Biomolecular Synthesis

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Access to Biomolecular Assemblies through One-Pot Triple Orthogonal Chemoselective Ligations**

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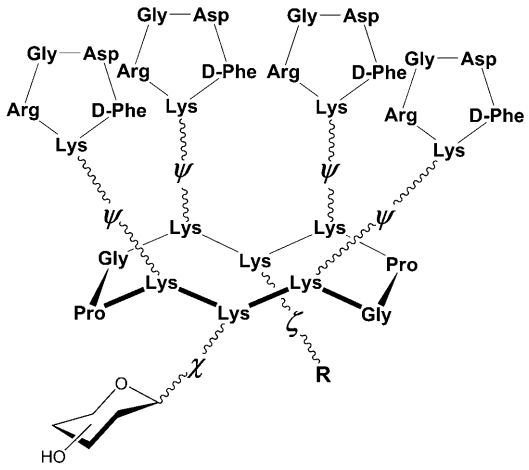
The adequate combination of chemoselective ligations is essential to have access to sophisticated biomolecular assemblies, thereby circumventing the problems associated with the incompatible chemistries of carbohydrates, peptides, and nucleic acids. In this regard, recent advances in the field of chemoselective ligation have proven to be particularly interesting for the construction of large biomolecular systems for biological applications.[1] Since the use of a single chemoselective ligation limits the design complexity of a macromolecule, new methods for the ligation of multiple biomolecular partners are required. The development of chemoselective ligations of unprotected peptides enables the routine chemical synthesis of proteins.[2] Very recently, Carell et al. have used the copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC)[3] to label nucleic acids with three different tags.[4] A critical point is the requirement of orthogonal protecting groups and purification steps. Ideally, sophisticated biomolecular assemblies can be generated by a combination of orthogonal chemoselective ligations. Recently, we described the combination of CuAAC and orthogonal oxime bond formation, which allows the one-pot synthesis of peptide conjugates.[5] Other groups have reported the combination of two orthogonal chemoselective ligations for the synthesis of neoglycopeptides,[6] and for the labeling of peptides.[7] Access to more sophisticated biomolecular compounds remains a challenging task.

Herein, we report that this chemistry can be extended to prepare sophisticated compounds encompassing a diversity of biomolecules, such as peptides, sugars, and nucleic acids (Scheme 1). The way to achieve such molecular assemblies is to use one-pot triple orthogonal chemoselective ligations through the combination of the CuAAC reaction, oxime ligation, and thioether bond formation. This methodology enables the ligation of up to four different molecular fragments without the requirement of protection schemes and/or tricky intermediate purifications. To illustrate this strategy, we constructed multifunctional Arg-Gly-Asp (RGD)-containing biomolecular systems encompassing a carbohydrate residue along with a molecular fragment that can be used for diagnostics (fluorescent probe) or for therapy (peptide or nucleic acids; see Scheme 2). The benefit of the RGD-containing cyclodecapeptides for tumor targeting was previously demonstrated,[8] and recent research efforts have shown that additional incorporation of a carbohydrate moiety within the RGD-containing compounds provided an enhanced compound solubility and clearance.[9]



Scheme 1. Structure of the RGD-containing biomolecular system. ψ: triazole, oxime; χ: oxime, thioether; ζ: thioether, triazole; R: peptide, nucleic acid, or dye.

To test the feasibility of our approach we first prepared a cyclodecapeptide scaffold **1** containing aldehyde, alkyne, and maleimide groups, which allowed the fully convergent synthesis of the RGD-containing neoglycopeptides **2** (Scheme 2a). To introduce aldehyde and alkyne functions, we incorporated the building blocks *N*-Fmoc-Lys[CO-$(CH_2)_2$-C≡CH] (Fmoc = 9-fluorenylmethoxycarbonyl) and *N*-Fmoc-Lys[*N*-Boc-Ser(O-*t*Bu)] (Boc = *tert*-butoxycarbonyl) into the decapeptide chain by using standard solid-phase peptide synthesis (SPPS; see the Supporting Information). These building blocks are very important because they considerably reduce the number of steps involved and the combination of protecting groups required for the synthesis of the functionalized peptides. As the maleimide function reacts rapidly with piperidine, which is commonly used during SPPS, the thiol-reactive maleimide group was consequently added at a lysine side chain after SPPS (see the Supporting Information). In parallel, the cyclopentapeptide cyclo[-Arg-Gly-Asp-DPhe-Lys(-CO-CH_2-N_3)-] **3** bearing the prerequisite azide function was prepared through a combination of solid- and solution-

