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UV Light Effects on Proteins: From Photochemistry to Nanomedicine

Maria Teresa Neves-Petersen[1,2], Gnana Prakash Gajula[3] and Steffen B. Petersen[1,2]
[1]*International Iberian Nanotechnology Laboratory (INL), Braga*
[2]*Aalborg University, Aalborg,*
[3]*Materials and Metallurgy Group, Indira Gandhi Centre for Atomic Research, Kalpakkam,*
[1]*Portugal*
[2]*Denmark*
[3]*India*

1. Introduction

Throughout 4.5 billion year of molecular evolution, proteins have evolved in order to maintain the spatial proximity between aromatic residues (Trp, Tyr and Phe) and disulphide bridges (SS) (Petersen et al, 1999). Aromatic residues are the nanosized antennas in the protein world that can capture UV light (from ~250-298nm). Once excited by UV light they can enter photochemical pathways likely to have harmful effects on protein structures. However, disulphide bridges in proteins are excellent quenchers of the excited state of aromatic residues, contributing this way to protein stability and activity. UV light excitation of the aromatic residues is known to trigger electron ejection from their side chains (Bent & Hayon, 1975a; Bent & Hayon, 1975b; Bent & Hayon, 1975c; Creed, 1984a; Creed, 1984b; Kerwin & Rammele, 2007, Neves-Petersen et al., 2009a). These electrons can be captured by disulphide bridges, leading to the formation of a transient disulphide electron adduct radical, which will dissociate leading to the formation of free thiol groups in the protein. This observation lead to the development in our lab of a new photonic technology, Light Assisted Molecular Immobilization (LAMI), used to functionalize surfaces with biomolecules. This technology is being used in order to create a new generation of biosensors with unsurpassed density (number of spots per mm^2). This technology is also being used in order to create nanoparticles based drug delivery systems relevant to nanomedical applications (Parracino et al., 2011).

In this chapter we will describe the effects of UV excitation of proteins. Furthermore, we will also describe the specific, conserved structural motif in protein molecules that can be activated by UV light, leading to the formation of reactive free thiol groups. The dynamics of formation and the lifetimes of transient species will be described. Afterwards, the different applications of LAMI will be shown. LAMI has been successfully used for the creation of protein microarrays and in order to immobilize proteins on a surface according

to any desired pattern, with submicrometer and nanometer resolution. LAMI has also been used for the creation of nanoparticle based drug delivery systems. An overview on different protein immobilization technologies will be given and the advantages of the new photonic technology will be highlighted. An overview of the use of nanoparticles in nanomedicine will also be given. Furthermore, a new light based cancer therapy which makes use of the knowledge derived from the effects of UV light on proteins will be described.

Light can change the properties of biomolecules and the number of drugs found to be photochemically unstable is steadily increasing. The effects of light on drugs include not only degradation reactions but also other processes, such as the formation of radicals, energy transfer, and luminescence. Adequate protection for most drug products during storage and distribution is needed. Indeed, proper storage conditions that secure protection from UV and visible radiation are essential for the efficacy of many common dermatologic drugs. If a drug is exposed to fluorescent tubes and/or filtered daylight for several weeks or months before it is finally administered to the patient, the drug may be altered. The most common consequence of drug photodecomposition is loss of potency with concomitant loss of therapeutic activity. It is therefore of interest to be aware of light induced reaction in biomolecules.

2. UV light induced photochemical reactions

Cells, their proteins and genes are sensitive to light. The vision process itself is initiated when photoreceptor cells are activated by light (photo-isomerization). Several papers report effects of UV light in cells and their proteins/genes. For example, UV-light is known to inhibit photosystem II activity in cyanobacterium and to enhance the transcription of particular genes (310nm light) (Vass et al., 2000). It is also known that near UV (290nm) exposed prion protein fails to form amyloid fibrils (Thakur & Rao, 2008). Nucleic acids in living cells are associated with a large variety of proteins. Therefore, it is logical to assume that the ultraviolet (UV) irradiation of cells could lead to reactive interactions between DNA and the proteins that are in contact with it. One reaction that does occur is the cross-linking between the amino acids in these associated proteins and the bases in DNA. Such reaction appears to be an important process that photoexcited DNA and proteins undergo *in vivo*, as well as in DNA-protein complexes *in vitro*. Since the crosslinking of DNA and protein by UV radiation is many times more sensitive than is thymine dimer formation, it was suggested that DNA-protein crosslinks may play a significant role in the inactivation of bacteria by UV radiation (Smith, 1962). The first amino acid shown to photochemically add to uracil was cysteine, to form 5-S-cysteinyl-6-hydrouracil (Smith and Aplin, 1966). The structure of the mixed photoproduct of thymine and cysteine was also determined (Smith, 1970). The first survey performed determined the ability of the 22 common amino acids to bind photochemically (upon 254nm excitation) to uracil. The 11 reactive amino acids were glycine, serine, phenylalanine, tyrosine, tryptophan, cystine, cysteine, methionine, histidine, arginine and lysine. The most reactive amino acids were phenylalanine, tyrosine and cysteine.

The three amino acid residues which side chains absorb in the UV range are the aromatic residues tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). Several reviews have been published on the photochemistry and photophysics of Trp (Bent & Hayon, 1975a; Creed, 1984b), Tyr (Bent & Hayon, 1975b; Creed, 1984a), Phe (Bent & Hayon, 1975c), and Cystine (name given to each bridged cysteine in a disulphide bridge) (Creed, 1984a).

Excitation to higher energy states is followed by relaxation to ground state (*e.g.* fluorescence, phosphorescence) or to excited state photochemical or photophysical processes, such as photoionization (Creed, 1984b).

Tryptophan

Flash photolysis studies have revealed two non-radiative relaxation channels from the singlet excited state of Tryptophan (Bent & Hayon, 1975a):

1. Electron ejection to the solvent, yielding solvated electrons, e^-_{aq}, which have a broad absorption peak centred at ~720 nm and the tryptophan radical cation $Trp^{\bullet+}$ which has its maximum absorption at ~560 nm. $Trp^{\bullet+}$ deprotonates rapidly, yielding the neutral radical $Trp^{\bullet}$ that has its maximum absorption at ~510 nm.

$$Trp + h\nu \rightarrow Trp^{\bullet+} + e^-_{aq} \tag{1}$$

$$Trp^{\bullet+} \rightarrow Trp^{\bullet} + H^+ \tag{2}$$

2. Intersystem crossing, yielding the triplet-state ^{3}Trp which has its maximum absorption at ~450 nm. The triplet state tryptophan can transfer an electron to a nearby disulphide bridge to give $Trp^{\bullet+}$ and the disulphide bridge electron adduct $RSSR^{\bullet-}$, where the latter has its maximum absorption at ~420 nm (Bent & Hayon, 1975a).

$$^1Trp + h\nu \rightarrow {}^1Trp^* \tag{3}$$

$$^1Trp^* \rightarrow {}^3Trp \tag{4}$$

$$^3Trp + RSSR \rightarrow Trp^{\bullet+} + RSSR^{\bullet-} \tag{5}$$

Tyrosine

Another aromatic residue with non-negligible absorption in the near-UV region is tyrosine (Tyr-OH). At neutral pH tyrosine has absorption maxima at 220nm (ε~9000 $M^{-1}cm^{-1}$) and 275nm (ε~1400 $M^{-1}cm^{-1}$) (Creed, 1984a). At alkaline pH the OH group of tyrosine side chain deprotonates. The resulting tyrosinate ($Tyr\text{-}O^{\bullet-}$) has a slightly red-shifted absorption compared to tyrosine, with maxima at 240nm (ε~11000 $M^{-1}cm^{-1}$) and 290nm (ε~2300 $M^{-1}cm^{-1}$) (Creed, 1984a). Photoexcited tyrosine can fluoresce, decay non-radiatively, or undergo intersystem crossing to the triplet state, from which most of the photochemistry proceeds. Alternatively, at neutral pH, tyrosine can be photoionized through a biphotonic process that involves absorption of a second photon from the triplet state. This results in a solvated electron (e^-_{aq}) and a radical cation ($Tyr\text{-}OH^{\bullet+}$) that will rapidly deprotonate to create the neutral radical ($Tyr\text{-}OH^{\bullet}$). Photoionization of tyrosinate at high pH is monophotonic and results in a neutral radical ($Tyr\text{-}O^{\bullet}$) and a solvated electron (e^-_{aq}).

$$^3Tyr - OH + h\nu \rightarrow Tyr - OH^{\bullet+} + e^-_{aq} \tag{6}$$

$$Tyr - OH^{\bullet+} \rightarrow Tyr - OH^{\bullet} + H^+ \tag{7}$$

$$Tyr - O^{\bullet -} + h\nu \rightarrow Tyr - O^{\bullet} + e_{aq}^{-} \quad (8)$$

The triplet state tyrosine is rapidly quenched by molecular oxygen or nearby residues like tryptophan or disulphide bridges (Bent & Hayon, 1975b):

$$^{3}Tyr - OH + RSSR \rightarrow Tyr - O^{\bullet} + H^{+} + RSSR^{\bullet -} \quad (9)$$

2.1 Important photochemical mechanism in disulphide bridge containing proteins

An important photochemical mechanism in proteins involves reduction of disulphide bridges (SS) upon UV excitation of Trp and Tyr side chains (Kerwin & Rammele, 2007, Neves-Petersen et al., 2002 & 2009a). As shown above, UV-excitation of tryptophan or tyrosine can result in their photoionization and to the generation of solvated electrons (Bent & Hayon, 1975a & 1975b; Creed, 1984b, Kerwin & Rammele, 2007, Neves-Petersen et al., 2009a). The generated solvated electrons can subsequently undergo fast geminate recombination with their parent molecule, or they can be captured by electrophillic species like molecular oxygen, H_3O^+ (at low pH), and cystines as summarized below:

$$e_{aq}^{-} + O_2 \rightarrow O_2^{\bullet -} \quad (10)$$

$$e_{aq}^{-} + H_3O^{+} \rightarrow H^{\bullet} + H_2O \quad (11)$$

$$e_{aq}^{-} + RSSR \rightarrow RSSR^{\bullet -} \quad (12)$$

In the case where the electron is captured by the cystine, the result can also be the breakage of the disulphide bridge (Hoffman & Hayon, 1972):

$$e_{aq}^{-} + RSSR \rightarrow RSSR^{\bullet -} \quad (10)$$

$$RSSR^{\bullet -} \Leftrightarrow RS^{\bullet} + RS^{-} \quad (11)$$

$$RSSR^{\bullet -} + H^{+} \Leftrightarrow RS^{\bullet} + RSH \quad (12)$$

The resultant free thiol radicals/groups can then subsequently react with other free thiol groups to create a new disulphide bridge. Reduction of SS upon UV excitation of aromatic residues has been shown for proteins such as cutinase and lysozyme (Neves-Petersen et al., 2009a, 2006 & 2002), bovine serum albumin (Skovsen et al., 2009a; Parracino et al., 2011) prostate specific antigen (Parracino et al., 2010), and antibody Fab fragments (Duroux et al., 2007). As mentioned in the introduction, this phenomenon has led to a new technology for protein immobilization (LAMI, light assisted molecular immobilization) since the created thiol groups can bind thiol reactive surfaces leading to oriented covalent protein immobilization (Neves-Petersen et al., 2006;Snabe et al., 2006; Duroux et al., 2007a, 2007b & 2007c; Skovsen et al., 2007, 2009a & 2009b; Neves-Petersen et al., 2009b; Parracino et al., 2010 & 2011).

