Translation of a phosphine- and azide-based reaction to chemical modification of biomolecules in ionic liquid

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Abstract The difference of reaction design principles between traditional, small molecule synthetic chemistry and biomolecular chemical reactions prevented the simple translation of small molecule chemistry into biomolecular reactions. One of the key challenges of bioconjugation, or reactions on biomolecules, are the necessity of aqueous solutions as the solvent. In this Synpact article, we describe our pursuit of using an ionic liquid as a nonaqueous reaction medium to conduct phosphine- and azide-based bioconjugation reactions.

Key words bioconjugation, ionic liquid, amine, azide, phosphine, phosphazide, iminophosphorane.

Introduction

Chemoselective modifications of functional groups on biomolecules or biomacromolecules such as proteins, nucleic acids, and carbohydrates represent unique selectivity and reactivity challenges that differ from traditional synthetic methods used in small molecule organic chemistry.[1] Within a single biomacromolecule, multiple copies of the target functional group are present, alongside a myriad of other functional groups. Therefore, an excess of modification reagents is often needed to effectively label biomacromolecules. Moreover, in order to fulfill their native functions in biological systems, biomacromolecules are typically coated with various nucleophilic functional groups (e.g. alcohols, amines, and thiols) and relatively inert electrophilic groups (e.g. carboxylates and amides). A standard chemical modification process typically differentiates the desired nucleophilic target from other nucleophiles within the biomacromolecule by using electrophilic labelling reagents.[2]

The limited number of reaction solvents compatible with biomolecules render the development of selective chemical reactions of biomolecules, or bioconjugation reactions, even more difficult. Unlike synthetic chemistry of small molecule substrates, many organic solvents are not practically usable for biomacromolecules, owing to not only their poor solubility in hydrophobic solvents, but also potential loss of their high-order structures and native activities through exposure of their inner hydrophobic pockets.[3] Thus, previous efforts for designing selective chemical modification methods for biomolecules have been focused on reaction development in aqueous environments. Although there are many reactions that favor aqueous conditions[4] and there have been significant efforts to develop aqueous chemical reactions from green chemistry perspectives,[5] the majority of synthetic chemistry reactions rely on organic solvents as media due to numerous reasons including reagent decomposition with moisture, decrease in reactivity of reagents in protic solvents, and poor reagent solubility in water. The general negative effects of aqueous environments on organic chemical reactions has made the development of novel bioconjugation methods challenging.

To circumvent the difficulties encountered by using aqueous solutions as the reaction solvent, we turned our attention to non-aqueous, aprotic media that would be compatible with biomacromolecules. Ionic liquids, which exist as liquids at temperatures <100 °C, have been used as an alternative solvent in differing fields of chemistry, and have also been shown to be effective in biomacromolecule studies[6,7] and as a substitute for organic solvents.[8] For instance, enzyme lipase-mediated acylation reactions were achieved in an ionic liquid reaction medium.[9] The preservation of the double helical structure of DNA by ionic liquids through electrostatic interactions has been also reported.[10] Dissolution of saccharides was effectively accomplished with ionic liquids as well.[11] Notwithstanding the recognition of the utility of ionic liquids for biomolecular studies, the chemical modification of biomolecules in ionic liquid has not been explored broadly for bioconjugation purposes, other than the traditional acetylation and acylation of polysaccharides.[12–14] With the increasing evidence of biomolecule compatibility with ionic liquids, our group initiated the Bioconjugation in Nonaqueous-Driven Reaction Solvent (BINDRS) program, which

aims to create alternative media to overcome the issues of the aqueous media-based bioconjugation. During the screening of chemical reagents incompatible with traditional aqueous bioconjugation reactions, we discovered that alkylazide reagents cause covalent bond formation with alkylamine groups on biomolecules in a phosphine-dependent manner (Figure 1).[15]

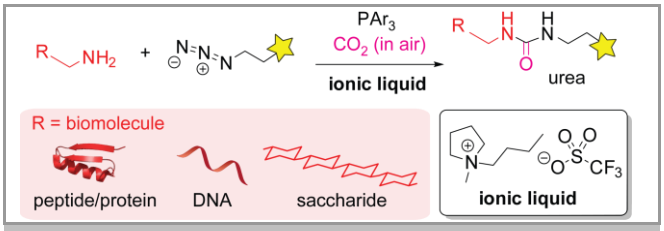

Figure 1 Urea-forming three-component coupling of biomolecules between alkylamines and alkylazides in ionic liquid (e.g. butylmethylpyrrolidinium trifluoromethanesulfonate, BMPy OTf) using atmospheric carbon dioxide.

