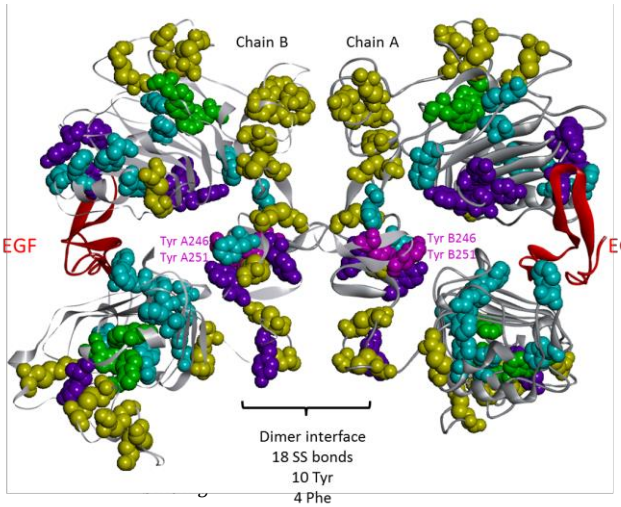
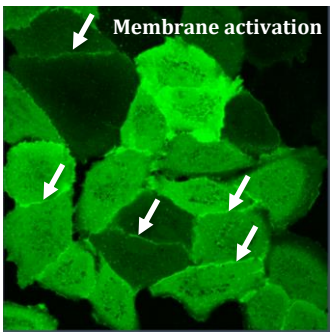


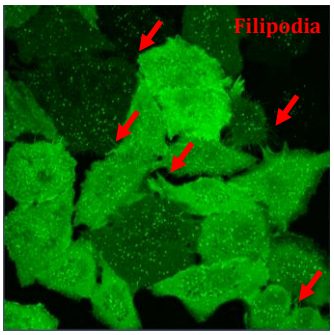
2015

Research Highlights and Perspectives

No UV

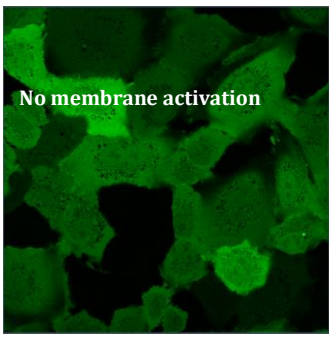


Lung carcinoma cells and EGF

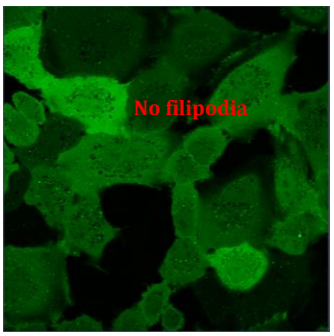


Lund carcinoma cells and EGF 30min later

UV



Lung carcinoma cells and EGF



Lung carcinoma cells and EGF 30min later

Medical Photonics Group

08/01/2015

Research Highlights

Executive summary

The Nanobio group is working in the interface of protein science, biophysics, molecular medicine, biophotonics and biological sciences. Our work always addressed protein structural stability and functionality studies. Key pharmaceutical proteins have been investigated (insulin, EGFR, plasminogen/plasmin). Protein bioinformatics has always been an important tool. Our focus is on the complex interplay between the protein molecule and light, on the dynamics of UV induced structural changes in protein and on UV induced electron transfer in protein. Our ultimate goal is to use such knowledge for modulating protein function and metabolic pathways with light, e.g. in order to modulate key ligand-receptor interactions at the cellular level (vide infra). Ultrafast spectroscopy has been used in order to document the dynamics of electron transfer in proteins and the lifetime of transient species formed after UV excitation of proteins. We have always been interested in monitoring the ultrafast fluorescence decay time in proteins and other biomolecules and to correlate the data with protein structural changes and molecular interaction. Aside from resulting in exciting new basic science insights, our work has led to the development of a new protein covalent photonic immobilization technique. The new photonic technology has successfully been used 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. We have also shown that the photonic immobilization technology achieved with 1-photon (UV) excitation can also be achieve with 3-photon (IR) excitation. We are also carrying out work on self-assembling functional nanoparticles with biomolecules creating bioconjugated nanoparticles with applications in biomedicine and biosensors.

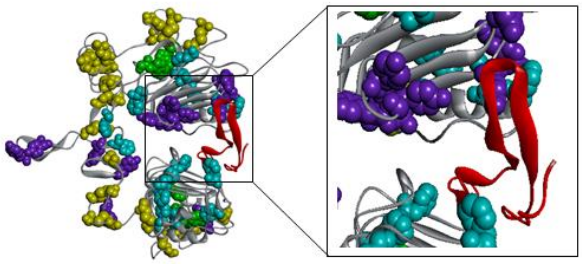
Furthermore we are working on the therapeutical effect of low dose UV light such as on cancer treatment and blood clot dissolution. Our work has resulted in important new knowledge concerning how UV light can disable or kill cancer cells. Pulsed UV illumination can halt activation of a specific cancer cell membrane receptor (Epidermal Growth Factor Receptor) 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. 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 have been able to prevent the formation of filipodia in cancer cells preventing this way the migration of such cells. Filipodia formation is a phenomenon observed after the activation of EGFR by the epidermal growth factor and contributes to cancer metastization. We believe that this holds promise for a totally new approach to treat some types of localized cancer.

Recently we have combined the knowledge that we have on the reaction mechanisms triggered by light on proteins with multiphoton excitation and nanoplasmonics effects in hotspot. We have succeed in immobilizing single protein molecules on nm sized plasmonic hot spots created on gold dimers. So far no group has been able to covalently immobilized protein molecules with such spatial accuracy. This works aims at the detection of single molecules that interact with sensor molecules immobilized onto plasmonic hotspot created e.g. in between gold dimers. The presence of single molecules in such hotspot will be efficiently detected since it will perturb the plasmonic resonance of the plasmonic structures.

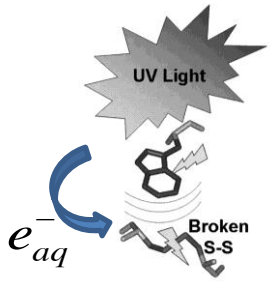

We will strive to develop an in-depth understanding of how and why protein structures respond to light exposure and how that can be used in order to modulate molecular interactions. Advanced data and imaging processing tools developed in the Nanobio group will be used in order to maximize our ability to extract relevant information from our experiments. The Nanobio group has been developing an alternative method that will allow us to achieve super-resolution and that will contribute to single molecule observation. At all times we will identify how the new knowledge may find relevant applications in the area of molecular medicine, molecular pharmacology and nanomedicine.
Yours sincerely
Steffen B. Petersen and Teresa Petersen

Modulating the structure of EGFR with UV light: a potential target for cancer treatment

The Nanobio group´s work has resulted in new knowledge concerning how low dose UV light can stop the EGF-EGFR activation of cancer cells. We have previously reported that pulsed UV illumination halts activation of cancer cell membrane receptors and thereby all downstream reactions that would lead to cancer, shutting down the cells' biological functions[1]. In particular, we have realized that UV light chemically modifies the receptor protein (EGFR, epidermal growth factor receptor) that many cancer therapeutic treatments target chemically. Our recent studies have given us insight into the structural changes and photochemistry induced in EGFR by UV-light. UV excitation of the aromatic residues in EGFR induces conformation changes in e.g. the region where EGF (epidermal growth factor) binds[2,3]. Such changes cause for the impaired correct binding of ligands to EGFR, halting this way the process of tumor growth.



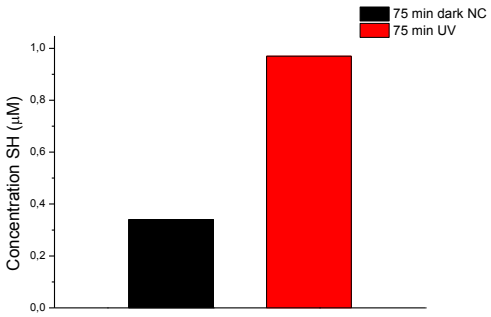
Crystal structure of human EGF (red) and EGFR extracellular domain (1ivo.pdb). Disulphide bridges and aromatic residues are displayed as CPK: SS bridges in yellow, Trp in green, Tyr in violet and Phe in cyan; 18 out of 25 SS bridges, 5 out of 6 Trp, 13 out of 16 Tyr and 17 out of 18 Phe residues are displayed (some residues are missing in the pdb file).



$$e^-_{aq} + RSSR \rightarrow RSSR^{\bullet -}$$

$$RSSR^{\bullet -} \Leftrightarrow RS^{\bullet} + RS^-$$

$$RSSR^{\bullet -} + H^+ \Leftrightarrow RS^{\bullet} + RSH$$



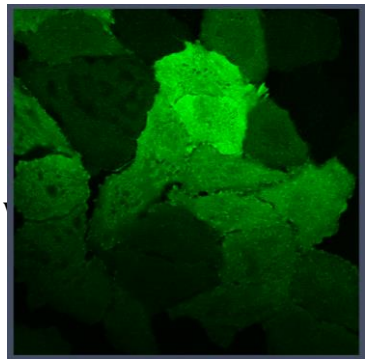
UV excitation of the extracellular domain of EGFR leads to the disruption of disulphide bridges (SS) in this protein. Free SH groups have been detected with Ellman´s reaction (right). Electron transfer from the aromatic residues to the SS bonds is a mechanism that leads to SS disruption (left).

