Synthesis and application of a new cleavable linker for "click"-based affinity chromatography†‡

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A new chemically-cleavable linker has been synthesised for the affinity-independent elution of biomolecules by classical affinity chromatography. This azo-based linker is shown to couple efficiently with "click" derivatised ligands such as biotin propargyl amide through a copper(I)-catalysed Huisgen 1,3-dipolar cycloaddition reaction. Binding to Affi-Gel matrices displaying ligands coupled to the new linker is both efficient and selective. The captured material may be readily released from the resin upon treatment with sodium dithionite. These mild elution conditions have allowed for the efficient isolation of the affinity partner from complex protein mixtures such as those found in fetal bovine serum.

Introduction

Affinity chromatography has relied historically on specific bioaffinity interactions between a resin-bound ligand and its biomolecular partner to effect the separation of that partner from a complex mixture of components (Fig. 1).[1] For successful application, the coupled ligand must retain its specific binding affinity for the target molecule and, once the unbound material has been removed by washing, the binding between the ligand and the target molecule must be reversible to allow the target molecule to be removed in an active form.[2] Perhaps one of the most notable early examples of the use of affinity chromatography for the identification of the binding partner of small-molecules was the capture of the rapamycin- and FK506-binding protein (FKBP) and other immunophilins from a human Jurkat T cell line extract.[3] More recently, affinity chromatography has been applied to the field of proteomics either in combination with small molecule probes for activity-based protein profiling to generate specific sub-proteomes,[4] or through amino acid-specific labelling of proteins (*e.g.* with biotin). This can be done either prior to, or post, trypsin digest to allow pre-fractionation of a complex mixture of peptides through an intermediate purification protocol (*e.g.* on streptavidin-coated beads) before MS analysis.[5,6] However, the efficient recovery of the separated biomaterial in a functionally active form can be a difficult process since it often requires elution using high salt concentrations and/or detergents [Fig. 1(a)].[2] For
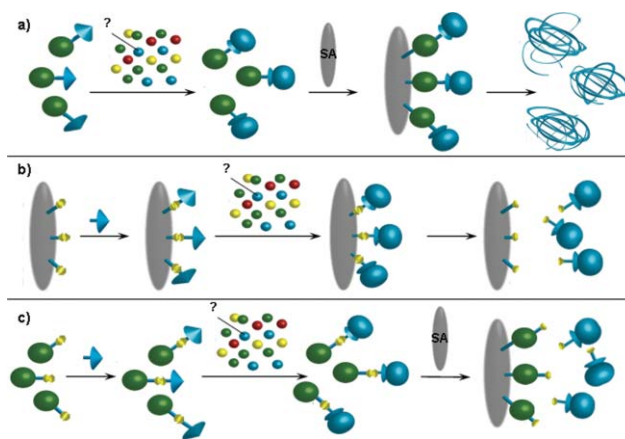
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‡ Electronic supplementary information (ESI) available: Details of the synthesis of linker **1**, preparation and affinity applications of derivatised Affi-Gel matrices **7**, **9** and **10**, together with spectra for the linker and azides. See DOI: 10.1039/b916693a

Fig. 1 Capture of an "unknown" affinity target for a small-molecule ligand: (a) capture of the affinity target in solution with a biotinylated ligand, immobilisation of the biotinylated ligand complex on a (strept)avidin coated matrix and elution under potentially denaturing conditions; (b) direct attachment of a "click" functionalised ligand to the matrix through a cleavable linker, capture of the affinity target and elution under affinity-independent, non-denaturing conditions; (c) attachment of biotin to the ligand through a cleavable linker, capture of the affinity target in solution, immobilisation of the biotinylated ligand complex on a (strept)avidin coated matrix and elution under affinity-independent, non-denaturing conditions.

this reason, general elution methods which are independent of the bioaffinity interaction (so-called affinity-independent elution protocols) are of particular interest.

