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Chemoselective precipitation of lactose from a lactose/sucrose mixture: proof of concept for a new separation methodology

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A new methodology for the facile isolation of biomolecules containing carbonyl groups has been developed. This methodology is based on tethering of 8-aminopyrene-1,3,6-trisulphonic acid (APTS) to a target molecule via amino or imino bonds, followed by treatment with cyclen–thiourea receptor (cyclen **1**). This receptor forms an insoluble complex with APTS conjugates, and therefore precipitates these conjugates from the solution. A sucrose/lactose mixture has been used for proof of concept and lactose was successfully isolated using this procedure. Whereas labelling of lactose via reductive amination resulted in the permanent attachment of the dye to the disaccharide, the labelling via an imine bond was reversible. Additional protocol developed for the lactose labelled with APTS dye via imine bond allowed us to recover all components in their unaltered form. Such an additional protocol not only allows isolation of target molecules without their modification, but also allows one to use recovered cyclen **1** and the dye for subsequent isolation cycles.

Keywords: chemoselective precipitation; reductive amination; supramolecular chemistry; 8-aminopyrene-1,3,6-trisulphonic acid

1. Introduction

Affinity chromatography (*1–4*), immunoprecipitation (*5–7*) and electrophoresis (*8–10*) remain the most common purification techniques for biological targets. Although the efficiency of these techniques has advanced to a level where virtually any biological substrate can be isolated in pure form, large-scale purification of such substrates is cost-prohibitive (*11*, *12*). Furthermore, nearly all techniques, except for immunoselective precipitation (*13*), require a substantial excess of gel matrices or solid phase, which may pose environmental problems in association with large-scale purifications. On the other hand, non-specific precipitation techniques such as protein salting-out (*14*, *15*) or precipitation with glycine (*16*) or trichloroacetic acid (*17*) do not require excessive amounts of reagents yet they provide rapid large-scale isolation of crude mixtures of proteins. Obviously, such precipitation methods show little selectivity as they rely on non-specific desolvation of proteins that are present in the mixture.

A precipitation methodology that does not require excessive amounts of components, yet is capable of targeting specific functional groups that are present or introduced into the substrate, may become a valuable addition to both highly selective microscale separations and non-selective large-scale separation techniques.

To our surprise, only a few examples of such chemoselective precipitation are known in the literature (*18*, *19*). Of most prominent examples, precipitation of C_{60} with calixarenes and related macrocycles, developed by Atwood and co-workers (*20*, *21*), serves as a proof of efficiency of such methodology. While being efficient, Atwood's protocol lacks versatility as it is only applicable to a specific pair of substrate and macrocycle, and is not applicable to biological substrates, which must be extracted from the aqueous medium. A precipitation methodology for the isolation of non-biological targets has been introduced by Wilcox et al. (*22–25*). In this methodology, a substrate of interest is allowed to react with a 'precipiton': an auxiliary stilbene-based molecule, solubility of which can be modulated by isomerisation from highly soluble *cis*-form to insoluble *trans*-form. After the reaction, the conjugate is converted into insoluble form, the precipitate is collected and the substrate is recovered through solvolysis. This methodology is applicable to the isolation of lipophilic compounds; however, it cannot be used for separations of highly polar biological substrates due to the reaction conditions that require organic solvents of low polarity.

While our new methodology utilises a purification approach similar to that described by Wilcox, this methodology is developed for aqueous medium and, therefore, especially suitable for the isolation of biological substrates.

Recently, we have identified a family of artificial cyclen–thiourea receptors that form complexes with the

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pyrene-based dyes under near-physiological conditions (*26*, *27*). These complexes feature micromolar stability and diminished solubility in water and methanol compared to the intrinsic solubility of the dye and receptor alone (*26*). More interestingly, the cyclen-based artificial receptors were highly selective for the pyrene scaffold, while at the same time tolerating considerable structural variations in peripheral positions on the dye. We reasoned that the conjugates of these dyes with biological substrates should form insoluble complexes with the cyclen–thiourea receptors and afford selective separation of the desired target.