[1] Olsen, B. B., Neves-Petersen, M. T., Klitgaard, S., Issinger O.-G. and Petersen, S. B., "UV light blocks EGFR signalling in human cancer cell lines," International Journal of Oncology 30(1), 181-185 (2007).
[2] Coutinho I., Correia M., Thiagarajan V., Gajula G.P., Petersen S.P, Neves-Petersen M.T. "Photonic cancer therapy: modulating cellular metabolism with light" Paper 8568-5, Proceedings paper, SPIE Photonics West 2013.
[3] Modulating the structure of EGFR with UV light: a potential target for cancer treatment, paper submitted.
[4] Modulating the Structure of EGFR with UV Light: New Possibilities in Cancer Therapy
By: Correia, Manuel; Thiagarajan, Viruthachalam; Coutinho, Isabel; et al.
PLOS ONE Volume: 9 Issue: 11 Article Number: e111617 Published: NOV 11 2014

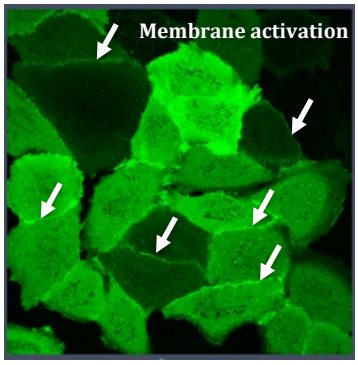
Photonic Arrest of Cancer Migration

In collaboration with *University of Minho, Department of Biological Engineering, Portugal.*

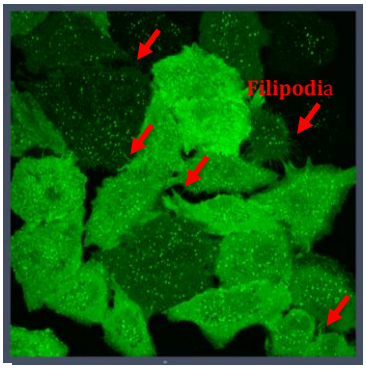
The Nanobio group has previously obtained data showing that UV light changes the structure of EGFR, preventing its activation by EGF. Now we show that low dose UV (280nm) prevents EGF induced migration of human lung cancer cells. UV illumination of the cancer cells prior to EGF activation prevents the phosphorylation of EGFR molecules located at the cell membrane, preventing cell-cell disaggregation. We have very clear confocal microscopy evidence for this. This research is being carried out on A549 cells (adenocarcinomic human alveolar basal epithelial cells). Furthermore, we have observed that low dose UV illumination of the cancer cells prior to EGF activation prevented the formation of filipodia, which are slender cytoplasmic projections (microspikes) that promote cellular migration. We always observe the formation of filipodia and cellular migration when we activate the cells with EGF. We are extremely excited about the potential of this observation in preventing cancer proliferation. The Nanobio group believes that this technology will be able of preventing or delaying the process of cancer metastization. The irradiance used in our study at 280nm is ***100x lower*** than the total irradiance of sunlight in the UVB region and 15min of illumination is sufficient to prevent cellular migration.



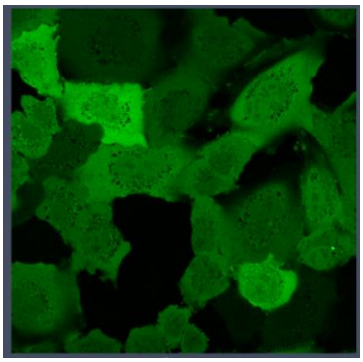
Confocal image of A549 cells (2 min after EGF addition)



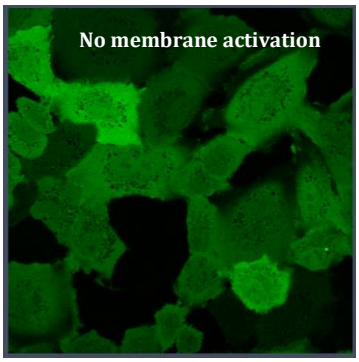
EGF induces autophosphorylation of the receptor EGFR located on the cell membrane. 11min after EGF addition.



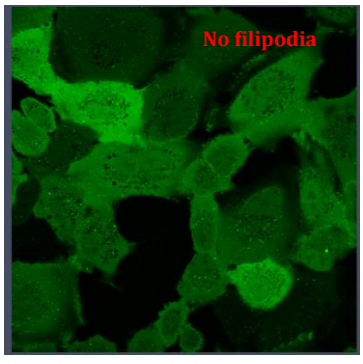
EGFR is internalized and the cells start forming filipodia which will enable them to migrate. 41min after EGF addition.



Confocal image of A549 cells previously illuminated with UVB (280nm) (2 min after EGF addition)



EGF does NOT induce autophosphorylation of the receptor EGFR located on the cell membrane. 11min after EGF addition.



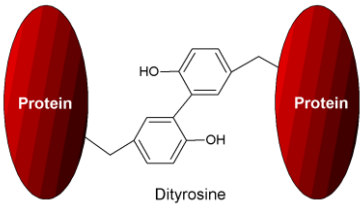
No formation of filipodia and no migration 41min after EGF addition.

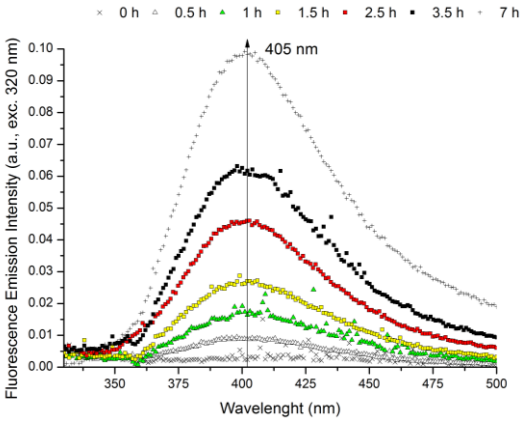
Photonic Arrest of Cancer Metastization. C. Botelho, A. Gomes, M.T. Neves-Petersen*. Full paper paper being written

UV-light exposure of Insulin: pharmaceutical implications

In collaboration with *Aarhus University Hospital, Dept. of Medicine and Endocrinology MEA, Aarhus, Denmark*

The observations provide a detailed interpretation of the effects of UV excitation of insulin preparations. The findings are highly relevant for the pharmaceutical industry. We report that exposure of insulin solutions to UV-light damages the peptide structure and function. Structural damage includes insulin dimerization via dityrosine cross-linking and disulphide bond disruption, which affect the peptide hormone's structure and activity. Our work advances conclusions of a global importance for the shelf life of pharmaceutical products and protein preparations. The majority of pharmaceutical peptide hormones contain tyrosine residues. If pharmaceutical preparations are exposed to ambient and artificial UV-light, inter-molecular aggregation may occur via dityrosine cross-linking. We estimate that direct long-term exposure of insulin to sunlight and common light sources for indoors lighting and UV-sterilization in industries can be sufficient to induce irreversible changes to the structure of human insulin. We conclude that preventive measures should be taken to avoid the photochemical damage of the preparations. The effects of exposing these samples to UV-light during routine biophysical lab checks should be taken in consideration since short exposure times of protein samples to UV-light has immediate consequences for protein structure and function.





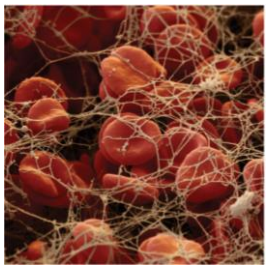
Fluorescence emission spectra recorded before and after 276 nm light continuous exc. (from 0.5 to 7 h) of human insulin in solution. There is a continuous increase in emission intensity at 405 nm upon 276 nm exc. time, showing dityrosine formation.

