2018 – Posters

Synthesis of two-photon sensitive pro-fluorescent Photo-removable Protecting Groups

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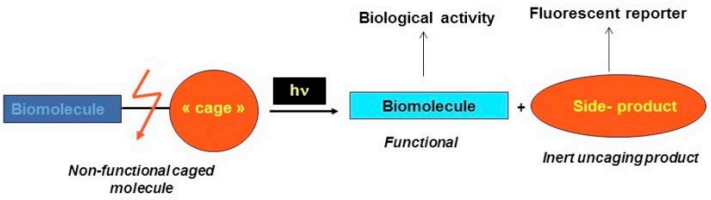
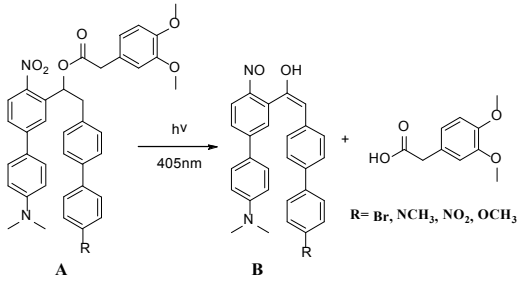
Photoremovable protecting groups (PPGs) have been able to provide spatial and temporal control over the release of various biological effector (neurotransmitters and cell signaling molecules), this process is called uncaging[1] (Scheme 1). The development of optical reporters of uncaging has only attracted little attention[2] as caged compounds and the uncaging secondary product exhibited similar low fluorescence in most cases. Only one example has been developed by using caging groups that were designed to release a fluorophore as a side product. This was achieved with the o-hydroxycinnamate photoremovable group introduced by Porter's group[3]. In order to acutely monitor uncaging event in cells, we wish to develop new caging groups with fluorescence uncaging reporting properties. Based on the photolytical release mechanism of our two-photon sensitive photoremovable groups, we will design new non-fluorescent PPG based on ortho-nitrobenzyl derivatives which release a fluorophore as a side product.[4] (Scheme 1)



Scheme 1: Mechanism of photo-irradiation (uncaging) of a PPG containing molecule

Recently, we have been able to develop a family of photoremovable protecting groups from the o-nitrobenzyl series. Using organozinc chemistry and Pd-catalysed coupling as key steps we were able to synthesize 4 different compounds A. After irradiation of A, an important increase in the fluorescence intensity of the sub-product B was observed and this increase depends on the electronic density of the conjugated system (R group) attached (Scheme 2).



Scheme 2: Mechanism of photocleavage of recently synthesized o-nitrobenzyl derivatives

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PALLADIUM-CATALYZED DOMINO REACTION AS A ROUTE TO MEDIUM- SIZED AZATHIACYCLE-FUSED BENZIMIDAZOLES

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The relevance of organosulfur compounds in medicinal chemistry can be evidenced by the high incidence rate of approved drugs containing a sulfur functional group, among them S-heterocycles that are typically 5- to 7-membered rings (thiophene, thiazolidinedione, dihydrothiazine, thiazepine).1 In general, the access to medium-sized rings (8 to 12 atoms) is more arduous in reason of entropy and enthalpy factors as well as transannular interactions. Nevertheless, overcome these unfavorable factors, aiming the development of cyclisation methods, is important to access novel ring systems like non-flat ones, by the introduction of sp3-carbon atoms. Considering a drug discovery program, based predominantly in aromatic fragments, the synthesis of non-aromatic scaffolds represents an increasing in chemical space and, potentially, in biological space. The more globular shape of structures containing a non-flat core can impact favorably in their pharmacokinetic and pharmacodynamic properties.2,3 Transition metal-catalyzed domino reactions represent a highly selective, step-economical and efficient synthetic strategy to increase structural complexity, from simple starting materials.4 Due to the possible interference during the catalytic cycle caused by the thiophilicity of some transition metals, the use of S-containing substrates often requires more efforts to find the right catalytic reaction conditions.

Our group successfully developed a cyclocarbopalladation/Suzuki and Stille coupling cascade reaction for sulfide substrates, achieving targeted benzene-fused 5- and 6-membered S-heterocycles.5-7 In this communication we will report our recent results in the extension of this method to the synthesis of 8- and 9-membered azathiacycle-fused benzimidazole compounds.

