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Synthesis, Characterization, and Utility of Thermoresponsive Natural/Unnatural Product Macroligands for Affinity Chromatography

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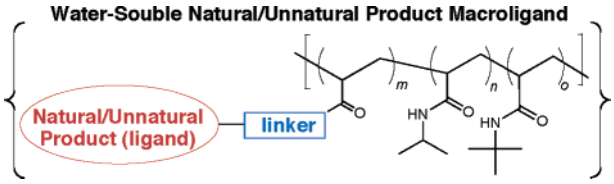
ABSTRACT



The synthesis and characterization of thermoresponsive, water-soluble poly-*N*-isopropyl acrylamide (PNIPAM) derived macroligands displaying cyclosporin A (CsA) and dexamethasone (Dex) for use as novel affinity resins are described. Characterization of these soluble macroligands, including ligand loading and integrity, was determined by ^{1}H NMR spectroscopy. One of the CsA macroligands was used in a protein affinity experiment to capture known binding proteins of CsA, the cyclophilins, from Jurkat T-cell lysates.

Organisms utilize biopolymers in the form of DNA for information storage and in the form of proteins for cellular structure and machinery. Most functional biopolymers respond to external stimuli such as heat and pH in an all-or-nothing or, at least, a highly nonlinear mode.[1] Most common among such biopolymers are proteins that precipitate or denature on heating. Recently, progress has been made toward the synthesis of functional polymers that mimic biopolymers by responding in a desired way to external environmental stimuli such as temperature, pH, and either electric or magnetic fields.[2] These highly nonlinear responses of synthetic polymers occur primarily in water but also in organic solvents[3] or polymer blends.[4] When this stimulus-responsive behavior occurs in aqueous solution, these polymers have potential as useful tools for biotechnological and medicinal research. Among the synthetic stimulus-responsive polymers, temperature-responsive polymers have been the most extensively studied.[5] This kind of polymer is soluble below its lower critical solution temperature (LCST) due to a combination of entropic and enthalpic effects. Above the LCST, the polymer precipitates out of solution presumably due to loss of hydration and exposure of hydrophobic chains.[6,7]

Copolymers of poly-*N*-isopropyl acrylamide (PNIPAM) and derived functionalized polymers have been shown to be thermoresponsive, exhibiting quantitative inverse temperature-dependent solubility in water.[8,9] These and other thermoresponsive polymers have enabled the design of smart

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chemical catalysts having activities that can be switched off and on by precipitation and redissolution based on reaction temperature.[8,9] In addition, these polymers are useful in syntheses using biological catalysts.[10] Most pertinent to the described studies herein are previous uses of PNIPAM-derived thermoresponsive polymers to purify known proteins. An imidazole−PNIPAM thermoresponsive polymer loaded with Cu(II) ions was used for the affinity purification of proteins from cereals (Figure 1).[11] PNIPAM coupled to an

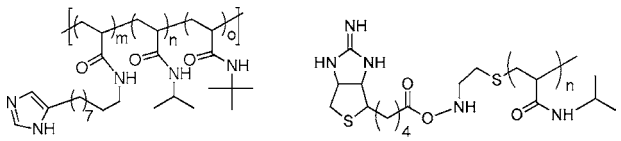


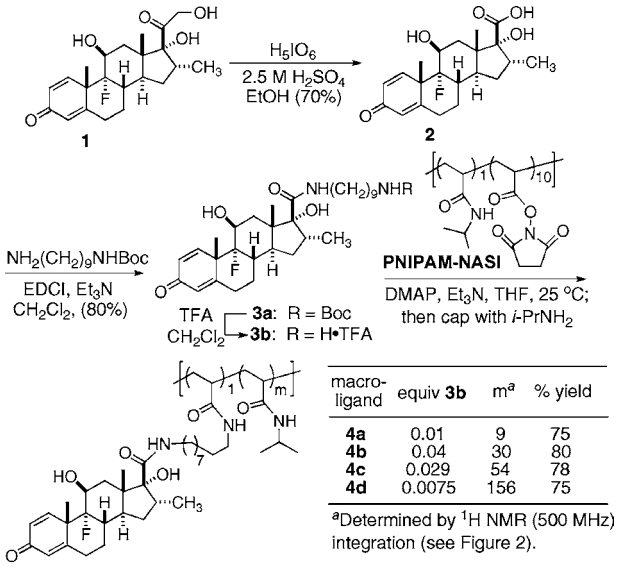
Figure 1. PNIPAM-derived macroligands previously employed for protein purification.

iminobiotin affinity ligand was used to isolate a lysozyme from fetal calf sera.[12] In connection with our interest in coupling natural products to their cellular receptors,[13] we considered the attachment of natural products to PNIPAM polymers to facilitate characterization of the immobilized natural product.[14]

