Nucleotide-mimetic synthetic ligands for DNA-recognizing enzymes
One-step purification of Pfu DNA polymerase

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Abstract

The commercial availability of DNA polymerases has revolutionized molecular biotechnology and certain sectors of the bio-industry. Therefore, the development of affinity adsorbents for purification of DNA polymerases is of academic interest and practical importance. In the present study we describe the design, synthesis and evaluation of a combinatorial library of novel affinity ligands for the purification of DNA polymerases (Pols). Pyrococcus furiosus DNA polymerase (Pfu Pol) was employed as a proof-of-principle example. Affinity ligand design was based on mimicking the natural interactions between deoxynucleoside-triphosphates (dNTPs) and the B-motif, a conserved structural moiety found in Pol-I and Pol-II family of enzymes. Solid-phase ‘structure-guided’ combinatorial chemistry was used to construct a library of 26 variants of the B-motif-binding ‘lead’ ligand X–Trz–Y (X is a purine derivative and Y is an aliphatic/aromatic sulphonate or phosphonate derivative) using 1,3,5-triazine (Trz) as the scaffold for assembly. The ‘lead’ ligand showed complementarity against a Lys and a Tyr residue of the polymerase B-motif. The ligand library was screened for its ability to bind and purify Pfu Pol from Escherichia coli extract. One immobilized ligand (oABSAd), bearing 9-aminoethyladenine (AEAd) and sulfanilic acid (oABS) linked on the triazine scaffold, displayed the highest purifying ability and binding capacity (0.55 mg Pfu Pol/g wet gel). Adsorption equilibrium studies with this affinity ligand and Pfu Pol determined a dissociation constant (KD) of 83 nM for the respective complex. The oABSAd affinity adsorbent was exploited in the development of a facile Pfu Pol purification protocol, affording homogeneous enzyme (>99% purity) in a single chromatography step. Quality control tests showed that Pfu Pol purified on the B-motif-complementing ligand is free of nucleic acids and contaminating nuclease activities, therefore, suitable for experimental use.

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Keywords: Affinity chromatography; Biomimetic ligand; Combinatorial chemistry; DNA enzymes; Enzyme purification; Ligand design; Pfu DNA polymerase

1. Introduction

Pyrococcus furiosus DNA polymerase (Pfu Pol) is an important enzyme for the recombinant DNA technology. The enzyme has gained considerable attention in DNA amplification, becoming an indispensable tool for many molecular biology techniques, including that of polymerase chain reaction (PCR). Pfu Pol, a member of the DNA polymerases II (family B, α-like DNA polymerases), is a thermophilic enzyme with 3′→5′ exonuclease activity (proofreading), in addition to 5′→3′ activity, able to correct errors introduced during substrate polymerisation [1,2]. The error rate for Pfu Pol is reported to be 7- to 10-fold lower than that of non-proofreading θ DNA polymerases [3,4]. Therefore, the development of a facile and effective purification protocol for Pfu Pol is a rewarding endeavour of practical significance and commercial interest.

Affinity chromatography [5–7] exploits the ability of bio-logically active macromolecules to form specific and reversible complexes with appropriate affinity ligands. Undoubtedly this is the most specific and effective enzyme purification technique. The design, synthesis and selection of affinity ligands has progressed rapidly because of the accumulated knowledge of structures obtained from X-ray crystallography, NMR and homology modelling studies, the impressive growth of bioinformatics and molecular docking techniques, the defined and combinatorial chemical synthesis and the technological advances in high-throughput screening [7].

Synthetic ligands can be generated by three methods [7]: (a) The rational method features the ‘functional approach’ [8–10] and the ‘structural template approach’ [11–17]. The former is
applied when enough structural data for the target protein are not available and/or an in silico reliable protein model cannot be built by homology modelling methods, whereas the latter assumes knowledge of the protein structure, existence or construction of an in silico model protein, and knowledge of the interaction between protein and its natural ligands. (b) The combinatorial method relies on the selection of ligands from a library of synthetic ligands randomly made. (c) The combined or structure-guided or directed combinatorial method, adopted in the present study, exploits both earlier methods, that is, the ligand is selected from an intentionally biased library based on a rationally designed ligand. This method foresees the following steps [7]: (i) create an in silico protein model, investigate known biological interactions involving the targeted protein site, and design a ligand using modelling techniques by mimicking, (a) a natural complex as a template [11–19], (b) an interaction where the ligand displays complementarity to exposed residues in the protein target site [20–22] (as employed in the present study) or (c) directly a naturally existing interaction [23–25]. (ii) Make up a limited ligand library of structures resembling the designed (in silico predicted) ‘lead’ ligand from step (i) by solid-phase synthesis. (iii) Evaluate the ligand library in immobilized form (affinity chromatography screening) using a biological extract containing the protein of interest.

