'Molecular-imprinting' in polyion complexes which creates the 'memory' for the AMP template

PERKIN 2

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The formation of a polyion complex precipitate was applied to molecular imprinting for the first time. Polyanion containing boronic acid units can sustain AMP by a boronate–*cis*-diol interaction. When this polyanion forms a polyion complex with polycation according to 1 : 1 cation–anion stoichiometry, the phosphate anionic charges introduced into the polyanion by the AMP complexation are also counted. Thus, after removal of AMP from the polyion complex a 'cleft' which has the memory for the AMP template is created. It was proved that this cleft shows high affinity with AMP. We also noticed that the removal and re-binding processes for AMP coincide with the swelling and deswelling phenomena of this polyion complex.

Introduction

'Foot-printing' is a technique to protect a specific segment of DNA (or RNA) from scission by complexation with its complementary oligonucleotides, intercalators or proteins. It occurred to us that this technique would be applicable to molecular imprinting (MI), to retain the memory of specific guest molecules. The conventional MI including a vinyl polymerisation process consists of three steps: (i) synthesis of functional vinyl monomers that can interact with a target template, (ii) radical polymerisation of the vinyl monomers with cross-linking reagents in the presence of the template, and (iii) removal of the template from the cross-linked copolymer resin.[1–10] Actually, this technique has already achieved success to some extent.[1,2] More recently, Kobayashi *et al.*[11] and our group[12] exploited new MI methods without the polymerisation process: that is, the 'memory' is directly created from the solution containing polymers sustaining the template molecule covalently or noncovalently by pouring it into a poor solvent[11] or casting it onto a polyethylene plate.[12] Such reprecipitation of the polymer solution is also possible, if the template-sustaining polymer is polyionic, by the formation of the polyion complex. The basic concept for the present methodology is illustrated in Fig. 1: that is, (A) the polyion complex formation between polyanion containing boronate groups and a polycation in the presence of an anionic template which can be bound to the boronate groups and (B) the removal of the anionic template by extensive extraction from the precipitate. The 'cleft' thus created in the polyion complex should show a 'memory' for the original anionic template molecule.

To test this working hypothesis we considered AMP as a template molecule. The ribose moiety has a *cis*-diol function which is bound to the phenylboronic acid group.[13,14] The complexation (to form a boronate ester) at basic pH yields an anionic charge.[15–19] Hence, this boronate anion site and the pendent phosphate anion site in addition to the carboxylate anion site in polyanion **1** are counted for the formation of a polyion complex with polycation **2**. After removal of AMP under acidic pH conditions, a 'cleft' is created which possesses a boronic acid group with the affinity for a *cis*-diol group and excess cationic charges with the affinity for the phosphate group. If this 'cleft' shows specific affinity with AMP, one can list this method as a new method of MI.

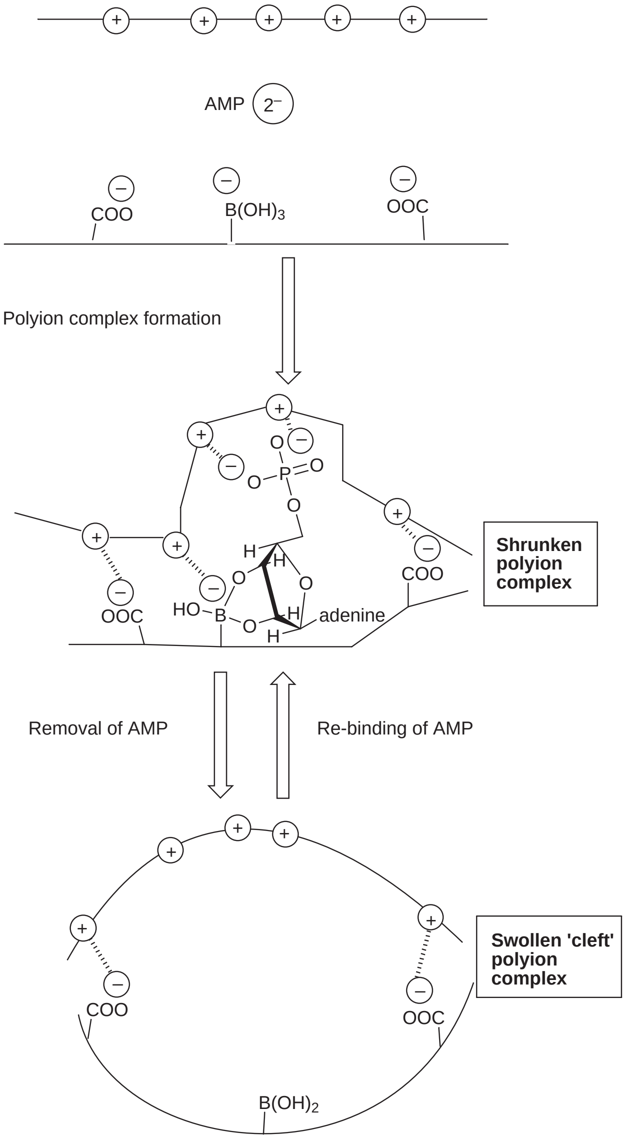
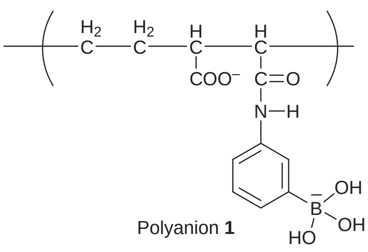
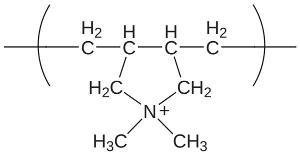