Rescoring docking poses by graph matching of protein–ligand interactions

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Molecular docking is a widely-used technique to predict the three-dimensional (3D) atomic coordinates of a protein-ligand complex. However, correctly scoring the docking solutions is a major issue and limitation of current scoring functions[1]. The docking community has therefore organized several resources to aid computational chemists to refine both their methods and protocols. One of them is the Drug Design Data Resource (D3R) that periodically proposes challenges aimed at predicting protein-ligand coordinates and binding energies prior to the release of their crystal structures and related experimentally determined affinity data. The D3R Grand Challenges were a good opportunity to test our algorithm GRIM[2] to rank docking poses. GRIM uses a knowledge-based approach to convert protein-ligand complexes in interaction pattern graphs and score docking solutions by similarity of predicted interaction patterns to that already visited in the Protein Data Bank.

Here we summarize the results achieved in two D3R Grand Challenges[3,4] (2015 and 2016) and discuss the strengths and the limitations of our method. When applied to the HSP90α data set, for which many protein-ligand X-ray structures were already available, GRIM provided very high quality solutions (mean rmsd = 1.06 Å, n = 6) as top-ranked poses, and significantly outperformed a state-of-the-art scoring function. In the case of MAP4k4 and FXR inhibitors, the accuracy of GRIM poses decayed due to two main factors: (i) scarce preexisting 3D knowledge and higher chemical diversity and (ii) hydrophobic nature of the active site. Nevertheless, GRIM still outperformed the docking energy-based scoring functions with a mean rmsd of 3.18 Å (n=30) for MAP4K4 and 3.25 Å (n=35) for FXR. Despite the limitations, our rescoring method is quite simple to implement, independent from a docking engine, and applicable to any target for which at least one holo X-ray structure is available. In addition to pose prediction, we established a simple scheme to rank 102 FXR agonists in the second challenge. Using GRIM to select the best pose and HYDE5 to estimate the Gibbs free energy of binding, we provided a fast protocol, yielding the third most accurate ranking method among 57 contributions.

This protocol is accurate enough and could be applied to post-process virtual screening data.

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Understanding cell adhesion and migration using rainbow cell coding by fluorescent polymer nanoparticles

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Fluorescence imaging is a valuable tool for tracking and identifying different cell populations in vitro or in vivo. Indeed, cell labelling technique can help to answer key questions such as cancer research, cell differentiation or regenerative medicine. Recently, we developed a new approach for long-term multicolor cell tracking in vitro and in vivo (1). This technique is based on dye-loaded fluorescent polymer nanoparticles (NPs), which combine biodegradability, low toxicity and superior brightness (1). NPs of three distinct colours were prepared by encapsulating three cyanine dyes (DiO, Cy3 or Cy5) with help of hydrophobic counterions. Combination of these three NPs in different proportions allowed us to generate multiple colour codes within living cells (2).

Here, we applied this approach to study cell adhesion and migration. First, the precision of the labelling technic for cell quantification has been determined by associating it to an automatic analysis of the microscope images. This method allowed us to observe fixed or living cells for a large range of cell density. Then, an adhesion test has been developed to monitor directly the behavior of several glioblastoma cell lines in a mixture, expressing at different level the α5 integrin, depending on the substrate. The obtained results have been compared and validated by the crystal violet assay which is a standard test of cell culture. We found that this cell labelling technique is easier to execute, less time-consuming because multiple cell lines can be studied together. Second, using a spheroid composed of five color-coded glioblastoma cell lines, a cell migration test has also been developed and followed by video-microscopy. This new test allows us to study and distinguish at least five cell populations at the same time with five different colours and observe direct competition between cell lines as well as to visualize their migration path and speed within complex systems.

To conclude, our nanoparticle-based multicolor cell labeling method can be applied to study cell adhesion and migration in complex multi-cellular systems and could in principle be applied to study of tumoral heterogeneity in vitro and in vivo.

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Prodrugs of alkyl phospholipids (pro-APLs) as a new therapeutic approach to cancer by anti-tumour lipids

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For several decades, stable analogs of lysophosphatidylcholines (lysoPCs) have been studied in the cancer field because of their ability to inhibit cell proliferation and a potential selectivity to tumor cells. Analogs such as miltefosine, erufosine or perifosine are part of the alkyl phospholipids family (APLs) which exhibit a targeted mechanism of action at the cell membrane (Figure 1).