In generating ligand libraries, syn-trichlorotriazine (cyanuric chloride) holds a central position and attracts increasing attention as a scaffold. There are several reports describing the rational design and/or selection of affinity ligands from libraries composed of sulfonated carbocyclics, (keto)carboxylates, dipptide-mimetics, amines and amino acid-analogues as subunits on a the triazine scaffold [7,18,26].

This is the first report describing nucleotide-mimetic synthetic ligands for a DNA-recognizing enzyme, the Pfu Pol. The philosophy of design relied on the ‘structure-guided combinatorial method’. Keeping synthetic routes simple, chemical building blocks were of commercial origin and were chosen on the basis of the ‘complementarity approach’ employing as a template, in the absence of the crystal structure of Pol-II family’s enzyme (Pol), the structure of Thermococcus gorgonarius DNA polymerase (PDB code 1tgo), with which Pfu Pol shares 80% sequence identity, was used as a template. An alignment of Pfu Pol and Thermococcus gorgonarius DNA polymerase was generated with Clustal W [29] and analysed by TTO [31]. An iterated protocol involving multiple model construction and rigorous protein structure quality assessment, using PROSA II [32] and Verify 3D [33], was used. PROSA II and Verify 3D both yield overall scores as well as local profiles, which can be used to localise areas of unusual packing and/or solvent exposure characteristics. The overall scores were used to choose the final model. Analysis of packing, solvent exposure and stereochemical properties suggest the final Pfu Pol model to be of good overall quality.

2.2. Bioinformatics and molecular modelling

Sequences homologous to Pfu Pol were sought in the NCBI using BLAST [27] and PSI-BLAST [28]. The resulting sequence set was aligned with Clustal W [29]. ESPript (http://prodes.toulouse.inra.fr/ESPript/cgi-bin/ESPript.cgi) was used for alignment visualization and manipulation. A model of Pfu Pol (NCBI accession number NP_577941) was constructed using MODELLER 6 [30] (run at http://www.infobiosud.cnrs.fr/bioserver). The determined X-ray crystal structure of Thermococcus gorgonarius DNA polymerase (PDB code 1tgo), with which Pfu Pol shares 80% sequence identity, was used as a template. An alignment of Pfu Pol and Thermococcus gorgonarius DNA polymerase was generated with Clustal W and analysed by TTO [31]. An iterated protocol involving multiple model construction and rigorous protein structure quality assessment, using PROSA II [32], and Verify 3D [33], was used. PROSA II and Verify 3D both yield overall scores as well as local profiles, which can be used to localise areas of unusual packing and/or solvent exposure characteristics. The overall scores were used to choose the final model. Analysis of packing, solvent exposure and stereochemical properties suggest the final Pfu Pol model to be of good overall quality.

2.3. Liquid-phase synthesis of 9-aminomethyladine

The method used for synthesizing AEAd (entry 8, Table 1) was an adaptation of a published one [34]. Dry potassium carbonate (0.6 g, 4.3 mmol) was added to an adenine solution (8 ml, 0.5 g, 3.7 mmol) in dry dimethyl formamide (DMF) and mixed for 1 h at 65 °C, before 2-bromoethylamine hydrobromide (1.1 g, 5.3 mmol) was added. The reaction progress was monitored by TLC (butanol/2-acetic acid, 97:3) using ninhydrin staining. After completion, the reaction mixture was separated by preparative TLC on Kieselgel 60 plates (2-propanol/ammonia/water 7:2:1, v/v). The product (Rf 0.76) was scraped off, extracted with water, filtered (0.45 μm cellulose membrane filter) and lyophilized, to give a pale yellow solid (0.2 g, 40% yield, w/w). Further purification of AEAd was effected by preparative TLC (n-butanol/water/acetic acid 5:3:2, v/v). The Rf 0.19 band was extracted with water, filtered and lyophilized, to give 9-aminomethyladine (0.045 g AEAd, 9% yield, w/w).

2.4. Liquid-phase synthesis of 9-aminomethylguanine

The method used for synthesizing AEGu (entry 9, Table 1) was an adaptation of a published one [35]. Sodium borohydride
Table 1
The structures of the ligands of the combinatorial library

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</table>

(0.15 g, 3.9 mmol) was added to a guanine solution (8 ml, 0.5 g, 3.3 mmol) in dry DMF and mixed for 1 h at 90 °C, before 2-bromoethylamine hydrobromide (1.0 g, 4.8 mmol) was added. The reaction progress was monitored by TLC (butanol-2/acetic acid, 97:3) using ninhydrin staining. After completion, the reaction mixture was separated by preparative TLC on Kieselgel 60 plates (2-propanol/ammonia/water 7:2:1, v/v/v). The product (Rf 0.41) was scraped off, extracted with water, filtered (0.45 μm cellulose membrane filter) and lyophilized, to give a yellow solid (0.14 g, 28% yield, w/w). Further purification of AEGu...
was effected by preparative TLC (n-butanol/water/acetic acid 5:3:2, v/v). The Rf 0.27 band was extracted with water, filtered and lyophilized, to give 9-aminoethylguanine (0.04 g AEGu, 8% yield, w/w).

