Mat. Res. Soc. Symp. Proc. Vol. 723 © 2002 Materials Research Society

Oligonucleotide Imprinting in Aqueous Environment

Dolly Batra and Kenneth J. Shea*
Department of Chemistry
University of California, Irvine
Irvine, CA 92697

INTRODUCTION

The development of synthetic receptors that recognize nucleotide bases and their derivatives is an important area of research [1-3]. Applications are envisioned in separation science, biosensors, drug therapy and genetic engineering. Previously in this laboratory, we have developed a molecularly imprinted synthetic receptor for 9-ethyladenine (9-EA). The network polymer has an affinity for adenine and its derivatives with an average association constant (K_a) of 75,000 M^{-1} in $CHCl_3$ [4]. When a 9-EA imprinted polymer was used as the chromatographic support, adenine eluted at 27 minutes using 92.5/5.0/2.5 $CH_3CN/H_2O/CH_3CO_2H$ as the mobile phase, while cytosine, guanine and thymine derivatives all eluted close to the void volume (2.0 min). In addition, imprinted polymers have been made with complementary binding sites for cytosine and guanine [5], as well as other nucleotide base analogues [6].

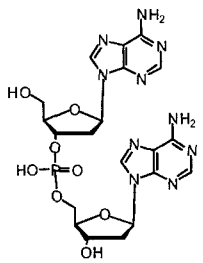
The extension of these results to construct robust receptors for *oligonucleotides* requires fundamental changes in imprinting strategies. Most importantly, since oligonucleotides are water soluble, strategies that employ EGDMA/MAA formulations in organic solvents will need to be replaced with those that do not compromise the interactions between template (the oligo) and functional monomer.

Initially, the imprinting of a 2'-deoxyadenosine dimer (**1**) was attempted. Due to the hydrophilicity of a DNA oligomer, it was difficult to find a suitable organic solvent that would solubilize the oligomers without disrupting the template's interaction with the polymer matrix [7]. To combat the solubility problems and to insure the homogeniety of the polymerization solution, we examined various polymer formulations with organic and/or aqueous-based solvents that would dissolve the template without disrupting these key interactions.

EXPERIMENTAL DETAILS

Synthesis of Template

The first phase of this research involved synthesis of an adenine dimer **1**. Since imprinting involves milligram quantities of template molecules, it was decided to synthesize these quantities using a solution phase technique. The synthesis utilized phosphoramidite methodology (Scheme 1) [8]. The coupling of individually protected nucleotides using a phosphoramidite linkage yields phosphite **2**. This linkage is later oxidized to the phosphate group, and, following deprotection with TFA, treatment with concentrated NH_4OH leads to the desired oligonucleotide (**3**).



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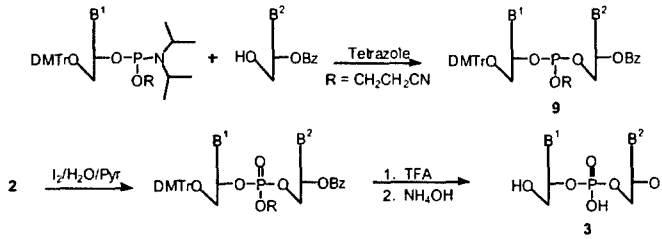
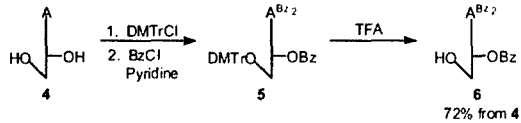
Scheme 1



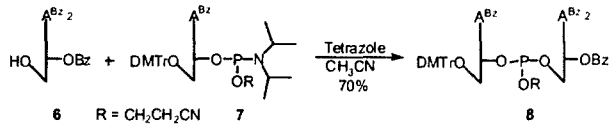
Protected nucleoside **6** was synthesized from 2'-deoxyadenosine (**4**, Scheme 2). Selective protection of the primary 5'-alcohol with dimethoxytrityl chloride (DMTrCl), followed by protection of the secondary 3'-alcohol with benzoyl chloride provided fully protected nucleotide **5**. Deprotection of the DMTr group with TFA furnished alcohol **6** [9].

