Molecular Recognition

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Small-Molecule Sensing: A Direct Enzyme-Linked Immunosorbent Assay for the Monosaccharide Kdo

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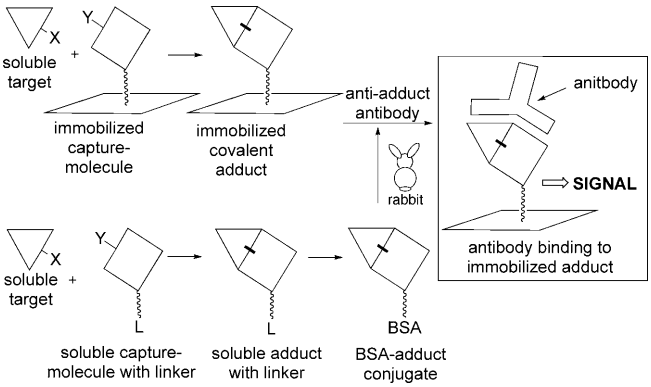
Identification and quantification of target small molecules in complex mixtures is a major goal of analytical chemistry, biochemistry, and clinical chemistry. The most commonly used methods rely on chromatographic separation (GC, HPLC, and CE) and subsequent identification and integration of the individual peaks. This is particularly challenging for small carbohydrates that have no chromophores, therefore refractive index, pulsed amperometric, or mass-spectral detection are frequently used.[1] Labeling of reducing sugars, especially with fluorescent tags, can be used for high-sensitivity detection.[2]

Recognition and reaction in solution with specific enzymes, if available, offers an alternative tool for simultaneously identifying and quantifying small molecules. The most prominent example in the case of sugars is the quantification of glucose in serum using the enzyme glucose oxidase.[3]

The methods noted above are stoichiometric in that the signal for each molecule is counted only once. Biological recognition of an immobilized ligand permits amplification, forming the basis of the enzyme-linked immunosorbent assay (ELISA).[4] In a typical ELISA, an antibody-enzyme adduct recognizes and binds to an immobilized ligand. The amount of enzyme remaining after washing therefore reflects the amount of immobilized ligand. The bound enzyme can catalytically turn over a spectroscopically active substrate resulting in a degree of amplification in the thousands that can be measured typically by visible absorption or fluorescence.

More recently, rapid advances in extracting a signal from a perturbation caused by the binding of a macromolecule (protein or DNA) to a surface-immobilized ligand formed the basis of a new generation of sensors. The most used of these methods is surface plasmon resonance,[5] where, in the most favorable instances, the binding of a macromolecule to a ligand immobilized on a chip is converted into a signal. Numerous other methods detecting the surface perturbation resulting from molecular recognition of an immobilized molecule are under development, two examples of which are surface acoustic wave and cantilever-based devices.[6]

Herein we describe a new approach to the development of sensors for small-molecule carbohydrates. The concept ideally requires a large protein (antibody) to recognize and bind to a surface only in the presence of the target small molecule. The concept for the assay is shown schematically in Scheme 1.



Scheme 1. Strategy for a small-molecule sensor. The target small molecule reacts with an immobilized capture molecule leading to an immobilized covalent adduct that is specifically recognized by an antibody. Signal generation can be monitored by ELISA or surface-perturbation techniques.

Implementation of the strategy requires identification of a functional group X on the target molecule that can react with group Y on a capture molecule to yield a stable covalent adduct. This adduct should ideally be unique, that is, not found in nature. If the capture molecule is immobilized on a surface by a linker (L), and if an antibody can be produced that specifically detects the immobilized adduct, then an ELISA or surface-perturbation assay can be devised (Scheme 1, top). We envisioned that the required antibodies can be produced by immunization of rabbits with a synthetic BSA-conjugate (bovine serum albumin; producing polyclonal antibodies; Scheme 1, bottom), or more ideally by production of monoclonal antibodies in mice.