While this bioconjugation reaction later proved to be a three-component coupling reaction of alkylamine and alkylazide with carbon dioxide to forge a urea linkage (Figure 1, vide infra), we initially hypothesized that the reaction provided a tetrazene group through a phosphazide intermediate. Tetrazene consists of four consecutive nitrogen atoms with an N-N double bond.[16] Although unsubstituted tetrazenes are thermally unstable at room temperature,[17] tetraalkyl-substituted tetrazene compounds are known to be stable even at temperatures higher than 100 °C (Figure 2A).[18,19] On the other hand, aryltetrazenes decompose relatively easily,[20] paralleling the well-known higher reactivity/lower stability of arylazide compounds compared to alkylazide groups.[21] Reactions of amine derivatives and azide compounds have been developed for some systems including the interconversion of ammonium azide salts to the parent tetrazene under high pressure[22] as well as nucleophilic attack of a metal amide group to organic azides to generate unstable tetrazene intermediates (Figure 2B).[23,24] The initial set of our data including mass spectrometry and $^{1}H/^{15}N$ NMR studies suggested tetrazene as a potential reaction product (though one of the assigned peaks in ^{15}N NMR turned out to be an artifact in the later study).[25,26] Based on the set of data, we proposed a phosphazide-based coupling process without loss of N_2 gas as a potential reaction pathway (Figure 2C).[15,27] This initial hypothesis was supported by the report of similar electrophilic behavior of phosphazide intermediates for an intramolecular reaction between alkylated phosphazide and enolate species.[28] Alkylazide compounds often behave as soft electrophiles to react with nucleophiles such as thiols and phosphines. We initially hypothesized that a phosphine reagent activated the alkyazide compound to cause an N-N bond formation with the amine groups without liberating nitrogen gas (i.e. without generating an iminophosphorane intermediate).[29]

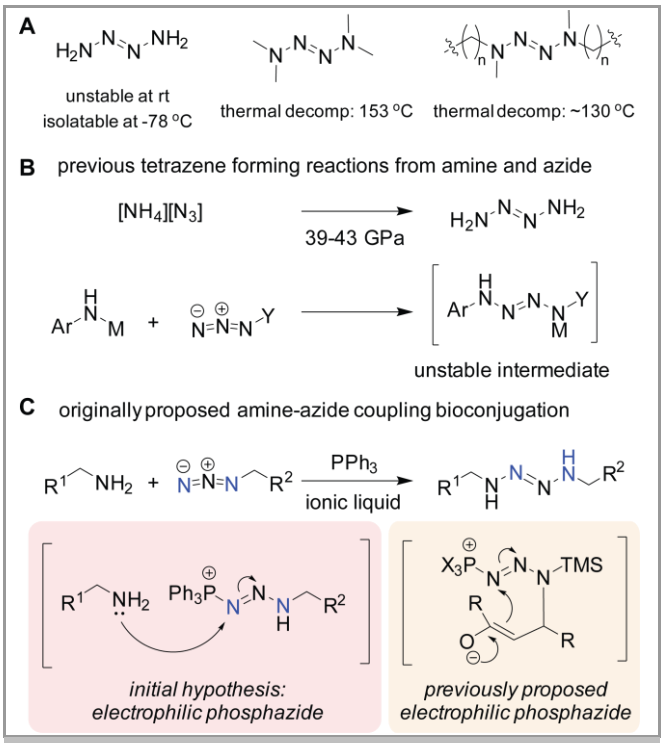

Figure 2 Previously reported tetrazene-forming reactions. (A) Stability comparison of tetrazene compounds: parent tetrazene (left),[17] tetramethyltetrazene (middle),[18] and tetraalkyltetrazene polymer (right).[19] (B) Top: Conversion of ammonium azide salt into parent tetrazene under high pressure.[22] Bottom: Formation of disubstituted aryltetrazene intermediates from anionic amine derivatives and azide compounds.[23,24] (C) Originally reported reaction scheme for the alkylamine-targeting bioconjugation with alkylazide and triarylphosphines. A protonated electrophilic phosphazide intermediate was proposed as a key reaction intermediate based on a previously reported alkylated electrophilic phosphazide intermediate.[28]