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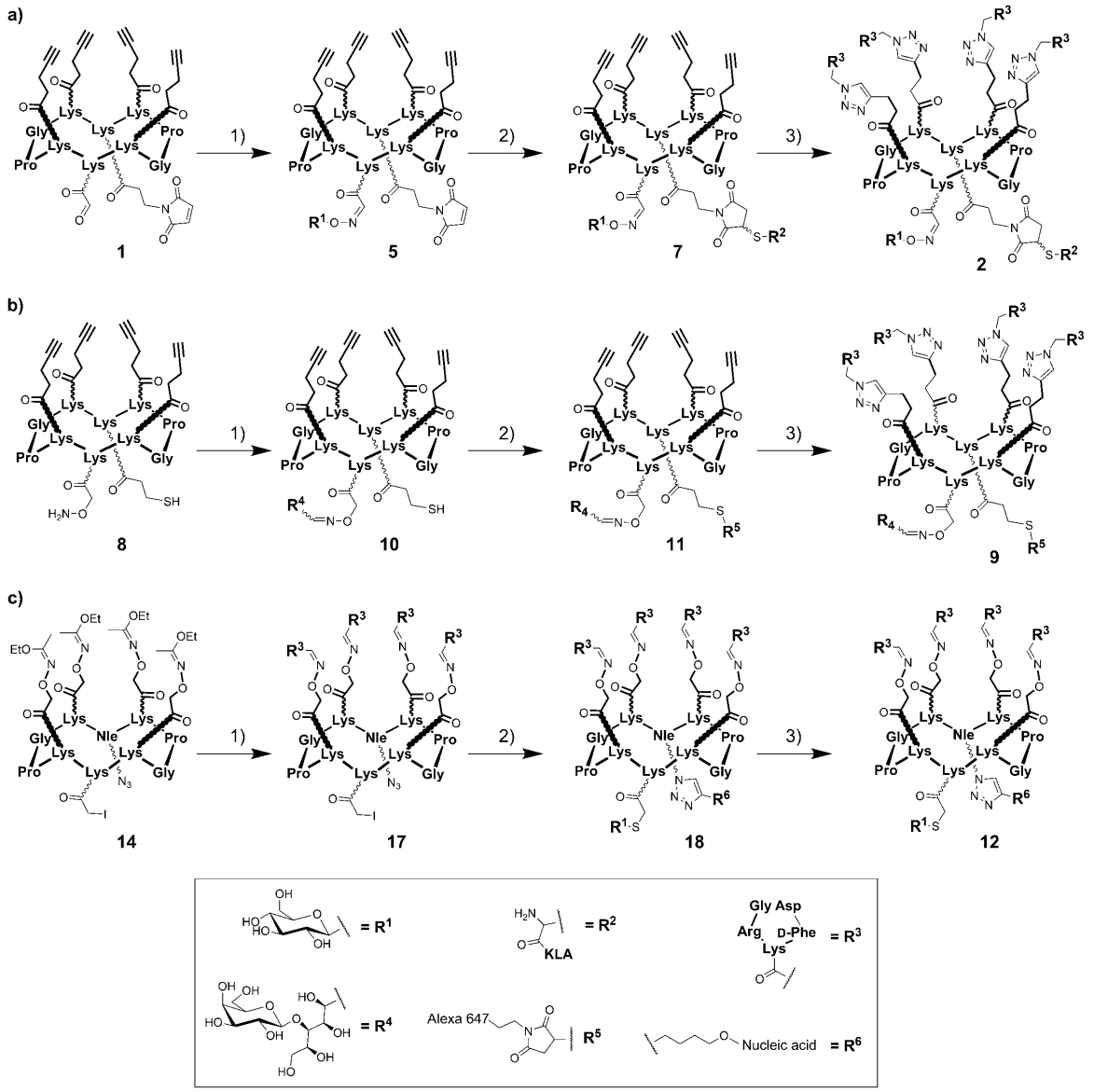