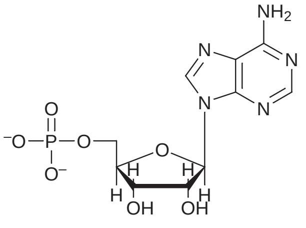
Fig. 1 Schematic representation of the polyion complex formation.



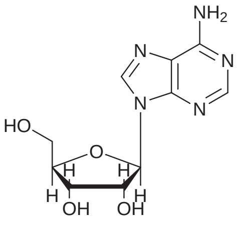
Polyanion **1**



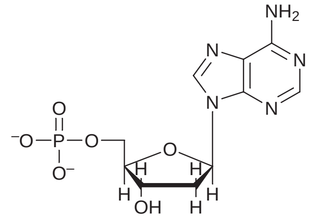
Polycation **2**



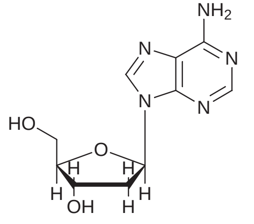
AMP



Ad



DAMP



DAd

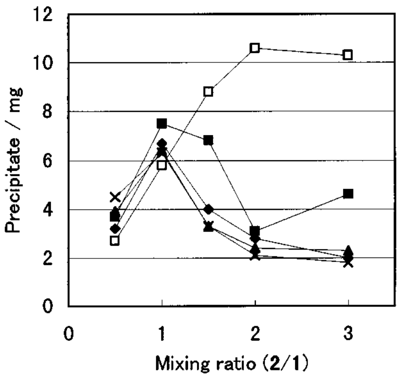


Fig. 2 Plots of the precipitate weight against the mixing ratio ([cation unit of **2**]/[anion unit of **1**]): × no additive, □ AMP, ■ Ad, ◆ DAMP, ▲ DAd. Here, the concentration of the anion unit of **1** is defined as the total concentration of [carboxylate unit] + [boronate unit].

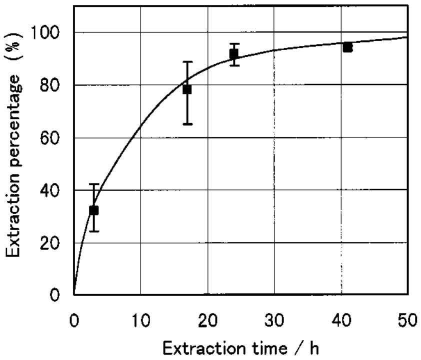


Fig. 3 Removal of AMP from the polyion complex prepared at the mixing ratio = 2.0 with an acidic aqueous solution (pH 5.5 with 100 mmol dm^{-3} acetate buffer). The reproducibility is shown by error bars. Rapid stirring or sonication destroyed the floc of the polyion complex. Hence, the mixture was slowly stirred once per hour.