2. Experimental section

2.1 General methods

The ^{1}H NMR spectra were recorded on a Varian Mercury instrument operating at 299.865 MHz. Chemical shifts are reported in ppm relative to the residual protonated solvent peak. The mass spectra were recorded on a Micromass Q-TOF™ 2 instrument (Manchester, UK) using the electrospray technique (negative mode was used). The sample was introduced into the mass spectrometer using a flow rate of 10 μl/min, the needle voltage was set at 3500 V with ion source at 110°C and the cone voltage at 35 V. Fluorometric experiments were carried out on a Fluoromax 3 (Jobin-Yvon/Horriba) spectrophotometer. All chemicals, solvents and dyes were purchased from Aldrich, Sigma (St Louis, Missouri, USA), Fluka (Buchs SG, Switzerland) and Invitrogen (Carlsbad, California, USA). Ion exchange chromatography was performed using DEAE Sephadex A-2S anion exchange resin, and 1% aqueous HCl eluent.

Experimental procedure for capillary electrophoresis is described in the Supporting Information, available online.

2.2 Synthesis and precipitation protocol

Cyclen **1** was synthesised as described in the literature (*26*).

2.2.1 Labelling of lactose with 8-aminopyrene-1,3,6-trisulphonic acid (APTS) dye via reductive amination

APTS trisodium salt (4.8 mg, 0.009 mmol) and lactose (50.6 mg, 0.140 mmol, 15-fold excess) were dissolved in 3 ml of 15% aqueous acetic acid; sodium cyanoborohydride (80 mg, 1.28 mmol, 142-fold excess) was added and the reaction mixture was refluxed at 70°C for 5 days. The reaction mixture was purified by ion exchange chromatography (DEAE Sephadex A-2S anion exchange resin, 1% HCl eluent). A sample of labelled lactose (3 mg, 0.0037 mmol) was mixed with sucrose (1.3 mg, 0.0037 mmol, 1 equiv.) and used in the next chemoselective precipitation experiment.

Labelled lactose ^{1}H NMR (D_2O, δ). 9.14 (s, 1H), 9.11 (d, 1H, $J = 8.9$ Hz), 9.02 (d, 1H, $J = 11.9$ Hz), 8.92 (d, 1H, $J = 8.9$ Hz), 8.51 (d, 1H, $J = 8.9$ Hz), 8.35 (s, 1H), 4.09–3.63 (m, 13H). MS (ESI $[M]^-$), monosodium salt: 804.27, calcd for $C_{28}H_{31}NNaO_{19}S_3$: 804.06.

Labelled lactose + sucrose ^{1}H NMR (D_2O, δ). 9.16 (s, 1H), 9.13 (d, 1H, $J = 8.9$ Hz), 9.07 (d, 1H, $J = 11.9$ Hz), 8.98 (d, 1H, $J = 8.9$ Hz), 8.51 (d, 1H, $J = 8.9$ Hz), 8.44 (s, 1H), 5.40 (d, 1H, $J = 2.9$ Hz), 4.20 (d, 1H, $J = 8.9$ Hz), 4.04 (t, 1H, $J = 8.9$ Hz), 3.90–3.15 (m, 23H).

2.2.2 Precipitation of labelled lactose

A solution of lactose–APTS conjugate (1 mM) in H_2O:MeOH (1:9) was mixed with equal volume of 2 mM solution of cyclen **1** in the same solvent system. An immediate precipitate formation was observed. The resulting solution was centrifuged for 15 min (14,000 rpm). The supernatant was removed, the precipitate was suspended in water and extracted with chloroform. The extraction caused partitioning of lactose–APTS conjugate into the aqueous layer and partitioning of cyclen **1** into the organic layer.

Supernatant ^{1}H NMR (D_2O, δ). 5.40 (d, 1H, $J = 2.9$ Hz), 4.20 (d, 1H, $J = 8.9$ Hz), 4.04 (t, 1H, $J = 8.9$ Hz), 3.90–3.15 (m, 11H).

Aqueous layer (after back extraction) ^{1}H NMR (D_2O, δ). 9.10 (s, 1H), 9.05 (d, 1H, $J = 8.9$ Hz), 8.92 (d, 1H, $J = 11.9$ Hz), 8.80 (d, 1H, $J = 8.9$ Hz), 8.54 (d, 1H, $J = 11.9$ Hz), 8.42 (s, 1H), 3.80–3.50 (m, 12H).