2.5. Construction of the ligand library by solid-phase synthesis

The method used for synthesizing the affinity adsorbents (Fig. 1) was an adaptation of published procedures [20,36]. An alkaline suspension of Sepharose CL-6B (50 g moist weight gel, 1 M NaOH, 40 ml) was treated with epichlorohydrin (1 M, 40 ml) for 2 h at 30 °C. Excess epichlorohydrin was removed by washing and the epoxy-activated gel (app. 30 μmol epoxy groups/g moist weight gel) was converted to its 6-aminohexyl-derivative (AH-gel) with the addition of hexane-1,6-diamine in 10-fold molar excess over free epoxy groups on the gel and shaking 18 h at 30 °C. Ninhydrin was used to visualize the presence of free amino-groups on the gel. The AH-gel was then washed with water before introduced in acetone–water mixture (1:1, v/v, 100 ml, 0 °C) whereupon a solution of 1,3,5-trichlorotriazine (6.4 mmol, 1.2 g) in acetone (15 ml, 0 °C) was added in small portions over a period of 2 h. The completion of the reaction was determined by ninhidrin test (no gel staining). The triazine-activated AH-gel was washed with increasing concentrations of acetone in water followed by decreasing concentrations of acetone in water, and finally with water. The activated AH-gel was divided in 26 equal portions (1 g each). In 24 of them were added solutions of the ten R1 substituents (0.048 mmol dissolved in water, 1 ml) and agitated gently at 30 °C, until no further decrease of the absorption (λ max) of the unreacted substituent was observed (R1, Table 2). Each R1-monosubstituted gel was recovered on a glass sinter (No. 2), washed exhaustively with increasing (50–100%, v/v) and decreasing (80–0%, v/v) concentrations of acetone solutions. To R1-monosubstituted gels and two of the AH-triazine gels were added solutions of the two R2 substituents (0.048 mmol dissolved in 0.2 M sodium carbonate, 1 ml) and agitated gently at 65 °C, until no further decrease of the absorption (λ max) of the unreacted substituent was observed (R2, Table 2). The disubstituted gels were filtrated and washed with water, before being packed to disposable polypropylene chromatographic columns (1 ml gel).

2.6. Preparation of pre-treated cell extract

Recombinant Pfu Pol was expressed in E. coli BL21(DE3) pLysS according to Lu and Erickson [37]. Cells were harvested (10,000 × g, 10 min) and stored at −20 °C. Cell paste (11 g) was suspended in 33 ml Tris–HCl buffer (50 mM, pH 7.5, 2 mM MgCl2) containing 1 mM PMSF. The cell suspension was disrupted by sonication on ice (5 min, 10 s pulse intervals) and centrifuged (13,000 × g, 15 min, 4 °C). The supernatant was incubated at 75 °C for 15 min, cooled on ice for 20 min and centrifuged (13,000 × g, 15 min, 4 °C) to remove denatured proteins. Nucleic acids were removed by adding a protamine sulfate solution (2%, w/v) to a final concentration of 5 mg/ml. The mixture was gently stirred for 1 h at 4 °C and centrifuged (13,000 × g, 15 min, 4 °C). The supernatant was kept at −20 °C for several months.

2.7. Screening of the library of immobilized ligands

Chromatographic procedures were performed at 4 °C using dialysed pre-treated cell extract (Tris–HCl buffer, 20 mM, pH 7.5, containing 2 mM MgCl2). The adsorbents of Table 1 (1 ml, 0.9 g moist weight gel) were equilibrated in the same buffer and loaded with pre-treated extract (0.8 ml, 2.5 mg protein), before being washed with equilibration buffer until effluent absorbance
2.8. Effect of pH on the purification of Pfu Pol from the affinity adsorbent oABSAd

Chromatographic procedures were performed at 4 °C. A column containing adsorbent No. 14 (oABSAd) (1 ml, 0.9 g moist weight gel) was equilibrated, in successive experiments, with 20 mM Tris–HCl buffer, pHs 7.0, 7.5, 8.0 and 8.5, containing 2 mM MgCl₂. A sample of pre-treated cell extract (0.8 ml, 2.5 mg protein) previously dialysed against the same equilibration buffer, was applied to the adsorbent. The column was washed with the equilibrating buffer until the effluent absorbance (A₂₈₀) was less than 0.01. The mixture was then centrifuged (5000 rpm, 2 min) and the amount of unbound protein in the supernatant was determined by the method of Bradford [39].