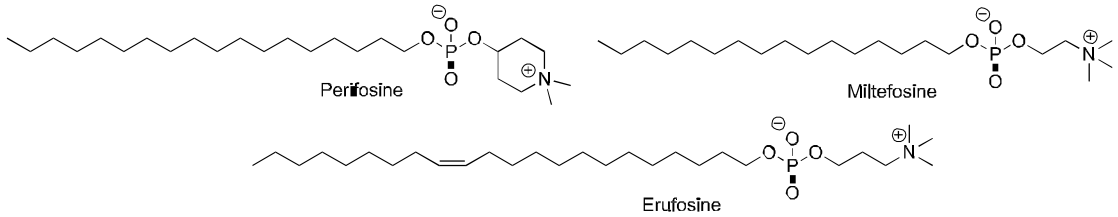

Figure 1. Molecular structure of the three investigated anti-tumour APLs.

From these three most promising APLs, we have synthesized various cationic pro-alkyl phospholipids (pro-APLs) and have investigated their potential in gene transfer applications. Indeed, the cationic and amphiphilic nature of these compounds makes it possible to complex and pack nucleic acid into discrete nanoparticles. Moreover, the biodegradability of the pro-APLs may lead to the in situ release of the anti-tumour APL precursors. Selecting a DNA plasmid coding for a pro-apoptotic protein (human tumour necrosis factor (TNF)-related apoptosis-inducing ligang, hTRAIL) is expected to offer a synergistic anti-tumour effect between gene transfer (hTRAIL-pDNA intracellular delivery by pro-APLs) and chemotherapy (intracellular release of anti-tumour APLs) (Figure 2).

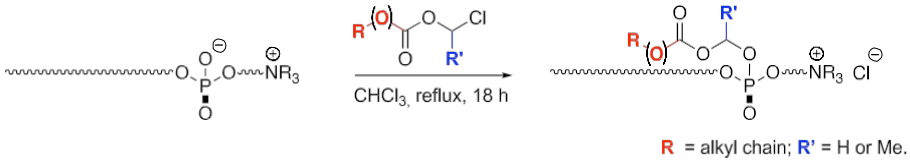

Figure 2. General synthetic route to pro-APLs.

The results obtained with a reporter gene encoding the luciferase protein conveniently allowed the identification of structure-activity relationships within a set of 33 pro-APLs, and various process parameters for the preparation of lipoplexes were systematically investigated to optimize efficiency of the formulations. The most potent pro-APLs are currently evaluated on healthy and malignant cell lines, using a therapeutic plasmid encoding hTRAIL.

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Dynamics of Methylated Cytosine Flipping by UHRF1

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The last decade has seen an explosion in our understanding of the underlying molecular mechanisms that govern gene expression, with epigenetics taking center stage. Epigenetics refer to the heritable phenotypic changes that occur without altering the DNA sequence. Major epigenetic markers include DNA methylation, post-translational modifications of histones, histone variants and nucleosome positioning.[1-3] In eukaryotes, DNA methylation is a heritable cytosine modification, critical for gene expression, is replicated by DNA methyltransferase 1 (DNMT1) and Ubiquitin-like containing PHD and RING Finger domains 1 (UHRF1) proteins. This replication is initiated by the recognition of hemi-methylated CpG sites and further flipping of methylated cytosines (mC) by the Set and Ring Associated (SRA) domain of UHRF1. Though crystallography has shed light on the mechanism of mC flipping by SRA, tools are required to monitor in real time how SRA reads DNA and flips the modified nucleobase.

To accomplish this aim, we have utilized two distinct fluorescent nucleobase surrogates, 2-thienyl-3-hydroxychromone (3HCnt) and thienoguanosine (thG), incorporated at different positions into hemi-methylated (HM) and non-methylated (NM) DNA duplexes. Large fluorescence changes were associated with mC flipping in HM duplexes, showing the outstanding sensitivity of both nucleobase surrogates to the small structural changes accompanying base flipping (Fig. 1). Importantly, the nucleobase surrogates marginally affected the structure of the duplex and its affinity for SRA at positions where they were responsive to base flipping, illustrating their promise as non-perturbing probes for monitoring such events. Stopped-flow studies using these two distinct tools revealed the fast kinetics of SRA binding and sliding to NM duplexes, consistent with its reader role. In contrast, the kinetics of mC flipping was found to be much slower in HM duplexes, substantially increasing the lifetime of CpG-bound UHRF1, and thus the probability of recruiting DNMT1 to faithfully duplicate the DNA methylation profile. The fluorescence-based approach using these two different fluorescent nucleoside surrogates advances the mechanistic understanding of the UHRF1/DNMT1 tandem and the development of assays for the identification of base flipping inhibitors.