Results and discussion

Preparation of polyion complexes

The polyion complex was prepared by dropping an aqueous polyanion (**1**) solution into an aqueous solution containing polycation (**2**) and AMP (or its analogues) at pH 10.3. The concentrations of **1** and AMP (or its analogues) were maintained at a constant level {[boronate unit]/[AMP (or its analogues)] = 1} while that of **2** was varied. The weight of the precipitated polyion complex is shown in Fig. 2. It is seen from Fig. 2 that in the absence of additive and in the presence of Ad, DAd and DAMP the maximum always appeared at the mixing ratio = 1.0. This implies that these additives scarcely have an influence on the formation of the polyion complex which features the 1 : 1 charge neutralisation. At the mixing ratio < 1.0 the solutions were clear whereas at the mixing ratio > 1.0 they became turbid. Presumably, excess **2** is bound onto the surface of 'apparently' neutral polyion complexes and yields colloidal particles.

In the presence of AMP, in contrast, the precipitate weight increased with increasing **2** concentration and the maximum appeared at around the mixing ratio = 2.0. The solutions were clear up to the mixing ratio = 2.0 and then became turbid above the mixing ratio = 2.0. It was confirmed by the UV measurement of the supernatant solution that the amount of AMP bound to the polyion complex corresponds to 53 mol% of the total boronate units. These results support the view that the *cis*-diol group in AMP forms the complex with the boronate group in **1** and additional anionic charges (*i.e.*, phosphate group) affect the formation of the polyion complex.

It is also seen from Fig. 2 that the precipitate weights for DAd and DAMP at the mixing ratio = 1.0 are comparable with that in the absence of additive. In fact, DAd and DAMP were not bound to the polyion complex (confirmed by quantifying DAd and DAMP concentration in the supernatant solutions by UV spectrophotometry). In contrast, the precipitate weight for Ad at the mixing ratio = 1.0 is heavier than that in the absence of additive. It was shown from the UV measurement of the supernatant solution that the amount of Ad bound to the polyion complex corresponds to 59 mol% of the total boronate units. This must be reflected in the heavier precipitate weight.

These results clearly indicate the importance of the *cis*-diol group and the anionic phosphate group: that is, DAd and DAMP without the *cis*-diol group are scarcely bound to **1** whereas Ad with the *cis*-diol group is significantly bound to **1** but does not influence the polyion complex formation because of the lack of an anionic charged group.

Removal of AMP from the polyion complexes

AMP was extracted out of the polyion complex prepared at the mixing ratio = 2.0 by immersing it in an acidic aqueous solution (pH 5.5 with 100 mmol dm^{-3} acetate buffer) at 25 °C. The acidic condition is useful for the extraction, because at pH 5.5 the boronate–*cis*-diol complex tends to be dissociated and the phosphate group should be monoanionic. The amount of AMP removed from the polyion complex by this treatment was estimated by an UV absorption spectroscopic method. The result (Fig. 3) indicates that the extraction rate gradually decreases with extraction time and is saturated at around 40 h. After 41 h more than 90% of AMP was extracted out of the polyion complex. This sample (the 'cleft' polyion complex) was used for the following re-binding experiment.

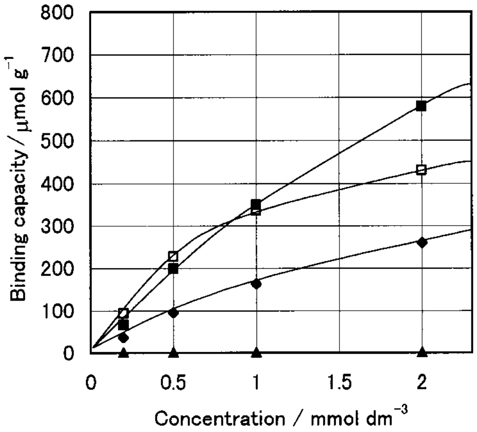


Fig. 4 Equilibrium binding capacity of the 'cleft' polyion complex in a basic aqueous solution (pH 9.3 with 10 mmol dm^{-3} ammonium buffer): □ AMP, ■ Ad, ◆ DAMP, ▲ DAd.

