Synthesis of Cyclic and Acyclic Oligocytidylates by Uranyl Ion Catalyst in Aqueous Solution

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Uranyl ion catalyses the oligomerization of cytidine-5'-phosphoroimidazolide in aqueous solution, yielding 3'–5'-linked cyclic di- and tri-cytidylates preferentially at high catalyst concentration, or 2'–5'-linked linear oligocytidylates at lower catalyst concentration. Addition of Ag^+ affects the uranyl ion-catalysed oligocytidylate formation and alters the product distribution.

A series of reactions of activated nucleotides in aqueous solution provides a non-enzymatic model of nucleic acid biosynthesis,[1] and affords a new attractive synthetic method for oligonucleotides.[2,3] Nucleoside 5'-phosphoroimidazolide, an imidazole-activated nucleotide, polymerizes to oligonucleotide in the presence of a complementary polynucleotide template[1] or a catalyst.[2,3] However, template-directed polymerizations of mononucleotides are limited to the system in which polymer–monomer complexes are stable.

We have studied the procedure for synthesizing oligonucleotides from activated nucleotides in aqueous solution. Previously we have shown that lead ion catalyses the oligomerization of nucleoside-5'-phosphoroimidazolide, giving 2'–5'-linked oligonucleotides along with small amounts of 3'–5'-linked oligonucleotides. The regioselectivity of the 2'–5' linkage was 85–95% in formation of the dimer. We carried out further searches for an efficient catalyst for the synthesis of oligonucleotides in aqueous solution and recently reported that uranyl ion catalyses the polymerization of adenosine 5'-phosphoroimidazolide very efficiently to form 2'–5'-linked linear oligoadenylates up to the hexadecamer.[3] The 2'–5' regioselectivity of the oligoadenylate obtained by using uranyl ion as catalyst was higher than that obtained by using lead ion as catalyst. The uranyl ion could play the role of a template for the oligomerization by co-ordination. To gain further knowledge of oligonucleotide synthesis by uranyl ion catalysis and of the catalytic role of the uranyl ion, we applied this reaction to the preparation of other oligonucleotides, and have found that the type of oligonucleotide resulting from cytidine 5'-phosphoroimidazolide was different from that of the corresponding oligoadenylate, and varied depending on the catalyst's concentration. The product distribution was also altered greatly by addition of Ag^+ which co-ordinates primarily to the base part of the cytidine nucleotide.[4] Here we report on the uranyl ion-catalysed synthesis of 3'–5'-linked cyclic oligocytidylates, along with 2'–5'-linked linear oligocytidylates.

Results and Discussion

Cytidine 5'-phosphoroimidazolide (ImpC) was prepared from cytidine 5'-monophosphate and imidazole.[5] The polymerization of 50 mmol dm^{-3} ImpC was carried out in the presence of 0.5 mmol dm^{-3} uranyl nitrate catalyst in 0.2 mol dm^{-3} *N*-ethylmorpholine buffer (pH 7.3) for 1 day at 20 °C. The reaction mixture was treated with ethylenediaminetetraacetic acid (EDTA) solution to stop the reaction and analysed by HPLC. Fig. 1 shows the elution profile on HPLC. Cyclic 3'–5'-linked dicytidylate was a main product as illustrated on the HPLC chart. In addition, the formation of 2'–5'-linked linear oligocytidylates from dimer to decamer was confirmed by HPLC. The shoulders to the right of the peaks of 2'–5'-linked oligocytidylates are linkage isomers containing one or more 3'–5' linkages. Digestion of the total mixture with nuclease P1 resulted in the disappearance of the peaks corresponding to the 3'–5'-linked oligomers, leaving only the peaks corresponding to the 2'–5'-linked oligomers. HPLC clearly demonstrated that the formation of 3'–5'-linked linear oligomers was very light. The products were separated by QAE-Sephadex anion-exchange column chromatography in large quantity. The separated products were further purified by paper chromatography when necessary. Isolated yields of the resulting