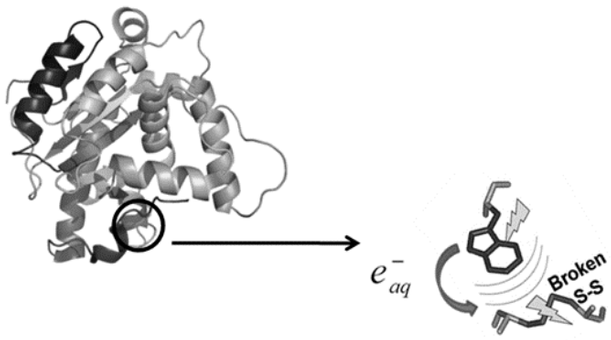

Fig. 1. The spatial proximity between aromatic residues and disulphide bridges (SS) has been conserved throughout molecular evolution. Trp is the preferred spatial neighbor of SS (Petersen et al., 1999). UV excitation of the side chain of aromatic residues leads to electron ejection. The electron can then be captured by disulphide bridges, leading to their dissociation.

2.2 Observing the solvated electron and other transient species formed upon UV excitation of proteins

In the flash photolysis experiments on lysozyme by Grossweiner and Usui (Grossweiner & Usui, 1971) it was shown that the initial photoproducts upon UV excitation of lysozyme are the photo-oxidized tryptophan residue, solvated electrons, and the cystine residue (disulphide bridge) electron adduct. In a more recent paper by Zhi Li *et al.* (Z. Li et al., 1989), experiments on a model system demonstrated that the fast electron transfer is consistent with direct electron transfer between the tryptophan triplet state and a nearby disulphide bridge (this is a very short range interaction that decays exponentially as the distance between the donor and acceptor increases). This process will result in a $RSSR^{\bullet -}$ radical, which again can result in breakage of the disulphide bridge, as shown above (scheme 12).

It is clear from the above that there are many possible pathways for the breakage of intra-molecular disulphide bridges in proteins upon UV excitation of aromatic residues, even in the absence of molecular oxygen. Breakage of the disulphide bridge can lead to conformational changes in the protein, not necessarily resulting in inactivation of the protein. Transient absorption data of *Fusarium solani pisi* cutinase has also been acquired, with supplemental experimental data on tryptophan and lysozyme as a reference (Neves-Petersen et al., 2009a). Cutinase is a good model protein for studying the UV induced breakage of disulphide bridges is since it contains only one tryptophan that is within van der Waals contact of a disulphide bridge (closest distance ~3.8 Å).

Data showed that UV excitation of cutinase lead to the formation of the solvated electron (transient species with absorption maximum around 710-720nm, see Fig. 2) and of the disulphide bridge electron adduct radical, $RSSR^{\bullet -}$ (transient species with absorption maximum around 420nm, see Fig. 2) (Neves-Petersen et al., 2009a). Figs. 2 and 3 show the kinetics of formation of the solvated electron. The increase in absorption of light at 710nm can clearly be seen following excitation at 266nm, which coincides with time zero in Figure 2. The data displayed in Fig. 3 is the intensity of absorbed light at 710nm (the intense peak displayed in Fig. 2) during the initial 43ns after excitation of cutinase with

266nm laser light. Light is absorbed at 710nm due to the presence of a new transient species created upon 266nm excitation of cutinase: the solvated electron. Solvated electrons are transient, i.e., short lived. One group that will capture them are disulphide bridges (RSSR). The capture of the solvated electrons by disulphide bridges leads to a decrease of the concentration of the solvated electron, and therefore, to a decrease in the intensity of absorbed light at 710nm. Such decay is displayed in Fig. 4. Furthermore, the combination of the solvated electron with the disulphide bridge leads to the formation of the disulphide-electron adduct radical, $RSSR^{\bullet -}$, a group which has its maximum absorption around 420nm. The presence of such group can be seen in Fig. 2, since absorption around 420nm can be observed.

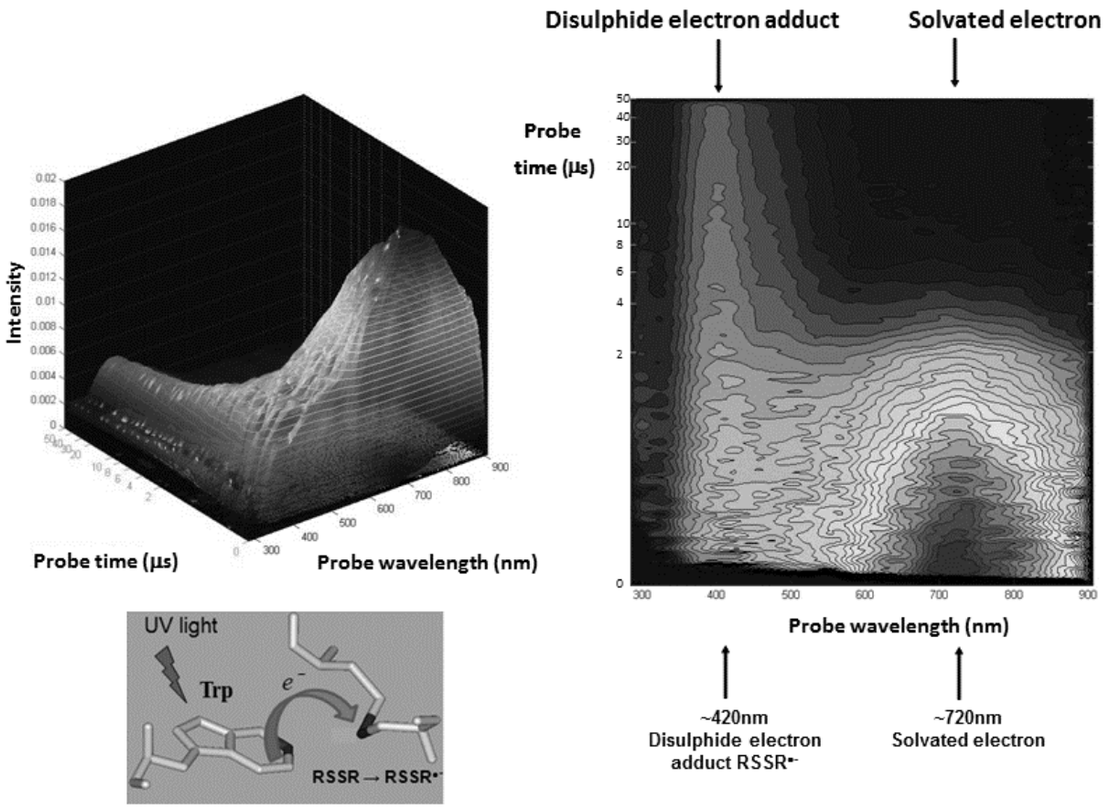

Fig. 2. Cutinase transient absorption data. Transient absorption data collected at probe times from 0 to 50μs. The transient solvated electron absorbs maximally light at ~710-720nm (intense peak) and the disulphide bridge electron adduct radical has its maximum absorption at ~420nm. The intensity of the peaks displayed in the 2D image to the right can be seen in the 3D image to the left.

Determination of the lifetimes of the different transient species formed upon UV excitation of proteins can be carried out by fitting the kinetic data displayed in Figs. 3 and 4. Upon fitting the initial increase of absorption at a 710nm and 420nm one recovers the rate of formation of solvated electrons and of the disulphide bridge electron adduct radical, respectively.

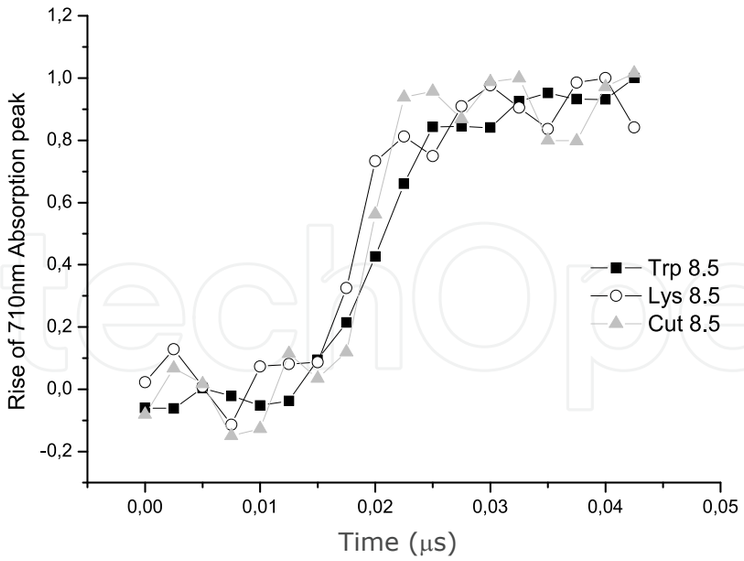

Fig. 3. Normalized transient absorption data at 710nm for 0-43ns probe times displaying the kinetics of formation of the solvated electron for tryptophan (Trp), lysozyme (Lys) and cutinase (Cut) samples at pH 8.5

Likewise, fitting the decay in the absorption peaks at 710nm and 420nm will allow us to recover the lifetime of the solvated electrons and of the disulphide bridge electron adduct radical, respectively. The lifetimes of the solvated electron in lysozyme and cutinase samples at different pH values can be found in Table I. Below is shown the decay kinetics of the solvated electron within 10 μs after 266nm excitation of cutinase (Neves-Petersen et al., 2009a).

