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Azide–alkyne cycloaddition for universal post-synthetic modifications of nucleic acids and effective synthesis of bioactive nucleic acid conjugates†

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The regioselective post-synthetic modifications of nucleic acids are essential to studies of these molecules for science and applications. Here we report a facile universal approach by harnessing versatile phosphoramidation reactions to regioselectively incorporate alkynyl/azido groups into post-synthetic nucleic acids primed with phosphate at the 5′ termini. With and without the presence of copper, the modified nucleic acids were subjected to azide–alkyne cycloaddition to afford various nucleic acid conjugates including a peptide–oligonucleotide conjugate (POC) with high yield. The POC was inoculated with human A549 cells and demonstrated excellent cell-penetrating ability despite cell deformation caused by a small amount of residual copper chelated to the POC. The combination of phosphoramidation and azide–alkyne cycloaddition reactions thus provides a universal regioselective strategy to post-synthetically modify nucleic acids. This study also explicated the toxicity of residual copper in synthesized bioconjugates destined for biological systems.

Introduction

Regioselective modifications of biomolecules with tags, probes or other biological molecules have been a critical tool which significantly advanced biomolecular studies for fundamental research and clinical application. In nucleic acids, site-specific modifications of smaller DNA/RNA such as oligonucleotides can be achieved by phosphoramidite chemistry to link predefined chemical moieties to positions in specific nucleotides during solid-phase oligonucleotide synthesis.[1] However, the solid-phase chemistry approach for regioselective modifications of oligonucleotides suffers from inherited drawbacks including limits on the length of synthesized oligonucleotides and on the variety of their incorporated chemical functionality.

To complement the shortcomings of solid-phase oligonucleotide synthesis, many site-specific post-synthetic modification methods for nucleic acids have been studied and adapted to any size of nucleic acid and a broad diversity of chemical groups integrated into the nucleic acids.[2–5] Nevertheless, recently developed post-synthetic modification methods for nucleic acids rely on enzyme catalysis to carry out chemical transformations but are unable to provide a universal strategy for both DNA and RNA modifications. Moreover, the required expensive enzymes and specific substrates in enzymatic reactions further stymie the efforts to modify nucleic acids with various chemical entities within reasonable costs.

Here we report a universal, economical post-synthetic modification method for nucleic acids involving orthogonal azide–alkyne cycloaddition reactions. The powerful copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction,[6,7] a prototype of click chemistry,[8] has found broad applications in science ranging from material studies to biomolecule research.[9] In addition, the copper-free variant of the CuAAC reaction, strain-promoted azide–alkyne cycloaddition (SPAAC), harnesses excellent reactivity of cyclooctyne derivatives and drastically expands the biocompatibility of the 1,3-dipolar cycloaddition reaction for studying biomolecules *in vivo*.[10,11] In the current study, we exploited the optimized site-specific two-step phosphoramidation reactions[12–14] to facilitate the introduction of azido and alkynyl groups into DNA/RNA. The acquired

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† Electronic supplementary information (ESI) available: Full details of figures referenced in the text, NMR and MS spectra for compounds (**6d**, **7**, **12**, **15**, **16** and **19**), and MALDI-TOF MS spectra for gel-purified nucleic acid conjugates. See DOI: 10.1039/c4ob01132e

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azide- and alkyne-modified nucleic acids set the stage for azide–alkyne cycloaddition and allowed effective and efficient conjugations with derivatives of biotin, fluorescein, and a cell penetrating peptide (CPP), the Tat peptide. As a proof of concept, we further demonstrated that the CuAAC-synthesized peptide–oligonucleotide conjugate (POC) was bioavailable and successfully trafficked into human cells.

Results and discussion

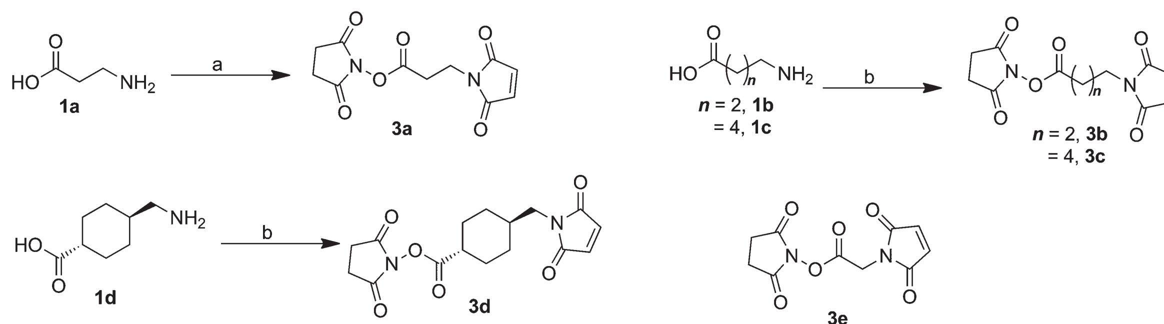
Potential of maleimide-assisted Michael addition for effective POC synthesis

Synthesis of POCs is an important strategy for the development of nucleic acid-based pharmaceuticals.[15] Short nucleic acids including antisense oligonucleotides, siRNA, miRNA and splice switching oligonucleotides have shown promise as therapeutic agents with the ability to modulate gene expression with excellent specificity.[15–17] However, the inability of oligonucleotides to permeate membranes and the propensity of these molecules to be entrapped in endosomes has raised concerns about the efficacy of DNA/RNA for clinical applications.[18,19] The conjugation of nucleic acids with CPPs in order to synthesize POCs could mitigate the delivery problems of oligonucleotides by promoting their translocation across membranes and their escape from endosomal entrapment.[15,20,21] CPPs enhance the cell permeability of POCs and significantly improve the uptake efficiency of oligonucleotides in POCs, qualities essential for POC applications in science and medicine.

We initially studied the phosphoramidation reaction-based Michael addition to achieve a more effective synthesis of nucleic acid conjugates such as POCs. Previous studies indicated that higher POC yield could be acquired by phosphoramidation, amidation and Michael addition reactions in sequence.[14] The successful POC preparation by the phosphoramidation-based Michael addition reactions inspired us to pursue the strategy for more effective synthesis of POCs. We first synthesized several *N*-maleoyl amino acid succinimidyl esters (**3a–d**; Scheme 1) which were reported to be more reactive, hetero-bifunctional, cross-linkers in Michael addition than **3e** used in a previous study.[2,14] We thus anticipated that the **3a–d**-assisted Michael addition reactions would deliver a better yield of nucleic acid conjugates. Unexpectedly, none of **3a–d** provided a POC yield better than **3e** (Fig. S1A†). Moreover, up to four of the cysteine-containing Tat peptide molecules were conjugated to DNA without prior modifications with cystamine and maleimides (the far right lane in Fig. S1A†) as indicated by MALDI-TOF MS analysis (Fig. S1B†). The observation of ineffective **3a–d**-based POC synthesis and the occurrence of multiple peptides conjugated to the DNA is perplexing as maleoyl-assisted Michael addition reactions are considered to be one of the major routes to modify biomolecules with thiols, a popular method of nucleic acid derivative synthesis for years.[2,22,23]