2.9. Purification of Pfu Pol on the affinity adsorbent oABSAd

Chromatographic procedures were performed at 4 °C. A column containing adsorbent No. 14 (oABSAd) (0.9 g moist weight gel) was equilibrated with potassium phosphate buffer 30 mM, pH 7.0, containing 2 mM MgCl₂. A sample of pre-treated cell extract (0.8 ml, 2.5 mg protein) previously dialysed against the same equilibration buffer was applied to the adsorbent. The column was washed with potassium phosphate buffer 30 mM, pH 7.0, containing 2 mM MgCl₂ until effluent absorbance (A₂₈₀) was less than 0.01. The column was then washed with 50 mM potassium phosphate buffer, pH 7.0 (4 ml), prior Pfu Pol was eluted with 80 mM potassium phosphate buffer, pH 7.0 (4 ml). Collected fractions (4 ml) were dialysed against water, lyophilized and analyzed by SDS-PAGE [38].

2.10. Adsorption equilibrium of Pfu Pol with the affinity adsorbent oABSAd

In a total volume of 1 ml of 30 mM potassium phosphate buffer, pH 7.0, containing 2 mM MgCl₂, varying amounts of purified Pfu Pol (5–60 μg), previously dialysed in the same equilibrium were mixed with 10 μg of affinity adsorbent (oABSAd), in the presence or in the absence of ATP (50 mM). The suspensions were shaken for 120 min in order for the system to reach equilibrium. The mixture was then centrifuged (5000 rpm, 2 min) and the amount of unbound protein in the supernatant was determined by the method of Bradford [39]. Bound protein was calculated by subtracting the amount of unbound protein from the total amount of protein added. The data were analyzed according to the method of Livingston and Chase [40].

2.11. Determination of the apparent capacity of affinity adsorbents AEAd, oABSAd, AESGu and AEGu-AEGu for Pfu Pol

Chromatographic procedures were performed at 4 °C. Columns containing adsorbents No. 8 (AEAd), No. 14 (oABSAd), No. 18 (AESGu) and No. 26 (AEGu-AEGu) (0.12 g moist weight gel) were equilibrated with Tris–HCl buffer (20 mM, pH 7.5, containing 2 mM MgCl₂) and a solution of purified Pfu Pol in the same buffer (0.08 mg/ml, 4 ml) was continuously applied on each column until effluent absorbance (280 nm) was constant. Bound Pfu Pol was eluted with 2 M KCl in the same equilibration buffer (2 ml).

2.12. Determination of protein concentration

The protein concentration of the pre-treated cell extract was determined by the method of Bradford [38], whereas that of purified Pfu Pol, additionally, by an extinction coefficient of A₂₈₀ = 0.78 (1 mg/ml) [37].

2.13. Assay for relative activity of Pfu Pol

The relative Pfu Pol activity was determined by comparing band intensities of PCR-amplified DNA obtained using heat-treated E. coli extract, Pfu Pol from the affinity adsorbent oABSAd and commercial recombinant Pfu Pol (Promega). A standard PCR protocol (sense primer, 5′-AGGAC-CTTAAATATAGAAGATGAG-3′; antisense primer, 5′-TTAATCAAGGCGATTGTTGTGCAG-3′) was applied to amplify a 1488-bp fragment of the pol gene of Moloney Murine Leukemia virus, using 30 amplification cycles (1.5 min at 94 °C, 2.5 min at 52 °C, 2.5 min at 72 °C), 50 ng of sample DNA and 5 pmol of each primer. The PCR products were visualized by agarose electrophoresis and analyzed using a Kodak 1D Image Analysis Software.

2.14. Quality control assays of Pfu DNA polymerase purified from the oABSAd adsorbent

The purified Pfu Pol from the adsorbent oABSAd was assayed for the presence of endonuclease and exonuclease activity using standard protocols (Promega). For endonuclease activity, 1 μg of lambda DNA was incubated with 12.5 units of purified Pfu Pol for 8 h at 45 °C followed by 8 h at 72 °C in nuclease testing buffer (100 mM KCl, 200 mM Tris–HCl, pH 8.0, 60 mM (NH₄)₂SO₄, 20 mM MgCl₂, 100 μg/ml nuclease-free BSA and 1% Triton X-100) containing 400 μM of each dNTP. Following incubation, the mixture was analyzed by agarose electrophoresis to verify the absence of visible cutting. For exonuclease activity, 1 μg of lambda DNA/HindIII marker was incubated with 5 units of purified Pfu Pol as described above. Following incubation, the DNA products were analyzed by agarose electrophoresis to verify the absence of visible smearing.
3. Results