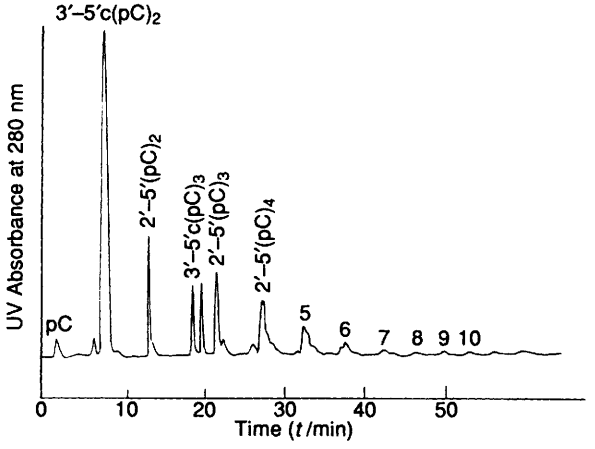


Fig. 1 HPLC profile of oligocytidylates obtained from ImpC by uranyl ion catalyst. The polymerization of ImpC (50 mmol dm^{-3}) was carried out for 1 day in the presence of uranyl ion (0.5 mmol dm^{-3}) at 20 °C and pH 7.3.

Table 1 Ion-exchange chromatography of polymerized products of ImpC by uranyl ion catalyst

Fractions pooled	A_{271}	Yield[a] (%)	Identification
185–195	40	1.1	pC
202–211	1350	38.5	3'–5'c $(pC)_2$
227–236	188	5.4	2'–5' $(pC)_2$
248–260	139	4.0	3'–5'c $(pC)_3$
267–275	229	6.5	2'–5' $(pC')_3$
279–288	197	5.6	unidentified
297–313	377	10.7	2'–5' $(pC)_4$
329–342	212	6.0	2'–5' $(pC)_5$
360–380	102	2.9	2'–5' $(pC)_6$
383–404	79	2.5	2'–5' $(pC)_7$

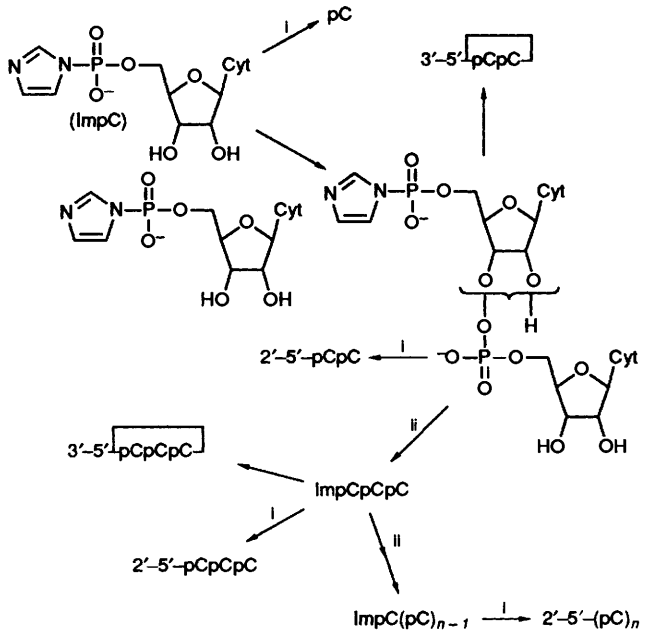
[a] Yield is expressed based on the starting amount of ImpC (0.39 mmol; A_{271} 3510). Hypochromicity correction was not applied to the yield data. Minor by-products are omitted.

Table 2 Uranyl ion-catalysed formation of oligocytidylates

[ImpC]/[UO_2^{2+}]	Time (days)	Yield (%)[a] pC	3′–5′ $c(pC)_2$	2′–5′ $(pC)_2$	3′–5′ $c(pC)_3$	2′–5′ $(pC)_3$	2′–5′ $(pC)_4$	2′–5′ $(pC)_5$	2′–5′ $(pC)_{6-11}$
50	1	0.6	55.7	1.7	7.8	9.9	6.8	5.6	7.4
100	1	1.8	40.8	6.9	5.5	7.4	13.5	8.9	8.0
500	5	0.3	24.4	36.5	2.4	19.9	5.9	2.2	0.7
1000	5	20.7	7.8	49.2	2.3	14.5	2.6	0.7	
5000	5	86.1	0.6	11.3	0.2	0.2			
None	5	98.1		0.2					

The oligomerization of ImpC (50 mmol dm^{-3}) was carried out at 20 °C in the presence of uranyl nitrate (0.01–1.0 mmol dm^{-3}) catalyst in 0.2 mol dm^{-3} *N*-ethylmorpholine–HNO_3 buffer (pH 7.3). [a] Yield, obtained by HPLC, is expressed based on the starting amount of ImpC. The pC includes the starting ImpC. The 3′–5′ cyclic trimer includes small amounts of a linkage isomer containing the 2′–5′ linkage. The 2′–5′ linear oligomers include a small quantity of linkage isomers containing a partial 3′–5′ linkage.

