Cholesteryl-Conjugated Ribonuclease A Exhibits Enzyme Activity in Aqueous Solution and Resistance to Dimethyl Sulfoxide

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ABSTRACT: Using bovine pancreatic ribonuclease A (RNase A) and cholesterol, we synthesized cholesteryl-conjugated ribonuclease A (CHRnase A) to evaluate the influence of a conjugated hydrophobic moiety on protein function. Nuclear magnetic resonance and matrix-assisted laser desorption/ionization time-of-flight spectrometry suggested that one cholesteryl group was conjugated to RNase A. Differential scanning calorimetry indicated that CHRnase A was denatured in the solid state but was folded in phosphate buffer (0.05 mol/L, pH 6.5). CHRnase A resembled RNase A in its secondary structure, but circular dichroism (CD) spectra revealed that the helical content of CHRnase A was decreased and the tertiary structure of CHRnase A differed from that of RNase A. Furthermore, fluorescence measurements, CD spectra, an 8-anilino-1-naphthalenesulfonic acid ammonium salt-based assay, and surface tension measurements suggested that cholesterol was conjugated to a tyrosine residue on the protein surface. The relative activity of CHRnase A to RNase A was 79 ± 7%, and the enzyme activity of CHRnase A by adding β-cyclodextrin (β-CyD) increased to 129 ± 7%. Therefore, we considered that the cholesteryl group interacted with substrate (cytidine 2′,3′-cyclic monophosphate monosodium salt) to inhibit the enzyme reaction. Finally, the environment around tyrosine residues in CHRnase A in dimethyl sulfoxide was similar to that of native RNase A in phosphate buffer (0.05 mol/L, pH 6.5). These results suggest that cholesterol conjugation to RNase A altered RNase A functionality, including improvement of RNase A resistance to dimethyl sulfoxide and modulation of the ability of β-CyD to control RNase A enzymatic activity.

1. INTRODUCTION

Recently, protein chemical modification (PCM) has emerged as a major technique in the chemical biology field and has found applications in industrial and pharmaceutical areas. The goal of PCM is to enhance the functionality of proteins by increasing their structural and functional diversity. This technique has also been used in the development of new drugs, including antibody–drug conjugates. Proteins can be chemically modified in several ways, including through conjugation to polyethylene glycol (PEG), which has proved useful in clinical applications. PEG conjugation not only improves the stability and solubility of proteins but also increases their retention time in the body. Many proteins are modified with hydrophilic groups; for example, polysaccharides may be conjugated to proteins in this manner, which improves their thermal stability against stress conditions. Although most proteins are modified with hydrophilic groups, some studies have introduced modifications with hydrophobic groups.

In natively structured proteins, hydrophobic residues are buried; however, protein denaturation, induced by physical stress, heat, or chemical exposure, allows these residues to become exposed. Aggregation of proteins is mediated by hydrophobic interactions between the newly exposed hydrophobic residues of denatured proteins. In addition, protein aggregation caused by denaturation can alter the biological activity of proteins, including changing their immunogenicity. Thus, protein aggregation is instead a factor to be avoided in the design and development of biological pharmaceuticals and must be appropriately controlled. Consequently, very few studies have explored the use of hydrophobic groups and their chemical modifications in the field of PCM. Typically, organic solvents are required for the conjugation of hydrophobic groups, resulting in protein denaturation. Furthermore, hydrophobic interactions between modified hydrophobic groups and hydrophobic residues, which were exposed by protein denaturation, could drive protein aggregation.

However, some studies have reported the successful conjugation of hydrophobic groups to water-soluble polymers, such as cholesterol-bearing pullulan (CHP) and cholesterol-bearing poly-L-lysine (CHPLL). These cholesterol-based...
materials have been reported to self-assemble into nanogels in an aqueous solution via the hydrophobic interactions between the conjugated cholesteryl groups. Functionally, CHP mimics natural molecular chaperones, and because of its ability to trap hydrophobic molecules, it has wide applications in drug delivery. Furthermore, the α-helical structures of CHPLL are controlled by host−guest interactions between cholesterol and cyclodextrin. Therefore, chemical modification of a hydrophobic group to water-soluble polymers may not only help enhance the existing functions of polymers but also introduce novel functions due to the formation of new intermolecular/intramolecular hydrophobic interactions.

In the present study, we used bovine pancreatic ribonuclease A (RNase A) as a model protein for conjugation with the hydrophobic group cholesterol and synthesized a novel cholesteryl-conjugated RNase A (CHRNase A). RNase A is a 124 amino acid, single-chain protein (molecular weight, 13.7 kDa) that catalyzes the cleavage of phosphodiester bonds in various types of single-stranded RNA. RNase A is an extensively studied and homogeneous enzyme, and proteins with structural homology to RNase A belong to the RNase A superfamily. Some RNases in this superfamily exhibit anti-HIV-1 activity and induce cytotoxic effects in tumor cells. Notably, RNase mimics that can irreversibly cleave or otherwise elicit damage to targeted mRNA transcripts represent a particularly promising therapeutic approach to combat various diseases.

2. RESULTS AND DISCUSSION

2.1. Confirmation of Cholesterol Conjugation to RNase A and the Number of Conjugated Cholesteryl Groups.

![Figure 1. 1H NMR spectra of the indicated samples in DMSO-d6 at 25 °C. (a) RNase A, (b) CHRNase A, and (c) physical mixture of RNase A and cholesterol. The red circle shows 1H signal from the cholesteryl group. RNase A and CHRNase A concentration: 21.4 mg/mL. Physical mixture of RNase A and cholesterol (RNase A [mol]/cholesterol [mol] = 1/12) concentration: 21.4 mg/mL.](https://dx.doi.org/10.1021/acsomega.0c05016)
confirm that cholesterol was successfully conjugated to RNase A. 1H NMR spectra of RNase A alone, CHRNase A, and a physical mixture of RNase A and cholesterol (RNase A [mol]/cholesterol [mol] = 1/12) in DMSO-d6 were acquired. Figure 1 shows that the 1H NMR spectrum of CHRNase A differed from that of the physical mixture. A 1H signal from the cholesteryl group in CHP (0.6−2.4 ppm) was observed in DMSO-d6. Therefore, the 1H signal in the CHRNase A spectrum at approximately 2 ppm likely corresponds to the cholesteryl group, as this 1H signal was not present in the spectra of either RNase A or the physical mixture. Cholesterol was conjugated to RNase A via the carbonyl carbon (C=O) of N,N′-carbonyldiimidazole (CDI). Figure 2 shows the 13C NMR spectra of RNase A and CHRNase A in DMSO-d6. The 30 and 200 ppm signals in the 13C NMR spectrum of CHRNase A correspond to the carbon in the cholesteryl group and the C=O group that connects RNase A to cholesterol, as these signals were not seen for RNase A. In addition, a 1H−13C heteronuclear multiple-quantum correlation (HMQC) NMR spectrum demonstrated a correlation between the 1H signal at approximately 2.0 ppm and the 13C signal at approximately 30 ppm (Figure S1). We thus concluded that we successfully synthesized RNase A conjugated to cholesterol.