2.2.3 Labelling of lactose with APTS in the presence of sucrose via reductive amination

Lactose (1 mg, 0.0029 mmol), sucrose (1 mg, 0.0029 mmol,) and APTS trisodium salt (4.4 mg, 0.0087 mmol, 3.2-fold excess) were dissolved in 2 ml of 15% aqueous acetic acid; sodium cyanoborohydride (24 mg, 0.381 mmol, 141-fold excess) was added and the reaction mixture was refluxed at 70°C for 2 h. Precipitation of conjugate was carried out in the same manner as described in the previous section.

$T = 0$ h ^{1}H NMR (D_2O, δ). 9.01 (s, 1H), 8.97 (d, 1H, $J = 11.9$ Hz), 8.85 (d, 1H, $J = 8.9$ Hz), 8.75 (d, 1H, $J = 11.9$ Hz), 8.36 (d, 1H, $J = 8.9$ Hz), 8.16 (s, 1H), 5.29 (s, 1H), 5.11 (s, 1H), 4.42 (d, 1H, $J = 8.9$ Hz), 4.11 (d, 1H, $J = 8.9$ Hz), 3.94 (t, 1H, $J = 8.9$ Hz), 3.90–3.40 (m, 23H).

$T = 2$ h ^{1}H NMR (D_2O, δ). 9.05 (s, 1H), 8.99 (d, 1H, $J = 8.9$ Hz), 8.85 (d, 1H, $J = 8.9$ Hz), 8.75 (d, 1H, $J = 11.9$ Hz), 8.39 (d, 1H, $J = 8.9$ Hz), 8.12 (s, 1H), 5.35 (d, 1H, $J = 2.9$ Hz), 4.42 (d, 1H, $J = 8.9$ Hz), 4.18 (d, 1H, $J = 8.9$ Hz), 4.0 (t, 1H, $J = 8.9$ Hz), 3.90–3.40 (m, 23H).

Supernatant ^{1}H NMR (D_2O, δ). 5.40 (d, 1H, $J = 2.9$ Hz), 4.20 (d, 1H, $J = 8.9$ Hz), 4.03 (t, 1H, $J = 8.9$ Hz), 3.90–3.40 (m, 11H).

Lactose ^{1}H NMR (D_2O, δ). 5.19 (d, 1H, $J = 5.9$ Hz), 4.42 (d, 1H, $J = 5.9$ Hz), 3.96–3.48 (m, 12H).

Sucrose ^1H NMR (D_2O, δ). 5.38 (d, 1H, $J = 2.9$ Hz), 4.19 (d, 1H, $J = 8.9$ Hz), 4.02 (t, 1H, $J = 8.9$ Hz), 3.90–3.40 (m, 11H).

2.2.4 *Labelling of lactose with APTS dye via imine bond*

A mixture of lactose (2 mg, 0.0058 mmol) and APTS (2.7 mg, 0.0114 mmol) was dissolved in the minimum amount of 10% aqueous acetic acid (0.2 ml). The reaction mixture was stirred for 12 h at 70°C. Then the reaction mixture was cooled to room temperature, the solvent was removed under reduced pressure and ^{1}H NMR for the solid residue was recorded in D_2O. The ^{1}H NMR spectrum showed some conjugated as well as some free APTS present in the reaction mixture.

2.2.5 *Precipitation of lactose–APTS imine conjugate*

The reaction mixture described in the previous section was dissolved in 0.2 ml of 80% aqueous methanol. A saturated solution of cyclen **1** in 1 ml of the same solvent was prepared. Approximately, 10 mg of cyclen **1** was dissolved. This saturated solution of cyclen **1** was added to the solution of lactose–APTS imine conjugate in small aliquots. Instantaneous precipitation was observed. The solution of cyclen **1** was added until green fluorescence of the reaction mixture disappeared. This precipitate was collected by centrifugation.

2.2.6 *Hydrolysis of lactose–APTS imine conjugate*

The collected solid was suspended in 10 ml of 20% aqueous acetic acid and stirred at 70°C for 12 h. A gradual dissolvation of precipitate was observed during the reaction. Then, the solution was evaporated to dryness under reduced pressure and the residual solid was suspended in 10 ml of water. The pH of the solution was raised to 9 by the addition of 1 N aqueous ammonia. Then, the solution was extracted with chloroform (5 × 25 ml) to remove cyclen **1**. The ^{1}H NMR spectrum of the aqueous layer showed only free APTS dye and free lactose peaks, indicating that hydrolysis was successful.

