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Nucleic Acid Separations Utilizing Immobilized Metal Affinity Chromatography

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Immobilized metal affinity chromatography (IMAC) is widely used for protein purification, e.g., in the isolation of proteins bearing the well-known hexahistidine affinity tag. We report that IMAC matrixes can also adsorb single-stranded nucleic acids through metal ion interactions with aromatic base nitrogens and propose that metal affinity technologies may find widespread application in nucleic acid technology. Oligonucleotide duplexes, plasmid, and genomic DNA show low IMAC binding affinity, while RNA and single-stranded oligonucleotides bind strongly to matrixes such as Cu(II) iminodiacetic acid (IDA) agarose. The affinity of yeast RNA for IDA-chelated metal ions decreases in the following order: Cu(II), Ni(II), Zn(II), and Co(II). Adsorption isotherms for 20-mer oligonucleotide homopolymers show that purines are strongly favored over pyrimidines and that double-stranded duplexes are not bound. IMAC columns have been used to purify plasmid DNA from *E. coli* alkaline lysates, to purify a ribozyme, to remove primers and imperfect products from PCR reactions, and to separate 20-mer oligonucleotide duplexes containing centered single-base mismatches. Potential further applications include SNP scoring, hybridization assays, and the isolation of polyadenylated messenger RNA.

Introduction

Immobilized metal affinity chromatography (IMAC) was introduced by Porath et al. (*1*, *2*) as a means of purifying proteins based on the affinity of their surface-exposed amino acids (especially histidines) for chelated metal ions. The method has found widespread application in the purification of recombinant histidine-tagged and native pharmaceutical proteins, most commonly using Cu(II)- and Ni(II)-charged iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) chromatographic adsorbents. Metal chelate ligands have also been immobilized on foams (*3*), membranes (*4*), and biosensor chips (*5*) and in electrophoresis gels (*6*); they have also been used as affinity precipitation agents (*7*).

While the interaction of metal ions with nucleic acids is a long-standing and active field of research (*8*−*13*), e.g., into the function of the widely used cancer drug cisplatin (*9*), IMAC has found surprisingly limited application in the purification of nucleic acids.. Adsorption of histidine analogue-conjugated PCR products and mononucleotides to IMAC resins has been demonstrated (*14*−*16*), but the potential applications of IMAC in nucleic acid separation and analysis remain largely unexplored.

We speculated that Cu(II)- and Ni(II)-charged IDA or NTA IMAC matrixes would selectively bind the imidazole functionalities of purine-containing, single-stranded nucleic acid molecules, such as oligonucleotides or unstructured RNA molecules. To avoid nonspecific interactions with backbone phosphates, only soft metals were evaluated (*1*). While the great variety of available ligands allows for extensive optimization, we chose to focus initially on those most widely used in protein purification. We found that the high, specific affinity of chelated soft metals for nucleic acid bases allows the use of IMAC in the purification of plasmid DNA and RNA, in the removal of contaminants and primers from PCR reaction products, and in the detection of mismatches in DNA heteroduplexes. The readily reversible nucleic acid affinity of small, robust metal chelate ligands may find widespread utility in high-throughput genomics, genetic medicine, and microanalytical devices.

Materials and Methods

Nucleic Acids. Plasmids used were pBGS19luxwt (6 kb (*17*)) and pCMV sport β gal (7.9 kb, Gibco BRL). Total baker's yeast RNA was from Sigma. Synthetic oligodeoxynucleotides were obtained from MWG Scientific. The 86-base ribozyme used was produced in an *E. coli* strain generously provided by Dr. Karl Drlica (*13*).

Equilibrium Adsorption Isotherms. Equilibrium adsorption isotherms were measured in duplicate in 1 mL microcentrifuge tubes (Fisher Scientific). Chelating Sepharose Fast Flow adsorbent (Amersham) was charged with metal by washing three times each with distilled water, 50 mM metal chloride solution, and IMAC binding buffer (20 mM HEPES (Sigma) with 250 mM NaCl at

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pH 7.0). Reagents were added in the following order: IMAC binding buffer, nucleic acid dissolved in IMAC binding buffer, and 20 μL of 50% adsorbent slurry in IMAC binding buffer.

