Pipette-tip selective extraction of glycoproteins with lectin modified gold nano-particle on a polymer monolith phase.

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The selective extraction of specific proteins (non-glycosylated, glycosylated or different glycoforms) from complex sample matrices utilising selective solid phase extration (SPE) is of significant interest within the fields of proteomics and glycoproteomics. Polymer monoliths have proven to be an excellent solid support for SPE applications in bio-analysis due to their excellent mass transfer characteristics for large biomolecules. Although biorecognition molecules such as lectins can be covalently immobilised directly onto the monolith surface using a variety of chemistries, the relatively low surface area of these monoliths results in a correspondingly low sample capacity. Recently, we have described the covalent attachment of 20 nm gold nanoparticles (AuNPs) upon a polymer monolith with excellent surface coverage leading to a significant increase in surface area. In this work therefore we describe the *in-situ* preparation of an ethylene dimethacrylate porous polymer monolith within the confines of 20 µL polypropylene pipette tips, onto which methacrylate anchor sites were previously grafted to ensure intimate monolith/wall contact. Then AuNPs were immobilised onto the monolith pore surface utilising azlactone cemistry. Field emission scanning electron microscopy was used to verify the high surface coverage of AuNPs. *Erythrina cristagalli* lectin (ECL) was immobilised upon the attached AuNPs via a biofunctional linker. The ECL-modified tip was successfully applied for the enrichment of galactosylated protein (desialated transferrin) versus a non-galactosylated protein (ribonuclease B) due to the specificity of ECL. Reversed-phase capillary HPLC was used to validate the efficiency and selectivity of the developed micro-extraction phase which resulted in an increase in extraction recovery of ~95 % in the presence of AuNPs. The specificity of the ECL-modified tip was further studied by using more complex mixture of proteins which included non-glycosylated proteins and glycosylated proteins with different terminal sugar structure. Finally, the lectin affinity extraction device was tested with a real sample (*E. coli* cell lysate) spiked with target galactosylated glycoproteins.

Introduction.

Most analytical methods require some form of sample preparation and quite often a significant amount of time is invested in the development of appropriate sample preparation strategies. Liquid-liquid extraction, ultracentrifugation, and solid phase extraction (SPE) are the most common techniques for sample preparation.[1] Among them, SPE is the simplest, most effective and most versatile technique. Generally, it is applied to the selective capture and preconcentration of the target analyte and/or the removal of potentially interfering components from the sample matrix.[1-4] SPE has been applied as a sample preparation strategy both for small molecules (pharmaceutical, pesticides etc) as well as larger biomolecules (peptides, proteins etc). SPE devices usually comprise a selective adsorbant material encased within a housing such that the sample and subsequent elution buffers can be passed through the bed for collection and analysis. Traditionally, the selective adsorbant is held within a flow-through cartridge device and the sample is manually loaded onto the top of the packed bed and eluted with the assistance of an applied vacuum. However when working with small volumes (<50 µL), SPE has increasingly been performed in different formats such as capillaries, the channels of a microfluidic device, or in commercial pipette tips. The advantages of SPE in pipette-tip formats include ease of use, small sample and solvent volumes as well as the possibility to process many samples at the same time using either multi-channel hand-held pipettes or robotic liquid handling systems.[5-7]

Traditional SPE sorption materials are beads packed in a cartridge or column. In addition, packed SPE-in-MPT (solid phase extraction in a micropipette tip) has been applied for enrichment, purification, desalting and fractionation of different biological samples using either home-made or commercially available tips.[8-14] For example, Kussmann *et al.* used a home-made tip that was prepared by filling a bottom-end squeezed GelLoader tip (Eppendorf, Hamburg, Germany) with a suspension of Poros materials (PerSeptive Biosystems, Framingham, MA, USA) to form a purification column[10] whereas Šalplachta *et al.* used the commercially available ZipTip C_{18} tips from Millipore (Billerica, MA, USA) for desalting tryptic digests of fractionated proteins.[12] However, there are some difficulties and drawbacks associated with the preparation of this kind of tips.[8,15-17], most notably the requirement for inclusion of retaining frits. As an alternative, many of these drawbacks can be overcome by the use of monolithic sorption beds. Since the early to mid-1990's great attention has been paid to monolithic sorption beds due to the various features that they have which include (i) the ease of fabrication, in particular, for microscale utilisation as there is no need for frits, (ii) operation at high linear velocity due to high permeability and good mass-transfer, (iii) different surface functionality and selectivity can be achieved and, (iv) chemical stability at a wide pH range.[18,19] Monolithic beds are generally classified into two categories: silica and organic polymer monoliths. Silica monoliths are manufactured by sol-gel technology with phase separation and have been used for fabricating SPE-in-MPT.[16,20-23] Miyazaki *et al.* prepared a monolithic silica bed fixed in a 200 µL pipette tip in which the silica surface was modified with a C_{18} phase or coated with titania phase and applied that for sample concentration, desalting and purification as well as selective extraction of phosphorylated peptides.[16] Hasegawa *et al.* [REFERENCE number here] and Kumazawa *et al.* [REFERENCE number here] demonstrated the effective use of the commercially available monolithic silica tips (MonoTip C_{18}) for the extraction of antihistamines from human plasma[20,21] as well as methamphetamine and amphetamine from human urine[22] and human whole blood.[23] On the other hand,

organic polymer monoliths are produced by one-step polymerisation of a mixture consisting of organic monomers, crosslinkers, porogenic solvents and initiators. The mixture is sonicated, purged under nitrogen, filled in a micropipette tip to a certain volume and irradiated with UV. Several studies have described the preparation and application of polymer monoliths in SPE-in-MPT for the analysis of the biological samples.[15,24-29] For example, Rainer and co-workers fabricated a divinylbenzene-based extraction tip for selective enrichment of phosphorylated peptides and successfully applied that to the study of *in vitro* phosphorylation.[24] Abdel-Rehim *et al.* introduced a set-up of polypropylene methacrylate-based monolithic tips for use with 96-well plates. It was applied for clean-up the beta-blocker pindolol and metoprolol in human plasma samples.[25]

Despite of all the advantages of the monoliths mentioned above, the low surface area of these materials relative to silica monolith somewhat restricts the applicability of these materials. Strategies to increase the surface area of polymer monoliths have mainly focussed on the incorporation of selected nanoparticleseither during [24, 29,30..also Krenkova, Anal Chem, 2010, 82 pp 8335] or after polymerisation [31]. Depending on the nature of the nanoparticle the selectivity of the final modified monolith can also be tuned for a specific application. For example, Krenkova *et al.* [Anal Chem, 2010, 82 pp 8335] recently incorporated hydroxyapatite nanoparticles into the polymerisation mixture during production of a polymer monolith which was subsequently used for separation of proteins and enrichment of phosphopeptides (by virtue of the hydroxyapatite nanoparticles exposed at the monolith surface).

