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Parallel *vs.* anti-parallel orientation in a curdlan/oligo(dA) complex as estimated by a FRET technique

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We already found that β-1,3-glucan polysaccharides form polymeric complexes with certain polynucleotides, but the parallel *vs.* anti-parallel orientation in those complexes had remained unsolved. In this paper, this controversial problem has been discussed for curdlan/oligo(dA) complexes utilizing two different energy transfer techniques. The first system consists of a combination of fluorescein-labeled curdlan and 3′-(or 5′-)tetramethyl-rhodamine (TAMRA)-labeled oligo(dA). The second system utilizes gold nanoparticles: that is, two curdlan chains were linked by a disulfide bond and after complexation with oligo(dA), the complex was immobilized on gold nanoparticles. In this system, TAMRA was attached to the 3′ (or 5′) end of oligo(dA) and the gold particle acted as a fluorescence quencher (energy acceptor). These experiments have led us to conclude that in the curdlan/oligo(dA) complex, parallel orientation is more favourable than anti-parallel orientation. These findings have enabled us to envision a clearer image for the complexation mode between β-1,3-glucan polysaccharides and polynucleotides.

Introduction

Biomacromolecules can self-assemble to construct hierarchically ordered structures, which frequently play a crucial role in biological events. For example, two DNA chains twine around each other through intermolecular hydrogen bonds to form a right-handed 10_1 double helix with 1.8 nm pitch. It is well-known that when they form a double strand, anti-parallel orientation is favourably adopted in the helical structure; that is, the 3′ end of one DNA chain and the 5′ end of another chain meet together at the same side of the helical structure. Structural analysis of the DNA double helix showed that anti-parallel orientation is an inevitable consequence from stereochemical viewpoints to maintain Watson–Crick-type hydrogen bonds.[1] The same principle of stereochemistry also determines the higher-order structures of polysaccharides. Curdlan is a natural polysaccharide consisting of a β-1-3-D-glucan main-chain structure. X-Ray crystallographic studies revealed that three curdlan chains adopt a parallel orientation to form a triple helix,[2] where all reducing ends of the polysaccharide chains are arranged to the same side of the triple helix. According to X-ray diffraction data, curdlan adopts a right-handed 6_1 triple helix with 1.8 nm pitch. Interestingly, curdlan's helix parameters are quite similar to those of DNA double strands.[3] This structural complementarity reasonably rationalizes our recent, original finding that curdlan forms polymeric complexes with certain polynucleotides. Curdlan's triple strand can be dissociated into single chains (s-CURs) by dissolving in dimethylsulfoxide (DMSO) (denaturation) and the s-CUR chains can retrieve the original triple strand by exchanging DMSO for water (renaturation).[4] Complexation can take place during the renaturation process, that is, when the renaturation is carried out in the presence of a homo nucleic acid such as poly(C), poly(A) or poly(dA); two curdlan chains and one nucleotide chain can form a complex instead of re-forming the curdlan triple strand.[5] This novel feature suggests the great potential of curdlan to act as a new type of DNA mimic.

Computational studies have clarified several interesting facets of curdlan/DNA complexes: that is, (1) two curdlan chains maintain the parallel orientation in the complex, (2) the 2-OH groups of curdlan bind to carbonyl or amine groups of the DNA nucleobase with intermolecular hydrogen bonds and (3) the complex is stabilized by hydrophobic interactions as well as hydrogen-bonding interactions.[6] Here, the most fundamental question arises for the curdlan/DNA triple strands: that is, what kind of orientation two curdlan chains and one polynucleotide chain take in the hetero-triple strand. As shown in Fig. 1, there are three possible orientations, *i.e.*, parallel, anti-parallel and alternate orientations. Here, parallel orientation denotes that the reducing end of the curdlan chains and the 3′ end of the polynucleotide chain are arranged at the same side of the triple strand. Anti-parallel orientation denotes that the polynucleotide chain is inserted inversely. On the other hand, alternate orientation denotes that two curdlan chains are arranged in an anti-parallel direction. Without clarifying

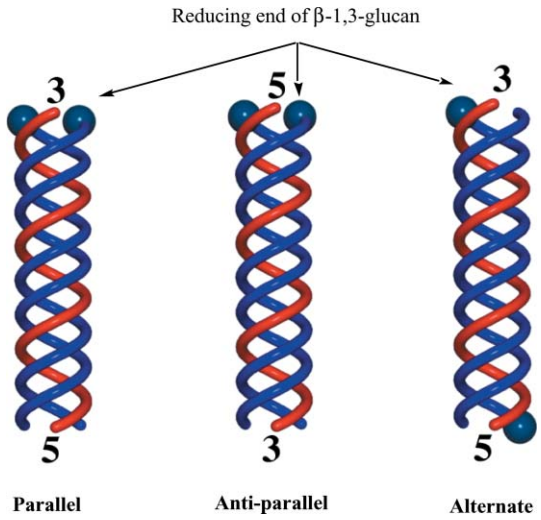

Fig. 1 Schematic illustration of parallel, anti-parallel and alternate orientations. Curdlan and polynucleotide are shown by blue and red chains, respectively.