3.1. Directed combinatorial ligand design

A molecular model of Pfu Pol was created using the structure of Thermococcus gorgonarius DNA polymerase (PDB code 1tgo) as template. This enzyme shares a common architecture with other DNA polymerases, resembling a cupped right hand built by the “palm”, “fingers” and “thumb” sub-domains [41,42]. The palm sub-domain forms a cleft flanked by the fingers and thumb sub-domains (Fig. 2). Together, these three sub-domains hold the primer template DNA and position the incoming dNTP for incorporation into DNA. The palm sub-domain contains the catalytic site where the chemistry of nucleotidyl transfer takes place. The fingers sub-domain interacts with and positions the template DNA strand and the incoming dNTP, while the thumb subdomain primarily binds the duplex DNA in a sequence-independent manner along the minor groove [41,42]. Most DNA polymerases also contain a 3′-5′ exonuclease domain (Fig. 2) within the same polypeptide, which proofreads newly synthesized DNA and corrects mismatched base pairs [43]. The available amino acid sequence of several DNA polymerases allowed the generation of sequence alignment of Pol-I and Pol-II families of DNA polymerases and the location of conserved and unique residues (Fig. 3). The fingers and thumb sub-domains are highly diverse among the different families (alignments not shown), whereas the palm sub-domains show similarity 63–69%. The topology of the palm sub-domain is conserved among polymerase families, with two long helices (Q and P, Fig. 2) packed against the five-stranded antiparallel β-sheet that contains the three conserved aspartate residues involved in nucleotidyl transfer. Helix P of the palm domain contains the strictly conserved B-motif (also called region III), which is related to the O helix of Pol-I family enzymes (Fig. 3). Amino acid residues from motif-B contribute to dNTP recognition and binding [43].

Considering the strictly conserved structural features of B-motif in Pol-I and Pol-II families, and taking into account the spatial proximity of the Tyr495 and Lys488 side chains (Fig. 4A), we thought that putative bifunctional ligands comprising a purine base and a negatively charged aliphatic or aromatic group, may function in a complementary fashion towards the two B-motif residues. Specifically, these ligands could develop aromatic stacking as well as electrostatic and hydrogen bond interactions with residues Tyr495 and Lys488, respectively. Computer-aided molecular modelling suggested that synthetic ligands based on a triazine scaffold and bearing such functionalities, provide a 2D complementarity to the target protein residues (Fig. 4B). For example, the distance between Tyr495 Cα and Lys488 Cα is approximately 12 Å, whereas that between the two substituents on the triazine scaffold falls in the range 10–13 Å.
Fig. 4. (A) Structure of the B-motif of Pfu DNA polymerase. Lys488 and Tyr495 residues are shown as ball-and-stick. (B) The structure of the lead B-motif-binding ligand.

Fig. 5. Adsorbent library screening with E. coli pre-treated extract containing Pfu Pol activity. SDS-PAGE was performed on a 0.75 mm-thick vertical gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. Protein bands were stained with Coomassie Brilliant Blue R-250. The chromatography was performed as described under Section 2. One hundred micrograms of the protein recovered from the adsorbent indicated was applied on each lane. The position of Pfu Pol is indicated with the arrow.
depending on the substituents. In the absence of data on any crystal structure of Pol-II enzymes bound with dNTP, molecular docking experiments of the putative affinity ligands were reckoned to be impossible, hence a purely rational design method could not be applied. Therefore, despite the conserved interactions found in DNA polymerase families, it became evident that for generating effective affinity ligands for Pfu Pol, one should employ directed combinatorial chemistry, for the ‘winner’ ligand had to be selected from a rational library. The structure of the ‘lead’ ligand for library construction was derived after applying the ‘complementarity approach’ with respect to aromatic Tyr495 and anionic Lys488 residues, whereas the ‘winner’ ligand was selected by the directed combinatorial approach. Accordingly, and anionic Lys488 residues, whereas the ‘winner’ ligand was the ‘complementarity approach’ with respect to aromatic Tyr495 and anionic Lys488 residues, whereas the ‘winner’ ligand was selected by the directed combinatorial approach. Accordingly, the combinations of Table 1 formed the basis for the solid-phase synthesis of the directed combinatorial library on beaded agarose.