Affinity chromatography is based on the principle of specific protein−ligand interactions and is a common tool for purification of proteins, antibodies, and receptor−ligand complexes.[15] However, traditional affinity chromatography techniques are plagued by nonspecific binding of proteins due to physical inclusion as a result of the solid state of the matrix and nonspecific interactions with the polymer backbone. In addition, it is typically not possible to assess the integrity of the natural product ligand following coupling to the insoluble matrix. In efforts to minimize these limitations, we considered the use of the soluble polymer, PNIPAM, for attachment of natural product ligands for isolation of their cellular receptors. There are several possible advantages of using PNIPAM. The parent polymer is soluble in aqueous solution at 4 °C and has an LCST of 32 °C. The LCST can be subtly increased or decreased by using more hydrophilic or hydrophobic comonomers, respectively.[16] This tactic could be used for more sensitive proteins that are unable to withstand higher LCSTs. Herein, we describe the synthesis and properties of two macroligands, with various ligand loadings, derived from coupling of a representative hydrophobic natural product derivative, dexamethasone (Dex), and a representative hydrophilic natural product, cyclosporin A (CsA), to a copolymer of *N*-isopropyl acrylamide and *N*-(acroyloxy)-succinimide (PNIPAM−NASI). In addition, a pull-down experiment with the CsA macroligand demonstrates the utility of these soluble macroligands in affinity experiments.

The synthesis of dexamethasone macroligands **4a**−**d** commenced with the known α-hydroxy acid derivative **2**[17] that was coupled with mono-*N*-Boc-1,9-diaminoundecane providing amide **3a** (Scheme 1). Following Boc deprotection

Scheme 1. Synthesis of Dexamethasone Macroligands



macro-ligand	equiv **3b**	m[a]	% yield
4a	0.01	9	75
4b	0.04	30	80
4c	0.029	54	78
4d	0.0075	156	75

[a]Determined by ^{1}H NMR (500 MHz) integration (see Figure 2).

under standard conditions, the trifluoroacetate amine salt **3b** was coupled to the PNIPAM−NASI copolymer, available by radical copolymerization.[18] The remaining unreacted *N*-acryloyloxy succinimide acid esters were capped by addition of excess isopropyl amine to provide the macroligands **4a**−**d** with various ligand loadings, accomplished by varying the stoichiometry of **3b** (0.01, 0.04, 0.029, and 0.0075 equiv of **3b** relative to the final *N*-^{i}Pr monomer, respectively) added during the coupling step (Figure 2a). The ratio of ligand to backbone monomer was verified by ^{1}H NMR analysis (Figure 2b).

The data in Figure 2 provide convincing evidence that covalent attachment of dexamethasone occurred. First, the water solubility of the polymer changes significantly. Incorporation of larger amounts of the hydrophobic dexamethasone lowered the water solubility of the product copolymer as expected. If the dexamethasone had not been incorporated as shown in Scheme 1, the NASI active esters

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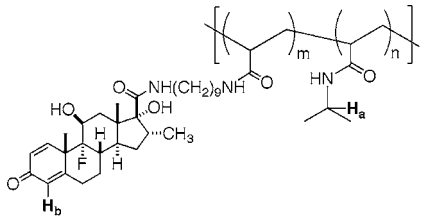
a)



macroligand	m:n[a]	H_2O solubility at 4 °C (mg/mL)
4a	1:10 (1: 9)	insoluble
4b	1: 25 (1: 30)	25
4c	1: 34 (1: 53)	27
4d	1: 133 (1: 156)	42

[a] Expected ratio based on equiv of **3b** employed. Ratio calculated based on ^{1}H NMR integration given in parentheses.

b)

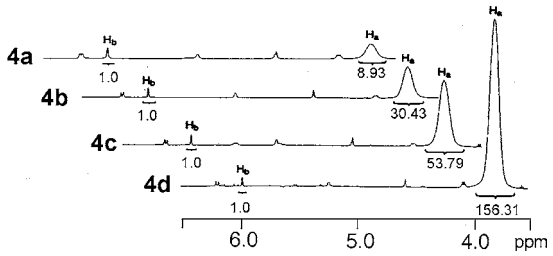


Figure 2. Water solubility data and ^{1}H NMR (500 MHz, DMSO-d_6) spectra of dexamethasone polymer macroligands **4a**−**d**. (a) Structure and water solubility of dexamethasone macroligands synthesized. (b) Expansion of ^{1}H NMR (δ 3.5−6.5) spectra showing the relative ratio of ligand to polymer based on relative integration of H_a and H_b.

