

# LIGHT-DEPENDENT, COVALENT IMMOBILIZATION OF BIOMOLECULES ON 'INERT' SURFACES

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**We describe a novel, versatile procedure for the light-dependent immobilization of ligands to 'inert' material surfaces. Covalent immobilization of ligands differing in chemical nature and complexity is accomplished under mild and non-destructive conditions. Topical interaction of ligands with organic or inorganic surfaces is mediated by photoactivable polymers with carbene generating trifluoromethyl-aryl-diazirines which serve as linker molecules. Light activation of aryl-diazirino functions at 350 nm yields highly reactive carbenes, and covalent coupling is achieved by simultaneous carbene insertion into both the ligand and inert surface. Thus, reactive functional groups are not required on either the ligand or the supporting material. These procedures are applicable whenever ligands, from molecules to cells—synthetically or genetically produced, or isolated from biological sources—need to be immobilized for improved performance.**

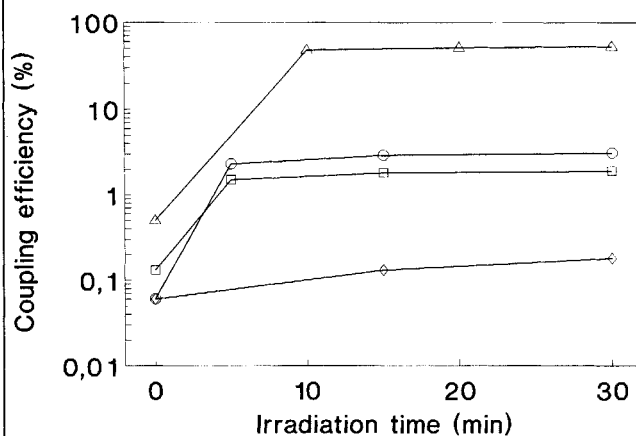
**C**urrent technologies for the molecular modification of 'inert' materials and their derivatization with macromolecules include thermochemical reactions, grafting, plasma techniques, photocoupling and molecular self-assembly. The photolabeling approach is unique in that highly reactive molecular species are generated on appropriately modified surfaces by topical irradiation. Photochemical reactions which initiate ligand immobilization have the following properties in common: Photogenerated carbenes (or nitrenes) insert into surface exposed parts of ligands and material surfaces and form covalent bonds. The photoreaction itself is independent of invasive coupling reagents and specific reaction conditions are not required, except light. Several investigators have proposed coupling thermochemical-photochemical bifunctional reagents to solid supports, the covalent coupling reaction being initiated by light activation of photochemical groups such as alkyl azides, acyl azides,  $\alpha$ -keto diazo compounds, diazirines and diazoalkanes<sup>1-5</sup>. These proposals have not proved practically useful, perhaps because the high reactivity of photogenerated carbenes and nitrenes which, when activated in the presence of solvents, promotes insertion primarily with abundant solvent molecules. In addition, the type of photoreagents which, until recently, were commercially available (alkyl-, arylazides and derivatives thereof) require activation energies that overlap with the absorption bands of proteins and nucleic acids. With the introduction of

diazirines as photolabels<sup>6</sup> new routes of biomolecule labeling and immobilization have been initiated<sup>7,8</sup>. Here we use trifluoromethyl-aryldiazirines which, upon photoactivation, do not undergo intramolecular rearrangement reactions, can be handled under normal laboratory conditions and, most importantly, absorb light at 350 nm. The utility of this approach for the non-destructive immobilization of macromolecules is demonstrated.

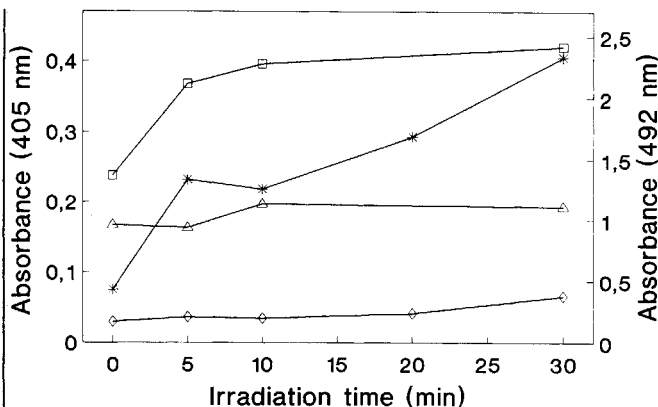
## RESULTS

The general photolinker polymer-mediated immobilization procedure includes the following steps. Clean material surfaces are coated with the photolinker polymer and dried. Macromolecules in solution are added to the photolinker-polymer coated surface and, upon solvent removal, the coated surface is exposed to activating light. This leads to photopolymer mediated immobilization of macromolecules. Excess ligand is then removed and the modified surface is ready for use. For photoactivation any light source providing sufficiently intense 350 nm radiation is applicable. Aryldiazirine absorption occurs in the range of 320 to 380 nm with an absorption maximum at 348 nm<sup>7</sup>. In general, unfiltered sunlight will generate carbenes from diazirines as well as the sophisticated laser instrumentation applied in nanolithography. In this study the commercially available Stratalinker (Stratagene, La Jolla, CA 92037, equipped with five Sylvania light bulbs, 350 Blacklight 8W F8T5/350 BL, emission band 300 nm to 420 nm, maximum emission 350nm) was used at an irradiance of 1mWcm<sup>-2</sup>. The  $\geq 320$  nm output of a high pressure mercury lamp (Osram 350 W, operating with a controlled 200 W output) served for specific applications, e.g. irradiation under argon<sup>9-12</sup>.