### 3.2. Construction of the ligand library

The route followed for the synthesis of the 26 affinity adsorbents is shown in Fig. 1. The strategy was based on the successive substitution of two chlorine atoms of the trichlorotriazine-activated AH-gel by different amines. The first chlorine was substituted by negatively charged alkyl- and arylamines (R1), i.e. aminomethanesulfonic acid, 2-aminoethylsulfonic acid, 3-aminopropylphosphonic acid (Table 1, entries 1–3, 10–12, 17–19), 4-aminobenzylphosphonic acid, sulfanilic acid, aniline-2-sulfonic acid and 3-aminobenzensulfonic acid (Table 1, entries 17–19), 4-aminobenzylphosphonic acid, sulfanilic acid, aniline-2-sulfonic acid and 3-aminobenzensulfonic acid (Table 1, entries 15–23), and by a purine base-analogue (R2), i.e. 9-aminoethyladenine and 9-aminoethylguanine (Table 1, entries 24–26). The second chlorine was substituted by two purine base-analogues (R2), i.e. 9-aminomethyladenine and 9-aminomethylguanine (Table 1, entries 8–26).

### 3.3. Screening of the adsorbent library and determination of binding capacity with Pfu Pol

All adsorbents were evaluated for their ability to bind and purify Pfu Pol from E. coli extract. Prior to affinity chromatography screening, the cell lysate was heat treated (75°C) in order to effect precipitation of contaminating cellular proteins. Protamine sulfate treatment was also examined and adopted for removing contaminating nucleic acids. Assessment of the purifying effectiveness of the adsorbents was based on SDS-PAGE analysis, using the same high amount of protein (100 μg) eluted from each adsorbent, thus ensuring direct comparability of bands. Visual examination of the gels (Fig. 5) revealed that all adsorbents exhibited appreciable binding for the target enzyme, with adsorbents No. 9 (AEAd), No. 14 (oABSAd), No. 18 (AESGu) and No. 26 (AEGu-AEGu) achieving the highest purification. Furthermore, these four adsorbents showed similar binding capacity for Pfu Pol (mg enzyme/g moist weight gel), i.e. AEAd, 0.40; AEGu-AEGu, 0.43; AESGu, 0.49; oABSAd, 0.55. Adsorbent No. 14 (oABSAd) exhibited slightly higher capacity and, therefore, was finally chosen for the purification protocol of Pfu Pol.

### 3.4. Adsorption equilibrium studies with affinity adsorbent oABSAd and Pfu Pol

Equilibrium adsorption studies were employed to characterize the interaction of Pfu DNA Pol with the adsorbent oABSAd. This approach provides a relationship between the concentration of the protein in solution and the amount of protein adsorbed to the solid phase, when the two phases are at equilibrium [21,40]. The model most often employed for affinity systems is based on a second-order reversible interaction, where the protein-ligand interaction has a characteristic binding energy [40] and proceeds in a monovalent fashion. At equilibrium, a familiar Langmuir isotherm model, described by Eq. (1), can be obtained [40]:

$$ q = \frac{q_{\text{max}}c}{K_d + c} \tag{1} $$

where \( q \) is the bound adsorbate concentration at equilibrium (μg/ml adsorbent), \( c \) the equilibrium liquid phase concentration (μg/ml), \( q_{\text{max}} \) the Langmuir isotherm maximum capacity constant (μg/ml adsorbent), and \( K_d \) is the apparent dissociation constant. The batch adsorption of Pfu Pol on the oABSAd adsorbent is shown in Fig. 6, in the absence and in the presence of ATP. The calculated dissociation constant was determined to be equal to 83 nM in the absence of ATP. This value falls within the range expected for a highly selective affinity ligand [16]. The results showed that ATP perturbs the immobilized ligand–enzyme complex, as evidenced by the decline of the amount of bound enzyme in the presence of ATP (Fig. 6).

#### 3.5. Purification of recombinant Pf u Pol from E. coli extract on the oABSAd adsorbent

Prior to developing the purification protocol, the influence of pH on the binding process and the desorption conditions were investigated. At pH 7.0, affinity adsorbent oABSAd (Fig. 7A) displayed the highest purifying ability and enzyme recovery. At higher pH values either the recovery (Fig. 7B, pH 7.5) or
The purifying ability (Fig. 7C and D, pHs 8.0 and 8.5, respectively) declined. Consequently, pH 7.0 was chosen for the enzyme purification protocol. With regard to the desorption conditions, the agents glycerol (50%), adenosine triphosphate (5 and 15 mM), EDTA (5 mM) and phosphate buffer (50 mM), were tested. Potassium phosphate buffer led to the highest purification and recovery and, therefore, was further investigated. All other agents failed to desorb the enzyme (results not shown). Application of stepwise desorption, using increasing concentrations of potassium phosphate buffer (20–100 mM) revealed that 80 mM buffer, pH 7.0, was the most effective and, therefore, adopted for the purification of Pfj Pol (Table 3). SDS-PAGE analysis of the purified enzyme showed a single protein band corresponding to 90 kDa (Fig. 8, Coomassie Blue R-250 staining). Furthermore, quality control tests revealed the absence of endonuclease and exonuclease activities (Promega’s standard protocols).

