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Yellowish and blue luminescent graphene oxide quantum dots prepared via a microwave-assisted hydrothermal route using H$_2$O$_2$ and KMnO$_4$ as oxidizing agents

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Development of “Dual-key-and-lock” Responsive Probes for Biosensing and Imaging

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The past few decades have witnessed the rapid development of responsive probes as the tools for sensing and imaging of biomarkers in situ in real-time. Among various responsive probes, “dual-key-and-lock” probes have been recently developed as an emerging approach for the detection of biomolecules in specific cell organelles and/or diseased tissues, such as tumours. In comparison with most commonly used “single-key-and-lock” probes, the new dual-key-and-lock” probes offer substantial advantages in biosensing and imaging, such as high accuracy and precision, minimal “false positive/negative” result, and high selectivity for biomolecules detection at targeted sites. This focus article presents recent advances in the development of “dual-key-and-lock” responsive probe for biosensing and imaging. Two approaches for the design of these probes, including “dual-key-and-one-lock” and “dual-key-and-two-lock” are highlighted, along with the discussions of the challenges and future research directions in the end.

These signal changes include “OFF-ON”, “ON-OFF”, colorimetric and ratiometric responses that are generated after the specific interaction between analyte and probes (“single lock-and-key” probes). Responsive probes provide higher selectivity and specificity for the detection of analyte because the interference from background signals could be minimized in most cases. Nevertheless, it remains challenging to use “single lock-and-key” probes to determine the levels of analyte in a specific diseased tissue and/or cellular organelle. This is mainly because most of analyte (single “key”) are not uniquely present in these target sites. Taken example of an analyte detection in cellular lysosomes (50-500 nm in diameter), most of molecular and nanomaterials-based responsive probes are able to be internalised and distributed in entire cells, which makes the lysosomal analyte detection impossible. Recent studies have revealed that the coupling of some functional groups (e.g., morpholine moiety) could guide the original probe and also the products of original probe reacted with analyte to be accumulated in lysosomes, thus providing a solution for the development of responsive probes for lysosomal analyte detection. This approach of lysosome targeting can be realised by three different mechanisms: i) the probe initially reacts with analyte in cytoplasm and other organelles and then accumulate in lysosomes; ii) the probe is directly internalised into lysosomes for lysosomal analyte detection; iii) mixed (i) and (ii) mechanisms. The issue of three different mechanisms is also true for the development of probes for analyte detection in a specific diseased tissue, such as cancer.

To improve the selectivity of the probes, another synergistic effect approach has recently been presented for an analyte detection in a specific sample with minimum false-positive/negative result. In this approach, the probe’s signal can be changed solely by dual and/or multiple factors stimulation, i.e., the detection of the target analyte can be done by the
assistance of another one or more exo-/endo-genus
stimulation. The one that is designed based on synergistic dual-
factor stimulations could be termed as “dual-key-and-lock”
probe, including “dual-key-and-one-lock” and “dual-key-and-
two-lock” probes(Scheme 1). For the “dual-key-and-one-lock”
probe (Scheme 1A), the two “keys” (dual-factor stimulation) are
required to open one “lock” (responsible unit) for the detection
of analyte. For “dual-key-and-two-lock” probes (Scheme 1B),
the signal of probes are suppressed by two “lock”, and the
detection of analyte can be only achieved after opening two
“locks”. The “dual-key-and-lock” probe, therefore, is capable of
analyte detecting accurately and precisely.

![Scheme 1. Schematic illustration of the mechanism for “dual-key-and-
one-lock” (A) and “dual-key-and-two-lock” probes.](image)

In this focus article, we highlight the recent advances in the
development of “dual-key-and-lock” probe. “Dual-key-and-one-
lock” probe for the detection of various analytes in a specific
acidic microenvironment is introduced, and then the “dual-key-
and-two-lock” probes that are activated by first “key” (acidity,
enzyme, light, etc.) and second “key” (analyte) are summarized.
The challenges and future research directions for the development
of “dual-key-and-lock” probe are discussed in the end.

