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“Recent Applications of N-Acyl Imidazole Chemistry in Chemical Biology.”

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Abstract

N-Acyl imidazoles are unique electrophiles that exhibit moderate reactivity, relatively long-half life, and high solubility in water. Thanks to their tunable reactivity and chemical selectivity, the application of N-acyl imidazole derivatives has launched to a number of chemical biology researches, which include chemical synthesis of peptide/protein, chemical labeling of native proteins of interest (POIs), and structural analysis and functional manipulation of RNAs. Since proteins and RNAs play pivotal roles in numerous biological events in all living organisms, the methods that enable the chemical modification of endogenously existing POIs and RNAs in live cells may offer a variety of opportunities not only for fundamental scientific study but also for biotechnology and drug development. In this review, we discuss the recent progress of N-acyl imidazole chemistry that contributes to the chemical labeling and functional control of endogenous proteins and RNAs under multi-molecularly crowded biological conditions of live cells.

Keywords

N-Acyl imidazole, Chemical synthesis of peptide/protein, Chemical modification of proteins, Ligand-directed acyl imidazole chemistry, RNA structural analysis and functional control

Introduction

N-Acyl imidazoles are unique electrophiles that exhibit moderate reactivity, relatively long half-life, and high solubility in aqueous media [1]. Currently, N-acyl imidazoles are known to play crucial roles in the activation process of some proteins and enzymes [2,3]. For instance, it is proposed that the histidine at position 1106 attacks an internal thioester to form an acylimidazole intermediate for the activation of human complement component C4B [2]. The released thiol then acts as a base to catalyze the transfer of the acyl group to amino and/or hydroxyl groups of corresponding substrates for eliminating the pathogens. As another example, it is reported that an acyltransferase HlyC from a pathogenic hemolytic *E. coli* strain forms an acyl imidazole intermediate to produce a mature bacterial toxin HlyA [3]. In the field of organic chemistry, on the other hand, acyl imidazole and its derivatives have long been utilized as moderately reactive acylation reagents. N,N'-Carbonyldiimidazole (CDI), a commercially available coupling reagent, is one of the representatives that enhances the amide and ester bond formation under mild conditions [4–6]. N-Acetyl imidazole has also been used for the chemical modification of proteins and carbohydrates in test tubes [7–9].

More recently, application of N-acyl imidazole derivatives to the field of chemical biology has launched as valuable electrophiles because the reactivity of N-acyl imidazole derivatives can be tuned by introducing the substituent to its imidazole ring and/or employing a variety of carbonyl group, and enhanced by metal binding to the imidazole moiety (Figure 1). Herein, we introduce recent examples how N-acyl imidazole can be used for the chemical biology research, which includes chemical synthesis of peptide/protein, chemical labeling of native proteins of interest (POI), structural analysis and functional manipulation of RNA. Both proteins and RNAs are central biopolymers that play pivotal roles in a variety of biological phenomena in all living organisms. Accordingly, the structural and functional studies of them have been fundamental subjects in all research of life science. Chemical synthesis of protein and peptide offers a powerful tool for exploring the structure and function of POI since it enables the introduction of any chemical modifications and functional units, such as post-translational modifications (PTMs), non-standard amino acid resides, glycans, and polyethylene glycols (PEGs) in a position specific manner without limitations as to number and kind of tethered tags. Chemical modification of endogenously expressed proteins with designed synthetic molecules also provides many opportunities for protein

research and applications. Acyl imidazole chemistry has now greatly contributed to the progress in protein/peptide synthesis and modification. In contrast to the advances in the chemical modification of proteins, there have been yet a limited number of strategies for the chemical labeling of existing long RNAs; however, recent efforts relying on acyl imidazole chemistry have enabled labeling and functionalization of endogenously transcribed RNAs in live cells. In this review, we describe the recent progress of N-acyl imidazole chemistry that greatly contributes to the chemical modification of endogenous proteins and RNAs under multimolecular crowded biological conditions of live cells.