Scheme 2



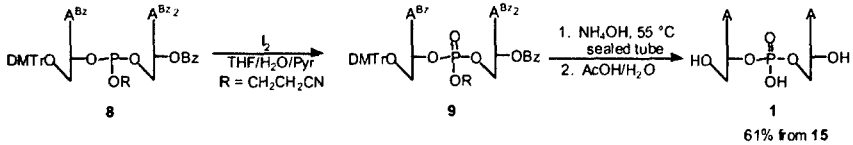
Protected 2'-deoxyadenosine **6** was then coupled with the commercially available phosphoramidite **7** using standard coupling conditions to provide 2'-deoxyadenosine dimer **8** (Scheme 6) [10].

Scheme 3



Phosphite **8** was then oxidized using I_2 to yield phosphate **9**[10]. The fully protected dimer **9** was treated with NH_4OH and AcOH deprotection, and following reverse-phase HPLC purification gave adenine dimer **1**.

Scheme 4



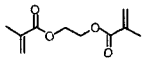
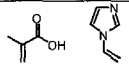
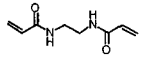
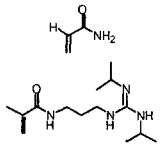
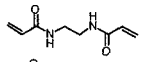
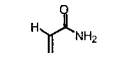
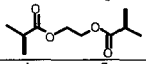
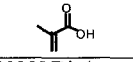
Polymerization Reactions

The 2'-deoxyadenosine dimer **1** was used as the imprint molecule for various organic and aqueous polymerization formulations (Table 1). The polymer formulations were selected on the basis of earlier work from our and other laboratories (**P1** and **P4**) or were newly developed (**P2** and **P3**). Buchardt and Mathew developed a molecularly imprinted polymer for adenine in an organic/aqueous medium similar to **P1** [11]. **P4** was synthesized using a polymerization

formulation similar to that used for 9-EA imprinting [4, 7]. A quaternary ammonium surfactant, which has been shown to be stable under the polymerization conditions used for 9-EA,was used to solubilize the oligonucleotide in an organic solvent [7] .

An aqueous formulation, **P2**, was based on the known interaction of a guanidine group with a phosphate salt [5]. Ethylene bisacrylamide[12] was used as the crosslinking monomer for its solubility in H_2O and its compatibility to co-polymerize with acrylamide, which was added for its interaction with the adenine base. A similar formulation, **P3**, was made without the guanidine functional monomer. Non-imprinted polymers **$P1_0$ - $P4_0$**, were also synthesized as controls to test for non-specific interactions (Table 1).

Table 1. Polymer formulations used to imprint adenosine dimer **1**. Molecularly imprinted polymer (MIP) using 1% dimer **1** as template (MIP) were made together with polymers made in the absence of template (Blank Polymer).

MIP	*Blank Polymer*	*Crosslinking Monomer*	*Functional Monomer(s)*	*Solvent*	*Splitting Yields*
P1	**$P1_0$**			95/5 MeOH/H_2O	73 %
P2	**$P2_0$**			95/5 H_2O/MeOH	40 %
P3	**$P3_0$**			95/5 MeOH/H_2O	60 %
P4*	**$P4_0$***			$CHCl_3$	90 %

*A quarternary ammonium surfactant (*N,N,N,N*-Bis(octadecyl)dimethylammonium bromide) was used to solubilize the oligonucleotide in an organic solvent.

Polymers **P1** – **P4** were prepared by thermal polymerization of degassed solutions of the above formulations for 24 h at 65 °C using AIBN (1 %) as initiator. The resulting polymers were coarsely crushed and Soxhlet extracted using MeOH. After the polymers were ground to 25 – 125 μm particles, rebinding studies with the dimer **1** were carried out in water, by studying the uptake of the dimer over a concentration range of 0.2 – 3 mM. The pH of the uptake solutions was ~3.5 and did not change significantly over time.

Rebinding Studies with Dimer 1

P1 and **P4**, along with their non-imprinted counterparts **$P1_0$** and **$P4_0$**, were found to rebind poorly with the dimer **1**. **P2** and **$P2_0$** bound the dimer equally well, with non-specific interactions drowning any specific interactions. MIP **P3**, however, showed imprinting effects as its uptake of dimer **1** was significantly better than the non-imprinted polymer **$P3_0$** (Figure 1, below).