would have been converted to isopropyl acrylamides and the water solubility of the polymer would have remained unchanged. These experiments also show that acceptable solubility of a macroligand can be achieved with 0.5−2.0 mol % of loaded polymer and also suggest that for hydrophobic natural products such as dexamethasone a loading of 1:30−50 may be ideal for precipitation of the macroligand around 25 °C. In addition, it should be noted that there is of course a limit for measurement of the ligand/polymer ratio by ^{1}H NMR integration at low ligand loadings (see Figures 2b, **4a**). Second, the amount of macroligand incorporated as measured by the change in the integral ratio for protons H_a and H_b in the polymer products **4a**−**d** is consistent with the equivalents of **3b** used in the synthesis of the macroligands. Finally, as was noted previously for other PNIPAM derivatives with groups tethered by modest-sized alkyl chains, there is a modest change in the ^{1}H NMR spectra for the immobilized amine. Figure 3 illustrates this, showing that the ^{1}H NMR spectrum of the macroligand **4c** has an H_b signal that is 0.43 Hz broader relative to the same proton in an admixture of dexamethasone salt **3b** and PNIPAM (Figure 3). Other procedures for analysis of the macroligand were less successful. Previously, analysis by thin-layer chromatography (TLC) could be used to demonstrate covalent attachment of dyes to PNIPAM.[19] However, in the case of macroligands **4a**−**d**, this analysis was frustrated by streaking of the macroligand on the TLC plate.

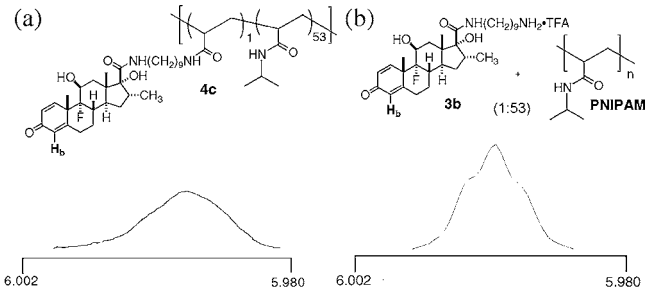
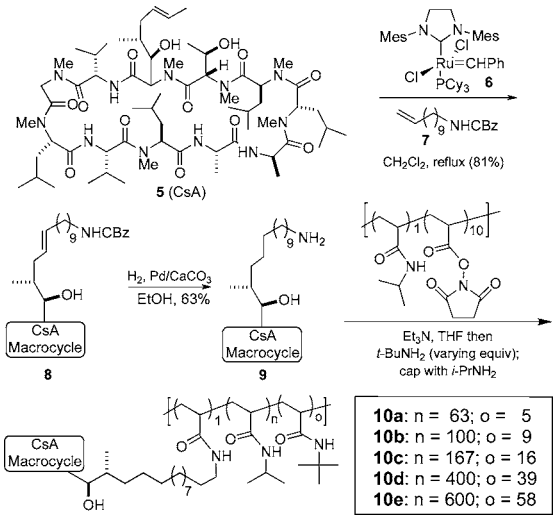


Figure 3. Comparison of the width at half-height in ^{1}H NMR (500 MHz, DMSO-d_6) of H_b in (a) macroligand **4c** ($w_{1/2}$ = 4.55 Hz) vs (b) a 1:53 mixture of dexamethasone amine **3b** and parent PNIPAM ($w_{1/2}$ = 4.12 Hz).

As a representative macroligand bearing a hydrophilic natural product, we synthesized microligands loaded with varying amounts of CsA (Scheme 2). Using the strategy

Scheme 2. Synthesis of Cyclosporin A Macroligands



reported by Diver, cross metathesis enabled coupling of the olefin in CsA to olefin **7**[20] to give modified CsA derivative **8** as a mixture of *E*/*Z* isomers.[21] Simultaneous removal of the Cbz group and reduction of the alkene under Lindlar conditions gave the CsA derivative **9** bearing an amino linker for subsequent attachment to the polymer. Varying amounts of amine **9** were reacted with the PNIPAM−NASI copolymer in the presence of triethylamine. To lower the LCST of the resulting polymer due to the hydrophilic nature of CsA, the unreacted NASI groups were capped with *tert*-butyl amine, to give various CsA macroligands **10a**−**e**. The degree of loading was again determined by ^{1}H NMR spectroscopy by comparing intensities of the N−CH_3 of cyclosporin and

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(20) This linker was synthesized in three steps from ω-undecenyl alcohol in 44% yield. See Supporting Information for details.

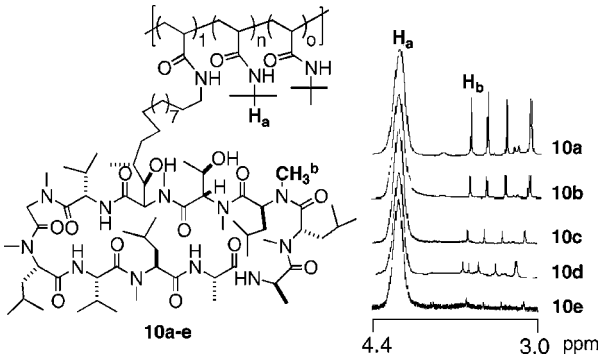

Figure 4. Expansion of ^{1}H NMR spectra (δ 3.0−4.4) showing the relative ratio of ligand to polymer based on relative integration of H_a of PNIPAM and N-$CH_3{}^b$ of CsA in macroligands **10a**−**e**.

