

# New light induced molecular switch allows sterically oriented micrometer sized immobilization of biomolecules

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## ABSTRACT

Fundamental knowledge on protein structures and the effect of ultraviolet light on these structures has paved the way for the development of a unique light-based immobilization technology that allows oriented protein immobilization onto micrometer sized spots. The methodology is considered to be a strong alternative to the conventional procedures which often include the use of harsh conditions such as strong chemicals and elevated temperatures.

The technology behind this immobilization technique – here termed “light assisted immobilization” - is based on the fact that disulphide bridges that are naturally present within the protein structure can be broken as a result of UV-illumination. The free thiol groups (-SH) created upon disruption of a disulphide bridge are very reactive and can be used as linkers for covalent attachment to a surface. The surface can for example be gold or thiol-derivatized silicon, making this technology extremely useful for a large range of application areas, including biosensors.

**Keywords:** UV, protein, immobilization, nanoarray, biosensor.

## 1 INTRODUCTION

In the area of photosynthesis an impressive amount of knowledge has been obtained on how photons from the visual part of the electromagnetic spectrum are converted into high energy metabolites. In contrast, comparatively little is known at the molecular level about the molecular effects of UV photons on proteins. Nevertheless, the use of light in the 280-300 nm range is well established in protein science for the excitation of the aromatic amino acids (Tyrosines, Tryptophans, and Phenylalanines). The absorption of UV-light can be used to estimate the protein concentration rapidly using simple absorption spectroscopy, as well as for gaining information on structure and protein-ligand interactions using fluorescence spectroscopy.

Another and rather serious effect of UV-light interaction with biological molecules in general is that such radiation may alter their biocatalytic function as well as their

molecular structure. Concerning proteins, one known impact (among others) of UV-radiation is that UV-radiation absorbed by aromatic residues as a secondary effect can induce the disruption of disulphide bridges. Disulphide bridges are important stabilizers in the protein architecture, and loss of these stabilizers can do serious damage to the stability and as well as the function of a protein.

The molecular mechanistic aspects of light mediated SS-disruption is not yet known in detail, but it is suggested that the excitation of the aromatic residues – especially Tryptophan - initiates a cascade of photophysical reactions which can lead to the breaking of nearby located disulphide bridges [1, 2] (Figure 1). Nevertheless, the effect is now recognized and has been shown experimentally with two different proteins, cutinase [3] and  $\alpha$ -lactalbumin [4]. Yet unpublished data showing the same effect include immunoglobulin F(ab) fragments, alkaline Phosphatase, and lysozyme.

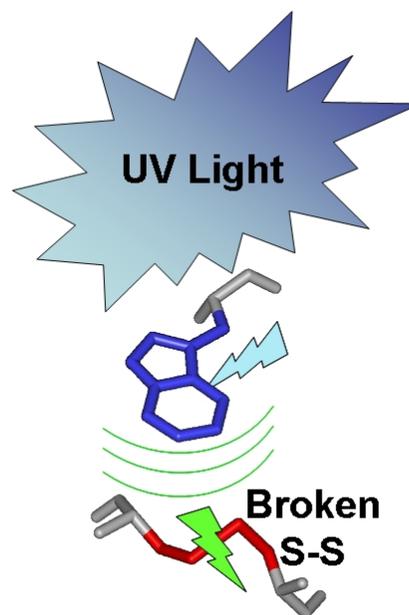


Figure 1: UV radiation can as a secondary effect (via aromatic amino acids) induce disruption of disulphide bridges in proteins.

## 2 LIGHT ASSISTED DISRUPTION OF DISULPHIDE BRIDGES IN PROTEINS FOR ORIENTED COVALENT IMMOBILIZATION

Disruption of disulphide bridges in the protein architecture can lead to serious damage of the protein structure and function. Nevertheless is reduction (opening) of protein disulphide bridges forced - commonly by addition of harsh chemicals - as a prelude for covalent protein immobilization, e.g. for use in biosensors [5]. The formed free thiols in proteins can then form new disulphide bonds to a thiol-derivatized surface, or directly to a clean gold surface, resulting in a strong covalent attachment.