Furthermore, we obtained the nuclear Overhauser effect (NOE) difference spectrum of CHRNase A through radio frequency irradiation of the 1H signal at approximately 2 ppm, corresponding to the signal of the cholesteryl group in CHRNase A (Figure S2). NOE measurement provides information about spatial proximity of protons in a molecule. If the spatial distance between the cholesteryl group and amino acids was close, negative signals would be observed in the NOE difference spectra. Conversely, if the spatial distance was far, signals would not be observed. The results suggested that the
spatial distance of conjugated cholesteryl group is close to many amino acid groups in DMSO.

2.1.2. MALDI-TOF MS. The NMR results suggested that cholesterol was conjugated to RNase A in our CHRNase A sample. However, we could not determine the number of conjugated cholesteryl groups in CHRNase A using this technique. Therefore, we next applied MALDI-TOF MS to confirm the number of conjugated cholesteryl groups in CHRNase A. The MALDI-TOF MS spectra of RNase A and CHRNase A, with α-cyano-4-hydroxycinnamic acid (CHCA) used as the matrix, were similar (Figure S3a). Therefore, the conjugated cholesteryl group may have been cleaved by an analytical process, such as sample preparation or irradiation by the laser during MALDI-TOF MS. Consequently, we conducted MALDI-TOF MS using another matrix (sinapinic acid, SA). Upon using SA as the matrix, the m/z of CHRNase A increased by approximately 400 Da compared with that of RNase A (Figure S3b), suggesting that the number of cholesteryl groups conjugated to RNase A was one.

2.2. Structural Characterization of CHRNase A.

2.2.1. Evaluation of Protein State in Solid and Buffer Solution by DSC. Proteins that are denatured because of thermal stress undergo conformational collapse. DSC measures the thermal stability of proteins by detecting changes in their conformation. The endothermic peak observed in DSC is indicative of a cooperative structural transition, which classically corresponds to a native-denatured transition and occurs in both solid and aqueous solutions. Furthermore, there is an increase in hydrophobic hydration when a protein transitions from the native (folded) state to the denatured (unfolded) state. Therefore, to evaluate the state of hydrophobic residues in CHRNase A, we measured the DSC in both solid state and phosphate buffer (0.05 mol/L, pH 6.5). Phosphate buffer was composed of Na₂HPO₄ and KH₂PO₄.

Figure 3a,b shows the DSC thermograms for RNase A and CHRNase A in the solid and buffer solutions, respectively. In the solid state (Figure 3a), an endothermic peak was observed for RNase A and for the physical mixture of RNase A and cholesterol. The maximum peak of the endothermic point (Tm) of RNase A was observed at 110°C, and that of the physical mixture was observed at 113 and 138°C. Conversely, an endothermic peak for CHRNase A was not observed within the measurement range. Both acetone and DMSO were used during the synthesis of CHRNase A, which may have resulted in denaturation of the protein and hence the absence of the endothermic peak. We also evaluated whether CHRNase A in the solid state was denatured using other methods. As Fourier transform infrared (FT-IR) spectroscopy can be used to evaluate protein conformation using solid samples, using the potassium bromide (KBr) pellet method, we applied FT-IR measurements of RNase A and CHRNase A (Figure S4). In FT-IR measurements of proteins, amide band I (near 1650 cm⁻¹) indicates C=O stretching and amide band II (near 1550 cm⁻¹) indicates N–H bending and C–N stretching. The intensity of the amide II band in proteins disappears upon unfolding, whereas the amide I band remains unchanged. Therefore, we confirmed whether CHRNase A was unfolded by evaluating the ratio of transmittance (intensity) for amide II to amide I (Figure S4) and found that the ratios in RNase A and CHRNase A were 2.1 and 1.4, respectively. In agreement with the DSC measurements, these results suggested that CHRNase A was unfolded in the solid state.

Figure 3b shows that in buffer solution, the Tm’s of RNase A and CHRNase A were detected at 67°C, indicating that CHRNase A was folded in buffer solution. Cholesterol-bearing polysaccharides and peptides, such as CHP24 and CHPLL, were previously shown to form hydrogel nanoparticles, suggesting that the conjugated cholesteryl group could affect the folding of CHRNase A in buffer solution. These results suggest that CHRNase A was folded in buffer solution, although it was denatured in the solid state.

2.2.2. Evaluation of the Secondary and Tertiary Structures by CD Spectroscopy. The results of the DSC and FT-IR indicated that CHRNase A was denatured in the solid state but folded in phosphate buffer (0.05 mol/L, pH 6.5). We measured CD to evaluate the secondary and tertiary structures of CHRNase A, which may have been affected by hydrophobic interactions mediated by the cholesteryl group.

