Synthetic Surfaces for Ribonuclease Adsorption

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Intact RNA and DNA are of central importance to biochemical research and biotechnology. The preservation of these nucleic acids requires the absence of nuclease activity. Here, radical-mediated polymerization of vinylsulfonate on resin and glass surfaces is shown to produce a high-density poly(vinylsulfonate) coating that sequesters ribonucleases from aqueous solutions quickly and completely. The adsorptive efficacy of this coating exceeds that of other known coatings by $\geq 10^7$-fold. Surfaces coated with poly(vinylsulfonate) could be used to maintain the integrity of ribonucleic acids in a variety of contexts.

Introduction

Biochemical research and biotechnology rely on polymeric nucleic acids, yet during their storage and use, nucleic acids encounter nucleases, both advertently and inadvertently. For example, nucleases are often added with the intent of destroying RNA in a DNA sample, or vice versa. Residual amounts of these nucleases can affect downstream steps in protocols.[1,2] Alternatively, human skin is an abundant source of nucleases that can be transferred accidentally to surfaces and solutions.[3] Moreover, reagents (including those labeled "nuclease free") are often contaminated with nucleases.[4]

Ribonucleases are perhaps the most problematic of nucleases because of their high natural abundance, prodigious catalytic activity, notorious conformational stability and resistance to proteolysis, and lack of requisite cofactors.[5,6] Although several ribonuclease inhibitors have been described,[1,6,7] each suffers from one or more undesirable attribute. For example, the ribonuclease inhibitor protein (RI[8,9]) binds ribonucleases with femtomolar affinity but is expensive and highly sensitive to oxidation.[10] In addition, RI inhibits only ribonuclease A (RNase A;[6,11] EC 3.1.27.5) and some of its homologues. Although diethylpyrocarbonate (DEPC) inactivates many nucleases, it is toxic and its use requires time-consuming procedures. Moreover, DEPC treatment results in the covalent modification of many proteins, nucleic acids, and small molecules.[12]

Recently, we discovered that oligo(vinylsulfonate) is an extremely potent inhibitor of catalysis by RNase A (as well as a contaminant in common buffers that contain sulfonylethyl groups).[13] Its inhibitory activity is due to its high density of anionic charges, which are displayed in a manner reminiscent of that in a polymeric nucleic acid (Figure 1A). Previous work had demonstrated that poly(vinylsulfonate) (PVS) is also a potent inhibitor of deoxyribonucleases.[14,15] Still, the utility of PVS as a general nuclease inhibitor has been limited by the difficulty of separating PVS from nucleic acids, which are likewise polyanionic.

We envisioned that surfaces coated with PVS could be useful in many contexts.[6,16,17] The addition of PVS-coated resin, followed by filtration or centrifugation, could be used to remove nucleases from solution. Alternatively, PVS-coated glassware or plasticware could adsorb a contaminating nuclease, thereby providing safe long-term storage for valuable nucleic acids. To test these hypotheses, we coated two distinct surfaces with PVS. To maximize the density of the coating, we chose to synthesize PVS directly on the surfaces rather than couple the polymer to a surface. The results indicate that PVS-coated surfaces provide an extraordinary means to sequester ribonucleases.

Experimental Procedure

Materials. Reagents were from Sigma-Aldrich (St. Louis, MO). Anhydrous THF, DMF, and CH_2Cl_2 were withdrawn from a CYCLETAINER solvent delivery system from Mallinckrodt−Baker (Phillipsburg, NJ). Other anhydrous solvents were from Sigma-Aldrich and were withdrawn from septum-sealed bottles.

Silica gel functionalized with 3-aminopropyltriethoxysilane was from Silicycle (Québec City, Canada). Glass slides functionalized with 3-aminopropyltriethoxysilane were from CEL Associates (Pearland, TX).

