RNA-Peptide Conjugation through an Efficient Covalent Bond Formation

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Abstract: Many methods for modification of an oligonucleotide with a peptide have been developed to apply for the therapeutic and diagnostic applications or for the assembly of nanostructure. We have developed a method for the construction of receptor-based fluorescent sensors and catalysts using the ribonucleopeptide (RNP) as a scaffold. Formation of a covalent linkage between the RNA and the peptide subunit of RNP improved its stability, thereby expanding the application of functional RNPs. A representative method was applied for the formation of Schiff base or dihydroxy-morpholino linkage between a dialdehyde group at the 3′-end of sugar-oxidized RNA and a hydrazide group introduced at the C-terminal of a peptide subunit through a flexible peptide linker. In this report, we investigated effects of the solution pH and contribution of the RNA and peptide subunits to the conjugation reaction by using RNA and peptide mutants. The reaction yield reached 90% at a wide range of solution pH with reaction within 3 h. The efficient reaction was mainly supported by the electrostatic interaction between the RNA subunit and the cationic peptide subunit of the RNP scaffold. Formation of the RNP complex was verified to efficiently promote the reaction for construction of the RNA-peptide conjugate.

Keywords: ribonucleopeptide (RNP); RNA-peptide conjugate; Schiff base; aptamer; fluorescent sensors

1. Introduction

RNA and peptide conjugates have been constructed for versatile therapeutic application, such as the delivery of siRNA [1–3] or the screening of a library of peptides in a mRNA display method [4,5]. Efficient reaction for the formation of covalent linkage between RNA and peptide is important for developing practical applications. Many methods have been reported to conjugate an oligonucleotide (RNA or DNA) with an oligopeptide by applying various chemistries [6–9]. Post-synthetic coupling of the respective oligonucleotide and peptide fragments is the common and reliable method to construct oligonucleotide-peptide conjugates because the stepwise solid-phase synthesis of the conjugates encounters difficulties in finding the compatible protecting groups for both nucleobases and amino-acid side chains. Several chemical reactions were applied for coupling the nucleotide and peptide, such as amidation [10,11], disulfide bond formation [12–14], the modification of the thiol group by haloacetyl [15,16], or the maleimide group [12,17,18], native chemical ligation [19–21], and click reaction [22,23]. These reactions are certainly useful for the selective coupling of oligonucleotides and oligopeptides. However, these reactions often associate with drawbacks, such as the instability of the linkage, the necessity for introducing additional chemical groups into both the oligonucleotide and the oligopeptide chains by multistep reactions, limitation for the sequence of oligopeptide due to the solubility or reactivity of the side chains, and/or the potential formation of the side products and the stereoisomers. In some cases, even applying the long reaction time, the yields of the product were
rather low. One of the alternative methods for the modification of nucleotide with functional molecules
was based on incorporating the aldehyde group into the nucleotide. Oxidation of the sugar moiety by
perchloric acid or periodate is one of the common methods for introducing the aldehyde group on DNA
or RNA [24–27]. By coupling the aldehyde group with alkoxyamine, cysteine, and hydrazine, formation
of the covalent linkage through oxime, thiazolidine, and hydrazine (or a morpholine-like structure),
respectively, has been reported [27–29]. These reactions proceed in mild conditions in a relatively short
time to provide high coupling yields in aqueous solution. While this strategy also displays some of
the limitations, as mentioned above, it has the advantage of having no need for the introduction of
unnatural nucleic acids into oligonucleotide and facile preparation of the reactive peptide.

