# Poly(vinylpyrrolidone) for Bioconjugation and Surface Ligand Immobilization

Alexander N. Zelikin,* Georgina K. Such, Almar Postma, and Frank Caruso

*Centre for Nanoscience and Nanotechnology, Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia*

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Poly(vinylpyrrolidone) (PVP), a nonionic and nontoxic polymer with antifouling properties, has been synthesized via RAFT polymerization to obtain thiol-terminated PVP. We demonstrate that when the polymer is adsorbed onto the surface of colloidal silica particles, the terminal thiol groups of PVP remain accessible for chemical modification and lend themselves to the immobilization of ligands. We show that ligand attachment onto the surface via conjugation to PVP is reversible, as the polymer can be desorbed from the surface for conjugate and surface recovery. We present the conjugation of a model peptide and an oligonucleotide to PVP via the polymer terminal thiol and demonstrate that conjugates remain functional in molecular recognition assay. The developed technique offers a novel method to functionalize low-fouling surfaces for a variety of biomedical applications and presents opportunities to use PVP as a macromolecular drug carrier.

## Introduction

Conjugation of biologically active molecules to surfaces and carrier systems is a vital technique for a variety of biomedical applications, such as targeted drug delivery and biosensing. Polymers are particularly important in bioconjugation due to the variety of properties accessible through a range of different polymer families, including biocompatibility and stimuli-responsive behavior.[1,2] Bioinert polymers are of special interest for biomedical applications such as drug carriers, yet functionality is particularly limited in these molecules. Poly(vinylpyrrolidone) (PVP) has gained considerable interest for biomedical applications due to its water-soluble, nonionic, and nontoxic nature and has been used as a plasma expander[3] and as a macromolecular drug carrier.[4,5] It adsorbs onto a variety of surfaces, endowing the surfaces with low-fouling properties.[6,7] However, the inertness of the lactam ring[8] restricts broader applications of PVP where modification of the chains or conjugation to other molecules is required.

Reversible addition−fragmentation chain transfer (RAFT) polymerization[9,10] presents an opportunity for postmodification of inert polymers.[11−13] Together with precise control over the polymer molecular weight and polydispersity, this technique provides each of the synthesized chains with a thiocarbonylthio end-group, which can be converted easily into a thiol.[14,15] Thiol groups lend themselves well to bioconjugation, as few other chemical moieties offer comparable availability and specificity for subsequent reactions. Recently, this has led to RAFT-derived polymers being explored for diverse conjugation applications.[11,15]

In this work, to biofunctionalize surfaces we combine the features of PVP, namely, adsorption onto surfaces and low-fouling properties, with those inherent in RAFT-derived polymers. We synthesize PVP via the RAFT technique[16] and demonstrate that (i) the terminal thiol groups remain accessible for chemical modification on the chains of PVP adsorbed onto template surface, (ii) the PVP conjugates are *reversibly* adsorbed onto the surface, and (iii) the conjugated biomolecules remain functional, as demonstrated via molecular recognition assays (Scheme 1). The significance of this study lies in the possibility to functionalize low-fouling surfaces for protein and DNA sensing, targeted drug delivery, and particle-based screening applications and presents novel opportunities to use PVP, a nonionic nontoxic polymer, as a macromolecular drug carrier.

## Experimental Section

**Materials.** The solvents were AR grade and were used as received. Dithiothreitol (DTT), 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent), and 2,2′-azobis(isobutyronitrile) (AIBN) were purchased from Sigma-Aldrich, and succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was from Molecular Probes; all chemicals were used as received. High-purity water with a resistivity greater than 18 MΩ cm was obtained from an in-line Millipore RiOs/Origin system. The 5′-thiolated ssDNA (GAGCTCCAGCTTTGTTCCC) was purchased from Sigma-Proligo and used without further purification. The oligopeptide, SIINFEKL, labeled with fluorescein isothiocyanate, was purchased from GL Biochem Ltd. (Shanghai, China) and was used as received.

**Gel permeation chromatography** was performed on a Shimadzu modular LC system equipped with a UV−vis and an RID-10A refractive index detectors using distilled $H_2O$ + 0.02% $NaN_3$ as mobile phase/eluent and poly(ethylene glycol) molecular weight standards (4600 to ∼400 000).