oligocytidylates are listed in Table 1. The 3′–5′-linked cyclic dimer and trimer were obtained in 38.5 and 4.0% yield, respectively. They were insensitive to bacterial alkaline phosphatase, while they were cleaved to cytidine 5′-monophosphate as the sole product with nuclease P1 and venom phosphodiesterase as expected. Alkaline hydrolysis of the 3′–5′-linked cyclic di- and tri-cytidylate gave cytidine 2′(3′)-monophosphate. Their NMR and FAB-mass spectra agreed with the assigned structure.[6] The coupling constant between the C-1′ and -2′ protons of the cyclic dimer was too small to be observed, which is a characteristic of the cyclic dinucleotides.[6] The 2′–5′-linked linear oligomers up to the heptamer were isolated. We identified the linear oligomers by enzyme digestion and by comparison of their HPLC elution times and TLC mobilities with those of the authentic samples.[5] Oligocytidylate formation proceeds in a stepwise manner as shown in Scheme 1.



Scheme 1 *Reagents:* i, water; ii, ImpC

We examined the effect of the concentration of catalyst on oligocytidylate formation. Table 2 lists the yield data for oligocytidylates calculated by HPLC. The cyclic 3′–5′-linked di- and tri-cytidylates were formed preferentially when concentrations greater than 1 mmol of dm^{-3} of uranyl nitrate was used as catalyst. No cyclic 2′–5′-linked dimer was obtained in the reaction. A catalyst concentration lower than 0.1 mmol dm^{-3} resulted in a decrease in formation of the cyclic oligomer, but in an increase in the 2′–5′-linked linear oligomers. The catalytic activity of uranyl ion remained even when the concentration was as low as 0.01 mmol dm^{-3}, though it could yield oligocytidylates of only very short chain length and in small amounts. The formation of linear 3′–5′-linked dimer was low under all conditions. The results suggest that the imidazolide of 3′–5′-linked dicytidylate formed from two molecules of cytidine 5′-phosphoroimidazolide tends to cyclize, while the imidazolide of 2′–5′ linked oligocytidylate tends to elongate and form longer oligomers in the presence of the uranyl ion catalyst.

In our previous experiment, very little or no cyclic oligoadenylate was formed from adenosine 5′-phosphoroimidazolide by uranyl ion catalyst.[3] The product distribution differed, depending on the differences in the base part of the starting nucleotides. This implies that modification of the base part may also alter the selectivity of the uranyl ion-catalysed oligomerization. The character of the base part can be modified by co-ordination with a metal ion. It was reported that Ag^+ co-ordinates primarily to the base part of nucleotides and DNA.[4] We therefore conducted the uranyl ion-catalysed formation of oligocytidylates in the presence of various concentrations of Ag^+. The molar ratio of ImpC to the uranyl ion was fixed at 80 for the reaction. Ag^+ had a profound effect on the product distribution as shown in Table 3. The Ag^+ ion alone gave only a hydrolysed product. A high concentration of Ag^+ accelerated hydrolysis of the phosphoroimidazolide bond, thereby suppressing oligocytidylate formation. However, a Ag^+ ion concentration of 5–12.5 mmol dm^{-3} enhanced the formation of 2′–5′-linked linear oligocytidylate by uranyl ion catalyst. The 2′–5′-linked linear oligocytidylates up to dodecamer were formed in substantial yields. Ag^+ is likely to co-ordinate to the exocyclic amino group or the N-3 of the cytosine[4] of ImpC, causing a change in the regioselectivity of the internucleotide bond formation. Ag^+ also co-ordinates to the imidazole group of ImpC, and its use may increase the reactivity of the phosphoroimidazolide bond.