Optimal azide–alkyne cycloaddition for universal modifications of nucleic acids

Since the *N*-maleoyl amino acid succinimidyl ester **3a–d** failed to provide a more effective approach for nucleic acid modifications, we turned our attention to the CuAAC reaction to acquire nucleic acid conjugates with higher yield (Scheme 2). We began with synthesis of additional hetero-bifunctional cross-linkers, Cu(I) ligands and other azido-/alkynyl-containing molecules for performing nucleic acid modifications by the CuAAC reactions. For example, in order to have azide-modified nucleic acids in one of the CuAAC reactions (Scheme 2A), we synthesized several azido carboxylic acid succinimidyl esters (**6a–d**; Scheme 3) to determine the appropriate azides in **6a–d** and to provide the best yield for the CuAAC reaction. In addition, we synthesized tris(3-hydroxypropyltriazolylmethyl) amine (THPTA) and tris(benzimidazol-2-ylmethyl)amine (NTB) as Cu(I)-stabilizing ligands to accelerate the CuAAC reactions and prevent biomolecule degradation.[24,25] However, studies of THPTA and NTB indicated that only THPTA was capable of stabilizing DNA/RNA structures in the CuAAC reactions (Fig. S2†) and THPTA was thus the sole ligand used in subsequent CuAAC reactions for nucleic acid modifications. NTB was not a good ligand in CuAAC for nucleic acid modifications because nucleic acids were completely degraded by the end of the reaction (Fig. S2†). To evaluate the effectiveness of the CuAAC reactions, we also synthesized alkynyl and azido derivatives of the tag



Scheme 1 Synthesis of the *N*-maleoyl amino acid succinimidyl esters (**3**). Synthesis of **3e** was reported in our previous study.[14] a: (1) Maleic anhydride (**2**), DMF, rt, 2 h, (2) NHS, DCC, 1–25 °C, 16 h. b: (1) Flask A: **2**, DMF, rt, 3 h; *sym*-collidine, 0 °C, (2) Flask B: NHS, TFAA, *sym*-collidine, 0 °C; (3) mixing solutions in flasks A and B within 1–2 h, 0 °C; (4) rt, 16 h.

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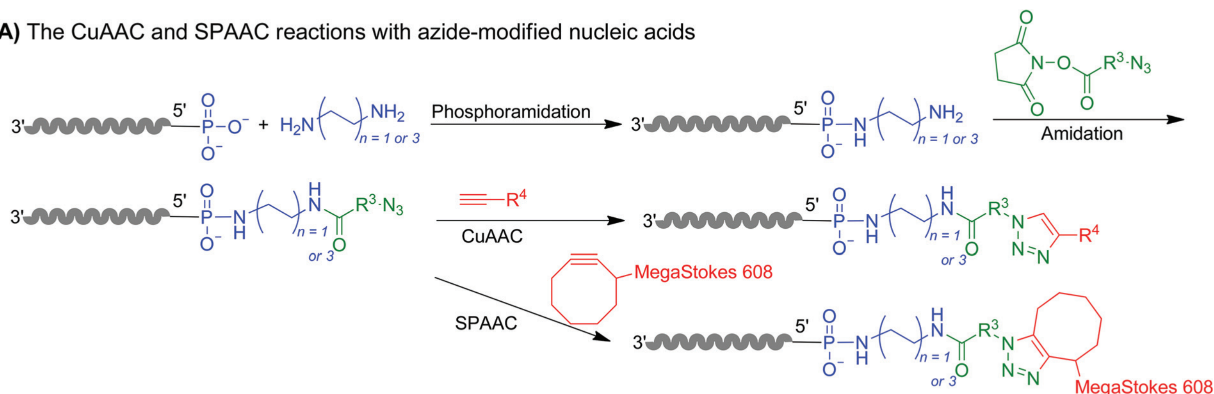
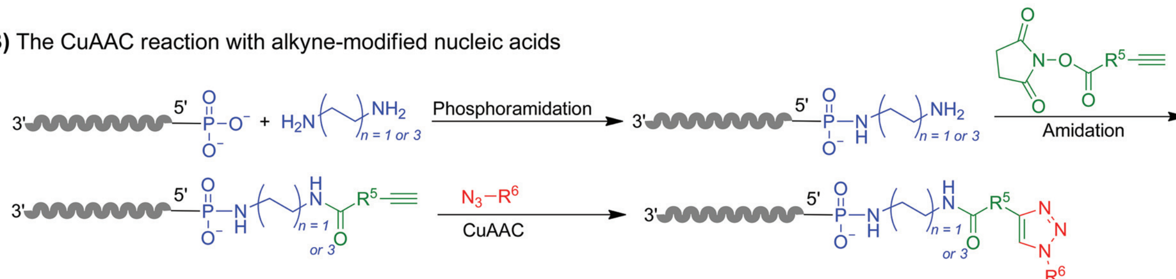
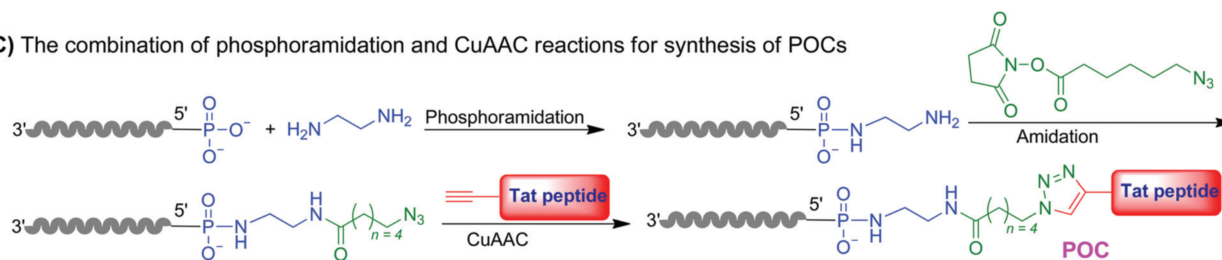
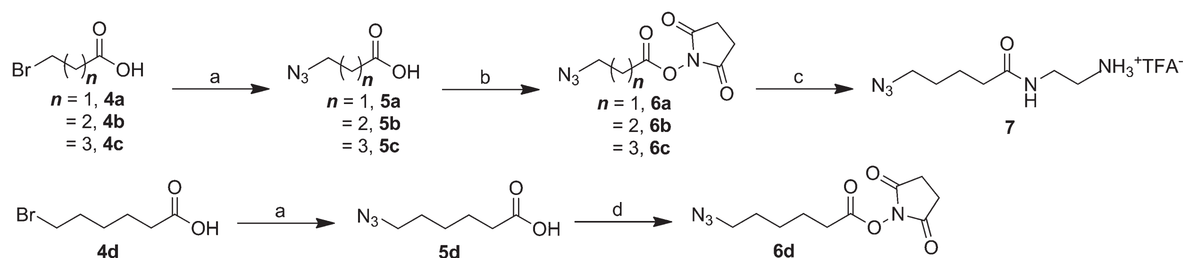






Scheme 2 Phosphoramidation reaction-based modifications of nucleic acids with azides and alkynes for subsequent synthesis of nucleic acid conjugates including peptide–oligonucleotide conjugates (POCs) by the CuAAC and SPAAC reactions. R^3 and R^6, substituents in azides; R^4 and R^5, substituents in alkynes.