Affinity-independent elution of the captured biomaterial [Fig. 1(b) & (c)] typically relies upon the introduction of a linker between the resin and the bound ligand. This linker may be enzymatically cleaved (*e.g.* the nuclease-cleavable linker described by Santala *et al.* for the efficient recovery of antibody-displaying phages that have been captured with a biotinylated antigen on streptavidin-coated magnetic particles),[7] photocleavable (*e.g.* the photocleavable linker applied to microbead-based affinity chromatography chips used to purify the hepatitis C virus RNA polymerase proteins described by Cho *et al.*),[8] or cleaved under mild chemical conditions (*e.g.* the use of an azobenzene linker to purify cathepsin peptides from rat liver homogenate described by Verhelst *et al.*).[9a] This last case was of particular interest to us as the cleavage reaction conditions (mild reduction with sodium dithionite, $Na_2S_2O_4$) are known to be compatible with biochemical systems,§ as exemplified by the use of azobenzenes as cleavable cross-linking reagents for proteins[10] and in the functionalisation of a tyrosine residue on the surface of viral capsids.[11] Thus in designing a cleavable support for affinity-independent elution methodology that was compatible with the extensive range of

propargyl functionalised "click" ligands that have emerged in recent years,[12,13] we chose to base our linker on this precedent.

Results and discussion

Azobenzene linker **1** was designed with orthogonal reactive functionalities: an amine for linking to a matrix support, and an azide for reaction with the propargyl-functionalised ligands of interest. The azobenzene unit (**2**) at the heart of this linker was found to be readily accessible through diazotisation of 4-aminobenzoic acid and reaction with phenol (Scheme 1).[14] Etherification of the phenol in the presence of the benzoic acid was found to give a mixture of products, but reaction of the acid with *N*-Boc-ethylenediamine[15] followed by phenol etherification with 1,3-dibromopropane[16] resulted in a good yield of alkyl bromide **3**. Bromide displacement (NaN₃, **3**→**4**) and Boc-deprotection, led to the preparation of a novel cleavable linker **1** for affinity applications which could be readily prepared on a gram scale (5 steps, 29% overall yield).
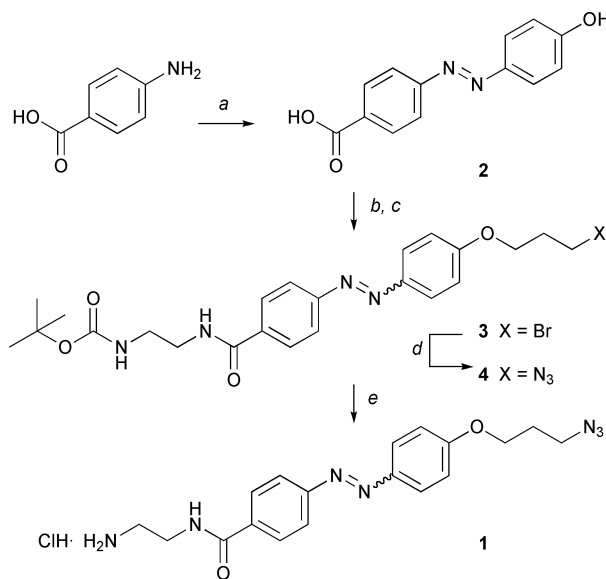
Scheme 1 Linker synthesis. Reagents and conditions: (a) i. NaNO₂, HCl, 0 °C, 20 min; ii. PhOH, NaOH, K₂CO₃, 0 °C to RT, 3 h, 90%; (b) BocNHCH₂CH₂NH₂, EDC, HOBt, DMF, 18 h, 60%; (c) 1,3-dibromo-propane, K₂CO₃, CH₃CN reflux, 5 h, 67%; (d) NaN₃, DMF, 18 h, 90%; (e) MeOH, CH₃COCl, 0 °C to RT, Et₂O, 1 h, 90%.