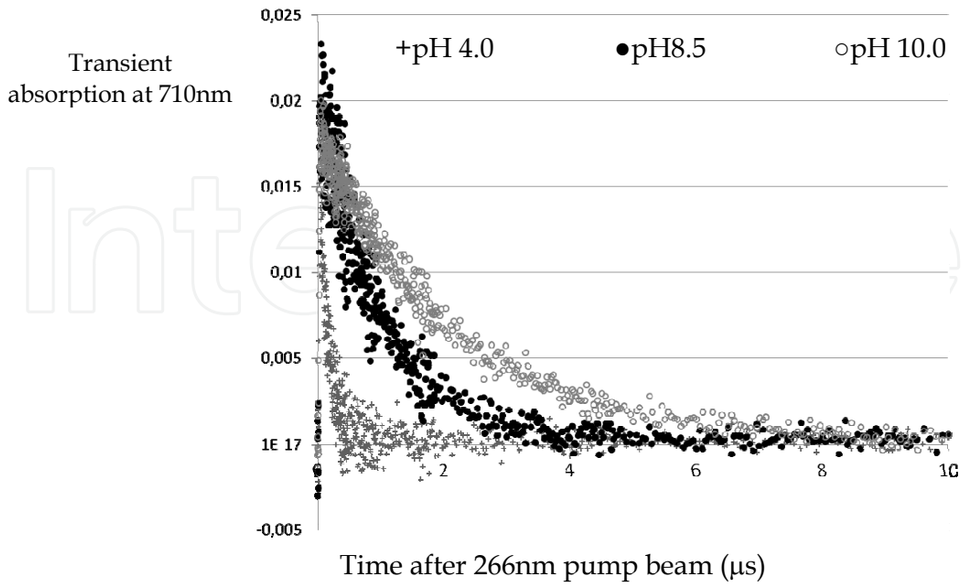

Fig. 4. Decay kinetics of the solvated electron absorption peak at 710nm within 10μs after excitation.

The governing equations for the time-resolved intensity decay data were assumed to be a sum of exponentials as in

$$Abs(t) = \sum \alpha_i \cdot exp\left(\frac{-t}{\tau_i}\right) \tag{13}$$

where *Abs*(t) is the intensity decay, α_i is the amplitude (pre-exponential factor), τ_i the lifetime of the i-th component and $\sum\alpha_i = 1.0$. Data was analysed using a global analysis approach.

The fractional intensity f_i of each decay time is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{14}$$

and the mean lifetime is

$$\langle \tau \rangle = \sum_i f_i \tau_i \tag{15}$$

It was observed that the solvated electron average lifetime is shorter at acidic pH values, which is correlated with the fact that H_3O^+ captures the solvated electron. Furthermore, the solvated electron lifetime is significantly shorter in protein systems as compared to from Trp alone in solution, thus indicating that a protein offers other pathways involving capture of the solvated electron.

	Tryptophan	Lysozyme	Cutinase
pH	<t> (μs)	<t> (μs)	<t> (μs)
4.0	1.0	0.3	0.3
7.5	1.1	0.7	
8.5	2.3	0.8	1.0
10.0	2.8	1.6	2.1

Table 1. Mean lifetime of the solvated electron in all samples at different pH values.

Data analysis shows that the solvated electron has ns and sub μs decay lifetimes (Neves-Petersen et al., 2009a). These different lifetimes can be explained due to different recombination pathways of the solvated electron: recombination with the parent molecule (geminate recombination), with the hydronium ion present in the solvent or with other electron acceptor, such as disulphide bridges and positively charged groups. The intensity of the solvated electron peak is clearly pH dependent (Neves-Petersen et al., 2009a). This is correlated with the fact that the hydronium ion H_3O^+ is an electron scavenger. Recombination happens according to the reaction (Spanel & Smith, 1995):

$$e_{aq}^- + H_3O^+ \rightarrow H^\bullet + H_2O \tag{16}$$

Therefore, the lower the pH the faster the rate of decay of solvated electrons formed upon UV excitation of Trp molecules. In a protein, besides H_3O^+, different groups can act as electron scavengers, e.g. positively charged residues, the carbonyl group of the peptide chain (Faraggi & Bettelheim, 1977) as well as disulphide bridges, according to the reaction below:

$$e_{aq}^- + RSSR \rightarrow RSSR^{\bullet -} \tag{17}$$

Data shows that the higher the pH the longer time it takes for the solvated electron to recombine with the parent molecule (geminate recombination) or another electron scavenger molecule, such as H_3O^+. The observed lifetime increase with pH can be explained since the lower the pH, the higher the concentration of H_3O^+ and therefore the larger the probability of recombination of the solvated electron with the hydronium ion. Furthermore, for proteins, the higher the pH of the solution, the larger the number of basic titratable residues that have lost their positive charge and became neutral (His, Lys, Arg) and the larger the number of acidic titratable residues that have acquired a negative charge (Asp, Gly, Tyr, Cys not bridged). This means that an increase of pH leads to a loss of positive charge in the protein and a gain of neutral and negative charged residues in the protein. This will lead to an increase of the areas in the protein that carry a negative electrostatic potential. Therefore, an increase in pH will decrease the efficiency of electron recombination with the molecule due to electrostatic repulsion. This will lead to an increase of the solvated electron lifetime, as observed in Fig. 4.

3. Protein Immobilization onto surfaces: An overview

The importance of immobilisation technology is demonstrated by the recent development of DNA microarrays, where multiple oligonucleotide or cDNA samples are immobilised on a solid surface in a spatially addressable manner. These arrays have revolutionised genetic studies by facilitating the global analysis of gene expression in living organisms. Similar approaches have been developed for protein analysis, where as little as one picogram of protein need be bound to each point on a microarray for subsequent analysis. The proteins bound to the microarrays, can then be assayed for functional or structural properties, facilitating screening on a scale and with a speed previously unknown. The biomolecules bound to the solid surface may additionally be used to capture other unbound molecules present in the mixture. Development of this technology, with the goal of immobilising a biomolecule on a solid surface in a controlled manner, with minimal surface migration of the bound moiety and with full retention of its native structure and function, has been the subject of intensive investigation in recent years (Veilleux & Duran, 1996). The simplest type of protein immobilisation exploits the high inherent binding affinity of surfaces to proteins in general. For example, proteins will physically adsorb to hydrophobic substrates via numerous weak contacts, comprising van der Waals and hydrogen bonding interactions. The advantage of this method is that it avoids modification of the protein to be bound. On the other hand, adsorbed proteins may be distributed unevenly over the solid support and/or inactivated since, e.g., their clustering may lead to steric hindrance of the active site/binding region in any subsequent functional assay.

Molecules can be immobilised on a carrier or solid surface either passively through hydrophobic or ionic interactions, or covalently by attachment to surface groups. In response to the enormous importance of immobilisation for solid phase chemistry and biological screening, the analytical uses of the technology have been widely explored. The technology has found broad application in different areas of biotechnology, e.g. diagnostics, biosensors, affinity chromatography and immobilisation of molecules in ELISA assays. Alternative methods of immobilisation rely on the use of a few strong covalent bonds to bind the protein to the solid surface (Wilson & Nock , 2001). Examples include immobilisation of biotinylated proteins onto streptavidin-coated supports, and immobilisation of His-tagged proteins, containing a poly-histidine sequence, to Ni^{2+}-chelating supports. Other functional groups on the surface of proteins which can be used for attachment to an appropriate surface include reacting an amine with an aldehyde via a Schiff-base, cross-linking amine groups to an amine surface with gluteraldehyde to form peptide bonds, cross-linking carboxylic acid groups present on the protein and support surface with carbodiimide, cross-linking based on disulphide bridge formation between two thiol groups and the formation of a thiol-Au bond between a thiol group and a gold surface. Amine groups in proteins are widely used for protein covalent immobilization via NHS (N-hydroxysuccinimide)-EDC (N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride) chemistry (Johnsson et. al., 1991). Following immobilisation, un-reacted N-hydroxysuccinimide esters on the support are deactivated with ethanolamine hydrochloride to block areas devoid of bound proteins. The method is laborious since the reagents, used at each step of a chemical immobilization method, usually need to be removed prior to initiating the next step.

Methods for the immobilization of biomolecules via disulphide bridges are described by Veilleux J (1996). Protein samples are treated with a mild reducing agent, such as dithiothreitol, 2-mercaptoethanol or tris(2-carboxyethyl)phosphine hydrochloride to reduce disulphide bonds between cysteine residues, which are then bound to a support surface coated with maleimide. Alternatively primary amine groups on the protein can be modified with 2-iminothiolane hydrochloride (Traut's reagent) to introduce novel sulfhydryl groups, which are thereafter immobilized to the maleimide surface. Immobilization of proteins on a gold substrate via SH groups formed upon intracellular reduction of surface engineered disulphide bridges is shown for cupredoxin protein plastocyanin (Andolfi et al., 2002). The disulphide bridge has been engineered upon mutating the solvent accessible residues Ile21 and Glu25. An alternative approach to engineering thiol-groups into a protein has been described for ribonuclease (RNaseA), which has four essential cystines (Sweeney et al., 2000). In this case a single cysteine residue was substituted for Ala19, located in a surface loop near the N-terminus of RNase A. The cysteine in the expressed RNase was protected as a mixed disulphide with 2-nitro-5-thiobenzoic acid. Following subsequent de-protection with an excess of dithiothreitol, the RNase was coupled to the iodoacetyl groups attached to a cross-linked agarose resin, without loss of enzymatic activity. Again, preparation of the protein for immobilization requires its exposure to both protecting and de-protecting agents, which may negatively impact its native structure and/or function.