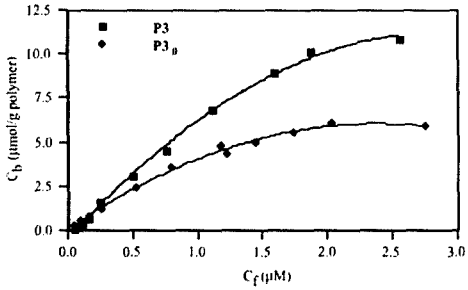


Figure 1. The rebinding of dimer **1** to MIP **P3** is compared to its rebinding with non-imprinted polymer $\mathbf{P3_0}$. C_b is the amount of dimer rebound to the polymer. C_f is the remaining concentration of the dimer in solution.

Rebinding studies were also performed in a buffer solution of 10mM K_3PO_4 buffer, but polymers showed a decrease in uptake of analyte in both imprinted and non-imprinted polymers. Previous results in this laboratory have shown that increased ionic strength due to buffers has an adverse affect on analyte uptake [13] . This phenomenon has been attributed to the sensitivity of dissolved salt on the solution conformation of high molecular weight polyacrylamides, with the salts causing contraction or collapse of polymer chains [14]. These changes in the polymer structure may cause changes in the microenvironment of a binding site giving decreased uptake of the analyte. Because of problems with using buffer solutions, further rebinding studies were performed in water alone.

To establish if an equilibrium is achieved at higher concentrations, the binding of polymer **P3** was evaluated as a function of concentration. As seen in Figure 2 below, instead of reaching an equilibrium, the bound dimer concentration reaches a maximum around 2.5 mM and then falls off. One possibility why this fall-off in binding occurs may be due to some self-association of the analyte at higher concentrations which may decrease the binding of the analytes to the polymers. Another possibility maybe that at the higher concentrations, the increased ionic strength due to the increased concentration of the ionic analyte **1** causes the same fall off in binding as observed in binding of the analytes with buffers (*vide supra*).

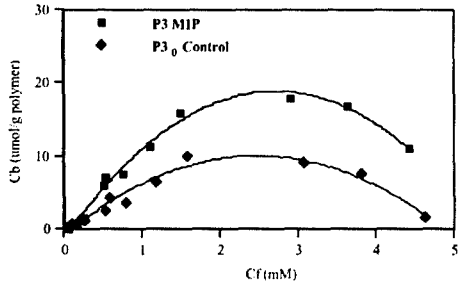
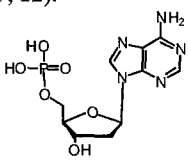


Figure 2. The rebinding of dimer **1** to MIP **P3** is compared to its rebinding with non-imprinted polymer $\mathbf{P3_0}$. C_b is the amount of dimer rebound to the polymer. C_f is the remaining concentration of the dimer in solution.

Rebinding Experiments with dAMP as analyte

Dimer imprinted polymer **P3** and the control polymer $\mathbf{P3_0}$ were then compared for the uptake of 2'-deoxyadenosine-5'-monophosphoric acid (dAMP, **11**) and 2'-deoxyguanosine-5'-monophosphate (dGMP, **12**).



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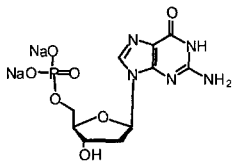
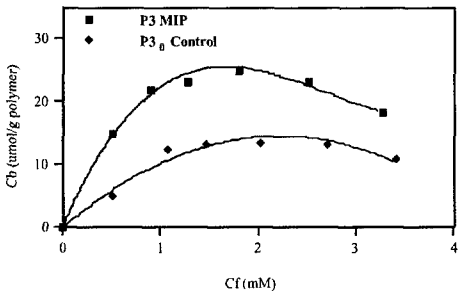


12

P3 showed selective uptake of dAMP, but not dGMP (Figures 3 and 4, below).



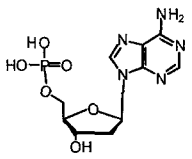


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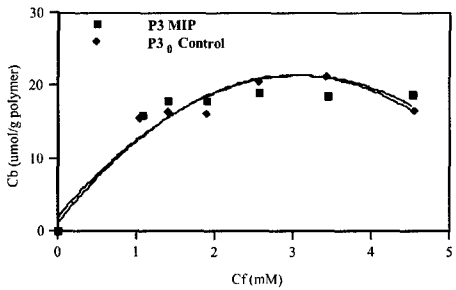
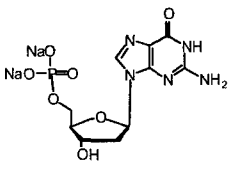
Figure 3. The rebinding of dAMP **11** to MIP **P3** is compared to its rebinding with non-imprinted polymer $\mathbf{P3_0}$. (**P3** was imprinted for adenosine dimer **1**).