We have developed a stepwise method for the construction of receptor-based fluorescent sensors by
using ribonucleopeptide (RNP) as a scaffold (Figure 1A). In this method, a complex of Rev peptide and
Rev Responsive Element (RRE) RNA [30] was utilized to construct the RNP library with a randomized
RNA sequence in the RNA subunit. RNP receptors [31] for a target molecule were selected from the
RNP library by applying the in vitro selection method [32,33]. The peptide subunit of the selected RNP
receptors was further modified with a fluorophore to construct a fluorophore-modified RNP receptor
(F-RNP) library [34–42]. By screening the F-RNP library, fluorescent RNP sensors showing measurable
fluorescent intensity changes upon binding the substrate were selected. Many kinds of RNP receptors
and sensors were constructed for various target molecules, such as ATP [34–38,41,42], GTP [34,35,41],
dopamine [39], and a tetra peptide containing a phosphorylated tyrosine residue [40]. Furthermore, the
RNA subunit and the peptide subunit of fluorescent RNP sensors were covalently linked to improve
the chemical and thermal stability [41]. The covalently linked RNP (c-RNP) sensors were applicable
for simultaneous detection of multiple target molecules in the solution. Time-course monitoring of
the concentration changes of the substrate and product in an enzymatic reaction was demonstrated
by simultaneous application of sensors for the substrate and the product [41,42]. As mentioned
above, for the covalent bond formation with the aldehyde group, the ribose moiety at the 3′-end of
RNA was oxidized by sodium periodate to a dialdehyde group (Figure 1B). A hydrazide group was
introduced at the C-terminal of peptide subunit through a ten-amino-acid flexible linker. Coupling of
the RNA dialdehyde group and the C-terminal hydrazide group of peptide formed a covalent linkage
between the RNA and peptide subunits of the Rev-RRE complex. This reaction proceeded rapidly in
a quantitative yield. It is likely that a proximity effect between the reactive groups on the RNA and
peptide subunit originated from the complex formation of RNP to assist the efficient coupling reaction.
Here, we investigated several conditions for covalent linkage formation between the dialdehyde group
of RNA and the hydrazide group of peptide within the ribonucleopeptide scaffold to elucidate the effect
of solution pH for the reaction yield. Furthermore, the role of proximity effect in the coupling reaction
was investigated by using mutants of RNA and peptide. Our results demonstrated that the efficient
coupling reaction within the RNP was indeed the outcome of the proximity effect of reactive groups
and provided an optimal condition for the construction of the covalently linked RNA-peptide complex.
2. Materials and Methods

PrimeSTAR HS DNA polymerase for PCR reactions was obtained from TaKaRa Bio Inc. (Shiga, Japan), and the T7-Scribe Standard RNA IVT Kit was obtained from CELLSSCRIPT (Madison, WI, USA). N-α-Fmoc-protected amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), distilled N,N-dimethylformamide (DMF), and 4-Hydroxymethylbenzoic acid-polyethylene glycol (PEG) resin (HMBA-PEG resin) were obtained from Sigma-Aldrich, Japan (T7 RNA promoter is underlined). N,N-diisopropylethylamine (DIEA), diisopropylcarbodimide (DIC), and 2',4',6'-trihydroxyacetophenone monohydrate (THAP) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). N,N-dimethyl-4-aminopyridine (DMAP), sodium periodate, hydrazine monohydrate, gel electrophoresis grade acrylamide, bisacrylamide, phenol, thioanisole, and 1,2-ethanedithiol were purchased from Wako Chemicals (Tokyo, Japan). Diammonium hydrogen citrate (DAHC) was obtained from Nacalai Tesque (Kyoto, Japan). A reversed-phase C18 column ULTRON VX-ODS (For analysis: 4.6 × 150 mm; For purification: 20 × 250 mm, particle size 5 µm) was purchased from Shinwa Chemical Industries (Kyoto, Japan).

2.1. Preparation of RNA

Forward and Reverse primers for the construction of the double-stranded template DNA for An16 RNA (5’-TCTAATACGACTCACTATAGGGTGTTGCCGCA-3’ and 5’-GGCCTGTACCGTCGAGGCTTCAGCTTCG-3’) and for scrAn16 RNA (5’-TCTAATACGACTCACTATAGGGTGTTGCCGCA-3’ and 5’-CACAC AACCGCCCGGGC-3’) were purchased from Sigma–Aldrich, Japan (T7 RNA promoter is underlined). The double-stranded DNA templates and RNAs were prepared as previously described [42]. RNA subunits of RNP receptors were purified by means of denaturing polyacrylamide gel
electrophoresis (8 M urea, 12%). The concentrations of purified RNA were quantified by measuring the absorption at 260 nm \((\text{An}16 = 439,500; \text{scrAn}16 = 438,800 \, \text{M}^{-1} \text{cm}^{-1})\).