For our preliminary affinity chromatography studies we chose to use the matrix Affi-Gel 10 (Bio-Rad), an agarose based affinity medium that contains a neutral 10-atom spacer arm and a reactive *N*-hydroxysuccinimide ester (Scheme 2). Complementary supported azides **5** and **6** (with and without the azo linker) were generated through coupling either 3-azidopropylamine, or linker **1** to the Affi-Gel 10 matrix. A ligand-assisted, copper(I)-catalysed Huisgen 1,3-dipolar cycloaddition, or "click", reaction conducted in the presence of tris-(benzyltriazolylmethyl)amine (TBTA)[17] allowed successful coupling of propargyl alcohol to azide **6** (Scheme 2). This derivatised matrix **7** was used to test the desired chemoselective reductive cleavage of the azo linker by sodium dithionite (Na₂S₂O₄)[18] which was easily followed since reduction of the azo group is accompanied by a dramatic loss of
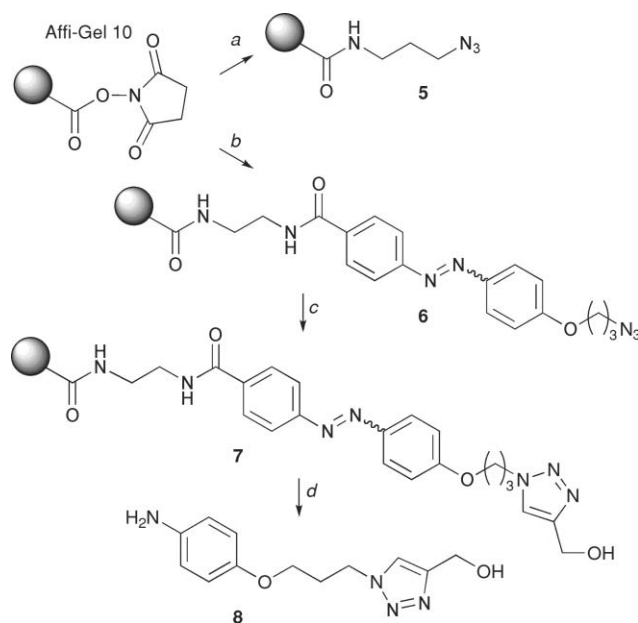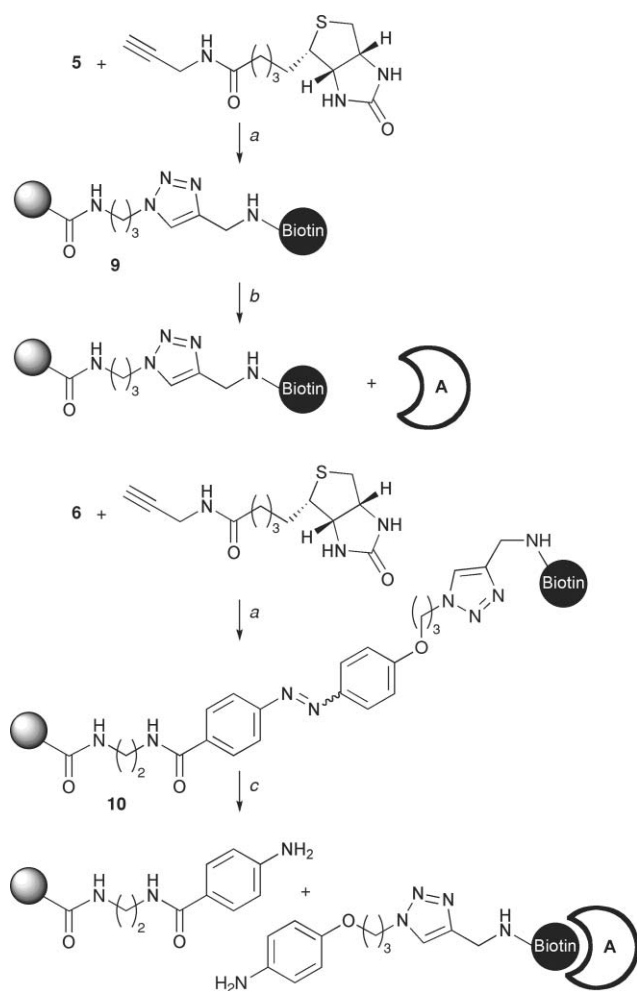

Scheme 2 Affi-Gel supported azide synthesis and reactions. Reagents and conditions: (a) 3-azidopropylamine, MeOH, 18 h; (b) linker **1**, Et₃N, H₂O/CH₃CN, 18 h; (c) propargyl alcohol, CuSO₄, sodium ascorbate, TBTA, H₂O, *t*-BuOH, 18 h; (d) Na₂S₂O₄.

colour of the agarose matrix. The corresponding aniline derivative **8** was eluted from matrix **7** in good yield (70%). This preliminary study demonstrates for the first time that the azo unit is compatible with the reductive conditions of the "click" coupling reaction.