Recently, we produced a high-density Au-immobilised monolith in which the AuNPs were immobilised on the surface of the preformed monolith utilising azlactone chemistry surface modification.[32] Using a different strategy Xu *et al.* prepared a GMA-*co*-EDMA monolithic capillary column modified with AuNPs for selective capturing of cysteine-containing peptides. The epoxy groups were first reacted with cysteamine to expose thiol moieties on the surface and then chloroauric acid was *in situ* reduced to form AuNPs attached to the thiol groups.[33] This work was quickly followed by Cao *et al.* [Anal Chem 2010, 82 pp 7416] who used the same gold modified monolith as an "exchangable" surface which could be readily functionalised with either reversed phase or ion-exchange functionalities for the separation of selected peptides and proteins.

Monolithic materials have shown excellent utility as support for affinity chromatography for separation of bimolecules due to the mass-transfer properties that they exhibited. Different bioligands have been immobilised on monolithic surface such as protein A for biospecific separation of immunoglobulins,[34] mannan for separation of mannose-binding proteins,[35] concanavalin A (Con A) and wheat germ agglutinin (WGA) for isolation and preconcentration of glycoconjugates,[36] carbohydrate for affinity of lectins[37] and trypsin immobilised on a monolithic pipette tip for rapid proteins digestion.[38]

In the work reported here, we describe the successful modification of a polymer monolith in pipette-tip format with gold nanoparticles and, via the use of a commercial bifunctional coupling agent DTSP, the covalent attachment of a selected lectin for affinity extraction of glycoproteins from samples of varying complexity.

Experimental

Reagents and materials

Benzophenone, 2,2-dimethoxy-2-phenylacetophenone (DPA), ethylene glycol dimethacrylate (EDMA), lauryl methacrylate (LMA), 1-decanol, ethylenediamine, gold(III) chloride trihydrate, 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DTSP), transferrin (human), ribonuclease B, insulin (bovine pancreas), insulin chain B (oxidized from bovine pancreas), enolase (S. *cerevisiae*), thyroglobulin from porcine thyroid gland, carbonic anhydrase (bovine), cytochrome c (equine), manganese (II) chloride tetrahydrate, calcium chloride hexahydrate, D-(+)-galactose (Gal), 1,4-butanediol, 1-propanol, Trizma hydrochloride, sodium citrate, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, ≥99.0%), dimethylsulphoxide (DMSO) and trifluoroacetic acid (TFA, 99%) were purchased from Sigma-Aldrich (Dublin, Ireland). Trizma base (≥99.0%), sodium chloride and bovine serum albumin were from Fluka (Buchs, Switzerland). 4,4-dimethyl-2-vinyl-2-oxazolin-5-one (vinyl azlactone) was purchased from TCI Europe (Zwijndrecht, Belgium). Unconjugated Erythrina cristagalli lectin (ECL) was provided by Vector Laboratories (Peterborough, UK). Clostridium perfringens neuraminidase was purchased from New England BioLabs (Hitchin, UK). PNGase was purchased from New England BioLabs. The 20-µL polypropylene (PP) tips used for in-situ fabrication of monolith were from Brand (Wertheim, Germany). Methanol, ethanol, acetone and acetonitrile were of HPLC grade and purchased from Fisher Scientific (Dublin, Ireland). All chemicals were used as received without further purification. Teflon-coated fused silica capillary (100 µm i.d.) was supplied by Composite Metal Services (Shipley, England).

Instrumentation

Photopolymerisation and photografting were carried out using a Spectrolinker XL-1000 UV Crosslinker at 254 nm (Spectronics Corp., Westbury, NY, USA). The balance used was a Sartorius Extend (Sartorius, Goettingen, Germany). The sonication bath used was from Branson Ultrasonics Corporation (Danbury, CT, USA). A KD Scientific syringe pump (KDS-100-CE, KD Scientific Inc, Holliston, MA, USA) was used for all washing and functionalisation of monoliths in pipette-tip formats as well as trap/release of protein mixtures. Scanning electron microscopy (SEM) was performed on a 1 mm long cross-section of the unmodified monolith using a Hitachi S-3400N instrument (Hitachi, Maidenhead, UK) after sputtering the sample with gold using a SputterCoater S150B (BOC Edwards, Sussex, UK). To visualise the coverage of AuNPs on cross-sections of gold-modified monoliths previously removed from their housings (as described later) and mounted on carbon grids, a Hitachi S-5500 Field emission SEM (Hitachi, Maidenhead, UK) was used. Chromatography was performed using a Dionex Ultimate 3000 Capillary LC system (Dionex, Sunnyvale, CA, USA) at a flow rate of 2 $\mu L.min^{-1}$. The injection volume was 1 µL, with detection by UV at 214 nm using a 3 nL flow-cell. Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in 90% acetonitrile. For the separation of ribonuclease B and desialated

transferrin a 10 minute gradient of 5 % B to 100 % B was applied at 25 °C. All other separations involved a 9 minute gradient (5 % B to 100 % B) at 40 °C. The monolithic column used was prepared by filling a 100 µm x 15 cm vinylised[39] fused silica capillary with a deoxygenated mixture of 24 wt% LMA, 16 wt% EDMA, 14.5 wt% 1,4 butandiol, 45.5 wt% propanol and 1 wt% DPA (w.r.t monomers) followed by irradiation with 2 $J.cm^{-2}$ UV energy at 254 nm. The resulting monolith was washed with MeOH for 2 hours at the flow rate of 2 $\mu L.min^{-1}$ to remove the porogen and unreacted monomers.

Modification of the polypropylene tip surface.