Light-induced immobilization techniques have also been explored, leading to the use of quinone compounds for photochemical linking to a carbon-containing support (European patent EP0820483). Activation occurs following irradiation with non-ionizing UV and visible light. Masks can be used to activate certain areas of the support for subsequent attachment of

biomolecules. Following illumination, the photochemically active compound anthraquinone will react as a free radical and form a stable ether bond with a polymer surface. Since anthraquinone is not found in native biomolecules, appropriate ligands have to be introduced into the biomolecule. In the case of proteins, this additional sample preparation step may require thermochemical coupling to the quinone and may not be site specific. A further development of light-induced immobilization technology is disclosed in US patents US 5,412,087 and US 6406844, which describe a method for preparing a linker bound to a substrate. The terminal end of the linker molecule is provided with a reactive functional group protected with a photo-removable protective group, e.g. a nitro-aromatic compound. Following exposure to light, the protective group is lost and the linker can react with a monomer such as an amino acid at its amino or carboxy-terminus. The monomer, furthermore, may itself carry a similar photo-removable protective group which can also be displaced by light during a subsequent reaction cycle. The method has particular application to solid phase synthesis, but does not facilitate orientated binding of proteins to a support. Bifunctional agents possessing thermochemical and photochemical functional substituents for immobilizing an enzyme are disclosed in US patent US 3,959,078. Derivatives of arylazides are described which allow light mediated activation and covalent coupling of the azide group to an enzyme, and substituents which react thermochemically with a solid support. The orientation of the enzyme molecules is not controlled. A method for oriented, light-dependent, covalent immobilization of proteins on a solid support, using the heterobifunctional wetting-agent N-(m-(3-(trifluoromethyl)diazirin-3-yl)phenyl)-4-maleimidobutyramine, is described by Collioud et al. (Collioud et al., 1993). The aryldiazirine function of this cross-linking reagent facilitates light-dependent, carbene-mediated, covalent binding to either inert supports or to biomolecules such as proteins, carbohydrates and nucleic acids. The maleimide function of the cross-linker allows binding to a thiolated surface by thermochemical modification of cysteine thiols. However this treatment may modify the structure and activity of the target protein. Light-induced covalent coupling of the cross-linking reagent to a protein via the carbene function, however, has the disadvantage that it does not provide controlled orientation of the target protein.

Common for most of the described immobilization methods is their use of one or more thermochemical/chemical steps, sometimes with hazardous chemicals, some of which are likely to have a deleterious effect on the structure and/or function of the bound protein. The available methods are often invasive, whereby foreign groups are introduced into a protein to act as functional groups, which cause protein denaturation, as well as lower its biological activity and substrate specificity. There is a need in the art of protein coupling and immobilization to improve the method of coupling, where the structural and functional properties of the coupled or immobilized component are preserved and the orientation of coupling can be controlled. We believe that LAMI represents a significant step in this direction.

3.1 Light Assisted Molecular Immobilization technology (LAMI)

Photochemistry, biosensor microarrays and drug delivery systems

Light assisted molecular immobilization technology provides a photonic method for coupling a protein or a peptide on a carrier via stable bonds (covalent bond or thiol-Au bond) while preserving the native structural and functional properties of the coupled protein or peptide. This technology avoids the use of one or more chemical steps, in contrast with traditional coupling methods for protein immobilisation, which typically involve several chemical

reactions. That can be costly, time-consuming as well as deleterious to the structure/function of the bound protein. Furthermore the orientation of the protein or peptide, coupled according to the method of the present invention, can be controlled, such that their functional properties, e.g. enzymatic, may be preserved. In comparison, the majority of known protein coupling methods lead to a random orientation of the proteins immobilised on a carrier, with the significant risk of lower biological activity and raised detection limits.

LAMI technology exploits an inherent natural property of proteins and peptides, whereby a disulphide bridge in a protein or peptide, located in close proximity to an aromatic amino acid residue, is disrupted following excitation of aromatic amino acids. The aromatic residues are actually the preferred spatial neighbours of disulphide bridges (Petersen et al., 1999). The thiol groups created by light induced disulphide bridge disruption in a protein or peptide are then used to immobilise the protein or peptide to a carrier. The formed free thiol groups in the protein can afterwards attach the protein onto a thiol reactive surface, such as gold, thiol derivatized glass and quartz, or even plastics (see Fig. 5). The new protein immobilization technology has led to the development of (Neves-Petersen et al., 2006;Snabe et al., 2006; Duroux et al., 2007a, 2007b & 2007c; Skovsen et al., 2007, 2009a & 2009b; Neves-Petersen et al., 2009b; Parracino et al., 2010 & 2011):

- microarrays of active biosensors and
- biofunctionalization of thiol reactive nanoparticles, aiming at engineering drug delivery systems

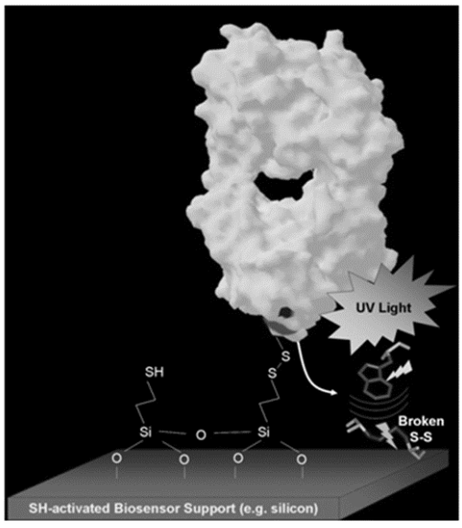

Fig. 5. The principle of light assisted molecular immobilization (LAMI) sketched with tryptophan near a disulphide bridge on a protein molecule. UV illumination of aromatic residues leads to disulphide bridge (SS) opening and to the formation of free SH groups, which will react to thiol reactive surfaces.

3.2 Bio-functionalization of surfaces with micrometer-resolution

With a beam of UV laser light we are able to open disulphide bonds in most SS-containing proteins. If this happens at or close to a thiol reactive surface, such as thiol derivatized glass,

quartz or a gold surface, the protein is immobilized onto the surface. Since this happens where the UV photons are present, the size of the focal spot e.g. in a simple focusing setup determines where immobilization takes place. We are able to control this process such that spot size is ~3-5 micron. The process is relatively fast, being determined by physical chemical parameters as well as the light fluency (power per unit area). Currently we are operating with 100 ms illumination per spot with ~1mW 280nm 8MHz femtosecond pulses (Duroux et al, 2007). With a pitch of 10 micron and spot size of 5 micron, this allows for about 40.000 spots per mm^2. We have verified that Fab anti prostate specific antigen can be immobilized with our technology and still remains biologically active (Parracino et al., 2010).

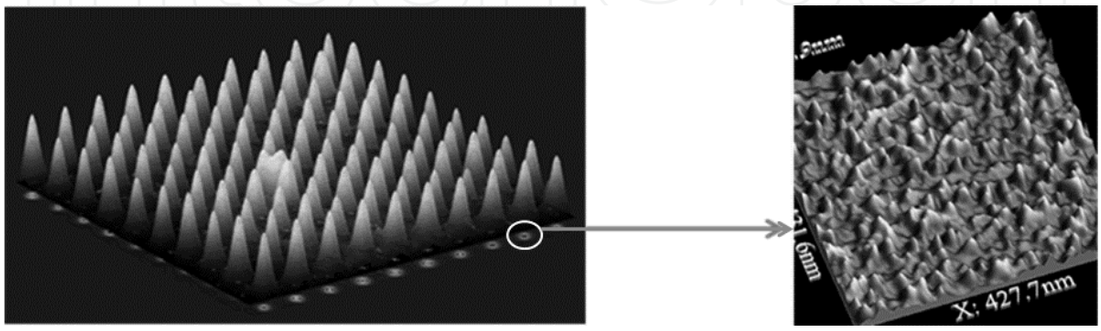

Fig. 6. (Left) Protein microarray engineered with LAMI using 280nm laser light focused to a spot size of 5 micron. The displayed array has 5 micron spots, 10 micron pitch leading to an array density equivalent to 10^4 spots per $1mm^2$ of sensor surface. (Right) Atomic Force Microscopy visualization of protein immobilized using LAMI, of the central area of 1 spot of the displayed array.

Although light assisted immobilization technology has obvious applications in the area of biosensor microarraying, any pattern of immobilized proteins can be generated. As such, we have successfully transferred several different bitmaps to selected surfaces. As seen in the figure a bitmap of a fullerene was printed into a protein pattern that retained almost all graphical details of the original bitmap (Neves-Petersen et al., 2009b). The size of the protein printed surface is $1mm^2$.