RNase A (type α + β) comprised both α-helix and β-sheet secondary structures, with a greater percentage of the composition made up of β-sheets than that of α-helices. Figure 4a shows the CD spectra of RNase A and CHRNase A in the far-ultraviolet (UV) (200–250 nm) region, providing information on protein secondary structure. Although the CD spectrum of CHRNase A was similar to that of RNase A, indicating that CHRNase A still exhibited a secondary structure comprising α-helices and β-sheets, the absolute value at 222 nm was lower than that of RNase A. As the ellipticity at 222 nm in CD spectra is correlated with a protein’s α-helical content, this therefore indicates that
the α-helical content in CHRNase A was lower than that of RNase A. The CD spectrum in the near-UV (250–320 nm) region reflects the environment of aromatic residues and provides information about the protein tertiary structure. Figure 4b shows the CD spectra of RNase A and CHRNase A in the near-UV region. The signal in the region around 275 nm, which arises from tyrosine (Tyr) residues, differed between CHRNase A and RNase A. RNase A has six Tyr residues: three are buried in the protein interior (i.e., Tyr25, Tyr92, and Tyr97) and three were partly exposed (i.e., Tyr73, Tyr76, and Tyr115), where the hydroxyl groups of the exposed Tyr interact with solvent. It has previously been shown that the intensity around 275 nm decreases because of the unfolding of RNase A by thermal stress. Therefore, the change in the CD spectrum of approximately 275 nm reflects the environment around buried and exposed Tyr residues, such as cholesterol conjugation to Tyr or exposure of Tyr to the protein surface. 

Altogether, the CD and DSC analyses suggest that CHRNase A existed in the folded state in buffer solution. Although the secondary structure of CHRNase A was similar to that of RNase A, its tertiary structure differed, which may have been induced by cholesterol conjugation to RNase A.

2.3.3. Evaluation of the Environment around Aromatic Residues and Surface Hydrophobicity Using Fluorescence Spectroscopy. Absorption and fluorescence emission at 280 nm are indicative of the environment around aromatic residues in proteins, especially tryptophan (Trp) and Tyr, which is derived from tyrosine (Tyr) residues, di near-UV region. The signal in the region around 275 nm, which arises from tyrosine (Tyr) residues, differed between CHRNase A and RNase A. RNase A has six Tyr residues: three are buried in the protein interior (i.e., Tyr25, Tyr92, and Tyr97) and three were partly exposed (i.e., Tyr73, Tyr76, and Tyr115), where the hydroxyl groups of the exposed Tyr interact with solvent. It has previously been shown that the intensity around 275 nm decreases because of the unfolding of RNase A by thermal stress. Therefore, the change in the CD spectrum of approximately 275 nm reflects the environment around buried and exposed Tyr residues, such as cholesterol conjugation to Tyr or exposure of Tyr to the protein surface.

Altogether, the CD and DSC analyses suggest that CHRNase A existed in the folded state in buffer solution. Although the secondary structure of CHRNase A was similar to that of RNase A, its tertiary structure differed, which may have been induced by cholesterol conjugation to RNase A.

Figure 5. Fluorescence measurements of each RNase A and ANS in phosphate buffer (0.05 mol/L, pH 6.5) at 25 °C. (a) Fluorescence emission spectra of RNase A and CHRNase A in 0.05 mol/L phosphate buffer (pH 6.5) at 25 °C. (1) RNase A and (2) CHRNase A. RNase A and CHRNase A concentration: 0.5 mg/mL. Excitation wavelength: 280 nm. (b) Fluorescence emission spectra of ANS and ANS with RNase A or CHRNase A in 0.05 mol/L phosphate buffer (pH 6.5) at 25 °C. (1) ANS, (2) ANS with RNase A, and (3) ANS with CHRNase A. ANS concentration: 1.0 × 10−4 mol/L. RNase A and CHRNase A concentration: 0.5 mg/mL. Excitation wavelength: 365 nm.

Figure 5a depicts the fluorescence emission spectra of RNase A and CHRNase A. RNase A has six Tyr and three phenylalanine (Phe) residues but no Trp residues. The maximum fluorescence emission spectrum (λmax) for both RNase A and CHRNase A occurred at 306 nm, attributed to Tyr (Tyr λmax = 305 nm), and the intensity of the CHRNase A spectrum was similar to that of RNase A. These results indicated that the environment around buried and exposed Tyr residues in CHRNase A was highly similar to that of RNase A. Fluorescence emission spectrum excited at 280 nm is attributed to the emission of the aromatic ring in aromatic amino acid residues.

The CD spectrum of approximately 275 nm reflects the environment of Tyr residues, and the y-axis of the CD spectrum shows ellipticity. Ellipticity suggests the difference in the extinction coefficient or absorbance of left and right circularly polarized light. If Tyr residues were exposed to the surface, λmax of CHRNase A reveals a longer shift (red shift) and the intensity changes. However, these changes were not observed in CHRNase A. Thus, we assumed that the alteration of CD spectrum of approximately 275 nm may have been induced by the conjugation of cholesterol to the Tyr residue.

Buried hydrophobic residues in RNase A could have been exposed to the protein surface during the synthesis process and/or cholesterol conjugation. Additionally, because cholesterol is highly hydrophobic, its conjugation to RNase A could have increased the surface hydrophobicity of the protein. Therefore, we applied fluorescence measurements at 365 nm using ANS to evaluate the hydrophobicity on the surface of CHRNase A. ANS is a fluorescent probe that binds to hydrophobic regions on protein surfaces with high affinity and has therefore been used for the characterization of protein binding sites and the study of folding pathways. The λmax value of ANS shifts to shorter wavelengths (blue shift) when it transits from a polar to a nonpolar environment, which also increases its fluorescence intensity. Figure 5b depicts the fluorescence emission spectra of ANS and ANS with RNase A or CHRNase A. The λmax value and intensity for ANS in the presence of RNase A differed slightly from those for ANS alone. Conversely, both values for ANS with CHRNase A were significantly altered compared with those for ANS alone and ANS with RNase A. These results suggest that the hydrophobic regions on the surface of CHRNase A were higher than the hydrophobic regions on the surface of RNase A, which may correspond to an increased exposure of...
hydrophobic residues on the protein’s surface because of the synthesis process and/or cholesterol conjugation. However, on the basis of the fluorescence and CD measurements, we could not determine whether the cholesteryl group was located on the interior or surface of the protein.

The surface tension of proteins is decreased by denaturation, as hydrophobic residues typically buried in the interior of proteins are exposed to the surface. Therefore, we measured the surface tension of RNase A and CHRNase A in phosphate buffer (0.05 mol/L, pH 6.5) and found that both RNase A and CHRNase A exhibited surface tensions of 59 mN/m. This suggests that the extent of hydrophobic residue exposure in CHRNase A was similar to that of RNase A. Thus, the apparent increase in hydrophobicity of CHRNase A likely corresponded to the presence of the cholesteryl group on the protein surface in buffer solution, indicating that the conjugated cholesteryl group was located on the surface of the protein. However, if this were the case, the cholesteryl group could induce protein aggregation. Therefore, we analyzed the particle size of RNase A and CHRNase A at a concentration of 1.0 mg/mL. The particle size for both RNase A and CHRNase A was approximately 119 nm, which indicated that the cholesteryl group was unlikely to drive the aggregation of the modified protein.