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Excited state dynamics of oxyluciferin and its derivatives in water

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Bioluminescence is a natural phenomenon by which living organisms convert chemical energy into light1. One of the most characterized reaction involves the firefly luciferase that catalyses the oxidation of the luciferin producing oxyluciferin in its first excited state. While relaxing to the ground state, oxyluciferin emits visible light with an emission maximum that can vary from 536 to 638 nm2. Despite the increasing number of studies, the exact nature of the emissive species involved in the enzymatic reaction is still an open question. Indeed, oxyluciferin can exist in six different forms as a result of ionization of two hydroxyl groups and the keto-enol tautomerism of the 4-thiazolone subunit.

By using oxyluciferin derivatives, together with steady state and nanosecond time-resolved spectroscopy, we recently characterized the optical properties of the six-chemical species in aqueous buffer3. In particular, we evidenced the important role played by excited state proton transfer (ESPT) reactions. However, due to the limited temporal resolution of fluorescence lifetime measurements (50 ps), it was not possible to time resolved the ESPT reaction. To further characterize the excited state reactions, femtosecond pump-probe spectroscopy was used to monitor the ESPT dynamics of oxyluciferin derivatives in aqueous buffer as a function of pH.

With the help of global analysis, it was possible to obtain the spectra of the transient species together with the characteristic time associated to the proton transfer in the first excited state.

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Development of high-resolution probes for electron microscopy based on antibody-gold nanocluster conjugates

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The objective of my PhD project is to synthesize novel electron microscopy (EM) probes for high resolution imaging of a desired protein inside living cells. This project relies on generating novel antibody-gold nanocluster conjugates and on developing novel observation procedures.

Over the last years there have been tremendous technical advances in the development of EM apparatuses leading to a drastic improvement in resolution. The analysis of biological samples usually requires contrasting agents for the electron beam and among other heavy metals gold nanoparticles represent a valuable tool for this purpose. However, the currently used gold nanoparticle-EM-probes are in the size range of 5-10 nm and are therefore not small enough to diffuse well inside tissues and to cope with the novel advances in EM instrumentation. To improve the quality of the EM probes and to expand the scope of action of EM to detection of an intracellular epitope inside a living cell, we propose to develop gold nanoclusters (AuNCs) having sizes between 2 and 4 nm and bioconjugation methods for cleanly linking these EM-contrasting agents to organic ligands such as antibodies, antibody fragments or nanobodies.

Up to now, I have synthesized novel organothiolate-protected gold nanoclusters and optimized ways to conjugate these clusters to peptides and full antibodies. The gold nanocluster synthesis is accomplished by a reduction of a gold(III) salt in the presence of an organothiolate ligand. The conjugation to biomolecules is then performed either by thiol exchange-, NHS-ester- or maleimide chemistry. For generating antibody-AuNC conjugates we selected two organothiolate protected AuNCs having a molecular weight of 25 kDa and 80 kDa respectively, as well as a monoclonal antibody directed against the nuclear RNA polII and a monoclonal antibody against the epidermal growth factor receptor (EGFR). Our next plan is to test those antibody-AuNC conjugates (EM probes) on living cells and to immunolocalize the desired proteins at the highest possible resolution.

This project will provide valuable ground for the development of antibody-gold nanomaterials for biomedical research and particularly for selective targeting and imaging of proteins in the dense and crowded cellular environment.

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Non-Peptidic Fluorescent Turn-on Probes for Live-Cell Imaging of GPCRs

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Fluorescent turn-on systems are probably the most powerful tools in biological sensing, enabling direct quantification of an analyte without removal of unreacted probe and providing the possibility of in vivo monitoring of molecular interactions.[1] Especially, turn-on probes will be of high interest to study G protein-coupled receptors (GPCRs), the largest and most diverse group of membrane receptors in eukaryotes, at the surface of living cells.

Recently peptidic turn-on probes have been developed for imaging and quantifying of GPCRs in living cells.[2]However such probes present several limitations for in vivo imaging because of their low metabolic stability, high molecular weight and limited passage through biological membranes.