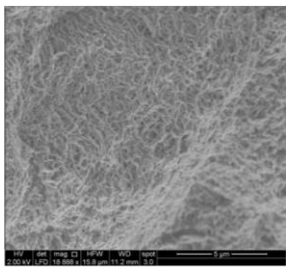
Correia M, Neves-Petersen* MT, Jeppesen PB, Gregersen S, Petersen SB (2012). "UV-Light Exposure of Insulin: Pharmaceutical Implications upon Covalent Insulin Dityrosine Dimerization and Disulphide Bond Photolysis". PLoS ONE 7(12):e50733.doi:10.1371/journal.pone.0050733

UV Induced modulation of Plasminogen and Plasmin function

Manuel Correia, Viruthachalam Thiagarajan, Maria Teresa Neves-Petersen, Torben Snabe, Allan Stenballe, Steffen B. Petersen

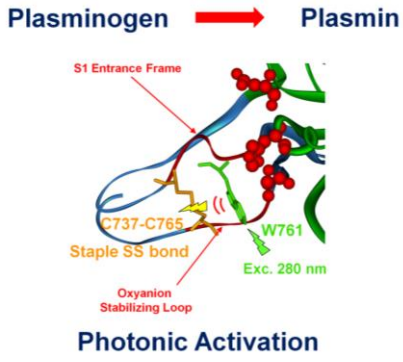
Human blood plasma contains a large range of proteins and enzymes that regulates e.g. thrombosis (blood coagulation) and thrombolysis (dissolution of coagulated blood). The key enzyme in thrombolysis is plasmin, formed after activation of the inactive proenzyme plasminogen. Activated plasminogen, plasmin, is a trypsin-like serine protease which degrades fibrin. Fibrin is a protein that spontaneously polymerises to form blood clots, e.g. as a mesh that covers a wound. Within the circulation, the role of plasmin is to maintain the blood fluidity by dissolution of fibrin thrombi (blood clots). Plasminogen activation in humans is predominantly catalysed by two serine proteases: the tissue-type Plasminogen Activator (tPA) or the urokinase-type Plasminogen Activator (uPA). Our studies show that low dose and brief (10 min) UV excitation of the aromatic residues in plasminogen leads to structural changes that lead to the activation of plasminogen into plasmin[1]. We report the consequences of UV light excitation on the absorption, fluorescence properties, structure and activity of this protein. A key allosteric disulphide bridge which blocks the active site of plasminogen is the most likely disulphide bridge to be disrupted upon UV illumination of the aromatic residues in plasminogen. This could explain the molecular reasons behind the UV induced activation of plasminogen into plasmin. MALDI-TOF is currently being used in order to identify which disulphide bridges are broken upon UVB illumination of plasminogen. The activity of the enzyme is reduced when UV illuminated for longer than 20min. This can have application in cancer research since proteolytic activity should be reduced in order to prevent tumor progression.





Left image was retrieved from http://medicalstate.tumblr.com/.
Right image: Fibrin imaged with Scanning electron microscopy at INL.

[1]Manuel Correia, Maria Teresa Neves-Petersen*, Torben Snabe, Steffen B. Petersen,. "UV Induced Activation of Plasminogen". Paper to be submitted September/October 2013.



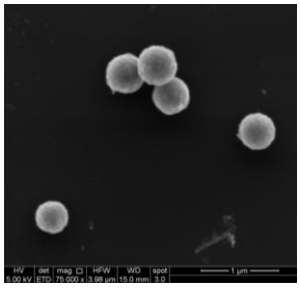
UVB excitation of Trp761 leads to electron transfer to Cys737-Cys765. This results in disruption of this SS bond, leading to a conformational change that renders the catalytic triad active.

"Photonic Activation of Plasminogen induced by low dose UVB", Correia Manuel, Torben Snabe, Thiagarajan Viruthachalam, Petersen Steffen B., Neves-Petersen Maria Teresa, Plos One , accepted December 23rd, 2014

Researchers: Teresa Petersen (PI), Viruthachalam Thiagarajan, Manuel Correia, Allan Stenballe , Steffen Petersen.

Developing drug delivery systems with therapeutical properties

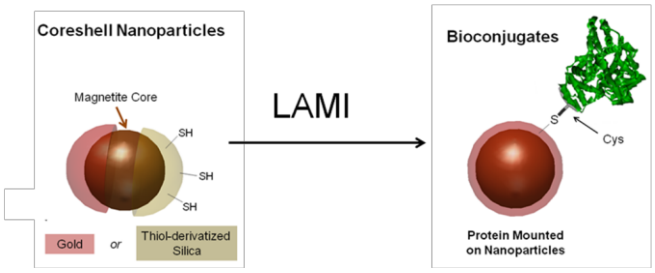
In collaboration with *Aarhus University Hospital, Dept. of Medicine and Endocrinology, Denmark*



Pharmaceutical relevant proteins are now being coupled to nanoparticles. In order to develop suitable functional nanoparticles for biomedical applications, magnetic nanoparticles have been prepared and characterized using scanning electron microscopy, X-ray diffraction, vibrating sample magnetometer and thermal gravimetric analysis. Spherical magnetite nanoparticles have been prepared with very precise different sizes ranging from 30 nm to 400 nm. The nanoparticles are functionalized with carboxylic group in order to allow for dispersion in water. We have successfully established a production of spherical super paramagnetic nanoparticle. Most of the particles are spherical and monodisperse. The nanoparticles will be biofunctionalised with medically relevant proteins, such as insulin in order to develop drug delivery systems with therapeutical properties.

New photonic methodology used to create functional nanoparticles

We are tagging biomolecules directly to the magnetic nanoparticles using classic immobilization protocols, like EDC-NHS chemistry, and using our new photonic technology, light assisted molecular immobilization (LAMI). The surface of these nanoparticles is thiol reactive, being gold or thiol derivatised silica. 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) has been succesfully immobilised with our new photonic technology. The photonic immobilization technique has created free and active thiol functional groups in BSA and insulin linked them to Fe_3O_4@Au core-shell nanoparticle's surface and thiol derivatised silica nanoparticles, respectively.



We have developed the necessary technology that allows us to produce a variety of nanoparticles, from gold and silica nanoparticles (right picture) to core-shell superparamagnetic nanoparticles. Furthermore, we can further derivatise the silica ourter layer of those nanoparticles with chemical finctional 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.

We are monitoring the interactions between the proteins carried by the nanoparticles with cellular components (e.g. receptor proteins in cell membranes) and to follow in vivo the physiological response induced.

1. Visualization and quantification of magnetic field induced 2D ordering of superparamagnetic nanoparticles. Gnana Prakash, Teresa Neves Petersen, Steffen Petersen. Applied Physics Letters, 97, 103103 (2010)
2. Towards Nanoscale Biomedical Devices in Medicine: Biofunctional Superparamagnetic Nanoparticles. A Parracino, GP Gajula, A Kold, MT Neves-Petersen, and SB Petersen. Journal of Fluorescence, 21(2), 663-672 (2011)
3. Photonic immobilisation of BSA onto superparamagnetic nanoparticles. Antonietta Parracino, Gnana Prakash Gajula, A Kold, MT Neves-Petersen, and SB Petersen. Biotechnology and Bioengineering 2011, 108(5): 999-1010.
4. Insulin biofunctionalised superparamagnetic nanoparticles: effects at the cellular level. Manuel Correia, Gnana Prakash Gajula, MT Neves-Petersen, and SB Petersen. To be submitted.
5. Biofunctionalizing superparamagnetic nanoparticles with antibacterial proteins. Gnana Prakash Gajula, Thiagarajan Viruthachalam, Maria Teresa Neves-Petersen. Paper being written.

Researchers: Gnana Prakash, Manuel Correia, Thiagarajan Viruthachalam, Steffen Petersen, Teresa Petersen (PI)

Plasmon-assisted delivery of single nano-objects in an optical hot-spot

M. T. Neves-Petersen, Steffen B. Petersen, Manuel Correia in collaboration with the group of Prof Romain Quidant at the Institute of Photonic Sciences (ICFO), Spain.