2.3. Enzyme Activity of CHRNase A. 2.3.1. Relative Activity and Enzyme Kinetic Constants of CHRNase A. The changes we observed in the conformation and hydrophobicity on the protein surface of CHRNase A may have affected its enzyme activity. Therefore, using cCMP as a substrate and monitoring turbidity at 286 nm, we evaluated the enzyme activity of RNase A and CHRNase A. Estimated enzyme activity was 100% (estimated relative activity to RNase A: about 100%). This suggested that the inhibition by interaction with cholesteryl group and cCMP could be eliminated by adding β-CyD. However, this experiment could not reveal the mechanism by which the enzyme activity of CHRNase A was altered with the addition of β-CyD. For example, β-CyD could have induced a conformational change of protein by trapping the cholesteryl group. Therefore, we conducted CD measurements of CHRNase A before and after adding β-CyD using cCMP. The relative enzyme activity of CHRNase A with β-CyD to CHRNase A without β-CyD was 129 ± 7% (estimated relative activity to RNase A: about 100%). This suggested that the inhibition by interaction with cholesteryl group and cCMP could be eliminated by adding β-CyD.

2.4. Evaluation of the Environment of Tyr Residues in DMSO. DMSO is used as protein denaturant at high concentrations and can induce conformational changes in proteins by binding to newly exposed hydrophobic and aromatic side chains upon protein unfolding. We used 100% DMSO as a solvent in the synthesis of CHRNase A, and acetone was used to obtain the powdered product, which induced the denaturation of CHRNase A in the solid state. However, we observed that CHRNase A was folded in phosphate buffer (0.05 mol/L, pH 6.5). This suggested that hydrophobic interactions with the conjugated cholesteryl group may promote the folding of CHRNase A in organic solvents such as DMSO. Although the NOE result (Figure S3) indicated that the conjugated cholesteryl group is located near...
amino acid residues in the presence of 100% DMSO, it did not elucidate whether CHRNase A is in the native state in DMSO.

As fluorescence measurements can evaluate the environment surrounding Tyr residues and indicate the folding state of proteins, we applied fluorescence measurements of CHRNase A in DMSO at 25 °C. Figure 7 shows the fluorescence emission spectra of RNase A and CHRNase A in phosphate buffer (in 0.05 mol/L, pH 6.5) and 100% DMSO. A red shift of λ_{max} and an increase in the fluorescence intensity imply that aromatic amino acids are exposed to the protein surface. The λ_{max} of RNase A in DMSO (310 nm) showed a red shift compared with that of RNase A in 0.05 mol/L phosphate buffer (pH 6.5, 307 nm), with a concomitant increase in fluorescence. Conversely, the λ_{max} of CHRNase A in DMSO (307 nm) was highly similar to that of the protein in 0.05 mol/L phosphate buffer (pH 6.5). However, the intensity of CHRNase A in DMSO was decreased compared with that of RNase A in 0.05 mol/L phosphate buffer (pH 6.5). The environment of Tyr in CHRNase A in both buffer and DMSO was similar to that of native RNase A, but RNase A was denatured in 100% DMSO. Thus, it was inferred that CHRNase A could form a folded state in DMSO and may have been resistant to DMSO.

3. CONCLUSIONS

We succeeded in synthesizing CHRNase A using CDI in DMSO. NMR measurements suggested that cholesterol was conjugated to RNase A through the carbonyl carbon (C=O) of CDI. Moreover, MALDI-TOF MS indicated that one cholesteryl group was conjugated to RNase A.

We conducted a structural characterization of CHRNase A by examining its folding state in a solid and in phosphate buffer (0.05 mol/L, pH 6.5), the secondary-tertiary structure, the environment around aromatic residues, and the hydrophobicity on the protein surface. DSC results suggested that CHRNase A was unfolded following the synthesis process but folded in buffer solution. The CD spectra indicated that the secondary structure of CHRNase A in buffer solution was similar to that of RNase A. However, the helical content of CHRNase A was less than that of RNase A, and the tertiary structure of CHRNase A differed from RNase A. On the basis of fluorescence analysis excited at 280 nm and CD spectra at 275 nm, we considered the cholesterol was conjugated to a Tyr residue in RNase A. The conjugated cholesteryl group is likely located on the protein surface according to the ANS fluorescence assay and measurements of surface tension.

Furthermore, we measured the enzymatic activity of CHRNase A to evaluate the effects of cholesterol conjugation on protein function. The relative activity of CHRNase A to RNase A was 79 ± 7%, and the Lineweaver–Burk plots implied that a decrease of the affinity to cCMP had occurred. We then evaluated the enzyme activity of CHRNase A before and after adding β-CyD and found that it increased to approximately 129 ± 7% (estimated relative activity to RNase A: approximately 100%); however, we observed no conformational change in CHRNase A before and after adding β-CyD. Therefore, we assumed that the enzyme activity of CHRNase A would be decreased by inhibition by interaction with cholesteryl group and cCMP, which could be eliminated by adding β-CyD, thus recovering enzyme activity. Additionally, although RNase A was denatured in 100% DMSO, fluorescence measurements indicated that the environment of Tyr residues in CHRNase A in 100% DMSO was similar to that of native RNase A. Thus, CHRNase A may form a folded state in DMSO and may be resistant to DMSO.

This study suggests that novel functions, including β-CyD-dependent control of enzyme activity and improved endurance to organic solvents like DMSO, can be added by cholesterol conjugation to RNase A. Such changes in protein function may be more widely applicable. Hence, we conclude that the techniques used in the present study have the potential to enhance industrial and pharmaceutical methods, including those used in the development of immobilized enzymes, allosteric enzymes, biosensors, and protein formulations.

