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Ribosomal Synthesis of Polypeptoids and Peptoid−Peptide Hybrids

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Peptoids are artificially designed synthetic oligomers composed of N-substituted glycine building blocks.[1] It has been shown that peptoids exhibit unique pharmacokinetic properties, different from ordinary peptides, with greater protease resistance and membrane permeability being attributed to the lack of protons on the amide bond.[2] The N-substituted groups represent the secondary structural diversity of peptoids like peptide sidechains, allowing for construction of libraries and leading to the discovery of aptamers (or inhibitors) against therapeutic targets.[3] However, peptoids have intrinsic poor plasticity to form a variety of 3D structures, and therefore the 3D structural diversity in the peptoid library would be limited. Alternatively, a peptoid−peptide hybrid would be an attractive framework to improve the plasticity perhaps without sacrificing the major pharmacokinetics of the peptoid. However, such an approach to date relied on a semirational design of hybrids based on an active peptide followed by their activity validation,[4] giving only a limited number of successes. Clearly, a new synthetic strategy of peptoids or peptoid−peptide hybrids, which is readily extendable to their diverse library construction and screening (or selection) of active ligands, is awaited for the discovery of potent molecules. We here report a novel method to express polypeptoids and peptoid−peptide hybrids by means of translation machinery under the reprogrammed genetic code, allowing for the mRNA-directed synthesis of such oligomers with linear and cyclic scaffolds.

Despite the fact that *N*-methyl-α-amino acids are difficult to efficiently incorporate without prematurely truncated products or oligomerize into a nascent peptide chain using the classical amber suppression method,[5] we and other groups recently found that nearly a dozen *N*-methyl-α-amino acids derived from proteinogenic or nonproteinogenic amino acids could be incorporated by means of translation machinery using genetic code reprogramming.[6] Success, at least in our case, relied on the integration of two systems. One is a reconstituted *E. coli* cell-free translation system in which certain proteinogenic amino acids and/or cognate aminoacyl-tRNA synthetases are withdrawn (referred to as *withdrawn* PURE system; *w*PURE), allowing us to diminish the competing background incorporation of the proteinogenic amino acids in the translation elongation event.[7] The other is a system involving artificial tRNA acylation ribozymes, referred to as flexizymes.[8] This has greatly facilitated the preparation of a wide variety of tRNAs charged with nonproteinogenic amino acids.[6g,8c,9] During our studies, we found the flexizyme system was able to charge N-substituted glycines (rGly) onto tRNAs efficiently (Supporting Information, Figure S1). We thus became interested in investigating if rGly could be incorporated into a nascent peptide chain under the reprogrammed genetic code.

To investigate the incorporation efficiencies of rGly into a peptide chain by translation machinery, we chose a systematic set of rGly bearing various *N*-substituted groups (Figure 1A). The N-substituted groups were divided into three families, (1) nonbranched alkyl chains (**1**−**8**), (2) branched alkyl chains (**9**−**14**), and (3) functionalized alkyl chains (**15**−**30**). These rGly were derived to 3,5-dinitrobenzyl esters or cyanomethyl esters, making them compatible to the flexizyme system,[8c] and then charged onto suppressor tRNA$^{Asn\text{-}E2}_{GGU}$[9a] (Figure S1). The individual rGly-tRNA$^{Asn\text{-}E2}_{GGU}$ were added to a *w*PURE system lacking threonine (Thr) and surveyed for their single incorporation into a model peptide upon

