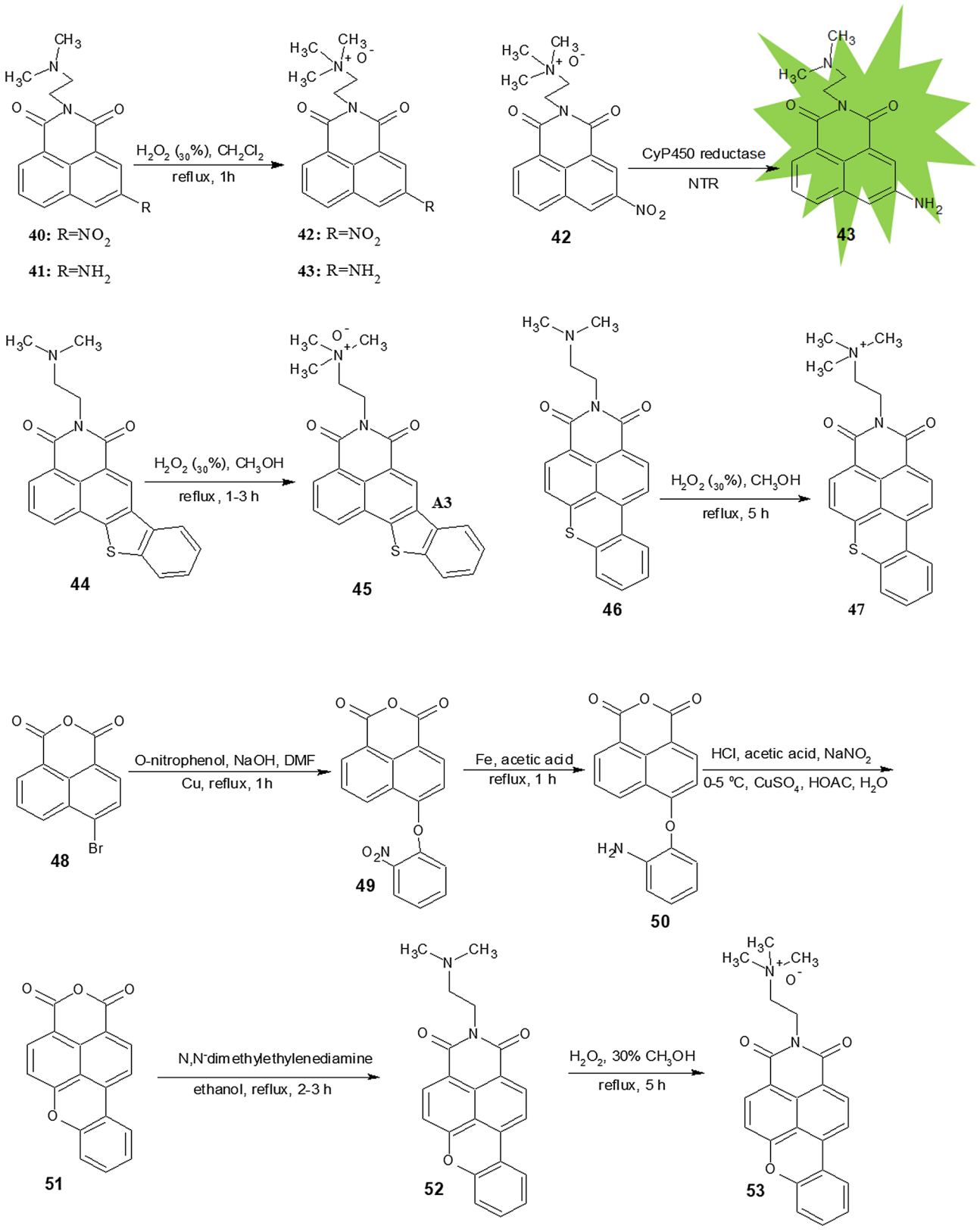


Fig. 10. Synthetic procedure of aliphatic *N*-oxide of naphthalimides as fluorescent markers for hypoxic solid tumours.

solution gave benzo(*k*,*l*)xanthene-3,4-dicarboxylic anhydride **51**, which was further mixed with *N*,*N*-dimethylethylenediamine to yield the intermediate **52**. Finally, the introduction of the NO group by oxidation with 30% hydrogen peroxide into the amine side arm linked to the flat naphthalimide chromophore as a bio-reductive hypoxic marker generated **53**. Although the cytotoxic profiles and ctDNA-binding affinities of **42**, **43**, **45**, **47** and **53** against tumour cell lines were lesser than the respective amines, the *N*-oxides **42** and **47** displayed hypoxia-responsive behaviour against A375 (human malignant melanoma) cells *in vitro* thereby acting as prodrug leads in hypoxic tumour cells. Later Yin and his team in 2011 explored the hypoxia-sensitive fluorescence properties of these naphthalimide derivatives **42**, **43**, **45**, **47** and **53** [114]. The incorporation of the weak electron withdrawing NO group into the side chains resulted in a greater electronic push-pull effect and caused about 4–5 nm red-shift than the respective naphthalimides. A bis-bioreduction mechanism was involved due to the presence of two different independent oxygen-sensitive redox centres such as the nitro and the NO groups. This entrusted derivative **42** with 17 times hypoxic-normoxic luminescence difference in V79 cells *in vitro*. The NO group was reduced by CYP3A (Cytochrome P450, family 3, subfamily A) isozyme of NADPH (reduced form of NADP): cytochrome C(P-450) reductase in the normoxic environment. Whereas in hypoxic cells, the NO_2 group was reduced to NH_2 by NTR in 2-electron steps, and the chromophore transformed from pull-pull arrangement to pull–push system, which led to distinct enhancement in fluorescence. The ability of these *N*-oxides to act as fluorescent markers is due to the PET (Photoinduced electron transfer) between the planar aromatic ring and the cationic tertiary amine side chain that ensured good cellular uptake, tight electrostatic DNA binding affinity and interfered with topoisomerase function. The bio-reduction of the NO unit resulted in less hypoxic-normoxic fluorescence differential for the remaining *N*-oxides **43**, **45**, **47** and **53**.