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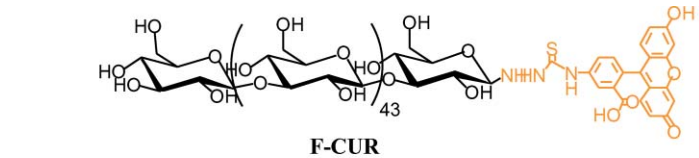
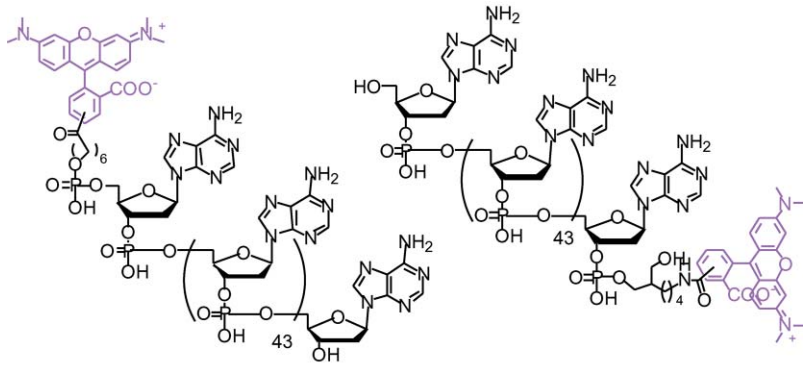


F-CUR



5'TAMRA-dA 3'TAMRA-dA

Fig. 2 Structures of fluorescein-labeled curdlan (F-CUR) and 3′ (or 5′)-end-TAMRA-labeled deoxyadenylic acid (3′ (5′) TAMRA-dA).

this orientational problem, it seems difficult to obtain further insights into curdlan–polynucleotide interactions. In this paper, we report our novel approach to find an answer for this question.

Results and discussion

The most distinct difference between the three orientations in Fig. 1 is the distance between the end groups. For example, in the parallel orientation, the distance from the reducing end of the polysaccharides to the 3′ end of the polynucleotide should be shorter than that to the 5′ end of the polynucleotide. The most convenient method to estimate the distance in solution is to utilize fluorescence resonance energy transfer (FRET).[7] Here, we utilize FRET between fluorescein and tetramethyl-rhodamine.

We prepared the complex from fluorescein-labeled curdlan (F-CUR) and tetramethyl-rhodamine (TAMRA)-3′(or 5′)-labeled deoxyadenylic acid (TAMRA-dA) (Fig. 2).[8] Here, the excited fluorescein can transfer its energy to TAMRA according to the FRET mechanism only when both dyes are located in close proximity. The complexation was accomplished by mixing an aqueous solution of 3′-TAMRA-dA (or 5′-TAMRA-dA) with a DMSO solution containing F-CUR according to a reported procedure.[5] The final composition of water–DMSO was adjusted to be 90 : 10 (v/v). After keeping the solution for 3 days at 4 °C, we measured the UV–Vis and fluorescence spectra. Fig. 3(a) shows the UV–Vis spectra for 3′TAMRA-dA/F-CUR and 5′TAMRA-dA/F-CUR complexes. Two peaks are seen at 490 and 560 nm, which can be assigned to the absorption bands of fluorescein in F-CUR and TAMRA in 3′TAMRA-dA or 5′TAMRA-dA, respectively. Fig. 3(b) shows the fluorescence spectra obtained by exciting the fluorescein group at 495 nm. A strong emission peak appears at around 520 nm, which is assigned to emission from the fluorescein group, however, almost no emission peak from the TAMRA group (which is expected to appear at around 580 nm) can be detected for both 3′TAMRA-dA/F-CUR and 5′TAMRA-dA/F-CUR complexes. We confirmed that the shoulder peak around 580 nm is assigned to an own emission from the TAMRA group (not the FRET peak) which can be excited even at 495 nm. These results suggest that the perceptible level of FRET does not take place suggesting that complex formation does not occur under the present conditions or, even if the complexes are formed, they do not have enough thermodynamic stability to provide FRET because of the short polymer chain length.

