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Huan Feng, Qingtao Meng, Hang T. Ta and Run Zhang

The past few decades have witnessed the rapid development of responsive probes as 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” results, and high selectivity for biomolecule detection at targeted sites. This focus article presents recent advances in the development of “dual-key-and-lock” responsive probes 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 discussions of the challenges and future research directions at the end.

Introduction

Biosensing and imaging is known as a multi-dimensional technique that converges bioassay, characterisation, and visualization of key biomarkers and their biological processes in the native microenvironment at the molecular level in vitro and in vivo. As a key component of the biosensing and imaging technique, probes serve as important tools that contribute significantly to the advances in bioassay, characterization, and biomedical and (pre)clinical investigations. Generally, probes can be developed by integrating a reporter (signalling unit) with a biomarker recognition unit. The most common signalling units are electrochemical sensors, nuclear magnetic resonance contrast agents, luminophores, and photoacoustic agents. After binding and/or interaction of biomolecules with the recognition unit, the signals from the receptor could be recorded for

Qingtao Meng received his PhD degree in Fine Chemicals from Dalian University of Technology, China in 2011. He started his independent academic career in the University of Science and Technology Liaoning as an associate professor, and was promoted to a professor in 2018. His research interests focus on molecular recognition and bioimaging. He has published about 50 peer-reviewed journal papers and two licensed patents.

Run Zhang received his PhD from the Dalian University of Technology in 2012. He was a postdoctoral fellow and then a three years Macquarie University Research Fellow (MQRF) at MQ in 2012–2015. He joined the Australian Institute for Bioengineering and Nanotechnology, The University of Queensland (AIBN, UQ), in 2016. Here, he was awarded an ARC DECRA Fellowship in 2017–2019 and is now a NHMRC Emerging Leadership Fellow. He is currently a leader of the biosensing and bioimaging team, working on the development of responsive molecules/nanomaterials for early disease diagnosis and treatment.
biomolecule detection. More importantly, rapid progress of bioimaging techniques, such as microscopes and animal imaging facilities, has catalysed the development of new probes for bioassay and imaging because imaging allows direct visualization of the biological process in situ and in real-time.

In contrast to the conventional labelling approach using always “ON” probes, responsive probes collect the signal changes of the reporter for detection, measurement, and observation. These signal changes include “OFF–ON”, “ON–OFF”, colorimetric and ratiometric responses that are generated after the specific interaction between analytes and probes (“single lock-and-key” probes). Responsive probes provide higher selectivity and specificity for the detection of analytes 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 analytes (single “key”) are not uniquely presented in these target sites. Taking the example of analyte detection in cellular lysosomes (50–500 nm in diameter), most molecular and nanomaterial-based responsive probes are able to be internalised and distributed in entire cells, which makes lysosomal analyte detection impossible. Recent studies have revealed that the coupling of some functional groups (e.g., the morpholine moiety) could guide the original probe and also the products of the original probe reacted with the 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 the analyte in the cytoplasm and other organelles and then accumulates in lysosomes; (ii) the probe is directly internalised into lysosomes for lysosomal analyte detection; and (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 probes, another synergistic effect approach has recently been presented for analyte detection in a specific sample with minimum false-positive/-negative results. In this approach, the probe’s signal can be changed solely by dual and/or multiple factor stimulation, i.e., the detection of the target analyte can be done by the assistance of another one or more exo/endogenous stimulations. The ones that are designed based on synergistic dual-factor stimulations could be termed as “dual-key-and-lock” probes, 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), two “keys” (dual-factor stimulation) are required to open one “lock” (responsive unit) for the detection of the analyte. For the “dual-key-and-two-lock” probe (Scheme 1B), the signal of the probe is suppressed by two “locks”, and the detection of the analyte can be only achieved after opening two “locks”. The “dual-key-and-lock” probe, therefore, is capable of detecting analytes accurately and precisely.