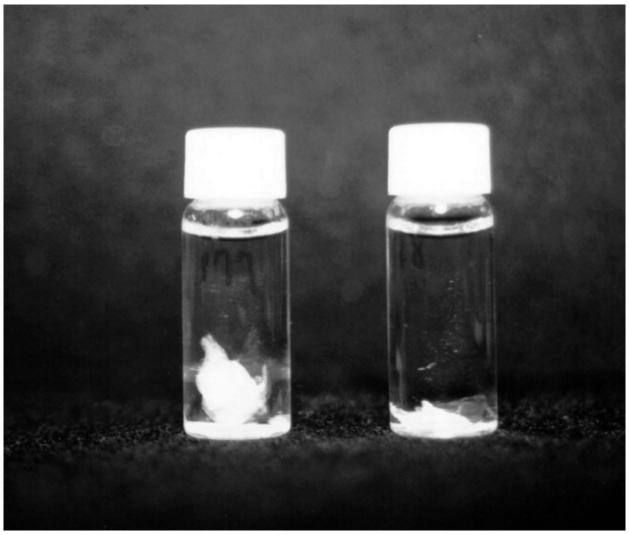


Fig. 5 Picture of the swollen (left) and shrunken (right) polyion complex.

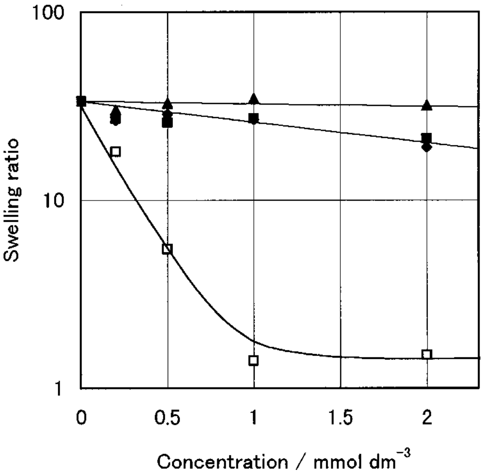


Fig. 6 Plots of the swelling ratio of the 'cleft' polyion complex *versus* the additive concentration: □ AMP, ■ Ad, ◆ DAMP, ▲ DAd. Here, the swelling ratio is defined as (the weight of swollen polyion complex − the weight of dried polyion complex)/the weight of dried polyion complex.

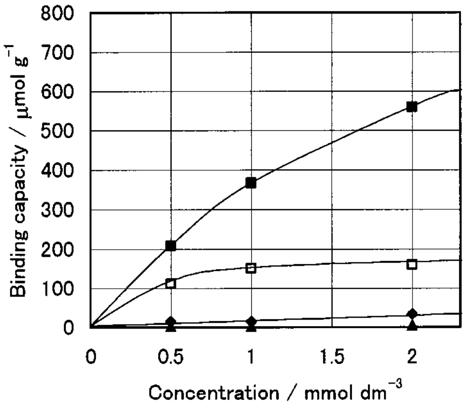


Fig. 7 Equilibrium binding capacity of the polyion complex prepared in the absence of additives (pH 9.3 with 10 mmol dm^{-3} ammonium buffer): □ AMP, ■ Ad, ◆ DAMP, ▲ DAd.

Binding of AMP to the 'cleft' polyion complex

The binding ability of the 'cleft' polyion complex was estimated in a basic aqueous solution (pH 9.3 with 10 mmol dm^{-3} ammonium buffer) at 25 °C. We plotted the equilibrium binding values against the additive concentration (Fig. 4). DAd having neither the *cis*-diol group nor the anionic group was not bound to this polyion complex. DAMP, which does not have the *cis*-diol group but does have the anionic phosphate group, was gradually bound to the polyion complex. Since this polyion complex retains an excess amount of cationic charges, this binding is attributed to the electrostatic interaction.