It is already known that β-1,3-glucan–polynucleotide complexes are thermodynamically more stabilized by decreasing the DMSO concentration.[5] Encouraged by this finding and to observe the FRET phenomenon under more favourable conditions, we prepared the sample with decreasing the DMSO concentration, *i.e.*, water–DMSO = 95 : 5 and 99.5 : 0.5 (v/v), respectively. Fig. 3(c) shows the fluorescence spectrum obtained from the solution containing water–DMSO = 95 : 5 (v/v). As shown in Fig. 3(c), a weak but significant emission peak newly appears at around 580 nm only for the 3′TAMRA-dA/F-CUR (blue line) complex, which would be attributable to emission from the TAMRA group. Furthermore, one can see that the emission peak becomes stronger in the composition of water–DMSO = 99.5 : 0.5 (v/v) (Fig. 3(d)). Here, it should be emphasized that the intensity of the FRET peak from the 3′TAMRA-dA/F-CUR complex (blue line) is significantly stronger than that from the 5′TAMRA/F-CUR complex (red line), suggesting that FRET takes place more effectively in the 3′TAMRA-dA/F-CUR complex.[9] Taking these results into consideration, we further removed DMSO from the water–DMSO (95 : 5 (v/v)) mixed solvent by extensive dialysis at 4 °C. The fluorescence spectral measurements were carried out for these aqueous solutions under the same conditions as described above (at 4 °C, excitation 495 nm).[10] As shown in Fig. 4(b), an emission peak attributed to TAMRA appears clearly and the difference in the FRET peak intensity between 3′TAMRA-dA/F-CUR (blue line) and 5′TAMRA/F-CUR (red line) becomes bigger. Moreover, we prepared the sample solutions from a 60 : 40 water–DMSO mixed solvent followed by extensive dialysis and confirmed that the final fluorescence spectrum is basically the same as that obtained from the 95 : 5 water–DMSO solvent (Fig. 4(c)). The complex formation after the dialysis was confirmed with gel electrophoresis (Fig. 5). Lanes 1 and 2 show the band for the 5′TAMRA-dA/F-CUR and 3′TAMRA-dA/F-CUR complexes, respectively: one can clearly recognize the non-migrated bands that are assigned to the complexes. Taking these facts into consideration, we can propose that the 3′ end of oligo(dA) and the reducing end of curdlan are located at the same side in the complex: that is, parallel orientation is more favourable than anti-parallel orientation.

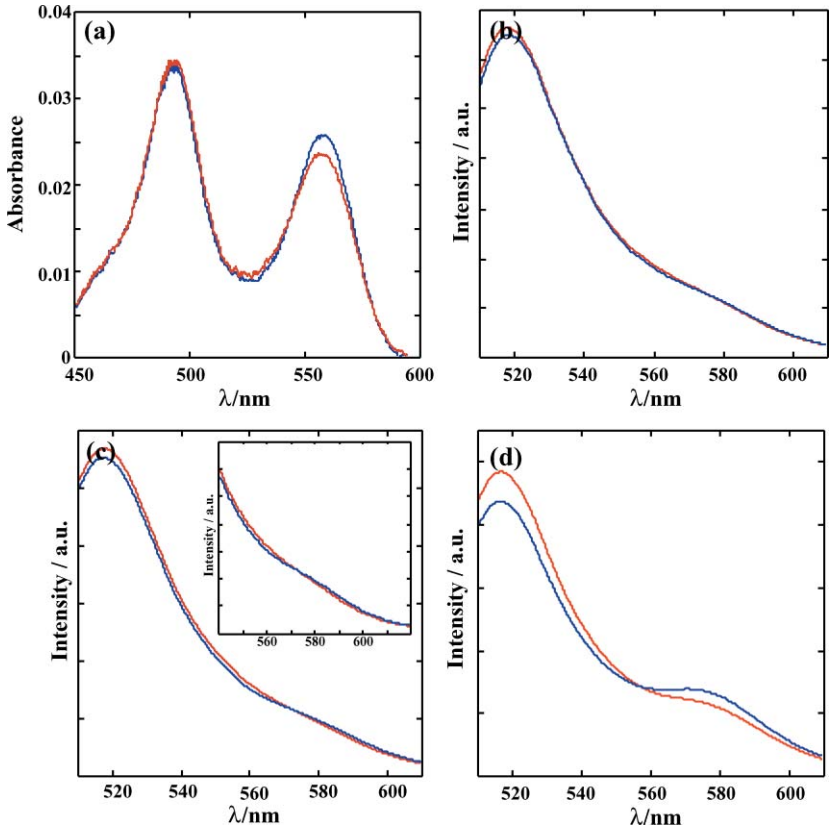


Fig. 3 Comparison of UV–Vis (a) and fluorescence spectra, (b) between 3′TAMRA-dA/F-CUR (blue line) and 5′TAMRA-dA/F-CUR (red line) complexes in water–DMSO = 90 : 10 (v/v), (c) 95 : 5 (v/v) and (d) 95.5 : 0.5 (v/v), respectively: 1.0 cm cell, excitation 495 nm. To avoid decomposition of the complexes, all measurements were carried out at 4 °C. The concentration of 3′TAMRA-dA/F-CUR and 5′TAMRA-dA/F-CUR was adjusted to be 3.85×10^{-7} M by the absorption peak intensity at 495 nm.