2.2.7 *Recovery of unlabelled lactose and APTS dye*

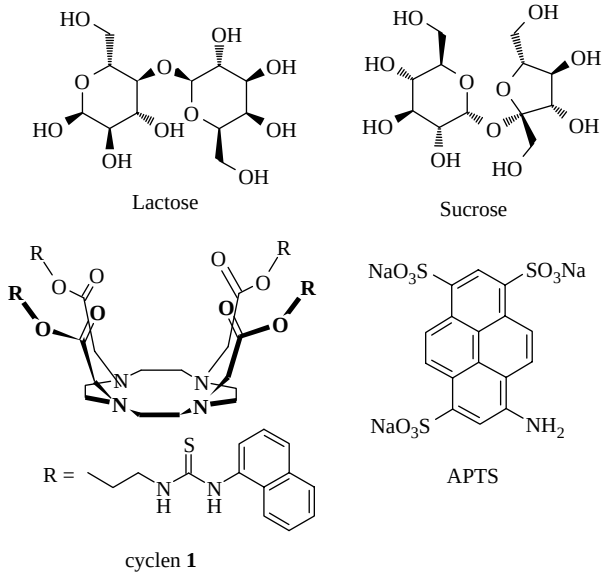
The aqueous layer from the previous experiment was evaporated and the residue was dissolved in 0.2 ml of 80% aqueous methanol. APTS dye was precipitated by the application of saturated solution of cyclen **1** in the same solvent. The precipitate was removed by centrifugation, and the supernatant was evaporated to dryness under reduced pressure. ^{1}H NMR analysis of the solid residue revealed only peaks consistent with label-free lactose. The remaining precipitate was dissolved in 10 ml of water, and back-extracted with chloroform (5 × 25 ml). This extraction caused partitioning of APTS-free cyclen **1** into the organic layer. Both organic and aqueous layers were evaporated to dryness and analysed by ^{1}H NMR. A solid residue from the aqueous layer revealed only peaks consistent with APTS, and organic layer revealed only peaks consistent with cyclen **1**. Overall, 0.4 mg of lactose, 0.9 mg of APTS and 7.2 mg of cyclen **1** were recovered.

3. Results and discussion

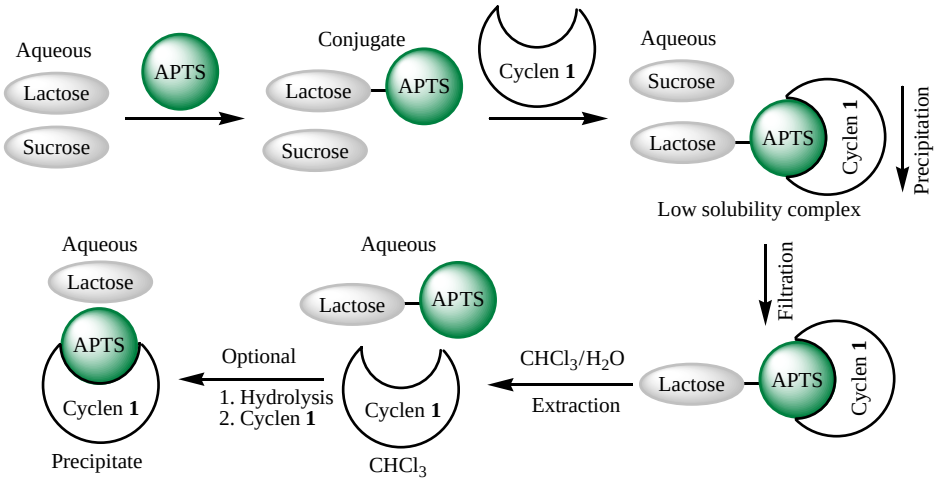
In this paper, we report the proof of concept for the chemoselective precipitation of biological substrates using a model system of two disaccharides: lactose and sucrose. While the physical properties of these sugars are very similar (*28*), which makes their separation by common techniques difficult (*29*, *30*), the presence of a reducing end in lactose allows a test of our principle. APTS is the dye that forms a stable complex ($K_a = 8 \times 10^5\ M^{-1}$) with the naphthyl thiourea cyclen receptor (cyclen **1**, Scheme 1) (*26*). The presence of an amino group in this dye implies that it can be readily tethered to the reducing end of lactose via reductive amination reaction.