2.2. Construction of a Covalently Linked RNP

The peptide subunit for the formation of a covalent linkage (Ac-TRQARRRRRWRERQRGGSPEGSG-HZ) was synthesized as follows. A HMBA-PEG resin was placed in a dry flask, and a sufficient amount of DMF was added to soak the resin; this mixture was allowed to swell for 30 min. \(N\)\(\alpha\)-Fmoc-glycine (10 equivalent relative to resin loading) dissolved in DMF was mixed with a solution of DIC (5 equivalent relative to resin loading) in dry DMF on ice and then incubated for 20 min. The solution of an activated first amino acid was added to the resin prepared above. A DMF solution of DMAP (0.1 equivalent relative to resin loading) was added to the resin/amino acid mixture and incubated at room temperature for 1 h with occasional swirling. This procedure of coupling the first amino acid residue was repeated twice. The subsequent synthesis was performed on an automated peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan) according to the Fmoc chemistry protocol using protected Fmoc-amino acids and HBTU. Acetylation of the \(N\)-terminal of peptide was performed by mixing with 1 M acetic acid anhydride and 1 M 1-methyl imidazole in DMF for 1 h at room temperature. The following procedures, cleavage of the protected peptide from the resin with hydrazine monohydrate, deprotection of the protected peptide, and purification of the peptide were performed as described previously [41]. The synthesized peptides were characterized by MALDI-TOF mass spectrometry (AXIMA-LNR, Shimadzu), as follows: Acetylated Rev peptide hydrazide (Ac-Rev-(GGS)\(_3\)G-HZ), \(m/z\) 3154.9 (calcd for \([M+H]^+\) 3155.5); Acetylated flexible peptide linker hydrazide (Ac-(GGS)\(_3\)G-HZ), \(m/z\) \([M+H]^+\) 735.3, \([M+Na]^+\) 757.8 (calculated for \([M+H]^+\) 734.3).

Crosslinking reaction of the RNA and the peptide subunits of RNP was carried out as described previously [41,42] with a slight modification of the conditions. Freshly prepared 0.01 M sodium periodate (5 µL; 50 nmol; 50 equiv) was added to 200 µM RNA (5 µL; 1 nmol) in 15 µL of 0.03 M sodium acetate (pH 5.2), and the reaction mixture (25 µL) was incubated for 1 h at 37 °C in the dark. After the reaction, 2.5 µL of 10 M glycerol was added to the reaction mixture to reduce an excess amount of sodium periodate. The resulting oxidized RNA was purified by ethanol precipitation. A coupling reaction between the 3′-modified RNA (40 µM) and Ac-Rev-(GGS)\(_3\)G-HZ (88 µM) was performed in 0.03 M sodium acetate (for pH 4 and 5) or 0.03 M sodium phosphate (for pH 6 and 7), containing 0.01 M NaCl (total 25 µL) at 37 °C in the dark. The reaction mixture was extracted by phenol/chloroform and purified by ethanol precipitation to remove unreacted peptide, then dissolved in TE (25 µL).

2.3. Evaluation of the Reaction Yields of Covalently-Linked RNPs

Denaturing polyacrylamide gel electrophoresis (PAGE) (8 M urea, 12%) was performed to separate the unreacted RNA and covalently linked RNP by loading the same volume of the purified samples. A total of 10 pmol of RNA was loaded on the gel as the control of RNA band mobility. The above stock solution of phenol/chloroform extracted RNP (25 µL) was further diluted with TE to 200 µL. From this RNP solution, 2 µL was analyzed by PAGE for each reaction. The acrylamide gel was stained with ethidium bromide to detect RNA and RNP. The yield of each reaction was evaluated from the ratio of intensity of bands corresponding to unreacted RNA and RNP. Actual isolation yields of RNP ranged from 30 to 40% after the PAGE purification and successive purification by ethanol precipitation.