**Flow Cytometry.** Flow cytometry experiments were performed on a Becton Dickson FACS Calibur flow cytometer using an excitation wavelength of 488 nm. At least 25 000 particles were analyzed in each experiment.

**RAFT Polymerization.** The synthesis of the *O*-ethyl *S*-(phthalimidylmethyl) xanthate RAFT agent (**1**) was reproduced on the same scale as described by Postma et al.[16] 1-Vinyl-2-pyrrolidone (1 g, 9.0 × $10^{-3}$ mol), **1** (13 mg, 4.6 × $10^{-5}$ mol), and AIBN (0.7 mg, 4.3 × $10^{-6}$ mol) were combined in 2 mL of dioxane in a Schlenk flask. The flask was purged with nitrogen for 45 min and then heated in a constant temperature oil bath at 60 °C for 6 h. The reaction mixture was diluted with water and dialyzed for 24 h, and the final product was isolated via freeze-drying.

To cleave the thiocarbonylthio group, the synthesized polymer was dissolved in water at 10 g $L^{-1}$. To this solution sodium borohydride

* Corresponding author. E-mail: azelikin@unimelb.edu.au.

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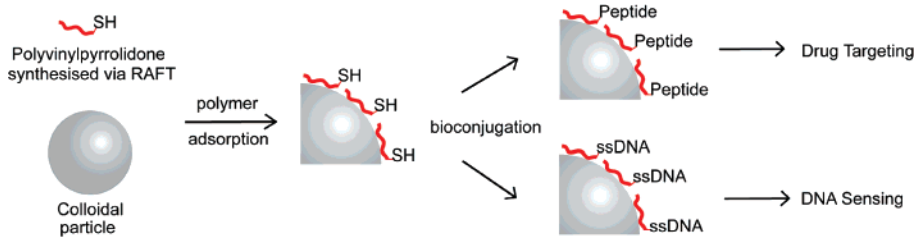
**Scheme 1.** Poly(vinylpyrrolidone) Synthesized via RAFT Polymerization Bears a Terminal Thiol Group, Which Remains Accessible for Bioconjugation When PVP is Adsorbed onto Colloidal Support Particles




was added to a 1 M concentration, and the reaction mixture was incubated at room temperature for 2 h. At this time, the excess borohydride was neutralized by concentrated hydrochloric acid, the mixture was supplemented with $K_2HPO_4$ to 0.1 M concentration, and the pH was adjusted to pH 8 using HCl and NaOH. To this solution, excess Ellman's reagent was added, and the reaction was allowed to proceed for 30 min. The resulting polymer, $PVP_{SSR}$, was isolated via column chromatography using an NAP-5 column (GE Healthcare) and recovered by freeze-drying to obtain an off-white powder. $\bar{M}_n$12 000 Da, PDI 1.2; $^1$H NMR ($D_2O$): $\delta$ 1.40−2.25 (br, 6H), 2.85−3.73 (br, 3H).

**Maleimide Labeling of $PVP_{SH}$ on the Surface of $SiO_2$ Particles.** $SiO_2$ particles of 1 $\mu$m diameter (10 $\mu$L of a 5 wt % suspension) were dispersed in a 1 g $L^{-1}$ solution of $PVP_{SSR}$ in 10 mM acetate buffer, pH 4, and adsorption was allowed to proceed for 30 min. The particles were then collected via centrifugation, and the supernatant was exchanged for distilled water. This represents a single washing cycle and was repeated at least 5 times. Finally, the particles were redispersed in 100 $\mu$L of 10 mM phosphate buffer, pH 8. To cleave the mixed disulfide and liberate the thiol moiety on PVP, the particles were treated with a 0.1 M solution of DTT for 15 min. Following this, the particles were collected and washed as described above and finally dispersed in pH 7.2 phosphate buffer. The obtained suspension was charged with 2.5 $\mu$g of Alexa Fluor 488 (AF488) maleimide and incubated for 2 h. The particles were collected and washed as described above, and the fluorescence of the particles was analyzed via flow cytometry. Control samples were prepared under the same conditions using a commercially available PVP sample (55 KDa), a $PVP_{SSR}$ sample bypassing the DTT step, and the noncoated silica particles.

**PVP Desorption Experiments.** The 1 $\mu$m $SiO_2$ particles coated with $PVP_{SH}$ and modified with AF488 were prepared as described above. The particles were incubated in test solutions for 90 min and washed (5×) with unbuffered water. The resulting particles were dispersed in unbuffered water and analyzed via flow cytometry.