Scheme 3 Synthesis of azido carboxylic acid succinimidyl esters (**6**) and an amino derivative (**7**). a: NaN_3, ACN, reflux, 3 h; b: NHS, EDC, DCM, 16 h; c: (1) mono-*t*-Boc-ethylenediamine, Et_3N, DCM, rt, 3 h, (2) TFA, 0 °C, 1 h; d: (1) Flask A: **5d**, DMF, *sym*-collidine, 0 °C, (2) Flask B: NHS, TFAA, *sym*-collidine, 0 °C, (3) mixing solutions in flasks A and B within 1–4 h, 0 °C; (4) rt, 16 h.

molecule biotin (**10** and **12**; Scheme 4) and the fluorophore fluorescein (**15–16**; Scheme 5). Finally, **19** was synthesized (Scheme 6) to enable preparation of alkyne-modified nucleic acids required in the other CuAAC reaction (Scheme 2B).

The optimal CuAAC reactions for azide- and alkyne-modified DNA/RNA derived from the two-step phosphoramidation reactions were effectively developed with all the required reagents at hand. We first determined that **6c** and **6d** were better electrophiles to react with amino-nucleic acids and provided higher yields of azide-modified nucleic acids. For the synthesis of alkyne-modified DNA/RNA, we originally used the commercially available propargylamine (**9**) as the substrate for nucleic acid modifications. The **9**-modified DNA/RNA, however, resulted in sluggish CuAAC reactions with low yield.

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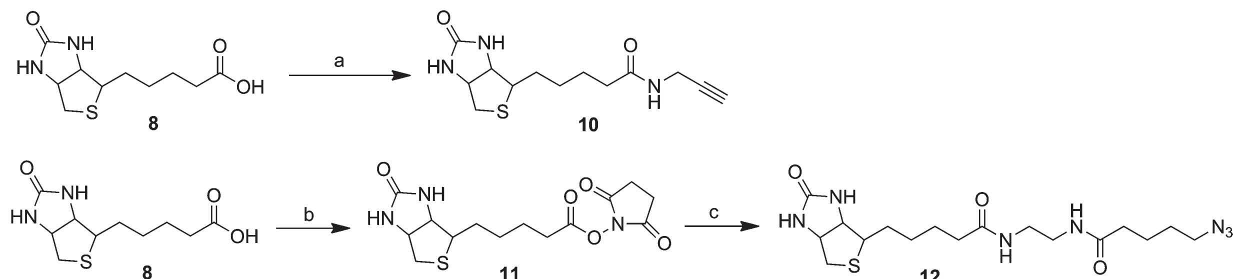
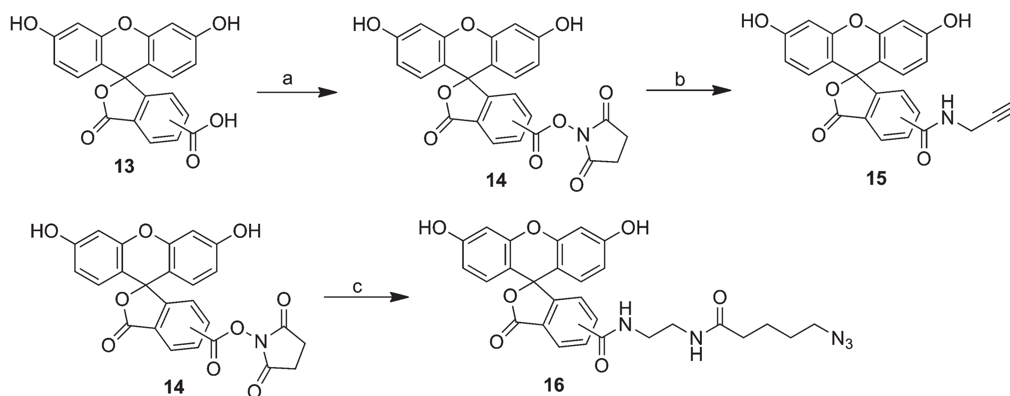
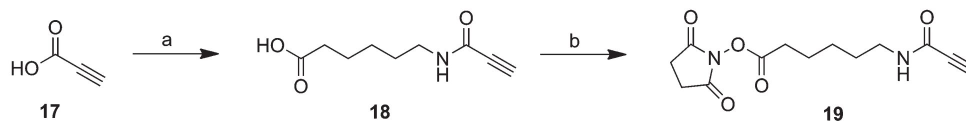


Scheme 4 Synthesis of the alkynyl (**10**) and azido (**12**) derivatives of biotin. a: HOBt, DCC, propargylamine (**9**), DMF, rt, 6 h; b: NHS, DCC, DMF, rt, 16 h; c: **7**, Et_3N, DMF, rt, 16 h.



Scheme 5 Synthesis of the alkynyl (**15**) and azido (**16**) derivatives of fluorescein. a: NHS, DCC, THF, rt, 2 h; b: **9**, Et_3N, THF, rt, 3 h; c: **7**, Et_3N, DCM, rt, 4 h.



Scheme 6 Synthesis of the alkynyl carboxylic acid succinimidyl ester **19**. a: 6-aminocarproic acid, EDC, 1 M Na_2CO_3–DMF, rt, 3 h; b: NHS, EDC, DCM, rt, 12 h.

We envisioned that the reaction yield could be improved by moving the alkynyl group away from the nucleic acids and introducing an electron-withdrawing group adjacent to the alkynyl group. Indeed, when substituting **19** for **9** in nucleic acid conjugates, the CuAAC reactions provided far better yield (results not shown). We then systematically surveyed the effects of nucleic acid concentration, the concentrations of **6c**/**6d** and **19**, copper concentration, THPTA concentration, reaction pH, the azide : alkyne ratio (Fig. S2†), and the Cu : THPTA ratio to obtain the optimal reaction conditions for the conjugation of azide-/alkyne-modified nucleic acids with the corresponding alkyne/azide substrates.

The optimal CuAAC reactions were successfully exploited to synthesize various nucleic acid conjugates. For instance, the azide-modified 3′ primer DNA was effectively conjugated with several alkynyl-containing substrates (Fig. 1A and Scheme 2A). Similar results were acquired using the azide-modified RNA in the optimal CuAAC reactions (Fig. 1B). Moreover, the alkyne-modified 3′ primer DNA was also effectively conjugated with azido-containing substrates by the other optimized CuAAC reactions (Fig. 2A and Scheme 2B). Again, a similar high yield was observed in the optimized CuAAC reaction when azides were reacted with the alkyne-modified 17-mer RNA (Fig. 2B). The presence of biotin and fluorescein moieties in the CuAAC triazole products was confirmed by streptavidin (SAv) gel-shift analysis and fluorescence imaging after electrophoresis (Fig. 2B and S3†). In addition, the modified nucleic acids were gel-purified and analyzed by MALDI-TOF MS to demonstrate the production of the expected DNA and RNA conjugates (ESI†). However, as revealed in Fig. 1 and 2, nucleic acids suffered noticeable degradation in the optimized CuAAC reactions, likely the result of the presence of Cu(I)-derived reactive species. We have ruled out the role of reactive oxygen species to trigger the undesired nucleic acid degradation because similar degradation of nucleic acid was detected when performing the CuAAC in the absence of oxygen (Fig. S4†). Nevertheless, the triazole nucleic acids were clearly the major products in the optimized CuAAC reactions.