RNase A was produced in *Escherichia coli* as described previously.[18] The A19C variant of RNase A[2] was labeled with

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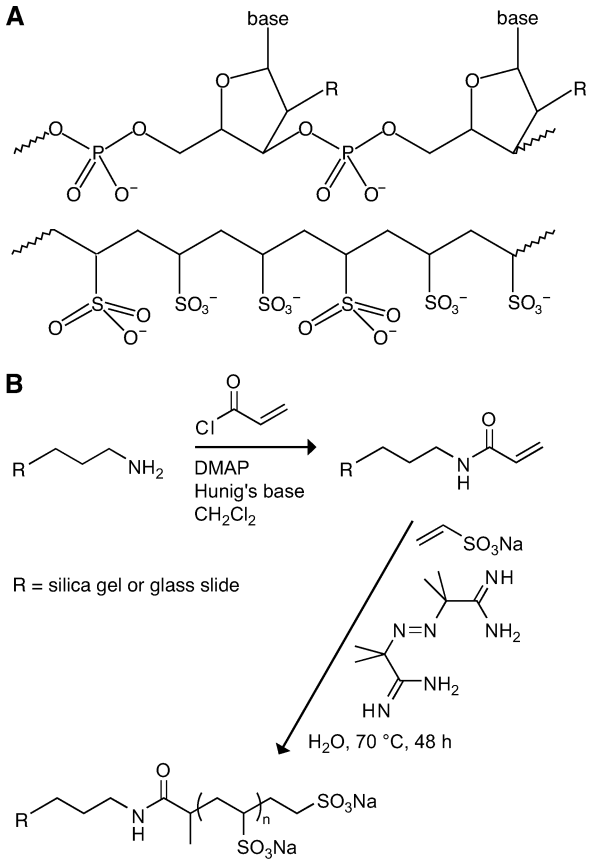

Figure 1. (A) Structure of DNA (R = H) and RNA (R = OH) (top) and PVS (bottom). (B) Scheme for the synthesis of poly(vinylsulfonate)-coated surfaces.

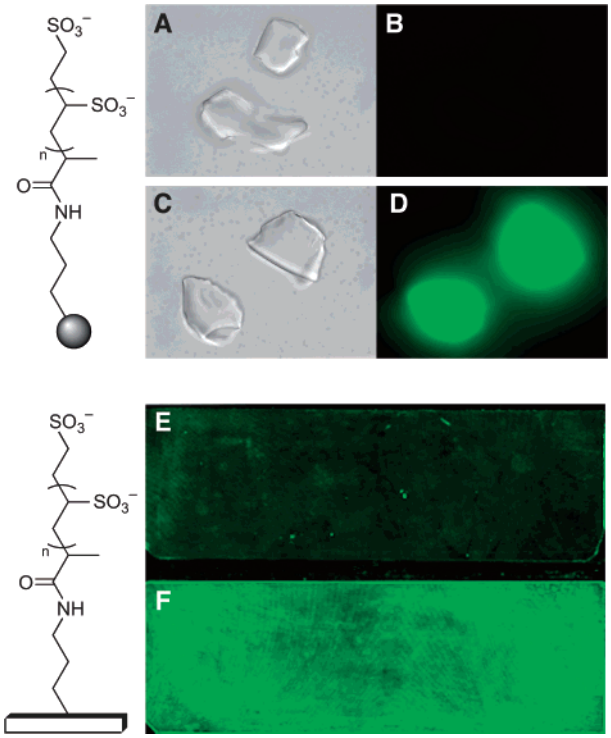

Figure 2. PVS-coated surfaces sequester a ribonuclease. (A) Bright-field image (60×) of PVS-coated silica gel. (B) Fluorescent image of panel A. (C) Bright-field image (60×) of PVS-coated silica gel in the presence of fluorescein−RNase A. (D) Fluorescent image of panel C. (E) Fluorescent image of an acrylamide-coated glass slide in the presence of fluorescein−RNase A. (F) Fluorescent image of a PVS-coated glass slide in the presence of fluorescein−RNase A.

fluorescein as described previously.[19] A fluorogenic ribonuclease substrate, 6-FAM-dArUdAdA-6-TAMRA,[20] was from Integrated DNA Technologies (Coralville, IA).