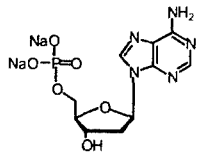


12

Figure 4. The rebinding of dGMP **12** to MIP **P3** is compared to its rebinding with non-imprinted polymer $\mathbf{P3_0}$. (**P3** was imprinted for adenosine dimer **1**).

Rebinding Experiments with dAMP as Template

To optimize the MIP formulation disodium salt of 2'-deoxyadenonsine monophosphate (dAMP disodium salt, **13**) was used as the imprint molecule in various formulations (Table 2). (The disodium salt of dAMP **13** was used because of the sodium salt's greater solubility in MeOH, which is used as the polymerization solvent for some of the formulations).



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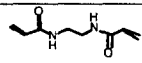
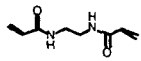
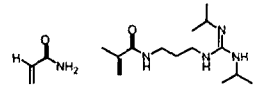
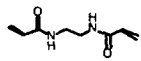
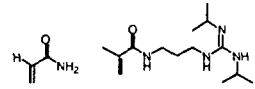
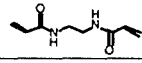
Polymer **P5** was based on the successful **P3** formulation, but H_2O, instead of MeOH was employed as porogen (Table 2). **P6** and **P7** formulations were similar to the formulation **P2**, except the guanidinum functional monomer concentration was reduced to 1% (from 14% in **P2**). Finally, **P8** was based on the successful imprinting formulation for the dimer, but dAMP **13** was used as template instead of the dimer. Non-imprinted polymers $\mathbf{P5_0}$ – $\mathbf{P8_0}$, were also synthesized as controls to test for non-specific interactions (Table 2)

Table 2. Polymer formulations used to imprint dAMP **13**. Molecularly imprinted polymers (MIP) using 1% dAMP **13** as template (MIP) were made together with blank polymers prepared in the absence of template.

MIP	*Blank Polymer*	*Crosslinking Monomer*	*Functional Monomer(s)*	*Solvent*	*Splitting Yields*
P5	$\mathbf{P5_0}$			H_2O	85 %
P6	$\mathbf{P6_0}$			H_2O	32 %
P7	$\mathbf{P7_0}$			MeOH	32 %
P8	$\mathbf{P8_0}$			MeOH	60 %

As in the previous polymerizations using template **1**, polymers **P5** – **P8** were prepared by thermal polymerization of degassed solutions of the above formulations for 24 h at 65 °C using AIBN (1 %) as initiator. The resulting solid polymers were coarsely crushed and Soxhlet extracted using MeOH. The extracted polymers were ground to 25 – 125 μm, and rebinding studies with various analytes were carried out in water (pH = 3.5, unadjusted).

It was found that when water was employed as the porogen as for polymers **P5** and $\mathbf{P5_0}$, the materials exhibited equal uptake of the dAMP template **13**. With a reduced amount of the guanidinium functional group in **P6**, it was anticipated that non-specific binding would be reduced during the rebinding studies. Uptake studies comparing **P6** and $\mathbf{P6_0}$, unfortunately, showed better uptake in the non-imprinted polymer $\mathbf{P6_0}$ than the MIP **P6**. This was perhaps due to the low splitting yield observed for the polymer (~30%); the guanidinium functional group in the MIP may be occupied by the remaining template molecule.

Since the **P7** formulation was very similar to **P6** except for the use of methanol in place of water as solvent, the results were very similar. Once again due to the low splitting yield (25%), the control polymer $\mathbf{P7_0}$ seemed to adsorb more template molecule than the corresponding imprinted polymer **P7**.

For **P8**, rebinding studies using dAMP **13** (pH of uptake solutions = 8.0) was not as successful as the uptake of the adenosine dimer **1** by MIP **P3** (Figure 1); in this case, both control and imprinted polymer adsorbed the template dAMP **13** equally well (Figure 5). This was surprising, as dAMP **13** was excepted to behave similar to its dimer counterpart **1**.