**Peptide Conjugation on Solid Supports.** Amounts of 200 $\mu$g of SIINFEKL and 400 $\mu$g of SPDP were combined in 60 $\mu$L of DMF and allowed to react for 2 h. After this time, the mixture was diluted with acetone to 250 $\mu$L and was spun down at 15 000 *g* for 15 min. The resulting pellet was washed (3×) with acetone and reconstituted in 25 $\mu$L of 0.1 M pH 8 phosphate buffer. $PVP_{SSR}$ was adsorbed onto the surface of 3 $\mu$m $SiO_2$ particles and converted into $PVP_{SH}$, as described above. Finally, the particles were dispersed in 50 $\mu$L of 0.1 M pH 8 phosphate buffer, to which 2.5 $\mu$L of solution of the SIINFEKL−SPDP was added. The resulting suspension was stirred in the dark at room temperature overnight, after which time the particles were extensively washed with 10 mM pH 8 phosphate buffer and analyzed via flow cytometry. The control experiment used PVP sample without thiol groups.

**Oligonucleotide Conjugation.** A volume of 40 $\mu$L of a 150 $\mu$M solution of 5′-thiolated ssDNA was combined with 40 $\mu$L of a 0.2 M solution of DTT in 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer, pH 9, and allowed to react for 2 h. After this, the mixture was charged with 8 $\mu$L of 3 M sodium acetate and 220 $\mu$L of cold ethanol and incubated at −20 °C overnight. The obtained ssDNA−SH was recovered via centrifugation, washed with cold ethanol, and reconstituted in 10 $\mu$L of 0.1 M phosphate buffer, pH 8.

The 3 $\mu$m $SiO_2$ particles with adsorbed $PVP_{SSR}$ were prepared as described above. The conjugation was carried out in a 30 $\mu$M solution of oligonucleotide for 24 h. The hybridization experiments were carried out using 100 nM solutions of probe sequences in saline sodium citrate (SSC) buffer for 90 min, after which the particles were washed with fresh SSC buffer and examined using a fluorescence microscope.

## Results and Discussion

The terminal thiocarbonylthio group on the RAFT-synthesized PVP has a characteristic absorption maximum at 305 nm (Figure 1, curve A). To obtain the polymer chains with a thiol end-group, $PVP_{SH}$, we cleaved the thiocarbonylthio moiety via reaction with sodium borohydride.[17,18] This was verified by the disappearance of the characteristic absorption band at 305 nm in the product. The resulting thiol was immediately reacted with Ellman's reagent to form a mixed disulfide with a characteristic absorption at 335 nm, $PVP_{SSR}$ (Figure 1, curve B). In this form, the terminal thiol is protected from side reactions and, more importantly, is activated toward thiol−disulfide exchange. These data demonstrate that the chain terminus of the RAFT-derived PVP can be modified with a thiol functionality.

Next, we sought to use the thiol group for further modification. The polymer with an activated terminal thiol group, $PVP_{SSR}$, was adsorbed onto 1 $\mu$m $SiO_2$ particles, treated with DTT to expose bare thiol groups, and incubated in a solution of AF488 maleimide at pH 7.2 for 2 h. The resulting particles, as analyzed by flow cytometry (Figure 2), exhibited a 100-fold increase in fluorescence compared to the control particles ($SiO_2$ particles without active thiol functionality). This result demonstrates that the PVP chains carry reactive thiol groups that are available for further modification. Importantly, the chemistry can be performed on the polymer adsorbed onto colloidal particles—a feature most attractive for modification of surfaces and syntheses on solid (colloidal) supports.

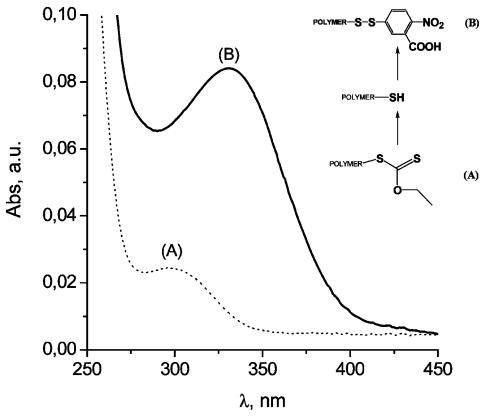



**Figure 1.** UV−vis spectra of the synthesized PVP with a characteristic RAFT absorption band at 305 nm (A) and PVP with a disulfide-protected terminal thiol group, $PVP_{SSR}$, with a corresponding absorption band at 335 nm (B).