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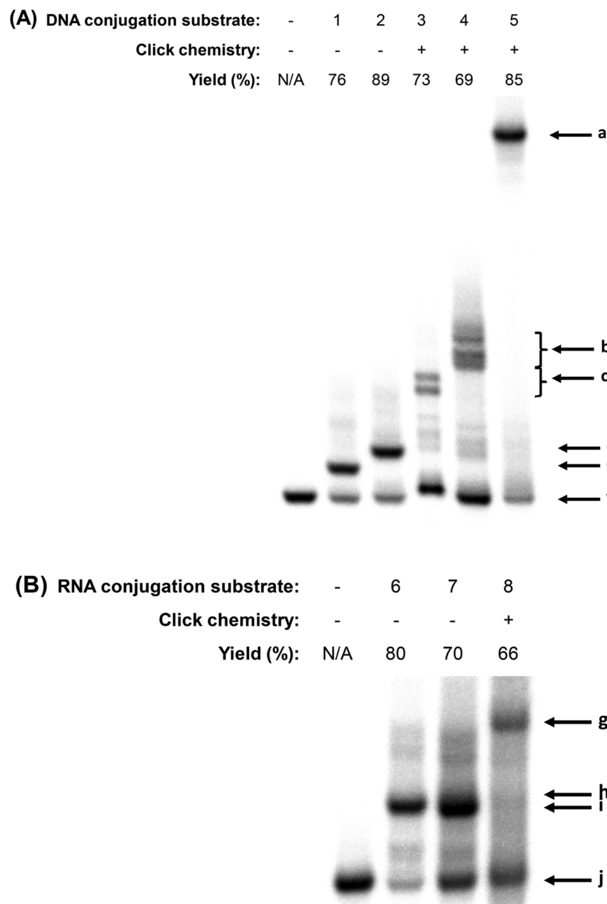

Fig. 1 Azide–alkyne cycloaddition for conjugations of the ^{32}P-labeled azide-modified (A) 3′ primer DNA and (B) 17-mer RNA with alkynyl-containing substrates. (1) Ethylenediamine; (2) ethylenediamine + **6d**; (3) ethylenediamine + **6d** + Alkyne MegaStokes dye 608; (4) ethylenediamine + **6d** + **15**; (5) ethylenediamine + **6d** + the alkynyl Tat peptide; (6) cystamine; (7) cystamine + **6c**; (8) cystamine + **6c** + **15**; (a) the azido-3′ primer DNA-alkynyl Tat peptide conjugate prepared by the CuAAC reaction; (b) the azido-3′ primer DNA-**15** conjugate prepared by the CuAAC reaction; (c) the azido-3′ primer DNA-Alkyne MegaStokes dye 608 conjugate prepared by the SPAAC reaction; (d) the 3′ primer DNA-ethylenediamine-**6d** conjugate; (e) the 3′ primer DNA-ethylenediamine conjugate; (f) the 3′ primer DNA; (g) the azido-17-mer RNA-**10** conjugate prepared by the CuAAC reaction; (h) the 17-mer RNA-cystamine-**6c** conjugate; (i) the 17-mer RNA-cystamine conjugate; (j) the 17-mer RNA.

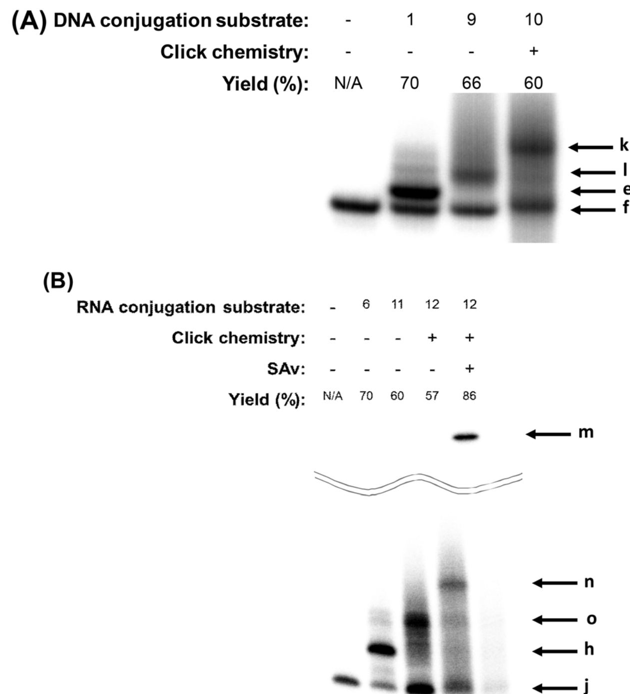

Fig. 2 Azide–alkyne cycloaddition for the ^{32}P-labeled alkyne-modified (A) 3′ primer DNA and (B) 17-mer RNA with azido-containing substrates. (1) ethylenediamine; (6) cystamine; (9) ethylenediamine + **19**; (10) ethylenediamine + **19** + **16**; (11) cystamine + **19**; (12) cystamine + **19** + **12**; (e) the 3′ primer DNA-ethylenediamine conjugate; (f) the 3′ primer DNA; (h) the 17-mer RNA-cystamine-**6c** conjugate; (j) the 17-mer RNA; (k) the alkynyl 3′ primer DNA-**16** conjugate prepared by the CuAAC reaction; (l) the 3′ primer DNA-ethylenediamine-**19** conjugate; (m) the SAv-shifted alkynyl 17-mer RNA-**12** conjugate prepared by the CuAAC reaction; (n) the alkynyl 17-mer RNA-**12** conjugate prepared by the CuAAC reaction; (o) the 17-mer RNA-cystamine-**19** conjugate. It is noted that the area between the wavy lines has been cut off from the original scan because it contains no detectable signals.

As copper-induced nucleic acid degradation can compromise efforts to accurately quantify nucleic acids *in vivo* by the optimized CuAAC reactions, we thus explored the potential of the copper-free SPAAC reaction for nucleic acid modifications. We again employed the two-step phosphoramidation reactions to easily synthesize azido-containing nucleic acids which could react with cyclooctynes in the SPAAC reaction (Scheme 2A). Congruent with the expectation, the cyclooctyne Alkyne MegaStokes dye 608 (DNA conjugation substrate 3 in Fig. 1A) was smoothly labelled to the azide-modified 3′ primer DNA without incurring detectable DNA degradation.[5] The presence of the fluorophore and the structural integrity of the DNA in the SPAAC triazole product were also visualized and confirmed by fluorescence imaging analysis after gel electrophoresis (Fig. S5†). Moreover, the identity of the gel-purified MegaStokes dye 608-modified DNA was affirmed by MALDI-TOF MS analysis (ESI). The results clearly explicate the reactivities of phosphoramidation reaction-derived azido-containing nucleic acids in the CuAAC and SPAAC reactions to provide the desired modified nucleic acids with good yield.