So far, we do not have a sufficient explanation of the catalytic mechanism of uranyl ion and of the different product distribution. We can, however, propose a model that should explain our findings. There are two requirements for efficient oligonucleotide synthesis by metal ion catalyst[3] or by template:[1d,1e] (1) orientation of the starting monomer that favours formation of an internucleotide bond, and (2) activation of the OH group of the ribose moiety of the monomer. ImpC co-ordinates to uranyl ions to form a polymeric complex similar to that claimed for the uranyl complex of 5′-mononucleotides.[7] The polymeric uranyl complex is likely to play the role of template in organizing the monomer and facilitates the oligonucleotide synthesis. The activation of the 2′-OH group of the ribose moiety of ImpC in the uranyl complex can induce

Table 3 Uranyl ion-catalysed formation of oligocytidylates in the presence of Ag^+

$[Ag^+]$ / [ImpC]	Yield (%)[a] pC	3′–5′ $c(pC)_2$	2′–5′ $(pC)_2$	3′–5′ $c(pC)_3$	2′–5′ $(pC)_3$	2′–5′ $(pC)_4$	2′–5′ $(pC)_5$	2′–5′ $(pC)_{6-12}$
0	2.7	43.6	3.7	7.1	6.2	8.0	7.7	8.8
1	61.0	7.0	18.0	true	6.7	2.7		
1/4	2.8	31.3	7.4	5.2	5.9	10.0	9.6	23.0
1/10	1.9	34.3	13.5	3.3	6.3	7.0	7.2	21.1
1/50	3.2	54.9	3.6	3.0	5.1	8.0	2.0	2.9
1/4[b]	98.0		0.7					

The oligomerization of ImpC (50 mmol dm^{-3}) was carried out at 20 °C in the presence of uranyl nitrate (0.63 mmol dm^{-3} catalyst and silver nitrate (0–50 mmol dm^{-3}) in 0.2 mmol dm^{-3} *N*-ethylmorpholine–HNO_3 buffer (pH 7.3). [a] As in Table 2. [b] No uranyl ion catalyst was used in this reaction.

the formation of the 2′–5′ internucleotide bond. The efficiency and the regioselectivity of the polymerization are sensitive to changes in the catalyst's concentration and to the identity of the base part of the monomer. Presumably, the orientation of monomers imposed by the uranyl complex varies depending on the identity of the base part of the monomer and the uranyl ion concentration, and induces the changes in the regioselectivity and efficiency of the formation of the internucleotide bond.

Whatever the mechanism, the uranyl ion has high catalytic activity in oligonucleotide synthesis. One target oligonucleotide cannot be prepared selectively by this process, as this reaction gives a series of 2′–5′-linked oligonucleotide and cyclic dinucleotides with a distribution that varies depending on the reaction conditions. On the other hand, a series of oligomers can be obtained simultaneously just in one simple reaction, though the yield of each oligonucleotide is modest. The resulting oligonucleotides can be purified by conventional anion-exchange chromatography. It was reported that some cyclic dinucleotides possess interesting biological activities.[8] We believe that the uranyl ion-catalysed oligomerization provides a very simple and efficient procedure for the preparation of cyclic dinucleotides and 2′–5′ linear oligonucleotides, as no protecting group is required and the reaction proceeds in neutral aqueous solution.

Experimental

Cytidine 5′-monophosphate (pC), *N*-ethylmorpholine, uranyl nitrate, imidazole, triphenylphosphine, 2,2′-dipyridyl disulfide, nuclease P1 (N.P1), snake venom phosphodiesterase (VPDase) and bacterial alkaline phosphatase (BAP) were obtained commercially. ImpC was prepared from pC and imidazole by using triphenylphosphine and 2,2′-dipyridyl disulfide as a condensation agent as described previously.[5]

Paper chromatography was performed on Whatman 3MM paper by a descending technique using propan-1-ol–conc. ammonia–water (55:10:35). TLC was carried out on PEI-cellulose-F plates in 0.25 mol dm^{-3} aq. ammonium hydrogen carbonate and on cellulose-F plates in the solvent systems (1), propan-1-ol–conc. ammonia–water (55:10:35) or (2), saturated aq. ammonium sulfate–0.1 mol dm^{-3} sodium acetate–propan-2-ol (79:19:2). HPLC was performed with a Hitachi 638 apparatus using a RPC-5 column (4 mm × 25 cm). RPC-5 was prepared from fine granular poly(chlorotrifluoroethylene) and Adogen 464 by a modification of the published method.[8,9] Elution was carried out with a linear gradient of $NaClO_4$ (0–0.03 mol dm^{-3}) buffered with 2.5 mmol dm^{-3} Tris-acetate (pH 7.5) and 0.1 mmol dm^{-3} EDTA during 60 min at a flow rate of 1.0 cm^3 min^{-1}. The eluate was monitored by UV absorption at 280 nm. Yields were calculated from the peak integrals of the oligocytidylate on the HPLC chromatogram. No correction for the hypochromicity of each oligocytidylate was made on the yield data.

