Organic &
Biomolecular
Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 4266

www.rsc.org/obc

PAPER

A pH-responsive carboxylic β-1,3-glucan polysaccharide for complexation with polymeric guests

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Received 24th January 2011, Accepted 7th March 2011
DOI: 10.1039/c1ob05114h

The helix-forming nature of β-1,3-glucan polysaccharides is a characteristic that has potential for producing gene carriers, bio-nanomaterials and other chiral nanowires. Herein, carboxylic curdlan (CurCOOH) bearing the β-1,3-polyglucuronic acid structure was successfully prepared from β-1,3-glucan polysaccharide curdlan (Cur) by one-step oxidation using a 4-acetamido-TEMPO/NaClO/$NaClO_2$ system as the oxidant. The resulting high-molecular-weight CurCOOH was proved to bear the 6-COOH group in 100% purity. The optical rotatory dispersion (ORD) spectra indicated that the obtained CurCOOH behaves as a water-soluble single-strand in various pH aqueous media. This advantage has allowed us to use CurCOOH as a polymeric host to form various macromolecular complexes. For example, complexation of CurCOOH with single-walled carbon nanotubes (SWNTs) resulted in a water-soluble one-dimensional architecture, which formed a dispersion in aqueous solution that was stable for several months, and much more stable than SWNTs complexes of the similar negatively-charged polyacrylic acid (PAA) and polymethacrylic acid (PMAA). It was shown that in the complex, SWNTs are effectively wrapped by a small amount of CurCOOH, enabling them to avoid electrostatic repulsion. This pH-responsive CurCOOH formed a very stable complex with cationic water-soluble polythiophenes (PT-1), which was stabilized not only by the hydrophobic interaction but also by the electrostatic attraction between trimethylammonium cations in PT-1 and dissociated anionic COO^- groups in CurCOOH. The included PT-1 became CD-active only in the neutral to basic pH region, and the positive Cotton effect suggested that the conjugated main chain is twisted in the right-handed direction. We also found that CurCOOH can interact with polycytidylic acid (poly(C)) only under high NaCl concentrations, the binding and release of which could be controlled by a change in the salt concentration. We believe, therefore, that CurCOOH bearing a dissociable COOH group can act as a new potential polymeric host to construct novel polymeric complexes applicable for gene carriers, biosensors, chiral polymer assemblies, *etc.*

Introduction

β-1,3-Glucan polysaccharides are well-known as a natural polymer family possessing features greatly different from other natural or synthetic helical polymers by their strong helix-forming nature and reversible interconversion between the single-strand random coil and the triple-stranded helical structure.[1] During this interconversion process, they can interact with certain polynucleotides to form triple-stranded helical macromolecular complexes consisting of two polysaccharide-strands and one polynucleotide-strand.[2] The most widely studied β-1,3-glucans are schizophyllan (SPG) and curdlan (Cur). Complex formation can occur only with the single-stranded random coil, so that the denaturing process of triple-stranded SPG (t-SPG) to single-stranded SPG (s-SPG), achieved by alkaline aqueous media or dimethylsulfoxide (DMSO),[3] is indispensable.

It is known that SPG and Cur are strongly relevant to the stabilization of the triple strand and to guest binding through the hydrogen-bonding interaction with the 2-OH group.[2,4] For the complex formation between polysaccharides and polynucleotides, for example, it has been proposed that the hydrogen-bonding interaction of the 2-OH group and the nucleic acid base is an essential factor influencing the complex stabilization.[2,5] On the other hand, the 6-CH_2OH group plays another important role in interactions with the surrounding environment. One may

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consider, therefore, that selective modification of the 6-CH_2OH group would scarcely reduce the above-mentioned helix-forming and guest-binding functions of β-1,3-glucan polysaccharides. It not only maintains the hydrogen-bonding effect in complexation with polynucleotides, but extends to other hydrophobic macromolecular guests to form one-dimensional architectures consisting of SWNTs,[6] conjugated polymers,[7] polysilanes,[8] functional molecules[9] and Au-nanoparticles,[10] to produce unique nanocomposites by their entrapment within the helical superstructure of β-1,3-glucans.

Previously, we reported a new methodology to introduce a variety of functional groups into the 6-CH_2OH group of Cur quantitatively and selectively by 'Click Chemistry'.[11] According to this method, we could introduce cationic trimethylammonium groups (Cur-N^+) and anionic sulfonate groups (Cur-SO_3^-) to obtain water-soluble ionic Cur derivatives.[6e,12] As these ionic Cur derivatives are able to wrap single-walled carbon nanotubes (SWNTs) or conjugated polymers, one can construct novel highly-ordered nanoarchitectures by just mixing two components or utilizing a layer-by-layer method.[13] Then, it occurred to us that direct oxidation of the 6-CH_2OH group might be applied to obtain one significantly dissociable carboxylic group on the Cur main chain (Fig. 1a). We found that this idea could be realized by using 4-acetamido-TEMPO/NaClO/$NaClO_2$ as an oxidant system to give entirely carboxylic curdlan (CurCOOH), simultaneously maintaining the high M_w.[14] Herein, the oxidation reaction conditions, inclusion abilities and self-assembling polymeric structure of CurCOOH were investigated for SWNTs, in comparison with analogous anionic polymers such as polyacrylic acid (PAA) and polymethacrylic acid (PMAA), cationic polythiophene (PT-1) and single-stranded homopolynucleotide (poly(C)). We have found that, being different from the SPG and Cur derivatives exploited so far, the present pH-responsive CurCOOH exhibits not only the wrapping inclusion properties but also the novel stimuli-responsive functions such as pH responsiveness and NaCl concentration dependence.

Results and discussion

Synthesis and properties of CurCOOH

Synthesis of CurCOOH. A scheme for direct oxidation of native curdlan to carboxylic curdlan (CurCOOH) is shown in Fig. 1a. The reaction was carried out according to a procedure reported by Isogai *et al.*,[14a] using excess $NaClO_2$ and NaClO as oxidants with respect to the catalyst, 4-acetamido-2,2,6,6-tetramethylpiperidine 1-oxyl free radical (4-acetamido-TEMPO), in a neutral medium.[15] The pH of the reaction mixture was kept stable in the pH range 6–7 by using aqueous 0.5 M HCl and 0.5 M NaOH during the oxidation process. The initially turbid Cur solution slowly became transparent after several hours, indicating the appearance of water-soluble polymer in the system.