Synthesis of Acrylamide-Coated Silica. Silica gel functionalized with 3-aminopropyltriethoxysilane (5.0 g, 7.3 mmol) was suspended in CH_2Cl_2 (50 mL). Dimethylaminopyridine (0.9 g, 7.3 mmol) was then added. The resulting suspension was flushed with Ar(g) and cooled to 0 °C. Finally, acrolyl chloride (2.6 g, 29.2 mmol) was added dropwise followed by the dropwise addition of Hunig's base (diisopropylethylamine; 7.55 g, 58.4 mmol). The reaction mixture was allowed to warm to room temperature and then stirred for 12 h under Ar(g). The resin was isolated by filtration and washed with CH_2Cl_2 (5 × 100 mL), DMF (5 × 100 mL), H_2O (5 × 100 mL), and Et_2O (5 × 100 mL). The resin was then dried under reduced pressure for 12 h and stored at 4 °C.

Synthesis of PVS-Coated Silica. Silica gel functionalized with acrylamide (1.0 g, 1.35 mmol) was suspended in degassed H_2O (30 mL). The resulting suspension was flushed with Ar(g). Sodium vinylsulfonate (17.5 g, 135 mmol, purified to remove MEHQ and dried to a white paste) was added, followed by 2,2′−azobis(2-methylpropionamideine) dihydrochloide (42 mg, 0.15 mmol). The reaction mixture was heated to 70 °C and allowed to stir for 48 h under Ar(g). The resin was isolated by filtration and washed with CH_2Cl_2 (5 × 100 mL), DMF (5 × 100 mL), H_2O (5 × 100 mL), and Et_2O (5 × 100 mL). The resin was then dried under reduced pressure for 12 h and stored at 4 °C.

Synthesis of Acrylamide-Coated Glass. A glass slide (75 mm × 25 mm) functionalized with 3-aminopropyltriethoxysilane was placed in CH_2Cl_2 (50 mL). Dimethylaminopyridine (0.9 g, 7.3 mmol) was then added. The slide was flushed with Ar(g) and cooled to 0 °C. Finally, acrolyl chloride (2.6 g, 29.2 mmol) was added dropwise followed by the dropwise addition of Hunig's base (7.55 g, 58.4 mmol). The reaction mixture was allowed to warm to room temperature and then stirred for 12 h under Ar(g). The slide was washed with CH_2Cl_2 (5 × 100 mL), DMF (5 × 100 mL), H_2O (5 × 100 mL), and Et_2O (5 × 100 mL). The slide was then dried under reduced pressure for 12 h and stored at 4 °C.

Synthesis of PVS-Coated Glass. An acrylamide-coated glass slide (75 mm × 25 mm) was placed in degassed H_2O (30 mL), which was then flushed with Ar(g). Sodium vinylsulfonate (17.5 g, 135 mmol, purified to remove MEHQ and dried to a white paste) was added followed by 2,2′-azobis(2-methylpropionamideine) dihydrochloride (42 mg, 0.15 mmol). The reaction mixture was heated to 70 °C and then allowed to stir for 48 h under Ar(g). The slide was washed with CH_2Cl_2 (5 × 100 mL), DMF (5 × 100 mL), H_2O (5 × 100 mL), and Et_2O (5 × 100 mL). The slide was then dried under reduced pressure for 12 h and stored at 4 °C.

Quantitative Analysis of the Adsorption of a Ribonuclease by PVS-Coated Silica. RNase A (1−128 μM) was incubated with PVS-coated silica (0.1 mg) for 2 h at room temperature in 0.10 mL of 50 mM MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M). The resin was collected by centrifugation at 5000*g* for 10 s. The supernatant was tested for ribonucleolytic activity with a sensitive assay based on a fluorogenic substrate: 6-FAM-dArUdAdA-6-TAMRA.[20] The concentrations of free and bound RNase A were determined and subjected to Scatchard analysis. Assays were performed in triplicate.