To overcome these drawbacks non-peptidic fluorescent turn-on probes were designed. The oxytocin receptor (OTR), a GPCR involved in the modulation of social behavior, was used as a model. A turn-on dye Nile Red was grafted onto an OTR selective non-peptidic antagonist[3] via PEG spacers of different lengths. Thereby, the modulation of the length of the spacer allowed us to fine-tune the turn-on properties and the non-specific interactions of the probes. One of them demonstrated a clear staining of the receptor in living cells expressing the OTR, with no detectable non-specific interactions with cellular membranes, thus being the first efficient non-peptidic turn-on probe for the OTR. This compound constitutes a promising tool for the imaging of the OTR in isolated tissues and eventually in whole animal. Furthermore the concept of non-peptidic turn-on probes could be extended to other GPCRs.

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PCBIS: Chemical libraries, biological models, technological tools and early ADMETox for laboratories

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High Throughput Screening (HTS) is the technology which best facilitates the search of new molecules with the potential of becoming the drugs of tomorrow. Until recently this expensive technology was only available in pharmaceutical companies.

Starting in 1999, the "Plate-forme de Chimie Biologique Integrative de Strasbourg" (PCBIS) developed the expertise in this field in order to be able to offer this technology in an academic context. One of our main goals is to offer our expertise to laboratories aiming to find new drugs to cure rare and/or neglected diseases. Our commitment to quality drove us to set up a quality management system granted by ISO 9001 and NF X50-900 certifications.

The PCBIS's expertise and equipment necessary for new drug discovery is now proposed to academic laboratories, start-ups and industries interested in a fast paced approach to screening.

We also propose to train people and give an access to our technologies to interested laboratories.

We will show some of the tools that PCBIS can propose to the scientific community.

www.pcbis.fr

Atrial endothelial cells senescence promotes thrombogenicity, inflammation and extracellular matrix remodeling: role of the local Ang II / AT1 receptor pathway

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Introduction: Ageing promotes atrial remodeling that paves way to atrial fibrillation and thrombogenicity. Preclinical studies on endothelial atrial cells are lacking.

Objective: This study aims to characterize phenotypical changes associated with atrial endothelial cells senescence and to decipher the link between ageing and thrombogenicity.

Methods: Atrial endothelial cells (AEC) were obtained from freshly harvested porcine left atria. Endothelial senescence was assessed by senescence-associated beta-galactosidase activity (SA-β-gal), using flow cytometry, protein expression by Western blot analysis and platelet aggregation using an aggregometer. Replicative senescence was induced by passaging AEC from passage P1 to P4, and premature endothelial cell senescence by exposing AEC to L-NAME, an endothelial NO synthase (eNOS) inhibitor or H2O2.

Results: AEC senescence was characterized by an increase in SA-β-gal activity and an up-regulation of p53, a key regulator of cellular senescence, and of p21 and p16, key cyclin-dependent kinase inhibitors. Senescent AEC phenotype was characterized by (i) cell thrombogenicity through an up-regulation of tissue factor expression, shedding of procoagulant microparticles, eNOS down-regulation and reduced NO-mediated inhibition of platelet aggregation, (ii) cell adhesion through up-regulation of ICAM-1, (iii) proteolysis and fibrosis remodeling through MMP-2, 9 and TGF-ß1 expression, and (iv) up-regulation of the local Ang II system through enhanced AT1 receptors (AT1R) and angiotensin-converting enzyme (ACE) expression. Losartan, an AT1 receptor antagonist, and Perindoprilat, an ACE inhibitor, prevented atrial endothelial cell senescence.

Conclusions: Thus, atrial endothelial senescence promotes thrombogenicity, inflammation, matrix remodeling and the up-regulation of the local Ang II system. They further suggest that targeting the Ang II/AT1R pathway may be a promising therapeutic strategy to delay atrial endothelial ageing.