The aqueous product solution was repeatedly precipitated by pouring into ethanol–water 6 : 4 (vol/vol), dialyzed for 3 days and dried under vacuum. The oxidation rate of 6-CH_2OH group in the Cur main chain and the averaged M_w of CurCOOH were characterized by NMR spectroscopy, FTIR spectroscopy, and size-exclusion chromatography (SEC). The ^{13}C NMR spectrum of CurCOOH (Fig. 2a) reveals the loss of the C6 resonance around 60–66 ppm and the appearance of a new peak at 175.6 ppm ascribable to the carboxyl group. The FTIR spectrum (Fig. 2b) also agrees with the ^{13}C NMR data, with the carbonyl group appearing as intense absorption bands at 1605 cm^{-1} and 1415 cm^{-1}, which were assigned to the symmetrical and asymmetrical carbonyl C=O stretching vibration bands, respectively. Through the comparison with other results described in the literature,[14] these results support the view that the 6-CH_2OH group is entirely oxidized to the carboxylate group. The SEC data (Fig. 2c) provide $M_w = 2.16 \times 10^5$ and PDI = 3.40 through the retention volume of the resulting CurCOOH at 17.85 min using pullulan as a calibration standard series in aqueous 100 mM $NaNO_3$ eluent (Table 1). We previously observed that TEMPO oxidation without careful pH adjustment of the reaction mixture induces the serious degradation

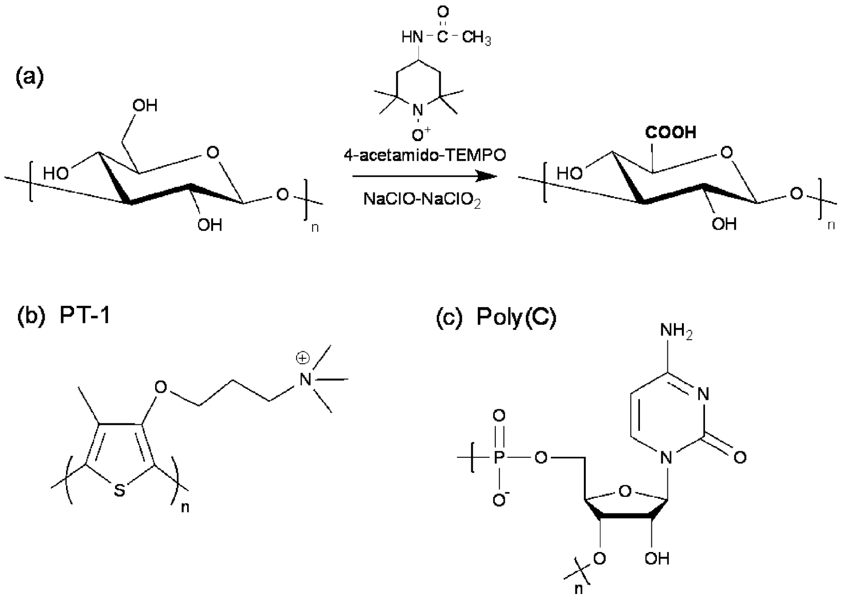

Fig. 1 (a) Schematic oxidation process from curdlan to carboxylic curdlan (CurCOOH) at 35 °C, magnetic stirring for 5 days; (b) cationic polythiophene (PT-1); (c) poly(C).

Table 1 Characterization of CurCOOH by SEC using pullulan as a calibration standard in 100 mM $NaNO_3$ solution at 40 °C

Sample	$M_w \times 10^{-5}$ [a]	$M_n \times 10^{-4}$ [b]	DP [c]	M_w/M_n [d]	Yield (%)
CurCOOH	2.16	6.35	1090	3.40	97.5

[a] Weight-average molecular weight. [b] Number-average molecular weight. [c] Degree of polymerization. [d] Polydispersity.

of Cur to low-molecular-weight compounds. The present result indicates that degradation of Cur in this oxidation process is moderately suppressed by preventing β-elimination in neutral medium, although the broadened polydispersity is inevitable.

Properties of CurCOOH in aqueous solution. The pK_a value of CurCOOH was evaluated by titration of CurCOONa at 25 °C with 0.10 M HCl aqueous solution. The aqueous solutions used for this experiment were all decarbonated with nitrogen and adjusted to μ (ionic strength) = 0.10 with KCl. The solution was slightly turbid at the pH region near pI and then became clear again at the pH region lower than pI. The titration data were analyzed according to the modified Henderson–Hasselbach equation (eqn (1)) frequently used for polymeric acid compounds.[16] The pK_a value corresponds to pH at the half-neutralization, and the deviation of n from unity is a qualitative measure of electrostatic interaction. These values were estimated from the linear plot (r = 0.996) to be pK_a = 3.11 and n = 2.44. The pK_a value is comparable with that of glucuronic acid (pK_a = 3.18, 20 °C, μ = 0.03).[17] On the other hand, the n value is considerably larger than unity, and even larger than those of related polymers.[18] This result suggests that the main chain of the CurCOOH is not so rigid, allowing the intra-polymer COOH–COOH (or COO^-) interaction.

$$\mathrm{pH} = \mathrm{p}K_a - n\log[(1-\alpha)/\alpha] \quad (1)$$

The obtained high-molecular-weight CurCOOH, being different from the high molecular weight of native curdlan, exhibits very good water-solubility but is entirely insoluble in pure DMSO, which is known to behave as a good solvent for native curdlan. One can expect that CurCOOH, as it contains dissociable carboxylic groups, should easily change its solubility depending upon the charge, controlled by the pH of the medium. This behaviour is quite new and different from familiar polysaccharide derivatives.