CuAAC for POC synthesis and causing undesirable cytotoxicity

Finally, we employed the optimized CuAAC reaction to more effectively synthesize POCs (Scheme 2C) and demonstrate the cell-penetrating ability of the acquired POCs. Click chemistry has been applied for POC synthesis.[26,27] However, past POC synthesis studies depended on solid-phase phosphoramidite chemistry to afford alkynyl- or amino-containing oligonucleotides and the CuAAC reaction to acquire POCs. Intriguingly, these synthesized POCs were never administered to biological systems nor did they demonstrate cell-penetrating activity. Here we successfully exploited the optimized CuAAC reaction and conjugated the azide-modified FITC-labelled 3′ primer DNA with an alkynyl-containing Tat peptide (DNA conjugation

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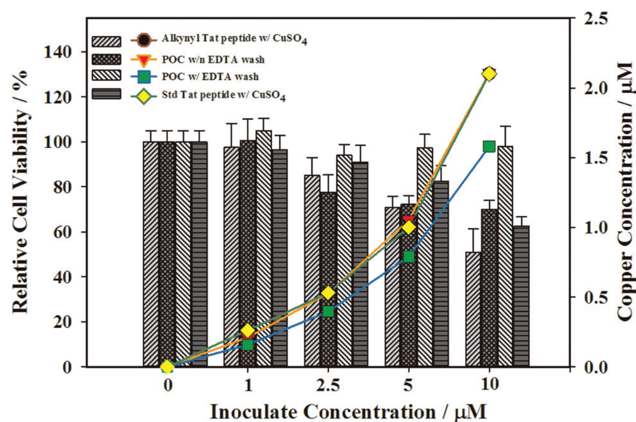

Fig. 3 MTT assays of human A549 cells in the presence of various inoculates under incremental concentrations to reveal the negative correlation of residual copper concentrations in inoculates (as line graph) and cell viability (as bar graph). The MTT assays for viability of A549 cells were performed on the cells previously inoculated with different concentrations of peptide-Cu(II) coordination complexes or the POC containing proportional residual copper concentrations. The copper concentrations in the POC inoculate stocks were determined by ICP-MS, but the copper concentrations in peptide-Cu(II) coordination complexes were estimated according to the assumption that all added free Cu(II) ions were chelated by peptides. Inoculates: alkynyl Tat peptide w/$CuSO_4$, the coordination complex of the alkynyl Tat peptide and $CuSO_4$; POC w/n EDTA wash, the CuAAC-prepared POC (the conjugate of the alkynyl Tat peptide and the azido 3′ primer DNA) purified by 20% urea-PAGE but not washed with EDTA; POC w/EDTA wash, the CuAAC-prepared POC purified by 20% urea-PAGE and further washed with EDTA; std Tat peptide w/$CuSO_4$, the coordination complex of the standard Tat peptide (no alkynyl group) and $CuSO_4$.

substrate 5 in Fig. 1A) with excellent yield (Fig. 1A). However, when inoculating human A549 cells with the gel-purified CuAAC-synthesized POC, significant cell death was observed (Fig. 3). As phosphoramidation bonds and the Tat peptides at the studied concentrations are non-toxic to human cells,[14] the detected cytotoxicity was attributed to a residual amount of copper catalyst in the POC or the newly formed triazole ring in the POC.

We further investigated the role of copper in cell death as copper ions have been documented to trigger nucleic acid modifications and cleavage.[28] We first confirmed the presence of copper in the POC by ICP-MS analysis, and determined that only 3.1% of total copper catalyst [1.91 nmol out of 60 nmol (acquired from 6 times of the scale-up 10× CuAAC reaction)] was retained in the purified POC (1.60 nmol). In addition, similar levels of cell death were observed when inoculating A549 cells with the Tat peptide and Cu(II) in which the concentrations of the peptide and copper were equal to those in the POC (Fig. 3). The results strongly supported that the residual copper from the CuAAC reaction trafficked into the human A549 cells through chelation causing significant cytotoxicity and cell death. Indeed, on extensively washing the POC with EDTA, we removed an additional 26% of copper in the POC and enhanced the viability of the POC-inoculated human A549 cells (Fig. 3). Moreover, we demonstrated translocation of the EDTA-treated POC into human A549 cells after inoculation by flow cytometry (Fig. S6†) and confocal laser scanning microscopy (Fig. 4) even though the deformation of A549 cells was visible (PC in Fig. 4A).

Experimental

Materials and methods

All reagent-grade chemicals were purchased from commercial sources (Sigma-Aldrich, Acros, Alfa Aesar, and Mallinckrodt Baker) except where noted, and were further purified as necessary. The standard Tat peptide (being the 48th to 57th amino acid residues in the Tat protein and having the sequence of H_2N-**GRKKRRQRRR**-COOH; each bold and capitalized letter stand for a specific amino acid residue)[14] and its alkynyl version (amidation with 5-hexynoic acid at the N terminus of the Tat peptide) were purchased from Peptide 2.0 (Chantilly, VA, USA) and used without further purification. THPTA was synthesized according to the method of Hong *et al.*[24] ^1H and ^{13}C NMR spectra were recorded using either a Varian 200 or 400 MHz spectrometer (Varian, Inc., Palo Alto, CA, USA) at Kaohsiung Medical University, Taiwan (KMU). NMR samples were prepared in CD_3OD, D_2O or $CDCl_3$, and the chemical shifts of ^1H signals were given in parts per million downfield from TMS. ^{13}C signals were given in parts per million based on the internal standard of each deuterated solvent. ESI high resolution mass spectra were acquired from Department of Chemistry, National Sun Yat-Sen University, Taiwan (NSYSU-Chemistry) using a Bruker APEX II Fourier-transfer mass spectrometer (FT-MS; Bruker Daltonics Inc., Taiwan). ICP-MS analysis for quantification of copper in POCs was also performed by NSYSU-Chemistry on a PE-SCIEX ELAN 6100 DRC mass spectrometer (PerkinElmer Taiwan, Kaohsiung, Taiwan). Radio-labeled or biotin-/fluorophore-modified nucleic acid conjugates were analyzed by urea polyacrylamide gel electrophoresis (urea-PAGE) or SAv gel shift assay in urea-PAGE, visualized and quantified using an Amersham Typhoon PhosphorImager (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at KMU. Molecular mass of purified nucleic acid conjugates was determined using an Autoflex III TOF/TOF analyzer (Bruker Daltonics) at KMU.[14] POC uptake by human A549 cells was analyzed using a BD FACSCalibur cytometer (BD, Franklin Lakes, NJ, USA) and a FluoView 1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) also at KMU.