Prior to *in-situ* fabrication of the monolith in the tip, the inner surface of the polypropylene tip was modified with grafted chains of EDMA. Firstly, the tip was washed with 10 µL EtOH (10 times) and 10 µL acetone (10 times) and dried using nitrogen to remove any impurities on the surface of the polypropylene. The tip was filled with 10 µL of deoxygenated 5 % benzophenone in methanol and irradiated with 1 $J.cm^{-2}$ UV energy at 254 nm followed by a thorough methanol rinse. The tip was then filled with deoxygenated 15 % EDMA in MeOH and irradiated using the same conditions Finally, the tip was washed thoroughly with MeOH and dried with nitrogen before use.

Preparation of the polymer monolith within the modified pipette-tip housing.

A polymerisation mixture consisting of EDMA (40 wt%), 1-decanol (60 wt%) and DPA (1 wt%, w.r.t monomer) was prepared, sonicated for 30 minutes and deoxygenated with a nitrogen flow (10 minutes). In order to ensure a constant and repeatable bed volume for each monolith, a guide ring of polypropylene (2 mm i.d.) was slipped over the outside of the modified tip, which was subsequently filled by capillary action until the meniscus reached the bottom of the guide ring. The filled tip was placed upright in a coned centrifuge tube (polypropylene) and irradiated with 3 $J.cm^{-2}$ UV energy at 254 nm, resulting in a monolith which was finally washed with MeOH at flow rate of 100 $\mu L.h^{-1}$ to remove the porogen and any unreacted monomers. In some instances, an attempt was made to prepare a main channel through the centre of the monolith to facilitate lower operating backpressures. This was achieved by inserting a section of either 150 µm o.d. or 360 µm o.d. fused silica capillary down the bore of the monomer-filled tip prior to polymerisation followed by the removal of this template afterwards as described by Hsu *et al.* [15]

Immobilisation of AuNPs on the monolithic surface.

Firstly, AuNPs (20 nm) were prepared using the citrate reduction method as described elsewhere by Frens.[40] Based on the measured absorbance at 520 nm and an extinction coefficient of 1 X 10^9 $M^{-1}.cm^{-1}$, the concentration of colloidal gold was 1.17 x 10^{-9} M (or 7.05 x 10^{11} $particles.mL^{-1}$).

The attachment of gold nanoparticles to the monolith surface first involved the amination of the monolith surface as described by Connolly *et al*.[32] The monolith was first conditioned with 50 µL MeOH followed by flushing the monolith with a deoxygenated solution of 5 % benzophenone in MeOH for 30 minutes. The monolith was then irradiated with 3 $J.cm^{-2}$ UV energy at 254 nm followed by washing with MeOH for 30 minutes. The monolith was then flushed with a deoxygenated solution of 15 % vinyl azlactone in MeOH for 30 minutes and subjected to the same irradiation cycle. After a preliminary wash with MeOH, the monolith was washed with water for 30 minutes prior to flushing with 1 *M* ethylenediamine for a nominal time of 4 hours. The monolith was again washed with water (until monolith rinsings were pH neutral) to remove free ethylenediamine. Finally, the aminated monolith was flushed with AuNPs (approx. 2 mL). Immobilisation of AuNPs was considered to be complete after the entire monolith bed had turned a deep red colour. The gold-modified monolith was washed with 1 mL water to remove unbound gold nanoparticles. The axial homogeneity of AuNPs coverage along the monolith bed was readily evaluated using an optical microscope. The coverage of AuNPs on the monolith was examined using FE-SEM as previously described.

Covalent attachment of ECL to immobilised AuNPs.

The AuNP-modified tip was conditioned with 50 µL of DMSO and then flushed with 25 m*M* DTSP in DMSO for 4 hours. The monolith was then washed with DMSO for 30 minutes to remove any unreacted coupling agent followed by deionised water for 30 minutes. Subsequently, the monolith was conditioned for 30 minutes with 10 m*M* HEPES buffer pH 8.2 containing 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+} followed by flushing a 1 $mg.mL^{-1}$ solution of ECL prepared in the same buffer for 4 hours at room temperature. Finally, in order to block any unreacted succinimidyl groups, the resulting ECL-modified monolith was flushed with a primary amine (1 *M* Tris buffer pH 7.4) followed by a 100 µL deionised water wash. When not in use, the ECL-modified monolith was kept immersed in 10 m*M* Tris buffer pH 7.4 containing 150 mM NaCl, 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+} at 4 °C. For the sake of clarity, this monolith shall be referred to hereafter as **Monolith A**. In order to investigate the effect of AuNP modification of the monolith, a comparison monolith (**Monolith B**) was also prepared which did not incorporate immobilised AuNPs. Instead, the monolith was grafted with vinyl azlactone as described, and then ECL was immobilised onto the resulting pendant azlactone moieties using the same immobilisation buffer followed by blocking with 1 *M* Tris buffer.

Additionally, in order to investigate the origin of unwanted non-specific interactions between test proteins and the gold-modified substrate, a further four monoliths were prepared (referred to as "blank" monoliths in that they did not include immobilised ECL). **Blank Monolith 1** did not include immobilised AuNPs but was grafted with polymer chains of vinyl azlactone and blocked with 1 *M* Tris buffer. **Blank Monolith 2** was modified with AuNPs which were not blocked with 1 M Tris buffer. **Blank Monolith 3** was modified with AuNPs which were subsequently blocked with 1 *M* Tris buffer. **Blank Monolith 4** was modified with AuNPs, functionalised with DTSP and the resulting succinimidyl groups blocked with 1 *M* Tris buffer. Figure 1 illustrates a schematic diagram of the different monoliths prepared.

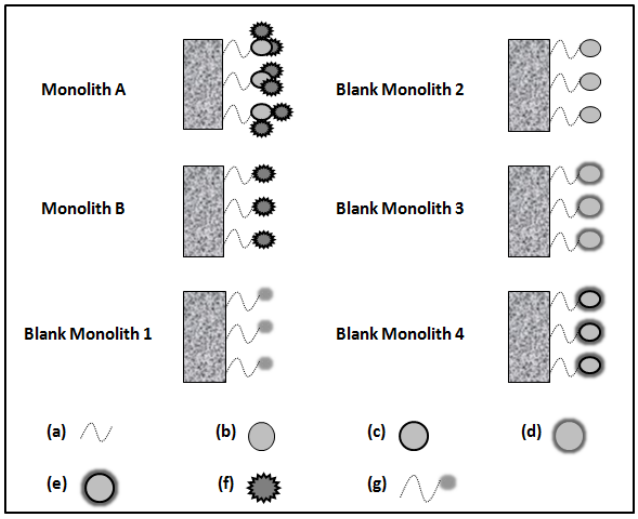

Figure 1: Schematic diagram of all affinity monoliths and blank monoliths prepared in this study. Key: (a): aminated azlactone polymer grafts, (b): bare AuNP, (c): AuNP modified with DTSP, (d): AuNP blocked with Tris, (e) AuNP modified with DTSP followed by Tris blocking, (f): ECL protein, (g): azlactone polymer grafts blocked with Tris.