Photophysical Study of an Isomorphic Fluorescent Nucleoside

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Isomorphic fluorescent nucleobases are extensively used to monitored nucleic acid structure, dynamics and recognition [1]. Among them, thienoguanosine (thG), a truly faithful emissive and responsive surrogate for G with reasonable quantum yield, actually reproduces the structural context and dynamics of the parent native nucleoside [2]. Photophysical properties of this nucleoside was examined prior to incorporation into oligonucleotides [3,4]. This fluorescent nucleoside in free state exists as two keto-amino tautomers in the ground state and excited state (thG-H1 and thG-H3). The equilibrium between the two tautomers was investigated in aqueous solution with variation of pH. In aqueous solution, thG has two pKa values with 4.5 and 10.1. We also identified the lifetimes associated with thG-H1 (20.5 ns) and thG-H3 (12.6 ns). It is noticeable that the red-shifted tautomer (thG-H1) is favoured when thG is incorporated into (-)/(+)PBS duplexes (18-mer primer biding site of HIV-1).

To further understand the photophysics of thG in duplexes, we have labelled (-) PBS DNA at 7th position and examined the dependency of its photophysical properties as a function of the neighbouring and opposite bases. To this end, we performed an integrated and systematic investigation combining steady-state and time-resolved fluorescence together with ab initio and molecular modelling. In case of match sequences, almost constant values of quantum yield (0.11-0.18) and long-lived lifetime (9.5-11.9 ns) were observed irrespective of the neighbours. In contrast, the quantum yield and lifetime values of thG are highly sensitive to the nature of the flanking bases in mismatched sequences, as a result of differences in charge transfer.

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Pan nuclear phosphorylation of histone H2AX reflects lethal replication stress induced by genotoxic drugs

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Gamma-H2AX (γ-H2AX) corresponds to the phosphorylated form of histone H2AX, a H2A histone variant. This phosphorylation, localized on serine 139 is induced by the protein kinases involved in the initiation of DNA damage response. γ-H2AX is a widely accepted DNA double-strand break (DSB) marker. Recently, it has been shown that this post-translational modification can also occur in response to the formation of single-stranded DNA, which arises after both DNA and RNA-polymerase stalling. Thus, γ-H2AX could be an excellent indicator of replication stress (RS), a biological process linked to abnormal progression of the DNA replication mechanisms.

Cancer cells are naturally experiencing mild levels of RS due to the down-regulation of cell cycle checkpoints and DNA repair pathways. Commonly used anticancer treatments aim to impair the DNA replication process thereby triggering RS to unsustainable levels. The accumulation of numerous DSBs due to the overwhelming of the DNA repair machinery brings cancer cells to death. Although the mechanisms of the replication stress response (RSR) are nowadays well characterized, currently there is no mean to evaluate precisely the efficiency of such treatments.

Our aim is to evaluate if pan nuclear phosphorylation of H2AX could be used as a readout of lethal RS following drug treatment. In particular, we are studying the dynamics of H2AX phosphorylation after treatment with different genotoxic drugs that target both the replisome and RSR. To monitor the RS induction, we have generated monoclonal antibodies that bind specifically to the phosphorylated form of H2AX. These new tools allowed us to visualize by immunofluorescence that γ-H2AX spreads to the whole nucleus after foci formation following treatment of a variety of cancer cells with defined combination of chemotherapeutic drugs. The spreading of the phosphorylation of H2AX under these conditions, often termed pan-nuclear γ-H2AX, was closely correlated with loss of survival. This was notobserved with untransformed cells.

We have observed that this pan-nuclear phosphorylation corresponds to a “point of no return” to physiological replicative stress in cancer. The co-expression of accurate biological activities triggered by identified molecular events such as p53 induction, DNA-PK kinase hyperactivation and 53BP1 foci collapse leads irreversibly to cell death. We are currently developing our VANIMA method [1] for realtimedetection of γ-H2AX in single cells that are not blocked in S-phase and progress to catastrophicmitosis.

We believe that this post-translational modification could be used in live cancer cells to screen new compounds that generate cancer-specific lethal RS.

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Invasive and migratory properties of human melanoma cells are correlated to the grade of malignancy.

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Melanoma is one of the most deadly cancers because of its high propensity to metastasis, a process that requires migration and invasion of tumor cells driven by the regulated formation of adhesive and invasive structures like focal adhesions and invadopodia. The metastatic spread of melanoma is a complex process involving several sequential events. Initially, normal melanocytes are transformed via genetic alterations leading to the local spreading of melanocytes into the epidermis in a Radial Growth Phase (RGP) which at this stage in not invasive and thus associated with good prognosis. The next stage called the Vertical Growth Phase (VGP) is associated with the dissemination of some melanoma cells away from the initial tumor. These cells display pronounced invasion properties, degrade the ECM, spread through the dermis and establish increasing vascularisation thus being able to enter the lymphatic and vascular network and metastasize into distant organs to form metastatic melanomas.