Of interest is the comparison of the binding ability between AMP and Ad. As shown in Fig. 4, the binding of AMP occurs more efficiently than that of Ad at low concentration but the binding of AMP was nearly saturated at high concentration while that of Ad is still increased. What does this difference mean? It is known that the boronate–*cis*-diol interaction generating an anionic charge is facilitated by the neighbouring cationic charge.[20] Hence, the boronate group situated in the cation-excess 'cleft' should show a high affinity with Ad even in the absence of an electrostatic interaction. On the other hand, one can expect both the boronate–*cis*-diol interaction and the electrostatic interaction for AMP. In fact, the binding efficiency is better than that of Ad at low concentration. Then, why is the saturation induced at high concentration? We noticed that the polyion complex in AMP solution shrinks with the binding time (Fig. 5). Similarly, when AMP in the polyion complex was extracted, it gradually swelled with the extraction time. These phenomena are rationalised by the charge balance of the polyion complex: that is, the removal of AMP results in the cation-excess polyion complex and the re-binding of AMP results in the charge-neutralised polyion complex. As shown in Fig. 6, the size of the cation-excess 'cleft' polyion complex was scarcely affected by added DAd and only to a smaller extent by added Ad and DAMP. In contrast, the same polyion complex shrank remarkably with increasing AMP concentration and the deswelling was saturated at [AMP] = 1.0 mmol dm^{-3}. The findings imply, therefore, that the binding of AMP neutralises the excess cationic charge and the shrinkage thus induced suppresses further binding of AMP at higher concentrations. Ad, which does not carry such anionic charge, does not induce the deswelling of the cation excess polyion complex. Hence, further binding of Ad at higher concentrations is still possible.

As a reference system, the same experiment was repeated using a polyion complex prepared in the absence of AMP (and its analogues). This charge-neutralised polyion complex kept its shrunken state during this experiment. Fig. 7 shows the binding ability of this polyion complex. It appears in the order of Ad > AMP ⩾ DAMP > DAd. When Fig. 7 is compared with Fig. 4, one can see some significant differences: (1) the binding of AMP in Fig. 4 is much higher than that in Fig. 7, (2) the binding abilities toward Ad are the same in both cases, (3) DAMP is bound to some extent in Fig. 4 whereas it is scarcely bound in Fig. 7. These differences support the view that in the 'cleft' polyion complex the excess cationic charge acts cooper-

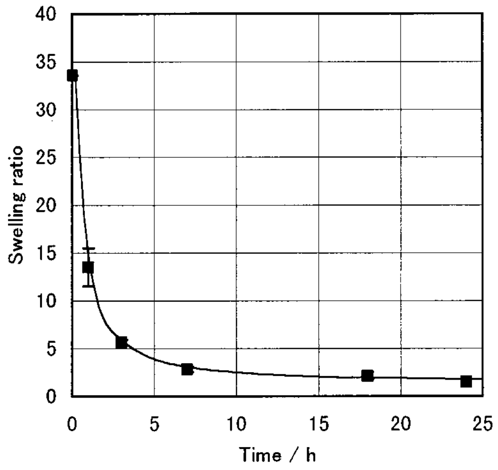


Fig. 8 Deswelling kinetics of the 'cleft' polyion complex in 2 mmol dm^{-3} AMP solution (pH 9.3 with 10 mmol dm^{-3} ammonium buffer). Error bars for the plots after 3 h are included in the range of the square marker.

atively with the boronate group to complex AMP. In other words, one can propose that the recognition site suitable to the AMP binding is created and retained through the polyion complex formation.

Deswelling kinetics of the 'cleft' polyion complex

Fig. 8 shows the deswelling kinetics of the 'cleft' polyion complex. Initially, it was equilibrated in ammonium buffer solution (pH 9.3) and then AMP was added to the solution. After 1 h, the swelling ratio became 1/2–1/3 of the initial state and then it became 1/6 of the initial state after 3 h. After that, the polyion complex shrank more slowly. The thermoresponsive poly(*N*-isopropylacrylamide) cross-linked gel shrinks in water above the LCST (lower critical solution temperature), but the deswelling rate is quite slow because a dense polymer skin layer formed at the gel surface suppresses the permeation of water trapped inside the gel.[21] The relatively rapid deswelling rate of the 'cleft' polyion complex suggests that the diffusion of water and ions inside the polyion complex is rather fast.

pH-Dependence of the AMP-binding

The binding ability of the 'cleft' polyion complex was also estimated at pH 7.2 and 4.6 (Fig. 9). At pH 7.2, the binding of Ad that occurs on the basis of the boronate–*cis*-diol interaction is markedly decreased. The binding of AMP is still high but it is comparable with that of DAMP. At neutral pH it is difficult to form the boronate–*cis*-diol complex.[15–19] Since AMP and DAMP behave similarly, one can consider that the major driving force for the binding is the electrostatic interaction. At pH 4.6, Ad is scarcely bound whereas AMP is still bound although weakly owing to the residual electrostatic interaction.