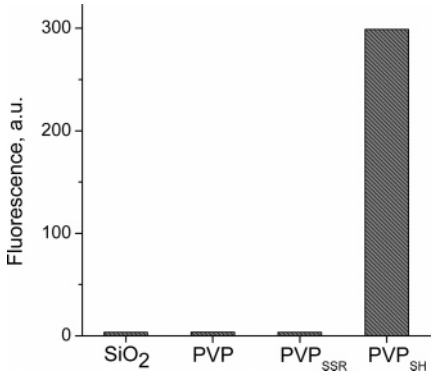



**Figure 2.** Fluorescence intensity of particles after exposure to a solution of AF488 maleimide: unmodified silica particles ($SiO_2$), $SiO_2$ particles coated with a commercial sample of PVP (PVP), PVP bearing disulfide-protected thiol ($PVP_{SSR}$), and PVP with a thiol group ($PVP_{SH}$).

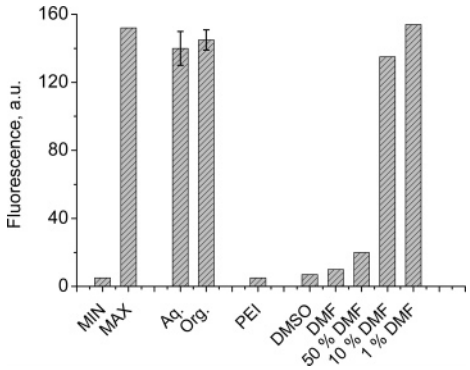



**Figure 3.** Fluorescence intensity of the silica particles with the $PVP_{SH}$–AF488 conjugate incubated in various aqueous and water-miscible solvents. Silica particles with no PVP−AF488 conjugate were used as the nonfluorescent control (MIN); $PVP_{SH}$–AF488-coated particles incubated in deionized water were used as the fluorescence reference (MAX). The data for all aqueous solutions and organic solvents are averaged and presented as respective single data points.

An important aspect in such applications is the stability of the polymer in the adsorbed state, i.e., the conditions under which the PVP chains remain adsorbed or are released from the surface into bulk solution. To address this, the particles with adsorbed fluorescently labeled PVP were incubated in a variety of aqueous solutions and water-miscible solvents and analyzed via flow cytometry (Figure 3). A range of aqueous buffered solutions covered the pH range from 4 to 8, concentrations of buffer salts and sodium chloride from 10 mM to 1 M, and included decimolar DTT, a common reagent in thiol modification reactions. None of the solutions caused desorption of PVP from the surface of the silica particles—the measured fluorescence intensity of the particles remained within the experimental error (<10%) compared with the parent particles. The same result was observed for macromolecular exchange with PVP in bulk solution and exposure to an anionic polyelectrolyte, poly(methacrylic acid). In contrast, exposing the particles to a solution of positively charged polymer, polyethyleneimine (PEI), resulted in immediate and complete removal of the PVP chains from the silica surface. The latter observation can be readily explained by the dominance of electrostatic interaction (PEI−silica) over other, lower energy interactions (e.g., hydrogen bonding) between PVP and the silica surface.

Of the water-miscible solvents tested, lower aliphatic alcohols, tetrahydrofuran (solvent for PVP), and acetone (nonsolvent) did not solubilize the polymer chains from the silica surface (Figure 3). In contrast, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF), strong hydrogen-bonding acceptors, desorbed the PVP chains from the surface. Diluting DMF with water restored the ability of the polymer to remain on the silica colloid, which was also achieved via saturating the 50 vol % DMF−water mixture with sodium chloride.

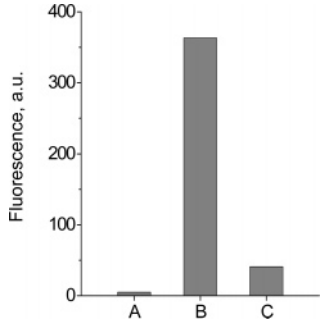



**Figure 4.** Flow cytometry analysis of the $PVP_{SH}$–SIINFEKL conjugation reactions: bare silica particles (A), coupling of SIINFEKL−SPDP to $PVP_{SH}$ (B), and conjugation control using the PVP sample without thiol groups (C).