4. Discussion

In the present work we report the design, synthesis and application of a directed (structure-guided) combinatorial library of dNTPs-mimetic affinity ligands.

DNA polymerases catalyse the synthesis of deoxyribonucleic acids in a template-dependent fashion that results in a copy of the original DNA molecule. The ubiquitous and essential nature of DNA predicts that enzymes responsible for DNA synthesis evolved early and share a common design and mechanism of action. Avaliable crystal structures for most known polymerase families, including the Pol-I and Pol-II family of DNA polymerases, have confirmed that such enzymes exhibit striking similarities in their overall architecture, the catalytic site, and the mechanism of nucleotidyl transfer [41–46]. For designing synthetic affinity adsorbents able to bind and purify the Pol-II archaeal DNA polymerase from Pyro-
with the arrow.

the participating moieties, the library included control-ligands, for comparison purposes and in order to assess the significance of library of nucleotide-mimetic triazine ligands (Nos. 10–23). For these considerations led to a rational (focused) para-acid) and configuration of the R1 aromatic sulfonate group serves bet-
tion with the target residue (e.g. Tyr495). A negatively charged moiety of the ligand to the solvent, thus facilitating the interac-
tion with the Lys488 side chain. In order to assess which group of the natural substrate (dNTP), and able for electrostatic
scaffold (R1) as a second substituent, mimicking the phosphate bases fulfils two roles. It provides a functional chemical site for attachment to the chlorotriazine scaffold of the activated AH-gel, and it acts as a ‘spacer’ that protrudes the recognition moiety of the ligand to the solvent, thus facilitating the interact-
tion with the target residue (e.g. Tyr495). A negatively charged phosphonate or sulfonate group was introduced on the triazine scaffold (R3) as a second substituent, mimicking the phosphate group of the natural substrate (dNTP), and able for electrostatic interaction with the Lys488 side chain. In order to assess which configuration of the R3 aromatic sulfonate group serves bet-
ter Pfu Pol, three isomers, having the sulfonate-group at the orortho- (oABS, sulfamic acid), meta- (mABS, aniline-2-sulfonic acid) and para-position (pABS, 3-aminobenzensulfonic acid) were evaluated. These considerations led to a rational (focused) library of nucleotide-mimetic triazine ligands (Nos. 10–23). For comparison purposes and in order to assess the significance of the participating moieties, the library included control-ligands, i.e. monosubstituted sulfonate- (Nos. 1,2,5–7), phosphonate- (No. 3), adenine- (No. 8) and guanine-triazines (No. 9), and bis-substituted base-triazines (Nos. 24–26).

The choice of triazine as a scaffold presents certain advanta-
geous features. This heterocycle, a structural element of the dye Cibacon blue 3GA, is located in the vicinity of the posi-
tion occupied by the nucleotide ribose when interacting with the NAD+-dependent horse liver alcohol dehydrogenase [47], thus pseudomimicking the nucleotide ribose. Furthermore, 1,3,5-chlorotriazine offers synthetic accessibility, when considering the temperature-dependent successive displacement of the chloro-
ride atoms by different nucleophiles [16–19]. The three chlorine atoms can be displaced in a sequential and controlled fashion by amines in aqueous solution at approximately 0°C, 30–50°C and 70–100°C, depending on the reactivity of the amino-compound.

The results obtained from screening the ligand library with pre-treated cell-extract appeared to verify the rationale of lig-
and design. Adsorbents having no adenine or guanine moiety (Nos. 1–7), bound the enzyme but presented low purifying ability (Fig. 5B and C). All other affinity ligands displayed sim-
ilar enzyme purifying ability with slight differences. The good behavior observed with affinity adsorbents AEAd and AESGu should be attributed to the presence of the purine moiety (Fig. 5).

Furthermore, combination of the sulfonate or phosphonate with the base-analogues appeared to work better, as for example, with adsorbents Nos. 10–23 (Fig. 5). It appears that these anionic and heterocyclic moieties are useful structural elements for the recognition and purification of the DNA-binding enzyme. This view is supported by the finding that, under the same experimen-
tal conditions, the selected oABSAd adsorbent exhibited clearly better purifying ability and higher binding capacity, compared to the monosubstituted adsorbent AEAD.