In order to test how robust the new linker would be in an affinity-independent elution protocol, the biotin/(strept)avidin affinity pair was selected. Biotin and its affinity partner (strept)avidin exhibit one of the strongest known non-covalent interactions with a dissociation constant of $K_D \approx 10^{-15}$ M and they are widely used in affinity based separations.[19] Although direct attachment of biotin to the matrix and isolation of its known binding partner, avidin, was chosen to investigate the new click linker properties (Fig. 1b, "unknown" target = avidin), this study would also probe the ability of the linker to provide an affinity-independent elution protocol where a click functionalised ligand was coupled to a biotinylated derivative of the linker and a (strept)avidin-coated matrix was used to isolate the unknown target of the ligand following incubation (Fig. 1c).¶ Indeed, the aqueous-based "click" coupling reaction of biotin-linker conjugates has been shown to have many advantages over conventional coupling techniques as a means of selective ligand biotinylation.[20]

The propargyl amide derivative of biotin[21] was coupled directly to the azido matrix **5** to generate affinity matrix **9**, as well as through the cleavable azobenzene unit in **6** to generate affinity matrix **10** (Scheme 3). In the first set of affinity experiments designed to test the new linker for its ability to bind the affinity partner without any competing non-specific interactions, both matrices **9** and **10** were incubated with avidin, whilst only matrix **10** with its novel azo "click" linker was incubated with albumin (negative control). The absence of a clear band for avidin (15 kDa) in the supernatant following incubation shows that both matrices bound avidin (Fig. 2, lanes A, B, D & E). Furthermore, matrix **10** was demonstrated not to exhibit any non-specific interactions for

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Fig. 2 SDS-PAGE for experiments with avidin and albumin: lanes A-E supernatants after incubation; lanes F-H supernatants following SDS elution; lanes I₁-J₃ supernatants following dithionite cleavage. M = marker lane; A & B = supernatant from incubation of matrix **9** with avidin; C = supernatant from incubation of matrix **10** with albumin; D & E = supernatant from incubation of matrix **10** with avidin; F = supernatant after SDS elution of matrix **9** incubated with avidin (Lane A); G = supernatant after SDS elution of matrix **10** incubated with albumin (Lane C); H = supernatant after SDS elution of matrix **10** incubated with avidin (Lane D); I_1-I_3 = supernatant after dithionite cleavage of matrix **9** incubated with avidin (Lane B) at pH 6.5, 7.5 & 8.5; J_1-J_3 = supernatant after dithionite cleavage of matrix **10** incubated with avidin (Lane E) at pH 6.5, 7.5 & 8.5.

could capture avidin selectively from complex protein mixtures. Gratifyingly, both matrices were shown to remove avidin (Fig. 3, lanes B & C). Furthermore, release of the bound material could be performed using the optimal cleavage conditions for each matrix: boiling with SDS for matrix **9** (Fig. 3, lane D); and dithionite cleavage at pH 6.5 for matrix **10** (Fig. 3, lane E),‖ with only marginally reduced efficiency for the chemical cleavage.

Scheme 3 Preliminary affinity studies with derivatised Affi-Gel matrices **5** and **6**. Reagents and conditions: (a) biotin propargyl amide, $CuSO_4$, sodium ascorbate, TBTA, H_2O, t-BuOH, 18 h; (b) incubation with avidin followed by elution with SDS; (c) incubation with avidin followed by cleavage with $Na_2S_2O_4$.