We evaluated the strategy presented in Scheme 1 using 3-deoxy-D-manno-2-octulosonic acid ("Keto-deoxy-octulosonic acid", Kdo) as the target small molecule (Scheme 2). Kdo is a monosaccharide present in cell-surface lipopolysaccharides, especially in Gram-negative bacteria.[7] It has also been found in plants and is most frequently quantified using the thiobarbituric acid assay, a process involving destructive periodate oxidation of the sugar.[8] This sugar is present in solution almost exclusively in the closed-ring pyranose form shown, but is in equilibrium with the open chain α-keto-carboxylic acid. The quantification of carbohydrate α-keto acids in solution has historically relied upon the reaction with *ortho*-phenylenediamine derivatives to yield fluorescent quinoxalinones (Scheme 2) which can be quantified by HPLC

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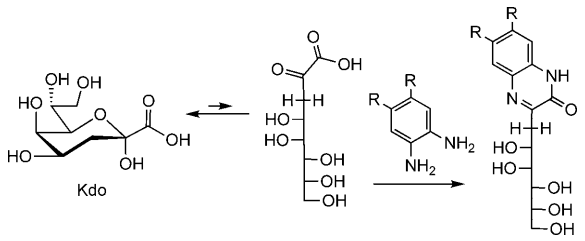
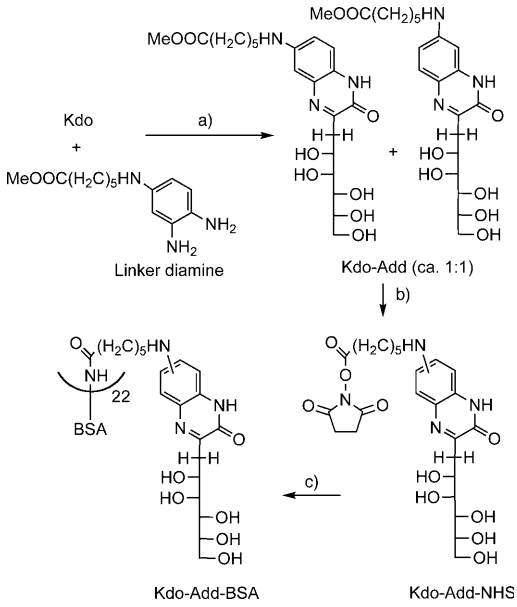


Scheme 2. The structure of Kdo and its reaction with a phenylenediamine derivative to give a fluorescent quinoxalinone adduct.

methods.[9] On the basis of this well-precedented reaction, we chose an *ortho*-phenylenediamine derivative as the capture molecule.

A phenylenediamine derivative (Scheme 3) containing the required linker was obtained in 2 steps from 2-nitro-1,4-phenylenediamine (see the Supporting Information). The reaction with Kdo under standard conditions in solution gave an isomeric mixture of Kdo adducts (Kdo-Add) in an approximate 1:1 ratio of isomers, and this mixture was used in all remaining steps. Conversion of the Kdo-Add methyl ester into the *N*-hydroxy-succinimide ester (Kdo-Add-NHS) allowed conjugation to BSA to give Kdo-Add-BSA with an average of 22 adducts per BSA, as estimated by MALDI analysis, that were ready for immunizing the rabbit.