The combined schematic representation of chemoselective precipitation protocols is shown in Scheme 2.

First, we demonstrated that the lactose–APTS conjugate can be precipitated out from the solution by interacting with cyclen **1** in the presence of sucrose. As detailed in the experimental section, the reductive amination reaction between lactose and APTS was performed according to the literature procedure (*31*, *32*), and the conjugate was purified on an anion-exchange



Scheme 1. Structures of lactose, sucrose, APTS dye and receptor cyclen **1**.



Scheme 2. General representation of protocols for chemoselective precipitation and isolation of lactose and sucrose from a lactose/sucrose mixture.

column. Then, we developed a procedure that allows precipitation of this conjugate through interaction with cyclen **1**. The most efficient results were achieved when a 1 mM solution of lactose–APTS conjugate in H_2O:MeOH (1:9) was mixed with an equal volume of 2 mM cyclen **1** solution in the same solvent system. At these concentrations, both lactose–APTS conjugate and cyclen **1** were completely soluble. However, the interaction between these two components resulted in the formation of a precipitate and almost complete discolouration of the originally bright greenish solution (Figure 1(a)). The fluorometric analysis suggested that at least 89% of the lactose–APTS conjugate was precipitated from solution (Figure 1(b)).

To test the selectivity of precipitation, a sample of APTS-labelled lactose was mixed with sucrose (1 equiv.), dissolved in H_2O:MeOH (1:9), followed by the addition of a solution of cyclen **1** (2 equiv.). A precipitate immediately formed, which was removed by centrifugation, suspended in water and back-extracted with chloroform. This back-extraction led to repartitioning of cyclen **1** into the organic layer, thereby releasing APTS-labelled lactose in the aqueous layer. Comparison of the ^{1}H NMR spectra (Figure 2) of the mixture of sucrose and labelled lactose (spectrum 1), the supernatant following precipitation (spectrum 2) and the back-extracted aqueous solution (spectrum 3) shows the chemoselective precipitation phenomenon. It is important to note that the precipitation and release are highly selective and almost quantitative as evidenced by the absence of APTS signals in spectrum 2 and cyclen **1** signals in spectrum 3. The aromatic signals observed in the supernatant correspond to a partly dissolved excess of cyclen **1**. The aromatic region shows signals of APTS label with no traces of cyclen **1**. Also note the absence of acetal signal for sucrose at 5.4 ppm in this spectrum.

To demonstrate that lactose can be selectively removed from the sucrose–lactose mixture via the two-step labelling – precipitation protocol, we then chose to carry out labelling with disaccharide mixture, rather than mix pre-labelled lactose with sucrose. Unfortunately, labelling

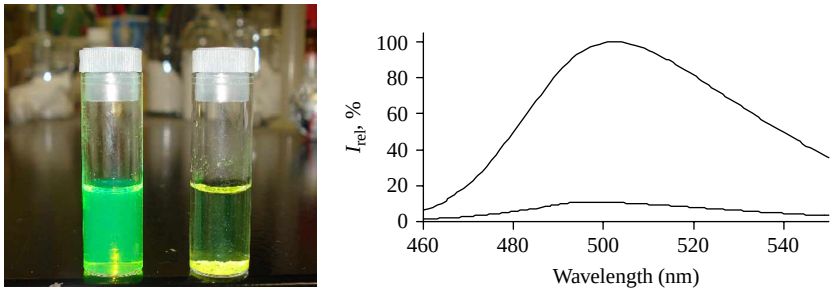


Figure 1. (a) Image of 1 mM solution of APTS–lactose conjugate in the absence (left vial) and in the presence (right vial) of 2 mM cyclen **1**. Note a precipitate formed in the right vial upon application of cyclen **1**. (b) Emission spectra of lactose–APTS conjugate (ex = 424 nm) before and after the treatment with cyclen **1**. Top spectrum was recorded for 1 μl of 1 mM solution of lactose–APTS conjugate in H_2O:MeOH (1:9) injected into 20 ml of water. Bottom spectrum was recorded for 2 μl of the supernatant formed upon mixing of lactose–APTS conjugate and cyclen **1** solutions, diluted into 20 ml of water.