Desialation of transferrin and thyroglobulin.

Desialated transferrin and thyroglobulin was prepared by treating them with *Clostridium perfringens* neuraminidase and following the supplier instructions. Briefly, 100 µg of the protein was prepared in 50 mM sodium citrate pH 6.0, mixed with 50 U neuraminidase and left to react at 37°C for overnight.

Deglycosylation of transferrin.

Transferrin was deglycosylated using *Flavobacterium meningosepticum* PNGase F following the supplier instructions. Briefly, 100 µg of the protein was prepared in 50 mM sodium phosphate pH 7.5 mixed with 500 U PNGase F and left to react at 37°C for overnight.

Bind and elute studies

A number of selected proteins were used to study the binding affinity of all fabricated monoliths and also to evaluate the blocking strategies used to eliminate non-specific interactions (ie: binding which was not due to strictly lectin-glycoprotein interactions). All lectin affinity monoliths and blank monoliths were tested using the syringe pump at a flow rate of 50 µL.hour^{-1}. The tip was first conditioned with 20 µL of loading buffer (10 m*M* Tris pH 7.4 containing 150 m*M* NaCl, 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+}). A test mix of selected proteins (total volume: 20 µL) was prepared in loading buffer and loaded from the top-end, pumped through the tip and collected in a clean 1.5 mL centrifuge tube followed by a 20 µL buffer wash which was collected in the same tube and combined with the extracted mixture. Preliminary binding studies and all evaluations of "blank monoliths" (Blank Monoliths 1 to 4) involved the use of a simple mix of two glycoproteins: desialated transferrin and ribonuclease B (20 µg.mL^{-1} each). Further more rigourous testing of the affinity monoliths involved the use of a more complex protein mixture containing up to nine proteins as described later. An E. coli cell lysate spiked with transferrin was also used to test the affinity monolith. In all cases, to elute any retained glycoprotein, 40 µL of 0.8 M galactose prepared in buffer solution was flushed through the tip and collected for analysis by LC.

Results and discussion

Fabrication of porous polymer monoliths in pipette tip format.

The fabrication of porous polymer monoliths in pipette-tip formats has been reported before [include REFerences] however in the work described herein, we report for the first time the in-situ covalent attachment of gold nanoparticles to the polypropylene-encased monolith resulting in two distinct benefits. Firstly, the surface area can be significantly increased resulting in increased loading capacity when a selective lectin is immobilised onto the gold surface. Secondly, the use of a gold surface and a commercially available bi-functional linker (DTSP) means that the extraction device is readily suited for the immobilisation of *any* bio-recognition molecule via the reaction between pendant succinimidyl groups and native lysine residues (from Protein A for trapping immunoglobulins to enzymes such as trypsin or PNGase for off-line enzymatic digestion). In our study however, we elected to immobilise a galactose-selective lectin (ECL) on the gold surface as a simple test-case, in order to examine the trap-and-release of various selected glycoproteins. Why was ECL chosen over other lectins? Include references to back up answer).The performance of the resulting ECL affinity monolith was also compared directly with that of an ECL affinity monolith which *did not* incorporate AuNP, but rather where the lectin was instead immobilised directly onto the lower surface area monolithic substrate itself via grafted chains of amine-reactive poly(vinyl azlactone).

The initial task was of course to form a porous polymer monolith *in-situ* within the confines of a selected plastic pipette tip and in this regard the strategies employed were largely similar to those described previously.[15,24-29] Pipette tips manufactured from polypropylene were selected since polypropylene is resistant to all solvents employed in this study and was found to be sufficiently UV transparent to facilitate all UV-initiated photografting and polymerisation events. Also, as described by Stachowiak *et al.* [Electrophoresis 2003, 24, 3689–3693] polypropylene is highly suited as a substrate for the photografting of selected monomers since it contains easily abstractable hydrogens (photografting is mediated by benzophenone and proceeds due to hydrogen abstraction from the surface of the substrate [Rånby, B., Yang, W. T., Tretinnikov, O., *Nucl. Instrum. Methods Phys. Res. Sect. B* 1999, *151*, 301–305] and [Rohr, T., Ogeltree, D. F., Svec, F., Fréchet, J. M. J., *Adv. Funct. Mat.* 2003, *13*, 264–270]. Therefore, polymer chains of EDMA were photografted to the walls of the pipette tip to ensure intimate covalent bonding between the EDMA monolith bed (subsequently polymerised *in-situ*) and the polypropylene wall, thereby preventing sample loss due to unwanted wall voids. This also conferred greater structural rigidity to the monolith, preventing it from detaching from its housing during operation.

The pipette tip housing was naturally cone-shaped, resulting in a limited degree of support for the monolith within, but the advantage of wall modification via photografting was readily

demonstrated by driving high pressure nitrogen through the monolith bed in the opposite direction i.e. from the tapered end. Resistance to this pressure was significantly higher for monoliths in wall-modified tips relative to monoliths in unmodified tips which easily became detached from their housings (which incidentally facilitated the evaluation of AuNP coverage via FE-SEM methods).

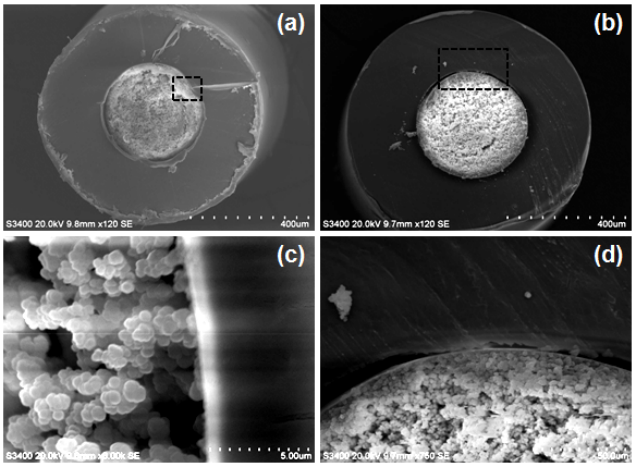

Figure 2. Scanning electron microscopy images of a porous polymer monolith formed within a polypropylene pipette tip which was modified by photografting (a,c) and unmodified (b,d) prior to monolith polymerization.