The positioning of molecules and nanoparticles at highly specific locations on a plasmonic structure has seen the focus of a considerable amount of interest in the last few years. By placing the particle of interest at the location where the local field enhancement is the strongest, light-matter interaction can be greatly increased. This has implications for many fields of research including biosensing, surface enhanced techniques like Raman (SERS) and fluorescence (SEF), as well as for coupling quantum emitters to plasmonic systems. In previous years, this has been somewhat achieved via complicated lithography procedures which can be extremely time consuming, and aggressive for the particle. Furthermore, it is questionable how effective and reproducible these techniques are for positioning extremely small numbers of particles down to single ones. Here, we propose a novel technique in which no lithography is required and the localized surface plasmon mode (LSPR) itself is the mechanism that creates the binding region. There are many advantages to such a technique: it is single step, it applies to any kind of pattern and automatically accounts for any defects, the binding region can be as small as the mode volume. Finally, there is no aggressive lift-off stage which may damage the nanoparticle.

The technique we propose makes use of a property of many proteins in which absorption in the UV leads to disulfide bond disruption and thiol formation [1,2]. These thiol groups can then bind thiol reactive surface, such as gold. However, the typical LSPR frequency for fabricated gold structures lies at the red end of the visible spectrum or the near infra-red. Consequently, in order to utilize intense plasmonic fields, higher order photon absorption is needed. While two-photon absorption may be used for colloidal plasmonic nano-particles, three-photon absorption (~840nm) is much better suited to top-down plasmonic systems designed by lithography. By exciting the plasmonic resonance with a femtosecond pulsed laser at 840nm, the LSPR will assist the protein binding on the gold surface. At relatively low exposure powers and irradiation times, the proteins will only bind in so-called ¨hot spot¨ regions where the local electric field is the strongest (Fig. 1). Once bound in the hot spot, the protein can be used as a scaffold to which additional particles can be attached. The particle of interest will then automatically bind to the region of highest field intensity. Towards these goals we have successfully demonstrated the three-photon absorption of Bovine Serum Albumin (BSA) labelled with the fluorophore Alexa488 to gold nanorod dimers. By measuring the fluorescence signal from the Alexa488 we have shown that the binding efficiency can be accurately controlled via the incident power/irradiation time and via the LSPR frequency. Furthermore, we have observed the localization of the BSA protein on the gold surface indirectly via SEM imaging and demonstrate that the binding originates from the gap region of the dimer. This is also in agreement with what we see from the LSPR frequency shift. Finally, the ability to use the protein as a scaffold is demonstrated by attaching single 20 nm gold colloids only in the region of highest enhancement.

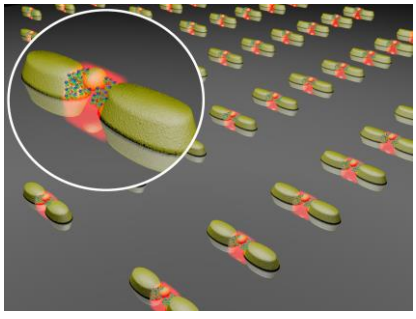


Fig. 1: *Visual representation of the binding of proteins to an array of gold dimers with a single gold colloid in the hot spot region.*

Work presented at the International Conference on Surface Plasmon Photonics, May 2013, Canada.

Plasmon-Assisted Delivery of Single Nano-Objects in an Optical Hot Spot
By: Galloway, Christopher M.; Kreuzer, Mark P.; Acimovic, Srdjan S.; et al.
NANO LETTERS Volume: 13 Issue: 9 Pages: 4299-4304 Published: SEP 2013

[1] M. T. Neves-Petersen *et al*., *Protein Science*. **15**, 343 (2006)
[2] M. Duroux, M.T. Neves-Petersen *et al*., *Proteomics* **7**, 3491 (2007)

Researchers: Teresa Petersen (PI), Prof. Steffen B. Petersen, Manuel Correia

Ultrafast Spectroscopy

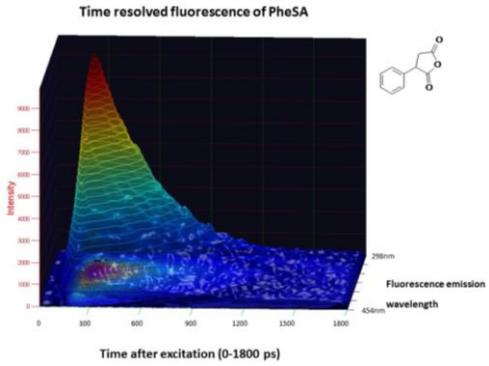
The establishment of the "Ultrafast Biospectroscopy laser lab" at AAU in 2004 was the enabling step to pursue the study of light induced ultra-fast reaction mechanisms in proteins. I have been following fs, ps and ns lasting events that occur in proteins upon UV illumination and their dynamics. Our studies have been focused on: 1) the photophysics and photochemistry of heme containing proteins upon UV illumination. The work done on horseradish peroxidase A2 has been published in Biophysical Journal and at the moment we investigate myoglobin and hemoglobin; 2) photophysics and photochemistry of UV light induced electron transfer in proteins and on the identification and decay kinetics of transient intermediates formed upon UV excitation of aromatic residues; 3) excited state deactivation of key eumelanin building blocks and its implications for melanin pigment photostability; 4) effects of fluorescence quenchers on protein fluorescence lifetime distribution. Some of these works are briefly described ahead.

Photophysics and photochemistry of horseradish peroxidase A2 upon UV illumination. Neves-Petersen, MT, Klitgaard, S., Carvalho, A.S., Aires-Barros, M.R., Melo, Petersen, S.B. **BioPhysical Journal**, 2007 Mar 15;92(6):2016-27.

Quenchers induce wavelength dependence on protein fluorescence lifetimes**.** Søren Klitgaard, Maria Teresa Neves-Petersen and Steffen B. Petersen. **Journal of Fluorescence**. 2006 Jul;16(4):595-609

Role of solvent, pH and molecular size in excited state deactivation of key eumelanin building blocks: implications for melanin pigment photostability" M. Gauden, A. Pezzella, L. Panzella, M.T. Neves-Petersen, E. Skovsen, S. B. Petersen, K. M. Mullen, A. Napolitano, M. d'Ischia and V. Sundström - **J. Am. Chem. Soc**., 2008, 130 (50), pp 17038–17043

Flash photolysis of cutinase: identification and decay kinetics of transient intermediates formed upon UV excitation of aromatic residues. Neves Petersen, Maria Teresa ; Klitgaard, Søren ; Skovsen, Esben ; Pascher, Torbjorn ; Polivka, Tomas ; Petersen, Steffen B. ; Yartsev, Arkady ; Sundström, Villy. I: **Biophysical Journal**, Vol. 97, Iss. 1; pg. 211, 2009.



Biophysical properties of phenyl succinic acid derivatised hyaluronic acid. Maria Teresa Neves-Petersen, Søren Klitgaard, Esben Skovsen, Steffen B Petersen, Kristoffer Tømmeraas, Khadija Schwach-Abdellaoui. **Journal of Fluorescence**, **Volume 20, Issue 2 (2010), Page 483**.

Electron Transfer in Proteins: Identification and Decay Kinetics of Transient Intermediates Formed upon UV Excitation of Aromatic Residues

In collaboration with Villy Sundström, Arkady Yartsev and Tomas Polivka from the Lund Laser Center, Lund University, Sweden

Aromatic amino acids play an important role in UV-induced photochemical reactions in proteins. In the present work we aim at gaining insight into the photochemical reactions induced by near-UV light excitation of aromatic residues that lead to breakage of disulfide bridges in our model enzyme, *Fusarium solani pisi* cutinase, a lipolytic enzyme. With this purpose, transient absorption data of cutinase has been acquired, with supplemental experimental data on tryptophan (Trp) and lysozyme as a reference. We here report formation and dynamics (lifetimes) of transient chemical species created upon UV-illumination of aromatic residues in proteins. Two proteins, lysozyme and cutinase, as well as the free amino acid Trp have been studied under acid, neutral and alkaline conditions. The shortest lived species is assigned to solvated electrons (few µs to ns lifetimes), whereas the longer-lived species are assigned to aromatic neutral and ionic radicals, Trp triplet states as well as radical ionic disulphide bridges. The lifetimes of each species and its pH-dependence is hereby reported. Solvated electrons ejected from the side chain of free Trp residues and aromatic residues in proteins have been observed from 12ns after excitation, reaching a maximum intensity value after approximately 40ns. Interestingly, its formation kinetics are not pH dependent and are similar in the different samples. On the other hand, upon analyses of the solvated electron kinetics, a clear increase of the solvated electron lifetime is observed with increasing pH. This observation is correlated with H_3O^+ being an electron scavenger. Prolonged UV illumination of the cutinase leads to a larger concentration of solvated electrons and to a larger absorption at 410nm (assigned to the disulphide electron adduct $RSSR^{\bullet -}$) with concomitant faster decay kinetics and almost disappearance of the $Trp^{\cdot}$ radical peak at 330nm, indicating possible additional formation of $TyrO^{\cdot}$ formed upon reaction of $Trp^{\cdot}$ with Tyr residues, since $TyrO^{\cdot}$ also absorbs at 410nm. Prolonged UV illumination of cutinase also leads to a larger concentration of free thiol groups, known to originate from the dissociation of the disulphide electron adduct $RSSR^{\bullet -}$. Additional possible mechanisms that lead to the almost disappearance of $Trp^{\cdot}$ are discussed. Our study provides insight into one key UV-light induced reaction in cutinase, i.e., light induced disruption of disulphide bridges mediated by the excitation of aromatic residues. Knowledge about the nature of the formed species and their lifetimes is important for the understanding of UV induced reactions in humans leading to light induced diseases, e.g., skin cancer and cataract formation.