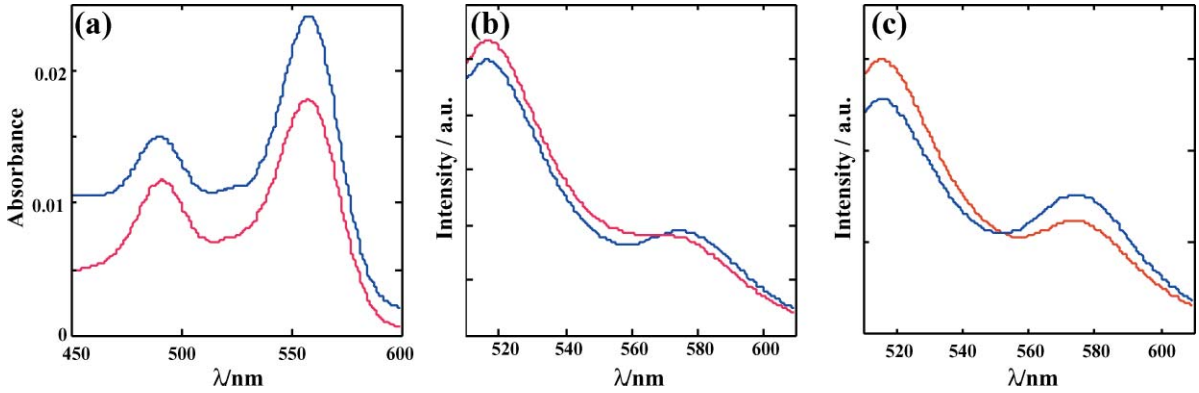


Fig. 4 Comparison of UV–Vis (a) and fluorescence spectrum, (b) between the 3′TAMRA-dA/F-CUR (blue line) and 5′TAMRA-dA/F-CUR (red line) complexes prepared in water–DMSO = 95 : 5 (v/v) followed by dialysis, (c) fluorescence spectrum from the sample prepared in water–DMSO = 60 : 40 (v/v) followed by dialysis: 1.0 cm cell, excitation 495 nm, 4 °C.

Here, we considered that a larger difference between 3′TAMRA-dA/F-CUR and 5′TAMRA/F-CUR might be obtained by using two curdlan chains preorganized in the same direction. Although the possibility of alternate orientation (Fig. 1) is pre-eliminated in this approach, this assumption seems to be acceptable because three chains in t-SPG adopt parallel orientation and this should also be the case in the complexation of the two chains. In addition, the preorganization of the two chains into parallel orientation would be advantageous for the formation of the stable complex with oligo(dA). Meanwhile, it is known that gold nanoparticles act as an effective fluorescence quencher for organic fluorophores and the surface can be easily modified by organic molecules through a gold–sulfide interaction.[11] Taking these lines of expectation into consideration, we designed a curdlan dimer (SS-CUR_2) in which the reducing ends of two curdlan chains are linked by a disulfide bond, as shown in Fig. 6(a). Thus, the complexes prepared from SS-CUR_2 and 3′(or 5′) TAMRA-dA were immobilized on the gold nanoparticle surface through the gold–disulfide interaction and the orientation of oligo(dA) in the complexes was examined with the emission intensity of the TAMRA group, as schematically illustrated in Fig. 6(b).

A DMSO solution containing SS-CUR_2 and an aqueous solution containing 3′TAMRA-dA (or 5′TAMRA-dA) were mixed according to the same procedure as described above. After removing DMSO by extensive dialysis, the resultant aqueous solution was subjected to spectroscopic measurements. Figs. 7(a) and (b) compare UV–Vis and fluorescence spectra between 3′TAMRA-dA/SS-CUR_2 and 5′TAMRA-dA/SS-CUR_2, respectively. An absorption peak at around 560 nm is attributed to the TAMRA group. When we excited the TAMRA group at 560 nm without (or before adding) gold

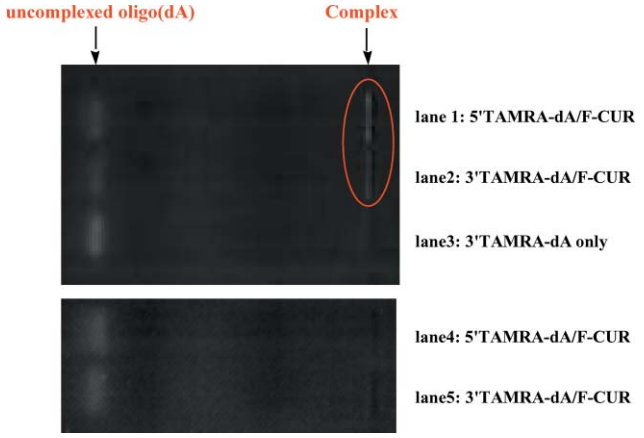


Fig. 5 Agarose gel electrophoresis of the 3′TAMRA-dA/F-CUR and 5′TAMRA-dA/F-CUR complexes after (lanes 1 and 2) and before (lanes 4 and 5) dialysis treatments. The 3.5% agarose gel was run for 60 min at 2.0 V cm^{-1} in MOPS buffer (4 °C, pH = 7.1). Oligo(dA) was stained by a gel star nucleic acid stain. We did not use any marker here. The migrated bands are assigned to the uncomplexed TAMRA-dA, which would be dissociated from the complex during electrophoresis.