4. EXPERIMENTAL SECTION

4.1. Materials. Bovine pancreatic RNase A, cCMP, CHCA, SA, and ANS were purchased from Sigma-Aldrich (St. Louis, USA). Cholesterol (Wako special grade), CDI (for organic synthesis), DMSO (guaranteed reagent), dehydrated DMSO (for organic synthesis), DMSO-d_6 (for NMR), and acetone (guaranteed reagent) were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). β-CyD was purchased from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). All other reagents were commercial products of analytical grade. Phosphate buffer (0.05 mol/L, pH 6.5) was prepared by Na_2HPO_4 and KH_2PO_4.

Figure 7. Fluorescence emission spectra of each RNase A in phosphate buffer (0.05 mol/L, pH 6.5) and DMSO at 25 °C. (a) RNase A in (1) phosphate buffer (0.05 mol/L, pH 6.5) and (2) DMSO. (b) CHRNase A in (1) phosphate buffer (0.05 mol/L, pH 6.5) and (2) DMSO. RNase A and CHRNase A concentration: 0.5 mg/mL. Excitation wavelength: 280 nm.
4.2. Synthesis of CHRNase A. CDI is used for conjugation with many organic compounds and polymers such as polysaccharides, peptides, and proteins.69-74 The imidazole in CDI is highly reactive, which allows it to be converted to an ester by reacting with alcohols or amines. Therefore, we used the following procedure and synthesized CHRNase A by conjugating cholesterol with hydroxyl and/or amino groups in RNase A. For conjugation to RNase A, cholesterol was agitated with CDI in dehydrated DMSO at 50 °C for 3 h and cooled at room temperature (RT). Then, RNase A was added based on the ratio mentioned here: CDI [mol]/RNase A [mol] = 10 and cholesterol [mol]/RNase A [mol] = 12. This solution was agitated at RT for 20 h to cause conjugation of the cholesteryl group to the hydroxyl and amino groups of RNase A. CHRNase A powder was obtained by first adding acetone, then washing with acetone several times, and finally, drying under reduced pressure at RT. The product was stored under −20 °C (Figure S7).

4.3. Confirmation of Cholesterol Conjugation to RNase A and the Number of Conjugated Cholesteryl Groups. 4.3.1. NMR Spectroscopy. RNase A (15 mg) and CHRNase A (15 mg) were separately dissolved in DMSO-d$_6$ (0.7 mL). Following filtration of the solutions through membrane filters (0.45 μm), they were analyzed using an NMR spectrometer (JNM-LA500, JEOL, Tokyo, Japan). NMR (500 MHz $^1$H-, 125 MHz $^{13}$C-, and $^1$H–$^{13}$C HMOC) spectra were all recorded at 25 °C. DMSO (2.49 ppm) was used as an internal standard in DMSO-d$_6$.

4.4. Structural Characterization of CHRNase A. 4.4.1. Evaluation of Protein State in a Solid State and Buffer Solution Using DSC. In the solid state, RNase A (5 mg), a physical mixture of RNase A and cholesterol (RNase A [mol]/cholesterol [mol] = 1/12) (5 mg), and CHRNase A (5 mg) were separately placed in aluminum sample pans, sealed, and analyzed by DSC (DSC8240D, Rigaku, Tokyo, Japan). RNase A and CHRNase A were thawed and dried in a desiccator before analyzing. The scan rate and scan range were set at 2 °C/min and 40–160 °C, respectively; alumina (Al$_2$O$_3$) was used as a reference material, and the analysis was conducted in air atmosphere.

In the buffer solution, RNase A (30 mg/mL) and CHRNase A (30 mg/mL) were separately dissolved using phosphate buffer (0.05 mol/L, pH 6.5). The solution (30 μL) was placed in stainless steel sample pans and sealed for analysis by DSC (DSC8240D). The scan rate and scan range were set at 2 °C/min and 40–100 °C, respectively. Phosphate buffer (0.05 mol/L, pH 6.5) was used as a reference material, and the analysis was conducted in air atmosphere.

4.4.2. Evaluation of the Secondary and Tertiary Structures by CD Spectroscopy. RNase A and CHRNase A were separately dissolved in phosphate buffer (0.05 mol/L, pH 6.5) at a concentration of 0.5 mg/mL and analyzed using a CD spectrometer (J-820, JASCO, Tokyo, Japan). The CD spectra were recorded at 25 °C from 200 to 320 nm.

4.4.3. Evaluation of the Environment around Aromatic Residues and Surface Hydrophobicity Using Fluorescence Spectroscopy. To evaluate the environment around aromatic amino acids, RNase A and CHRNase A were separately dissolved in a phosphate buffer (0.05 mol/L, pH 6.5) at a concentration of 0.5 mg/mL and analyzed using a fluorescence spectrometer (FP-750, JASCO, Tokyo, Japan) with the following parameters: excitation wavelength = 280 nm, band pass = 10 nm, and emission band pass = 10 nm. Fluorescence emission spectra were recorded at 25 °C from 260 to 400 nm.

RNase A, CHRNase A, and ANS were each dissolved in phosphate buffer (0.05 mol/L, pH 6.5) to evaluate the hydrophobic regions on the protein surface. Each RNase A and ANS solution was mixed to achieve concentrations of 0.5 mg/mL and 1.0 × 10$^{-5}$ mol/L, respectively, and then analyzed using a fluorescence spectrometer with the following parameters: excitation wavelength = 365 nm, band pass = 10 nm, and emission band pass = 10 nm. Fluorescence emission spectra were recorded at 25 °C from 400 to 600 nm.

4.5. Enzyme Activity of CHRNase A. 4.5.1. Relative Activity and Enzyme Kinetic Constants of CHRNase A. 4.5.1.1. Relative Activity of CHRNase A to RNase A. RNase A and CHRNase A were separately dissolved in phosphate buffer (0.05 mol/L, pH 6.5) to a concentration of 0.5 mg/mL (enzyme solution). cCMP was dissolved in phosphate buffer (0.05 mol/L, pH 6.5) to a concentration of 0.1 mg/mL (substrate solution); 0.3 mL of enzyme solution (3 h post-preparation) was added to 2.7 mL of the substrate solution. The change in absorbance was analyzed using a UV spectrometer (V-560, JASCO) for 10 s at 286 nm at 25 °C. The relative activity of CHRNase A was calculated by the following formula:

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\text{relative activity of CHRNase A} = \frac{(\text{initial reaction rate of CHRNase A})}{(\text{initial reaction rate of RNase A})} \times 100
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4.5.1.2. Enzyme Kinetic Constants of CHRNase A. RNase A and CHRNase A were prepared similarly as for measuring the relative activity, but the cCMP substrate solution was formulated to have a concentration range of 0.02–0.1 mg/mL. Enzyme solution (0.3 mL, 3 h post-preparation) was added to 2.7 mL of the substrate solution, and the change in absorbance was recorded as the relative activity at 286 nm. The initial reaction rate for each RNase A was computed using the initial slope. Using a Lineweaver–Burk plot, the Michaelis constant ($K_m$) and the maximum rate ($V_{max}$) for each RNase A were calculated.