After vortexing, tubes were rotated end-over-end in a Roto-Torque heavy duty rotator (Cole-Palmer) for 10 min, a time found sufficient for equilibration in control experiments. After equilibration, the tubes were centrifuged in an Eppendorf microcentrifuge for 2 min and the supernatant was removed for nucleic acid concentration measurement by absorbance at 260 nm.

Homopolymer isotherms were measured as detailed above except in 0.6 mL microcentrifuge tubes containing 4 μL of Chelating Sepharose in a total liquid volume of 200 μL. The tubes were eluted with 385 μL of 500 mM imidazole in IMAC binding buffer added to the loaded adsorbent along with precisely 15 μL of residual supernatant.

Dynamic Adsorption. RNA solutions and plasmid-containing alkaline lysates were stripped of RNA by continuous column adsorption using an FPLC system (Amersham Pharmacia Biotech) with a 20 mL Amicon FPLC column (1 cm $\times$ 15 cm) packed with 15 mL of Chelating Sepharose (Amersham Pharmacia Biotech). The column was first equilibrated with 10 column volumes of deionized water and then saturated with 50 mM metal chloride as determined by effluent absorbance at 254 nm. The column was next washed with 2 volumes of water and equilibrated in IMAC binding buffer over 10 column volumes. The IMAC binding buffer wash was used to remove weakly bound metal ions from the adsorbent to reduce metal ion leaching during column operation. Plasmid samples were then flowed continuously onto the column for measurement of breakthrough behavior. After use, columns were regenerated with 50 mM EDTA at pH 8.0 and cleaned with 1 M NaCl in 1 M NaOH.

PCR Product Purification. PCR reactions (100 μL) were run on a Perkin-Elmer GeneAmp 2400 PCR system using 2 units Taq polymerase, 10 μL of 10X reaction buffer with $MgCl_2$, and 800 μM dNTPs (all from Promega). The target was 40 pg/reaction of pCMV sport β gal (Gibco) with 0.1 nmol each of the forward and reverse PCR primers 5′ TAA TTG TTG CCG GGA AGC TAG AG 3′ and 5′ TCG CAT TGA ATT ATG TGC TGT GTA G 3′, which amplified an 800 base region of the plasmid DNA encoding β-lactamase. PCR reactions were run for 25 cycles of denaturation at 94 °C (45 s), annealing at 55 °C (30 s), and extension at 72 °C (3 min).

Promega mini-prep microfuge snap-on columns filled with 100 μL of Chelating Sepharose were used as spin columns. The columns were washed with water, charged with metal chloride solution (50 mM metal chloride in deionized water), and finally washed with IMAC running buffer. The columns were then switched to clean microcentrifuge tubes, and the PCR reactions were applied directly onto the spin columns.

Mismatch Detection. Duplexes of oligodeoxynucleotides with single-base mismatches at position 10 were all based on the same 50% G + C 20-mer oligonucleotide (5′ CAG ACG ATA GTC CTA GTT GC 3′) and its complement. IMAC mismatch detection used a 7 mm $\times$ 7 cm Toso Haas HPLC chelating column (charged with Cu(II)) on a Waters 600E HPLC system.

Oligonucleotides were resuspended in IMAC running buffer, allowed to hybridize at 42 °C for 10 min, and loaded using a 400 μL injection loop. Elution was by four column volumes of IMAC running buffer and a gradient from 0 to 14 mM imidazole in IMAC running buffer at 1 mL/min. The column was regenerated with 10 mM EDTA followed by a solution of 3 M NaCl in 0.1 N NaOH.

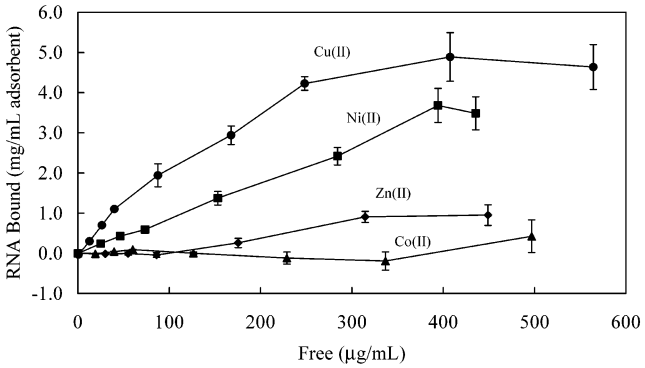

Figure 1. Equilibrium adsorption isotherms for baker's yeast RNA on Chelating Sepharose charged with various metals in 20 mM HEPES, pH 7.0, with 250 mM NaCl.