UV absorption spectra were recorded on a Hitachi 3200 instrument. ^1H NMR spectra were taken by a Varian XL200 FT NMR spectrometer with sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate as internal standard. *J*-Values are given in Hz. The sample for ^1H NMR analysis was purified by paper chromatography, passed through a Dowex 50WX-8 (Na^+-form) column, evaporated under reduced pressure several times with D_2O, and dissolved in D_2O (0.01 mol dm^{-3} phosphate buffer, pD 6.7). ^{31}P NMR spectra were recorded on a Hitachi R-90H FT NMR spectrometer with 85% H_3PO_4 as external standard. The sample was purified by paper chromatography, passed through a Dowex 50WX-8 (Na^+-form) column, evaporated under reduced pressure, and dissolved in 50% D_2O buffered with 0.01 mol dm^{-3} Tris-HCl (pH 6.7). FAB-mass spectra were kindly taken by staff at the Research Institute of Kirin Brewery Co. on a Hitachi M-80-B instrument.

Polymerization of ImpC and Separation of the Products.—To a stirred aq. solution (4.5 cm^3) of ImpC (0.39 mmol, A_{271} 3510) were added *N*-ethylmorpholine buffer (3.14 cm^3; 0.5 mol dm^{-3}, pH 7.3) and uranyl nitrate (0.156 cm^3). The stirred reaction mixture was kept at 20 °C for 1 day. Aq. EDTA (0.1 cm^3; 0.25 mol dm^{-3}, pH 7.0 was added to the reaction mixture to stop the reaction. The formation of oligocytidylates was confirmed by HPLC. The solution was applied to a QAE-Sephadex A-25 column (30 mm × 45 cm). The column was eluted with a stepwise linear gradient of triethylammonium hydrogen carbonate buffer, (i) water–0.66 mol dm^{-3} (2 × 3 dm^3), (ii) 0.66–1.0 mol dm^{-3} (2 × 0.6 dm^3). Fractions (16 cm^3) were collected every 10 min. Main UV-absorbing fractions were pooled, and evaporated under reduced pressure below 30 °C. Triethylammonium hydrogen carbonate was removed by coevaporation with water and lyophilization. The products were further purified by paper chromatography when necessary. Yields of the resulting oligocytidylates were calculated from their UV absorption at 271 nm based on the starting ImpC, without making a correction for their hypochromicity.

Characterization of Oligocytidylates.—Identification of 2′–5′- and 3′–5′-linked linear oligocytidylates up to the pentamer was carried out by comparison of the HPLC elution times and TLC mobilities with those of authentic samples.[5] TLC mobilities of oligocytidylates are listed in Table 4. 3′–5′-Linked cyclic oligocytidylates were identified by digestion using N.P1, BAP, and 0.5 mol dm^{-3} aq. NaOH. The cyclic di- and tri-cytidylates were unchanged by BAP. Digestion of the cyclic dimer and trimer with N.P1 afforded pC as the sole product. Alkaline hydrolysis of the cyclic oligomers with 0.5 mol dm^{-3} NaOH gave cytidine 2(3′)-monophosphate. The digested solution was analysed by HPLC or TLC. The structure of the cyclic oligomer was further confirmed by ^1H NMR, ^{31}P NMR, and FAB-mass spectra.

Table 4 TLC mobilities of oligocytidylates

Compound	R_f Value relative to pC		
	System I	System II	System III
pC	1.0	1.0	1.0
$c(pC)_2$	1.15	0.47	0.88
$c(pC)_3$	1.01	0.37	0.74
$2'–5'(pC)_2$	0.90	0.89	0.84
$2'–5'(pC)_3$	0.78	0.80	0.70
$2'–5'(pC)_4$	0.74	0.70	0.56
$2'–5'(pC)_5$	0.70	0.56	0.48
$2'–5'(pC)_6$	0.62	0.49	0.31
$3'–5'(pC)_2$	0.89	0.80	0.67
$3'–5'(pC)_3$	0.77	0.74	0.67
$3'–5'(pC)_4$	0.73	0.64	0.54

System I: cellulose F, propan-1-ol–conc. ammonia–water (55:10:35); System II– cellulose F, saturated aq. ammonium sulfate–0.1 mol dm^{-3} sodium acetate (pH 6.5)–propan-2-ol (79:19:2); System III: PEI-cellulose F, 0.25 mol dm^{-3} ammonium hydrogen carbonate.