Synthesis of *N*-maleoyl amino acid succinimidyl esters (3). Compound **3a** was synthesized according to previously reported procedures.[29] Synthesis of **3b–d** was also performed by following a published method.[30]

Synthesis of azidocarboxylic acid succinimidyl esters (6). Synthesis of **6a** and its precursor 3-azidopropionic acid (**5a**) from 3-bromopropionic acid (**4a**), and synthesis of **6b** from 4-bromobutyric acid (**4b**) were achieved by following the procedures of Grandjean *et al.*[31] The synthesis of **6c** generally adhered to the method of Seo *et al.*[32]

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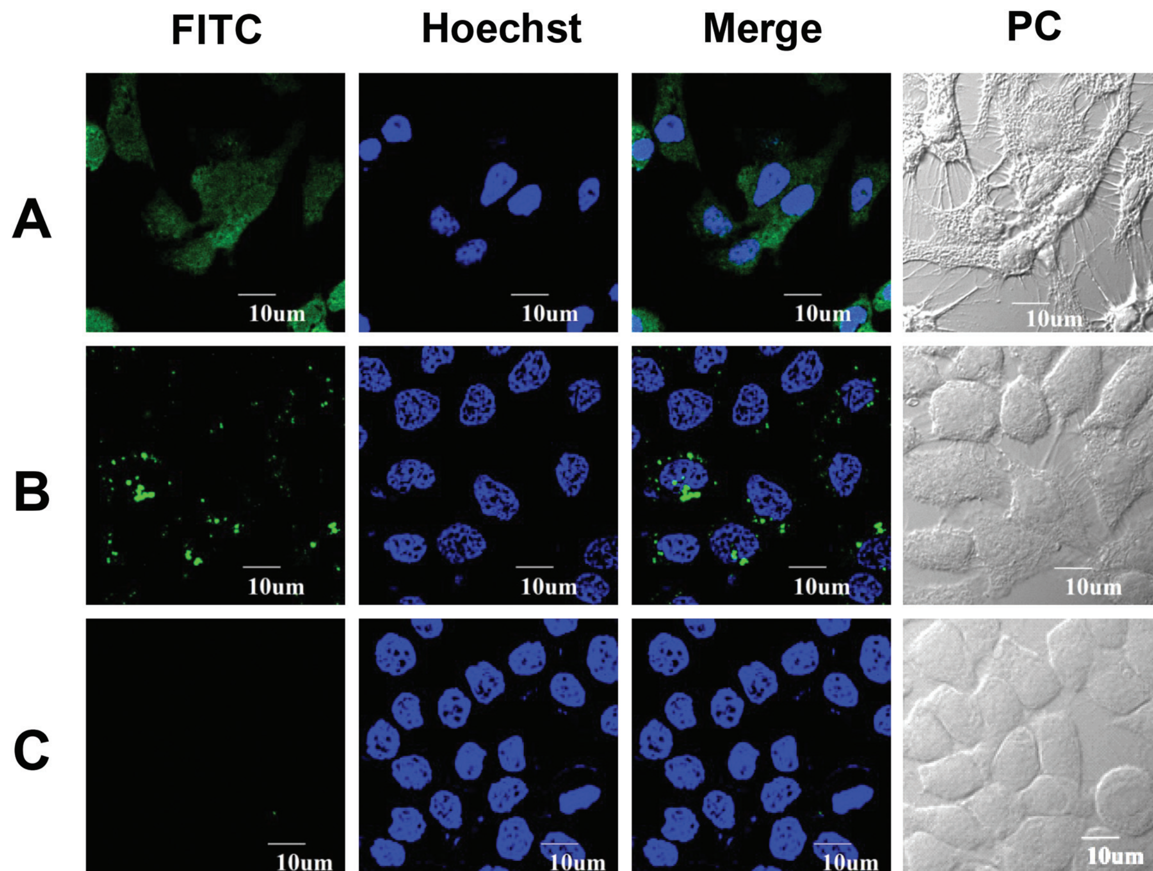

Fig. 4 The combination of phosphoramidation and CuAAC reactions to synthesize POCs successfully taken up by human A549 cell. (A) the alkynyl Tat peptide-azido 3′ primer DNA conjugate (the POC) with FITC labelled to the DNA; (B) the FITC-labelled alkynyl Tat peptide; (C) the FITC labelled 3′ primer DNA. PC, phase contrast.

6-Azido-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (6d). Synthesis of **6d** was achieved by modifying a published method[30] and briefly described below. First, the required 6-azidohexanoic acid (**5d**) was synthesized by dissolving and reacting 6-bromohexanoic acid (**4d**; 3.0 g, 15.4 mmol) with sodium azide (2.0 g, 30.8 mmol) in DMF (10 mL) at 85 °C for 3 h. The resulting reaction mixture was diluted with DCM, extracted with 0.1 N HCl, dried over Na_2SO_4, and concentrated under reduced pressure to give the colorless oil of **5d** (80%).

Without further purification, the acquired **5d** (1.352 g, 8.8 mmol) was dissolved and stirred in a DMF solution (20 mL) submerging in an ice-water bath, followed by slow addition of *sym*-collidine (2.5 mL, 18.5 mmol) in 10 min to give the Flask A solution. Immediately, the Flask B solution was prepared by dissolving NHS (4.048 g, 35.2 mmol) in the other DMF solution (20 mL) also immersed in an ice-water bath, followed by slow addition of TFAA (4.93 mL, 35.2 mmol) while stirring for 10 min, and finally adding *sym*-collidine dropwise (4.66 mL, 34.5 mmol) for 10 min to give the solution. The Flask B solution was then slowly dripped into the Flask A solution for 1.5 h while maintaining both solutions at 0 °C. The resulting mixture was returned to rt and stirred overnight. The final reaction mixture was diluted with DCM (60 mL), extracted with 1 N HCl (50 mL) three times, dried over Na_2SO_4, concentrated under reduced pressure, and further washed with Et_2O (40 mL) three times to afford the white-colored solid **6d** (2.14 g, 76%). ^1H NMR (400 MHz) ($CDCl_3$) δ: 3.30 (t, 2H), 2.84 (br s, 4H), 2.64 (t, 2H), 1.79 (q, 2H), 1.68–1.60 (m, 2H), 1.55–1.48 (m, 2H). ^{13}C NMR (100.67 MHz) ($CDCl_3$) δ: 169.1, 168.4, 51.0, 30.7, 28.3, 25.8, 25.5, 24.1. HRMS (ESI) calculated for $C_{10}H_{14}N_4O_4$, $[M + Na]^+$ 277.09073 (calcd), 277.09081 (found).

Synthesis of 2′-aminoethyl 5-azido-pentanamide, TFA salt (7). A reaction mixture for synthesis of **7** was prepared by dissolving **6c** (0.29 g, 1.2 mmol) in DCM (2 mL) first and followed by addition of Et_3N (0.16 mL, 1.16 mmol) and mono-*t*-Boc-ethylenediamine (0.24 g, 1.5 mmol)[14] to the DCM solution. After reacting at rt for 7 h, the final reaction mixture was diluted with DCM, and extracted with 1 N HCl, 5% $NaHCO_3$ and sat. NaCl, sequentially. The resulting organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to give the Boc-protected **7** which was deprotected by dissolving in 1 mL of TFA while stirring at 0 °C for 1 h, removing TFA under reduced pressure, and washing with Et_2O in sequence to afford the colorless oil-like **7** (0.17 g, 75%). ^1H NMR (400 MHz) ($CDCl_3$) δ: 3.32 (t, 2H), 2.41 (t, 2H), 1.78–1.60 (m, 4H).