Chemically induced opening of the disulphide bridges in multi-disulphide containing proteins is however unspecific since all solvent accessible disulphide bridges will be attacked. This is problematic since the orientation and the functionality of the immobilized protein cannot be predicted.

In contrast, the disulphide opening mediated by UV-radiation is specific in that sense that only disulphide bridges that are in close proximity to an aromatic residue are attacked [6] (Figure 2). Using bioinformatics it is possible to predict exactly which disulphide bridges that can be attacked, and how this will affect the orientation and the function of the immobilized protein.

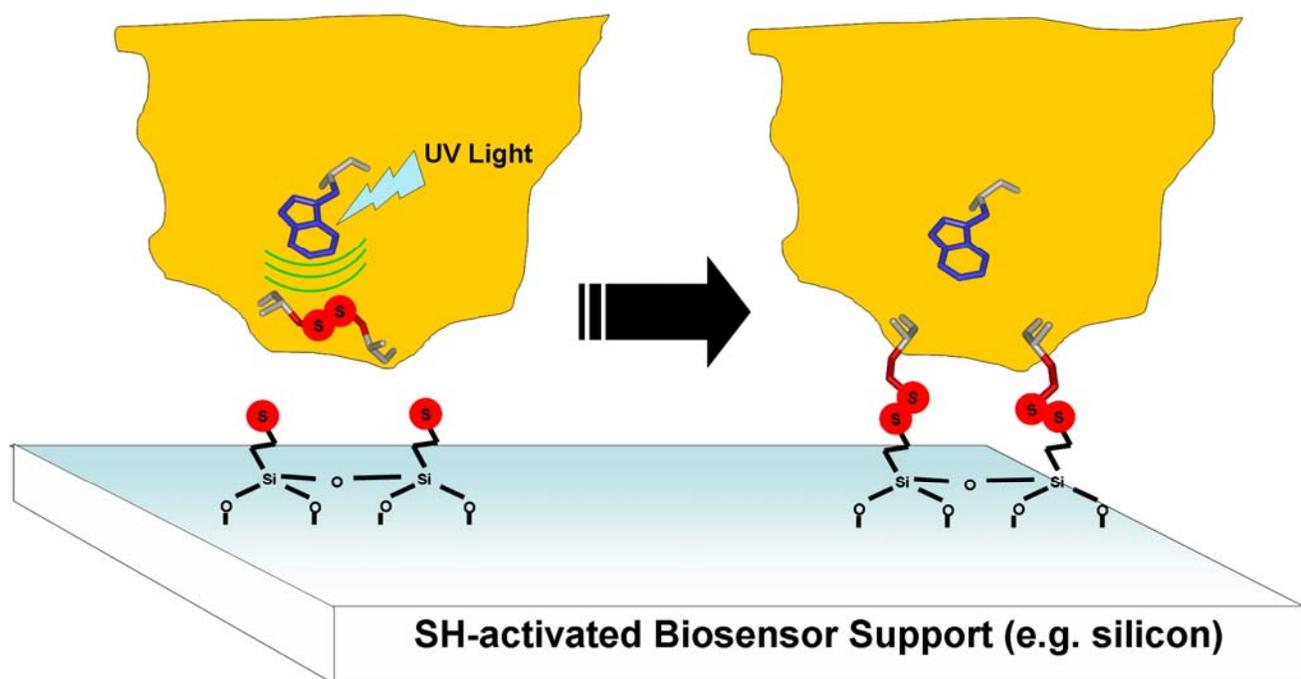


Figure 2: The principle of Light Assisted Immobilization sketched with tryptophan (blue) near a disulphide bridge (red) in a protein molecule (yellow). Sulphur atoms are highlighted as red spheres. The surface can be gold or – as illustrated– a thiol-derivatized surface that results in the formation of a new disulphide bond between the surface and the protein.