The higher-order conformation of helix-forming polysaccharides, as well as many other polymers such as polypeptides or synthetic polymers, has been evaluated by measurements of the optical rotatory dispersion (ORD).[2a,12,19,20] In these studies, triple-stranded SPG or Cur show a positive value at wavelengths from 600 to 200 nm, whereas a single-stranded random coil shows a negative value in the similar wavelength region.[19,20] Fig. 3 shows the ORD spectra of CurCOOH (1.26 mM) in pure water and in various phosphate buffer solutions. In all cases, the ORD signals of CurCOOH clearly afforded a negative value from the visible to the ultraviolet region, regardless of the dissociation of CurCOOH to $CurCOO^-$ in media with a wide range of pH. In our previous study on trimethylammonium derivative Cur–N^+ bearing only positive charges, the negative value of the ORD spectra was confirmed. This result was ascribed to electrostatic repulsion among the cationic charges on the side chains, which made formation of the triple-stranded helical structure energetically unfavourable.[12]

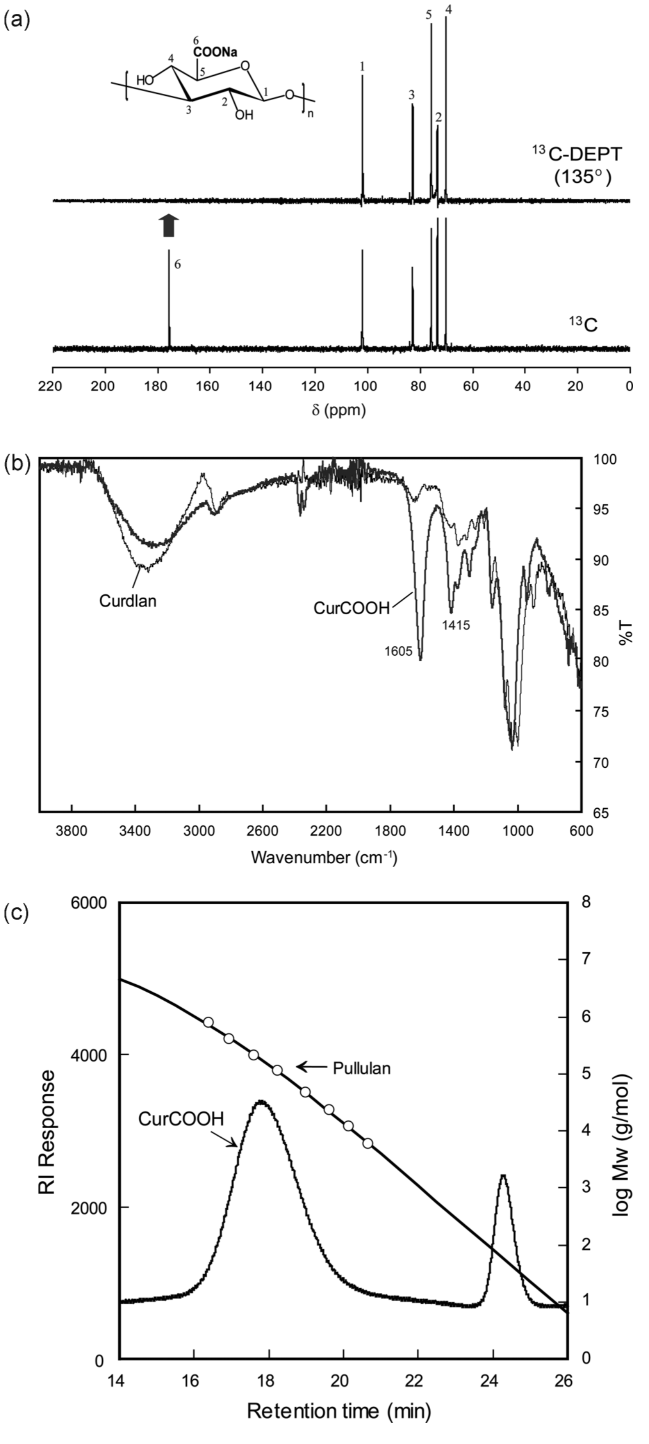

Fig. 2 (a) ^{13}C NMR and ^{13}C-DEPT (135°) spectra of CurCOOH in D_2O at 25 °C; (b) FT-IR spectra of CurCOOH and native curdlan; (c) GPC curve of CurCOOH determined by using pullulan as a calibration standard in 100 mM $NaNO_3$ aqueous solution at 40 °C.

In the present study, at high pH, single strand formation is also rationalized in terms of electrostatic repulsion among dissociated anionic carboxylate groups. In low pH media, on the other hand, CurCOOH can become 'neutral'; nevertheless, it still adopts the single-strand form. Presumably, conversion of 6-CH_2OH to 6-COOH largely reduces the main-chain hydrophobicity which is indispensable to stabilize the triple strand.[7d,12]

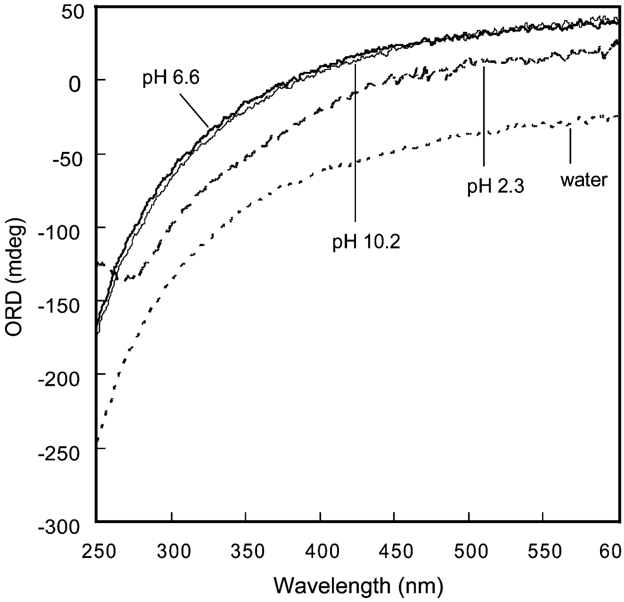

Fig. 3 ORD spectra of CurCOOH (1.25 mM) in pure water and in 50 mM phosphate buffer at pH 2.3 (H_3PO_4/NaH_2PO_4), pH 6.6 (NaH_2PO_4/Na_2HPO_4) and pH 10.2 (Na_2HPO_4/Na_3PO_3), respectively.