(Figure 1)

N-Acyl imidazoles in chemical synthesis of peptides and proteins

Imidazole can work as an effective additive for promoting the peptide ligation reaction, owing to its suitable nucleophilicity and good leaving group property along with high water solubility. The native chemical ligation (NCL) is one of the most important methods for the chemical synthesis of peptides and proteins [10]. In the NCL reaction, an unprotected peptide thioester intermolecularly reacts with an N-terminal Cys peptide to confer a thioester intermediate which is followed by a rapid S-to-N acyl shift, resulting in the formation of native amide bond. In the general protocol of NCL, an excess amount of 4-mercaptophenylacteic acid (MPAA) is required to facilitate the ligation reaction (Figure 2(a)) [11]. Although effective, co-elution of MPAA sometimes hampers the purification of the ligation product by RP-HPLC. In addition, contamination of MPAA disturbs subsequent desulfurization (Cys to Ala conversion at the ligation site). Yoshida and co-workers reported that imidazole can be employed as an alternative to MPAA in the NCL reaction (imidazole aided NCL, Im-NCL) (Figure 2(b)) [12]. In the proposed reaction mechanism, acyl imidazolium, generating as a result of an equilibrium with a relatively stable acyl imidazole intermediate, would function as an effective acyl donor for the reaction. Interestingly, 1-methyl imidazole cannot activate the alkylthioester possibly because the N-methyl group prevents the formation of acyl imidazole intermediate. Using Im-NCL, Yoshida and co-workers successfully synthesized adiponectin (19–107) and [Ser(PO_3H_2)65]-ubiquitin with negligible epimerization (<2%).

The requirement of a Cys residue on the N-terminus of one of the peptide fragments in NCL sometimes limits the possible retrosynthesis of target peptides/proteins because Cys residues relatively lack in naturally occurring proteins (1.8% abundance). If the non-cysteinyl residues become available, the versatility of NCL may be largely expanded. Tam and co-workers demonstrated His residue instead of Cys is able to work as a nucleophile for the fragment coupling of unprotected peptides [13]. They performed the orthogonal coupling of the acyl peptide segment containing C-terminal thiocarboxylate and N-terminal His peptide using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as an activation reagent for the thiocarboxylate. After activation of thiocarboxylic acid with DTNB, the acyl segment is captured by the N-terminal His to form an acyl imidazole intermediate, subsequently leading to N-im to N-α acyl transfer to form a peptide bond. More recently, Lin and co-workers also explored a peptide ligation method utilizing the His side chain (Figure 2(c)) [14]. They reported the coupling reaction between amino acid thioesters and His derivatives proceeded in the presence of relatively strong base, such as DBU. It was claimed that imidazolate acts as internal acyl transfer catalyst during ligation, and the transesterification with amino acid or peptide thioesters followed by N-im to N-α acyl transfer led to the ligated peptides. They synthesized a range of His-containing dipeptides in moderate and good yields and used this method to tripeptide synthesis *via* a long-range N-im to N-α acyl transfer. Although these His mediated ligation strategies have been applied to the synthesis of relatively short peptides to date, further optimization of reaction conditions and reagents may expand the utility and versatility of chemical synthesis of peptides and proteins.

(Figure 2)

N-Acyl imidazole for endogenous protein labeling in live cells: Ligand directed acyl imidazole (LDAI) chemistry

The reactivity of N-acyl imidazole derivatives can be tuned by introducing the substituent to its imidazole ring and/or employing a variety of carbonyl group (Figure 1(b)). We demonstrated that the alkyloxyacyl imidazole motif is significantly useful as a reactive group for the chemical modification of a POI under live cell conditions [15–19]. Chemical modification of proteins provides powerful tools allowing for a broad range of