nanoparticles, both solutions gave a strong fluorescence peak at 580 nm and the intensities were almost identical, indicating that disulfide linkage does not affect the emission properties of the TAMRA group. When gold nanoparticles were added to the solutions, drastic fluorescence quenching was observed only for the 3′TAMRA-dA/SS-CUR_2 solution. Fig. 7(b) compares the emission spectra between 3′TAMRA-dA/SS-CUR_2 and 5′TAMRA-dA/SS-CUR_2. Here, the final concentrations of the complexes and gold nanoparticles were 3.85×10^{-7} and 1.93×10^{-8} M, respectively, and the mixed solution was kept at 4 °C for 24 h before the fluorescence measurements. The emission intensity for the 3′TAMRA-dA/SS-CUR_2 complex is dramatically suppressed, whereas that of the 5′TAMRA-dA/SS-CUR_2 complex is reduced only by less than 10%. As reference experiments, we mixed 3′TAMRA-dA and 5′TAMRA-dA (without SS-CUR_2) with gold nanoparticles under the same conditions. We confirmed that no fluorescence quenching is induced in these systems. These results clearly support the view that in the complex, the 3′ end of oligo(dA) exists close to the gold nanoparticle: that is, the 3′ end of oligo(dA) and the reducing ends (disulfide bond) of curdlan are located at the same side in the complex according to parallel orientation. This conclusion is consistent with that obtained from the FRET studies as mentioned above.

To obtain supporting evidence for the experimental results, we carried out a MOPAC (semi-empirical molecular-orbital package) calculation. The calculation results support the experimental results described above: parallel orientation shows a more favourable symmetric and ordered structure than anti-parallel orientation.[12] Moreover, the heats of formation (HOF) clearly show the significant energy difference between the parallel and anti-parallel orientations in the complexes; for example, in the complex constructed from 6 repeating units, the parallel-orientation is more stable than the anti-parallel orientation according to a lower binding energy of 21 kcal mol^{-1}, and the difference of HOF becomes larger with the increase in the number of repeating units. Taking these theoretical studies into consideration, we can presume that the hydrogen-bonding

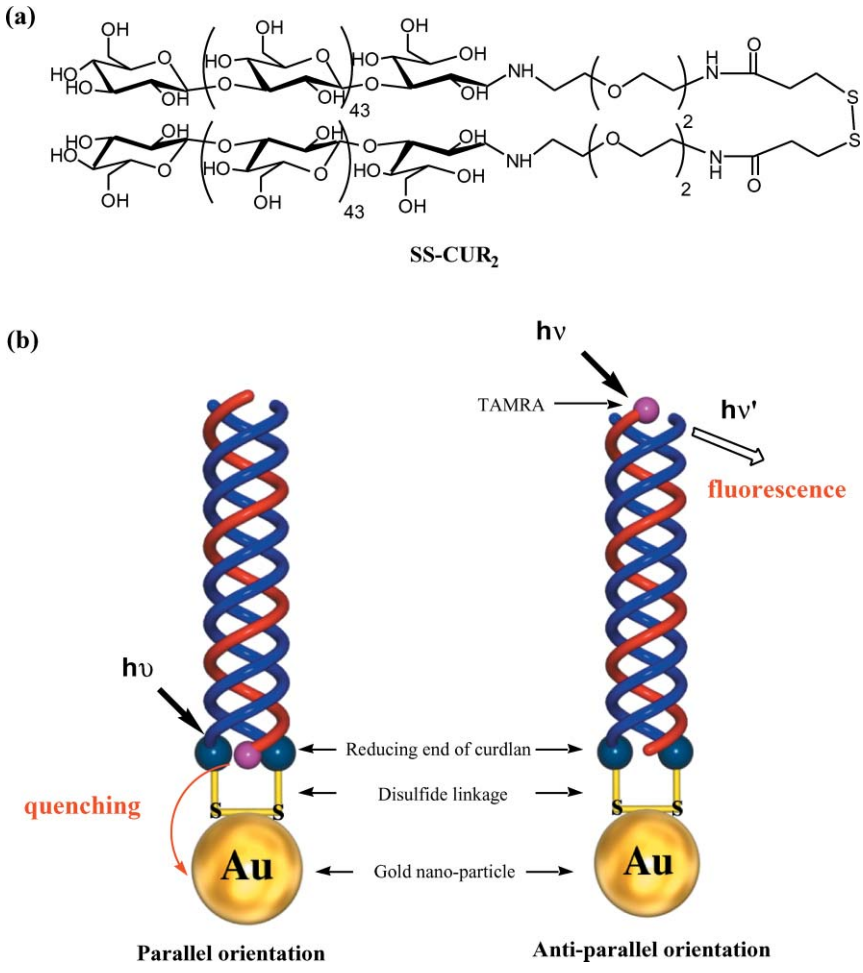


Fig. 6 Structure of disulfide linked curdlan dimer (SS-CUR_2) (a) and immobilization of 3′ (or 5′) TAMRA-dA/SS-CUR_2 complexes on the gold nanoparticle (b).