On the other hand, our crystallographic evidence suggested that the reaction product of the amine-azide bioconjugation contained a branched linkage (Figure 3A). The crystal structure was obtained for a reaction product between methylamino- and methylazido-pyrene. The crystals were formed in the reaction vessel, and their poor solubility in any solvents precluded solution-based characterization of this compound. We considered diamino-isodiazene as the alternative reaction product, due to fact that the functional group is a constitutional isomer of the tetrazene group. Isodiazene with ionic and neutral nitrene resonance contributors would be extremely reactive in general and are used as a reaction intermediate for versatile synthetic strategies.[30] However, there have also been reports of crystal structures of analogues of isodiazene species. Examples of crystal structures of such isodiazene derivatives include the triazene imine synthesized through an amination reaction of a cyclic triazene precursor[31] and a phosphorus analogue, as well as phosphinonitrenes formed through the azide decomposition reaction (Figure 3B).[32] Although both examples had similar bond length and angles to our crystallographic data, previous ^{15}N NMR data of tetrazene and isodiazene suggested that one of the nitrogen atoms of isodiazene has a distinctive chemical shift (917 ppm), and this peak was not observed in our initial ^{15}N NMR study (Figure 3C).[33]

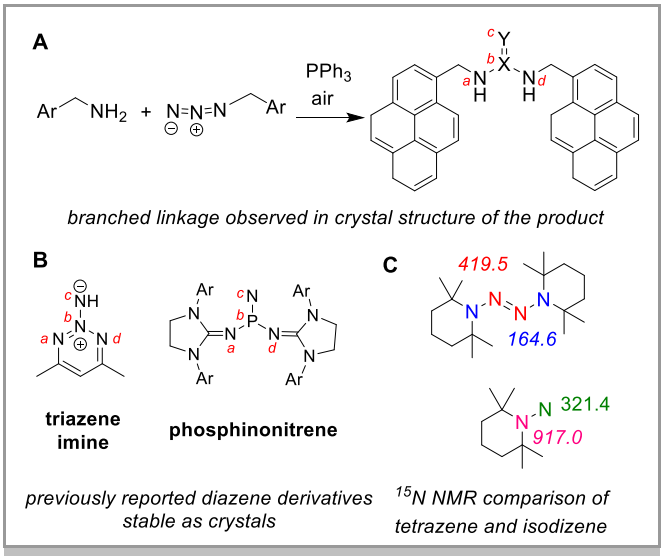

Figure 3 The diamino-isodiazene group is a constitutional isomer of a tetrazene group. (A) Reaction of aminomethyl-pyrene and azidomethyl-pyrene produced insoluble crystals with a branched linkage, unlike the proposed tetrazene structure with a linear linkage. Further studies confirmed that a C=O group corresponds to the X=Y group of the linkage.[34] Bond lengths (Å) and angles (deg): ^{a}N-^{b}X 1.355(2), ^{d}N-^{b}X 1.355(2), ^{b}X-^{c}Y 1.237(4), ^{a}N-^{b}X-^{d}N 115.6(3), ^{a}N-^{b}X-^{c}Y 122.21(13), ^{d}N-^{b}X-^{c}Y 122.21(13). (B) Previously reported crystal structures of diamino-isodiazene derivatives. Left: triazene-imine.[31] Bond lengths (Å) and angles (deg): ^{a}N-^{b}N 1.36, ^{d}N-^{b}N 1.35, ^{b}N-^{c}N 1.28, ^{a}N-^{b}N-^{d}N 125, ^{a}N-^{b}N-^{c}N 115, ^{d}N-^{b}N-^{c}N 116. Right: phosphinonitrene.[32] Bond lengths (Å) and angles (deg): ^{a}N-^{b}P 1.629, ^{d}N-^{b}P 1.618, ^{b}P-^{c}N 1.457, ^{a}N-^{b}P-^{d}N 101.8, ^{a}N-^{b}P-^{c}N 129.5, ^{d}N-^{b}P-^{c}N 128.6. (C) Previously reported ^{15}N NMR studies of tetrasubstituted tetrazene and dialkyl isodiazene. Values are in ppm, calibrated with ^{15}N- enriched nitromethane as the external standard at 380.7 ppm.[33]