Macromolecular complex formation

Inclusion of SWNTs. Discovery of carbon nanotubes has led us to development of various potential nanomaterials because of their unusual electronic and mechanical propreties.[21,22] However, the isotropic and bundled random cohesive SWNTs are scarcely soluble in most solvents, and this inherent drawback has hampered extension of their practical applications.[23] Many researchers have thus devoted efforts towards dissolution of SWNTs by amphiphilic polymeric materials.[24] Among them, helical natural polymers such as amylose and DNA were proved to act as effective dissolution agents.[25] We have also succeeded in wrapping SWNTs by helix-forming polysaccharides such as SPG, Cur and other modified Cur derivatives, which resulted in a new class of supramolecular structural nanoarchitectures.[6,12,13]

Here, SWNTs (~2 μm in length, 1–2 nm in diameter) were dispersed in water by a sonication probe in the presence of single-stranded CurCOOH according to a reported procedure.[12] A homogeneous aqueous solution of SWNTs/CurCOOH composite (Fig. 4a) was obtained after two centrifugations to remove unwrapped SWNTs (the details are described in the Experimental section). The clear solution of SWNTs/CurCOOH composite was stable for more than 2 months without phase separation. TEM images in Fig. 4b and its magnified image in Fig. 4c reveal that SWNT bundles are dispersed into SWNT fibrils of only several nanometres diameter. The finding allows us to conclude that CurCOOH can be classified as a new potential solubilizer for SWNTs.

It is already established that β-1,3-glucan polysaccharides tend to self-assemble to the helical structure on the SWNTs surface through rearranging intra-chain hydrogen-bonding interaction of OH groups to form stable macromolecular complexes.[6c] Thereby, the inner hydrophobic surface plays an important role to stabilize the complexes, whereas the outer hydrophilic surface exists for interacting with the aqueous medium. In order to obtain further insight into the influence of the hydrophilicity–hydrophobicity balance of the β-1,3-glucan derivatives on the stability of SWNTs complexes, we compared CurCOOH ($M_w = 2.16 \times 10^5$, pK_a 3.11) with simple anionic polymers consisting of carboxylic acid groups similar to CurCOOH, such as PAA ($M_w = 2.5 \times 10^5$, hydrophilic polymer, pK_a of corresponding monomer (acrylic acid) = 4.35)[26a] and PMAA ($M_w = 1.0 \times 10^5$, somewhat more hydrophobic polymer, pK_a of corresponding monomer (methacrylic acid) = 4.65).[26b] Under similar preparation conditions (details described in the Experimental section), 1.0 mg SWNTs were dissolved in various aqueous polymer solutions. Fig. 5a shows the UV-Vis spectra of composites after purifying and leaving at room temperature for 24 h. As the absorbance of polysaccharide, PAA and PMAA aqueous solutions at around 275 nm is almost negligible, the absorption of these complexes can be attributed to the SWNTs content dissolved in the aqueous media. The plotted variation of Fig. 5b reveals different behaviours of each polymer in the complexation with SWNTs. It is clearly shown that the absorption

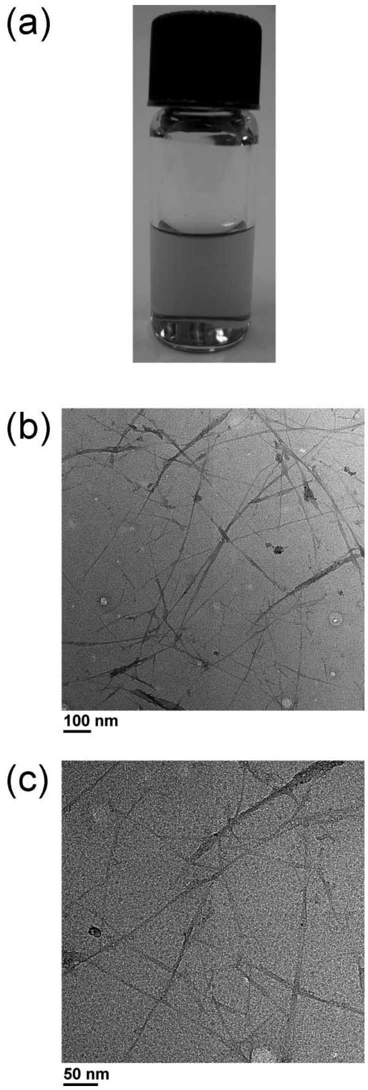

Fig. 4 (a) Photograph; (b) TEM image of SWNTs/CurCOOH composite in an aqueous solution after purification; (c) magnified picture.