For biofunctionalization of the surfaces, we first examined the conjugation of PVP to a fluorescently labeled model oligopeptide, SIINFEKL. The amine-to-thiol conjugation is a classic technique in bioconjugation and typically involves a divalent linker such as SPDP. The linker is first reacted with an amine functionality via the succimidyl ester, and the resulting product is further reacted with a thiol group. In this work, we performed the conjugation of the oligopeptide to a thiol group using the $PVP_{SH}$ adsorbed onto the silica particles and analyzed the conjugation via flow cytometry (Figure 4).

Reaction of a thiol group on the PVP, preadsorbed onto a silica particle, results in covalent immobilization of the reporter peptide to the polymer chain on the surface of the particle and is reflected by an increased fluorescence of the particle (Figure 4). The particles with adsorbed $PVP_{SH}$ bearing reactive thiol groups exhibited an order of magnitude higher intensity of fluorescence, compared with the control sample, where peptide was adsorbed onto silica particles coated with PVP without active thiol groups. This result reflects successful immobilization of the peptide onto the particles via conjugation to the PVP. The described technique provides a novel approach to the surface immobilization of peptides, which is important in targeted drug delivery and other biomedical applications.[1]

The second conjugation technique involved conjugation of $PVP_{SH}$ to an oligonucleotide. For this reaction, the activated terminal thiol in $PVP_{SSR}$ was reacted with the 5′-thiol-functionalized 20-mer oligonucleotide ssDNA via thiol−disulfide exchange. The reaction was conducted on the surface of silica particles with adsorbed $PVP_{SSR}$. We then probed the conjugation reaction using 100 nM solutions of fluorescently labeled sequences, including complementary, single nucleotide mismatch, and a noncomplementary sequences (Figure 5).

Hybridization of the probe sequence to the ssDNA−PVP conjugate was registered via an increase in the particle fluorescence. This was most pronounced for the fully complementary sequence (Figure 5A) and was not observed for the noncomplementary sequence (Figure 5C) or the particles with no conjugated target (Figure 5D). Importantly, the conjugated target was able to distinguish the single mismatch sequence hybridization (Figure 5B), which was less pronounced than for the complementary sequence. The data provide evidence that the immobilized DNA remained in its native conformation and can be used in sensing and other hybridization-based applications.

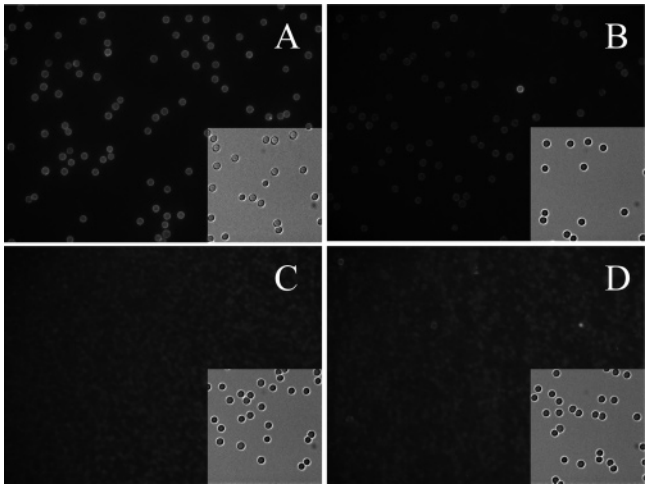



**Figure 5.** Fluorescence microscopy images of the hybridization assay using PVP−ssDNA conjugates on 3 $\mu$m $SiO_2$ particles and complementary (A), single mismatch (B), and noncomplementary (C) probe sequences; image D shows hybridization of complementary sequence on the particles without target ssDNA. Bottom right corners are bright-field images for the corresponding fields of view.

In conclusion, we have demonstrated that PVP synthesized via RAFT can be adsorbed onto the surface of silica particles and used for functionalization of the particles using the polymer terminal thiol groups (Scheme 1). This method of ligand attachment is reversible, as the conjugate can be removed from the surface for surface and/or conjugate recovery. Both peptides and oligonucleotides can be immobilized onto the surface via conjugation to the adsorbed PVP. The potential applications for this technique range from surface modification and functionalization to targeted drug delivery, biosensing, and other biomedical applications.

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