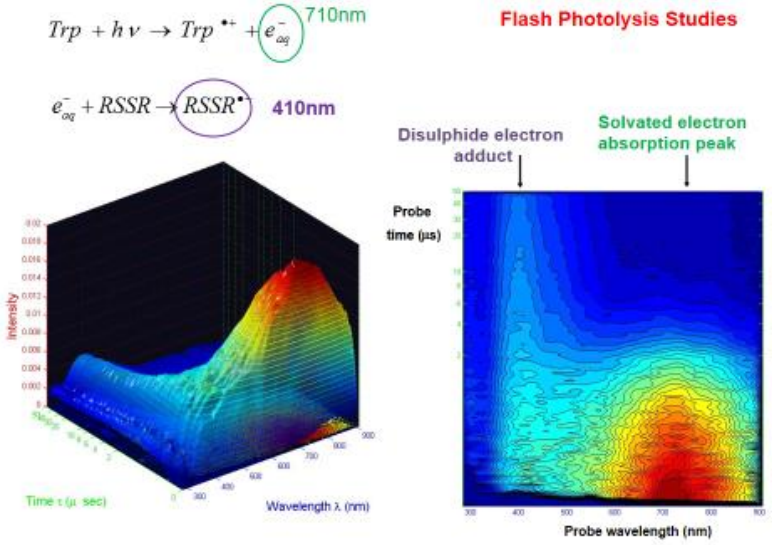


Fig. 1: Cutinase transient absorption data were collected at different probe times (from 0 to 50µs) after 266nm excitation at pH10: The intensity of each spectrum at a particular probe time is colour coded. Red codes for the highest intensity and dark blue for the lowest intensity. Putative transient absorption species assigned to each peak are listed

Neves-Petersen MT, Klitgaard S, Skovsen , Pascher T, Polivka T, Petersen, Steffen B. ; Yartsev A ; Sundström V. Biophysical Journal, Vol. 97, Iss. 1; pg. 211, 2009.

Excited state deactivation of key eumelanin building blocks: implications for melanin pigment photostability

In collaboration with Villy Sundström from the Lund Laser Center, Lund University, Sweden

Ultrafast time-resolved fluorescence spectroscopy has been used to investigate the excited state dynamics of the basic eumelanin building block 5,6-dihydroxyindole-2-carboxylic acid (DHICA) its acetylated, methylated and carboxylic ester derivatives as well as two oligomers, a dimer and a trimer in the O-acetylated forms. The results show that: 1) excited state decays are faster for the trimer relative to the monomer; 2) for parent DHICA, excited state lifetimes are much shorter in aqueous acidic medium (380 ps) as compared to organic solvent (acetonitrile, 2.6 ns); 3) variation of fluorescence spectra and excited state dynamics can be understood as a result of excited state intramolecular proton transfer (ESIPT). The dependence on DHICA oligomer size of excited state deactivation and its ESIPT mechanism provides important insight into the photostability and photoprotective function of eumelanin. Mechanistic analogies with the corresponding processes in DNA and other biomolecules are recognized.

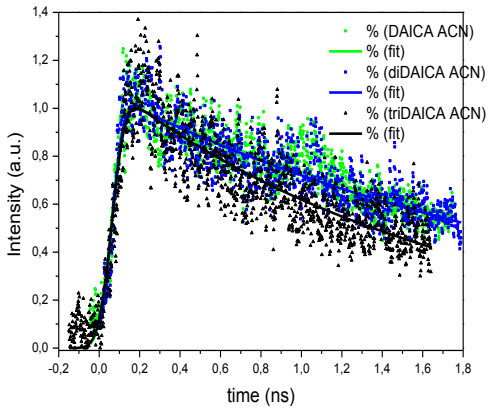


Fig. 1. Fluorescence kinetic traces of DAICA, DAICA-dimer and DAICA-trimer in acetonitrile

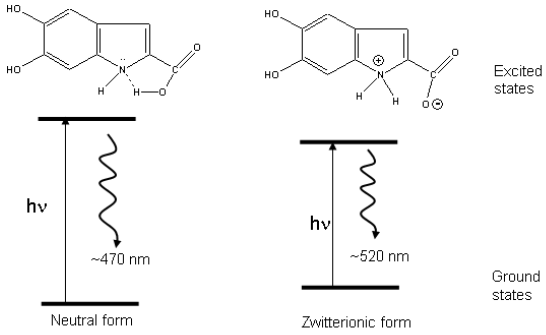


Fig. 2. Proposed energy level diagram for an excited state proton transfer in DHICA.

"Role of solvent, pH and molecular size in excited state deactivation of key eumelanin building blocks: implications for melanin pigment photostability" M. Gauden, A. Pezzella, L. Panzella, M.T. Neves-Petersen, E. Skovsen, S. B. Petersen, K. M. Mullen, A. Napolitano, M. d'Ischia and V. Sundström - J. Am. Chem. Soc., 2008, 130 (50), pp 17038–17043

Photophysics and photochemistry of horseradish peroxidase A2 upon UV illumination
In collaboration with the Institute of Molecular Pathology and Immunology of the University of Porto, Portugal, the Biological Engineering Research Group, Technical University of Lisbon and with the Center of Structural and Molecular Biomedicine from the University of Algarve, Portugal.

Detailed analysis of the effects of ultraviolet (UV) and blue light illumination of horseradish peroxidase A2, a heme-containing enzyme that reduces H_2O_2 to oxidize organic and inorganic compounds, is presented. The effects of increasing illumination time on the protein's enzymatic activity, Reinheitzahl value, fluorescence emission, fluorescence lifetime distribution, fluorescence mean lifetime, and heme absorption are reported. UV illumination leads to an exponential decay of the enzyme activity followed by changes in heme group absorption. Longer UV illumination time leads to lower Tm values as well as helical content loss. Prolonged UV illumination and heme irradiation at 403 nm has a pronounced effect on the fluorescence quantum yield correlated with changes in the prosthetic group pocket, leading to a pronounced decrease in the heme's Soret absorbance band. Analysis of the picosecond-resolved fluorescence emission of horseradish peroxidase A2 with streak camera shows that UV illumination induces an exponential change in the pre-exponential factors distribution associated to the protein's fluorescence life- times, leading to an exponential increase of the mean fluorescence lifetime. Illumination of aromatic residues and of the heme group leads to changes indicative of heme leaving the molecule and/or that photoinduced chemical changes occur in the heme moiety. Our studies bring new insight into light-induced reactions in proteins. We show how streak camera technology can be of outstanding value to follow such ultrafast processes and how streak camera data can be correlated with protein structural changes.