4.5.2. Effect of the Addition of β-CyD on the Enzyme Activity of CHRNase A. CHRNase A was dissolved in phosphate buffer (0.05 mol/L, pH 6.5) to achieve a concentration of 0.5 mg/mL (CHRNase A solution without β-CyD). β-CyD was dissolved in phosphate buffer (0.05 mol/L, pH 6.5) to a concentration of 0.2 × 10$^{-3}$ mol/L; 0.1 mL of 0.2 × 10$^{-3}$ mol/L β-CyD or the phosphate buffer (0.05 mol/L, pH 6.5) was added to 3.0 mL of CHRNase A solution (CHRNase A solution with or without β-CyD). cCMP was freshly dissolved in phosphate buffer (0.05 mol/L, pH 6.5) to achieve a concentration of 0.1 mg/mL in each measurement (substrate solution); 0.3 mL of CHRNase A solution after 24 h post-preparation was added to 2.7 mL of the substrate solution. The change in absorbance was analyzed using a UV spectrometer for 10 s at 286 nm at 25 °C. The relative activity of CHRNase A before and after the addition of β-CyD was calculated using the following formula.
relative activity of CHRNase A before and after addition of β

\[-\text{CyD} \equiv \frac{\text{(initial reaction rate of CHRNase A with } \beta)}{\text{(initial reaction rate of CHRNase A without } \beta)} - \text{CyD} \times 100\]

4.6. Evaluation of the Environment of Tyrosine Residues to DMSO. RNase A and CHRNase A were separately dissolved in DMSO at a concentration of 0.5 mg/mL. The solutions were analyzed using a fluorescence spectrometer with the following parameters: excitation wavelength = 280 nm, band pass = 10 nm, and emission band pass = 10 nm. Fluorescence emission spectra were recorded at 25 °C from 260 to 400 nm.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05016. 1H-13C HMOC and NOE NMR spectrum of CHRNase A in DMSO-d6, MALDI-TOF MS spectra of RNase A and CHRNase A with different matrices; FT-IR spectra of RNase A and CHRNase A; Lineweaver–Burk plots of RNase A and CHRNase A in phosphate buffer; CD spectra of CHRNase A before and after addition of β-CyD; synthetic procedure of CHRNase A; and \(K_m\) and \(V_{max}\) of each RNase A calculated from Lineweaver–Burk plots (PDF)

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REFERENCES

(1) Lutz, S.; Iamurri, S. M. Protein Engineering: Past, Present, and Future. In Methods in Molecular Biology, 2018; Vol. 1655, pp 1–12.
(2) Reetz, M. T. What are the limitations of enzymes in synthetic organic chemistry? Chem. Rec. 2016, 16, 2449–2459.
(3) Tsuchikama, K.; An, Z. Antibody-drug conjugates: recent advances in conjugation and linker chemistries. Protein Cell 2018, 9, 33–46.
(4) Hedrich, W. D.; Fandy, T. E.; Ashour, H. M.; Wang, H.; Hassan, H. E. Antibody-Drug Conjugates: Pharmacokinetic/Pharmacodynamic modeling, preclinical characterization, clinical studies, and lessons learned. Clin. Pharmacokinet. 2018, 57, 687–703.
(5) Yu, L.; Yao, Y.; Wang, Y.; Zhou, S.; Lai, Q.; Lu, Y.; Liu, Y.; Zhang, R.; Wang, R.; Liu, C.; Gou, L.; Chen, X.; Yu, Y.; Chen, Q.; Yang, J. Preparation and anti-cancer evaluation of promizimab-MMAE, an anti-CDS6 antibody drug conjugate, in small cell lung cancer cell line xenograft models. J. Drug Target. 2018, 26, 905–912.
(6) Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. Effective drug delivery by PEGylated drug conjugates. Adv. Drug. Deliv. Rev. 2003, 55, 217–250.
(7) Hamidi, M.; Azadi, A.; Rafiei, P. Pharmacokinetic consequences of pegylation. Drug Deliv. 2006, 13, 399–409.
(8) Pozzoli, L.; Distefano, M. D. Site-specific pegylation of therapeutic proteins. Int. J. Mol. Sci. 2015, 16, 25831–25864.
(9) Aowod, S.; Ginn, C.; Brocchini, S. 2—The Case for Protein PEGylation. In Engineering of Biomaterials for Drug Delivery Systems, Parambath, A.; Ed.; Woodhead Publishing: Cambridge, England, 2018; pp 27–49.
(10) Nakamura, S.; Kato, A. Multi-functional biopolymer prepared by covalent attachment of galactomannan to egg-white proteins through naturally occurring Maillard reaction. Nahrung 2000, 44, 201–206.
(11) Nakamura, S.; Ban, M.; Kato, A. Preparation of bioactive and surface functional oligomannosyl neoglycoprotein using extracellular pH-sensitive glycosylation of mutant lysozyme having N-linked signal sequence in yeast. Bioconjugate Chem. 2006, 17, 1170–1177.
(12) Gaber, M.; Mabrouk, M. T.; Freag, M. S.; Khiste, S. K.; Fang, J. Y.; Elkhodairy, K. A.; Elzoghby, A. O. Protein-polsaccharide nanohybrids: Hybridization techniques and drug delivery applications. Eur. J. Pharm. Biopharm. 2018, 133, 42–62.
(13) Gershkovich, A. A.; Verevka, S. V.; Kibirev, V. K.; Demchenko, A. P. Solid-state conjugation of proteins with hydrophobic compounds in non-denaturing conditions. I. Acylation of proteins by dansyl proline using a polymeric N-hydroxysuccinimide ester. J. Biochem. Biophys. Methods 2000, 45, 183–191.
(14) Lillo, A. M.; Lopez, C. L.; Rajale, T.; Yen, H. J.; Magurudeniya, H. D.; Phipps, M. L.; Balog, E. R. M.; Sanchez, T. C.; Iyer, S.; Wang, H. L.; Michalczzyk, R.; Rocha, R. C.; Martinez, J. S. Conjugation of amphiphilic proteins to hydrophobic ligands in organic solvent. Bioconjugate Chem. 2018, 29, 2654–2664.
(15) Huang, E. S.; Subbiah, S.; Levitt, M. Recognizing native folds by the arrangement of hydrophobic and polar residues. J. Mol. Biol. 1995, 252, 709–720.
(16) Malleshappa Gowder, S.; Chatterjee, J.; Chaudhuri, T.; Paul, K. Prediction and analysis of surface hydrophobic residues in tertiary structure of proteins. Sci. World J. 2014, 2014, No. 971258.
(17) Arakawa, T.; Kita, Y.; Timasheff, S. N. Protein precipitation and denaturation by dimethyl sulfoxide. Biophys. Chem. 2007, 131, 62–70.
(18) van Dijk, E.; Hoogenveen, A.; Abeln, S. The hydrodynamic temperature dependence of amino acids directly calculated from protein structures. PLoS Comput. Biol. 2015, 11, No. e1004277.
(19) Moskovitz, Y.; Srebnik, S. Conformational changes of globular proteins upon adsorption on a hydrophobic surface. Phys. Chem. Chem. Phys. 2014, 16, 11698–11707.
(20) Das, U.; Hariprasad, G.; Ethayathulla, A. S.; Manral, P.; Das, T. K.; Pasha, S.; Mann, A.; Ganguli, M.; Verma, A. K.; Bhat, R.; Chandrayan, S. K.; Ahmed, S.; Sharma, S.; Kaur, P.; Singh, T. P.; Srinivasan, A. Inhibition of protein aggregation: supramolecular assemblies of arginine hold the key. PLoS One 2007, 2, No. e1176.
ACS Omega  
http://pubs.acs.org/journal/acsodf

