A method for derivatizing surfaces with aldehyde groups by employing a new alkoxy aldehydic silane.

Coyne, Ann, N.[1], Benner, Lauren.[1], MacMillan, John H.[1], Telepchak, Michael.[1]
[1]*United Chemical Technologies, Inc., 2731 Bartram Road, Bristol, PA, 19007*
phone: +1-215-781-9255, fax: +1-215-785-1226, www.unitedchem.com

The use of immobilized bio-molecules is an essential technique required for many biological applications, from nucleotide purification to immunography[1,2]. A common protocol for covalent attachment is through the use of glutaraldehyde. This method involves the activation of an amine-functionalized surface with a glutaraldehyde solution to create aldehyde groups[3,4,5]. These aldehydes then readily react with the primary amine groups of biological molecules resulting in covalent attachment. Although popular, this method has numerous disadvantages. For example, it is labor-intensive, two Schiff bases are present in the covalent linkage, and glutaraldehyde is an unstable compound that is difficult to purify[6]. Additionally, the Schiff bases that are formed are susceptible to hydrolysis and thus have the potential for ligand leaching[6]. Treatment with reducing agents such as sodium borohydride is often performed to remove the Schiff bases, adding an additional step to the process.

A new line of trialkoxy aldehydic silanes, manufactured by United Chemical Technologies, Inc. under the trademark BIO-CONEXT™, allows the one-step addition of an activated surface directly to a matrix, thus circumventing the steps and reagents required by other methods. In addition, the covalent linkage formed by this method has only one Schiff base, yielding a much more stable product. Furthermore, this method can be used with any matrix that has either naturally occurring or synthetically incorporated hydroxyl groups that can be synthetically modified with trialkoxy aldehyldic silanes[7,8] (Figure I). Included are some of the most popular and useful matrix materials, such as glass, agarose, silica, glass-coated ELISA plates, metals such as nickel and paramagnetic iron, and some commercially available resins[1]. In addition, incorporation of trialkoxy aldehydic silanes with different chain lengths can be used to prevent crowding of large bio-molecules and to allow access to active sites[1,2]. In this report, we describe and evaluate the BIO-CONEXT™ method for covalent attachment of bio-molecules.

As a first step in the evaluation of this method, four duplicate samples of silica gel were derivatized with triethoxy aldehydic silane. 250 ml of hexanes (Fisher Scientific) was added to 25 grams of raw 40-60um silica (United Chemical Technologies, Inc.) in a 250 ml beaker. 2.0ml DI water and 0.30 ml of glacial acetic acid (Mallinckrodt Baker) were then added to the beaker, the mixture was recovered and allowed to equilibrate by mixing for 30 minutes. Over a 2-3 hour period, 7.5 ml of BIO-CONEXT™ PSX1055, triethoxy aldehydic silane (United Chemical Technologies, Inc.), was added to the silica suspensions in 0.5ml aliquots. After each addition of the silane, the suspension was purged with nitrogen and was recovered. The suspension was then allowed to mix for five hours. The silica was then washed with 600 ml of isopropyl alcohol (Mallinckrodt Baker) and one liter of deionized water. The resulting aldehydic silica was stored in a glass container and immersed in purged deionized water under nitrogen at 4°C. Three grams were put aside for percent organic loading testing.

Organic loading of the aldehydic silica was performed to quantify the polymeric coating on the surface of the silica particles. Samples of derivatized silica were dried under nitrogen at 103℃ for four hours and then cooled to room temperature in a desiccator. The samples were weighed before and after being placed in a 950℃ furnace (Thermolyme). Heating of the samples caused all material on the silica particles, except the SiO_2, to be volatilized. By examining the change in mass of the samples before and after heating, the organic loading can be expressed as a percent mass change:

% organic loading=1-(grams ash/grams sample) x 100

Grams sample=Mass sample before ignition

Grams ash=Mass sample after ignition

The four samples, run in duplicate, had average % organic loadings of 8.20%, 8.13%, 8.23%, and 8.29%, resulting in an overall average % organic loading of 8.21% with a standard deviation of 0.067% and coefficient of variation of 0.82%. These values demonstrate the successful attachment of the alkoxy aldehydic silane to the silica particles. A silica gel blank shows a carbon loading of 3.5%.