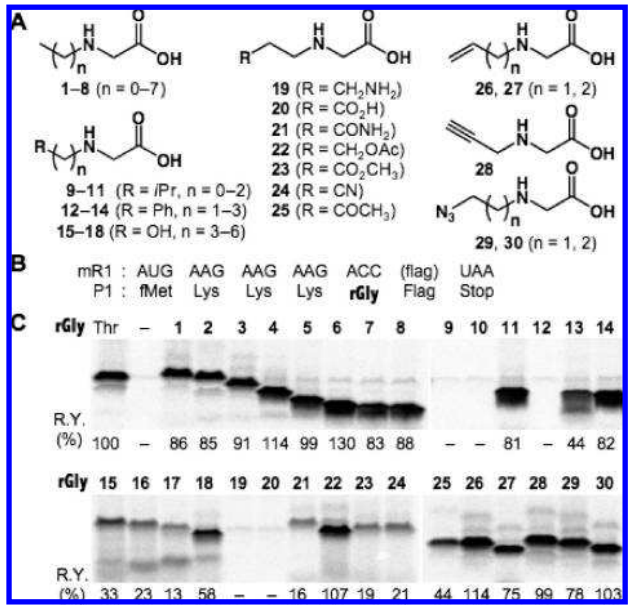

Figure 1. Tolerance of N-substituted glycines (rGly) in ribosomal peptide synthesis. (A) Chemical structure of rGly used in this study. **1**, *N*-methylglycine (***N*me**); **2**, *N*-ethyl-glycine (***N*et**); **3**, *N*-*n*-propyl-glycine (***N*pr**); **4**, *N*-*n*-butyl-glycine (***N*bu**); **5**, *N*-*n*-pentyl-glycine; **6**, *N*-*n*-hexyl-glycine; **7**, *N*-*n*-heptyl-glycine; **8**, *N*-*n*-octyl-glycine; **9**, *N*-isopropyl-glycine; **10**, *N*-i-butyl-glycine; **11**, *N*-isopentyl-glycine; **12**, *N*-benzyl-glycine; **13**, *N*-(2-phenylethyl)-glycine; **14**, *N*-(3-phenylpropyl)-glycine; **15**, *N*-(3-hydroxypropyl)-glycine; **16**, *N*-(4-hydroxybutyl)-glycine; **17**, *N*-(5-hydroxypentyl)-glycine; **18**, *N*-(6-hydroxyhexyl)-glycine; **19**, *N*-(3-aminopropyl)-glycine; **20**, *N*-(2-carboxylethyl)-glycine; **21**, *N*-(2-carbamoylethyl)-glycine; **22**, *N*-(3-acetoxypropyl)-glycine (***Naco***); **23**, *N*-[2-(methoxycarbonyl)ethyl]-glycine; **24**, *N*-(2-cyanoethyl)-glycine; **25**, *N*-(3-oxobutyl)-glycine; **26**, *N*-allyl-glycine (***N*all**); **27**, *N*-3-butenyl-glycine; **28**, *N*-propargyl-glycine; **29**, *N*-(2-azidoethyl)-glycine; **30**, *N*-(3-azidopropyl)-glycine. (B) Sequences of mRNA (mR1) that express an rGly-containing-peptide (P1). Flag in parentheses indicates the RNA sequence encoding the Flag peptide (DYKDDDDK). (C) Tricine-SDS-PAGE analysis of the expressed peptides labeled with [^{14}C]-Asp detected by autoradiography. Lane 1, the wildtype peptide expressed in the Thr-containing ordinary PURE system where ACC assigns Thr; lane 2, a negative control using Thr-withdrawn PURE (*w*PURE) system in the presence of uncharged tRNA$^{Asn\text{-}E2}_{GGU}$; lanes 3−32, expression of the peptide containing a single rGly in the presence of designated rGly-tRNA$^{Asn\text{-}E2}_{GGU}$ prepared by flexizyme system. Each expression yield (R.Y.) relative to wildtype was determined by a mean score of triplicates.

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suppression of the Thr ACC codon in the designed mRNA sequence (Figure 1B, mR1 and P1). Tricine-SDS-PAGE analysis of the resulting [^{14}C]-Asp-labeled peptides showed that the first family of rGly bearing a nonbranched alky chain were all incorporated as efficiently as Thr (Figure 1C, **1**−**8**). The incorporation of the designated rGly was also confirmed by MALDI-TOF analysis, giving the expected molecular mass in all cases (Figure S2).