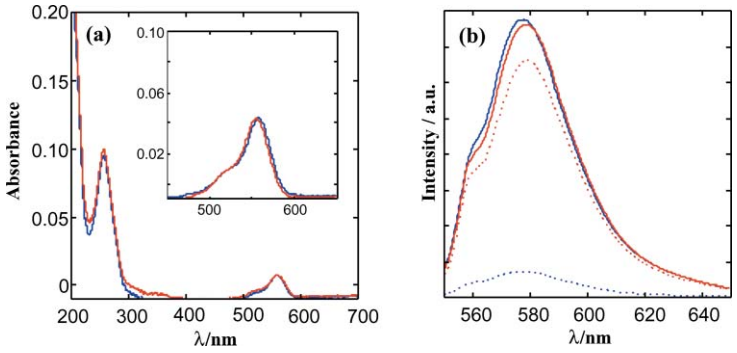


Fig. 7 Comparison of UV–Vis (a) and fluorescence spectra (b) between the 3′TAMRA-dA/SS-CUR$_2$ (blue line) and 5′TAMRA-dA/SS-CUR$_2$ (red line) complexes. Solid lines: without gold nanoparticles, dotted lines: after addition of gold nanoparticles, 1.0 cm cell, excitation 560 nm, 4 °C.

interactions and the steric complementarity between curdlan chains and oligo(dA) play an important role in the orientation of the polymeric complexes.[13] Since the main chain structures of β-1,3-glucan polysaccharides are more or less similar to that of curdlan,[6,14] we believe that the conclusion obtained from a combination of curdlan and oligo(dA) can be extended more generally to other β-1,3-glucans and polynucleotides.

Conclusions

In conclusion, we have examined a controversial problem related to parallel *vs.* anti-parallel orientation in β-1,3-glucan–polynucleotide complexes. In the FRET system, a combination of F-CUR and 3′ (or 5′) TAMRA-labeled oligo(dA) was used for the comparison of three different orientation modes. In the fluorescence quenching of the TAMRA group by gold nanoparticles, a very large difference was observed for their quenching efficiencies. Importantly, two experimental results support the view that in the curdlan/oligo(dA) complexes, parallel orientation is more favourable than anti-parallel orientation. This conclusion is further supported by our computational studies. From these findings, it is now clear that the specific hydrogen-bonding interaction and the shape complementarity between curdlan chains and oligo(dA) play a critical role for polymer orientation. These lines of new information are very useful to design novel polysaccharide-based gene carriers,[15] mRNA recovery systems,[16] polysaccharide–polynucleotide nanocomposites, *etc.*

Experimental

General

Curdlan was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and acid-hydrolyzed to reduce the molecular weight according to the reported method.[5c] This procedure was necessary to make the curdlan soluble in water. Here, we used a curdlan sample with $M_w = 8100$ and $M_w/M_n = 1.2$, which were determined with GPC (see below).[5c] Two kinds of TAMRA-labeled oligo(dA) (3′TAMRA-dA and 5′TAMRA-dA) were purchased from Hokkaido System Science. Spectroscopic grade DMSO was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Fluorescein-5-thiosemicarbazide was obtained from Molecular Probes. Gold colloid (5 nm diameter) was purchased from BB International (Cardiff, UK). UV–Vis absorption and fluorescence spectra were measured on a Shimadzu UV-2500PC spectrometer and a Hitachi F-4500 spectrometer, respectively. ^{1}H NMR spectra were recorded on a Bruker DRX600 spectrometer at 25 °C. Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were recorded on a PerSeptive Biosystems Voyager-DERP biospectrometry workstation. M_n and M_w were determined with a GPC (gel permeation chromatograph) system, with a JASCO PU-1580 pump, a JASCO RI-2031 RI detector, and a TOSOH TSKgel α-4000 column, in which DMF was used as a mobile phase. The molecular weight distribution (M_w/M_n) was also determined with this system.

Synthesis of F-CUR

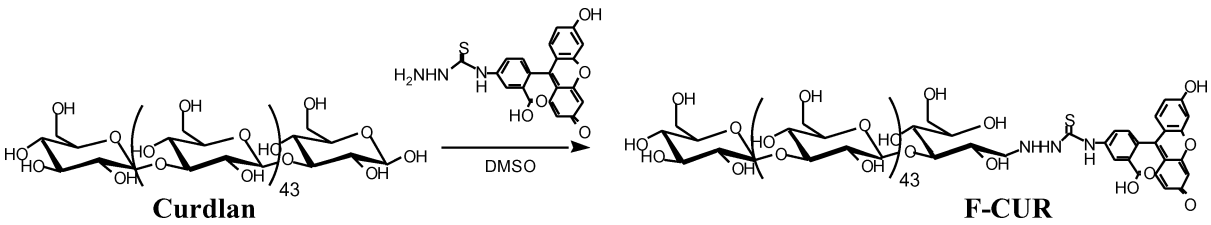
Fluorescein-labeled curdlan (F-CUR) was synthesized according to the following procedure (Scheme 1). The curdlan sample (50 mg, 6.2 × 1 mmol, $M_w = 8100$, $M_w/M_n = 1.2$) was dissolved in 5 mL of DMSO. To the curdlan DMSO solution, fluorescein-5-thiosemicarbazide (13 mg, 3.1 × 10^{-2} mmol) was added and the mixture was stirred under N_2 at room temperature. After 5 days, the resultant mixture was subjected to dialysis (MWCO = 3500) against distilled water to remove unreacted fluorescein-5-thiosemicarbazide. The freeze–dry process of the resultant solution gave desired F-CUR as a yellow powder (47 mg). The introduction of a fluorescein group was evidenced by elemental analysis (calc: C, 45.28; H, 6.09; N, 0.54, found: C, 41.70; H, 6.24; N, 0.31).