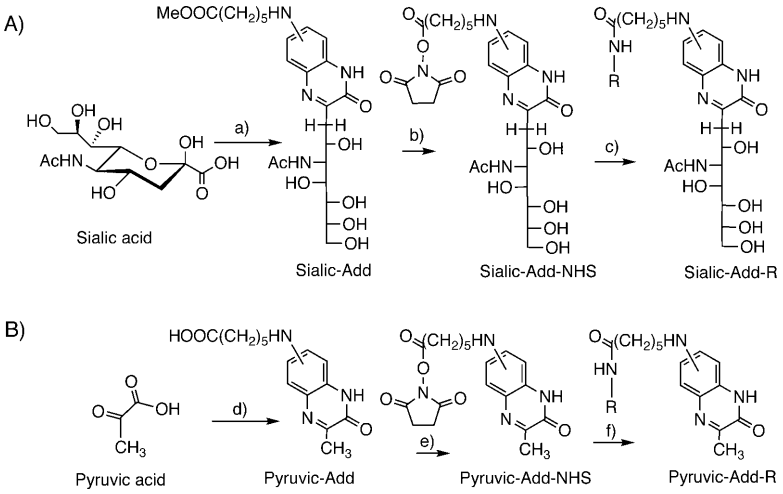


Scheme 3. Reaction of Kdo with linker-functionalized phenylenediamine to give the Kdo adduct (Kdo-Add) and it's elaboration into the Kdo-Add-NHS and Kdo-Add-BSA. a) $Na_2S_2O_4$, mercaptoethanol, H_2O/AcOH (9:1), 55 °C, 1.5 h, 63 %; b) 1. NaOH (0.03 M), 86 %; 2. TSTU, dioxane/DMF/H_2O (2:2:1), RT, 16 h, 55 %; c) BSA, phosphate buffer (0.1 M, pH 7.1), RT, 16 h. TSTU = 2-succinimido-1,1,3,3,-tetramethyluronium tetrafluoroborate.

It was critical early on in this work to determine if antibodies specific for Kdo-Add could in fact be obtained. Of particular concern was the possibility that other naturally occurring α-keto acids might be present in an analysis sample and that these would also react with the phenylenediamine capture group (Scheme 3) to give structurally similar qunioxalinone adducts. To evaluate this possibility, we selected 1) the related sugar *N*-acetylneuraminic acid (sialic acid), which would have an adduct with polyol side chain, and 2) pyruvic acid, which contains no carbohydrate unit, but would still form the quinoxalinone. These compounds were converted into their adducts as was done for Kdo, and then converted into their NHS-esters (Sialic-Add-NHS and Pyruvic-Add-NHS). They were finally coupled to BSA to give Sialic-Add-BSA and Pyruvic-Add-BSA (Scheme 4).

A rabbit was immunized with Kdo-Add-BSA and both the preimmunization serum and the postimmunization serum were assayed for antibodies capable of binding to Kdo-Add-BSA, Sialic-Add-BSA, and Pyruvic-Add-BSA, which were coated onto a plastic microtiter plate. Bound antibodies (IgG) were detected by a standard ELISA protocol. The results obtained with successively diluted serum are presented in Figure 1.

The preimmune serum did not react with any of the BSA conjugates. There was a good response against the immunizing conjugate Kdo-Add-BSA where a 6000-fold dilution resulted in half-maximum signal, and a cross-reaction with the other two conjugates could clearly be seen using less-diluted serum. It was hoped that these cross-reacting antibodies could be removed by affinity adsorption.



Scheme 4. Preparation of the quinoxalinone adducts of sialic acid (A) and pyruvic acid (B) and their elaboration into the NHS esters, and either the BSA conjugates (R = BSA) or affinity supports (R = sepharose). a) $Na_2S_2O_4$, diamine, AcOH/H_2O (9:1), 50 °C, 2 h, 52 %; b) 1. NaOH (0.03 M), 97 %; 2. TSTU, dioxane/DMF/H_2O (2:2:1), RT, 16 h, 42 %; c) BSA or amino-sepharose, phosphate buffer (0.1 M, pH 7.1), H_2O, RT, 16 h; d) 1. (see Scheme 3) with diamine, HCl (0.5 M), 50 °C, 1 h; 2. NaOH (0.03 M), RT, 30 min, 68 % (over two steps); e) EDC, NHS, DMF, RT, 16 h, 37 %; f) (see Scheme 3) with BSA or amino-sepharose, H_2O/dioxane (10:1), RT, on. EDC = 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, NHS = *N*-hydroxysuccinimide.