Fig. 3 SDS-PAGE for experiments with FBS: M = marker lane; A = FBS + avidin (control lane); B = supernatant after incubation of matrix **9** with FBS + avidin; C = supernatant after incubation of matrix **10** with FBS + avidin; D = supernatant after SDS elution of matrix **9** incubated with FBS + avidin (Lane B); E = supernatant after dithionite cleavage of matrix **10** incubated with FBS + avidin (Lane C).

albumin as shown by its quantitative elution; clear band at 66 kDa (Fig. 2, lane C) and the absence of this band following cleavage (Fig. 2, lane G).

The efficiency of a classical SDS elution procedure (for matrix **9**) and chemoselective cleavage with sodium dithionite (for matrix **10**) were then compared. Gratifyingly, both elution procedures showed the expected bands for avidin (Fig. 2, lanes F & J₁₋₃). The efficiency of dithionite cleavage was determined at a range of pH values (Fig. 2, lanes J₁₋₃); incubation with a phosphate buffer solution at pH 6.5 and a large excess of sodium dithionite were identified as optimal cleavage conditions (Fig. 2, lane J₁). These mild cleavage conditions were shown not to release avidin from matrix **9** in the absence of the azo linker (Fig. 2, lanes I₁₋₃) demonstrating the chemoselectivity of this protocol. Conversely, the presence of the linker does not interfere with a classical SDS elution protocol, as avidin is released from matrix **10** under these conditions (Fig. 2, lane H).

In the second set of experiments, matrices **9** and **10** were incubated with avidin enriched fetal bovine serum (FBS) (Fig. 3, lane A) to determine whether the derivatised Affi-Gel matrices
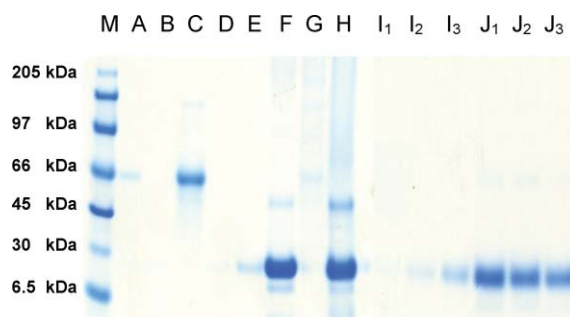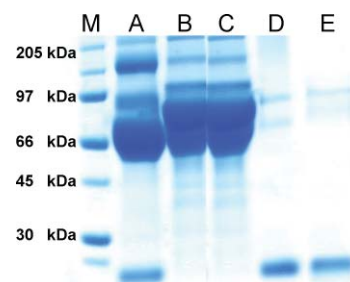
Conclusion

A new azobenzene-based linker for affinity chromatography applications has been developed; it may be readily cleaved under mild non-denaturing conditions (elution with $Na_2S_2O_4$) making it suitable for affinity-independent elution protocols. Affinity ligands functionalised with a bioorthogonal propargyl label may be readily attached to the terminal azide of the linker using the copper(I)-catalysed Huisgen 1,3-dipolar cycloaddition reaction (or "click" reaction). Affinity capture by matrices displaying ligands coupled

to the new linker has been shown to be both efficient and selective. The chemical cleavage of the ligand plus its bioaffinity partner from the gel has been shown to be comparable to a classical cleavage process under denaturing conditions; and indeed the two processes are both compatible with the new linker. Although the use of this linker has been demonstrated using the agarose matrix Affi-Gel 10, we believe that it will find widespread application on a range of activated matrix and/or chip surfaces. It is anticipated that the use of this azo linker as means of coupling biotin to the ligand of interest will also allow an affinity-independent version of the more classical manifold for target identification based upon immobilisation on a (strept)avidin coated matrix, as well as allowing *in vivo* experiments. Investigations with several ligands of interest (whose propargyl derivatives retain the activity of the lead compound) with known, or potential, affinity partners are currently under progress in our research group.

Acknowledgements

Notes and references

§ Whilst this reduction has found widespread use in biochemical applications, the concomitant reduction of some amino acids such as nitrotyrosine to aminotyrosine has been reported under these conditions.[9b]

¶ Synthetically this would require coupling of the amino group of the azo linker **1** to NHS-biotin, followed by click coupling of the biotin-linker conjugate to the ligand.

‖ The weak bands at 66-97 kDa are likely to be serum albumin and similar prominent serum proteins.

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