Synthesis of SS-CUR$_2$

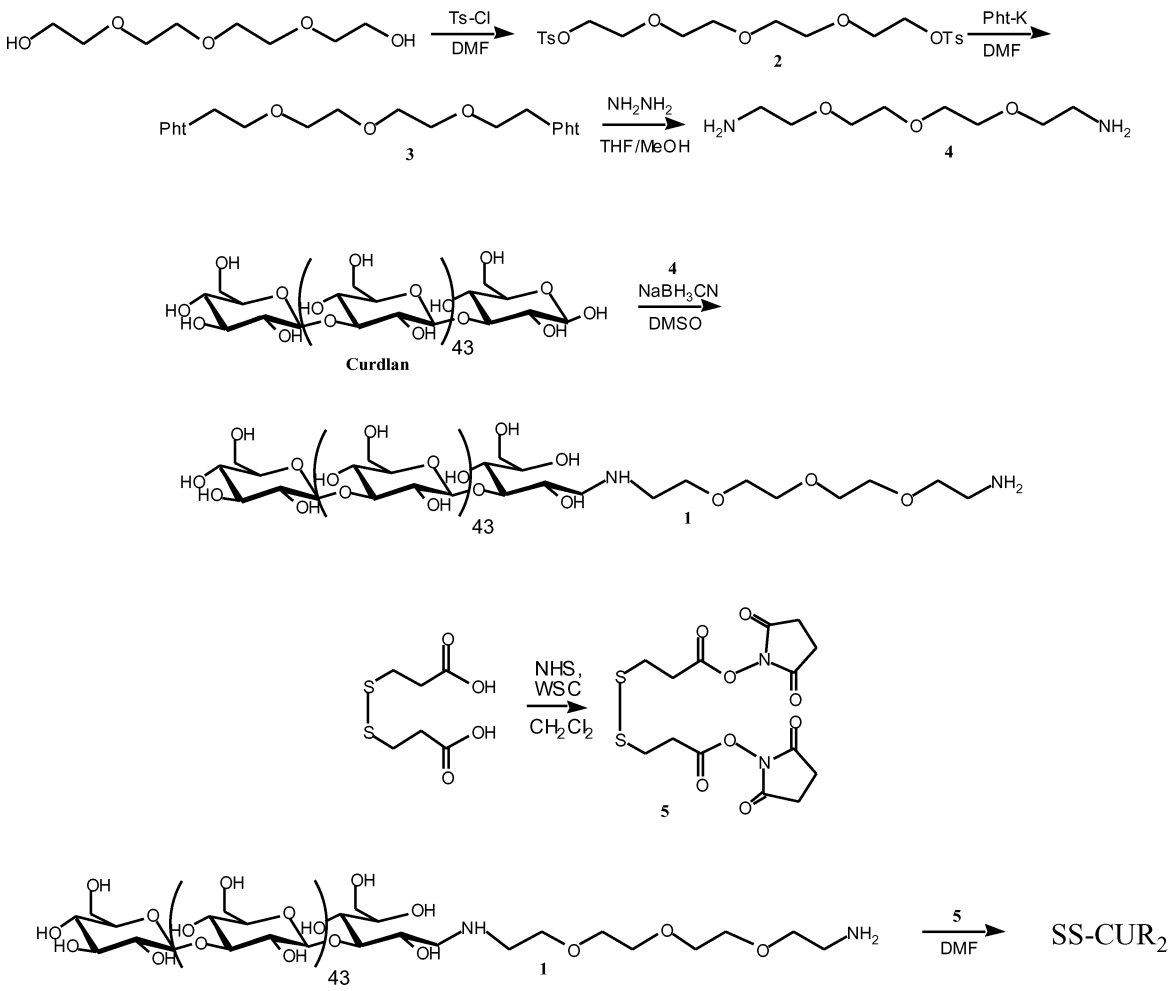
The synthetic route is shown in Scheme 2.

Synthesis of 3

2 (5.0 g, 11 mmol) and potassium phthalimide (4.9 g, 13 mmol) were suspended in 100 mL of anhydrous DMF and the mixture was stirred at 80 °C for 12 h. After 12 h, DMF was removed under reduced pressure and the residual oil was subjected to separation using silica gel column chromatography



Scheme 1 Synthetic scheme for fluorescein-labeled curdlan (F-CUR).