The ability of PVS-coated silica to adsorb RNase A was also assessed in Dulbecco's phosphate-buffered saline (PBS), which is KCl (2.68 mM), KH_2PO_4 (1.47 mM), NaCl (136.89 mM), and Na_2HPO_4 (8.06 mM).

Time Course for the Adsorption of a Ribonuclease by PVS-Coated Silica. PVS-coated silica (100 mg/mL) was preequilibrated with buffer for several days. The resin (0.1 mg, 0.4 nmol of tight-binding sites) was then mixed with 0.10 mL of 50 mM MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M) and RNase A (2 μM, 0.2 nmol). At several time points, the resin was

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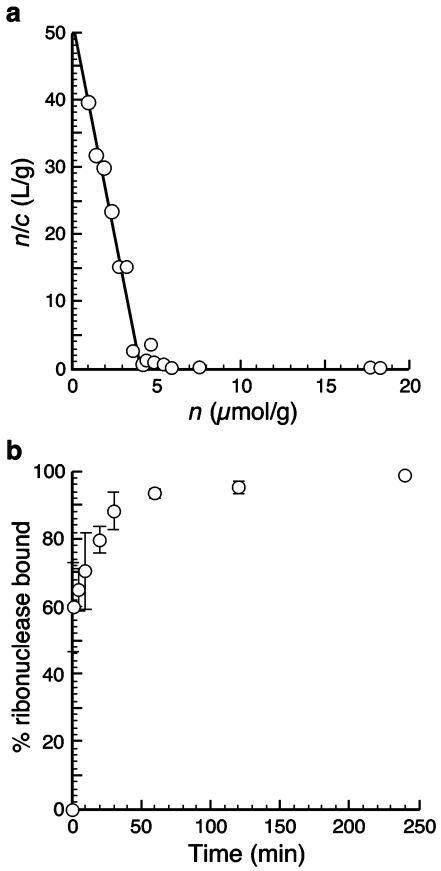

Figure 3. Quantitative analysis of the adsorption of a ribonuclease by PVS-coated silica. (A) Scatchard plot for the adsorption of RNase A by PVS-coated silica in 50 mM MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M). Data are for tight-binding sites. (B) Time-course for the adsorption of RNase A by PVS-coated silica that has a 2-fold molar excess of tight-binding sites.

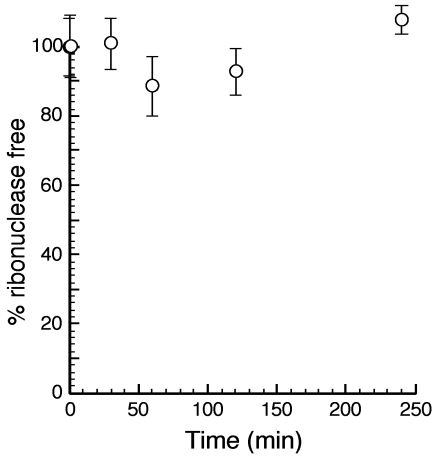

Figure 4. Nonadsorption of a ribonuclease by acrylamide-coated silica. Acrylamide-coated silica (0.1 mg) was incubated at room temperature with RNase A (2 μM) in 0.10 mL of 50 mM MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M). At known times, the resin was collected by centrifugation and an aliquot of supernatant was assayed for ribonucleolytic activity.

collected by centrifugation at 5000*g* for 5 s and an aliquot of the supernatant was assayed for ribonucleolytic activity.[20]

Reuse of PVS-Coated Silica. PVS-coated silica (10 mg) was mixed at room temperature for 2 h with RNase A (1.0 μM) in 1.0 mL of 0.05 M MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M). The resin was collected by centrifugation at 5000*g* for 10 s. The supernatant was removed, and an aliquot was assayed for ribonucleolytic activity.[20] The resin was then washed with