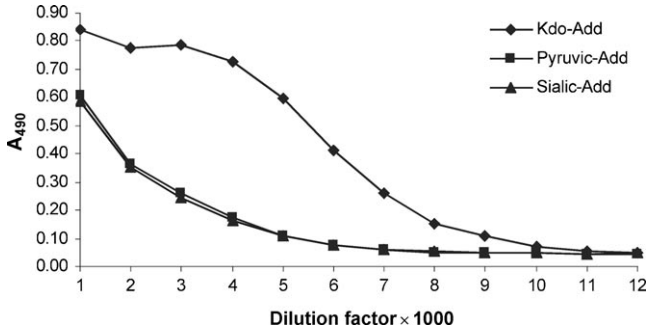


Figure 1. ELISA of crude serum from a rabbit immunized with Kdo-Add-BSA against the immunizing antigen and the structurally related Pyruvic-Add-BSA and Sialic-Add-BSA.

To this end, Sialic-Add-NHS and Pyruvic-Add-NHS (Scheme 4) were reacted with amino sepharose, and the serum was incubated sequentially with the two resulting affinity resins to remove the cross-reacting antibodies (see the Supporting Information). The binding data from the resulting refined serum (Figure 2) confirmed that the remaining antibodies were now active only against Kdo-Add-BSA.

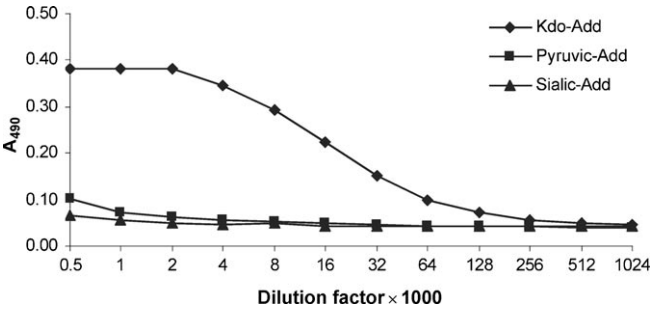
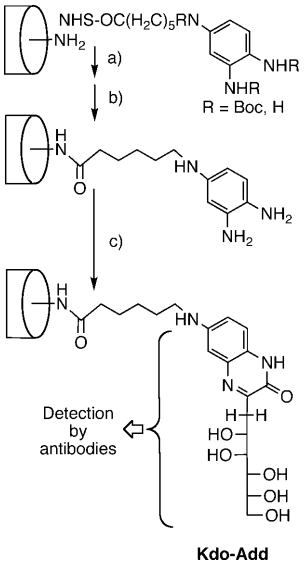


Figure 2. ELISA of refined serum from a rabbit immunized with Kdo-Add-BSA against the immunizing antigen and the structurally related Pyruvic-Add-BSA and Sialic-Add-BSA.

Two challenges remained: immobilizing the capture group and capturing the Kdo. The main difficulty was to generate an amine-protected form of the required linker diamine (Scheme 3) that could be deprotected on the solid surface used. All attempts with plastic plates failed, resulting in either the destruction of the plate or release of any capture group from the plate when using even standard Fmoc or Boc protection/deprotection strategies. Finally, we became aware of the availability of plastic microtiter plates coated with a 200 micron layer of glass that would permit the use of the harsh chemistry required.

Amino groups were grafted onto the glass-coated plastic plates using 3-aminopropyl triethoxysilane in ethanol at room temperature, resulting in the incorporation of 1.7 nmol per well (see the Supporting Information). The aminated wells were reacted with Kdo-Add-NHS, which was expected to result in the immobilization of the preformed Kdo-adduct. The ELISA confirmed that the antibodies recognized and bound the resulting plates as well as they did to the previous plastic plates coated with the Kdo-Add-BSA, and there was acceptably low nonspecific binding. More importantly, treating these antibody-active plates with TFA, conditions used for Boc-removal, did not affect the ELISA (data not shown).