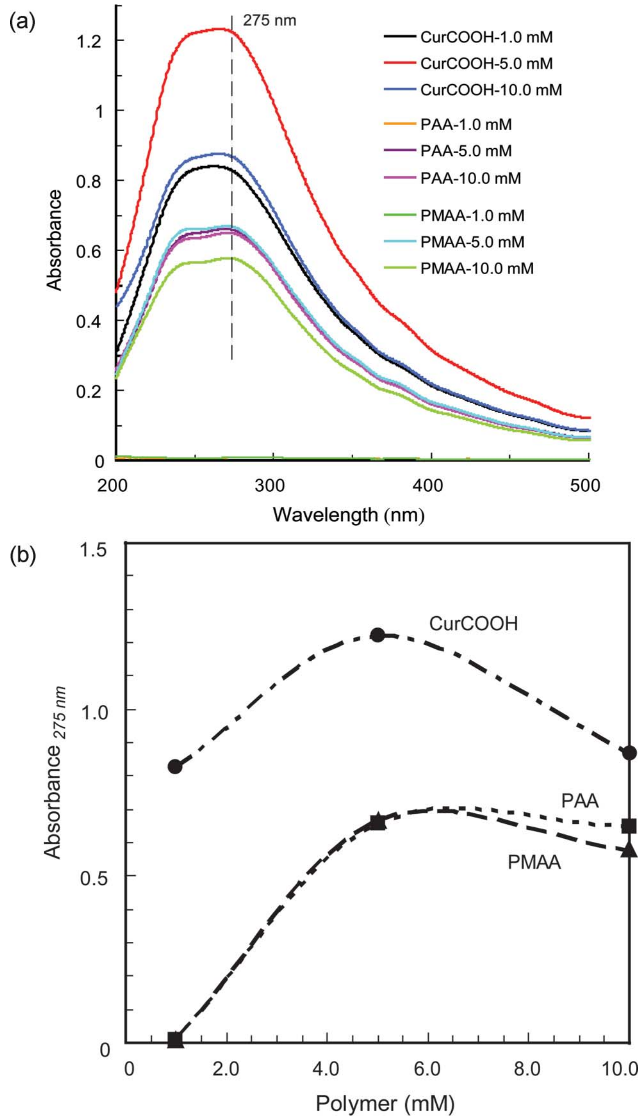

Fig. 5 (a) UV-Vis spectra of SWNTs/CurCOOH, SWNTs/PAA and SWNTs/PMAA composites in aqueous solution (1 cm cell) at 25 °C. (b) UV-Vis absorbance at $\lambda = 275$ nm against polymer concentration (at 1.0 mM, 5.0 mM, and 10.0 mM monomer units) in the complexation between SWNTs and CurCOOH, PAA and PMAA, respectively.

intensity of the SWNTs/CurCOOH complex is much higher than that of the SWNTs/PAA and SWNTs/PMAA complexes at similar polymer concentrations. At low polymer content (1.0 to 4.0 mM monomer units), there is no significant difference between PAA and PMAA, regardless of their different structure and hydrophobicity. In addition, the fact that no SWNT was dissolved up to 1.0 mM suggests that the dissolution of SWNTs is not due to wrapping by a single polymer chain, but due to adsorption of the hydrophobic domain in the polymer chains. These results agree with the previous studies reported for complex formation between PAA or PMAA with SWNTs in neutral solution: that is, SWNTs dissolution is ascribable to the mere adsorption of random coils of polymer chains onto the nanotube surface.[28–29]

In contrast, this result also means that the dissolution ability observed for the low CurCOOH concentration region is not only due to the adsorption, but also more effective actions. As already pointed out for other β-1,3-glucan polysaccharides,[2,24a,27] this is due to the helix-forming nature trapping guest macromolecules through reconstruction of the hydrogen-bonding interaction inside the helix strand. Strangely, the amount of dissolved SWNTs decreased in the higher polymer concentration region. Presumably, there are three different polymer–polymer interactions competing in this system: they are SWNT–SWNT, SWNT–anionic polymer and anionic polymer–polymer interactions. In the higher polymer concentration region, the contribution of the anionic polymer–polymer interaction (*i.e.*, self-aggregation of polymers) becomes relatively large and the amount of dissolved SWNTs decreases. In fact, the decrease in hydrophobic PMAA is more conspicuous than that in hydrophilic PAA. From this context, one may regard CurCOOH to be a flexible and relatively hydrophobic polymer, because the decrease at higher polymer concentrations is quite large. This conjecture is in line with the large n value obtained from the titration experiment. These findings support the view that the highest dissolution ability for SWNTs is obtained on the basis of a delicate balance between polymer hydrophilicity and hydrophobicity, in addition to the higher-order structure of the polymer chain. We also measured the Raman spectra of SWNTs themselves and the SWNTs/CurCOOH complex, but there was no significant difference.

Inclusion of conjugated polymer PT-1

The helix–random coil structural transition of polysaccharides is usually monitored by ORD, viscometry and light scattering. However, these methods are not so sensitive to the conformational transition, particularly in the low concentration region.[30] Thereby, the binding between a certain polysaccharide with a chromophoric macromolecular guest to form a supramolecular chiral complex can be easily detected by typical UV-Vis and CD spectroscopic methods.[7d,31] From these methods, one can recognize even the mode of helical higher-order structures. Herein, complexation of charge-changeable CurCOOH and a water-soluble polythiophene (PT-1) (Fig. 1b) was conducted in various pH media. It is known that PT-1 itself adopts a random coil conformation in mixed solvents and has no CD activity detectable by optical methods.[31a] The pH dependence of the obtained ellipticity of CD spectra (Fig. 6) indicates that the PT-1/CurCOOH complex is formed at pH 6.6–10.2 (above the pK_a), whereas no or very little complex formation occurs at low pH region (below the pK_a). This result clearly shows that PT-1/CurCOOH complex is stabilized not only by the hydrophobic interaction but also by the electrostatic attraction between trimethylammonium cations in PT-1 and dissociated anionic COO^- groups in CurCOOH. In other words, complexation and decomplexation can be easily controlled by a moderate pH change. Furthermore, the positive sign of the Cotton effect indicates that the CurCOOH-wrapped SWNTs maintain the right-handed helicity of the original triple-stranded SPG chains.[3]

Macromolecular complexes of CurCOOH with RNA

Development of reagents that can recognize a specific sequence of RNA or DNA is important in genetic engineering. Among those reagents, our group has found that an s-SPG molecule

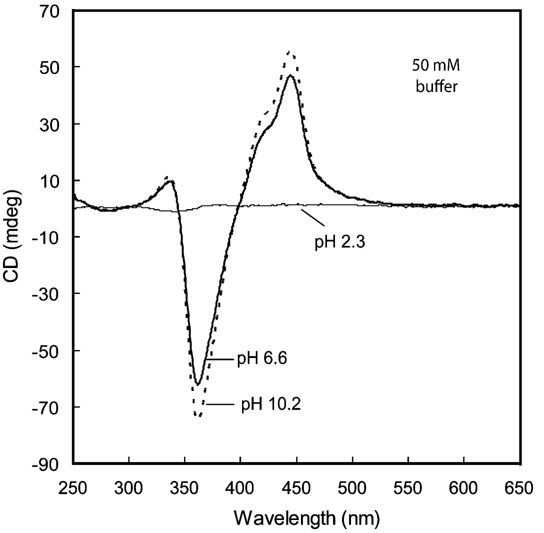

Fig. 6 pH-dependence of the CD spectra in the complexation of CurCOOH (1.26 mM monomer units) and PT-1 (0.10 mM monomer units) in various phosphate buffers (50 mM) at pH 2.3 (H_3PO_4/NaH_2PO_4), pH 6.6 (NaH_2PO_4/Na_2HPO_4), and pH 10.2 (Na_2HPO_4/Na_3PO_3), respectively, using a 1 cm cell at 25 °C.

behaves as if it were a complementary polynucleotide chain for the corresponding polynucleotide through the hydrogen-bonding interaction and the hydrophobic interaction, enhancing the base stacking in the complexes.[2,5,12–13] In particular, a curdlan derivative bearing trimethylammonium cationic groups (CUR-N$^+$) can form a very stable complex with poly(C), because three different forces are operating cooperatively: they are the hydrogen-bonding interaction between the 2-OH group in CUR-N$^+$ and the hydrogen-bonding site of cytosine ring,[2c] the electrostatic interaction between the trimethylammonium cation and the DNA (or RNA) phosphate anion[32] and the background hydrophobic interaction.[12] In this study, complexation of polyanionic CurCOOH toward poly(C) (Fig. 1c) was investigated by CD and UV-Vis spectra, changing the aqueous NaCl concentration.