(21) Fink, A. L. Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Folding Des.* 1998, 3, R9–R23.

(22) Kotarek, J.; Stuart, C.; De Paoli, S. H.; Simak, J.; Lin, T. L.; Gao, Y.; Ovanesov, M.; Liang, Y.; Scott, D.; Brown, J.; Bai, Y.; Metcalfe, D. D.; Marszal, E.; Ragheb, J. A. Subvisible particle content, formulation, and dose of an erythropoietin peptide mimetic product are associated with severe adverse postmarketing events. *J. Pharm. Sci.* 2016, 105, 1023–1027.

(23) Ratanji, K. D.; Derrick, J. P.; Dearman, R. J.; Kimber, I. Immunogenicity of therapeutic proteins: influence of aggregation. *J. Immunotoxicol.* 2014, 11, 99–109.

(24) Akiyoshi, K.; Deguchi, S.; Moriguchi, N.; Yamaguchi, S.; Sunamoto, J. Self-aggregates of hydrophobized polysaccharides in formation and characteristics of nanoparticles. *Macromolecules* 1993, 26, 3062–3068.

(25) Akiyoshi, K.; Ueminami, A.; Kurumada, S.; Nomura, Y. Self-Association of cholesteryl-bearing poly(L-lysine) in water and control of its secondary structure by host–guest interaction with cyclodextrin. *Macromolecules* 2000, 33, 6752–6756.

(26) Nomura, Y.; Sasaki, Y.; Takagi, M.; Narita, T.; Aoyama, Y.; Akiyoshi, K. Thermoresponsive controlled association of protein with a dynamic nanogel of hydrophobized polysaccharide and cyclodextrin: heat shock protein-like activity of artificial molecular chaperone. *Biomacromolecules* 2005, 6, 447–452.

(27) Hashimoto, Y.; Mukai, S. A.; Sasaki, Y.; Akiyoshi, K. Nanogel tectonics for tissue engineering: protein delivery systems with nanogel core–shell hybrid macromolecular nanoparticles. *Adv. Healthcare Mater.* 2018, 7, No. 1800729.

(28) Kobayashi, H.; Katakura, O.; Morimoto, N.; Akiyoshi, K.; Kasuga, S. Effects of cholesterol-bearing pullulan (CHP)-nanogels in combination with prostaglandin E1 on wound healing. *J. Biomed. Mater. Res., Part B* 2009, 91B, 55–60.

(29) Smyth, D. G.; Stein, W. H.; Moore, S. The sequence of amino acids in bovine pancreatic ribonuclease: revisions and confirmations. *J. Biol. Chem.* 1963, 238, 227–34.

(30) Okorokov, A. I.; Panov, K. I.; te Poele, R. H.; Breukelman, H. J.; Furia, A.; Karpeisky, M.; Beintema, J. An efficient system for active bovine pancreatic ribonuclease expression in Escherichia coli. *Protein Expr. Purif.* 1995, 6, 472–480.

(31) Stelea, S. D.; Pancoska, P.; Benight, A. S.; Keiderling, T. A. Thermal unfolding of ribonuclease A in phosphate at neutral pH: deviations from the two-state model. *Protein Sci.* 2001, 10, 970–978.

(32) Cucillo, C. M.; Nogues, M. V.; Raines, R. T. Bovine pancreatic ribonuclease: fifty years of the first enzymatic reaction mechanism. *Biochemistry* 2000, 39, 7835–7841.

(33) Koczera, P.; Martin, L.; Marx, G.; Schuerholz, T. The guest interaction with cyclodextrin. *Phys. Chem. Chem. Phys.* 2004, 6, 3271–3279.

(34) By, J. W.; Falconer, R. J. Thermal stability of lysozyme as a function of ion concentration: a reappraisal of the relationship between the Hofmeister series and protein stability. *Protein Sci.* 2013, 22, 1563–1570.

(35) Mehta, R.; Kundu, A.; Kishore, N. 4-Chlorobutanol induces unusual reversible and irreversible thermal unfolding of ribonuclease A: thermodynamic, kinetic, and conformational characterization. *Int. J. Biol. Macromol.* 2004, 34, 13–20.