5. Conclusions

Though different detection methods for imaging hypoxia exist, their practical utilities are limited owing to their invasive nature. There are evident advantages while using fluorescence based techniques as they are non-invasive and exhibit high sensitivity and spatiotemporal resolution. The detection of fluorescent metabolites produced through characteristic reduction of nitro-aryl compounds in hypoxic cells forms the basis of the non-invasive diagnostic test for hypoxia in solid tumours. The criteria for such suitable nitro-compounds include highly sensitive and selective fluorescent detection response between hypoxic and normoxic cells, which are weakly fluorescent until reduced. Small structural modifications in the nitro-derivatives of fluorescent naphthalimide skeleton can provide molecular entities that display diverse chemical as well as biological properties that can offer the design of simple molecular probes tailored for specific imaging applications.

6. Critical analysis and limitations of naphthalimide probes in tumour hypoxia imaging

The structural design of a hypoxia detection probe based on off-on fluorescence mechanism of nitro-naphthalimides is very crucial. The extent of intra-molecular photo-induced electron transfer from the amino metabolites formed by the reduction of nitro entities to the naphthalimide core is an essential factor in deciding the fluorescence intensities of the hypoxia probes in normoxic and hypoxic environments. The nature of the substituents and the length of the alkyl spacers linking the substituents also play a key role in the hypoxia sensitivity, NTR detection and cellular uptake of the napthalimide derivatives.

The fluorescence probe approach of imaging hypoxic regions is still evolving and the present testing level of these probes is yet to be advanced from the *in vitro* and *in vivo* laboratory scenario to clinical settings. Although the optical imaging modality is advantageous in terms of single-cell sensitivity, its spatial resolution declines rapidly with rise in imaging depth. Hence it is advisable to shift the development of these fluorescent probes to NIR as it offers the feasibility to achieve deeper tumour penetration, minimal photobleaching of the biological samples and lesser background signals due to auto-fluorescence from live cells. Detection of hypoxia through NIR fluorescence imaging probes have shown successful results and can be translated from laboratory testing level to clinical settings.

In the present review, the developments in the design and construction of naphthalimide-based functional molecules for the sensitive detection of tumour hypoxia have been discussed in detail. Despite many research investigations that endorse the applicability of nitro naphthalimides as potential molecular probes for fluorescence imaging of hypoxia, various associated factors such as photo-stability, biocompatibility, biodegradability, pH responses, pharmacokinetic features, and toxicity screening need to be essentially and carefully monitored for fabricating *in vivo* bioimaging probes that are applicable in clinical situations. In addition, certain other critical aspects such as reproducibility, repeatability, and variability in measuring the hypoxia status using nitro naphthalimide sensors within tumours need to be explored.

7. Future prospects

Although tumour hypoxia offers great challenges in cancer treatment, it also provides an ideal target with great opportunities that could be utilized for hypoxia-responsive interventions for anticancer therapy. The present review encompasses the recent advancements in the design and construction of naphthalimide-based functional sensors for the detection of tumour hypoxia. The nitro-naphthalimides demonstrate highly sensitive detection of their reduced fluorescent metabolites. However, poor bioavailability is an undesired consequence, and hence further research efforts can be directed to modify these systems for better bioavailability. Hypoxic imaging probes greatly support our better understanding of tumour diagnosis & subsequent treatment. Yet, the severe micro-pharmaco-kinetic requirements for their effective targeting to hypoxic areas of malignant cells away from tumour vasculature to ensure that they achieve their full potential is a major challenge that needs to be addressed. Most of the hypoxia imaging fluorophores have their absorption and excitation wavelengths within the visible or ultraviolet region, and are not favorable for their *in vivo* performance as they cause tissue damage easily and rapid diminution of excitation wavelengths. Hence, developing hypoxia sensors with absorption and excitation wavelengths within the NIR (near-infrared) region of around 650–1100 nm (biological window) where penetration depth of light in tissues is high or interference of –OH or –CH or –NH group absorption is minimum is highly attractive. Phosphorescence lifetime imaging and ratiometric fluorescence imaging could also be explored along with the conventional luminescent imaging for enhanced accuracy of the output signals. Thus, to accomplish the optimal success in the design and real-world utility of sophisticated probes for hypoxia detection and cancer diagnosis, it is highly desirable to pursue continuous interdisciplinary research efforts amongst various areas such as materials science, chemistry, nanomedicine, and bioimaging.

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