Scheme 2. a) Reagents and conditions for the synthesis of compounds **2**: 1) R^1-ONH_2 **4** (3 equiv), CH_3CN/PBS (pH 2.2, 40:60), 1 h; 2) R^2-SH **6** (1.3 equiv), pH adjusted to 7 using $NaHCO_3$, 30 min; 3) R^3-CH_2-N_3 **3** (6 equiv), Cu^0 (5 equiv), *t*BuOH. b) Reagents and conditions for the synthesis of compounds **9**: 1) lactose (5 equiv), CH_3CN/ammonium acetate buffer (pH 4.5, 40:60), 50 °C, 5 h; 2) Alexa Fluor 647 C2-maleimide (1.3 equiv), pH adjusted to 7 using $NaHCO_3$, 30 min; 3) R^3-CH_2-N_3 **3** (6 equiv), Cu^0 (5 equiv), *t*BuOH. c) Reagents and conditions for the synthesis of compounds **12**: 1) R^3-CHO **15** (5 equiv), TFA/CH_3CN/H_2O (5:45:50), 1 h; 2) pH adjusted to 7 using $NaHCO_3$, R^1-SH **16** (2 equiv), 30 min; 3) R^6-C≡CH **13** (0.8 equiv), Cu^0 (5 equiv), *t*BuOH, 50 °C, 5 h. PBS = phosphate-buffered saline, TFA = trifluoroacetic acid.

phase syntheses.[5] Glc-β-ONH_2 **4** was prepared by glycosylation of *N*-hydoxysuccinimide using glycosyl fluoride as donor and BF_3·Et_2O as promoter.[10]

With the trifunctional compound **1** in hand, we then carried out the one-pot sequential chemoselective ligations of the different biomolecules (Scheme 2a). We decided to perform the CuAAC after oxime ligation and thiol addition as the aldehyde residue is not stable under the CuAAC conditions,[9] and oxidation of thiopeptides was observed in the presence of copper. Chemoselective ligations were carefully monitored by HPLC analysis (Figure 1). Addition of Glc-β-ONH_2 **4** (3 equiv) to the aldehyde-containing cyclopeptide **1** was carried out under acidic conditions providing successfully within 1 hour the glycopeptide intermediate **5** (Figure 1a). The pH was then adjusted to neutral conditions and KLA peptide **6**[11] (1.3 equiv) bearing an N-terminal cysteine residue was added to the reaction mixture. Rapid thiol addition was observed at room temperature providing within 30 minutes the intermediate **7** (Figure 1a). It is worth mentioning that under neutral or acidic conditions, the addition of the remaining aminooxy-carbohydrate **4** (ca. 2 equiv) to the maleimide moiety was not detected. The subsequent third chemoselective ligation was carried out by adding Cu^0 microsize powder (5 equiv)[12] and azido RGD-peptide **3** (1.5 equiv per site) to the reaction mixture. The alkyne–azide cycloaddition furnished the expected RGD-

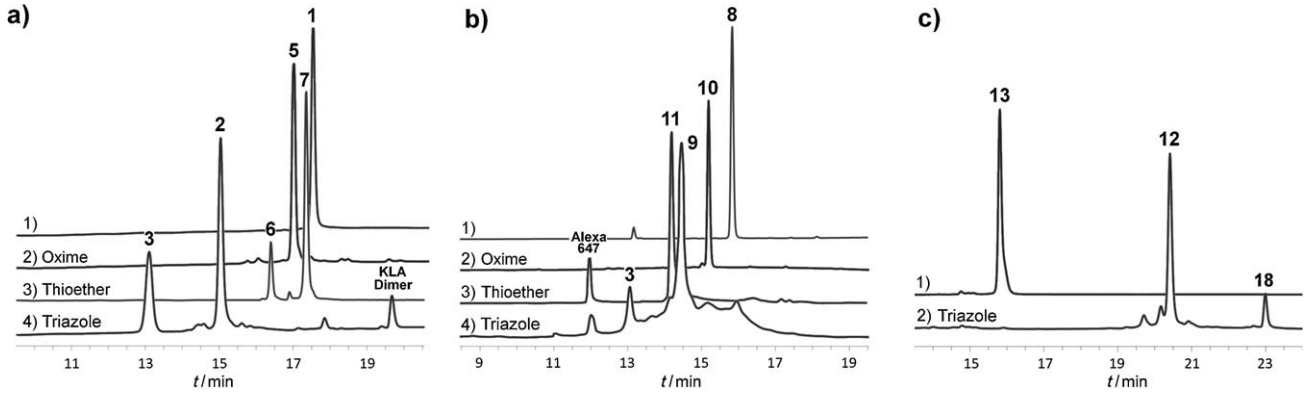

Figure 1. a) Chemoselective assembly of compound **2**. HPLC profiles (214 nm) for 1) scaffold **1**, and for 2) oxime ligation of carbohydrate **4**, 3) thioether addition of peptide **6**, and 4) CuAAC of peptide **3**. b) Chemoselective assembly of compound **9**. HPLC profiles (214 nm) for 1) scaffold **8**, and for 2) oxime ligation of lactose, 3) thioether addition of Alexa Fluor 647, and 4) CuAAC of peptide **3**. c) Chemoselective assembly of compound **13**. HPLC profiles (260 nm) for 1) free oligonucleotide **13** and 2) CuAAC of oligonucleotide **13** to intermediate **18**.