“Dual-key-and-one-lock” probe

In contrast to the neutral pH of biological fluid and most of
tissues, cellular lysosomes (pH 4.5-6.0) and some diseased
tissues, such as tumour (pH 6.7-7.1) are typical acidic condition
in live organisms. The intrinsically low pH of these compartments
facilitated the development of “dual-key-and-lock” probes for various analyte detection, where the acidity was served as one “key” for probe’s signal activation. For example, Xie et al. reported a probe, Lyso-TPFP, for the
detection and two-photon imaging of lysosomal formaldehyde
(Fig. 1A). Lyso-TPFP is able to react with formaldehyde in
acidic buffer (pH 4.0-6.0). The liberation of coumarin moiety led
to blue shift of emission wavelength and enhancement of
coumarin’s fluorescence intensity. Lyso-TPFP showed high
selectivity and sensitivity to formaldehyde (detection limit-Dol: 
3 μM). The capability of Lyso-TPFP for lysosomal formaldehyde
detection was attributed to: i) the morpholine of Lyso-TPFP
guide the probe to accumulate in lysosomes; ii) exclusive
response of Lyso-TPFP to formaldehyde in acidic condition. This
specific formaldehyde (firs “key”)-triggered_2aza-Cope
rearrangement reaction in acidic microenvironment (second
“key”) inspired us to develop “dual-key-and-lock” probe, Ru-FA,
for the detection of lysosomal formaldehyde (Fig. 1B). The
reaction between formaldehyde and Ru-FA occurs at pH < 6.0,
resulting in cleavage of 2,4-dinitrobenzene (DNB) electron
acceptor to form Ru-NR and thus turn “ON” luminescence (Dol:
19.8 nM). Different with the morpholine-guided lysosome
targeting, this “dual-key-and-lock” probe precludes false
positive signals derived from the probe being triggered “ON” in
other organelles and then accumulated in lysosome. In
Considering the acidic microenvironment of tumour, tumour-
derived endogenous formaldehyde and its scavenging by
exogenous drug were then monitored by luminescence imaging
using Ru-FA as the probe. Molecular probe for ratiometric
fluorescence detection and two-photon imaging of lysosomal
ATP, Lyso-ATP, was also developed by Jun et al. Similarly,
Lyso-ATP responds ATP only in the acidic pH range of lysosomes
over other organelles.

![Fig. 1. Response mechanisms of “dual-key-and-lock” probes, Lyso-TPFP
(A) and Ru-FA (B) for formaldehyde detection in acidic conditions.](image)
dye, NRM, and its self-assembling nanoprobe, DATN for PA and photothermal imaging of tumour associated with inflammation (Fig. 3). Upon reaction of NRM with NO (first “key”) in acidic condition (second “key”), triazole derivative (NRM-NO) was formed. The triazole is stronger electron acceptor than that of diamine, which promotes the donor (D)-π-acceptor (A)-π-donor based intramolecular charge transfer (ICT). Therefore, the absorption at 680 nm was increased while the one at 950 nm remained, allowing for ratiometric PA response of DATN for NO in acidic buffer (DoL: 326 nM). DATN was then successfully used specific for tumour-associated inflammation PA imaging.

**“Dual-key-and-two-lock” probe**

There are two approaches for dual “keys” to open the two “locks” in the “dual-key-and-two-lock” probe system. The first approach is sequential opening the two “locks”, i.e., the second “lock” for the detection of analyte can be only activated after opening of the first “lock”. For another approach, the two “locks” are unlocked separately without any sequence, but the reporter’s signal can only be observed after both “locks” open. The sequence-dependent approach has been previously demonstrated by the NO detection and biotihols-triggered drug release in acidic microenvironment.45, 46 Li et al. reported the development of responsive nanoprobe, Nanolab, for the NO detection in acidic condition (Fig. 4).45 The NO responsive rhodamine dye, Rhod-H-NO, was loaded into mesoporous silica nanoparticle, following by sealing the pores with β-cyclodextrinas “gatekeeper”. Interestingly, the NO responsive unit, o-phenylenediamine, was locked by imine bond that can only be unlocked in acidic condition. After H+ activation, the Rhod-NO in Nanolab showed rapid fluorescence “OFF-ON” response to NO (DoL: 100 nM), allowing for fluorescent imaging of lysosomal NO in Hela and RAW 264.7 macrophage cells. Similar activation by C=N bond cleavage reaction was explored by Zheng et al. to develop a iridium(III) complex probe for the visualisation of tumour acidity and hypoxia.47 The luminescence of iridium complex was switched “ON” after successive activations: i) tumour acidity triggered cleavage of C=N bond and ii) amplifying emission under tumour hypoxic microenvironment. In another sequential activation example, Yan et al. described a pH and biotihols sequence-dependent response nanosystem for drug release monitoring (Fig. 5).46 At pH >pK<sub>a</sub>(6.3), the pH-responsive block polymer, modified with camptothecin (CPT)-cyanine, can form nanoscale micelle in water. The micelle dissociation could occur at pH <pK<sub>a</sub>, leaving the block polymer dispersion in acidic condition and subsequently release of CPT anticancer drug molecules after reacting with overexpressed biotihols in tumour. The fluorescence at 830 nm was quenched in micelle, and was switched “ON” after pH-mediated micelle dissociation, and then was blue-shifted to 650 nm after release of CPT. The unique fluorescence signal changes enabled sensing of CPT drug release in A549 cancer cells and tumour-bearing mice. Zhang’s group recently demonstrated two endogenous enzymes-mediated activation of “dual-key-and-two-lock” probe, NML, for accurate bioimaging and hepatopathy differentiation.48 After successive activations by leucine aminopeptidase (LAP, first “key”) and monoamine oxidase (MAO, second “key”) to form NF, the absorption was red-shifted and fluorescence at 720 nm was switched “ON”.