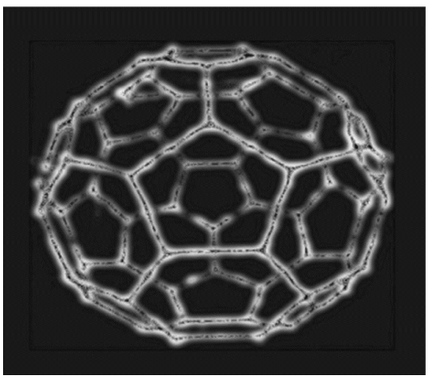

Fig. 7. Fluorescence is emitted from proteins that have been immobilized with LAMI technology. A film of protein has been illuminated according to a fullerene pattern. The illuminated proteins (280nm excitation) were immobilized onto the thiol reactive surface. The image has 10μm resolution and dimensions ~1260μm x 1220 μm

In Fig. 8 is displayed the optical setup used in order to immobilize the proteins according to a particular bitmap:

- The slide surface is covered with a protein film
- A bit map is loaded into the computer
- The surface is illuminated according to the bitmap, i.e., light will hit the surface reproducing the image in the bitmap
- Molecules will only be immobilized on the surface if they have been illuminated
- The slide is washed, removing the non-illuminated and therefore non-immobilized proteins
- The fluorescence of the immobilized molecules can be observed

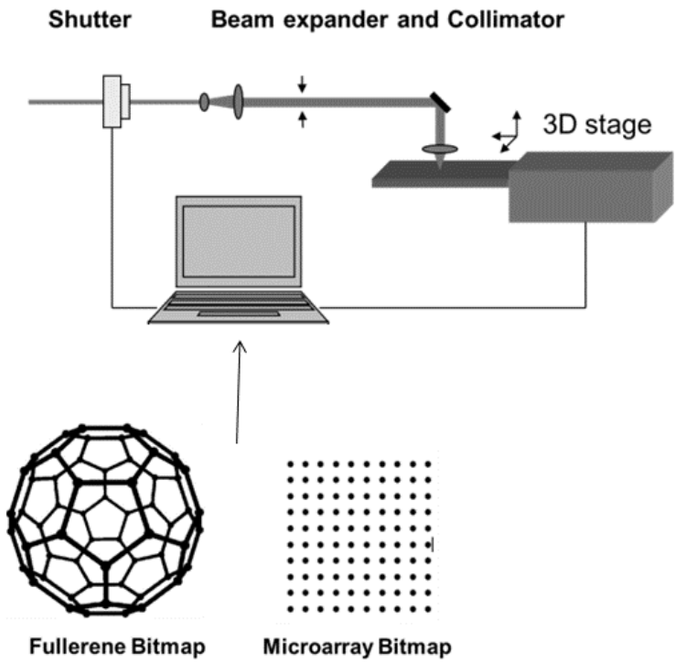

Fig. 8. Optical setup used in order to immobilize the proteins according to a particular bitmap. The slide were the protein film is placed on is optically flat and derivatised with thiol (SH) groups using silane chemistry.

3.3 Biofunctionalization of surfaces with nm/submicrometer-resolution

The technique of UV-light assisted immobilization of disulphide containing proteins has been combined with the Fourier transforming properties of lenses as well as with a simple mm scale feature size spatial mask. Theory predicts that when light passes through a spatial mask placed in the back focal plane of a focusing lens, we should obtain an intensity pattern in the front focal plane corresponding to the Fourier transform of the spatial mask (see Fig. 9).

A spatial mask consisting of eight holes arranged in a square with a hole in each corner and in the middle of each side is displayed below (Fig. 10). This pattern was then Fourier transformed in order to evaluate the diffraction pattern that the simplified eight-hole mask would generate after being Fourier transformed by a lens. The diffraction patter is displayed in Fig. 10

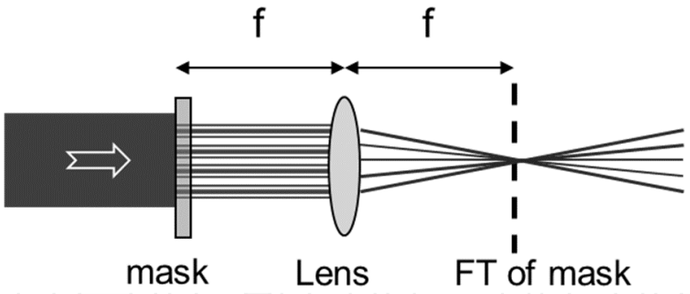

Fig. 9. Consider a thin lens illuminated by a monochromatic plan wave. According to the theory of Fourier optics, an aperture (transmission mask) placed in the back focal plane of a lens will generate a diffraction pattern in the front focal plane that will be identical to the FT of the aperture.

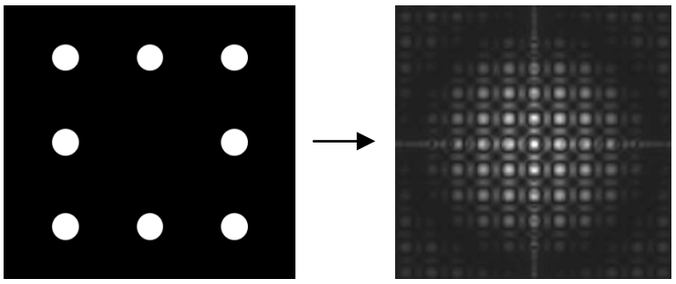

Fig. 10. A spatial mask of eight holes arranged in a square (total dimension of mask is 1cmx1cm) placed in the back focal plane of a lens, will give rise to a diffraction pattern displayed to the right (central part of the diffraction pattern).

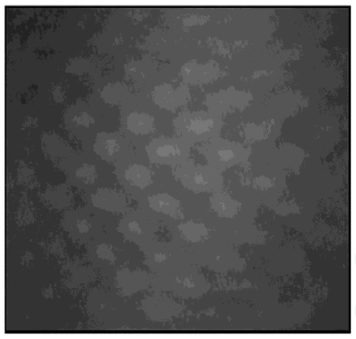

Fig. 11. Fluorescence image of the fluorescently labeled proteins immobilized using the simple eight-hole mask, depicting the fluorescence emission from FITC. The peaks in the array are interspaced by ~1.5µm and have a FWHM of 750nm. The pitch of 1.5µm corresponds to a spot density of ~4.5x10^5 spots per mm^2.

Using the light pattern produced by this simple mask with 8 x 1mm sized holes, in a single shot we produce multiple spots with immobilized protein with a spot size of ~700 nm (Fig. 11 and Skovsen et al., 2009b). With this spot density, we can populate 1 mm^2 with close to 1 million sensor spots, which represents an improvement of 10 fold over existing commercially available high density protein arraying methods. Our approach bypasses the use of micro dispenser techniques – and the technical difficulties associated with the use of

such. It is simple, and fast. With our current ability to generate immobilized patterns with a spot size down to 700 nanometer, we believe that we can design spatial patterns of binding proteins which could identify and bind, e.g., specific cells such as stem cells.

Our previous works report that we do see patterns similar to what theory predicts (Skovsen et al., 2009b; Petersen et al., 2010) but not always identical (Petersen et al., 2010). We have also shown that the presence of biomolecules on the slide surface can break the diffraction pattern of light (Petersen et al., 2010).

4. Biofunctionalization of thiol reactive nanoparticles, aiming at the development of drug delivery systems

For the last few decades, research efforts have been focused on the development of new materials at the atomic, molecular and macromolecular levels, on the length scale of approximately 1-100nm in order to build materials at the nanoscale, and to explore structures, devices and systems that have novel properties. It has been shown that it is possible to tune precisely the physico-chemical properties of nano-materials by modifying the crystal size, shape and composition. Among various nano materials, magnetic core-shell particles have been broadly used in many technological applications, especially for biological applications such as drug targeting and delivery, cell labeling and separation, cancer therapy, magnetic resonance image (MRI) contrast agents, bio-sensors and bio-imaging. Core-shell particles result from the combination of different metals that together display new properties compared to their monometallic counterparts. Therefore, the combination of both metal's properties such as optical, electrical, magnetic and catalytic can be used for technological applications. Among the core-shell nanoparticles, Fe_3O_4@Au and Fe_3O_4@SiO_2 particles are widely used not only for its magnetic, optical and chemical properties but also for chemical stability, good biocompatibility, low toxicity, easy dispersibility, affinity towards biomolecules with amine/thiol/carboxylic terminal groups and convenient preparation techniques. Magnetite nanoparticles of size below 26.1 nm are super-paramagnetic in nature which means that these particles can be controlled by external magnetic field but retain no coercivity value (no residual magnetism) once the field is removed (Gnanaprakash et al., 2007). This magnetic property is being used extensively for biosciences in various applications, including bioseparation and imaging. Since biomolecules are highly sensitive to pH, temperature and chemical environment, immobilization protocols should be developed in order to secure high molecular activity and stability.

4.1 New photonic methodology used to create functional nanoparticles

Recently, many protocols have been proposed for the immobilization of various biomolecules on to the particle surfaces for novel properties and various applications. The review paper on "Chemical Strategies for Generating Protein Biochips" by Jonkheijm et al. (Jonkheijm et al., 2008) describes different approaches using covalent and non-covalent immobilization chemistry are reviewed. Recent studies demonstrated that the incorporation of chiral molecules onto nanoparticles provides new opportunities for achieving specificity in the recognition of protein surfaces (You et al. 2008). Immobilization of trypsin on super-paramagnetic nanoparticles allows using higher enzyme concentrations, leading to shorter

digestion time than free enzyme molecules and easy separation from the solution (Y.Li et al. 2007). Earlier studies have shown that immobilization of biomolecules not only makes separation easier but also increases the stability of enzyme towards pH, temperature, chemical denaturants and organic solvents (Z. Yang et al. 2008, H.Yang et al., 2004). Water soluble carbodiimide was used to activate the direct adsorption of glucose oxidase, streptokinase, chymotrypsin, dispase, BSA and alkaline phosphatase on magnetic particles (Koneracka et al. 2002). Recently, decanthiol capped gold nanoparticles were modified with dithiobis (succinimidyl propionate) for BSA coupling by ligand exchange (H.-Y.Park et al. 2007). Ma and colleagues used condensation product of 3-glycidoxypropyltrimethoxysilane and iminodiacetic acid charged with Cu^{2+} to immobilize BSA onto the silica coated magnetic nanoparticles through metal ion affinity towards protein (Ma et al., 2006).

We are tagging biomolecules directly onto magnetic nanoparticles using our new photonic technology, light assisted molecular immobilization. The surface of these nanoparticles is thiol reactive, being gold or thiol derivatised silica. These particles provide very high surface area to tag protein efficiently and also there is no need to use other reagents to enhance the binding. The surface affinity towards the thiol groups present in the protein will be used to immobilize the protein molecule onto the nanoparticle. Bovine serum albumin (BSA, a carrier protein) and insulin have been successfully immobilized with our new photonic technology. Recently, LAMI technology has been used to create free and active thiol functional groups in BSA to be linked to Fe_3O_4@Au core-shell nanoparticle (Parracino et al., 2011).