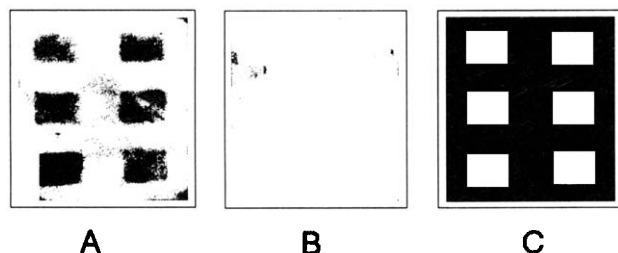
Figure 1 demonstrates the light-dependent immobilization of proteinaceous ligands (protein, peptides) and a polymeric carbohydrate (inulin) to polystyrene supports. Bovine serum albumin, multiply substituted with aryl-diazirines (TBSA), is used as the polymeric photolinker



**FIGURE 1** Photocoupling of biomolecules to polystyrene. Initially applied quantities (= 100%) of biomolecules were: [<sup>35</sup>S]-Streptavidin ( $\Delta$ ) 0.1 pmol per well; [<sup>3</sup>H] inulin ( $\circ$ ) 1.25 pmol per well; [<sup>3</sup>H] encephalin ( $\square$ ) 20.8 pmol per well; BSA control ( $\diamond$ ).



**FIGURE 2** Enzyme coupling to glass supports. (□) Alkaline phosphatase immobilized via TBSA. (△) BSA control with alkaline phosphatase (absorbance 405 nm). (\*) Horseradish peroxidase photoimmobilized via TBSA, and (◇) BSA control (absorbance 492 nm).



**FIGURE 3** Spatially selective immobilization of alkaline phosphatase to polyvinylidene difluoride. (A) The densitometric enzyme pattern (spacing 4 mm) obtained after exposure of an alkaline phosphatase coated membrane with the mask (C). (B) An identically treated, but not light-exposed membrane.

to effect coupling. Control experiments were carried out with unmodified BSA. Light activation of TBSA/streptavidin coated polystyrene surfaces leads to covalent binding of 50% of the applied ligand. The polycarbohydrate inulin and the enkephalin, Tyr-Ala-Gly-Phe-Leu, are linked to 3% and 2% respectively. Photocoupled streptavidin retains its ability to complex biotin, and both alkaline phosphatase and horseradish peroxidase remain enzymatically active upon photoimmobilization on polystyrene (data not shown).

The data depicted in Figure 2 demonstrates light-dependent immobilization and retention of enzymatic activity of catalysts photoimmobilized on plain glass. Glass discs were coated with the photolinker-polypeptide (TBSA) and either alkaline phosphatase or horseradish peroxidase. Dried samples were irradiated for various times, and following removal of excess protein, the photoimmobilized enzymes were incubated with their respective substrates and activities were recorded spectrophotometrically.

Spatially-selective enzyme immobilization has been achieved by irradiating TBSA/alkaline phosphatase coated polyvinylidene difluoride membranes through a mask (Fig. 3). Enzyme activity was recovered within light exposed areas after extensive washing of the photoactivated membrane and incubation with substrates of alkaline phosphatase which form colored insoluble products. A digitized camera recording of such an 'enzyme design'

is shown in Figure 3A. This and experiments in progress set the guidelines for future multiple-surface coating, with areas of immobilization being reduced to the nanometer scale<sup>13</sup>.

## DISCUSSION

In earlier work we have investigated the reactivity of photogenerated (aryl-diazirine derived) carbenes with individual amino acids<sup>9</sup>. More recently, a similar study has been carried out with monosaccharides. The results show that the mean reactivity of the sugars is ten-fold lower than the amino acids. It is probable that tightly bound water molecules are not removed by drying and thus compete for carbene binding (photolysis) with the (poly)carbohydrates. Similar arguments may apply to enkephalin, whose mean carbene philicity equals Ser/Thr. The molecular mass of enkephalin is smaller and the probability of carbene coupling is thus reduced. Moreover, with the exception of the N- and C-termini, enkephalin lacks polar groups, and ionic interactions with the underlying TBSA are therefore not optional. All these elements, either individually or in combination, may influence the coupling yield.