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^{13}C NMR (100.67 MHz) ($CDCl_3$) δ: 178.1, 51.0, 33.2, 28.3, 28.2, 21.8. HRMS (ESI) calculated for $C_7H_{16}N_5O$, $[M + H]^+$ 186.13494 (calcd), 186.13490 (found).

Synthesis of biotin derivatives, 10 and 12

*5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid prop-2-ynylamide (**10**).* The method of Poole *et al.*[33] was adopted to synthesize **10**.

*5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid [2-(5-azido-pentanoylamino)-ethyl]-amide (**12**).* The reaction mixture for synthesis of **12** was prepared by dissolving **7** (0.098 g, 0.58 mmol), Et_3N (82 μL, 0.58 mmol) and (+)-biotin *N*-hydroxysuccinimide ester[13] [**11**; 0.19 g, 0.55 mmol; synthesized from (+)-biotin (**8**)] in 5 mL of DMF while stirring at rt for 5 h. The final reaction mixture was concentrated under reduced pressure, recrystallized in IPA, and washed with EA to give the white-colored solid **12** (0.371 g, 80%). ^{1}H NMR (400 MHz) (CD_3OD) δ: 4.49 (1H, dd), 4.31 (1H, dd), 2.93 (1H, dd), 2.70 (1H, d), 1.60–1.31 (4H, m), 1.25 (2H, q). ^{13}C NMR (100.67 MHz) (CD_3OD) δ: 176.4, 176.1, 63.4, 61.6, 57.0, 52.1, 47.9, 41.0, 40.1, 36.8, 36.5, 29.8, 29.5, 29.4, 26.8, 24.1. HRMS (ESI) calculated for $C_{17}H_{29}N_7O_3$, $[M + Na]^+$ 434.1950 (calcd), 434.1947 (found).

Synthesis of fluorescein derivatives, 15 and 16

*5(6)-(N-Propargyl)amidofluorescein (**15**).* 5(6)-Carboxyfluorescein (**13**; 1.13 g, 3 mmol) was first dissolved in 10 mL of THF and followed by slow addition of a 10 mL THF solution containing *N*-hydroxysuccinimide (0.414 g, 3.6 mmol) and DCC (0.70 g, 3.6 mmol). The resulting reaction mixture was stirred at rt for 2 h, concentrated under reduced pressure, and resuspended in pentane (5 mL) to precipitate the orange-red-colored 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester (**14**; 0.145 g, 0.3 mmol). Without further purification, the acquired **14** was dissolved in 10 mL THF, and followed by addition of propargylamine (**9**; 39 μL, 0.6 mmol) and Et_3N (45 μL, 0.32 mmol) to initiate the reaction at rt for 3 h. The final reaction mixture was concentrated under reduced pressure, redissolved in EA (10 mL) and extracted with 1 N HCl, water and sat. NaCl, sequentially. The afforded organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to give **15** (0.08 g, 59%). ^{1}H NMR (400 MHz) (CD_3OD) δ: 8.01 (2H, s), 7.68 (1H, s), 7.33 (2H, dd), 6.58–6.57 (4H, m), 4.13 (2H, d), 2.59 (1H, t). ^{13}C NMR (100.67 MHz) (CD_3OD) δ: 181.4, 160.5, 135.7, 132.3, 131.2, 130.6, 129.7, 129.2, 129.0, 123.7, 113.6, 72.2, 34.8. HRMS (ESI) calculated for $C_{24}H_{15}NO_6$, $[M + Na]^+$ 436.0797 (calcd), 436.0795 (found).

*5(6)-[N-(5-Azido-N′-ethylpentamido)]amidofluorescein (**16**).* Synthesis of **16** started from crude **14** which was synthesized as described above and also used without further workup. A reaction mixture for synthesis of **16** was prepared by dissolving crude **14** (260 mg, 0.55 mmol), **7** (0.98 mg, 0.58 mmol) and Et_3N (82 μL, 0.58 mmol) in a 6 mL DCM–DMF (5 : 1) solution while stirring at rt for 4 h. The final reaction mixture was concentrated under reduced pressure, redissolved in DCM (20 mL) and extracted with 1 N HCl, water and sat. NaCl, sequentially. The resulting organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to give **16** (0.08 g, 61%). ^{1}H NMR (400 MHz) (CD_3OD) δ: 8.47 (s, 1H), 8.11 (s, 1H), 7.63 (s, 1H), 7.32 (d, 1H), 6.69 (t, 4H), 6.60 (d, 1H), 6.58 (t, 2H), 6.56 (d, 1H), 4.17–4.04 (m, 2H), 2.35 (t, 2H), 2.27–2.22 (m, 2H), 1.70–1.56 (m, 2H). ^{13}C NMR (100.67 MHz) (CD_3OD) δ: 103.8, 71.1, 66.5, 64.0, 52.1, 36.5, 34.9, 33.1, 30.8, 29.4, 26.7, 26.0. HRMS (ESI) calculated for $C_{28}H_{25}N_5O_7$, $[M + Na]^+$ 566.16462 (calcd), 566.16482 (found).

Synthesis of 6-propynoylamino-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (19). Propiolic acid (**17**; 98.4 μL, 1.6 mmol) was activated in an EDC (0.33 g, 1.6 mmol)-dissolved DMF solution (2 mL) at 0 °C while stirring for 15 min, and followed by adding a 6-aminohexanoic acid (0.212 g, 1.61 mmol)-containing 1 M Na_2CO_3–DMF mixture (1 M Na_2CO_3–DMF = 1 : 2; 3 mL) and reacting at rt for 3 h. The final reaction mixture was diluted with DCM, extracted with water twice and sat. NaCl once. The resulting organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to give light orange-colored oily crude **18** (0.183 g, 1 mmol). Without further purification, the obtained **18** was dissolved in a DCM solution (5 mL) containing EDC (0.24 g, 1.2 mmol) and followed by addition of NHS (0.18 g, 1.2 mmol) to allow reaction at rt while stirring for 12 h. The final reaction mixture was diluted with DCM, extracted with water twice and sat. NaCl once. The afforded organic phase was again dried over Na_2SO_4 and concentrated under reduced pressure to provide golden-colored **19** (0.31 g, 82%). ^{1}H NMR (400 MHz) (D_2O) δ: 3.11 (t, 2H), 3.08–2.98 (m, 4H), 2.79 (s, 4H), 2.69 (s, 1H), 1.84–1.77 (m, 2H), 0.98 (t, 2H). ^{13}C NMR (100.67 MHz) (D_2O) δ: 176.7, 174.5, 160.7, 55.4, 42.8, 36.5, 35.1, 25.5, 25.3, 25.1, 14.5. HRMS (ESI) calculated for $C_{13}H_{16}N_2O_5$, $[M + Na]^+$ 303.09514 (calcd), 303.09503 (found).