In the second family, three rGly (Figure 1C, **11**, **13**, and **14**) were efficient elongators similar to those in the first family, whereas the other three (Figure 1C, **9**, **10**, and **12**) having the branched methyl or phenyl group closer to the α-amino group were not. Most rGly in the third family exhibited acceptable incorporation efficiencies (Figure 1C, **15**−**18** and **21**−**30**), in which the MALDI-TOF was consistent with the expected molecular mass (Figure S2). The exceptions were those with a negatively or positively charged alkyl group (Figure 1C, **19** and **20**). In summary, our survey has shown that a wide variety of rGly, in which the *N*-substituent has no bulky group near the α-amino group and no charged functional group, act as elongators for translation machinery. This observation is similar to that for *N*-methyl-α-amino acids in which bulky or charged side chains were unfavorable elongators;[6g] a short list of unfavorable elongators recently reported by Zhang et al. also fell into in this trend, where *N*-substituted α-amino acids were bulky near the α-amino group due to the combination between a N-substituent and α-carbon side chain.[6f] Therefore, this seems to be a common trend for N-substituted α-amino acids.

We next attempted consecutive elongations of rGly to express a short stretch of polypeptoid sequences fused with a KK-Flag peptide tag to facilitate its isolation. In this study, we chose three rGly, ***N*et**, ***N*aco**, and ***N*all** (Figure 1A, **2**, **22**, and **26**) that were assigned to arbitrarily chosen ACC, UUC, and CUC codons, respectively, with aid of the flexizyme system. Five mRNA templates (Figure 2A, mR2−6) were designed to elongate the above rGly in succession ranging from 2 to 6 times (Figure 2A, P2−P6). We also expressed peptides (Figure 2A, wt2−6) from the respective mRNA templates using the ordinary PURE system to compare the expression level of peptoids. Tricine-SDS-PAGE analysis of [^{14}C]-Asp-labeled peptoids and peptides indicated that the expression level of peptoids gradually decreased with increasing peptoid length (lanes 2, 4, 6, 9, and 11), in contrast to the observation that peptides were expressed at a nearly consistent level independent from the length (Figure 2B, lanes 1, 3, 5, 8, and 10). Although this observation is somewhat similar to the successive elongation of *N*-methyl-α-amino acids,[6g] the expression level of peptoids was hampered more seriously by an increase in length. In fact, when we expressed a tetra-*N*-methyl-peptide using the mR4 template (Me4, see the box in Figure 2A and B), the expression level of Me4 was nearly double that of P4 (Figure 2B, lane 6 vs 7). Both ribosome and EF-Tu may have an effect on their elongation efficiencies.[10] In particular, peptoidyl elongation may be attributed to steric hindrances of the peptoidyl-tRNA in ribosome peptidyl-tRNA (P) site or/and the rGly-tRNA in aminoacyl-tRNA (A) site, probably making the peptoidyl elongation less efficient than *N*-methyl-peptidyl elongation.

To demonstrate the expression of designer peptoids, we prepared two sequence variants of the tetrapeptoid P4 (Figure 3A, P4a and P4b) and expressed them from the respective mRNA templates (Figure 3A, mR4a and mR4b). The expression levels of these tetrapeptoids were similar to each other, and MALDI-TOF analysis of each peptoid showed a single major peak that is consistent with its expected molecular mass according to the corresponding mRNA templates (Figure 3B). To the best of our knowledge, this result