containing neoglycopeptide **2** in yields of about 55% after purification. During CuAAC, KLA dimer that results from the oxidation of the residual thiopeptide **6** was detected.

To allow the grafting of a reducing sugar, we decided to exploit an alternative one-pot approach allowing the synthesis of the fluorescent neoglycopeptide **9** (Scheme 2b). We then prepared the scaffold **8** containing aminooxy, alkyne, and thiol groups. These functions are easily incorporated within the peptide during the SPPS (see the Supporting Information). *N*-Fmoc-Lys[-CO-CH_2-O-N=C-CH_3(OEt)] including 1-ethoxyethylidene (Eei) as protecting group for the aminooxy moiety was preferred because the Eei group is fully compatible with standard SPPS conditions.[13] Starting with the functionalized scaffold **8**, we first performed the ligation of lactose (Scheme 2b). Oxime formation was achieved with exclusive specificity under mild acid conditions after 5 hours at 50 °C (Figure 1b). Taking note of a recent report,[14] further investigation revealed that the aniline Schiff base (100 mM) decreased the reaction time to 1 hour. However, this reagent was found to be incompatible with the next ligations. Rapid aniline addition to maleimide was observed. The resulting glycopeptide **10** was subsequently converted to the fluorescent intermediate **11** simply by adjusting the pH to neutral conditions and by adding the maleimide-containing Alexa fluorescent probe. After 30 minutes, the orthogonal thiol addition to maleimide was complete (Figure 1b). The last ligation of the azido RGD-peptide **3** and the alkyne-containing intermediate **11** furnished the desired triply modified compound **9**. The fluorescent compound **9** was readily purified by reversed-phase HPLC methods and characterized by mass spectrometry (see the Supporting Information).

To demonstrate the broad utility of our method, we decided to extend the scope of this approach to nucleic acids. A last strategy was then adopted for the synthesis of the biomolecular conjugate **12** (Scheme 2c). Numerous works have demonstrated the effectiveness of the CuAAC reaction for oligonucleotide conjugation.[15] For this reason, we synthesized the alkyne-containing oligonucleotide **13**[16] by using the commercially available hexyn-5-yl phosphoramidite. In parallel, the scaffold **14** encompassing the complementary azido function was prepared by means of *N*-Fmoc-ε-azidonorleucine. The latter was obtained from the diazo transfer of imidazole-1-sulfonyl azide to *N*-Fmoc-lysine.[17] Aminooxy functions and the iodoacetamide group were chosen to graft respectively the homing peptide cyclo[-Arg-Gly-Asp-DPhe-Lys(-CO-CHO)-] **15** and the β-D-thioglucose **16**. With the scaffold **14** in hand, we then carried out the one-pot sequential chemoselective ligations of peptide **15**, sugar **16**, and oligonucleotide **13** (Scheme 2c). As the nucleic acids are sensitive to acidic conditions, we first performed simultaneous Eei removal and oxime ligation of the aldehyde-containing peptide **15**.[13] The resulting RGD peptide intermediate **17** was then converted to the neoglycopeptide **18** under neutral conditions (see the Supporting Information). Finally, the ligation of the oligonucleotide **13** and the intermediate **18** was obtained through CuAAC (Figure 1c). Reversed-phase HPLC methods furnished the expected biomolecular compound **12** in yields of about 60%. This result emphasizes the utility of this method for the synthesis of complex biomolecular assemblies.

In conclusion, we have shown for the first time that consecutive combination of three chemoselective reactions could be carried out in a one-pot approach. This strategy allows efficient and rapid syntheses of biomolecular compounds without intervening isolations and protection schemes. We believe that this strategy should pave the way for multifunctionalized macromolecular systems with tailor-made properties. To design immunoactive glycoconjugates,[18] we are currently implementing this strategy for the construction of a new generation of synthetic vaccine prototypes combining multiple cancer antigens within the same molecule. Immunological evaluation will be reported in due course.

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