biological applications, such as the development of new classes of protein-based biopharmaceuticals and functional studies of individual proteins in complex biological systems. In particular, a valuable method capable of chemically labeling endogenous proteins in live cells is indispensable. We expected that a strategy based on recognition driven labeling may allow for selective modification of endogenous target protein in live cell with minimal perturbations of cellular habitats without any genetic manipulations (Figure 3(a)). This approach relies on the specific interaction of a POI with a designer labeling-reagent via the ligand-protein recognition, which enhanced the reactivity due to the proximity effect. The labeling reagent is composed of an affinity ligand, a reporter tag, and a reactive linker moiety. Upon the selective binding to the ligand binding site of a POI, the reactive linker of labeling reagent can be placed in the vicinity of binding site, allowing the selective modification of an amino acid side-chain existing around the binding site of POI. The ligand moiety can be easily removed from the ligand binding pocket after labeling because the ligand is not covalently attached to the POI due to the cleavable nature of the linker moiety. Overall, this ligand-directed chemistry carried out chemical labeling/modification of a target protein without loss of the original activity and function. To date, we have discovered a few of the cleavable electrophilic linkers useful for the ligand-directed chemistry, including acyl imidazole (LDAI), tosyl (LDT) [20], dibromophenyl benzoate (LDBB) [21], N-sulfonyl pyridone (LDSP) [22], and N-acyl-N-alkyl-sulfonamide (LDNASA) [23] (Figure 3(b)). These cleavable electrophiles exhibit distinct features each other in the stability, reactivity and amino acid selectivity for protein modification [18]. In LDAI chemistry, it turned out that alkyloxyacyl group in the N-acyl imidazole unit affords the sufficient stability in aqueous solution such as cell culture medium, compared to a simple acyl imidazole, with retaining moderate reactivity. We also unveiled that LDAI chemistry can react with amino (Lys), hydroxyl (Ser), and phenol (Tyr) functionalities around the ligand binding pockets of POI, the selectivity of which is sharply different from that of LDT chemistry which is preferable to imidazole (His), phenol (Tyr), carboxylate (Glu and Asp), and thiol (Cys) *via* an S_N2 reaction of tosylate. The pseudo second-order rate constants of LDAI-mediated protein labeling are determined to be ~$10^{1\text{-}2}$ $M^{-1}s^{-1}$ [23]. These values are reasonably fast and are almost comparable to those of copper-catalyzed azide-alkyne cycloaddition (CuAAC) which has been used in many significant applications in the field of chemical biology. Using LDAI chemistry, we modified natural proteins/enzymes with a variety of functional molecules

such as fluorescent probes, affinity tag, and photo-activatable modules for imaging of endogenous proteins in live cells and tissues, pulse-chase analysis of membrane receptor proteins, photocontrol of enzymatic activities, and *in situ* construction of fluorescent biosensors for drug screening [24]. In the following sections, we briefly discuss several recent examples of LDAI chemistry for exploring the protein functions in live cells.

Visualization of membrane proteins in live cells and tissues by LDAI chemistry

Our recent efforts clarified that LDAI chemistry is powerful for chemical labeling of a variety of membrane proteins in a highly specific fashion in live cell environments, partially owing to the proper amino acid selectivity and stability of acyl imidazole (Figure 3(c)). It is well recognized that the functions of membrane proteins are tightly controlled by their dynamics such as protein trafficking, internalization, degradation, and recycling. The LDAI-based fluorophore labeling of the membrane proteins (i.e. bradykinin receptor 2 (B_2R), carbonic anhydrase 12 (CA12), and folate receptor (FR)) enabled the pulse-chase analysis of them and determined their half-lives as well as the degradation pathways through confocal laser scanning microscope (CLSM) imaging [25]. LDAI chemistry has been applied to chemical labeling of more complicated proteins localized under highly complex multimolecular crowding conditions. Glutamate (Glu) is a major excitatory neurotransmitter in the central nervous system (CNS), and fast excitatory neurotransmission is mediated mainly *via* AMPA-type Glu receptors (AMPARs). We have succeeded in the chemical labeling of AMPARs using designer LDAI reagents [26] not only in the cultured neurons but also in more complicated brain slices without affecting receptor functions, which enables us to characterize the diffusion dynamics of endogenous AMPARs both in cultured neurons and hippocampal slices. Other groups have also recently reported the application of LDAI method for visualization of other neurotransmitter receptors. Arttamangkul *et al.* have reported the fluorescent labeling of endogenous opioid receptors, G-protein coupled neurotransmitter receptors, in live neurons of brain slices [27].