Results and Discussion

RNA Adsorption. Preliminary experiments using Zn(II)-charged IDA Sepharose with total baker's yeast RNA and pBGS19luxwt plasmid DNA showed effective binding of RNA but no detectable capture of plasmid DNA.

The adsorption of RNA on IDA Sepharose loaded with various metal ions is illustrated in Figure 1. With Cu(II), Ni(II), Zn(II), and Co(II) respectively, 5, 3.5, 1, and 0 mg of RNA/(mL of matrix) bound at 0.4 mg/mL equilibrium free RNA concentration. The dynamic RNA capacity of Cu(II)-charged IDA Sepharose was found by breakthrough curve analysis to be 5−7 mg of RNA/mL at 100 cm/h.

Homopolymer Adsorption Isotherms. The relative IMAC affinities of the nucleic acid bases in polynucleotides were examined using 20-mer oligodeoxynucleotide homopolymer isotherms on Ni(II)-charged IDA Sepharose (Figure 2). A_{20} bound with the highest affinity while G_{20} had an affinity approximately 10 times lower, based on a Langmuirian fit. The affinities of the pyrimidines were much lower than those of the purines (C_{20} and T_{20} approximately 60- and 300-fold lower than A_{20}, respectively). The A_{20}/T_{20} heteroduplex had no detectable binding affinity, ruling out the phosphate backbone as a major source of adsorption affinity. The relative affinities of the homopolymers confirm the nucleotide monomer results of Fanou-Ayi and Vijayalakshmi (*5*) and Hubert and Porath (*6*).

Plasmid and RNA Separation. The nucleic acid discrimination achieved with IMAC suggests application to purification of plasmid DNA from RNA-rich bacterial lysates. Figure 3 illustrates repeated batch stripping of a plasmid-containing *Escherichia coli* (*E. coli*) alkaline lysate (lane 1) with Cu(II)-charged IDA Sepharose. Metal-free control lane 2 shows that the interactions are strictly dependent on the presence of metal ions. Lanes 3−6 trace one supernatant repeatedly exposed to Cu(II)-charged IDA Sepharose. RNA and damaged plasmid DNA are effectively removed without loss of the closed circular plasmid, presumably through interactions with exposed, single-stranded regions. This removal of damaged DNA is difficult to achieve by other methods.

A Cu(II)-charged IDA Sepharose adsorbent column was used to strip RNA continuously from an *E. coli* alkaline lysate containing the plasmid pCMV sport β gal. The plasmid initially passed through the column with an undetectable amount of contaminating RNA. Initial RNA breakthrough was observed after 1 mg/mL of RNA was

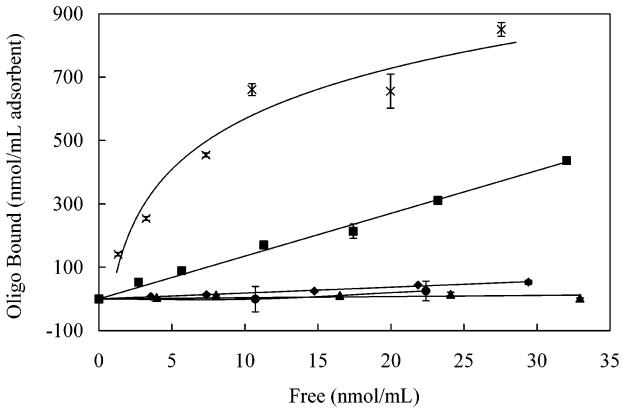

Figure 2. Equilibrium adsorption isotherms of 20-mer homopolymer oligodeoxynucleotides on Ni(II)-charged Chelating Sepharose matrix with 250 mM NaCl in 20 mM HEPES at pH 7.0 (×, A_{20}; ■, G_{20}; ◆, C_{20}; ●, T_{20}; and ▲, A_{20}/T_{20} duplex).