In addition to the endogenous acidity and enzyme activation, remote light-controlled analyte detection in live cells and animals has also been recently reported.49, 50 The most commonly light-controllable fluorescence probe is developed based on the spiropyran photochromic dye. Under alternative UV/vis light irradiation, reversible isomerization could be occurred, resulting in formation of merocyanine (MR) and spiropyran (SP) isomers.51-53 After UV-light activation, the formed MR isomer is capable of reacting with bisulphite, resulting in quenching of intramolecular charge transfer-based...
the absorption and fluorescence of MR. On the basis of this mechanism, probe, SP-Gal, have been reported by Fu et al. for light (first “key”) controlled bisulphite (second “key”) detection (Fig. 6).54 SP-Gal was designed using naphthalimide as the Förster resonance energy transfer (FRET) donor and MR isomer as the acceptor. Conjugating with a D-galactose (Gal) terminated polyethylene glycol (PEG) endows the SP-Gal water solubility and cell target ability. The probe SP-Gal showed ability to detect bisulphite only after UV-light activation. More interestingly, the amphiphilic SP-Gal can form micelles in water, which promoted the fluorescence response under UV/vis irradiation and bisulphite detection in PBS buffer and lysosomes. Through replacing Gal-PEG with morpholine derivative, Zhang et al. reported a light controlled fluorescence probe, Ly-NT-SP, for bisulphite (sulphur dioxide-SO2 in water) detection in lysosomes (Fig. 6).55 The Ly-NT-SP was then used as the probe for imaging of SO2 in lysosomes during heat shock. The same group has also reported a light-controlled single-/dual-site fluorescence probe for the discrimination and detection of H2S and SO2.56 In addition to the light-controlled imaging of SO2 in live cells, the UV-light activation of SP to MR was successfully demonstrated in a mouse model, enabling the visualization of bisulphite in mouse.

In another light-mediated sequential activation example, NIR remote light controlled “lock-unlock” nanoprobe system (DSAP-AuNS) has been developed by Cui et al. for the detection of potassium ions (K+).57 The DSAP-AuNS was developed by coupling recognition molecules, dual-stranded aptamer precursor (DSAP), on the gold-silica core-shell nanoparticles (AuNS). The NIR light-mediated photothermal effect led to the increase of local temperature, followed by the dehybridization of DSAP and K+ detection. In comparison with UV-activation of photochromic platform, NIR light activation enabled lower photo-toxicity and deeper tissue penetration. Through caging the resorufin-based fluorescence substrate with photolabile 2-nitrobenzyl group, Yang et al. recently reported a photocaged probe, FTFP, for monitoring of intracellular tyrosinase activity.58 FTFP is capable of fluorescence response to tyrosinase only after UV light-mediated activation. The probe exhibited high selectivity and sensitivity (DoL: 0.08 U/mL) for tyrosinase, allowing it used for fluorescence imaging of tyrosinase activity in B16 cells.