Construction of protein-based semisynthetic biosensors by LDAI chemistry

Chemical modification of membrane receptors by LDAI chemistry provides a powerful tool for the creation of protein-based semisynthetic biosensors in live cells. Proteins/enzymes that are often causative factors of many diseases such as cancer, diabetes and neurological disorders are regarded as attractive drug targets, as well as biomarkers. Conversion of such drug-target proteins to biosensors should allow for the construction of ligand-binding assay systems. These systems are also powerful for in-depth quantitative evaluation of interactions between the protein and a specific ligand not only *in vitro* but also in live cells (or more complex biological samples). Such a unique platform for drug screening would be crucial for the discovery of new therapeutics and diagnostics. Traceless protein labeling by LDAI chemistry may provide native (membrane) protein-based semisynthetic biosensors applicable for drug screening in live cells with minimal perturbation of cellular conditions. We labeled endogenous FRs with several fluorophores on the cancer cell surface by LDAI chemistry and showed the fluorescein-labeled FR changed its fluorescence with and without ligands [15]. FR is overexpressed in many of cancer cell lines, and thus is considered to be a drug target and biomarker for cancer diagnosis. The quantitative evaluation of the binding kinetics and dissociation constants of several FR ligands (folic acid (FA) and its analogues (dihydrofolic (H2FA) acid, tetrahydrofolic acid (H4FA), pterine (PT) and methotrexate (MTX)) was readily conducted under live cell conditions using the fluorescent biosensor function of the modified FR. As another example, we reported the fluorophore-labeled AMPARs act as turn-on-type biosensors that allow the selective detection and quantitative analysis of AMPAR ligands under live cell conditions (Figure 3(d)) [28]. We successfully determined the affinities of a variety of agonists or antagonists for AMPARs, and revealed that the agonist-binding properties of AMPARs are remarkably different between live-cell environments and non-cellular conditions, suggesting that biosensors constructed from full-length membrane-receptors in natural environments would be critical for precise analysis of drug binding. Unlike Glu, γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the CNS, and inhibitory neurotransmission in the brain is mediated *via* ion-channel-type $GABA_A$ and GPCR-type $GABA_B$ receptors ($GABA_A$Rs and $GABA_B$Rs). $GABA_A$Rs are therefore regarded as important targets for a number of pharmaceuticals, such as anticonvulsants, anesthetics, sedatives, anxiolytics and antidepressants. A total of 19 different subunits of $GABA_A$Rs have been identified and they assemble in limited combinations to form functional heteropentamers. By a

combination of LDAI chemistry and the bimolecular fluorescence quenching and recovery (BFQR) strategy [29] (Figure 3(e)), we successfully constructed $GABA_AR$-based turn-on fluorescent biosensors in live cells [30]. High-throughput screening for $GABA_AR$ ligands was then conducted using this fluorescent biosensor. Four hit compounds were newly discovered from a library of pharmacologically active compounds (LOPAC1280), among which two compounds were newly identified as negative allosteric modulators of $GABA_ARs$. This example indicated us that $GABA_AR$-based biosensors are able to sensitively detect the subtle conformational changes of $GABA_AR$ induced by ligand-binding. Very recently, Sames and co-workers have succeeded in modification of native AMPARs with dextran-6000 tethered voltage sensitive fluorescent dyes (VSDs) in live brain tissues using LDAI chemistry [31]. This group demonstrated the feasibility of the optical voltage recording in the proximal to AMPAR in live brain slices. These exciting examples demonstrate the versatility of LDAI chemistry applicable for the construction of protein-based biosensors, as well as endogenous protein labeling in live cell and tissue conditions.