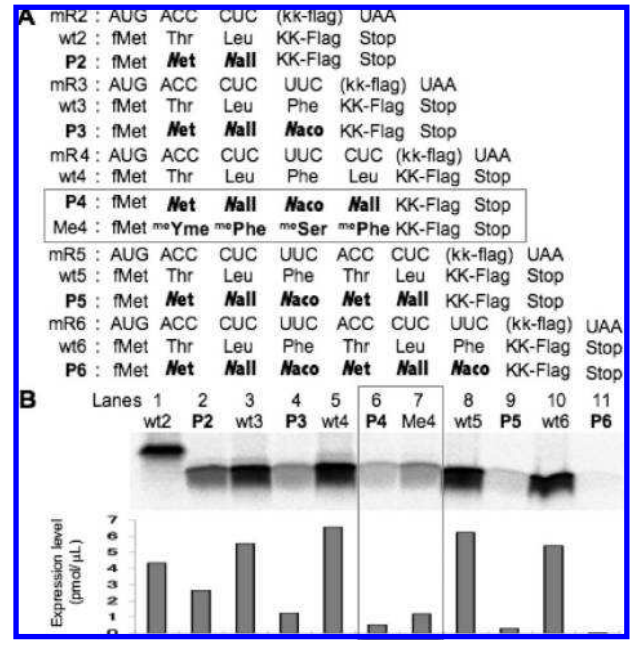

Figure 2. mRNA-programmed synthesis of di-, tri-, tetra-, penta-, and hexapeptoid. (A) Sequences of mRNA templates (mR2−6), control wildtype peptides (wt2−6), a tetra-*N*-methyl-peptide (Me4), and peptoids containing ***N*et**, ***N*all**, and ***N*aco** (P2−6). ^{me}Yme, *N*-methyl-L-*p*-methoxyphenylalanine; ^{me}Phe, *N*-methyl-L-phenylalanine; ^{me}Ser, *N*-methyl-L-serine. (B) Tricine-SDS-PAGE analysis of control wildtype peptides, a tetra-*N*-methyl-peptide, and peptoids expressed from the respective mRNA. The wildtype peptides were expressed in the ordinary PURE system, while peptoids were expressed in the *w*PURE system containing ***N*et**-tRNA$^{Asn\text{-}E2}_{GGU}$, ***N*all**-tRNA$^{Asn\text{-}E2}_{GAG}$, and ***N*aco**-tRNA$^{Asn\text{-}E2}_{GAA}$. Expression level of each peptide labeled with [^{14}C]-Asp based on its observed radioisotope counts is shown in the graph.

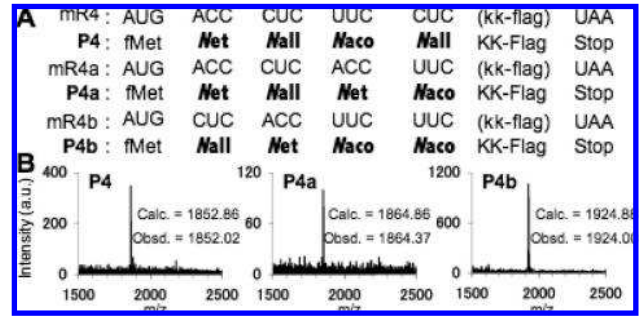

Figure 3. mRNA-programmed synthesis of linear tetrapeptoids (A) Sequences of mRNA templates (mR4, mR4a, and mR4b) and encoded linear peptoids containing ***N*et**, ***N*all**, and ***N*aco** (P4, P4a, and P4b). The kk-flag in parentheses indicates the RNA sequence encoding a KK-Flag peptide (KKDYKDDDDK). The expression level of each tetrapeptoid was estimated by tricine-SDS-PAGE as follows: P4, 0.52 pmol/μL; P4a, 0.37 pmol/μL; P4b, 0.51 pmol/μL. (B) MALDI-TOF-MS spectra of the Flag-purified translation products. The calculated mass (Calc) and observed mass (Obsd) for singly charged species $[M+H]^{+1}$ are shown in each spectrum.

records the first demonstration of mRNA-directed expression of polypeptoids using the translation machinery.