Since photocoupling is carried out in the dried state, encounter frequencies are ill defined. Carbene insertion occurs within bond distance only and molecular motion is restricted to BSA-bound diazirines. The effects of biomolecule concentration on coupling yields have not yet been investigated, and the lifetime of the excited state of the photogenerated trifluoromethyl-aryl carbene remains to be determined.

Preliminary data on DNA immobilization have been obtained using a high pressure mercury lamp as light source (irradiance > 100 mW/cm<sup>2</sup>) and an appropriate filter combination. Despite the high energy applied, enzyme-catalyzed restriction was not affected when DNA fragments were irradiated in solution, and PCR procedures could be successfully applied after short irradiation times. The analysis of DNA binding and hybridization capabilities of photoimmobilized (low irradiance) PCR products is the subject of a current study.

The successful covalent immobilization of various biomolecules illustrates the versatility and potential of this coupling procedure. It may be easily integrated into existing analytical routines (e.g. ELISA), and its independence from ligand-borne functional groups, favorable reaction conditions, multiple use of covalently modified surfaces and the spatial-addressable immobilization of macromolecules make it particularly attractive. Non-invasive, light-initiated immobilization may find applications in a number of fields, including nanotechnology, diagnostics, fermentation, separations, bioanalytics and medicine.

## EXPERIMENTAL PROTOCOL

**Preparation of aryldiazirine derivatized bovine serum albumin (TBSA).** Bovine serum albumin (240 mg) dissolved in 42 ml 0.1% (w/v) triethylamine in H<sub>2</sub>O, pH 11, was combined with 17.5 mg 3-(trifluoromethyl)-3-(m-isothiocyanophenyl) diazirine<sup>7</sup> in chloroform and 0.9 ml ethanol. The disperse solution was sonicated until homogeneous and incubated at 50°C for 2 hours. Upon cooling to room temperature, the reaction mixture was chromatographed on Sephadex G-25 medium in 1.5 mM NaCl, 0.05 mM sodium phosphate buffer, pH 7.4. The modified protein (photolinker-peptide) eluted in the void volume. It was pooled and stored at -20°C until use. The degree of substitution with the photolabel was 8-10 mol diazirine per mol bovine serum albumin.

**Photoimmobilization of biomolecules to polystyrene.** The general procedure for protein immobilization is described for [<sup>35</sup>S]-streptavidin. Photoimmobilization of other biomolecules has been carried out analogously. Microtiter plates (Nunc, Immunomodule Polysorp F8) were coated with TBSA providing 10 nmole diazirine per well. BSA (instead of TBSA) was used in control wells. [<sup>35</sup>S]-streptavidin was added and, upon

drying, the wells were irradiated for the indicated length of time with the Stratalinker light source. Wells were then repeatedly (5 times) washed with phosphate buffered saline, water (twice) and ethanol (twice). [<sup>35</sup>S]-streptavidin immobilization was quantitated by liquid scintillation counting.

**Enzyme coupling to glass supports.** Round (24 mm diameter) cover slips (Assistant) were cleaned with concentrated HCl (24 hours). Glass surfaces were thoroughly rinsed with bidistilled water and acetone. Glass discs were placed in wells of Falcon plates (Falcon 3064) and coated with 60  $\mu$ l of either TBSA (88  $\mu$ g/ml) or BSA (84  $\mu$ g/ml, both in ethanol/water 1:1 by volume). After drying, enzymes (alkaline phosphatase 1  $\mu$ g/ml phosphate buffered saline/ethanol 1:1 by volume or horseradish peroxidase 500  $\mu$ g/ml phosphate buffered saline/ethanol 1:1 by volume) were spotted on TBSA or BSA impregnated surfaces (40  $\mu$ l each) and dried under reduced pressure at ambient temperature. Enzyme/TBSA coated glass cover slips and control samples were then exposed to the 350 nm light (Stratalinker) for indicated lengths of time. Upon removal of excess enzyme, catalytic activities were assayed according to published procedures<sup>14,15</sup>.

**Spatially selective immobilization of alkaline phosphatase to polyvinylidene difluoride.** Disc shaped (30 mm diameter) polyvinylidene difluoride membranes (Immobilon-P, Millipore) were immersed in 0.7 ml photolinker-polymer (TBSA, 0.44 mg/ml 1.5 mM NaCl, 0.05 mM sodium phosphate buffer, pH 7.4) and incubated for 4 hours at 37°C in Falcon wells. Soaked membranes were transferred to new wells and dried at 37°C. TBSA coated membranes were then spotted with 0.5 ml alkaline phosphatase (1  $\mu$ g enzyme per ml phosphate buffered saline, pH 7.4) and incubated for 4 hours at 4°C. Upon drying at ambient temperature enzyme coated membranes were irradiated through a patterned mask (Fig. 3C) for 20 min). To remove noncovalently-linked enzyme, membranes were rinsed four times with 300 mM NaCl, 0.04% Tween 20 (v/v) and seven times with H<sub>2</sub>O. Alkaline phosphatase activity was detected by procedures which lead to insoluble stain deposition<sup>16</sup>.

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