(Figure 3)

Metal-directed AI chemistry for conditional proteomics approach

The imidazole ring of N-acyl imidazole derivatives can be coordinated to various metal cations, by which the electrophilicity is largely modulated. Metal-directed AI chemistry was very recently developed for conditional proteomics of living cell. Not only a single target protein but also a protein ensemble (proteome) bearing a common character are significant objectives in current chemical biology research. We have developed a ”conditional proteomics“ approach in order to unveil mobile zinc-related biological events. We elaborately designed zinc-activatable labeling reagents in which N-alkyloxyacyl imidazole is employed as a moderately reactive electrophile (Figure 4(a)) [32]. Zinc signaling and dynamics are considered to play significant roles in many physiological responses and diseases. To understand the physiological roles of zinc in detail, comprehensive identification of proteins that localize in spaces accumulating high concentrations of zinc ions is crucial. In the designer labeling reagents, AIZin, a Zn^{2+} binding chelator (dipicolylamine, DPA) and a reporter probe are connected with an acyl

imidazole moiety (Figure 4(b)). Importantly, DPA and acyl imidazole modules are closely connected through one methylene unit, resulting the imidazole unit can be involved in Zn^{2+} complexation. Upon binding to Zn^{2+} ion, the electron density of imidazole ring is reduced, which enhances the reactivity of the acyl group to facilitate the covalent labeling of surrounding proteins. The AIZin-based conditional proteomics approach clearly revealed dynamic changes of the zinc related proteomes and successfully enhanced our elucidation on zinc dyshomeostasis induced by nitric oxide stimuli in glioma cells. Moreover, this method identified zinc rich vesicles originating from an ER-Goldi intermediate component for the first time. Recently, Chang and co-workers have extended the conditional proteomics approach to characterize the labile copper ion pools in mouse brain [33]. Like zinc cation, copper is an essential metal for life and particularly important for the function of CNS. Chang and co-workers designed copper-responsive labeling reagents which are composed of a thioether-based copper chelator, an acyl imidazole unit, and a coumarin or Si-rhodamine fluorophore. Similar to AIZin, the coordination of Cu ion increases the reactivity of acyl imidazole electrophiles, providing the proximal labeling of proteins around areas of elevated labile copper in live cells. Using the designer Cu-directed acyl imidazole dyes, Chang's group visualized labile copper in live cells and revealed the copper homeostasis is distinct among the major cell lines of the brain, that is neurons, astrocytes and microglia. These examples indicated the conditional proteomics strategy could be powerful to characterize unknown or poorly understood biological processes. The conditional proteomics is now under expansion by further design of labeling reagents in response to other cellular microenvironmental factors, such as ROS [34], RNS [35], pH, or other metals.

(Figure 4)

Acyl imidazole reagents for RNA structure analysis and functional control

Thanks to the unique reactivity of acyl imidazole electrophiles capable of acylating the hydroxyl groups even in aqueous conditions, acyl imidazole based reagents have recently been used for the RNA modification to obtain the structural information of a particular RNA. Secondary and tertiary structure of RNA has important roles in its function in every facet of gene regulation. Since the 2'-hydroxyl group of ribose ring is a universal