Figure 2 shows SEM images of a monolith formed within a pipette tip with and without wall modification. Clearly the wall modified tip (a,c) resulted in direct covalent attachment of the monolith whereas the unmodified tip (b,d) resulted in a monolith which is shaped like a truncated cone merely by virtue of the shape of its housing, but around which a large void is clearly visible.

Previous studies have suggested creating a main channel through the monolith[15,29] to reduce backpressure and facilitate the use of a simple laboratory autopipette. However, two issues occurred during the fabrication and modification process of the monolithic tip when the channel was created. Firstly, the channel was close to the tip wall and it was difficult to reproducibly position it in the centre of the monolithic bed. It was considered that this issue could negatively affect the trap-elution of the analyte due to uneven fluid flow through the monolith pore network. Secondly, the subsequent modification (with AuNP) of monoliths incorporating a main channel did not occur evenly throughout the entire monolith. Rather, the coverage of AuNP was centred around the bore of the main channel such that the radial homogeneity of immobilised AuNP was poor.

Consequently, the absence of a main flow-through channel resulted in high backpressures which prohibited the use of hand-held auto-pipettes for sample loading and rinsing, necessitating the use of a syringe pump. Efforts were made to reduce the backpressure by varying the monomer concentration between 20% and 40% prior to polymerisation, but all monoliths with < 40% EDMA had very poor mechanical rigidity. However the main focus of our efforts in this work was to demonstrate the use of gold-modified monoliths in pipette tips for affinity applications and thus the means by which the monolith was flushed was considered incidental. Finally, the reproducibility of monolith bed size was acheived by the use of an o-ring placed on the outside of the tip such that each empty photografted tip could be accurately filled with a given volume of monomer mixture. Other studies have reported the placement of o-rings inside the tip for this purpose[15] but this strategy was avoided due to the possible generation of a void volume between the top of the monolith bed and the o-ring. The monolith bed size was measured under an optical microscope using a vernier caliper and a % RSD of 8 % achieved for 8 separately prepared monoliths.

Modification of the monolith with gold nanoparticles.

The covalent attachment of gold nanoparticles onto the polymer monolith with a high density of coverage was achieved using a protocol originally described by Connolly *et al*.[32] with minor modifications. This protocol was broadly divided into two steps; amination of the monolith surface by reacting grafted polymer chains of vinyl azlactone with ethylenediamine, and the subsequent flushing of the monolith with 20 nm citrate-stabilised gold nanoparticles. One particular matter of concern was the possibility that the UV radiation (during grafting of azlactone) might not penetrate through the entire bulk of the monolith (due to the screening effect of UV absorbing monomers, solvents and the monolith itself) leading to an unwanted radial gradient of graft density. Rohr and Ogletree [Rohr, T., Ogeltree, D. F., Svec, F., Fréchet, J. M. J., *Adv. Funct. Mat.* 2003, *13*, 264–270]. have previously demonstrated that homogeneous grafting can be achieved through a 200 µm thick layer of monomer and also investigated the effect of rotating the substrate during grafting events [Rohr et al. (Macromolecules, Vol. 36, No. 5, 2003] In their study, Rohr et al. (Macromolecules as above) used electron probe microscopy to evaluate the homogeneity of graft density (for sulphonated polymer grafts). However, since we were immobilising 20 nm AuNP via aminated azlactone graft sites, we elected to evaluate AuNP coverage by FE-SEM which we took to be indicative of graft homogeneity since each nanoparticle is very strongly attached by multi-point interactions to the grafted surface via primary amine lone-pair electrons (Svec and Connolly).

Figure 3 illustrates the excellent coverage of AuNP. Figure 3a shows that the edge of the monolith (adjacent to the wall) had rather a sparse coverage of AuNP's which extended some 7 µm into the centre of the monolith.

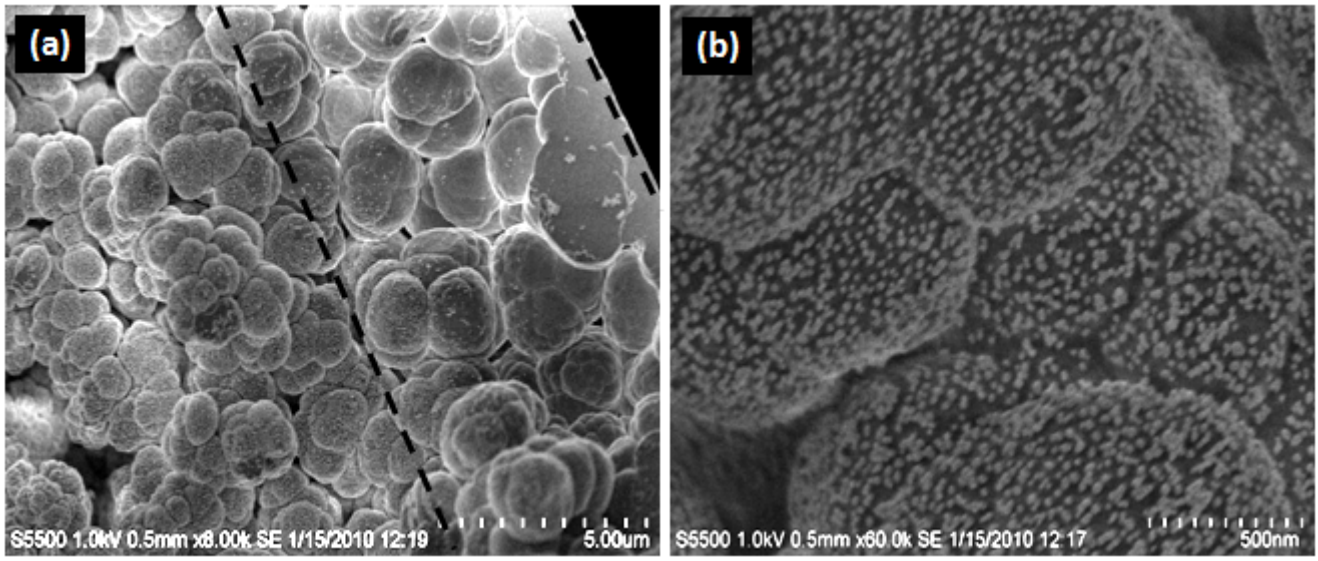

Figure 3. Field emission scanning electron microscopy images of a porous polymer monolith agglomerated with covalently attached 20 nm AuNP. (a): 8,000X magnification. The dashed lines represent a 7 µm region of monolith with lower AuNP coverage. (b): 60,000X magnification.