At this stage the clinical outcome is seriously compromised. We investigated the mechanism involved in melanoma dissemination, by analyzing the cell migration/invasion properties of melanoma cells derived from different grades of malignancy. In a 3D-invasion assay, RGP cells were less invasive than VGP and metastatic cells. To decipher further the underlying cellular behaviour, cell polarity was explored using MTOC/Golgi positioning in fixed and living cell. Our results show that melanoma cells are capable of orienting their Golgi toward the front of cells. Moreover, VGP cells appear to display higher polarization efficiency compared to other melanoma grades, which correlates with a higher persistence of directional migration. These results suggest that migration directionality and polarization, but not migration speed, are stimulated when cells escape from their original tissue. Furthermore, investigating invadopodia localisation and function, we surprisingly found that both VGP, RGP and metastic melanoma displayed invadopodia as indicated by actin/cortactin co-staining.

Nevertheless, in-situ zymography revealed that only invadopodia from VGP and metastatic melanoma but not RGP are capable of matrix degradation suggesting that maturation of invadopodia is a critical step in the molecular switch from RGP to VGP grade.

RPL7, a novel partner of HIV-1 Gag polyprotein enhancing its nucleic acid chaperone activity

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HIV-1 infection is characterized by Gag-mediated virion formation during the late phases of viral replication (1). During assembly, genomic RNA (gRNA) recruitment and dimerization requires specific interaction between Gag and gRNA, relying on the nucleic acid chaperoning activity (NAC) of Gag (2). As shown earlier, Gag recruits also cellular proteins including human ribosomal protein L7 (RPL7) which also possesses substantial activity as demonstrated by in-cell and in-vitro studies (3). Hence, the limited chaperoning activity of HIV-1 Gag (4) could be enhanced by cellular co-factors to reach optimal level.

To understand the RPL7 alone or associated with Gag (Gag-RPL7 complex) chaperones nucleic acids, we investigated the annealing reaction between the DNA sequence corresponding to the viral transactivation element and its complementary cTAR sequence. We used fluorescence-based assays to follow the extended duplex training (ED) in real time (5, 6). dTAR mutants were also used to provide information regarding reaction pathways.

We showed that the annealing of cTAR to dTAR proceeds through a two-step mechanism involving the formation of a complex transient and its slow conversion into the final duplex (ED). This annealing is mediated by the stems in the presence of Gag and RPL7 alone. CTAR / dTAR annealing follows both stem-stem and loop-loop pathways. These results indicate that Gag and RPL7 act in concert in the annealing reaction favoring the notion that Gag recruits RPL7 to boost its chaperone activity in order to initiate the assembly process.

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FRET Based Polymeric Nanoparticles for Ratiometric Detection of Molecular Oxygen

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Optical sensing of molecular oxygen has attracted a great deal of scientific attention since the determination of oxygen concentration is essential in diverse areas ranges from life sciences to environmental sciences. It is particularly important for biomedical applications because tissue hypoxia has been found to be closely related to the clinical course of a variety of diseases, such as tumor growth, rheumatoid arthritis etc[1].Fluorescent polymer nanoparticles (NPs), composed of conjugated polymers and organic dyes, have received great attention due to a number of unique properties, such as exceptional brightness, color tuning from visible to NIR region, potential biodegradability and low toxicity[2]. We previously reported the formulation of ultrabright dye-loaded fluorescent NPs based on biocompatible polymers such as PLGA, PMMA, etc loaded with cationic dyes and bulky hydrophobic counterions[3].