### 3 BIOINFORMATIC RECRUITMENT OF PROTIEN CANDIDATES

Previous studies have shown that surprisingly many proteins widespread in nature have a disulphide bridge in close proximity to an aromatic residue [7]. Numerous proteins have thereby the potential to be adaptable for light assisted immobilization, but effective procedures is needed to pick the best candidates that also experimentally will be adaptable to light assisted immobilization. We have developed procedures finding these candidates, with an experimental success rate of approximately 80-90%.

The election procedure is based on bioinformatic studies using relevant criteria for election of candidates. The most essential election criterions that each protein has to fulfill includes:

- Distance from the S-S bridge to an aromatic residue must be less than 20 Å, preferably less than 10 Å.
- The S-S bridge must be partially solvent accessible (access to environment /immobilization surface)
- S-S must not be near the active site of the protein (avoids damaging and/or blocking of the active site)

The most interesting proteins that are adaptable for light assisted immobilizations - and which also passed experimentally with proved functionality - include:

- Immunoglobulin F(ab) fragments (see illustration)
- Alkaline Phosphatase
- Lysozyme
- Cutinase (a triacylglycerol lipase)

Especially the F(ab) fragment from immunoglobulin G is a very interesting candidate since it is the constant region that is used for immobilization, while the variable region (where the antigen binding site is located) is oriented away from the immobilization surface. This means that arrays of different F(ab) fragments can be immobilized using this technology.

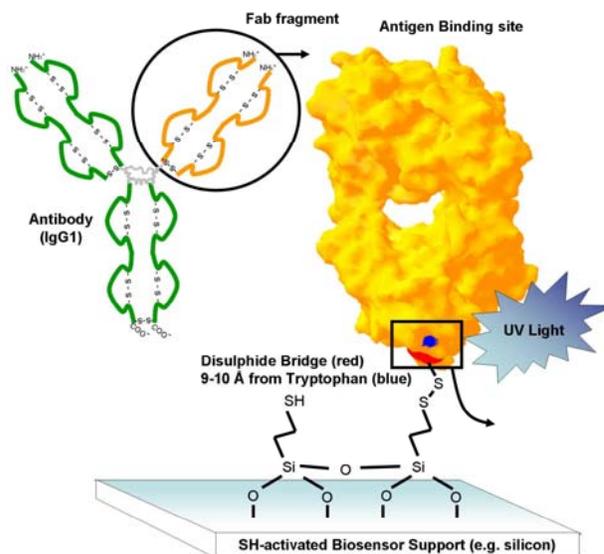


Figure 3: Light assisted immobilization illustrated with an Immunoglobulin F(ab) fragment. Note that the disulphide bridge (red) located near a Tryptophan (blue) is located far away from the antigen binding site.

### 4 NANOARRAY BIOSENSOR

The immobilization of proteins can also be spatially controlled since the light assisted coupling of each biomolecule to a support surface can be limited to the focal point of illumination. Present day laser technology allow for focal spots with dimensions of 1 micrometer or less. This approach allows for an extremely dense packing of identifiable and different molecules on a support surface ideal for charging microarrays with molecules.

Figure 4 shows the initial result from a 6-spot array based on light assisted immobilization using laser technology. The six peaks on the surface represent the activity of cutinase (the preferred model protein for initial immobilization experiments). Cutinase is an enzyme that degrades ester bonds in lipids, and the presentation shown is based on data extracted from an image recorded 5 minutes after the addition of a droplet of a lipidic substrate (4-methylumbelliferyl-butyrate) which fluoresces blue when degraded.