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

**“Dual-key-and-one-lock” probes**

In contrast to the neutral pH of biological fluid and most tissues, cellular lysosomes (pH 4.5–6.0) and some diseased tissues, such as tumours (pH 6.7–7.1), are typically acidic 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 served as one “key” for the 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 the coumarin moiety led to a blue shift of the emission wavelength and enhancement of coumarin’s fluorescence intensity. Lyso-TPFP showed high selectivity and...
sensitivity to formaldehyde (detection limit – LoD: 3 μM). The capability of Lyso-TPFP for lysosomal formaldehyde detection was attributed to: (i) the morpholine of Lyso-TPFP guides the probe to accumulate in lysosomes; and (ii) the exclusive response of Lyso-TPFP to formaldehyde in acidic conditions. This specific formaldehyde (first “key”)-triggered 2-aza-Cope rearrangement reaction\(^\text{37}\) in an acidic microenvironment (second “key”) inspired us to develop “dual-key-and-lock" probe Ru-FA for the detection of lysosomal formaldehyde (Fig. 1B).\(^\text{38}\) The reaction between formaldehyde and Ru-FA occurs at pH < 6.0, resulting in cleavage of the 2,4-dinitrobenzene (DNB) electron acceptor to form Ru-NR and thus turn “ON” the luminescence (LoD: 19.8 nM). Different from 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 lysosomes. In considering the acidic microenvironment of tumours, tumour-derived endogenous formaldehyde and its scavenging by an exogenous drug were then monitored by luminescence imaging using Ru-FA as the probe. A molecular probe for ratiometric fluorescence detection and two-photon imaging of lysosomal ATP, Lyso-ATP, was also developed by Jun et al.\(^\text{39}\) Similarly, Lyso-ATP responds to ATP only in the acidic pH range of lysosomes over other organelles.

In light of the important roles of reactive oxygen/nitrogen species (ROS/RNS) in live organisms,\(^\text{40–42}\) “dual-key-and-one-lock" nanoprobe have also been developed recently for the detection of hydrogen dioxide (H\(_2\)O\(_2\)) and nitric oxide (NO).\(^\text{43,44}\) A polymer–MnO\(_2\) nanoparticle-based nanoprobe, PBP@MnO\(_2\) NP, was developed by Hu et al. for photoacoustic (PA) and magnetic resonance (MR) bimodal imaging of H\(_2\)O\(_2\) in living mice (Fig. 2).\(^\text{43}\) PBP@MnO\(_2\) NP was developed by wrapping positively charged MnO\(_2\) nanoparticles with negatively charged PBP polymer. The degradation of MnO\(_2\) nanoparticles by the H\(_2\)O\(_2\)–pH synergistic reaction led to the liberation of Mn\(^{2+}\) for MRI. The MnO\(_2\) nanoparticles’ absorbance at 680 nm was decreased after degradation while the absorbance at 825 nm of BODIPY was retained, which enabled ratiometric PA detection in acidic conditions (Fig. 4).\(^\text{45}\) The NO responsive unit, DATN, was described by NO detection and biothiol-triggered drug release in an acidic microenvironment.\(^\text{45,46}\) Li et al. reported the development of responsive nanoprobe Nanolab for NO detection in acidic conditions (Fig. 4).\(^\text{45}\) The NO responsive rhodamine dye Rhod-H-NO was loaded into mesoporous silica nanoparticles, followed by sealing the pores with a β-cyclodextrin “gatekeeper”. Interestingly, the NO responsive unit, o-phenylenediamine, was locked by an imine bond that can only be unlocked

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**Fig. 2** Self-assembled “dual-key-and-lock” nanoprobe PBP@MnO\(_2\) NP for H\(_2\)O\(_2\)/pH-mediated bimodal PAI and MRI.

**Fig. 3** Self-assembled “dual-key-and-lock” nanoprobe DATN for NO detection in acidic conditions. (A) Chemical structures of NRM and NIM and the response reaction mechanism of NRM to NO. (B) Changes of absorption spectra for ratiometric PA detection of NO. Adapted from ref. 44 with permission from the American Chemical Society.

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**“Dual-key-and-two-lock” probes**

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 of the two “locks”, i.e., the second “lock” for the detection of the analyte can only be 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 NO detection and biotin-triggered drug release in an acidic microenvironment.\(^\text{45,46}\) Li et al. reported the development of responsive nanoprobe Nanolab for NO detection in acidic conditions (Fig. 4).\(^\text{45}\) The NO responsive rhodamine dye Rhod-H-NO was loaded into mesoporous silica nanoparticles, followed by sealing the pores with a β-cyclodextrin “gatekeeper”. Interestingly, the NO responsive unit, o-phenylenediamine, was locked by an imine bond that can only be unlocked
in acidic conditions. After H\(^+\) activation, the Rhod-NO in Nanolab showed a rapid fluorescence “OFF–ON” response to NO (LoD: 100 nM), allowing for fluorescence imaging of lysosomal NO in HeLa and RAW 264.7 macrophage cells. Similar activation by a C= N bond cleavage reaction was explored by Zheng et al. to develop an iridium(ii) complex probe for the visualisation of tumour acidity and hypoxia.\(^{47}\) The luminescence of the iridium complex was switched “ON” after successive activations: (i) tumour acidity triggered cleavage of the C=N bond and (ii) the emission was amplified under the tumour hypoxic microenvironment. In another sequential activation example, Yan et al. described a pH and biothiol sequence-dependent response nanosystem for drug release monitoring (Fig. 5).\(^{46}\) After successive activations by leucine aminopeptidase (LAP, the first “key”) and monoamine oxidase (MAO, the second “key”) to form NF, the absorption was red-shifted and the fluorescence at 720 nm was switched “ON”.