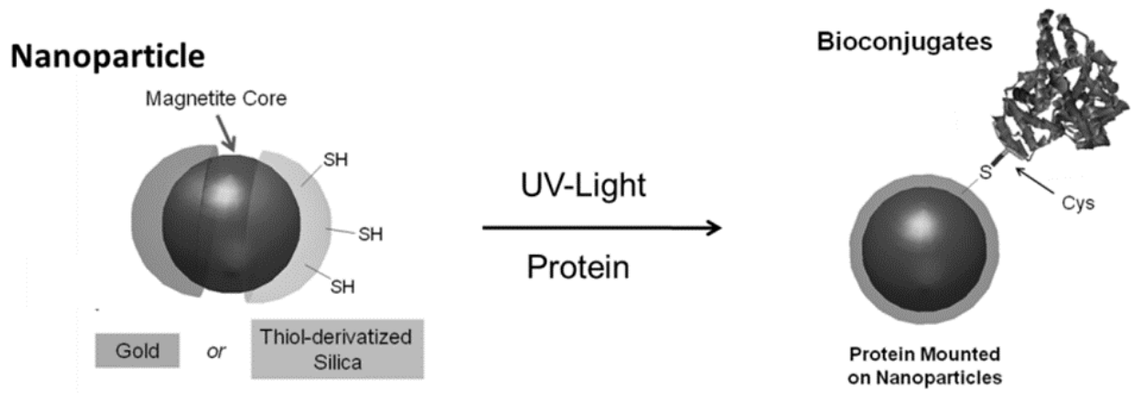

Fig. 12. Covalent immobilization of proteins onto the thiol reactive surface of nanoparticles using light assisted molecular immobilization (LAMI) technology, in order to engineer drug delivery systems.

We have developed the necessary technology that allows us to produce a variety of nanoparticles, from gold and silica nanoparticles to core-shell superparamagnetic nanoparticles. Furthermore, we can further derivatise the silica outer layer of those nanoparticles with chemical functional groups, such as thiol, amino and carboxylic groups. The combination of such knowledge with our new photonic immobilization technology allows us to build protein bioconjugates in a new way. Our new photonic immobilization technology is ideal to couple drugs, proteins, peptides, DNA and other molecules to nanoparticles such as gold or biopolymer nanospheres, which can subsequently be used as molecular carriers into cells for therapeutic purposes.

4.2 Medical applications of bio-functionalized nanoparticles

Nanomedicine is the medical application of nanotechnology and related disciplines, which mostly include biocompatible nanoparticle platforms that contain both therapeutic and/or imaging components. Since biomolecules such as individual cells, mRNA, DNA and proteins are nanoscale sized, probes of equivalent dimensions can provide very effective detection of individual chemical interactions of biomolecules, understanding of the chemical reactions and manipulation of the same. Therefore, the integration of nanotechnology and medical sciences has led to fundamental understanding in molecular biology, as well as new advanced technological applications such as drug targeting and delivery, cell labeling and separation, cancer therapy, magnetic resonance image (MRI) contrast agents, bio-sensors and bio-imaging. Nanoparticles may carry chemo-, radio-, and gene therapeutics or combination of these. They can be inorganic nanoparticles (noble metal, metal oxide, silica, mesoporous silica and combination of these components), lipid aggregates, and synthetic surfactant-polymer systems (such as vesicles, micelles). Inorganic nanoparticles that have unique physicochemical properties allow applications in nanomedicine after proper synthesis, coating, surface functionalization and bioconjugation. These nanosized materials provide a robust framework in which two or more components can be incorporated to give multifunctional capabilities (Wang et al., 2008; Salgueiriño-Maceira & Correa-Duarte, 2007). The combination of metals or polymer molecules should provide suitable and tunable magnetic, optical and chemical properties, chemical stability, low toxicity, easy dispersibility, affinity towards biomolecules with amine/thiol/carboxylic terminal groups and convenient preparation techniques.

Among the various compositions, gold composites are used for the development of various clinical diagnosis methods (Baptista et al., 2008; Raj et al., 2011) because of its size dependent optical properties. Time-resolved single-photon counting fluorescence studies on porphyrin monolayer-modified gold clusters revealed resonant energy transfer between the porphyrin and the gold surface, which is a phenomenon of considerable interest in biophotonics (Imahori & Fukuzumi, 2001). The ability to control the size and shape of gold nanoparticles and their surface conjugation with antibodies allow for both selective imaging and photo-thermal killing of cancer cells by using light with longer wavelengths for tissue penetration (Gobin et al., 2007). Similar success was also demonstrated with polymer-coated superparamagnetic iron oxide nanoparticles conjugated with, e.g., fluorescent molecules, tumor-targeting moieties and anticancer drugs which aim targeting human cancers. Imaging inside the body can either be done using magnetic resonance or fluorescence imaging (Kohler et al., 2006). Quantum dots optical properties including bright emission, photostability, size dependant luminescence and long fluorescence lifetimes make them also suitable for bioimaging applications. In combination with superparamagnetic nanoparticles and surface modification with peptides or other functional groups, these multifunctional particles are being used in bioimaging, bioseparation and in order to understand the behaviour of nanoparticles in cells such as tracking the particles, cell uptake of particles, drug dose evolution at targeted site (Janczewski et al., 2011; Summers H.D et al., 2011). Many biosensors and bioseparation protocols were also demonstrated using such multifunctional nanoparticles (Rossi et al., 2006; Liu and Xu, 1995; Fan et al., 2003). Figure 13 shows the example of functional nanoparticles. Silica nanoparticles can be linked to functional groups like carboxylic, thiol, amine or hydroxide, in order to attach dye

molecules and therapeutic molecules to track particles inside the human body and targeted drug delivery. However, intense research studies are needed in order to overcome the barriers in the human body that challenge the efficiency of nanoparticle delivery such as walls of blood vessels, physical entrapment of particles in organs and removal of particles by phagocytic cells. Therefore, the ideal nanoparticle system to be used in nanomedicine should not only overcome such barriers but also allow for real time visualisation of particles, detection of the damaged tissues, selective and rapid accumulation at diseased tissue, effective drug delivery or effective therapy (in the case of hyperthermia). Hence, pioneering works are being carried out at related interdisciplinary fields such as chemistry, biology, pharmacy, nanotechnology, medicine and imaging.

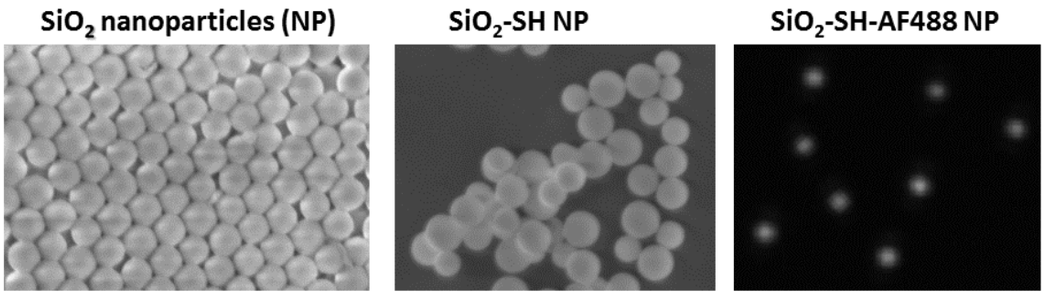

Fig. 13. The surface of silica nanoparticles can be functionalized with different chemical groups which can be used to bind fluorophores (e.g. in this example the fluorophore AF 488 has been coupled to the SH groups introduced on the surface of bare silica nanoparticles), proteins or other molecules.

Nanoparticles based drug delivery is particularly advantageous when planning cancer therapy because the leaky blood cells in tumours mean that particles of certain size tend to accumulate more in cancer tissues than in normal tissues (Peer D et al, 2007). By coating nanoparticles with biomolecules that recognize receptors on cell membranes, a wide range of drugs and imaging agents can enter cells (endocytosis). Drug loaded magnetic particles can be localized to the specific site by an external magnetic field (Hu et al., 2008). This allows more concentrated doses of the anticancer drugs to be delivered to the cancer cells and keep them on site for longer periods of time. In order to prevent dangerous agglomeration of the particles in the blood stream, the particles must be of a small size relative to the dimensions of the capillaries, monodisperse and spherical in shape. In addition, the particles must have a high magnetic moment and switch their magnetisation quickly at low fields (Ankamwar et al., 2010; Shah et al., 2011). Nanoparticles surface modified with amphiphilic polymeric surfactants such as poloxamers, poloxamines or polyethylene glycol derivatives (S.-M. Lee et al., 2010), folic acid derivatives (Das et al, 2008; Landmark et al., 2008), silica derivatized with different functional groups or with porous structure (Wang et al., 2008; Slowing et al., 2007) are used for drug targeting and delivery. Moreover, triggerable drug delivery systems enable on-demand controlled release of drugs that may enhance therapeutic effectiveness and reduce systemic toxicity. Recently, a number of new materials have been developed that exhibit, e.g., sensitivity to UV and visible light, such that irradiation can release covalently bound drugs from dendrimers or dendrons with photocleavable cores or photoactivated surfaces (C. Park et al., 2008), temperature (Bikram et al., 2007; Peng et al., 2011), near-infrared light (S.-M.Lee et al., 2010), pH (Benarjee & Chen, 2008), ultrasound (Bawa et al.,

2009), or external magnetic fields (Hu et al., 2008). Long-wavelength light such as radio frequency radiation and microwave radiation have also been used to trigger drug release. This responsiveness can be triggered remotely to provide flexible control of dose magnitude and timing. Mann et al. (Mann et al., 2011) demonstrated enhanced delivery of therapeutic liposomes carried in E-selectin thioaptamer conjugated porous silica particles to the bone marrow tissue. These particles can also be utilised to deliver imaging agents and growth factors (eg. colony stimulating factor) for the protection of bone marrow against chemotherapy and radiation.