Extensive chromatographic and spectroscopic studies finally revealed that the azide- and phosphine-based conjugation of biomacromolecules in an ionic liquid is dependent on carbon dioxide to generate a urea group through liberation of N_2 gas (Figure 4A).[34] A new set of ^{15}N NMR data with ^{15}N isotopes on all the four possible nitrogen atoms of amine and azide groups disclosed that one of the peaks at ~300 ppm previously assigned to the N=N group of tetrazene was an artifact,[25,26] and introduction of only two ^{15}N isotopes out of the four possible nitrogens was observed. The formation of the urea group was confirmed through NMR, mass spectrometry, and infrared spectroscopy experiments. The studies supported the loss of N_2 gas during the reaction to generate an iminophosphorane intermediate.[34] Indeed, recent reports have described an iminophosphorane-mediated urea forming reaction with CO_2 even at low concentrations of the CO_2 gas,[35] which is consistent with our reaction conditions relying on atmospheric levels of CO_2. Taking into account the previously proposed reaction mechanism for small molecule substrates, we propose that the bioconjugation reaction proceeds through a reactive isocyanate intermediate, which is known to be an excellent alkylamine selective modification of biomolecules (Figure 4B).[36]

The exquisite chemoselectivity of the urea-forming reaction toward alkylamine groups in ionic liquids was successfully applied to biomolecules with various functional groups. In ionic liquids, the urea-forming reaction was able to modify protein substrates at lysine or N-terminal amine groups through use of excess alkylazide reagents with triphenylphosphine, despite the presence of numerous other nucleophiles including guanidines, imidazoles, alcohols, and phenols (Figure 4C).[15] Quite interestingly, the bioconjugation reaction in ionic liquid showed selectivity for alkylamines over arylamines, whereas the small molecule reaction showed selectivity for arylamines.[35] This attribute facilitated site-specific modification of external alkylamine groups of DNA without appreciable side reactivity on the arylamine groups of the nucleobase (Figure 4D).[27] Additionally, the incorporation of alkylazides with various functional groups into DNA demonstrated the wide substrate tolerance of the reaction. Modification of azide-tagged biomolecules such as azide-tagged hyaluronic acid proved successful as well. Because many biomolecules are only soluble in aqueous solutions, our ionic liquid-mediated bioconjugation setup contains <6% v/v water. Due to the presence of water in the reaction solution, we believe a significant amount of the iminophosphorane intermediate undergoes the Staudinger reduction pathway to an alkylamine group. In that sense, the bioconjugation of azide-tagged biomolecules in the presence of excess alkylamine reagents should have been challenging. However, our study demonstrated the successful modification of azide-containing saccharides, showing the advantage of the method (Figure 4E).[34]