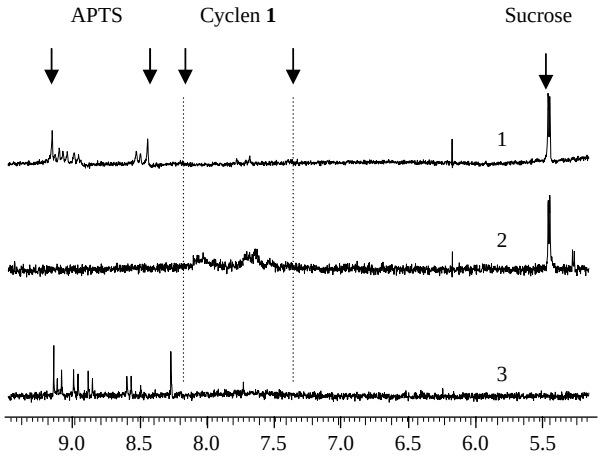


Figure 2. ^{1}H NMR spectra (D_2O) of (1) labelled lactose and sucrose mixture; (2) supernatant formed upon the treatment of the mixture with cyclen **1** and (3) aqueous portion of precipitate after back extraction with $CHCl_3$.

of lactose with equimolar amounts of APTS dye was too slow, and sucrose underwent acid-catalysed hydrolysis during this time. When a five-fold excess of APTS was used in the reductive amination, a rapid (within 2 h) labelling of lactose took place without any detectable sucrose degradation. Even though the resulting mixture was more complex than a mixture of labelled lactose and sucrose, the additional component in this mixture was APTS dye, which was also a target substrate for cyclen **1**. Application of two-fold excess of cyclen **1** (with respect to the total amount of APTS) resulted in a complete removal of APTS and APTS–lactose conjugate, leaving sucrose as the only soluble component in this mixture (Figure 3). The capillary

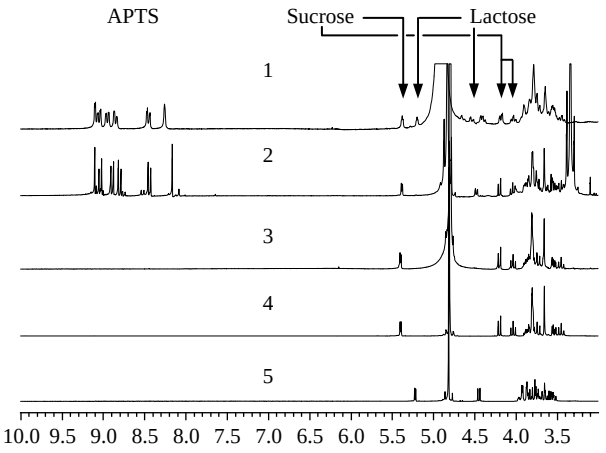


Figure 3. ^{1}H NMR spectra (D_2O) of (1) APTS, lactose, sucrose and $NaBH_3CN$ mixture prior to the reductive amination reaction; (2) same mixture after 2 h of the reduction; (3) same mixture after treatment with cyclen **1**. Please note the disappearance of all peaks except those produced by sucrose; (4) sucrose and (5) lactose.

electrophoresis analysis of the supernatant also confirmed that the only component present in the supernatant was unaltered sucrose (see Supporting Information, available online). Almost 70% of sucrose was recovered.

The protocol for chemoselective precipitation that relies on the reductive amination reaction leads to the irreversible labelling of the target molecule. Formation of an imine bond from the same carbonyl group and the amino group of the dye would introduce the label that could be hydrolysed back to the original substrate after chemoselective precipitation. This Schiff-base chemistry has been recently used for the reversible attachment of proteins to the solid matrix (*33*). In order to test such a possibility, we have developed an additional protocol to label lactose with APTS via an imine tether. Due to the reversibility of imine bond formation, the labelling of carbonyl compounds is an equilibrium process, and an excess of the amino compound is required in order to label most of the substrate. Even though such an approach results in a mixture comprising at least thee components (free excess label, a conjugate and label-free substrate), our new protocol solves this problem.