The use of biocompatible iron oxide particles for hyperthermia is increasing in cancer therapy. Iron oxide magnetic nanoparticles exposed to an alternating magnetic field act as localized heat sources at certain target regions inside the human body. The heating of magnetic oxide particles with low electrical conductivity in an external alternating magnetic field is mainly due to either loss processes during the reversal of coupled spins within the particles or due to frictional losses if the particles rotate in an environment of appropriate viscosity. Magnetic nanoparticles coated with amphipathic polymer pullulan acetate (food additive) were examined for their cytotoxicity and cellular uptake. Moreover, in vitro hyperthermia treatment of KB cells produced therapeutic efficacies of 56% and 78% at 45°C and 47°C, respectively, indicating the great potential of surface modified magnetic nanoparticles as magnetic hyperthermia mediators (Gao et al., 2010). Gonzalez-Fernandez and co-workers have presented a study on the magnetic properties of bare and silica-coated ferrite nanoparticles with sizes between 5 and 110 nm (Gonzalez-Fernandez et al., 2009). Their results show a strong dependence of the power absorption with particle size, with a maximum around 30 nm, as expected for a Neel relaxation mechanism in single-domain particles. Recently, in order to enhance the heat conversion capacity of nanoparticles from electromagnetic energy into heat, core-shell nanoparticles were designed to exploit the advantage of exchange coupling between a magnetically hard core and magnetically soft shell to maximize the specific loss power (J.H. Lee et al., 2011). However, in order to avoid the risk of overheating during the hyperthermia effect, curie temperature tuning is done by designing the composition of the core magnetic nanoparticles (Kaman et al., 2009).

MRI is one of the most useful diagnostic tools for medical sciences. MRI contrast agents are chemical substances introduced to the region being imaged to increase the contrast between different tissues or between normal and abnormal tissue, by altering the relaxation times. Generally, gadolinium or manganese salts as well as superparamagnetic iron-oxide based particles are by far the most commonly used materials as MRI contrast agents. Superparamagnetic iron oxide based contrast agents have the advantage of producing an enhanced proton relaxation in MRI better than those produced by paramagnetic ions. Consequently, lower doses are needed which reduce to a great extent the secondary effects in the human body. Particle's negligible remanence after removing the magnetic field (minimizes the particles aggregation) and low toxicity makes them beneficial for in vivo applications (Kinsella et al., 2011; Y.Park et al., 2009).

Various biosensors were developed exploiting novel properties of nanomaterials. Rossi and co-workers have shown the utility of fluorescent nanospheres to detect the breast cancer marker HER2/neu in a glass slide based assay (Rossi et al., 2006). For the detection of cholesterol, plasmon resonance properties of gold nanoparticles were used after conjugating digitonin onto the surface of gold nanoparticles (Raj et al., 2011).

4.3 Self-organization of magnetic nanoparticles

The magnetic properties and self-organization of magnetic nanoparticles has to be understood in order to use these particles for biological applications. Extensive studies have been carried out in this direction. Superparamagnetic nanoparticles consisting of single-domain magnetite nanoparticles are randomly dispersed in liquid in the absence of external magnetic field. Under an external magnetic field, these particles are assembled to form chain-like structures along the magnetic field lines. An attractive magnetic force due to the magnetic dipole is balanced by repulsive electrostatic and solvation forces. Upon removal of the external magnetic field, thermal energy dominates and the particles disintegrate (Fig. 14). A wide range of studies were carried out for quantitative investigation of the temporal self-organization of superparamagnetic composite particles in the presence of an external magnetic field. The kinetics of field-induced self-organization into linear chains, time-dependent chain-size distribution, resolved growth steps condensation, polarization, colinearity, and concatenation, the average chain growth rate, and inter-particle interaction length were calculated in the presence of external magnetic fields (Gajula et al., 2010). These studies give us valuable information relevant to hyperthermia treatment, MRI contrast agents, bioseparation, drug targeting studies and is relevant for making 2D and 3D biocompatible structures for tissue engineering. Moreover, the LAMI technique will enhance the nanomedical applications by creating strong, covalent bond interactions between the nanoparticles and therapeutical biomolecules.

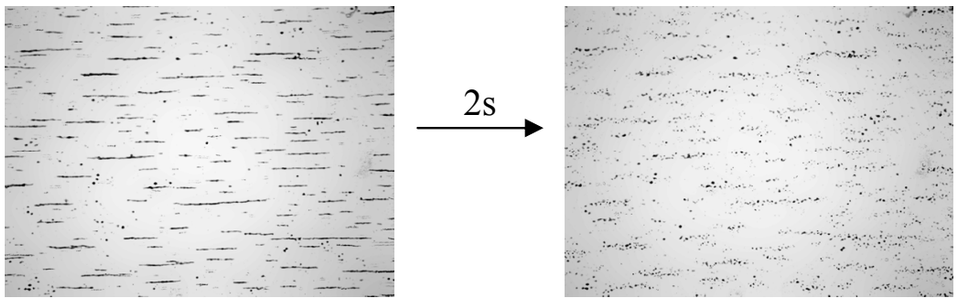

Fig. 14. Bright field microscope images of superparamagnetic nanoparticles in the (left) presence and (right) absence of an external magnetic field (2s after field removal).

Most tissues in organisms are composed of repeating basic cellular structures that are embedded in an extracellular 3D matrix. Tissue functionality arises from these components and the relative spatial locations of these components (Nichol & Khademhosseini, 2009). Tissue engineering enables to recreate the native 3D architecture *in vitro*. Artificial biocompatible 3D structures enable researchers to create organs for transplantation and to understand the structure/function relationship, to characterize cell-membrane mechanical properties, enables the theoretical analyses and to model cellular events and diseases. Xu and co-workers fabricated magnetic nanoparticles loaded cell-encapsulating microscale hydrogels and assembled these gels into 3D multilayer constructs using magnetic fields (Xu et al., 2011). A three-dimensional tissue culture based on magnetic levitation of cells in the presence of a hydrogel consisting of gold, magnetic iron oxide nanoparticles and filamentous bacteriophage has been exploited for direct cell manipulation and cell sorting. By spatially controlling the magnetic field, the geometry of the cell mass is manipulated (Souza et al., 2010). Ito and co-workers have demonstrated the successful construction and

delivery of human retinal pigment epithelial cell sheets using magnetic nanoparticles (Ito et al., 2005). Biomimetic nanopatterns can also be explored for analysis and control of live cells growth (Kim et al., 2010). Richert and co-workers observed the control over the growth of the osteogenic cells, fibroblastic cells and smooth muscle cells using nano-porous titanium alloy explaining that nanotopography may modulate cytoskeletal organization and membrane receptor organization (Richert et al., 2008). This work indicates the impact of nanoscale engineering in controlling cell-material interfaces, which can have profound implications for the development of tissue engineering and regeneration medicine.

Structural colour originates from physical configurations of materials e.g. upon light interaction the lattice spacing of the melanin rods generate various colours in the feathers of a peacock. Structural colour is free from photobleaching, unlike traditional pigments or dyes. Owing to its unique characteristics, there have been many attempts to make artificial structural colour through various technological approaches such as colloidal crystallization, dielectric layer stacking and direct lithographic patterning. However, these techniques are time consuming, cost, and great effort is needed to produce multicoloured patterns on a substrate. Using the self-organization of nanoparticles, external magnetic field tunable bandgaps can be created on various substrates within a few seconds (Philip et al., 2003; H. Kim et al., 2009). During the chaining process under external magnetic field, the combination of attractive and repulsive forces determines the interparticle distance, and the interparticle distance in a chain determines the colour of the light diffracted from the chain, which can be explained by Bragg diffraction theory. Thus, the colour can be tuned by simply varying the interparticle distance using external magnetic fields. The spacing between the particles is sensitive towards charges over the particle's surface. This property can be used to develop biosensors. For example, the specific molecular recognition of cholesterol by digitonin on nanoparticles may result in a reduction of the inter-particle spacing due to the enhanced hydrophobicity of the surface after cholesterol binding. Therefore, the light diffracted from the chain varies indicating the adsorption of cholesterol onto the nanoparticles surface. Cassagneau et al., have shown that the reversible colour tuning of a colloidal crystal could be potentially adopted for biosensing. Biospecific binding of avidin with biotin is demonstrated by monitoring changes in the bandgap spectral peak position caused by Bragg-diffraction of electromagnetic waves within the structure **(**Cassagneau & Caruso, 2002). The same chaining technique is explored for the DNA separation (Doyle et al., 2002).

5. Photonic cancer therapy

The epidermal growth factor receptor (EGFR), also known as HER1/Erb-B1, belongs to the ErbB family of receptor tyrosine kinases (RTKs) (Riese & Stern, 1998; Yarden & Sliwkowski, 2001; Olayioye et al., 2000). Binding of ligands such as EGF and TGF, leads to homo- and heterodimerization of the receptors (Olayioye et al., 20003). Dimerization in the case of EGFR leads to autophosphorylation of specific tyrosine residues in the intracellular tyrosine kinase domain. EGFR activation results in cell signaling cascades that promote tumor cell proliferation, survival and inhibits apoptosis (Fig. 14). EGFR is expressed or highly expressed in non-small-cell lung cancer (NSCLC) and in a variety of common solid tumors, and has also been associated with poor prognosis. High EGFR expression is generally associated with invasion, metastasis, late-stage disease, chemotherapy resistance, hormonal

therapy resistance and poor outcome. Therefore, inhibition of EGFR function is a rational treatment approach.