The deswelling behaviour of the polyion complex is shown in Fig. 9. At pH 7.2, the polyion complex does not shrink in the solution containing low AMP concentration but does in the solution containing high AMP concentration (2.0 mmol dm^{-3}). At pH 4.6, the shrinkage does not take place at all. These results again indicate that both the electrostatic interaction and the boronate–*cis*-diol complex formation are indispensable for the deswelling of the polyion complex.

Concluding remarks

The basic idea utilised in the present study is the phenomenon that the polyion complex always precipitates holding a 1 : 1 cation–anion stoichiometry. Hence, if an anionic guest is appended to the polymer chain *via* a reversible covalent bond, the complementary number of opposite charges is '*imprinted*' in the resultant polyion complex precipitate. We found that this idea really works for the AMP template by using the boronate–*cis*-diol interaction. Unexpectedly, we noticed that the removal

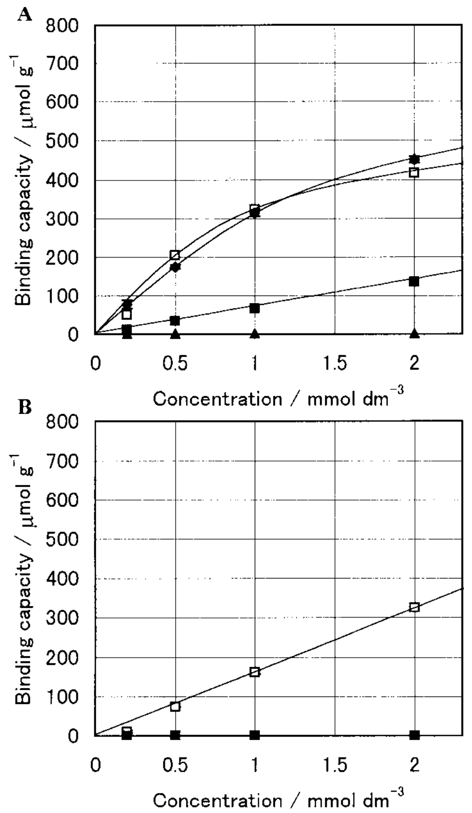


Fig. 9 Equilibrium binding capacity of the 'cleft' polyion complex at (A) pH 7.2 (10 mmol dm^{-3} MOPS buffer) and (B) pH 4.6 (10 mmol dm^{-3} acetate buffer): □ AMP, ■ Ad, ◆ DAMP, ▲ DAd.

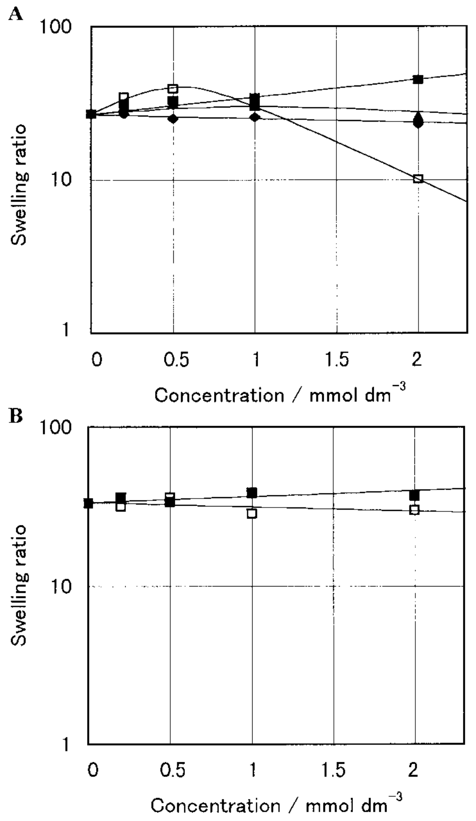


Fig. 10 Plots of the swelling ratio of the 'cleft' polyion complex *versus* additive concentration at (A) pH 7.2 (10 mmol dm^{-3} MOPS buffer) and (B) pH 4.6 (10 mmol dm^{-3} acetate buffer): □ AMP, ■ Ad, ◆ DAMP, ▲ DAd.

and re-binding processes for AMP coincide with the swelling and deswelling phenomena of this polyion complex. This provided a new possibility that this polyion complex can be applied to a novel mechanochemical system, drug delivery system, sensing system, *etc*. Further studies are currently continuing.[22]