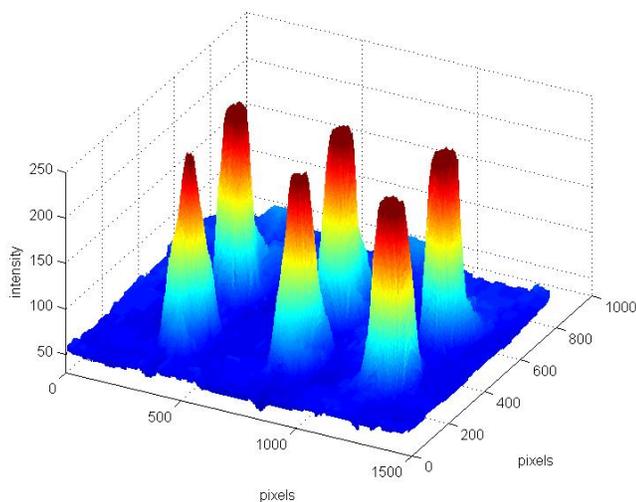


Figure 4: Functionality data from a protein array immobilized using light assisted immobilization (see text above for further explanation)

## 5 PERSPECTIVES

A possible advantage of this technology is increased sensitivity by using sensor molecules that can be immobilised in a sterically favourable way. Thereby better signals can be collected or one could settle with less sensor molecules, which could reduce either cost or size of the sensor. Furthermore this technology would be environmentally preferable since the use of hazardous chemicals for immobilization can be avoided.

We believe this new technology will find important use in the fields of biosensors, catalysis, and other fields where immobilized proteins are used. Application areas are broad, ranging from biosensors in immunochemistry to immobilization of enzymes used for industrial bioprocesses.

## 6 THE DISCOVERY OF LIGHT ASSISTED IMMOBILIZATION

As mentioned in this text is the use of chemicals to reduce (open) protein disulphide bridges, and the use of the formed free thiol groups for covalent immobilization, a well known technology for proteins immobilization (known and used for decades). The invention of light assisted immobilization is the result of knowledge picked up through years of work in the field of protein science and the intense use of fluorescence spectroscopy. Despite the knowledge of light-induced opening of disulphide bridges in proteins forming free thiol groups has been public since 1970 [1], it is somehow peculiar that the opportunity using those formed free thiol groups for

immobilizations has not been applied commercially before.

Nevertheless, the technology of light assisted immobilization is now protected [6] and is currently investigate further in a university-based venture company, BioNanoPhotonics ([www.bionanophotonics.com](http://www.bionanophotonics.com)), located at Aalborg University, Denmark.

## REFERENCES

- [1] J.J. Prompers, C.W. Hilbers, and H.A.M. Pepermans, Tryptophan mediated photoreduction of disulphide bond causes unusual fluorescence behavior of *Fusarium solani* pisi cutinase. *FEBS Lett.* 45(6), 409–416, 1999.
- [2] M.T. Neves-Petersen, Z. Gryczynski, J. Lakowicz, P. Fojan, S. Pedersen, E. Petersen, S.B. Petersen, High probability of disrupting a disulphide bridge mediated by an endogenous excited tryptophan residue. *Protein Sci.* 11, 588-600, 2002.
- [3] Y.A. Vladimirov, D.I. Roshchupkin and E.E. Fesenko, Photochemical reactions in amino acid residues and inactivation of enzymes during UV irradiation: A review. *Photochem. Photobiol.* 11, 227–246, 1970.
- [4] A. Vanhooren, B. Devreese, K. Vanhee, J. Van Beeumen, I.A. Hanssens, Photoexcitation of tryptophan groups induces reduction of two disulfide bonds in goat alpha-lactalbumin. *Biochemistry* 41(36), 11035-11043, 2002.
- [5] J. Veilleux, *IVD Technology*, 26-31, March 1996.
- [6] S.B. Petersen and M.T. D.C.A. Neves-Petersen, Light induced immobilisation, WO2004065928/DK20030000081.
- [7] Petersen MTN, Jonson PH, Petersen SB. Amino acid neighbours and detailed conformational analysis of cysteines in proteins. *Protein Engineering* 12(7), 535 – 548, 1999.