Nucleic acid preparation and radio-labeling

The single-stranded 3′ primer DNA (5′-TACCCCTTGGGG-ATACCACC-3′) was purchased from Purigo Biotech, Inc., Taiwan and purified by 20% urea-PAGE. The $TW17_{1-17}$ RNA (5′-GGGAUCGUCAGUGCAUU-3′), which is the first 17 nucleotides in the TW17 RNA,[34] was purchased from Bioneer (Daejeon, South Korea) and used without purification. Both the 3′ primer DNA and the $TW17_{1-17}$ RNA were ^{32}P-labeled at the 5′-end according to the previously reported methods.[14]

Modifications of nucleic acids with azides and alkynes

DNA. As prepared in *N*-hydroxysuccimide esters, either azides **6** (Scheme 3) or alkynes (**9** and **19**; Scheme 6) were covalently linked to the ethylenediamine-modified 3′ primer DNA by the amidation reaction reported previously.[14] The ethylenediamine-conjugated 3′ primer DNA was synthesized according to the optimized two-step phosphoramidation reaction of DNA in which ethylenediamine was the nucleophile in the reaction.[12,14] Acquired DNA conjugates were purified by ethanol precipitation and analyzed by 20% urea-PAGE.

RNA. Similarly, *N*-hydroxysuccimide esters of either azides (**6**) or alkynes (**9** and **19**) were coupled to the cystamine-modified $TW17_{1-17}$ RNA by the same amidation reaction for DNA

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modifications indicated above.[14] The cystamine-conjugated $TW17_{1-17}$ RNA was also prepared by the optimized two-step phosphoramidation reaction of RNA in which cystamine served as the nucleophile in the reaction.[14] Acquired RNA conjugates were again purified by ethanol precipitation and analyzed by 20% urea-PAGE.

Copper-catalyzed 1,3-dipolar azide–alkyne cycloaddition for nucleic acid modifications

The previously reported CuAAC reaction[24] was further studied to modify nucleic acids with appropriate substrates and to attain the best yield. The acquired optimal CuAAC reactions were briefly stated below. Either azido- or alkynyl-modified DNA/RNA (80 pmol) and the corresponding alkynyl- or azido-containing molecules (1.6 nmol) were dissolved in 8.85 μL phosphate buffer (100 mM potassium phosphate, pH 7.0) and followed by addition of 0.15 μL of a $CuSO_4$-THPTA premix solution (prepared by mixing one part of 20 mM $CuSO_4$ in water and two parts of 50 mM THPTA in water), 0.5 μL of 100 mM aminoguanidine, and 0.5 μL of 100 mM fresh-prepared sodium ascorbate in sequence to give the final reaction mixture of 10 μL. After the reaction at rt for 1 h, the reaction products were purified by ethanol precipitation and analyzed by 20% urea-PAGE.

In addition, the optimal CuAAC reaction was scaled up ten times to more efficiently synthesize enough quantities of the Tat peptide-3′ primer DNA conjugate (a POC) for bioactivity studies of the POC in human A549 cells. The scale-up CuAAC reaction (10×) generally gave the triazole product yield similar to that of the optimal CuAAC reaction (1×).

Copper-free strain-promoted 1,3-dipolar azide–alkyne cycloaddition for the modification of azido nucleic acids with the cyclooctyne substrate

The method of Winz *et al.*[5] was studied to modify the azido nucleic acids with the cyclooctyne Alkyne MegaStokes dye 608 and was briefly described below. The azide-conjugated DNA/RNA (80 pmol) was dissolved in 76 μL of phosphate buffer (50 mM potassium phosphate, pH 7.0) to give an 1 μM nucleic acid solution. The SPAAC reaction was initiated by adding in 0.4 μL of the Alkyne MegaStokes dye 608 (10 mM in DMSO) and proceeded at 35 °C for 2 h. The final reaction products were purified by ethanol precipitation and analyzed by 20% urea-PAGE.

Cytotoxicity by MTT assay

The cytotoxicity of inoculates was determined by MTT assays against A549 cells. In brief, A549 cells were seeded in 96-well tissue culture plates at a density of 5×10^3 per well in a medium containing 10% FBS before treating inoculates. The cytotoxicity of inoculates was evaluated by determining cell viability after 24 h of incubation with various concentrations of inoculates (1–10 μM). The number of viable cells was acquired by estimating their mitochondrial reductase activity using the tetrazolium-based colorimetric method (MTT conversion test).[35]

Flow cytometry analysis of cellular uptake

In order to observe the cellular uptake efficiency of inoculates, A549 cells were seeded in 6-well culture plates at a density of 2×10^5 per well in a medium containing 10% FBS for 24 h. The medium containing 5 μM concentration of inoculates was added to cells. After 24 h of incubation, cells were washed, trypsinized, centrifuged, and resuspended in 1 mL of cold PBS, and then analyzed using the flow cytometer. The fluorescein-labeled inoculates (peptide, DNA and POC) used in flow cytometry and confocal laser scanning microscopy were prepared according to the published methods[14] and the method developed in the current study.

Confocal laser scanning microscopy (CLSM)

The intracellular delivery of inoculates was observed using CLSM. A549 cells were seeded at a density of 1.0×10^5 per well in 12-well plates containing one glass coverslip per well in RPMI supplemented with 10% FBS, and then incubated for 24 h. Each inoculate of 5 μM was added to cells for 24 h at 37 °C. After incubation, the inoculate-containing medium was removed and washed gently with 1 mL of 0.1 M PBS at pH 7.4. The cell nuclei was then stained with 5 μg mL^{-1} Hoechst 33342 (Invitrogen, Carlsbad, CA) for 30 min. The cells on the coverslips were washed 3 times with 0.1 M PBS and mounted with a fluorescence mounting medium on glass slides. Cell imaging was obtained by CLSM (Fv 1000; Olympus, Tokyo, Japan) and analyzed using the Olympus CLSM software.

Discussion

In summary, a novel strategy consisting of versatile two-step phosphoramidation reactions and the powerful CuAAC and SPAAC reactions enabled effective and selective modifications of post-synthetic DNA and RNA, and has produced various nucleic acid conjugates including a POC. The POC was effectively trafficked into human cells with the potential to alleviate the delivery challenge of therapeutic nucleic acids in clinical applications. The combination of the phosphoramidation and azide–alkyne cycloaddition reactions thus establishes a universal regioselective approach to modify any 5′ phosphate-primed nucleic acids with diverse molecules ranging from proteins and peptides to lower molecular mass tags and probes. The effective modification strategy will facilitate administration, detection and quantification of nucleic acids *in vivo* and *in vitro*. However, the results of this study have raised concerns about the use of the CuAAC reaction for synthesis of bioconjugates such as POCs destined to be delivered into biological systems. Residual copper chelated to the bioconjugates may cause damage and reduce cell viability. Ongoing research is in progress to extend azide–alkyne cycloaddition for more effective synthesis of modified nucleic acids with bioactivity not adversely affected by a residual copper catalyst.

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