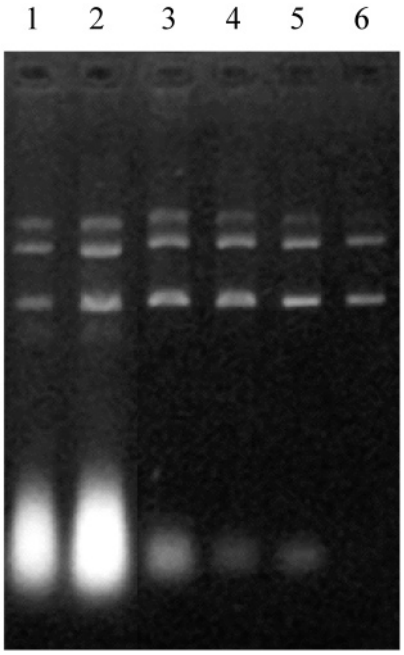

Figure 3. Repeated Cu(II) IDA agarose stripping of RNA from a plasmid DNA-containing alkaline lysate. Ethidium bromide stained 1% agarose gel of Cu(II)-charged Chelating Sepharose batch adsorption of *E. coli* alkaline lysate with plasmid pBGS19luxwt. A 1 mL aliquot of an IPA-precipitated alkaline lysate resuspended in 1 mL of IMAC running buffer was contacted with 50 μL of Chelating Sepharose per batch experiment. Lane 1 is the original lysate; lane 2 is lysate contacted with 50 μL of metal-free IDA matrix; lane 3 is the unbound material after a single batch adsorption with 50 μL of Cu(II)-charged matrix; and each of lanes 4−6 is the previous lane after exposure to 50 μL of fresh Cu(II)-charged matrix.

bound, and approximately 80% of the contaminating RNA was still bound at a loading capacity of 5 mg/mL.

Compaction precipitation of DNA from RNA readily yields plasmid containing only traces of RNA as the major contaminant without column chromatography (*7*). IMAC adsorption offers a very high effective capacity for polishing of this plasmid DNA by selective adsorption of the minor (1−3%) RNA component, while plasmid passes through unretained. A similar approach to purification of conventional alkaline lystes is also possible. A β ribozyme, partially purified by compaction precipitation (*8*), was readily separated from small *E. coli* RNA contaminants on a 1 mL Cu(II)-charged Hytrap chelating column using a gradient of 0−2 M ammonium chloride.

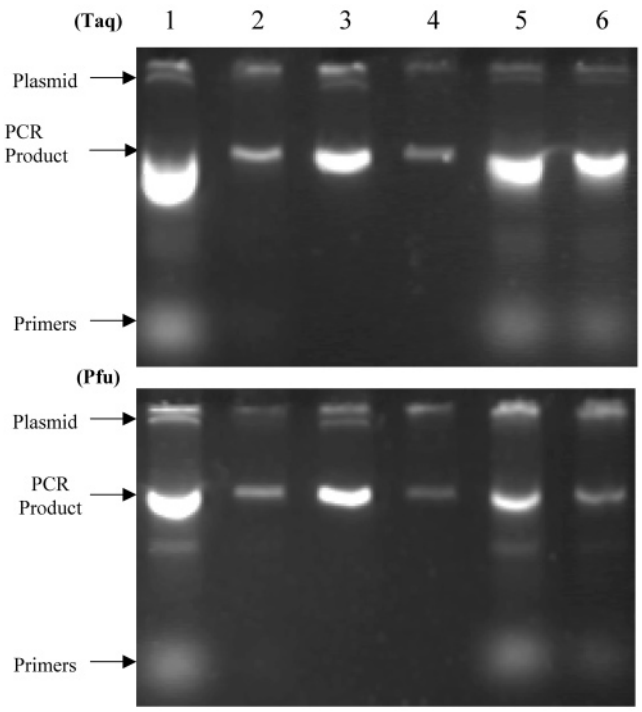

Figure 4. PCR product cleanup by IMAC. (Upper) 1.5% agarose gel stained with SYBR Gold (Molecular Probes). Lane 1 is an overload (approximately 1300 ng) of Taq PCR product amplifying an 800 bp fragment of pCMV sport β gal; lane 2 is a diluted loading (200 ng) of lane 1; lane 3 is an overload (200 ng) of the PCR product mixture from lane 1 run through a Cu(II)-charged spin column; lane 4 is a diluted loading (30 ng) of lane 3; lane 5 is an overload of the elution (with 500 mM imidazole + 250 mM NaCl in 20 mM HEPES, pH 7.0) of the Cu(II)-charged spin column from lanes 3 and 4; and lane 6 is a 2-fold dilution of lane 5. (Lower) Identical to the upper panel except Pfu polymerase was used in the PCR reactions.