2.4. MALDI-TOF Mass Analysis of the Reaction Solution

The reaction solution removed the unreacted peptide by phenol/chloroform extraction, and ethanol precipitation was characterized by MALDI-TOF mass spectrometry (AXIMA-Confidence, Shimadzu, Kyoto, Japan). A matrix solution was prepared as a mixture \((v/v: 9/1)\) of 10 mg/mL of THAP in acetonitrile/H\(_2\)O \((v/v: 1/1)\) and 50 mg/mL of DAHC in pure water. The sample solution was mixed with the same volume of matrix solution (1 µL each). DNA oligonucleotides (molecular weight: 5828.9,
The effect of solution pH for the formation of covalent linkage between the 3′-dialdehyde group of the oxidized RNA subunit and the hydrazide group at the C-terminal of peptide subunit of the Rev-RRE complex was investigated by carrying out the reaction at various pH levels. The RNA subunit of ATP-binding of the RNP receptor, An16, was oxidized by using sodium periodate in a sodium acetate buffer at pH 5. The reaction was performed at 37 °C for 1 h. After isolation of the oxidized RNA by ethanol precipitation, acetylated Rev peptide, modified with a C-terminal hydrazide group through a flexible linker (GGS)₃G (Ac-Rev-(GGS)₃G-HZ), was mixed with the oxidized RNA in sodium acetate buffer at pH 4 or 5 or in a sodium phosphate buffer at pH 6 or 7. The reaction mixtures were incubated for 3 h at 37 °C, then the reactions were stopped by the addition of phenol/chloroform and vigorously mixed. The water layer was condensed by ethanol precipitation. The reaction yield of c-RNP in each solution was evaluated by quantitation of the band intensity, corresponding to free RNA and conjugated c-RNP in a denaturing polyacrylamide gel electrophoresis (PAGE) (Figure 2). Over 80% of RNA was reacted with peptide in all conditions. The yields of reaction were 92 ± 4% at pH 4, 92 ± 5% at pH 5, 86 ± 3% at pH 6, and 82 ± 3% at pH 7 (Figure S1A). These results indicated that the solution pH ranging from 4 to 7 did not affect much to the yield of RNA-peptide conjugate. Prolonged incubation over 15 h at pH 5 did not show any difference in the ratio of the band intensity for c-RNP and unreacted RNA (data not shown).

Figure 2. A denaturing polyacrylamide gel electrophoresis (PAGE) analysis (8 M Urea) of the conjugation reaction products of c-RNPs at different pH conditions. The yield was calculated from the ratio of intensities of the bands corresponding to c-RNP and RNA. Lane 1: An16 RNA only; Lane 2: An16 and Ac-Rev-(GGS)₃G-HZ reacted at pH 4; Lane 3: An16 and Ac-Rev-(GGS)₃G-HZ reacted at pH 5; Lane 4: An16 and Ac-Rev-(GGS)₃G-HZ reacted at pH 6; Lane 5: An16 and Ac-Rev-(GGS)₃G-HZ reacted at pH 7.
3.2. Contribution of the RNA-Peptide Interaction for the Reaction Yield

RNA sequence dependency of the reaction was next evaluated to address the proximity effect of reactive groups. The specific interaction between the Rev peptide and RRE RNA of the RNP scaffold was expected to have an influence on the efficiency of covalent linkage formation. A sequence-scrambled scrAn16 RNA that contained the same number of each nucleotide to An16 RNA was prepared (Figure 3A). The reaction of the oxidized scrAn16 with Ac-Rev-(GGS)₃G-HZ was performed at pH 5. The MALDI TOF MS analysis using the reaction solution after removing the unreacted peptide was performed to characterize the products. The production of covalently linked RNP was confirmed by observing respective MS peaks (Figures S2 and S3). In the five trials, scrAn16 showed a similar averaged reaction yield (92 ± 5%) to that of the parent An16 (92 ± 5%) when conjugated with the hydrazide group of Ac-Rev-(GGS)₃G-HZ (Figure 3B and Figure S1B,D). The result showed that the RNA sequence did not affect the efficiency of reaction. The non-specific electrostatic interaction between the RNA and the cationic Rev peptide would relate to the reaction efficiency. To verify this notion, the reaction for the formation of covalently linked RNP was carried out by using a truncated peptide of Ac-Rev-(GGS)₃G-HZ. A truncated peptide possessing only the GGS peptide link moiety, Ac-GGSGGGSGSG-HZ, was designed by deleting the Rev peptide sequence from Ac-Rev-(GGS)₃G-HZ. The truncated peptide showed a significant decrease in the yield to 57 ± 1%, even after a prolonged reaction time for 14 h (Figure 4 and Figure S1C). This result supported the notion that the electrostatic interaction between RNA and the peptide mainly contributed to increasing the reaction yield. Thus, the RNP scaffold was useful for the efficient production of RNA-peptide conjugates.