Protein A (Prozyme) was then attached to the functionalized support to evaluate the activity of the silica surface as well as the loading capacity of the protein A. 0.4ml of the aldehydic silica was added to a Poly-Prep fritted column (Bio-Rad) and washed with five columns full of 0.01M PBS buffer, pH 7.4 (Sigma). The aldehydic silica was then incubated with 3.41mg of protein A in PBS buffer (Sigma) overnight. The column was then allowed to drain into a test tube, the volume was noted, and the absorbance of the supernatant at 280nm and 320nm was recorded. The column was then washed with 1ml of PBS buffer. The wash was collected and the volume recorded as well as the absorbances at 280nm and 320nm. This wash procedure was repeated until there were no significant absorbances at 280nm or 320nm, insuring that no unbound protein A remained. The amount of protein A present in the washes and supernatant was calculated as follows:

mg in solution=((A_{280}-A_{320})/0.14) x volume solution (ml)

A_{280}=Absorbance at 280nm

A_{320}=Absorbance at 320nm

0.14=Extinction coefficient ($E^{0.1\%}$) of protein A at 280nm

The absorbance at 320nm represents light scattering due to the presence of particles, notably silica, in the solution, while protein A does not have significant absorbance at this wavelength. These particles will also cause absorbance at 280nm. By subtracting the absorbance at 320nm from the absorbance at 280nm, a true measure of the absorbance due to protein A is obtained. The amount of protein A bound to the silica support was calculated by subtracting the amount of protein A collected in the supernatant and the washes from the amount of protein A originally loaded onto the column (3.41mg). Four samples of 0.4ml of aldehydic silica support were used and yielded results of 1.59mg, 1.50mg, 1.51mg, and 1.56mg of bound protein A, corresponding to an average loading of 1.54mg protein A. The standard deviation was 0.04mg and the coefficient of variation was 2.60%.

The activity of the protein A columns was then confirmed by a qualitative enzymatic assay[9,10]. 2mg of peroxidase conjugated rabbit IgG whole molecule (Rockland) was diluted in 2ml cool 0.01M phosphate, 0.5M NaCl PBS coupling buffer. The enzyme solution was then added to the protein A columns and incubated with gentle end-to-end mixing for 1.5 hours at 4°C. A negative control consisted of the enzyme solution added to 0.4ml of aldehydic silica (without protein A attached); this control was also incubated with gentle end-to-end mixing for 1.5 hours at 4°C. After incubation, the columns were drained and washed with PBS buffer. The washes were collected and their absorbance read at 280nm and 320nm. The columns continued to be washed until there was no significant absorbance at 280nm or 320nm, insuring that no unbound conjugated IgG remained. 2.90ml of 9.1mM 2,2´-Azino-bis (3-Ethylbenzthiazoline-6-sulfonic Acid) (ABTS) in 100mM potassium phosphate buffer, pH 5.0, and 0.05ml of 0.3% (w/w) hydrogen peroxide solution in deionized water, both freshly prepared, were then added to each of the columns. Peroxidase will catalyze the following reaction:

$$H_2O_2 + \text{ABTS} \qquad 2H_2O + \text{oxidized ABTS}$$

Oxidation of ABTS causes the development of a dark blue color, caused by an increase in absorbance at 405nm. During the

qualitative assay, the protein A columns displayed instant and intense color development while the negative control showed an extremely slight color change over several minutes. The intense blue color observed in the protein A columns indicates the presence of active peroxidase conjugated IgG. Since the negative control demonstrated only slight color change, the activity observed in the protein A columns indicates the presence of bound conjugated IgG. The color change observed in the negative control was most likely due to minute amounts of peroxidase conjugated IgG that was nonspecifically bound to the aldehydic silica that was not removed by the repeated washes.

A preliminary evaluation of the use of this method to attach BIO-CONEXT™ trialkoxy aldehydic silanes to 4% crosslinked agarose was performed. Two duplicate samples of agarose were derivatized as before with the following method modifications: 1) 4% crosslinked agarose (XC Corporation) was used in place of silica; 2) 50ml instead of 250ml of hexanes was used; 3) 20 ml of hexanes was added 3 hours into the reaction; 4) After the addition of the silane, the reaction was allowed to run two hours instead of five; 5) The % Organic Loading test could not be done with agarose. The protein A was also bound to the agarose as before, but the protein A columns were made with 0.5 ml of settled derivatized agarose, while 0.4ml of the aldehydic silica was used previously. Protein A coating values of 1.53mg and 1.34mg of protein A per 0.5ml agarose were obtained. This preliminary evaluation suggests that this method can be successfully used to covalently attach bio-molecules to agarose. Further work will be done to confirm this result as well as to try this method on other surfaces, such as glass slides and ELISA plates.