3′–5′-Linked cyclic dicytidylate [$c(pC)_2$]: $\delta_H(D_2O$; pD 6.7) 8.06 (6-H, d, *J* 7.5, 1 H), 5.99 (5 H, d, *J* 7.5, 1 H), 5.84 (1′-H, s, 1 H), 4.55–(3′-H, ddd, *J* 4.4, 9.5, and 9.5, 1 H), 4.35–4.45 (2′-, 4′-, and 5′-H, nonresolved due to overlap of adjacent proton signals, 3 H) and 4.06 (5″-H, dd, *J* 3.0 and 11.9, 1 H); δ_P[50% D_2O; pH 6.7 (85% H_3PO_4 ext. ref.)] −1.8; FAB-mass 654 ($M^{2-} - 2\,Na^+$).

3′–5′-Linked cyclic tricytidylate [$c(pC)_3$]: $\delta_H(D_2O$; pD 6.7) 7.84 (6-H, d, *J* 7.4, 1 H), 6.10 1′-H, d, *J* 6.1, 1 H), 6.05 (5-H, d, *J* 7.4, 1 H), 4.63 (3′-H, ddd, *J* 5.4, 4.0, and 6.9, 1 H), 4.49 (4′-H, m, 1 H), 4.37 (2′-H, dd, *J* 6.0 and 5.4, 1 H) and 4.18–4.26 (5′-H_2, m, 2 H); δ_P[50% D_2O; pH 6.7 (85% H_3PO_4 ext. ref.)] −0.6; FAB mass 981 ($M^{3-} - 3\,Na^+$).

Since no authentic sample of long 2′–5′-linked oligocytidylate was available, the products were identified by degradation with enzymes. N.P1 degrades the 3′–5′ internucleotide linkage but is insensitive toward the 2′–5′ internucleotide linkage. After treatment of an aliquot of the reaction mixture with Chelex-100 to remove the uranyl ion, digestion of the resulting oligonucleotide with N.P1 gave a series of fully 2′–5′-linked oligocytidylates (as demonstrated by HPLC). Digestion with N.P1 was carried out for 2.5 h at 37 °C in a mixture (50 mm^3) containing the oligocytidylate (A_{271} 0.1–10) 0.03 mol dm^{-3} veronal–acetate buffer (pH 5.75) and enzyme solution (5 mm^3 in 50 mm^3).

VPDase degrades both 2′–5′ and 3′–5′ internucleotide linkages. Digestion with VPDase was carried out at 37 °C for 2.5 h in a mixture (50 mm^3) containing the substrate (A_{271} 0.1–1), 0.01 mol dm^{-3} Tris-acetate (pH 8.8), 0.01 mol dm^{-3} $MgCl_2$ and the enzyme solution (0.02 units).

Digestion with BAP was carried out for 2.5 h at 37 °C in a mixture (50 mm^3) containing the substrate (A_{271} 0.1–1), 0.1 mol dm^{-3} Tris-HCl (pH 8.05), 0.01 mol dm^{-3} $MgCl_2$ and the enzyme solution (0.1 unit).

Alkaline hydrolysis was carried out at room temperature for 1 day in a mixture (10 mm^3) containing the substrate (A_{291} 0.1–0.2) in 0.5 mol dm^{-3} NaOH.

Effect of Uranyl Ion Concentration on the Polymerization of ImpC.—Reactions were carried out in an Eppendorf tube. The reaction mixture was prepared on an ice-bath, agitated vigorously, and kept at 20 °C for various periods of time. A typical reaction mixture (100 mm^3) contained ImpC (50 mmol dm^{-3}) and uranyl nitrate (0.01–1 mmol dm^{-3}) in *N*-ethylmorpholine–HNO_3 buffer (0.2 mol dm^{-3}, pH 7.3). The reaction was stopped by addition of 0.25 mol dm^{-3} aq. EDTA (20 mm^3) and stored in a freezer until analysis by HPLC.

Effect of Ag^+ *on the Uranyl Ion-catalysed Polymerization of ImpC.*—Reactions were carried out in the same way as described above. A reaction mixture (100 mm^3) containing ImpC (50 mmol dm^{-3}), uranyl nitrate (0.63 mmol dm^{-3}) and silver nitrate (1–50 mmol dm^{-3}) in *N*-ethylmorpholine–HNO_3 buffer (0.2 mol dm^{-3}, pH 7.3) was kept at 20 °C for 1 day, and was then treated with 0.25 mol dm^{-3} aq. EDTA (20 mm^3) and analysed by HPLC.

Acknowledgements

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