Fig. 7a shows the CD and UV-Vis spectra of poly(C) in the absence and the presence of CurCOOH in 200 mM NaCl aqueous solution at 25 °C. Likewise, Fig. 7b shows the CD and UV-Vis spectra of poly(C) itself and with CurCOOH in water at 25 °C as reference experiments. As the absorption intensity of CurCOOH itself is negligible in the observed CD band region, the CD band is ascribable to the poly(C) origin. In water, both the CD and UV-Vis spectra scarcely changed, indicating that the complexation of poly(C) and CurCOOH does not occur. In 200 mM NaCl solution, on the other hand, the mixture of poly(C) and CurCOOH clearly shows a positive CD band at 285 nm with a similar intensity to poly(C) itself, and a new negative peak appears at around 264 nm. According to the previous studies,[2] the poly(C)+s-SPG complex often induces a few characteristic spectral changes: for example, (i) decrease in the UV absorbance (hypochromism), (ii) increase in the CD signals and (iii) appearance of a new CD band due to intensified base-stacking through the hydrophobic and hydrogen-bonding interactions. In the present study, the CD band of poly(C)+CurCOOH is red-shifted from 275 nm (the original CD band of poly(C)) to 285 nm, being different from those of cationic CUR-N$^+$ case[12] and SPG case,[2b] in which the CD peak

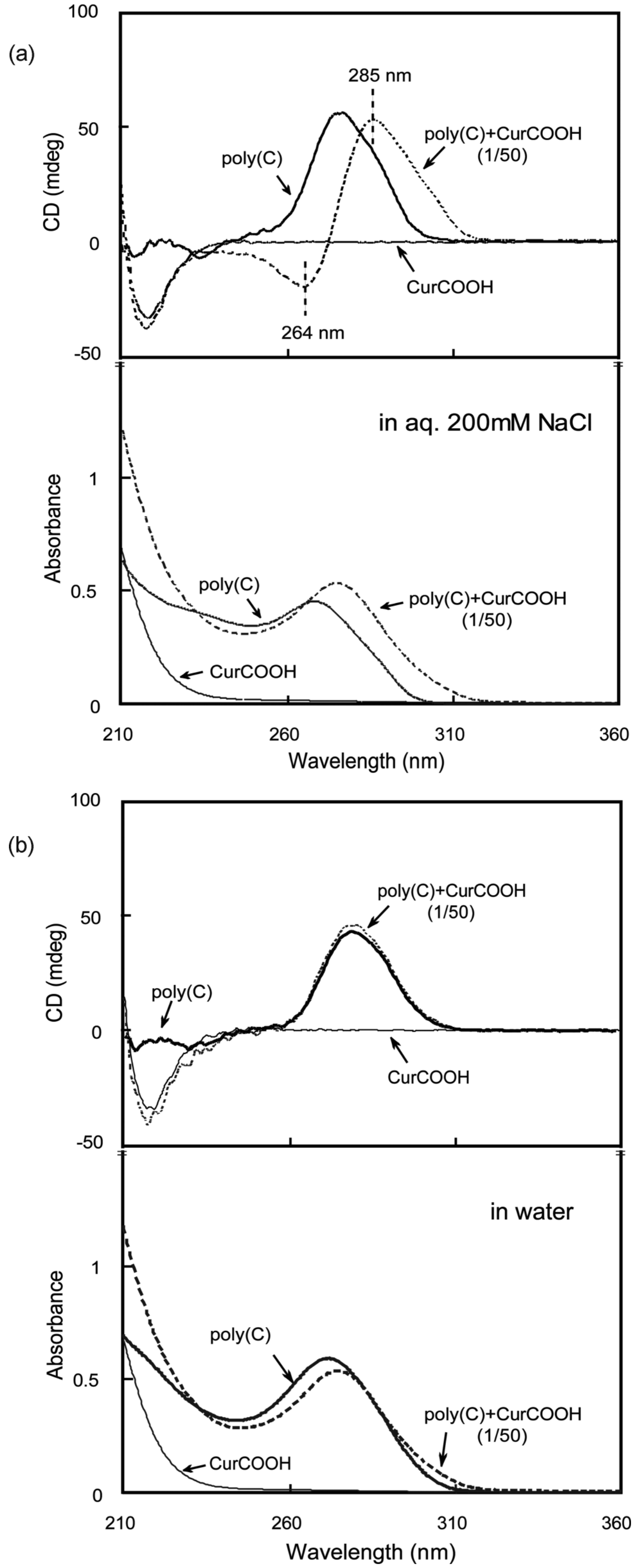

Fig. 7 CD and UV-Vis spectra of poly(C) (0.10 mM monomer units) and poly(C)+CurCOOH (1/50) complex (0.10 mM monomer units, 5.0 mM monomer units) in (a) 200 mM NaCl aqueous solution and (b) in water at 25 °C with a 1 cm cell.

is blue-shifted toward the shorter wavelength. In addition, it was found that the CD intensity at 285 nm continuously increases up to a molar ratio of poly(C)+CurCOOH ~1/50 (Fig. 8) without the decrease in the UV-Vis absorbance in the similar wavelength range. This behaviour is also different from those of cationic CUR-N$^+$ [12] and SPG[2b] which tend to form the 1 : 2 or 1 : 1 stoichiometric complexes. The decrease in the 285 nm CD intensity at the higher CurCOOH is ascribable to self-aggregation of CurCOOH, as already discussed for the SWNTs complexes. The appearance of the new negative CD band around 264 nm provides evidence for the formation of a new helical complex, which is induced by strong base-stacking of cytosine on poly(C) intensified by the high NaCl concentration.