Adsorption equilibrium studies with adsorbent oABSAd and Pfu Pol (Fig. 6) showed that the respective ligand–enzyme complex appears to have a fairly strong \( K_D \) (83 nM), falling within the range expected for a highly selective affinity ligand [16–19]. Furthermore, obtaining a Langmuir isotherm is indica-
tive of interaction at a fixed number of enzyme sites each of which can only hold one molecule. In spite of the fact that Pols reject NTPs as substrates, ATP (50 nM) is able to perturb the oABSAd–enzyme complex, indicative of an interaction between Pfu Pol and the adenine moiety. Furthermore, experimental evi-
dences are indicative of the presence of specific and non-specific ionic interactions in the oABSAd–enzyme complex. For exam-
ple, while phosphate (20–50 mM) fails to desorb the enzyme from the AEAd adsorbent, ATP (5 mM) and KCl (50 mM) lead separately to enzyme elution (results not shown). In contrast, enzyme desorption from the oABSAd adsorbent is successful with phosphate and KC1 but not with ATP. It may be possible that the presence of the sulphonate moiety on the oABSAd lig-
and, results in a different mechanism of Pfu Pol desorption, as compared to the mechanism operating with the AEAD ligand. Pfu Pol has been purified before by different methods, all incorporating at least a pre-treatment step prior to chro-
matography. Employment of a protocol combining heat pre-
treatment followed by metal chelate, anion exchange (S-Sepharose) and gel filtration (Superdex 200) chromatogra-

**Fig. 8.** Pfu Pol purified from affinity adsorbent oABSAd. SDS-PAGE was performed on a 6.75 mm-thick vertical gel containing 12.5% (w/v) polyacy-
lamide (running gel) and 2.5% (w/v) stacking gel. Protein bands were stained with Coomassie Brilliant Blue R–250. The chromatography was performed as described under Section 2. Lane 1, pre-treated cell extract; lane 2, flow through from the column after loading; lane 3, eluted fraction with 50 mM phosphate buffer pH 7.0; lanes 4 and 5, eluted fraction with 80 mM phosphate buffer, pH 7.0 (10 and 20 µg total protein, respectively). The position of Pfu Pol is indicated with the arrow.

**coccus furiosus** (Pfu Pol), we initially focused on finding a conserved structural motif present on both Pol-I and Pol-II families. Structural features of the B-motif, the strictly con-
served motif found on Pol-I and Pol-II molecules, were exploited for assembling the ligand library. The chemical nature and spatial arrangement of Tyr495 and Lys488 residues of the B-
motif have rationally guided the construction of the combina-
torial ligand library. One of the chlorine atoms of the 1,3,5-
trichlorotriazine-activated AH-gel was substituted by purine analogues, 9-aminoethyladenine or 9-aminoethylguanine (R2). These substituents were assumed to mimic the purine functions within the range expected for a highly selective affinity ligand (A and G) of the enzyme’s natural substrate (dNTP). Although polymerase requires primer-templates for binding dNTPs in a catalytic fashion, for the present purpose, it may be sufficiet a}

Furthermore, obtaining a Langmuir isotherm is indica-
tive of interaction at a fixed number of enzyme sites each of which can only hold one molecule. In spite of the fact that Pols reject NTPs as substrates, ATP (50 nM) is able to perturb the oABSAd–enzyme complex, indicative of an interaction between Pfu Pol and the adenine moiety. Furthermore, experimental evi-
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phy, leads to enzyme of a specific activity 7500 units/mg [1]. Another purification protocol includes heat-treatment and DNase digestion before cellulose phosphate and Mono Q anion exchange chromatography, leading to Pfu Pol of specific activity 22 500 units/mg [37]. Recently, a purification procedure combining heat-treatment, heat-exchange followed by DEAE-Sepharose and heparin-Sepharose chromatography [43]. Heat-treatment prior to metal chelate affinity chromatography is also an effective approach for purifying recombinant His-tagged Pfu Pol to a specific activity of 31,000 units/mg. However, the metal chelate adsorbent is restricted to His-tagged enzymes. Furthermore, the purification protocol incorporates enterokinase for hydrolyzing the His-tag from the purified enzyme [48]. Applying the present strategy (Table 3) led in one chromatography step to high purity Pfu Pol (specific activity 32,000 units/mg) with good recovery (75%), using low-cost elution conditions (80 mM phosphate buffer, pH 7.0). Although the 6.2-fold purification achieved numerically is not impressive, nevertheless, it is sufficient for an overexpressed enzyme. Purified Pfu Pol was kept at −20 °C for more than 2 years in 50 mM Tris–HCl, pH 7.0, containing 20% glycerol, without appreciable loss of its activity. The in-house purified Pfu Pol has been used in routine applications, e.g. PCR screening, cloning, mutagenesis and DNA shuffling protocols.

In conclusion, in this report we described the design, synthesis and application of a novel library of ligands from which a dNTP-mimetic triazine ligand was selected for the purification of recombinant Pfu Pol from E. coli extract. The purification method described is simple and effective, yielding pure enzyme suitable for application to molecular biology techniques. The new library is under investigation with respect to other DNA-recognizing enzymes.

References