In summary, a simple method was developed, using basic laboratory equipment, for the successful and consistent binding of the BIO-CONEXT™ triethoxy aldehydic silane to silica and agarose, creating aldehyde-functionalized surfaces. Protein A was then successfully attached to the surface, demonstrating the following: A the aldehyde groups were retained on the matrix and remained available for reaction with primary amine groups on the protein A; B) the high loading of protein A indicates a high aldehyde activity level on the support. An IgG-peroxidase qualitative assay demonstrated that the protein A columns were able to function as affinity columns. This method offers numerous advantages to the glutaraldehyde method, including fewer steps and reagents as well as a more stable linkage.

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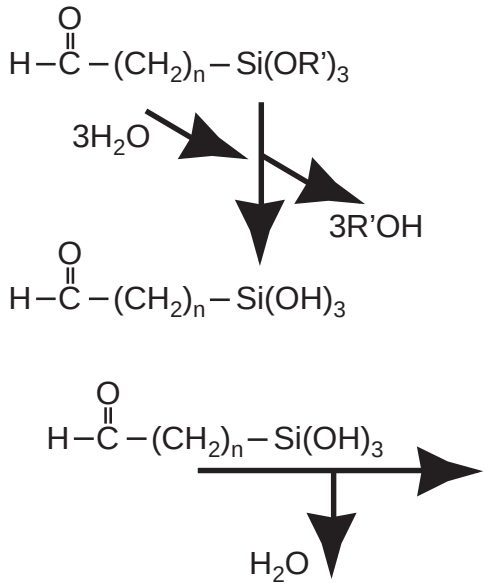
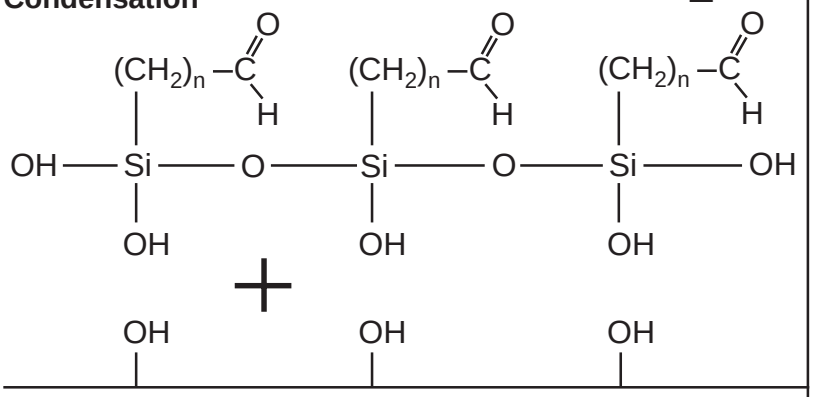
Deposition of Aldehydic Silanes

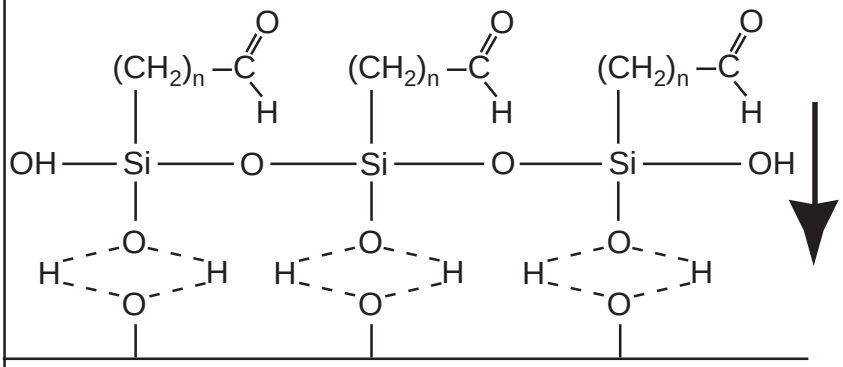
Hydrolysis **1**



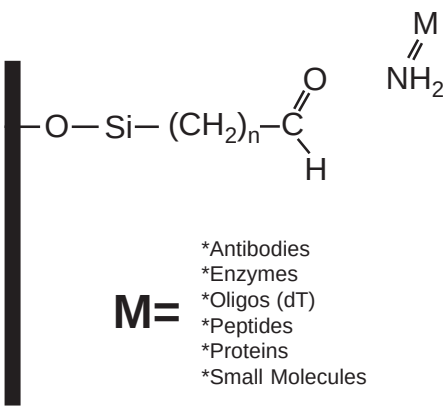
Condensation **2**



Hydrogen bonding **3**



5



M=

*Antibodies
*Enzymes
*Oligos (dT)
*Peptides
*Proteins
*Small Molecules

Derivatized Aldehydic Surface

4

Bond formation