Scheme 2 Synthetic scheme for the disulfide linked curdlan dimer (SS-CUR_2).

(MeOH–chloroform = 3 : 1 (v/v)). Solvent was removed under reduced pressure to give **3** (4.1 g, 83%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85 (d, 2H, J = 5.16 Hz), 7.84 (d, 2H, J = 5.28 Hz), 7.17 (d, 2H, J = 5.16 Hz), 7.12 (d, 2H, J = 5.28 Hz), 3.88 (t, 4H, J = 5.86 Hz), 3.71 (t, 4H, J = 5.86 Hz), 3.52–3.58 (3.8H); $[M + H]^+$ = 460.20 (calc. 458.55).

Synthesis of 4

The product **3** (3.0 g, 6.6 mmol) obtained above was dissolved in anhydrous THF–MeOH (1 : 1 (v/v)). To the THF–MeOH solution, 4.0 mL of hydrazine monohydrate were added and the resultant mixture was stirred at room temperature. After 12 h, the white precipitate was removed by filtration. This filtrating process was repeated at least three times to remove the precipitate. The filtrate was concentrated under reduced pressure to give **4** as yellow oil (815 mg, 64%). ^{1}H NMR (600 MHz, $CDCl_3$–CD_3OD = 1 : 4 (v/v)) δ 3.61–3.66 (m, 16H); $[M + Na]^+$ = 216.31 (calc. 192.26).

Synthesis of amine-modified curdlan 1

To 5 mL of a DMSO solution containing curdlan (M_w = 8100, M_w/M_n = 1.2, 26 mg, 3.2 × 10^{-3} mmol)), **4** (150 mg, 0.8 mmol) and $NaBH_3CN$ (20 mg, 0.8 mmol) were added and the resultant suspension was stirred for 1 week. To remove an excess amount of **4** and inorganic products, the solution was subjected to extensive dialysis with a cellulose membrane (MWCO = 3500). The freeze–dry treatment of the resultant aqueous solution afforded **1** as a white powder (17.4 mg). The introduction of an amino group was evidenced by elemental analysis (calc: C, 44.67; H, 6.38; N, 0.40, found: C, 43.48; H, 5.77; N, 0.41).

Synthesis of 5

3,3′-Dithiodipropionic acid (3.0 g, 14 mmol) was dissolved in 20 mL of dry dichloromethane. To this solution, *N*-hydroxysuccinimide (4.0 g, 17 mmol) and WSC HCl (water soluble carbodiimide HCl) (6.5 g, 34 mmol) were added and the mixture was stirred at room temperature for 24 h. The resultant solution was washed with water and the organic layer was concentrated to dryness. The oily residue was purified by column chromatography (silica gel, chloroform). Solvent was removed under reduced pressure to give activated 3,3′-dithiodipropionic acid **5** (4.2 g, 73%). ^{1}H NMR (600 MHz, CD_3OD) δ 3.07 (t, 4H, J = 6.4 Hz), 3.03 (t, 4H, J = 6.4 Hz), 2.85 (s, 4H); $[M + H]^+$ = 405.2 (calc. 404.4).

Synthesis of SS-CUR_2

Amine-modified curdlan **1** (17 mg, 2.2 × 10^{-3} mmol) and activated 3,3′-dithiodipropionic acid **5** (50 mg, 2.2 × 10^{-3} mmol) were dissolved in 3 mL of DMSO and the mixture was stirred at room temperature for 3 days. To remove unreacted **5** from the reaction mixture, the solution was first treated with $NaBH_4$ to cleavage the disulfide bond, and then the resultant mixture was subjected to extensive dialysis against water with a cellulose membrane (MWCO = 3500). In this process, unreacted **5** can be removed through the membrane, while thiol modified curdlan remains in the membrane. The freeze–dry treatment of the aqueous solution afforded thiol-modified curdlan (curdlan monomer). Subsequently, the reaction mixture was dissolved in DMSO again and treated with an excess amount of I_2 to give SS-CUR_2. To remove I_2, the solution was subjected to gel chromatography (elute DMSO). Dialysis with a cellulose

membrane (MWCO = 3500) against water followed by the freeze–dry treatment afforded pure SS-CUR_2 as a white powder (7.8 mg). The introduction of an amino group was evidenced by elemental analysis (calc: C, 44.80; H, 6.43; N, 0.76, found: C, 43.39; H, 5.78; N, 0.38). GPC chromatography showed that the molecular weight of the obtained curdlan is twice that of the curdlan monomer, indicating that the two curdlan chains are linked by a disulfide bond.

Acknowledgements

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9 We assumed that two curdlan chains take a parallel orientation in the complex. This assumption is basically reasonable because if two curdlan chains take an anti-parallel orientation in the complex, FRET should be observed from both 3′dA-CUR and 5′dA-CUR complexes, so that both systems should give the same fluorescence spectra.
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