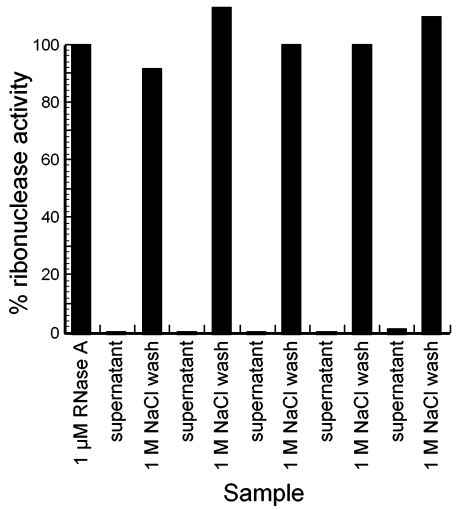

Figure 5. Adsorption of a ribonuclease during repeated use of a poly(vinylsulfonate)-coated surface. PVS-coated silica (10 mg) was mixed at room temperature for 2 h with RNase A (1.0 μM) in 1.0 mL of 50 mM MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M). The resin was collected by centrifugation. The supernatant was removed, and an aliquot was assayed for ribonucleolytic activity. The resin was then washed with 1.0 M NaCl and collected by centrifugation. The supernatant was again assayed for ribonucleolytic activity. The resin was then washed thoroughly with H_2O. A new aliquot of RNase A was added, and the process was repeated five times.

1.0 M NaCl (2 × 1.0 mL). The resin was again collected by centrifugation, and the supernatant was assayed for ribonucleolytic activity.[20] The resin was then washed thoroughly with H_2O. A new aliquot of RNase A was then added, and the entire process was repeated five times.

Adsorption of Contaminating Human Ribonucleases by PVS-Coated Silica. MES−NaOH buffer (0.05 M), pH 6.0, containing NaCl (0.10 M) was contaminated with secretory ribonucleases by swiping a human finger on the inside of the tube. The buffer was passed through a 0.2-μm sterile filter to remove any human or microbial cells. Ribonuclease-contaminated buffer (1 mL) was mixed with PVS-coated resin (10 mg), acrylamide-coated resin (10 mg), or no resin for 2 h at room temperature. The resin was collected by centrifugation at 5000*g* for 10 s. The supernatant was removed and assayed for ribonucleolytic activity as follows. Each sample was incubated with 16S and 23S rRNA (4 μg; Roche) overnight at 37 °C. As controls, a sample of noncontaminated buffer and a sample of RNase A (10 pM) were incubated likewise. An aliquot (10 μL) of each sample was then mixed with loading dye and subjected to electrophoresis for 15 min at 100 V through a 1.0% (w/v) agarose gel in TAE buffer (40 mM Tris−acetic acid, 1 mM EDTA) containing ethidium bromide (6 μg/mL).

Results and Discussion

We synthesized PVS on silica gel and glass slides (Figure 1B) that had been modified so as to display amino groups.[21] Reaction of the surface amino groups with acryolyl chloride converted the amino groups into acrylamide groups, which have electron-deficient alkenes that undergo facile polymerization. We then performed radical-mediated polymerization of vinylsulfonate (CH_2=$CHSO_3^-$) on the acrylamide-coated surfaces. The resulting surfaces were washed until free PVS was no longer detectable with a sensitive assay for ribonuclease inhibition.[13]

PVS-coated surfaces sequester a fluorescently labeled ribonuclease (Figure 2). We quantified the ability of PVS-coated silica to remove a ribonuclease from 50 mM MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M). A