We believe that the poor coverage in this very narrow zone (representing only 3.5 % of the entire 400 µm monolith radius) was due to poor convective fluid flow at the extremities of the monolith compared with flow through the remaining monolith bulk. In relation to the work of Rohr et al (Adv Funct Mater paper and Macromolecules paper) regarding the radial homogeneity of grafting, for the work presented here, no gradient of grafting density was observed, either axially or radially despite the fact that our monolith was of considerably larger dimensions (400 µm at the tip). The high surface coverage of AuNP likely led to a significant increase in surface area however the very small monolith bed volume and the photografting methods used precluded any possible use of BET analysis to accurately quantitate the surface area increase. The number of individual gold-agglomerated monoliths required to provide a high enough mass of material for BET analysis was prohibitively high. In addition, fabricating a larger single monolith in a bigger mould for BET analysis was not considered since complete coverage of AuNP's throughout the entire bulk of the larger monolith would likely not be achieved due to excessively large monolith diameters inhibiting homogeneous grafting as previously discussed.

Modification of the AuNP-modified monolith with ECL

A number of different strategies were considered for the immobilisation of ECL on the gold-modified surface. The simplest method (which was not adopted here) would be to flush the gold surface with a solution of ECL as suggested by Storri et al [43] such that the lectin is physically adsorbed on the bare gold surface. However, this interaction is known to be weak and the adsorbed lectin could easily leach from the Au surface during the subsequent use of the extraction device.[43]. An alternative method resulting in covalent attachment to the gold surface involved the use of a bi-functional linker, DTSP as described by Katz [Katz paper on DTSP Katz, E.Y., 1990. *J. Electroanal. Chem.* **291**, pp. 257–260.] Figure 4 shows the DTSP coupling reaction between the gold surface and ECL. The AuNP-modified monolith was first flushed with DTSP to form a self-assembled monolayer of N-succinimidyl-3-thiopropionate (NSTP).[44][also, Katz] via strong gold-sulphur bonds. NSTP has an exposed active NHS group that is reactive towards primary amines; therefore covalent immobilisation of ECL was achieved by virtue of lysine residues on the exterior of the protein molecule[45].

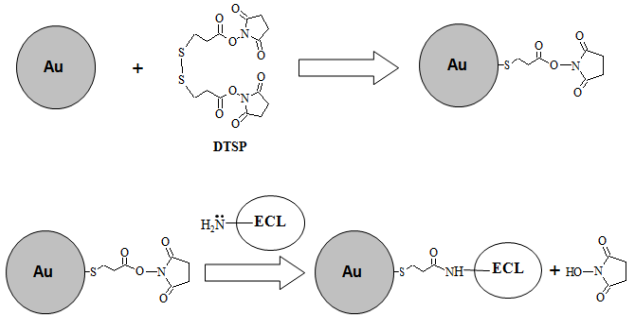

Figure 4. Reaction scheme for immobilisation of ECL via DTSP coupling reagent. (For simplicity, the gold nanoparticles are illustrated as isolated particles rather than showing their multi-point attachment to aminated azlactone grafts on the monolith surface).

The immobilisation buffer was selected to have a pH close to the optimum pH for lectin/glycan binding as dictated by the vendor and also incorporated 1 mM each of Ca^{2+} and Mn^{2+} which are known to be required metals for optimum protein folding/substrate binding/or binding site integrity....[reference?]. Therefore, by maintaining optimum buffer conditions for ECL during immobilisation and subsequent testing of the affinity monoliths, the risk of deactivation of ECL was minimised.

Binding studies for ECL monolith: Ribonuclease B and transferrin.

ECL is known to be selective for glycoproteins with terminal galactose residues and for this reason two test glycoproteins were initially chosen for selectivity studies. Ribonuclease B was selected as the negative control since its glycan structures are terminated by mannose residues and thus this glycoprotein was not expected to be retained by the ECL affinity monolith. Conversely, transferrin was selected as the positive control protein. The glycan of transferrin is terminated by neuraminic acid residues. This necessitated their removal via neuraminadase to reveal underlying galactose residues. Trap and release studies involved loading a mixture of (conc?) both proteins onto the affinity monoliths followed by a rinse step. All fractions and combined monolith rinsings were immediately collected and subjected to capillary LC analysis to determine if selective binding had occured.

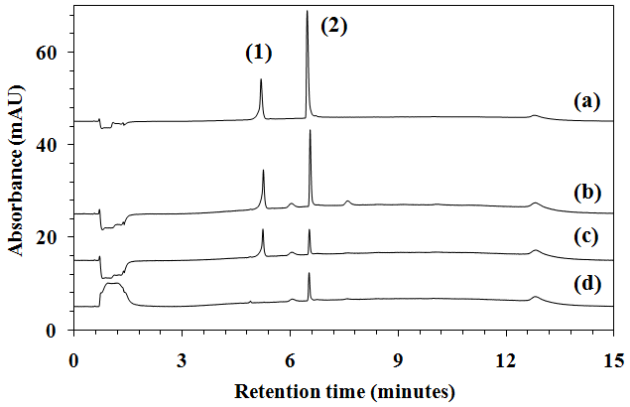

Figure 5. Trap and release studies for ribonuclease B and transferrin on ECL affinity monoliths. (a) Ribonuclease B and transferrin standard, (b) standard mixture after passage through Monolith B, (c) standard mixture after passage through Monolith A, (d) Release of bound transferrin from Monolith A with a galactose rinse. Chromatographic conditions: Column: 100 µm x 15 cm LMA-co-EDMA monolith, Gradient: 10 minute gradient from 5 % B to 100 % B at 2 µL/min, Column temperature: 25 °C, Injection volume: 1 µL, Detection: UV at 214 nm. Peaks: (1) Ribonuclease B, (2) Transferrin (desialated).