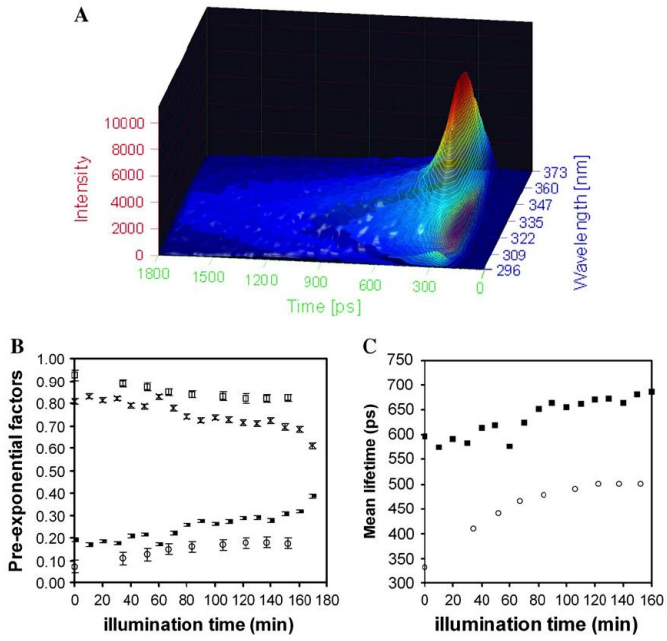


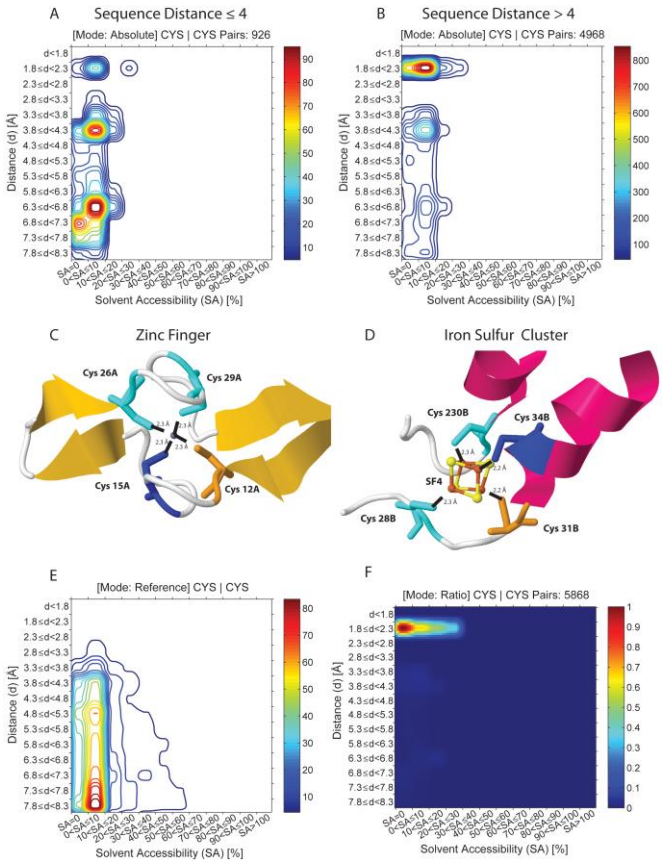
Fig. 1 (A)Three-dimensional representation of a temporally and spectrally resolved streak camera image of the fluorescence emission decay of HRPA2 upon 280nm excitation. (B) Distribution of the pre-exponential factors associated with the two fastest lifetime components of HRPA2 (100 ± 1 ps, open squares; 746 ± 34 ps, open circles) as a function of 280nm illumination time; Distribution of the pre- exponential factors associated with the two fastest lifetime components of HRPA2 (83 ± 0.8 ps, cross and 819 ± 37 ps horizontal bar) as a function of 290nm illumination time; (C) Mean fluorescence lifetime of HRPA2 as a function of 280nm (open circles) and 290nm (solid squares) illumination times.

Photophysics and photochemistry of horseradish peroxidase A2 upon UV illumination. Neves-Petersen, MT, Klitgaard, S., Carvalho, A.S., Aires-Barros, M.R., Melo, Petersen, S.B. **BioPhysical Journal**, 2007 Mar 15;92(6):2016-27.

Hyperdimensional Analysis of Amino Acid Pair Distributions in Proteins

Svend B. Henriksen, Rasmus J. Mortensen, Henrik M. Geertz-Hansen, Maria Teresa Neves-Petersen*, Omar Arnason, Jón Söring, Steffen B. Petersen

Our work presents a novel approach to protein structure analyses. We have organized an 8-dimensional data cube with protein 3D-structural information from 8706 high-resolution non-redundant protein-chains with the aim of identifying packing rules at the amino acid pair level. The cube contains information about amino acid type, solvent accessibility, spatial and sequence distance, secondary structure and sequence length. We are able to pose structural queries to the data cube using program ProPack. The response is a 1, 2 or 3D graph. Whereas the response is of a statistical nature, the user can obtain an instant list of all PDB-structures where such pair is found. The user may select a particular structure, which is displayed highlighting the pair in question. The user may pose millions of different queries and for each one he will receive the answer in a few seconds. In order to demonstrate the capabilities of the data cube as well as the programs, we have selected well known structural features, disulphide bridges and salt bridges, where we illustrate how the queries are posed, and how answers are given. Motifs involving cysteines such as disulphide bridges, zinc-fingers and iron-sulfur clusters are clearly identified and differentiated. ProPack also reveals that whereas pairs of Lys residues virtually never appear in close spatial proximity, pairs of Arg are abundant and appear at close spatial distance, contrasting the belief that electrostatic repulsion would prevent this juxtaposition and that Arg-Lys is perceived as a conservative mutation. The presented programs can find and visualize novel packing preferences in proteins structures allowing the user to unravel correlations between pairs of amino acids. The new tools allow the user to view statistical information and visualize instantly the structures that underpin the statistical information, which is far from trivial with most other SW tools for protein structure analysis.



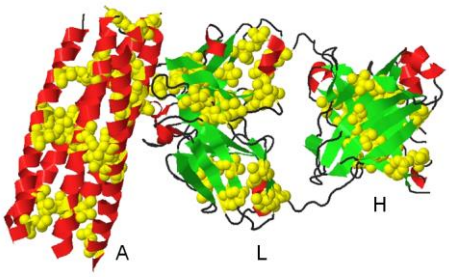
Distribution of the observed spatial distance (Å) and solvent accessibility of the protein shell where 923 Cys-Cys pairs located at a sequence distance ≤4 residues (4A) and at a sequence distance >4 residues (4B) are found. The intensity map is color coded (blue to red) and next to the color bar is displayed the number of pairs corresponding to each color code. Protein structures containing Cys-Cys pairs representative of the two major peaks displayed in 4A have been retrieved with the ProPair program and are displayed in 4C and 4D. Figure 4C shows that Cys-Cys pairs with distances peaking between 3.8–4.3 Å are part of a classical zinc finger motif in proteins. The Cys-Cys pair is displayed in dark blue and yellow. Two other Cys residues are displayed in cyan. Zn is displayed as a blue sphere. Figure 4D shows that Cys-Cys pairs with distances peaking between 6.3–7.3 Å are part of yet another classical cluster, the iron sulfur cluster. The Cys-Cys pair is displayed in dark blue and yellow. In the Fe4S4 cluster, Fe is displayed in orange and S in yellow. Figure 4E shows the distribution of the observed spatial distance (Å) and solvent accessibility of the protein shell where 923 Cys-Cys pairs located at a sequence distance >4 residues are found in a randomized reference database (see Methods section). Figure 4F was obtained by dividing the absolute data for Cys-Cys pairs with a sequence separation larger than 4 residues (Fig. 4B) by the reference dataset data (Fig. 4E), this way displaying the statistically relevant peaks.

Hyperdimensional Analysis of Amino Acid Pair Distributions in Proteins. Svend Henriksen, Rasmus Mortensen, Henrik Geertz-Hansen, Maria Teresa Neves-Petersen, Omar Arnason, Jón Söring, and Steffen B. Petersen. PLoS ONE PLoS ONE, vol. 6(12), pp. 1-19, December 2011, e25638

Protein folding information at the amino acid pair level

Steffen B. Petersen, Maria Teresa Neves-Petersen, Svend B. Henriksen, Rasmus J. Mortensen, and Henrik M. Geertz-Hansen

Our work brings evidence for a scale free organization of protein structure. Our data is based on a detailed and comprehensive analysis of the precise fold environment around amino acid pairs in 8706 protein 3D structures. To our knowledge this is first time this has been reported. There is a clear analogy to e.g. the structure of the World Wide Web, where hubs (e.g. Google) have millions of links and nodes with only a few links. The hubs we identify in the protein fold consist of a small subset of the 20 amino acids: Ala, Ile, Leu and Val. Buried pairs of these amino acids, in close proximity 3.8-4.3 Å and in a sequence distance >4 residues constitute the hubs in the protein fold. Surprisingly we find no aromatics in these hubs and only relatively few Gly and Pro.
The composition of the 'nodes' provide quantitative information relevant for the nucleation-condensation as well as hydrophobic collapse model for protein folding. It also strengthens the proposed reduced alphabet model. In our opinion it creates an important new framework for discussing protein fold models. Our paper also suggests that our data to some extent may explain why simple (sequence based) prediction schemes have failed to convincingly predict protein folds. Simply put, the protein "nodes" may help in defining folding rules, but they only constitute around 20% of the total amount of amino acid pairs – the rest are found in more unique fold environments.