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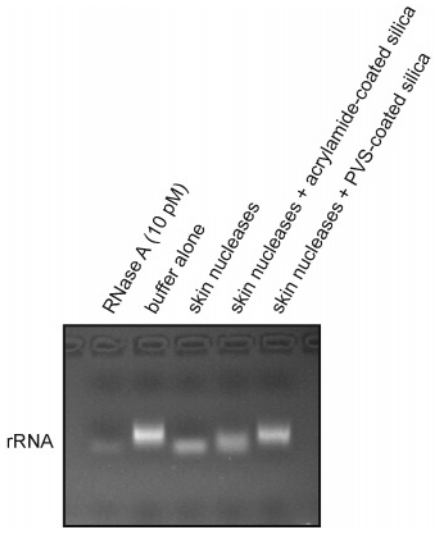

Figure 6. Adsorption of human secretory ribonucleases by a poly(vinylsulfonate)-coated surface. Buffer was intentionally contaminated with secretions from human skin. The ability of PVS-coated silica to sequester secretory ribonucleases and protect the integrity of rRNA was assessed qualitatively by agarose gel electrophoresis.

Scatchard plot of the data is curvilinear (Figure 3A), as expected for the binding of a ligand to a lattice of sites.[22] We determined the equilibrium dissociation constants for the tightest binding sites from the limiting slope of the Scatchard plot.[17] The PVS-coated silica has 4.1 μmol/g of tight-binding sites for RNase A and $K_d = 80$ nM for these sites. This K_d value is in gratifying agreement with the inhibition constant observed using free PVS ($K_i = 120$ nM).[13] In the presence of PVS-coated silica with excess tight-binding sites, residual ribonucleolytic activity is not detectable (<0.01%).

PVS-coated surfaces sequester a ribonuclease quickly. The enzymatic activity of an added ribonuclease was measured at known times after exposure to PVS-coated silica in which the number of tight-binding sights was only 2-fold greater than the amount of ribonuclease. Half of the ribonuclease was bound within 1 min; maximum binding occurred within 1 h (Figure 3B). In contrast, acrylamide-coated silica did not remove any ribonuclease after 4 h (Figure 4).

PVS-coated surfaces far exceed the capacity and affinity for nucleases of other known surfaces. In the most-effective prior example, poly[2′-O-(2,4-nitrophenyl)]poly(A) [DNP-poly(A)] was attached to acrylic beads.[17] This material has 0.017 μmol/g of binding sites for RNase A (which is 240-fold less than PVS-coated silica) and $K_d = 0.41$ μM in a solution of low salt concentration (which is 3.7×10^4-fold greater[13]). Accordingly, the adsorptive efficacy of PVS-coated silica exceeds that of other known surfaces by $\geq 10^7$-fold.

PVS-coated silica is also able to remove RNase A from PBS. This buffer has a pH of 7.4, which allows for only weak Coulombic interactions between the two active-site histidine residues of RNase A and a polyanion.[23] Moreover, PBS contains inorganic phosphate, which like PVS binds to the active site of RNase A.[24] Still, PVS-coated silica was able to sequester RNase A from PBS (data not shown), though less effectively than from 50 mM MES−NaOH buffer, pH 6.0, containing NaCl (0.10 M).

PVS-coated surfaces can be regenerated and reused many times. PVS-coated silica was incubated with RNase A such that the amount of free RNase A was <0.5%. After the incubation, nearly 100% of the bound RNase A was eluted by washing with 1.0 M NaCl. This incubation/elution cycle was repeated a total of five times with no detectable change in the amount of ribonuclease sequestered or released (Figure 5).

Finally, PVS-coated surfaces bind the most-prevalent human ribonuclease, RNase 1. Under conditions similar to those used for RNase A, a solution of human RNase 1 is >99.5% bound by PVS-coated silica (data not shown). In addition, migration rates during electrophoresis in an agarose gel indicate that PVS-coated silica diminishes RNA degradation by secretory ribonucleases from human skin (Figure 6).

Conclusions. Surfaces can be coated with PVS by a facile chemical synthesis. Such surfaces extract ribonucleases from solution quickly and completely and can be used repeatedly. PVS-coated surfaces have many advantages over other known means to sequester ribonucleases and thereby preserve the integrity of RNA.

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