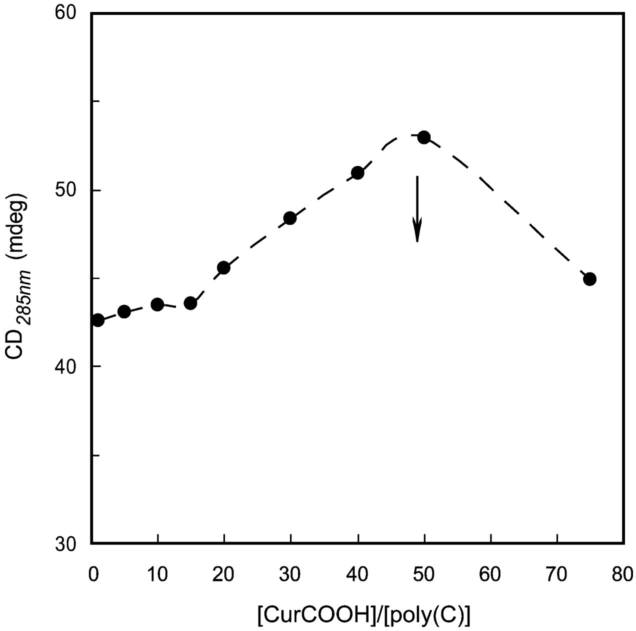

Fig. 8 Ellipticity of the CD intensity at 285 nm for the various molar ratios of CurCOOH against poly(C) at 25 °C.

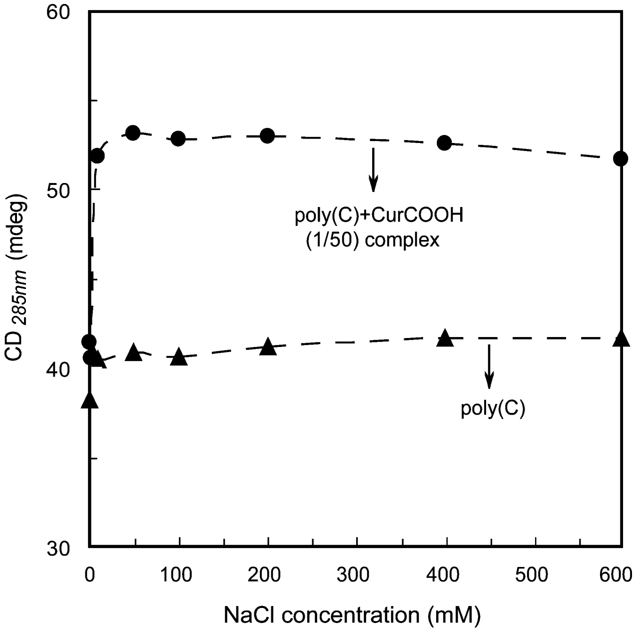

Fig. 9 Influence of the NaCl concentration on the CD spectra monitored at 285 nm for poly(C) itself and poly(C)+CurCOOH (1/50 molar ratio) complex ([poly(C)] = 0.10 mM).

Complex formation between Cur-N$^+$ and poly(C) is greatly stabilized by the electrostatic attraction between the trimethylammonium cation and the DNA (or RNA) phosphate anion.[12,32] On the other hand, the DNA (or RNA) binding to CurCOOH is destabilized by the electrostatic repulsion. Therefore, shielding of the electrostatic repulsion by the high salt concentration becomes indispensable to stabilize the complex. This disadvantage does, however, lead to an advantage – the binding and release of DNA (or RNA) is possible by the control of the salt concentration. Fig. 9 indicates the CD intensity at 285 nm plotted against the NaCl concentration. For poly(C) itself, a small CD intensity increase occurs at very low salt concentrations, and it becomes almost independent of the salt concentration. On the other hand, it is clearly seen for poly(C)+CurCOOH that the CD intensity increases even at low NaCl concentrations, and reaches a plateau above 50 mM. The result indicates that salt shielding of the electrostatic repulsion between polyanionic CurCOOH and polyanionic poly(C) is very effective for complex formation. This salt effect is clearly the reverse of that observed for poly(C)+Cur-N$^+$ complex formation supported by electrostatic attraction.[12] It is rather surprising that the polyanionic CurCOOH can bind polyanionic poly(C) at such low salt concentrations. Presumably, compatibility of the helical pitch length between CurCOOH bearing the β-1,3-glucan main chain and the poly(C) main chain[33] is advantageous for stabilization of the complex, in spite of electrostatic repulsion.[34] We have attempted the determination of the stoichiometry for the poly(C)+CurCOOH complex using a molar ratio plot or a Job plot, but have so far failed, because complex formation can be recognized only in the presence of a large excess of CurCOOH.

Conclusions

In conclusion, we have synthesized CurCOOH by one-step oxidation using 4-amido-TEMPO/NaClO/NaClO$_2$: the obtained CurCOOH is a high-molecular-weight polymer bearing the 6-COOH group in 100% purity upon the β-1,3-glucan polysaccharide chain. This negatively charged curdlan can be easily dissolved in water, and behaves as a random coil in various pH aqueous solutions. It has been shown that pH-responsive CurCOOH can bind to SWNTs by simple mixing of two components with sonication to form a macromolecular complex with a fibrous one-dimensional architecture. The resultant complex is very stable in water – no precipitation occurs for several months. One can conclude, therefore, that the helix-forming feature of β-1,3-glucan polysaccharides retaining a hydrophobic helix core shielded by a hydrophilic carboxylate surface is an important factor for maintaining high stability in complexes with polymeric guests. In complexes with polynucleotide poly(C), it also shows that the complex formation is affected by hydrogen-bonding, electrostatic and hydrophobic forces. An important finding is that the negative factor, electrostatic repulsion, can be removed by high-concentration NaCl solution, which makes the salt-depending binding and release of polynucleotides possible. We believe, therefore, that CurCOOH bearing a dissociable functional group is a potential candidate for dispersion of fibrous nanomaterials, delivery of functional polynucleotides, layer-by-layer adsorption of these ionic complexes, *etc*. In particular, the easily changeable charge of CurCOOH in media of various pH may provide novel applications in biosensors and chiral polymer assemblies.