![Figure 3](image-url)

**Figure 3.** (A) Nucleotide and peptide sequence of An16 RNA, scrAn16 RNA, Ac-Rev-(GGS)₃G-HZ peptide, and Ac-(GGS)₃G-HZ peptide; (B) a denaturing PAGE (8 M Urea) analysis of the conjugation reaction products of c-RNPs constructed from An16 or scrAn16 RNA with Ac-Rev-(GGS)₃G-HZ. Lane1: An16 RNA; Lane 2: An16 and Ac-Rev-(GGS)₃G-HZ reacted for 3 h; Lane 3: scrAn16 RNA; Lane 4: scrAn16 and Ac-Rev-(GGS)₃G-HZ reacted for 3 h; Lane 5: scrAn16 and Ac-Rev-(GGS)₃G-HZ reacted for 14 h.
4. Discussion

A Schiff base formation between the hydrazide and aldehyde group rapidly progressed at a mild acidic condition although the stability was less than that at a neutral pH. Because the reaction efficiency of this crosslinking method was based on the balance of such properties, the assays under the wide range of pH helped us to understand a limitation and the applicability of this reaction under the physiological condition for RNA-protein or RNA-peptide conjugation. Formation of the covalent linkage between RNA and the Rev peptide subunit in the Rev-RRE complex quantitatively proceeded in a mild acidic to a neutral pH condition within 3 h. The result indicated the versatility of this reaction in a physiological condition for the crosslink formation of peptide and RNA. Unlike the sequence-specific nature for the stable noncovalent RNP complex formation, formation of the covalent linkage between the 3’-dialdehyde group of RNA and the hydrazide group at the C-terminal of Rev peptide required no specific RNA sequence for the RNA binding of Rev peptide. Even the nonspecific RNA-peptide complex formation driven by the electrostatic interaction was sufficient for exerting the proximity effect of reactive groups, thereby resulting an efficient formation of covalent linkage between the Rev peptide and RNA. The significant decrease of the reaction efficiency by deletion of the cationic moiety in the peptide sequence also showed the contribution of the electrostatic interaction for the efficient proceeding of the crosslinking reaction.

We designed a DNA sequence-specific protein-tag that underwent a proximity-driven intermolecular crosslinking between protein and DNA [43–49]. Selective DNA modification by a self-ligating protein tag conjugated with a DNA-binding domain, termed as a modular adaptor, was achieved by relying on the chemoselectivity of the protein tag [50–52]. By tuning the alkylation kinetics for the protein-tag and its substrate, the sequence-specific crosslinking reaction of the modular adaptor was exclusively driven by the DNA recognition when the dissociation rate of the DNA complex was much larger than the rate constant for the alkylation reaction [45–49]. In connection with these findings, a sequence-specific crosslinking reaction between the 3’-dialdehyde group of RNA and the hydrazide group of RNA-binding peptide would be realized by tuning the reaction conditions, such as the concentration of sodium salt. As we have reported previously, the crosslinking reaction between the 3’-dialdehyde group of RNA and the hydrazide group of peptide was applied for the facile construction of covalently crosslinked fluorescent RNP sensors not only realized an improved stability of RNP sensors
of covalently crosslinked fluorescent RNP sensors [41,42]. The covalently crosslinked fluorescent RNP sensor not only realized an improved stability of RNP sensors but also expand its application, such as the simultaneous detection of multiple targets in the solution. The noncovalent RNP scaffold was effective for the library-based selection and the cooperative functionalization of RNP receptor, such as the fluorescent sensors and catalysts [33]. Once a functional noncovalent RNP was obtained, formation of a covalent linkage within the RNA-peptide complex provided stable RNP for a wide range of applications. A natural RNA oligonucleotide and facile preparation of the reactive peptide was only needed to perform this reaction. Our investigations regarding the effect of the pH, RNA, and a peptide sequence for the reaction efficiencies in this reaction would help us to understand the limitation and applicability of this method for construction of RNA-peptide or protein conjugate. It would also be helpful for the development of an emerging application of oligonucleotide-peptide conjugates, such as a self-assembled scaffold of nanostructure or an efficient delivery system of functional RNA into the cell.

**Supplementary Materials:** The following are available online at [http://www.mdpi.com/2076-3417/10/24/8920/s1](http://www.mdpi.com/2076-3417/10/24/8920/s1), Figure S1: The results of band intensity analysis of denaturing PAGE, Figure S2: MALDI TOF MS analysis of the reaction solution, Figure S3: Summary of the results of MALDI TOF MS analysis.

**Author Contributions:** S.N. and T.M. conceived and designed the experiments; S.N., T.S. and Z.Z. performed the experiments; T.M. supervised the project. All authors have read and agreed to the published version of the manuscript.

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