One of the advantages of using a AuNP-agglomerated monolith as a substrate for lectin affinity extractions is the obvious increase in available surface area when the test lectin (ECL) was immobilised onto the functionalised gold surface (Monolith A). Therefore for the sake of comparison, the binding capacity of Monolith A was compared directly with that of Monolith B which did not incorporate AuNP but instead had ECL covalently attached via amine-reactive azlactone polymer grafts, resulting in an anticipated lower binding capacity. As illustrated in Figure 5 (using relative peak areas in standard Chromatogram (a) as a reference), transferrin was partially extracted by both Monolith A and Monolith B, relative to ribonuclease B whose peak area remained completely unaffected. This is clearly indicative of the selective retention of transferrin over ribonuclease B due to lectin-galactose interactions. However ~ 95 % more transferrin was retained on Monolith A relative to Monolith B, clearly showing the advantage of AuNP-modified monoliths as high surface-area substrates for lectin affinity devices (or indeed *any* affinity or solid-phase enzymatic digestion applications). A subsequent monolith rinse of Monolith A with 40 µL of 0.8 *M* galactose in loading buffer revealed that the bound transferrin could be readily recovered, appearing as a single peak in chromatogram (d).

Investigation and mitigation of non-specific protein binding.

The use of a AuNP-modified polymer monolith as a substrate for affinity extraction presents the obvious possibility that non-specific binding of protein would occur, either due to hydrophobic interactions with the polymer monolith, gold-sulphur/gold-amine interactions with the immobilised gold nanoparticles, or irreversible covalent attachment of proteins with exposed unreacted succinimidyl groups.

After immobilisation of ECL on the functionalised AuNP, tris(hydroxymethyl)aminomethane was used to block both the remaining succinimidyl groups and any bare gold adsorption sites. For the sake of completion therefore, four blank monoliths were prepared as described in Figure 1 and the ribonuclease B/transferrin standard was used to evaluate the extent of unwanted protein binding to the AuNP-monolithic substrate. The base monolith comprised EDMA as monomer and so hydrophobic interactions might be expected. However, further modification of this surface by azlactone grafting followed by extensive amination (to facilitate AuNP attachment) had the added advantage of rendering the monolith extremely hydrophilic. Since tris was used as a blocking agent throughout this work, Blank Monolith 1 (Figure 1) was prepared to demonstrate that this small hydrophilic amine was effective in eliminating any hydrophobic interactions as evidenced by the complete lack of retention of either test protein on the monolith surface after exposure to the test mix. Conversely, Blank Monolith 2 resulted in almost 100 % retention of both proteins due to the presence of bare unfunctionalised AuNP, presumably due to adsorption of protein on the gold surface as described by Storri et al. [43]. However, when the bare AuNP was blocked with tris to produce Blank Monolith 3, no detectable retention of either test protein was observed. Finally, the DTSP-functionalised AuNP monolith was blocked with tris and again no retention was observed (Blank Monolith 4), suggesting that tris, as a small hydrophilic amine was highly suited to providing efficient blocking of unreacted succinimidyl groups.

It should be noted that the loading buffer selected in this study was 10 m*M* Tris pH 7.4 containing 150 m*M* NaCl, 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+}. The buffer pH was selected to closely match the optimum binding pH of ECL. In addition, 150 m*M* NaCl was included in an effort to minimise any non-specific protein-protein interactions and thus limit all possible interactions to lectin/galactose. The composition of this loading buffer was not subject to exhaustive optimisation due to the observed absense of unwanted protein interactions in the blank monolith study.

Extraction of two glycoproteins from complex mixtures.

Based upon the performance of the affinity monolith when tested with a relatively simple mixture of two glycoproteins, more complex test samples were prepared. The first sample included a mixture of nine selected proteins which included six non-glycosylated proteins, (insulin chain B, insulin, cytrochrome C, bovine serum albumin, enolase and carbonic anhydrase) as well as glycoproteins which had either terminal mannose (ribonuclease B), or terminal galactose (desialated transferrin and

desialated thyroglobulin). In order to mimic a real sample, the non-glycosylated proteins were specifically selected to span a wide range of molecular masses from insulin chain B (3.5 kDa) to enolase (82 kDa to 100 kDa, depending on the isoform). A separate chromatographic method was developed for this mixture of nine test proteins. Using the same reversed phase monolith, a nine minute gradient (as described in the Experimental) was considered optimum. Column temperature (from 25 ^{o}C to 75 ^{o}C) however had a significant effect upon selectivity, particularly for three adjacent peak pairs. The optimised chromatogram of the standard mix is shown in Figure 6. Resolution between Peak 2 and 3 increased from 0.9 at 25 ^{o}C to 2.5 at 75 ^{o}C, resolution of Peak 3 and 4 decreased from 3.6 at 25 ^{o}C to 1.2 at 75 ^{o}C and resolution of Peak 7 and 8 decreased from 2.0 at 25 ^{o}C to complete co-elution at 75 ^{o}C. As a compromise, a column temperature of 40 ^{o}C was selected which resulted in resolution between Peaks 2/3, 3/4 and 7/8 of 1.4, 2.5 and 1.7 respectively. The optimised gradient conditions resulted in a peak capacity of 102.

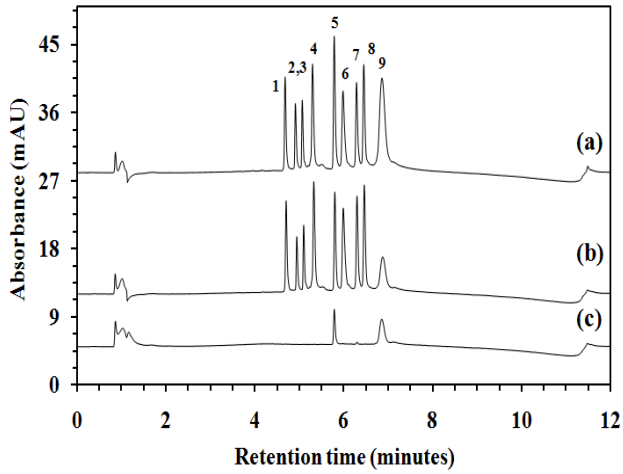

Figure 6. Test mix of nine proteins before (a) and after (b) extraction with Monolith A. Chromatogram (c) is after a galactose wash step. Chromatographic conditions as for Figure 5 except: Gradient: 9 minute gradient from 5 % B to 100 % B. Column temperature: 40 ^{o}C. Peaks: (1) 20 µg/mL ribonuclease B, (2) 20 µg/mL insulin chain B, (3) 20 µg/mL insulin, (4) 20 µg/mL cytochrome C, (5) 25 µg/mL transferrin, (6) 25 µg/mL BSA, (7) 20 µg/mL carbonic anhydrase, (8) 40 µg/mL enolase, (9) 80 µg/mL thyroglobulin.