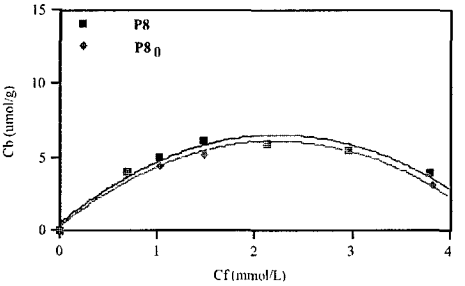


Figure 5. The rebinding of dAMP **13** to MIP **P8** is compared to its rebinding with non-imprinted polymer **P8**$_0$. (**P8** was imprinted for dAMP **13**).

Adjusting the pH of the uptake solution with HCl to 3.5 did not make a significant difference in the uptake of the template in either the control (**P8**) or imprinted (**P8**$_0$) polymers (Graphs not shown). Finally, uptake with the free acid form of dAMP **11** (pH = 3.5) gave a differential uptake in the imprinted polymer **P8** and the non-imprinted control polymer **P8**$_0$ (Figure 6).

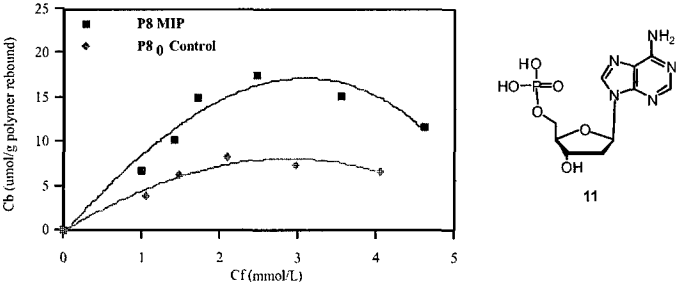


Figure 6. The rebinding of dAMP **11** to MIP **P8** is compared to its rebinding with non-imprinted polymer **P8**$_0$. (**P8** was imprinted for dAMP **13**).

This uptake study was compared to the uptake of free acid of dGMP (**14**) on the same polymer (Figure 7). As seen in the graph below, dGMP **14** showed greater scatter in the binding data but still bound to the polymer less selectively than dAMP.

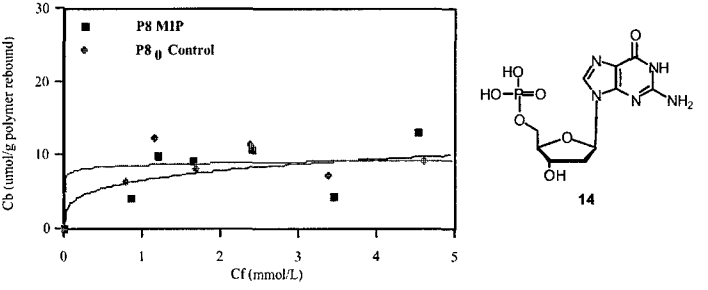


Figure 7. The rebinding of dGMP **5** to MIP **P8** is compared to its rebinding with non-imprinted polymer **P8**$_0$. (**P8** was imprinted for dAMP **4**).

CONCLUSIONS

We have found that imprinting of a single nucleotide base could be extended to the imprinting of a short DNA fragment. Several polymer formulations were examined to develop adenine receptors. Of these, one polymer formulation (**P3**), was found to have a higher capacity for 2'-deoxyadenosine dimer (**1**) than the corresponding non-imprinted polymer in water. The MIP also showed greater selectivity for the adenine base than guanine, when the free acid of the monophosphate salts were used for uptake experiment.

In addition, this polymer (**P3**) is composed of hydrophilic monomers enabling binding studies to be performed in aqueous solution. Binding studies done in 10mM K_3PO_4 buffer solutions showed decreased binding of the analyte to the polymers most likely due to a change in the solvation and conformation of the polymer and hence the microenvironment of the binding sites in a MIP.

Optimizations of the **P3** formulation using dAMP **13** established that the phosphate group of the analyte must be in the free acid form to observe a differential binding between the imprinted and control polymers, during uptake studies. The origin of this observation may be due to difference in the pH of the solutions. Using the sodium salt of dAMP (**13**), the pH of the solution is ~8; however, using the free acid of dAMP (**11**), the pH of the solution is ~3.5. Better rebinding is observed using the free acid; however, adjusting the pH of dAMP **13** to 3.5 does not improve rebinding. These results once again indicate that the pH and/or the ionic strength [15] of the uptake solutions in the rebinding studies has a significant effect on the binding of the analyte to the polymer, and the uptake studies are best performed in water alone.

ACKNOWLDGEMENTS

The authors would like to thank NIH for financial support of this work.

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