(36) Brauner, J. W.; Flach, C. R.; Mendelsohn, R. A. Quantitative reconstruction of the amide I contour in the IR spectra of globular proteins: from structure to spectrum. *J. Am. Chem. Soc.* 2005, 127, 100–109.

(37) Long, G.; Ji, Y.; Pan, H.; Sun, Z.; Li, Y.; Qin, G. Characterization of thermal denaturation structure and morphology of soy glutenin by FTIR and SEM. *Int. J. Food Prop.* 2015, 18, 763–774.

(38) Vinu, A.; Miyahara, M.; Ariga, K. Biomaterial immobilization in nanocomposite carbon molecular sieves: influence of solution pH, pore volume, and pore diameter. *J. Phys. Chem. B* 2005, 109, 6436–6441.

(39) Kelly, S. M.; Price, N. C. The use of circular dichroism in the investigation of protein structure and function. *Curr. Protein Pept. Sci.* 2000, 3, 349–384.

(40) Kelly, S. M.; Jess, T. J.; Price, N. C. How to study proteins by circular dichroism. *Biochim. Biophys. Acta* 2005, 1751, 119–139.

(41) Greenfield, N. J. Using circular dichroism spectra to estimate protein secondary structure. *Protein Sci.* 1995, 4, 89–109.

(42) Moorthy, S. R.; Chakrabarti, S.; Giri, S. Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Forster resonance energy transfer techniques. *Curr. Protein Pept. Sci.* 2013, 16, 22518–22538.

(43) Teale, F. W.; Weber, G. Ultraviolet fluorescence of the aromatic amino acids. *Biochem. J.* 1957, 65, 476–482.

(44) Protein Fluorescence. *Principles of Fluorescence Spectroscopy*, Lakowicz, J. R., Ed.; Springer US: Boston, MA, 2006; pp 529–575.

(45) Ghisaidoob, A. B.; Chung, S. J. Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Forster resonance energy transfer techniques. *Int. J. Mol. Sci.* 2014, 15, 22518–22538.

(46) Fitter, J.; Haber-Pohlmeier, S. Structural stability and unfolding properties of thermostable bacterial alpha-amylases: a comparative study of homologous enzymes. *Biochemistry* 2004, 43, 9589–9599.

(47) Duy, C.; Fitter, J. How aggregation and conformational scrambling of unfolded states govern fluorescence emission spectra. *Biochem. Biophys. Acta* 2006, 1760, 3704–3711.

(48) Kirk, W. R.; Kurian, E.; Prendergast, F. G. Characterization of the sources of protein–ligand affinity: 1-sulfonato-8-(1′)-anilino naphtalene binding to intestinal fatty acid binding protein. *Biophys. J.* 1996, 70, 69–83.
(61) Pastukhov, A. V.; Ropson, I. J. Fluorescent dyes as probes to study lipid-binding proteins. Proteins 2003, 53, 607−615.
(62) Gasymov, O. K.; Glasgow, B. J. ANS fluorescence: potential to augment the identification of the external binding sites of proteins. Biochim. Biophys. Acta 2007, 1774, 403−411.
(63) Izutsu, K. I. Applications of Freezing and Freeze-drying in Pharmaceutical Formulations. In Advances in Experimental Medicine and Biology, 2018; Vol. 1081, pp 371−383.
(64) Kato, A.; Tsutsui, N.; Matsudomi, N.; Kobayashi, K.; Nakai, S. Effects of partial denaturation on surface properties of ovalbumin and lysozyme. Agric. Biol. Chem. 2014, 45, 2755−2760.
(65) Jiang, L.; Wang, Z.; Li, Y.; Meng, X.; Sui, X.; Qi, B.; Zhou, L. Relationship between surface hydrophobicity and structure of soy protein isolate subjected to different ionic strength. Int. J. Food Prop. 2015, 18, 1059−1074.
(66) Moussauoi, M.; Nogues, M. V.; Guasch, A.; Barman, T.; Travers, F.; Cuchillo, C. M. The subsites structure of bovine pancreatic ribonuclease A accounts for the abnormal kinetic behavior with cytidine 2′,3′-cyclic phosphate. J. Biol. Chem. 1998, 273, 25565−25572.
(67) Akiyoshi, K.; Sasaki, Y.; Kuroda, K.; Sunamoto, J. Controlled association of hydrophobized polysaccharide by cyclodextrin. Chem. Lett. 1998, 27, 93−94.
(68) Jackson, M.; Mantsch, H. H. Beware of proteins in DMSO. Biochim. Biophys. Acta 1991, 1078, 231−235.
(69) Hosseinkhani, H.; Aoyama, T.; Ogawa, O.; Tabata, Y. Liver targeting of plasmid DNA by pullulan conjugation based on metal coordination. J. Controlled Release 2002, 83, 287−302.
(70) Koner, A. L.; Krdnija, D.; Hou, Q.; Sherratt, D. J.; Howarth, M. Hydroxy-terminated conjugated polymer nanoparticles have near-unity bright fraction and reveal cholesterol-dependence of IGF1R nanodomains. ACS Nano 2013, 7, 1137−1144.
(71) Hermanson, G. T. Zero-Length Crosslinkers. In Bioconjugate Techniques, 2nd ed.; Hermanson, G. T., Ed.; Academic Press: New York, 2008; Chapter 3, pp 213−233.
(72) Varshosaz, J.; Hassanzadeh, F.; Sadeghi Aliabadi, H.; Nayebsadrian, M.; Banitalebi, M.; Rostami, M. Synthesis and characterization of folate-targeted dextran/retinoic acid micelles for doxorubicin delivery in acute leukemia. Biomed. Res. Int. 2014, No. 52684.
(73) Hussain, M. A.; Abbas, K.; Lodhi, B. A.; Sher, M.; Ali, M.; Tahir, M. N.; Tremel, W.; Iqbal, S. Fabrication, characterization, thermal stability and nanoassemblies of novel pullulan-aspirin conjugates. Arab. J. Chem. 2017, 10, S1597−S1603.
(74) Sakamoto, S.; Nagamitsu, R.; Yusakul, G.; Miyamoto, T.; Tanaka, H.; Morimoto, S. Ultrasensitive immunoassay for monocrotaline using monoclonal antibody produced by N, N′-carbonimidazole mediated hapten-carrier protein conjugates. Talanta 2017, 168, 67−72.