Crystal structure of the N-heptad repeat of HIV-1 gp41 mimetic 5-helix complexed with two antibody fragments (3MA9.pdb). Amino acid pairs containing Ala, Ile, Leu or Val residues are highlighted in yellow and as CPK. Alpha-helices are colored red and beta-sheets green. The three different chains are displayed: A(HIV-1 gp41 5-helix), L and H (Fab fragments). From [1].

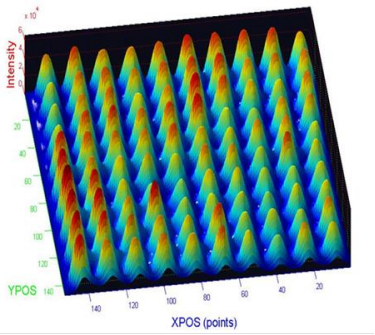
Svend Henriksen, Rasmus Mortensen, Henrik Geertz-Hansen, Maria Teresa Neves-Petersen, Omar Arnason, Jón Söring, and Steffen B. Petersen. ProPack, a program for hyperdimensional analysis of amino acid distribution in proteins: case studies involving cysteines and salt bridge forming amino acid residues. **PLoS ONE**, vol. 6(12), pp. 1-19, December 2011, e25638

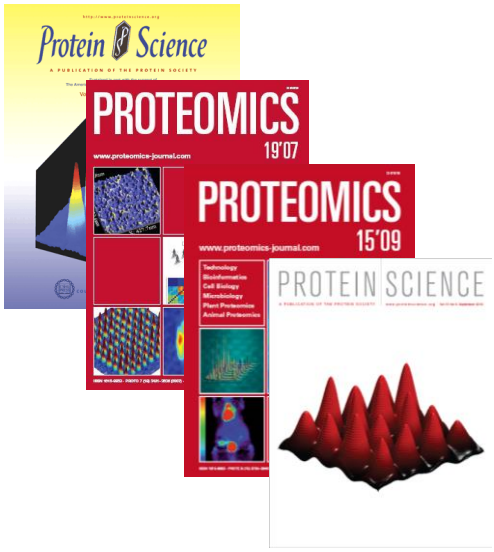
[1]Petersen SB, Neves-Petersen MT, Henriksen SB, Mortensen RJ, Geertz-Hansen HM (2012) Scale-Free Behaviour of Amino Acid Pair Interactions in Folded Proteins. **PLoS One**, July 2012 | Volume 7 | Issue 7 | e41322

Researchers: Teresa Petersen (PI), Prof. Steffen Petersen

Nanoscale Protein Immobilization: Biosensors

Our research activities are focused on both the use and effects of laser light on biomolecular function. With a beam of laser generated UV 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 a glass, quartz or a gold surface, the protein immobilizes onto the surface. Since this happens where the UV photons are present, the size of the focal spot determines where immobilization takes place. We are able to control this process such that spot size is ~1-5 micron. The process is relatively fast – it is determined by physical chemical parameters, as well as the number of photons per second per mm^2. Currently we are operating with around 100 ms per spot. With a pitch of 10 microns and spot size of 5 micron, this allows for about 40.000 spots per mm^2. Almost all proteins are biologically active after this process – and notably so for Fab fragment. We have verified that about 50% of all proteins are likely to respond well to our immobilization approach. We have verified that Fab anti prostate specific antigen can be immobilized with our technology, remaining active. Over the last year we have studied a highly interesting extension to this approach – we are inserting spatial masks in the UV beam prior to focusing on the image plane. Theory predicts that we should obtain an intensity pattern corresponding to the Fourier transform of the spatial mask. We do see patterns similar to what theory predicts – but not identical. The interesting feature is that we in a single shot produces multiple spots (25-36) with a spot size of ~500 nm. With this spot density, we can populate 1 mm^2 with more than 1 million sensor spots. This we believe is a world record. Industrial meso- or macro scale production appears realistic. Our approach bypasses the use of micro dispenser techniques – and the technical difficulties associated with the use of such. It is simple, and fast. We have published our findings in several papers, four of which appeared as front page illustrations on Protein Science (February 2006), Proteomics (October 2007), Proteomics (July 2009) and Protein Science (September 2010).





4 Front Page publications

1. Photonic activation of disulfide bridges achieves oriented protein immobilization on biosensor surfaces. ***Protein Science 2006*** *; vol. 15, s. 343-351*

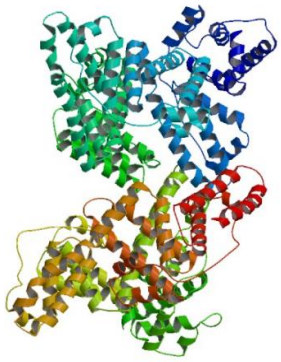
2. Light-induced immobilisation of biomolecules as an attractive alternative to microdroplet dispensing-based arraying technologies. ***Proteomics 2007****, Vol. 7, No.19, October 2007.*

3. Photonic Immobilization of High Density Protein Arrays Using Fourier Optics. ***Proteomics 2009****, 9, 1–4*

4. Arraying prostate specific antigen PSA and Fab anti-PSA using light-assisted molecular immobilization technology. ***Protein Science 2010,*** *19 (9) 1751—1759.*

The new technology has recently been listed in the review paper on "Chemical Strategies for Generating Protein Biochips" by Jonkheijm et al. (2008) as the key technology to achieve covalent and uniform orientation of biomolecules onto surfaces.

Protein Structure/Function Relationship and Protein Stability Bioinformatics and Data mining



The Nanobio groups work has been focused on the role of electrostatic interactions, molecular surface composition, and light in protein structure and function and in molecular recognition. One of my goals has been to underline the common electrostatic features, as a function of pH, of the lipase/esterase family and how such properties influence their pH-dependent activity and structural stability. I have provided knowledge about the common molecular surface composition features of this large family of enzymes, believed to be essential in order to better understand molecular interactions. Such analyses have offered crucial necessary information to propose models of protein interaction and structure-function relationship.

The Nanobio group has indetifed some of the structural preferences that nature has preserved when designing proteins. We have speculated on the reasons why nature has such preferences, in particular what could be the mechanisms that such assembly could provide for successful protein function and stability. Such preferences observed, e.g., in the active site of lipases and esterases gave me insight into the causes of the pH-activity and pH-stability profiles observed among several members of this large family. My studies provided an overview of the common features of molecular surface composition of lipases and esterases, their electrostatic features as a function of pH, and their structure and function were correlated. A model of hydrolytic activity emerged from these studies: the "Electrostatic Catapult Repulsion Model" was born, as well as a model describing what is happening at the lipid/water interface upon catalysis. The "Electrostatic Catapult Repulsion Model" made possible engineering/changing the pH optimum of lipases. Predictions successfully lead to pH optimum changes.

When studying the interactions between light and matter, we have ample evidence that electrostatic interaction play a key role in such interactions. Also, we observed that the structural preferences of nature when designing protein structures is providing the protein with the needed architecture that will enhance protein stability and function when interacting with light. Furthermore, protein database mining lead to the original observation that throughout evolution proteins have kept the close proximity between disulphide bridges and aromatic residues. This has been the observation that later on in my career led to the development of the new photonic immobilization technique, light assisted molecular immobilization (LAMI) described at the beginning of this document. Recently the Nanobio group has developed a novel approach to protein structure analyses. We have organized an 8-dimensional data cube with protein 3D-structural information from 8706 high-resolution non-redundant protein-chains with the aim of identifying packing rules at the amino acid pair level. The programs can find and visualize novel packing preferences in proteins structures allowing the user to unravel correlations between pairs of amino acids. The new tools allow the user to view statistical information and visualize instantly the structures that underpin the statistical information, which is far from trivial with most other SW tools for protein structure analysis. Furthermore, our work brings evidence for a scale free organization of protein structure. Our data is based on a detailed and comprehensive analysis of the precise fold environment around amino acid pairs in 8706 protein 3D structures.

It has been a constant throughout my entire career the use of different spectroscopic techniques in order to investigate protein structure stability and activity. Techniques such as steady state and time resolved fluorescence spectroscopy, total internal reflection fluorescence, circular dichroism, differential scanning calorimetry, and fourier transform total infrared spectroscopy. The philosophy of our group has always been that that deeper a more complete insight will be obtained if we look at a system with different spectroscopic techniques.