First, a mixture of lactose and APTS dye was dissolved in a small volume of 1 M solution of acetic acid and stirred for 12 h, producing lactose–APTS imine conjugate and excess of free dye, as evidenced by ^{1}H NMR. Then, an excess of cyclen **1** was applied to this solution, causing precipitation of labelled lactose and lactose-free dye. This precipitate was collected and subjected to hydrolysis with a large volume (3 ml) of 2 M aqueous acetic acid. Under such conditions, the precipitate was immediately dissolved. After 2 h of stirring, the solvent was removed under vacuum, and 1 ml of 1 N aqueous ammonia was added to the solid residue and back-extracted with chloroform. The ^{1}H NMR analysis of organic and aqueous layers showed that only cyclen **1** was present in chloroform, and a mixture of lactose and APTS was found in the aqueous layer. Finally, lactose was separated from the APTS dye by the second application of cyclen **1**, which resulted in precipitation of cyclen **1** and APTS dye, and left lactose as the only component in aqueous layer. Resuspension of the precipitate in water and back extraction with chloroform separated cyclen **1** from APTS (Figure 4).

With this new protocol, not only could lactose be isolated in an unaltered form, but the dye and cyclen **1** were recovered as well.

An attempt of separation of lactose from sucrose in an unmodified form reached only partial success. While the isolated precipitate gave pure lactose after hydrolysis and back extraction, the supernatant showed sucrose contaminated with some residual lactose. Apparently, precipitation of lactose was not complete because of the reversible nature of the imine bond, which set the equilibrium between labelled and label-free disaccharide. Although the methodology of separation that relies on the temporary

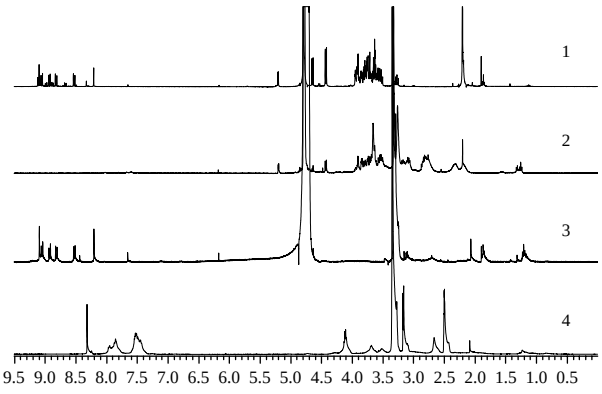


Figure 4. ^{1}H NMR spectra of (1) reaction mixture comprising lactose and APTS dye in D_2O; (2) recovered lactose in D_2O; (3) recovered APTS dye in D_2O and (4) recovered cyclen **1** in DMSO-d_6.

labelling via imine bond did not allow us to separate both sugars in the pure form, it can still be used for isolation of reducing sugars. In case if non-reducing sugars are the target substrates, they can be separated from the reducing components via permanent labelling of the latter via amine bond.

Since many sugars possess reducing ends, a similar methodology can be applied to their separation. One example of practical value is the separation of low molecular weight heparin (*34*). Heparin oligomers contain reducing ends (*35*) and, therefore, can be labelled with APTS dye and complexed with cyclen **1**. The overall solubility of such complexes should depend on the length of the heparin oligomers, where the complexes of the shortest oligomers will be the least soluble and the complexes of the longest oligomers will be the most soluble. Our preliminary studies indicated that labelling of a heparin digest with APTS followed by the treatment with cyclen **1** resulted in a partial precipitation of heparin conjugates, the extent of which was dependent on the solvent composition. Chemoselective precipitation of heparin oligomers will be the focus of our future studies.

4. Conclusions

We developed a facile method for separation of biomolecules via tethering them to the APTS dye and subsequently applying to these conjugates a synthetic receptor cylen **1**, which binds to the APTS label. Due to the low solubility of the APTS–cyclen **1** complex, the APTS conjugate precipitates out from the solution upon interaction with cyclen **1**, thus leaving unlabelled molecules as the only soluble components in the mixture. Whereas tethering of carbonyl-containing compounds to APTS via the reductive amination reaction makes them permanently labelled with APTS, the attachment of APTS through an imine bond allows for the release of the precipitated molecule in the label-free form. Subsequent extraction of supernatant with organic solvent provides a means for the recovery of all components used for the chemoselective precipitation. This feature makes our new approach especially attractive for the large-scale separations of biological molecules. Separation of low molecular weight heparin oligomers is the focus of our future studies.

Supporting Information

Experimental details for capillary electrophoresis experiments and electropherograms, ^{1}H NMR spectra of cyclen **1** and APTS.

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