Due to the above success, we extended our methodology to the ribosomal synthesis of peptoid−peptide hybrids. With a future application to drug discovery in mind, we attempted to express peptoid−peptide hybrids with a framework in which peptoid bonds are scattered in cyclic peptides closed by a nonreducible thioether bond.[9b] We reprogrammed the genetic code in which CGC, ACC, CAC, and UCC were assigned to ***N*me**, ***N*et**, ***N*pr**, and ***N*bu** (Figure 1A, **1**−**4**), respectively, for the peptoid synthesis and AUG was

assigned to *N*-chloroacetyl-α-^{L}Phe or *N*-chloroacetyl-α-^{D}Phe[9d] (ClAc-^{L}Phe or ClAc-^{D}Phe) for the formation of cyclic scaffolds (Figure S3B). Four mRNA templates (Figure S3A, mR7−10) were designed to express cyclic peptoid−peptide hybrids with different sequence compositions attached to the C-terminal KK-Flag (Figure 4A, P7−P10). MALDI-TOF analysis of each cyclic peptoid−peptide

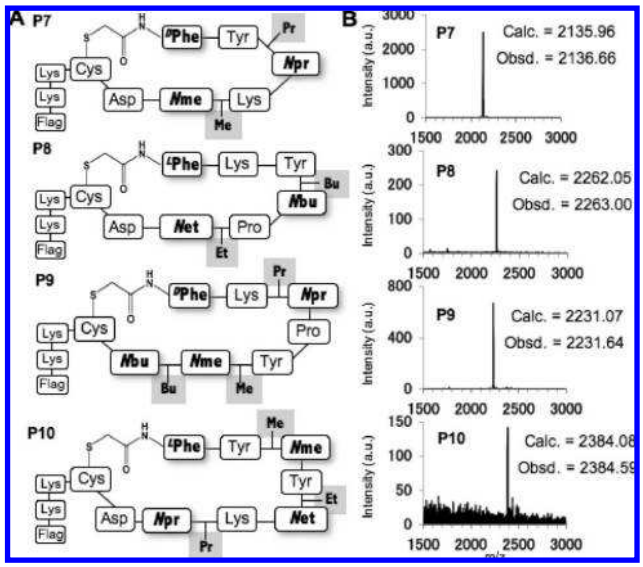

Figure 4. mRNA-programmed synthesis of cyclic peptoid−peptide hybrids. (A) Schematic structure of cyclic peptoid−peptide hybrids containing ***N*me**, ***N*et**, ***N*pr**, and ***N*bu** (P7−10). The locations of *N*-substituted groups on the peptoid bond are highlighted in gray. The expression level of each peptoid−peptide hybrid was estimated by tricine-SDS-PAGE as follows: P7, 1.97 pmol/μL; P8, 2.63 pmol/μL; P9, 0.21 pmol/μL, and P10, 0.84 pmol/μL. (B) MALDI-TOF-MS spectra of the Flag-purified translation products. The calculated mass (Calc) and observed mass (Obsd) for singly charged species $[M+H]^{+1}$ are shown in each spectrum.

hybrid expressed from the respective mRNA template exhibited the expected molecular mass as a single major peak (Figure 4B, P7−P10). This firmly demonstrates the mRNA-directed expression of cyclic peptoid−peptide hybrids under the reprogrammed genetic code.

In conclusion, we have demonstrated mRNA-directed synthesis of linear polypeptoids and cyclic peptoid−peptide hybrids. The translation machinery surprisingly accepts a variety of rGly for elongation, including those with alkyl chains and functional groups that are orthogonal[7d,9c,11] to proteinogenic side chains, such as the ketone, alkene, azide, and alkyne. We have shown consecutive elongations of rGly and synthesis of cyclic peptoid−peptide hybrids. Particularly, the latter hybrids contain two or more peptoid bonds dispersed in the sequence and consist of cyclic structures closed by a physiologically stable thioether bond. Since we have already shown that the thioether cyclization is spontaneously formed and applicable to a variety of ring sizes independent from the sequence compositions of peptide,[6g,9b,c,12] the methodology reported herein offers us a new means of the mRNA-programmed library synthesis of cyclic peptoid−peptide hybrids. This has already directed our current research to running screenings or selections of a new class of peptidic drugs against therapeutic targets by the integration with appropriate techniques.[13] Such efforts are underway in our laboratory.

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Supporting Information Available: Experimental details and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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