In addition to endogenous acidity and enzyme activation, remote light-controlled analyte detection in live cells and animals has also been recently reported.\(^{49,50}\) The most common light-controllable fluorescence probe is developed based on the spiropyran photochromic dye. Under alternating UV/vis light irradiation, reversible isomerization could occur, 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. The intramolecular charge transfer process in MR is disrupted, resulting in decreases of absorption and fluorescence of MR. On the basis of this mechanism, probe SP-Gal has 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 the MR isomer as the acceptor. Conjugating with D-galactose (Gal) terminated polyethylene glycol (PEG) endows SP-Gal with water solubility and cell targeting ability. Probe SP-Gal showed the 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 a morpholine derivative, Zhang et al. reported a light controlled fluorescence probe, Ly-NT-SP, for bisulphite (sulphur dioxide, SO\(_2\), in water) detection in lysosomes (Fig. 6).\(^{55}\) Ly-NT-SP was then used as a probe for imaging of SO\(_2\) 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 H\(_2\)S and SO\(_2\).\(^{56}\) In addition to the light-controlled imaging of SO\(_2\) in live cells, the UV-light activation of SP to MR was successfully demonstrated in a mouse model, enabling the visualization of bisulphite in mice.
In another light-mediated sequential activation example, a NIR remote light controlled “lock–unlock” nanoprobe system (DSAP–AuNS) has been developed by Cui et al. for the detection of potassium ions (K+). DSAP–AuNS was developed by coupling recognition molecules, a dual-stranded aptamer precursor (DSAP), on 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 a photochromic platform, NIR light activation enabled lower photo-toxicity and deeper tissue penetration. Through caging a resorufin-based fluorescence substrate with photolabile 2-nitrobenzyl groups, Yang et al. recently reported a photocaged probe, FTFP, for monitoring of intracellular tyrosinase activity. FTFP is capable of a fluorescence response to tyrosinase only after UV light-mediated activation. The probe exhibited high selectivity and sensitivity (LoD: 0.08 U mL⁻¹) for tyrosinase, allowing it to be used for fluorescence imaging of tyrosinase activity in B16 cells.

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

Conclusions and outlook

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 analyte detection. These probes are particular useful when detection is needed in specific biological samples, e.g., biomolecule detection in lysosomes and tumours under acidic conditions. Of the two “dual-key-and-lock” probe strategies, “dual-key-and-one-lock” probes offer more advantages than “dual-key-and-two-lock” probes. For “dual-key-and-two-lock” probes, in particular the non-sequential activation approach, the first “lock” could be unlocked at one site and then the second “lock” opened at another site, which makes analyte detection potentially inaccurate. This issue could be addressed using remote light-controlled systems because the first activation is achieved exogenously. As a result, the probes are capable of detecting biomolecules at the desired time in target sites. Due to the high photo-toxicity and shallow tissue penetration of UV light, recent research has developed 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 biomolecule detection in situ in deep tissue.

Despite the fact that “dual-key-and-lock” probes offer substantial advantages in biomolecule detection, “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 to acidic conditions (e.g., lysosomes and tumours) and light accessible sites (<1 cm tissue depth). Therefore, it is highly required to develop new “dual-key-and-lock” probes that can be activated by other factors, such as exogenous ultrasound, and endogenous enzymes and other biomolecules; examples are the HISSNP probe, which can be activated by Hyal and biothiols, and probe NML, which 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 the lysosome, mitochondria, and nucleus of live cells. Moreover, despite the fact that some probes (e.g., DSAP-AuNS) have been reported as NIR light-controlled “dual-key-and-lock” probes, MRI 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 “dual-key-and-lock” probes were developed on the basis of optical signals. Although responsive MR reporter-based “dual-key-and-lock” probes (e.g., PBP NPs) have also been reported, the low resolution of MRI could be another issue for these probes. Considering the high resolution of fluorescence bioassay and imaging, MRI–fluorescence bimodal reporter based probes could be a solution for this issue. In summary, enormous effort is required in this emerging research field in developing “dual-key-and-lock” probes with better capability in biosensing and imaging. As more research is focused on the development of “dual-key-and-lock” probes, it is believed that these remaining issues will be addressed in the not-too-distant future.

Conflicts of interest
There are no conflicts to declare.

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