Next, a Boc-protected capture molecule (Scheme 5) was attached to the aminated glass-coated plastic plate which was then treated with TFA. A series of solutions containing



Scheme 5. Coupling of a partially Boc-protected phenylenediamine to glass-coated microliter plates and subsequent reaction with Kdo. a) H_2O/DMSO (20:1), RT, 16 h; b) TFA (80% aq), RT, 45 min; c) Kdo, $Na_2S_2O_4$, mercaptoethanol, H_2O/AcOH (9:1), 60°C, 1.75 h.

decreasing amounts of Kdo was prepared and each added to the wells under conditions similar to those used for the solution preparation of Kdo-Add. After washing, the plate was probed for the presence of immobilized Kdo-Add using the refined antibodies described above. The results are presented in Figure 3 and clearly show that antibody binding is dependent upon the concentration of Kdo in the capture solutions, thus having detection limits in the 1 nmol per well range for a solution of pure Kdo.

Kdo was originally discovered in the cell wall lipopolysaccharide (LPS) of *E. coli* O111-B_4 from which it was

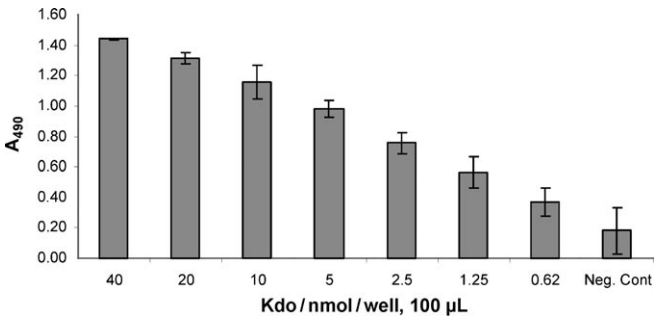


Figure 3. ELISA detection of decreasing amounts of Kdo captured by the linker-diamine attached to glass-coated microtiter wells.

released by mild acid hydrolysis and purified.[7] We were therefore interested in seeing if Kdo could be detected in the crude hydrolysis product of the *E. coli* O111 LPS using our new adduct/ELISA technique described above. The commercially available LPS was hydrolyzed as originally described (0.2 N H_2SO_4, 95 °C, 15 min), neutralized, and then the crude hydrolysate incubated with the phenylenediamine plates under our standard capture conditions. Figure 4 shows that the refined anti-Kdo-Add antibodies did indeed bind to the plates in a manner proportional to the amount of LPS hydrolysate.

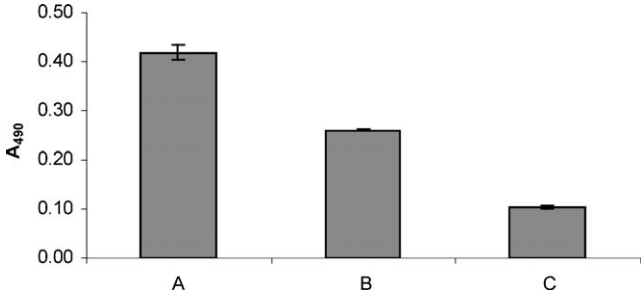


Figure 4. An ELISA detects Kdo captured from the crude hydrolysate at 490 μg (A) and 245 μg (B) of *E. coli* lipopolysaccharides. C) Blank (no LPS).

In conclusion, we have presented a proof-of-principle study in which a small-molecule carbohydrate can be covalently captured on a solid surface, and the resulting adduct can be detected by specific antibodies. Issues of surface selection, capture efficiency on the surface, potential benefits of using a monoclonal antibody, potential interference by (or analysis of) other α-keto acids in crude samples are important objectives of our ongoing investigation. However, notably, even in the present format, Kdo was identified in the crude acid hydrolysates of *E. coli* LPS.

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