Experimental

Synthesis of polymers

Polyanion **1** was synthesised by reacting poly(ethylene-*alt*-maleic anhydride) (Aldrich Co., Ltd. M_w: 100 000–500 000) and 3-aminophenylboronic acid (Tokyo Chemical Industry Co., Ltd.) in DMF at 70 °C for 20 h. Formation of polyion **1** was confirmed by FT-IR and ^{1}H NMR spectra. Poly(ethylene-*alt*-maleic anhydride) had a $\nu_{C=O}$ band at 1790 cm^{-1} due to the acid anhydride group. This peak disappeared after the reaction and new peaks assignable to the carboxylic acid and the amide group appeared at 1710 cm^{-1} and 1670 cm^{-1}, respectively. In the ^{1}H NMR spectrum polyion **1** showed broad signals at around 7.5 (consists of four peaks), 2.7 and 1.7 ppm which were assignable to aromatic protons, CH protons and CH$_2$ protons in the vinyl group, respectively. The integral intensity ratio was 4:2:4, which supports the view that 3-aminophenylboronic acid quantitatively reacted with the acid anhydride group. Polycation **2** [poly(diallyldimethylammonium chloride) M_w: 400 000–500 000] was purchased from Aldrich Co., Ltd.

Preparation of polyion complexes

An aqueous solution (1.0 cm^3: pH 10.3 with 100 mmol dm^{-3} carbonate buffer) containing AMP or its analogues (10 mmol dm^{-3}) and an aqueous solution (0.5–3.0 cm^3: pH 10.3 with 100 mmol dm^{-3} carbonate buffer) containing polycation **2** (20 monomer unit mmol dm^{-3}) were mixed and the solution was stirred at 25 °C for 1 h. To this solution was added dropwise an aqueous solution (1.0 cm^3: pH 10.3 with 100 mmol dm^{-3} carbonate buffer) containing polyanion **1** ([carboxylate unit] = [boronate unit] = 10 mmol dm^{-3}) [thus the ratio of AMP (or its analogues) *vs.* boronate unit is maintained as 1:1] and then the mixture was stirred at 25 °C for 1 h. The precipitate formed during this period was collected by centrifugation and dried under vacuum. To estimate the amount of bound AMP or its analogues, UV spectra of the supernatant solutions were measured (adenine has an absorption maximum at 260 nm).

Extraction of AMP from the polyion complex

The AMP-containing polyion complex prepared at the mixing ratio = 2.0 was immersed in a 100 mmol dm^{-3} acetate buffer (pH 5.5) at 25 °C for 41 h. The amount of extracted AMP was determined by measuring the UV absorption of the solution phase at 260 nm. After that, the polyion complex was dried under vacuum.

Binding of AMP to the 'cleft' polyion complex

The 'cleft' polyion complex was immersed in aqueous solutions (pH 9.3 with 10 mmol dm^{-3} ammonium buffer, pH 7.2 with 10 mmol dm^{-3} MOPS buffer, pH 4.6 with 10 mmol dm^{-3} acetate buffer) of AMP and its analogues at 25 °C for 24 h with occasional stirring. The polyion complex was taken out of the solution and weighed after removing excess water from the polyion complex surface. The swelling ratio is defined as (the weight of swollen polyion complex − the weight of dried polyion complex)/the weight of dried polyion complex. The amount of bound AMP was determined by the UV measurement of the solution.

Deswelling kinetics of the 'cleft' polyion complex

The 'cleft' polyion complex was immersed in a 10 mmol dm^{-3} ammonium buffer (3.0 cm^3: pH 9.3) at 25 °C for 24 h. To this solution was added an aqueous AMP solution (5 mmol dm^{-3}, 2.0 cm^3: pH 9.3 with 10 mmol dm^{-3} ammonium buffer). Thus the AMP concentration in the solution was 2.0 mmol dm^{-3}. After a certain period, the weight of the polyion complex was measured.

Equipment

UV spectra were recorded on a Jasco V-570 spectrophotometer using a quartz cell with a 10 mm pathlength.

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22 Response for nucleosides was studied. Guanosine and uridine which exist as monovalent anionic species at pH 9.3 shrunk the 'cleft' polyion complex, whereas adenosine and cytidine which are non-charged species did not.

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