chemical feature of RNA, the method to examine the reactivity of 2'-hydroxyl group provides the information related to the dynamics and solvent exposure of local RNA structure. Currently, selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) method has become the gold standard for monitoring the secondary structures of complex RNAs [36]. In the SHAPE profiles, single-strand or flexible RNA regions have high reactivity of 2'-hydroxyl group, whereas RNA nucleotides engaged in base pairing or other interactions show the lower reactivity. Chang, Kool and co-workers developed new acyl imidazole-based electrophiles, FAI and NAI, which selectively react with hydroxyl groups, highly soluble in aqueous solution and amenable to RNA modification inside live cells (Figure 5(a)) [37]. Importantly, acylation of other functionalities including base portions of RNA strands are scarcely observed with these reagents under the experimental conditions. Using these SHAPE reagents, they succeeded in reading out RNA structure in live cells at single-nucleotide resolution and addressing tertiary contacts and RNA-protein interactions (Figure 5(b)). Further, the same group also designed an acylation reagent, NAI-N_3, in which an azide group is added to NAI for the purification handle of modified RNA using streptavidin beads after the Cu-free click reaction with DIBO-biotin (*in vivo* click SHAPE, icSHAPE) [38]. The use of NAI-N_3 enabled the global view of RNA secondary structures in live cells. By comparing the icSHAPE profiles *in vitro* and in live cells, the authors successfully carried out dynamic RNA structural footprinting, which enables accurate prediction of RNA-protein interactions and N^6-methyladenosine genome wide.

In addition to the RNA structural analysis, the acylating reagent NAI-N_3 was utilized to the reversible control of RNA hybridization, folding and enzyme interactions (Figure 5(c)) [39,40]. Kool and co-workers have shown that the reaction of RNA with NAI-N_3 (100–200 mM) yields several 2'-OH acylations per RNA strand within 10 min. This poly-acylated ("cloaked") RNA is strongly blocked from hybridization with complementary nucleic acids, from cleavage by RNA-processing enzymes, and from folding into active aptamer structures. The treatment with a water-soluble phosphine triggers a Staudinger reduction of the azide groups resulting in spontaneous decomposition of acyl groups ("uncloaking") to recover the RNA folding and its biochemical activity. Using the RNA cloaking strategy, they successfully demonstrated the reversible control of the Spinach RNA aptamer's function in test tube and the control of CRISPR-Cas9 gene editing in live cells [39,41]. Moreover, the same group also designed an RNA acylation reagent

bearing a photocleavable group, which allows the photocontrol of structure and function of hammerhead ribozyme and Broccoli RNA aptamer in test tube and in live cell, respectively (photo-cloaking strategy) [42]. These RNA cloaking approach based on acyl imidazole chemistry is extremely simple, and applicable to both chemically synthesized short RNAs and transcribed long RNAs. Thus, these new methods could potentially find widespread use for study of chemistry and biology of RNAs.

(Figure 5)

Conclusions

In this review, we briefly described recent advances in the application of N-acyl imidazole electrophiles in the field of chemical biology. Thanks to their tunable reactivity, chemical selectivity, and stability in water, N-acyl imidazole reagents can be uniquely employed in live cells for chemical modification of not only endogenous protein but also RNA. Indeed useful, modification and functional control of biomolecules in live tissues and whole organisms yet remains challenging. We believe that further optimization in various aspects of acyl imidazole groups could produce invaluable tools that are applicable to chemical biology study in more-complicated multimolecular crowding systems.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure legends

Figure 1. Application of N-acyl imidazole derivatives for the modification of biopolymers. (a) Reaction scheme of chemical modification of biopolymer using N-acyl imidazole. (b) Tunable reactivity and solubility of N-acyl imidazole by the introduction of suitable substituents and/or metal coordination to the imidazole group.

Figure 2. Application of N-acyl imidazole in chemical synthesis of peptides and proteins. (a) Scheme of native chemical ligation (NCL) using MPAA as an additive. (b) Scheme of imidazole aided NCL (Im-NCL). (c) Scheme of a peptide ligation method utilizing His side chain at N-terminal of a peptide fragment.

Figure 3. Ligand directed acyl imidazole (LDAI) chemistry for chemical labeling of protein. (a) Chemical modification of POI using ligand directed (LD) chemistry. (b) Structure of cleavable electrophiles for LD chemistry developed by Hamachi's laboratory. (c) Scheme of chemical labeling of natural membrane protein using LDAI chemistry. (d) Construction of AMPAR-based fluorescent biosensor using LDAI chemistry. (e) $GABA_AR$-based semi-synthetic biosensor constructed by a combination of LDAI chemistry and BFQR strategy. Lg, ligand moiety; FL, fluorophore; Nu, nucleophilic amino acid residue; Q, quencher.