In another approach, the signal of probe is locked by two “locks” that are unlocked separately without any sequence. This approach provides more flexibility in designing of responsive probes than aforementioned sequential activation approach. This approach has been demonstrated by the development of responsive probes for imaging at tumour tissues. Zhao et al. reported a nanoprobe, Pep-Acy/Glu@AuNRs, for precision tumour targeting and fluorescence guided photothermal therapy (Fig. 7).59 The Pep-Acy/Glu@AuNRs was developed by attaching asymmetric cyanine to glycosyl-functionalized gold nanorods (AuNRs) through matrix metalloproteinases (MMPs)-specific peptide linker. The asymmetric cyanine is able to respond acidity through the protonation. Due to the intense absorption of AuNR, the fluorescence of protonated cyanine can only be switched “ON” after cleavage the peptide linker by MMPs. This characteristic allowed Pep-Acy/Glu@AuNRs for fluorescence response in the tumour tissues with minimal “false positive” result and good signal-to-noise ratio. In 2018, Tang et al. reported the development of “dual-key-and-two-lock”
nanoprobe, HISSNPs, for NIR II fluorescence imaging of tumours. The fluorescent NIR II dye, IR-1061, was conjugated to hyaluronic acid (HA), and this IR-1061 pendent HA forms HINPs nanoparticles through self-assembly. The HISSNPs was then formed after crosslinking the surface HA through disulphide linkers. The fluorescence of IR-1061 was quenched at the aggregation state in HISSNPs nanoparticles. Upon the biothiols (first “key”) and hyaluronidase (Hyal) (second “key”) activation, the dissociation of HISSNPs occurred, accompanied with the “OFF-ON” NIR II fluorescence response. Moreover, such bonds (two “locks”) cleavage-mediated fluorescence response is independent to the sequence of “key” (biothiols and Hyal) activation. This HISSNPs nanoprobe was then used as the NIR II fluorescence probe for specific imaging of tumours because the high expression of these two “keys” in tumour tissues.

In summary, we highlighted the recent advances in the development of “dual-key-and-lock” probes for biosensing and imaging. As we have seen in the course of this focus article, in comparison with the most commonly used “single lock-and-key” probes, “dual-key-and-lock” probes are able to provide higher accuracy and precision for the analyte detection. These probes are particular useful when the detection is needed in a specific biological samples, e.g., biomolecules detection in lysosomes and tumours of acidic condition. Among the two strategies of “dual-key-and-lock” probes, “dual-key-and-one-lock” probe offers more advantages than “dual-key-and-two-lock” probe. For “dual-key-and-two-lock” probes, particular the non-sequential activation approach, the first “lock” could be unlocked at one site and then open the second “lock” at another site, which makes the analyte detection potentially inaccurate. This issue could be addressed using remote light-controlled system because the first activation is achieved exogenously. As a result, the probes are capable of detecting the biomolecules at desired time in target sites. As the high photo-toxicity and shallow tissue penetration of UV light, recent research has developed the probes (e.g., DSAP-AuNS) that can be activated by NIR light. This NIR light activation could promote the applications of remote light-controlled “dual-key-and-lock” probes for biomolecules detection in-situ in deep tissue. Despite the fact that “dual-key-and-lock” probes offer substantial advantages in biomolecules detection, the “single-key-and-lock” probes remain the most popular tools in biological investigations. Currently available “dual-key-and-lock” probes are developed using acidity/light as one “key”. As a result, the applications of these probes are limited in acidic condition (e.g., lysosomes and tumours) and light accessible sites (< 1 cm tissue depth). Therefore, it is highly demanded to develop new “dual-key-and-lock” probes that can be activated by other factors, such as exogenous ultrasonic, and endogenous enzyme and other biomolecules, examples of the HISSNPs probe can be activated by Hyal and biothiols and probe NML can be activated by LAP and MAO enzymes. For light-controlled “dual-key-and-lock” probes, it remains challenging to control the light irradiation in situ in a very small site, such as lysosome, mitochondria, and nucleus of live cells. Moreover, despite some probes (e.g., DSAP-AuNS) have been reported as the NIR light-controlled “dual-key-and-lock” probe, NIR II light-activatable probes are more desirable due to their capability of deeper tissue penetration. In terms of the reporters’ signals to be collected for analyte detection, most of “dual-key-and-lock” probes were developed on the basis of optical signals. Although responsive MR reporter-based “dual-key-and-lock” probe (e.g., PBP NPs) has also been reported, the low resolution of MRI could be another issue for these probes. Considering the high resolution of fluorescence bioassay and imaging, the MRI-fluorescence bimodal reporter based probes could be a solution for this issue. In summary, enormous effort is required to this emerging research field in developing “dual-key-and-lock” probe with better capability in biosensing and imaging. As more research focused on the development of “dual-key-and-lock”
probes, these remaining issues are believed to be addressed in the not-too-distant future.

**Conflicts of interest**

There are no conflicts to declare.

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**References**


“Dual-key-and-lock” probe

164x105mm (300 x 300 DPI)