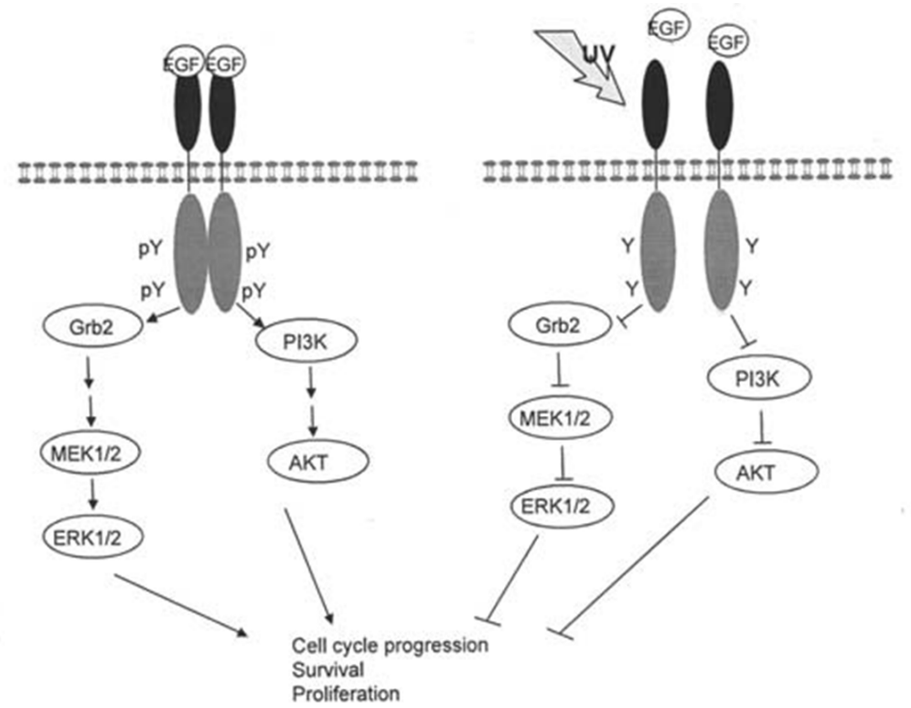

Fig. 15. Overview of the cellular pathways affected by the laser-pulsed UV illumination of the EGF (epidermal growth factor) receptor leading to attenuation of the EGFR signalling cascade. Photoactivation of aromatic residues within the extracellular domain of the EGF receptor, leads to disruption of nearby disulphide bridges. This prevents the ligand, e.g. EGF, from binding to the receptor and activating the EGFR pathways. In addition, it is possible that laser-pulsed UV illumination targets the intracellular domain of the EGF receptor causing photodegradation of phosphorylation-targeted tyrosine residues again preventing the proteins from binding to the phosphorylated tyrosine residues.

Bioinformatics studies show that the extracellular domain of human HER3 (ErbB3), a member of the epidermal growth factor receptor (EGFR) family, is exceedingly rich in SS bridges and aromatic residues (Petersen et al, 2008, Fig. 15). The structure of the extracellular domain consists of four domains. Tethered domains II and IV are displayed. A total of 22 disulphide bridges can be seen in one domain and 25 in the other domain. On the other hand, biophysical studies show that UV illumination of aromatic residues nearby disulphide bridges leads to the SS disruption (Neves-Petersen et al, 2002, Fig. 1). Therefore, it is likely that we induce structural changes in the 3D structure of EGFR upon prolonged UV excitation. If the UV light fluency (power per unit area) is above a certain threshold it is likely that 3D structural changes occur that will prevent the correct binding to, e.g., EGF. We have shown that relatively low intensity UV light (0.273 mW 200fs femtosecond pulses at 280nm diffused onto a petri dish area) can be used to induce cancer cell death (apoptosis) in skin cancer cells in culture (Olsen B.B. et al., 2007, Petersen S.B. et al., 2008). We have also shown that most likely the epidermal growth factor receptor in cancer cell membranes upon

absorption of the UV photons changes its molecular structure as a consequence. The net result is that phosphorylation and cell signaling is abolished, which in turn leads to apoptosis. The technology may lead to an important new modality in the treatment of various cancers. Pulsed UV illumination can halt activation of cancer cell membrane receptors and thereby all downstream reactions that would lead to cancer, shutting down the cells' biological functions. Moreover, this new treatment activated the cell's own cell death program. This has been documented on two human epidermal cancer cell lines (Olsen B.B. et al., 2007). The photonic dosage necessary for therapeutical results has additionally been determined.

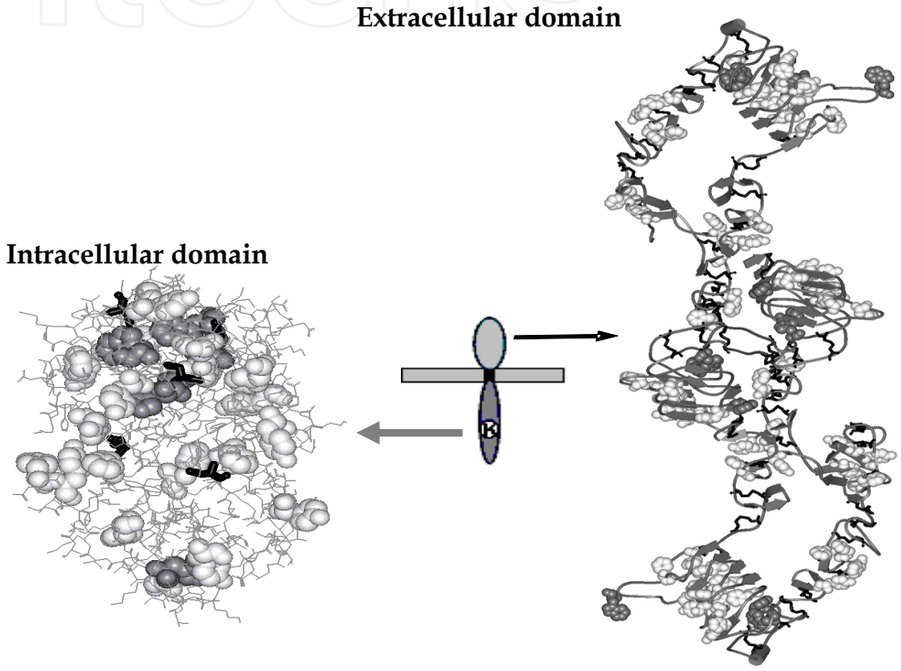

Fig. 16. Molecular structure of the intracellular and extracellular domains of human HER3 (ErbB3), a member of the epidermal growth factor receptor (EGFR) family. Tethered extracellular domains II and IV are displayed. The pdb code of the extracellular domain is 1m6b.pdb. The extracellular domain is extremely rich in disulphide bridges. Tethered extracellular domains II and IV are displayed. A total of 22 disulphide bridges can be seen in each of the displayed domains. The intracellular domain is a protein tyrosine kinase. In the extracellular domain, disulphide bridges are depicted as black sticks, the tryptophan amino acids as rendered grey CPK models, and the tyrosine and phenylalanine residues are depicted as white CPK models. As expected, no disulphide bridges are observed in the intracellular domain due to the reduction environment of cells. However, this domain is also rich in aromatic residues.

This technology is applicable to the treatment of various forms of cancer. Using optical fibers it is possible to illuminate localized areas in any region of the human body. Therefore, both external and internal tumors can be treated. This method may also offer a better treatment of a surgical wound cavity prior to its closure in order to prevent cancer reappearance. This new photonic method differs from the classical photodynamic therapy

(PDT) which requires the use of a photosensitizer molecule that upon excitation and interaction to molecular oxygen leads to the formation of singlet oxygen, which kills the cells. The new photonic cancer therapy does not require the use of photosensitizer molecules. UV light induced 3D structural changes in the EGFR protein prevents binding/activation by EGF, halting this way the phosphorylation of the intracellular domain of EGFR and stopping metabolic pathways that lead to cancer proliferation. The new photonic cancer therapy can be used in combination to PDT and other cancer therapies.

Several reports describe how UV light can activate the EGF receptor hence activating the AKT and MAPK pathway (Warmuth et al., 1994; Coffer PJ at al., 1995; Huang et al., 1996; Katiyar, 2001; Wan et al., 2001; Iordanov et al., 2002; Matsumura & Ananthaswamy, 2004; El-Abaseri at al., 2006). Our observations seem to point at another effect of UV light, in apparent contrast with those results. The reason for this discrepancy could be found in the illumination power per unit of illuminated area (fluency). In our experiments the total integrated power over a second is significantly less than the average solar UV output but comparing the actual output during a pulse event we have 1000-fold higher intensity during the 200 femtosecond long pulse event (Olsen et al., 2007, Petersen et al., 2008).

6. Conclusion

Our work on the interplay between the protein molecule and UV light has resulted in new basic science insights. It has also led to the development of a new protein covalent immobilization technique. The new photonic technology has been used successfully to design and engineer drug delivery systems and biosensors at the micro and nanoscale relevant to nanomedicine. The new engineering principle is made possible due to the presence of a conserved structural motif in proteins conserved by nature throughout evolution.

As a surprising spin-off, our work has resulted in new knowledge concerning how UV light can stop skin cancer. Pulsed UV illumination can halt activation of cancer cell membrane receptors and thereby stop all downstream reactions that would lead to cancer, shutting down the cells' biological functions. Moreover, this new treatment activated the cell's own cell death program (apoptosis). In particular we have realized that UV light chemically modifies the same receptor protein that many cancer therapeutic treatments are trying to target chemically. We believe that this holds promise for a totally new approach to treat some types of localized cancer. We will strive to develop an in-depth understanding of how and why nanometer-sized protein structures respond to light exposure.

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8. References

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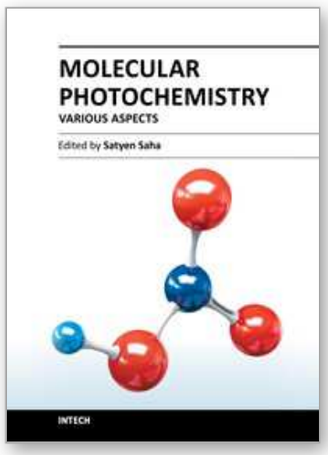

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There have been various comprehensive and stand-alone text books on the introduction to Molecular Photochemistry which provide crystal clear concepts on fundamental issues. This book entitled "Molecular Photochemistry - Various Aspects" presents various advanced topics that inherently utilizes those core concepts/techniques to various advanced fields of photochemistry and are generally not available. The purpose of publication of this book is actually an effort to bring many such important topics clubbed together. The goal of this book is to familiarize both research scholars and post graduate students with recent advancement in various fields related to Photochemistry. The book is broadly divided in five parts: the photochemistry I) in solution, II) of metal oxides, III) in biology, IV) the computational aspects and V) applications. Each part provides unique aspect of photochemistry. These exciting chapters clearly indicate that the future of photochemistry like in any other burgeoning field is more exciting than the past.

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University Campus STeP Ri
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51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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