Torwards a Molecular Level Understanding of Protein Stabilization: The Interaction Between Lysozyme and Sorbitol. R Wimmer, M Olsson, MT Neves Petersen, R Hatti-Kaul, SB Petersen, and N Müller. J. of Biotech 1997 Jun 13;55(2):85-100.

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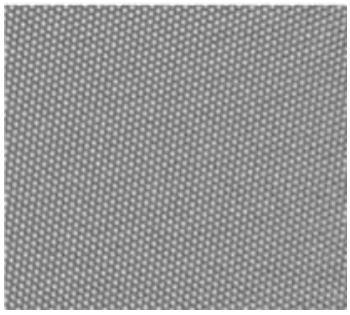
What distinguishes a lipase from an esterase: a new practical approach. Fojan P, Jonson PH, Petersen MTN, Petersen SB (2000). Biochimie 82, 1033-1041.
The thermal stability of the Fusarium solani pisi cutinase as a function of pH. S.B. Petersen, P. Fojan, E. Petersen, MT Neves Petersen (2001). Journal of Biomedicine & Biotechnology, Vol 1., Issue 2, pages 1-8.
Engineering the pH-optimum of a triglyceride lipase: From Predictions Based on Electrostatic Computations to Experimental Results. MT Neves Petersen, E Petersen, P Fojan, M Noronha, RG Madsen and SB Petersen (2001) J. of Biotechnology, May 18;87(3):225-54.
Protein Electrostatics: A review of the equations and methods used to model electrostatic equations in biomolecules. Applications in Biotechnology. M.T. Neves-Petersen, S.B. Petersen (2003). ***Biotechnology Annual Review****, Vol. 9, Pages: 315-395 Elsevier.*
Hyperdimensional Analysis of Amino Acid Pair Distributions in Proteins. Svend Henriksen, Rasmus Mortensen, Henrik Geertz-Hansen, Maria Teresa Neves-Petersen, Omar Arnason, Jón Söring, and Steffen B. Petersen. PLoS ONE PLoS ONE, vol. 6(12), pp. 1-19, December 2011, e25638
Petersen SB, Neves-Petersen MT, Henriksen SB, Mortensen RJ, Geertz-Hansen HM (2012) Scale-Free Behaviour of Amino Acid Pair Interactions in Folded Proteins. **PLoS One**, July 2012 | Volume 7 | Issue 7 | e41322

Advanced Image processing improves the definition of structural information with a factor of ~100



In a 2D self-organized crystalline structure more than 1000 unitcells can be observed in a single image. Here we exploit the benefits from having a large number of observations of the same unit cell utilizing an image processing methodology. We obtain sub-picometer resolution data from a 50 pm image of graphene, revealing a 1% axial elongation and a 3 fold symmetry, indicating a chair conformation.
The close proximity of neighboring building blocks modulates the magnetic, optical and electronic properties of self-assembled structures as compared to the monomers. Precise knowledge about structural details of the self-assemblies is therefore crucial for design and engineering. Electron and atomic force microscopies currently achieve 50 and 3 pm resolution, respectively. However, functional details would be better understood if we could obtain structural details at even higher resolution. Graphene-based electronic devices are intensely studied but the band gap of large area single-layer epitaxial graphene is the subject of controversy. Many authors have reported that the band gap of graphene is zero.8 However, other authors have reported that the band gap depends on the sample thickness (number of graphene layers) on a substrate, the interfacial atomic structure between graphene and substrate, the existing strain due to substrate stretching, and different graphene nanostructures such as narrow graphene na graphene. On the other hand, experimental graphene may also have topological defects, vacancies, edges, cracks and adsorbed impurities. Here we report an image analysis tool that allows us to de□ne structural details of graphene with sub-picometer precision.

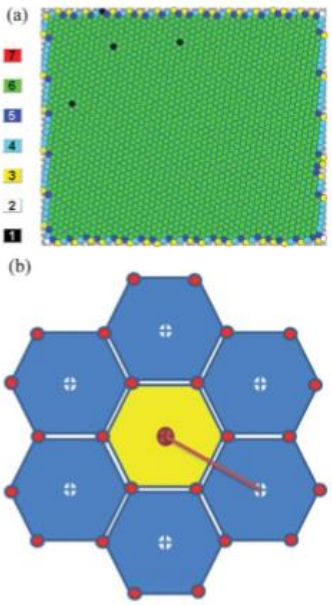


Fig. 2 (a) Co-ordination plot of the graphene structure. The number of nearest neighbors is the co-ordination number for each individual graphene core object. The color box shows the co-ordination number for each graphene core. (b) Schematic diagram of the hexagonal cell around a graphene core (in yellow). The centroid of this core is shown in red while the centroids of the 6 neighbouring cores (in blue) are shown in white. Atom locations are shown as red spheres. The centroid-to-centroid distance is indicated by a line.

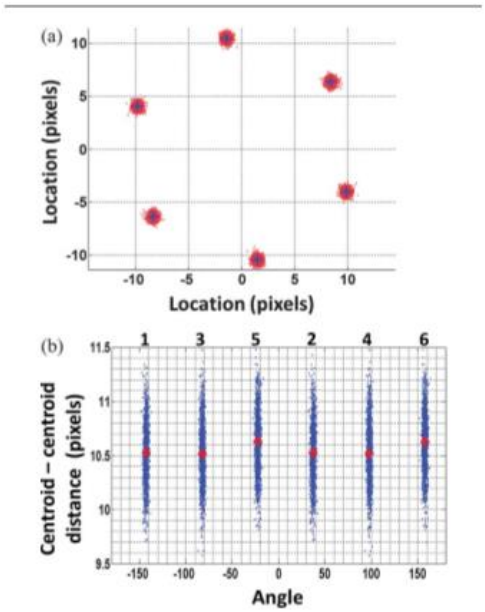


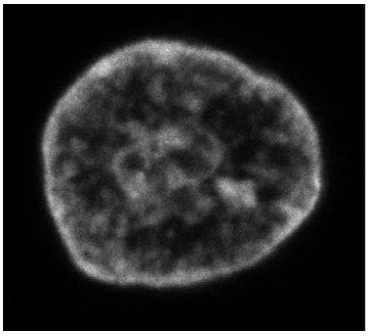
Fig. 3 (a) Cartesian plot of the location of each of the 6 nearest neighbor centroids (geometrical mean given by blue crosses) and (b) polar plots after translational fit (the centroid for each of the 6 distributions are indicated by a red cross). The numbers above each distribution are described in Fig. 4. The centroid-to-centroid distance in each of 6 distributions (in pixels) is: distribution 1 (10.5270), distribution 3 (10.5160), distribution 5 (10.6316), distribution 2 (10.5283), distribution 4 (10.5173) and distribution 6 (10.6310).

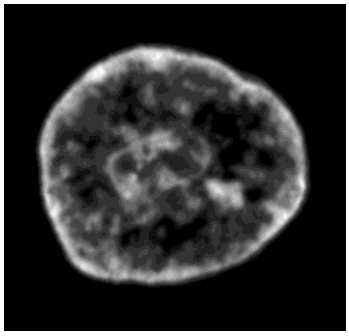
Sub-picometer structural information of graphene hidden in a 50 pm resolved image By: Petersen, Steffen B.; Gajula, Gnana Prakash; Neves-Petersen, Maria Teresa NANOSCALE Volume: 5 Issue: 19 Pages: 8874-8878 Published: 2013

Image processing for drift compensation in fluorescence microscopy

My group at INL has been developing an alternative method that will allow us to achieve super-resolution. The work has started in 2012.

Fluorescence microscopy is characterized by low background noise, thus a fluorescent object appears as an area of high signal/noise. Thermal gradients may result in apparent motion of the object, leading to a blurred image. We have developed an image processing methodology that may remove/reduce blur significantly for any type of microscopy. We can quantity the drift in X and Y using the sub pixel accuracy computed centroid location of an image object in each frame. We can measure drifts down to approximately 10 nm in size and a drift-compensated image can therefore be reconstructed on a grid of the same size using the "Shift and Add" approach leading to an image of identical size as the individual image. We have also reconstructed the image using a 3 fold larger grid with a pixel size of 10 nm. The resulting images reveal details at the diffraction limit[1]. We believe that our results are of general applicability in microscopy and other types of imaging.





Low resolution (left) image of a cell nucleus converted into a high (right) resolution image after drift-compensation and being reconstructed on a grid of the same size. Both images are 15.36 micron by 15.36 micron.

Image Processing for Drift Compensation in Fluorescence Microscopy
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