Selective IMAC binding of RNA allows orthogonal separations using a different principle than anion exchange (*19*), hydrophobic interaction (*20*), boronate (*21*), and size-exclusion chromatography (*22*).

PCR Product Purification. Taq polymerase PCR reaction products were loaded directly onto Cu(II)-charged, 100 μL spin columns for rapid removal of primers and stunted or mismatch-containing products. The IMAC column captured the primers and some defective products, leaving primarily the double-stranded PCR product and the plasmid DNA template (Figure 4, top, lane 3). Partial elution (Figure 4, top, lane 5) shows that the column binds not only the primers and fragments, but also some of the Taq PCR product itself, presumably through mismatched bulges, partially single-stranded products of stalled extension, or the 3′A overhang commonly left by Taq polymerase. Processing of a Pfu (*23*) polymerase PCR reaction product mixture amplifying the same sequence gave similar results (Figure 4, bottom, lane 5), but products of this proofreading polymerase are not as readily retained on the IMAC column. IMAC purification of Taq PCR product greatly improved sequencing fidelity over a non-IMAC purified control sample, comparable to that achieved with a silica-based commercial kit.

Mismatch Detection Using IMAC. Higher-resolution IMAC HPLC can separate mismatch-bearing oligonucleotide heteroduplexes, presumably through interactions with bases in the disordered region. As shown in Figure 5, retention correlates well with the binding affinities of the homopolymers (Figure 2). With the

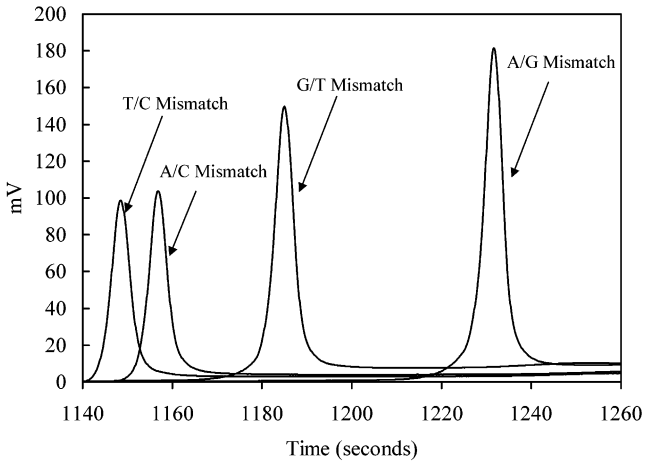

Figure 5. HPLC chromatogram of four 20-mer oligodeoxynucleotide heteroduplexes, each bearing a single internal mismatch at position 8, 9, or 10. The mismatches examined were T/C, G/T, A/C, and A/G. The gradient was run over 2l mL (at 1 mL/min) from 0 to 40 mM imidazole in 20 mM HEPES with 250 mM NaCl on a 7 mm × 7 cm Toso Haas metal chelating HPLC column charged with Cu(II).

enhanced resolution of metal affinity capillary electrophoresis (*24*), this mismatch separation could serve as the basis of PCR product cleanup, SNP scoring, or hybridization assays. Efficient full-length sequence confirmation might be achieved by fragmentation, denaturation, and reannealing in the presence of wild-type DNA, followed by metal-affinity capillary electrophoresis in an analysis similar to "tryptic mapping" of proteins.

Conclusions

The results presented here suggest a variety of applications and extensions. Variations in operating pH, ligand structure, metal ion, and competitive eluant concentration may allow tailoring of affinity and selectivity for specific purposes. Potential metal ion leaching has not prevented the use of IMAC in the manufacture of recombinant pharmaceuticals, but can be minimized by washing of the charged adsorbent, or operation with higher-affinity ligands or with some ligands uncharged (either by incompletely loading the column or by use of a second, unloaded bed in series to capture released metal ions (*1*, *25*)). In some applications, the potential for oxidation can be greatly reduced by selection of ions other than Cu(II), inclusion of reductants, or exclusion of oxygen.

Chelated soft metal ions interact with exposed bases of nucleic acids, and this interaction can serve as the basis of a variety of preparative and analytical methods in nucleic acid technology. In addition to those demonstrated here, further applications may include eukaryotic (polyA) mRNA isolation, improvement of the quality, sequenceability, and clonability of PCR products, and economical SNP scoring and sequence confirmation. Supports other than porous particles may be useful in separations of large nucleic acids.

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