Experimental

Materials

Native curdlan (M_w ~ 10^6), NaClO, $NaClO_2$, HCl and NaOH were purchased from Wako Co. Ltd., and 4-acetamido-TEMPO was supplied from Tokyo Chemical Industry Co. Poly(C) was purchased from Amersham Pharmacia, and detailed information is provided in a previous report.[12] All were used as received without purification.

Instruments and measurement

IR spectra were recorded on a JASCO FTIR-4200 attached with a universal ATR sampling accessory. ^{13}C NMR and ^{13}C NMR DEPT (135°) spectra were performed at 25 °C on a JEOL 400 MHz (JNM-ECS400) instrument using D_2O as a solvent. Chemical shifts were reported in ppm (δ) relative to tetramethylsilane (TMS). UV-Vis absorbance was recorded on a JASCO V-570 spectrophotometer. ORD and CD spectra were performed on a JASCO J-720WI spectropolarimeter using a 1 cm quartz cell. Transmission electron microscopy (TEM) was conducted on a JEOL JEM-2010 with accelerating voltage 120 kV. TEM samples were prepared from depositing one droplet of the sample solution upon a carbon-coated film supported on a copper grid. After adsorbing few minutes upon the surface of grid, the excess solution was removed with filter paper and the samples were dried under vacuum over night before observation. The averaged molecular-weight of the resultant CurCOOH was determined by size exclusion chromatography (SEC). A Jasco PU-980 liquid chromatography system was used with a refractive-index detector (RI-2031 Plus), a column oven (CO-2060 Plus) and a UV detector (UV-970). This system was connected with a column (Shodex: OHpak SB-860M HQ) and used $NaNO_3$ 100 mM as the eluent with a flow rate 0.50 mL min^{-1} at 40 °C. Elution volume in SEC was calibrated with a series of pullulan standard (Shodex).

Synthesis of CurCOOH. 2.0 g native curdlan was dispersed in 200 mL pure water in a 500 mL two-necked bottom flask kept in a thermoregulated bath at 35 °C by magnetic stirring for 30 min. Then 4-acetamido-TEMPO (0.20 g, 0.94 mmol) and one half of the $NaClO_2$ (2.17 g, 24.0 mmol) were added. A 12.0% solution of NaClO (12.7 mL, 24.0 mmol) was prepared, and oxidation of curdlan occurred when NaClO droplets were slowly added. The NaClO and remaining $NaClO_2$ were added in 10 steps over a period of 10 h. The medium was kept stable and adjusted to neutrality (pH 6–7) using 0.5 M HCl and 0.5 M NaCl. The reaction was complete after 5 days, giving a transparent yellow solution.[15]

Purification of CurCOOH. The mixture of products was precipitated in EtOH–H_2O 6 : 4 (vol/vol) with magnetic stirring for 1 h, centrifuged to remove the soluble part, taken up in pure water and precipitated into EtOH–H_2O. This procedure was performed 3 times. The resultant polysaccharide then was dialyzed using Spectra/Por 5000 in pure water at room temperature for 3 days, filtered and freeze-dried under reduced pressure (yield 97.5 wt%). The final white solid product was characterized by IR, ^{13}C NMR, ^{13}C NMR-DEPT, GPC and ORD.

Preparation of SWNTs. SWNTs were prepared according to the method reported by M. Sano *et al.*,[35] by ultrasonicating commercially-available pristine SWNTs (CNI® Carbon Nanotechnologies, Inc.) in concentrated H_2SO_4/HNO_3, to cut the SWNTs into many short pieces. The cut SWNTs then were etched lightly in H_2SO_4/H_2O_2 to introduce oxygen-containing groups at both ends, filtered, washed and dried. The final product was determined by AFM (length: 1–3 μm).[6a] We have confirmed that SWNTs prepared according to this method are very pure.

Complexation of CurCOOH with SWNTs. 1.0 mg SWNTs were dispersed in 1.8 mL pure water for 10 min using a homogenizor probe, then mixed with the stock aqueous solution of CurCOOH (5.0 mg mL^{-1}, 200 μL) for 30 min. The mixture was centrifuged (13 000 rpm, 15 min, 20 °C) to separate the unreacted SWNTs from the solution. The supernatant, containing the complex of SWNTs/CurCOOH, was pipetted off. This centrifugation procedure was repeated twice in order to remove almost all the unreacted SWNTs. The solution of SWNTs/CurCOOH was stable, no precipitation occurring for about 2 months at room temperature. The dispersion of SWNTs was observed by TEM.

Complexation of CurCOOH with PT-1. Various aqueous phosphate buffers (200 mM, 250 μL), consisting of H_3PO_4/NaH_2PO_4 (pH 2.1), NaH_2PO_4/Na_2HPO_4 (pH 6.0), and Na_2HPO_4/Na_3PO_3 (pH 10.0), respectively, were diluted with pure water (650 μL). An aqueous solution of CurCOOH (25.25 mM monomer units, 50 μL) was added into the diluted buffer. Finally, 50 μL of PT-1 aqueous solution (2.0 mM monomer units) was added to form the complex. The pH of the resultant mixture was confirmed and the CD, UV-Vis spectra were measured.

Complexation of CurCOOH with RNA. Poly(C) (1.0 mM unit, 100 μL) was mixed with a high concentration of aqueous NaCl (500 mM, 400 μL), and diluted with pure water (200 μL). The desired volume of aqueous CurCOOH solution (25.0 mM monomer units) then was added, and the mixture was adjusted to 1000 μL by pure water. The samples were left at room temperature for 30 min and measured by CD and UV-Vis.

Acknowledgements

We thank Dr Shuichi Haraguchi for valuable discussions and comments. We thank Professor Masashi Nishida (Sojo University) for his experimental help on the titration and Professor Nobuo

Kimizuka (Kyushu University) for measurement of Raman spectra. We gratefully acknowledge the City Area Program of the Ministry of Education, Culture, Science, Sports, and Technology (Japan) for the financial support. This work was partially supported by the MEXT, Grant-in-Aid for Scientific Research on Innovative Areas "Emergence in Chemistry".

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