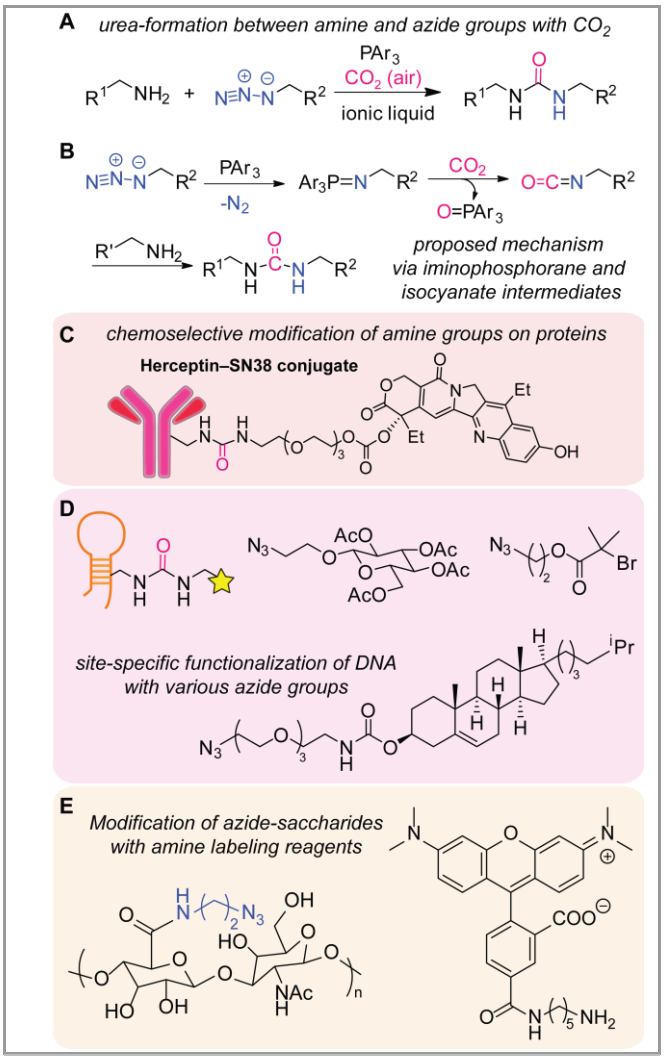

Figure 4 The corrected reaction product and mechanism for the amine-azide coupling reaction through the formation of urea with atmospheric carbon dioxide. (A) A reaction scheme of the urea-forming bioconjugation reaction. (B) The proposed reaction mechanism of the urea-forming bioconjugation, based on a

previously reported mechanism for a small molecule reaction. (C–E) Substrate scopes of the urea-forming bioconjugation in ionic liquid with high tolerance to broad types of functional groups on proteins (C), DNAs (D), and saccharides (E).

Outlook

The successful translation of a water-sensitive iminophosphorane-based synthetic approach into bioconjugation strategies using ionic liquid demonstrated the potential of nonaqueous chemical modification of biomolecules. Given that there are countless numbers of reagents that are incompatible with protic solvents, ionic liquid-based methods would revolutionalize bioconjugation by enabling use of such reagents. For the case of the iminophosphorane-based amine-azide coupling reaction, both the aprotic nature of the ionic liquid and the higher pKa of the solvent contributed significantly to the success of the reaction. The pKa of typical imidazolium salts are ~30 in MeCN.[37] The higher pKa in an aprotic solvent most likely elongated the lifetime of the iminophosphorane intermediate compared to a protic solvent (e.g. pKa of conjugate acid of triphenylphosphine-based iminophosphorane is ~22 in MeCN).[38] The wide compatibility of ionic liquids towards stronger nucleophiles is in stark contrast with aqueous media; usable reagents in aqueous bioconjugation usually have a pKa value of less than 12–13 (for the conjugate acid), otherwise the high pH of aqueous media would cause the destruction/hydrolysis of the biomolecule backbone. The tunable nature of ionic liquids presents the possibility of creating novel tailor-made ionic liquids for chemical modification of biomolecules with even stronger chemical resistance and higher biomolecule compatibility than existing ones. Besides serving as an alternative medium, ionic liquids are also occasionally known to participate in the chemical reactions such as *N*-heterocyclic carbene-mediated chemistry,[39–41] and such capability could be explored to develop novel bioconjugation strategies as well.

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Conflict of Interest

The authors declare no conflict of interests.

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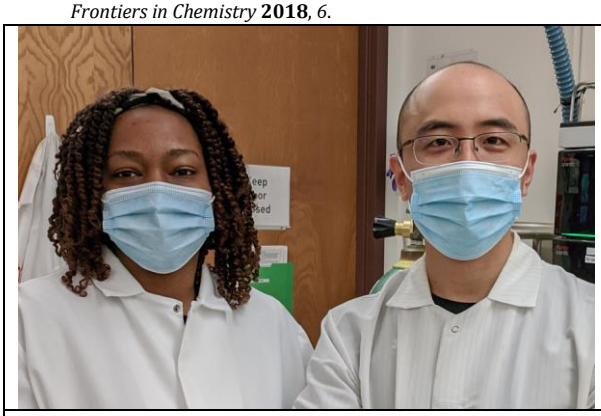

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Jun Ohata (right) was born and raised in Japan. He received his B.S. in 2011 and his M.S. in 2013 from Osaka Prefecture University, where he worked with Prof. Hiroyuki Matsuzaka studying the reactive carbon species on diruthenium complexes. He earned his Ph.D. in 2018 in Prof. Zach Ball's group at Rice University studying protein bioconjugation by transition-metal catalysis in aqueous buffers. He conducted his post-doctoral work with Prof. Christopher Chang at the University of California—Berkeley as a Postdoctoral Fellow of the Japan Society for the Promotion of Science, developing chemical probes for the detection of cellular metal ions using protein labeling methods. In 2020, he took up his current position at North Carolina State University as an assistant professor developing novel strategies for bioconjugation.