H_a of PNIPAM in the polymer (Figure 4). In preparation for affinity chromatography experiments, the solubility of macroligand **10b** in pH 7.4 lysis buffer was determined to be ∼22 mg/mL and the LCST was ∼10 °C.

Finally, we tested the ability of the CsA macroligand to retain and capture cyclophilins from Jurkat T-cell lysates. In our initial studies, we considered the possibility that binding of proteins to the macroligand may alter the solubility properties of the macroligand−protein(s) complex. We also considered the loading required to detect binding proteins and settled initially on high loading of CsA to ensure detection. A comparison was run simultaneously with a CsA−sepharose resin that has been used previously to pull down cyclophilins.[21] CsA−PNIPAM **10b** was incubated with Jurkat T-cell lysates with or without sanglifehrin A (as a positive control to compete with cyclophilin binding) at 4 °C in pH 7.4 lysis buffer (20 mM Tris·HCl, 0.1 M KCl, and 0.2% Triton X-100) for 1 h. The CsA−PNIPAM was precipitated by incubation at 32 °C (to ensure complete precipitation) for 5 min. After recovering the polymer by centrifugation, the pellets were washed by redissolving in lysis buffer at 4 °C, and the precipitation cycle was repeated once. Bound proteins in the pellets were solubilized in denaturing buffer (pH 7.5, 20 mM Tris·HCl, 2 mM DTT, and 8 M urea), followed by heating with gel loading buffer containing sodium dodecyl sulfate in a boiling water bath for 5 min, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 5). These polymers clearly retained the 18 kDa cyclophilin and other less-abundant cyclophilins and did so to the exclusion of other nonspecific binding proteins. However, we found that the amount of protein captured is less than what might be anticipated on the basis of initial protein concentration in the cell lysates and on comparison with CsA−sepharose under similar conditions (Figure 5). This may be due to the precipitation/redissolution cycles which may also remove some of the lower-abundance cyclophilins that may bind

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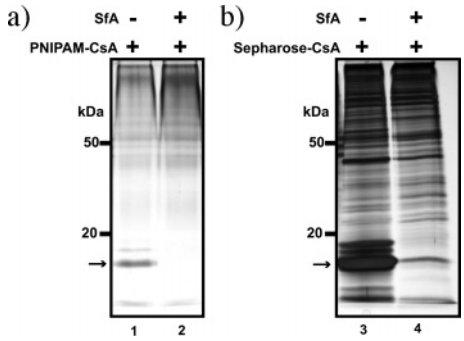

Figure 5. Pull-down experiments to isolate cyclophilins from Jurkat T-cell lysates using (a) CsA macroligand **10b** and (b) CsA-sepharose resin. Protein samples were mixed with equal volumes of SDS-PAGE sample buffer, loaded, and run on 12% SDS-PAGE gels which were silver stained. (a) Lane 1: denatured proteins released from CsA−PNIPAM showing cyclophilin bands (18−20 kDa). Lane 2: competition experiment; same as lane 1 but in the presence of 100 μM sanglifehrin A. (b) Lane 1: denatured proteins released from CsA−sepharase showing cyclophilin bands (18−20 kDa). Lane 2: competition experiment; same as lane 1 but in the presence of 100 μM sanglifehrin A.

initially but may be incompatible with the hydrophobic interior of the precipitated polymers. These results suggest avenues to explore for improving pull-down capacity.

In summary, we have synthesized thermoresponsive macroligands from dexamethasone and cyclosporin A by coupling to PNIPAM polymers. Characterization including ligand loading and integrity of the ligand was determined by solution-phase ^{1}H NMR spectroscopy, which highlights a very useful aspect of these affinity resins. Successful pull-down experiments with the CsA macroligand indicate that nonspecific binding can indeed be minimized with these soluble affinity resins; however, the amount of protein captured is less compared to that with the use of sepharose resin with an equivalent CsA loading. These proof of principle studies lay the groundwork for potential application of these polymers for isolation of unknown natural product cellular receptors and have provided general guidelines for optimal ligand loading for both hydrophobic and hydrophilic ligands.

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Supporting Information Available: General experimental procedures and characterization data for compounds **3a**,**b** and **7**−**9**. Representative ^{1}H NMR spectra for macroligands **4a**−**d** and **10a**−**e** and procedure for the affinity experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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