When the test mix was passed through the affinity monolith (Monolith A), a comparison of chromatogram (a) and (b) in Figure 6 reveals that all of the non-glycosylated proteins (Peaks 2-4 and 6-8) were unretained as expected. In addition, Peak 1 (ribonuclease B) did not diminish in area since this glycoprotein has terminal mannose residues rather than terminal galactose. Conversely, significant levels of both desialated transferrin and thyroglobulin were extracted (decreased peak areas in Chromatogram b) and subsequently recovered with a galactose wash step. The lack of interfering peaks in the galactose wash is indicative of the specificity of binding. Interestingly, the affinity monolith was demonstrated to simultaneously extract both transferrin and thyroglobulin even in the presence of other proteins and glycoproteins, This was despite the considerable difference in size of both target glycoproteins (80 kDa and 660 kDa respectively) and also the difference in glycan structure and coverage.....?????

In a further effort to verify that transferrin had indeed been retained due to lectin-glycan interactions rather than non-specific protein-protein interations, two further experiments were performed. Firstly, transferrin was not treated with neuraminidase enzyme such that the transferrin glycan presented terminal sialic acid residues rather than terminal galactose. Incorporation of this untreated transferrin into a test mix (of all proteins except thyroglobulin) resulted in no extraction of transferrin when passed through the affinity monolith. Similarly, transferrin was treated with PGNase enzyme for removal of the entire glycan resulting in deglycosylated transferrin. Again, the deglycosylated transferrin was not extracted (data not shown).

It should be noted that throughout all of the extraction studies described in this report, the flow rate through the extraction device during loading and elution steps was not subjected to rigourous optimisation. Rather, the operational flow rate was chosen based on a compromise between one of the lowest possible flow settings on the syringe pump, and a flow rate at which reasonable volumes (> 20 µL) of eluted fractions could be collected in a reasonable timeframe. No doubt the recovery could have been significantly improved by optimisation of the flow rate and thus contact time between analyte protein and the immobilised lectin, however in this work our focus was largely on the qualitative assessment of the affinity monolith.

Affinity monolith stability studies.

During the course of this work a large number of affinity monoliths were prepared and some preliminary studies conducted on their short-to-medium term stability upon storage. As discussed, all monoliths were stored in 10 m*M* Tris pH 7.4 containing 150 m*M* NaCl, 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+} at 4 ^{o}C when not in use. Although a rigourous study of the stability of prepared affinity monoliths was not conducted, it was observed that affinity monoliths could retain their extraction capabilities after numerous consecutive periods of storage at 4 ^{o}C and re-use at room temperature. For example immediately after preparation, a particular monolith was stored for 12 hours at 4 ^{o}C, used for one extraction cycle (bind/elute), stored for a further 5 days at 4 ^{o}C, re-used for 6 consecutive extraction cycles, stored for 36 hours at 4 ^{o}C and finally used for three extraction cycles. In all cases, the performance of the extraction device was excellent. More detailed stability testing shall be the subject of future studies although current data suggests that the extraction performance dropped off significantly after prolonged storage (> 1 month).

Extraction of glycoprotein from spiked real samples: E. coli.

Results presented thus far involved the use of protein standards prepared in the laboratory on the basis of their molecular weight, presence/absence of glycan structures and the nature of the terminal sugar unit on the glycan. As a final examination of the performance of the affinity monolith with real samples, an E. coli cell lysate (filtered and passed through a 10 kDa spin cartridge) was spiked with 20 µg/mL desialated transferrin. Figure 7 shows that the transferrin peak decreased significantly as a result of extraction despite the presence of unknown matrix protein. Indeed the peak profile of the E. coli proteins was remarkably

unchanged before and after extraction which indicates that unwanted non-specific protein binding or protein-protein interactions were largely eliminated. Chromatogram (c) clearly shows that transferrin could readily be recovered from the extraction device and there was a marked absence of interfering proteins in this galactose elution step. Future work shall involve a more detailed examination of extraction recovery, but these preliminary results are extremely promising in terms of the development of a novel AuNP-modified monolithic substrate for affinity applications.

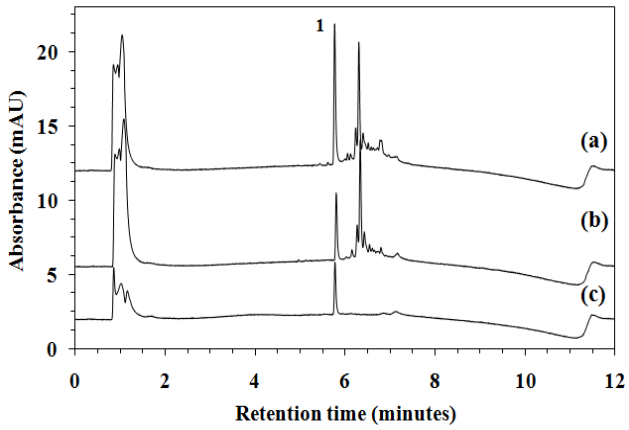

Figure 7. Spiked E.coli cell lysate before (a) and after (b) extraction with Monolith A. Chromatogram (c) is after a galactose wash step. Chromatographic conditions as for Figure 6. Peaks: (1) 20 µg/mL transferrin.

Conclusions

A novel monolithic extraction device has been described which incorporates covalently attached gold nanoparticles resulting in a significant increase in surface area. By using the well studied bifunctional coupling agent DTSP, a selected lectin was successfully immobilised on the gold surface while retaining its activity. The obvious advantage of this new AuNP-modified substrate is that using DTSP chemistry, any bio-recognition molecule (lectins, enzymes, Protein A etc) can be immobilised depending upon the required application while taking full advantage of the excellent mass-tranfer characteristics afforded by the underlying porous polymer monolith. Although our evaluation of binding capacity was semi-quantitative at best, in our hands we nevertheless observed a clear increase in binding capacity by virtue of the increased surface area due to immobilised gold. Further studies in this area shall involve a more comprehensive evaluation of protein recovery, both in pipette tip format for offline extraction as well as in capillary formats for online sample cleanup.

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