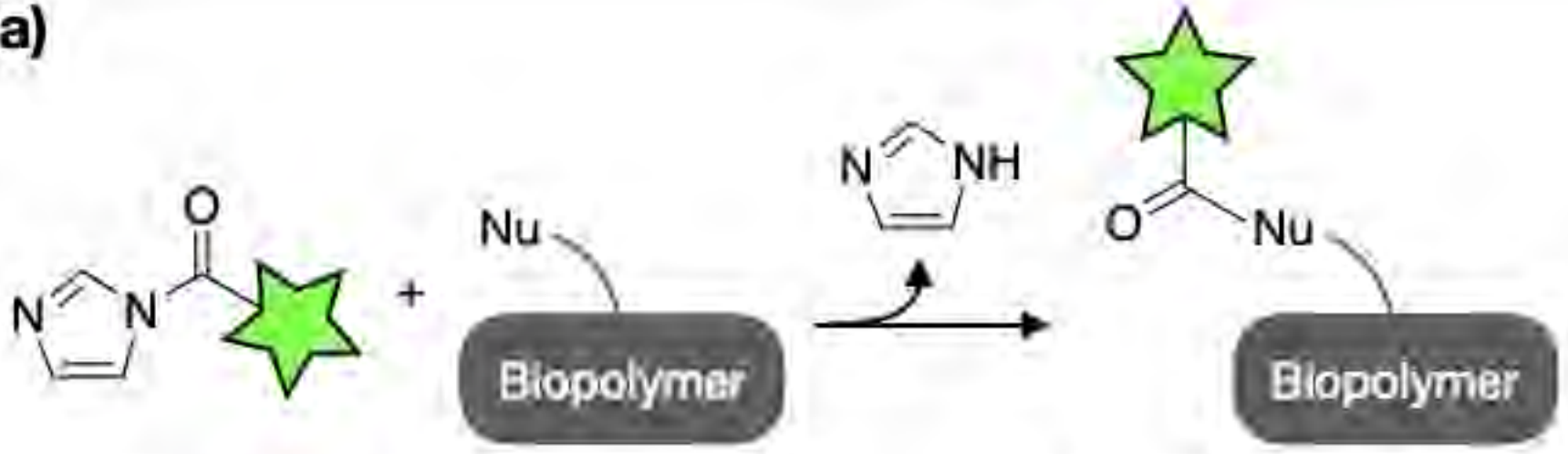
Figure 4. Conditional proteomics approach using N-acyl imidazole chemistry. (a) Schematic illustration of Zn^{2+}-enhanced chemical labeling of proteins in living cell. (b) Schematic scheme of Zn^{2+}-promoted protein labeling.

Figure 5. N-Acyl imidazole reagents for structure analysis and functional control of RNAs. (a) Chemical structures of NAI and FAI. (b) RNA acylation using the NAI reagent for in cell SHAPE analysis. (c) Functional control of RNA by cloaking and un-cloaking strategy using the NAI-N_3 reagent.

Graphical abstract caption

The tunable reactivity and unique chemical selectivity of N-acyl imidazole promote their application in the field of chemical biology.

(a)



N-Acyl imidazole derivative

Chemical modification of biopolymer

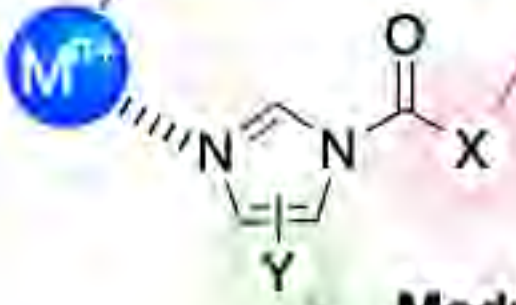
(b)

Tunable reactivity

Enhancing reactivity by metal binding.

Modulating reactivity/stability.

Increasing water solubility.



Modulating reactivity.