With an objective to develop a FRET based ratiometric oxygen sensor, we made fluorescent polymeric nanoparticles containing a cyanine-based donor dye (D) with a fluorinated counter ion and a porphyrin acceptor (A) as the oxygen sensitive moiety. The preparation of the NPs was achieved by charge-controlled nanoprecipitation[3b], by varying the ratio between D and A concentrations in order to optimize their ratiometric emission response. Steady state experiments suggest the efficient energy transfer from cyanine (D) to porphyrin (A) in the PMMA matrix. However, the phosphorescence emission of the porphyrin at 650 nm was suppressed by the dissolved oxygen, which recovered with increasing concentrations of sodium sulfite, a well-known oxygen scavenger. By varying the oxygen concentration in the mixture from 0 to 95%, we could able to observe the enhancement of acceptor emission whereas the donor emission remained same, which facilitated the ratiometric detection. Time-resolved measurements also suggest the decrease in phosphorescent life time of the acceptor with increase in oxygen concentration. Further, we investigated the potential of the probe in single particle fluorescence imaging, after immobilizing NPs on a glass coverslip. Interestingly, we could observe bright emission from donor channel and negligible emission from acceptor channel in the presence of oxygen, and the enhancement in acceptor emission upon oxygen scavenging. The cellular uptake of NPs and ratiometric imaging of oxygen in cells are currently in progress. The small sizes, high brightness, and the ratiometric emission response make these NPs a good candidate for the quantitative detection and imaging of molecular oxygen in biological samples.

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Design of polydiacetylenic nanofibers as new siRNA vehicles for in vitro and in vivo delivery, with high pharmacodistribution in target tissues

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Polydiacetylenic nanofibers (PDA-Nfs) obtained by photopolymerization of a diacetylenic surfactant bearing primary amine groups (1) were optimized for intracellular delivery of small interfering RNAs (siRNAs).

In this work we were able to show for the first time that PDA-Nfs can be promising delivery agents for siRNAs. Surfactant (1) allows for the generation of relatively small nanofibers that are able to internalize efficiently into cells. The PDA nanofibers associated with a siRNA targeting a reporter gene (luciferase gene in A549-Luc and 786-O-Luc cell lines) lead to a very efficient silencing.

In vitro, the PDA-Nfs/siLim1 efficiently silenced by more than 80% the expression of the Lim-1 oncogene in the 786-O human CCC cell line. Furthermore, when administered intra-peritoneally, the PDA-Nfs were able to deliver siRNA into sub- cutaneously grown tumors, where they led to specific silencing of the targeted Lim-1 oncogene. By analyzing the biodistribu- tion of the PDA-Nfs, we proved that these nanofiber systems diffuse in the bloodstream and reach various organs as well as the targeted tumors. They were cleared from the body 48 hours post-injection presumably by renal excretion, as the PDS-Nfs could then be detected in urine samples.

Taken together, we found that PDA-Nfs are a promising and highly efficient new class of self-organized siRNA vectors for in vitro and in vivo applications, with good biodistribution pro- files in the body.

Upregulation of sodium-glucose cotransporter 2 (SGLT2) expression in cultured senescent endothelial cells and in arterial sites at risk in vivo in rats

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Introduction: Endothelial senescence is thought to promote endothelial dysfunction and the subsequent development of cardiovascular diseases. The EMPA-REG trial has shown that sodium-glucose cotransporter 2 (SGLT2) inhibition is associated with a reduced risk of cardiovascular mortality in type 2 diabetic patients, but the protective mechanism remains unclear. SGLT2 mRNA has not been detected in control endothelial cells (ECs).

Aim: This study examined the possibility that SGLT2 contributes to endothelial senescence and dysfunction and, if so, to characterize the underlying mechanism.

Methods: Endothelial cells isolated from porcine coronary arteries were used at passage 1. Senescence was assessed using senescence-associated beta-galactosidase activity (SA-beta-gal activity), protein level by Western blot analysis, oxidative stress using dihydroethidium, and Nitric oxide (NO) formation by electronic paramagnetic resonance.

Results: Exposure of ECs to High glucose (HG) (25 mM) for 96 h increased the level of SA-beta-gal activity and of senescence markers (p21 and p16) and oxidative stress, decreased eNOS expression and NO formation, and increased the expression of VCAM-1 and tissue factor (TF). Both HG and H2O2induced the appearance of SGLT2 mRNA, increased SGLT2 protein level and SGLT2-mediated glucose entry in ECs. An increased expression level of SGLT2 and VCAM-1, and a down-regulation of eNOS were observed at arterial sites at risk (aortic cross) compared with those at low risk (thoracic aorta) in young rats.

Conclusion: These findings indicate that premature ECs ageing is characterized by the down-regulation of NO formation and the expression of pro-atherothrombotic factors, and is associated with the redox-sensitive upregulation of SGLT2 expression promoting excessive glucose entry. The fact that an increased SGLT2 expression level is observed in vivo at arterial sites at risk, suggests that inhibition of SGLT2 might be an attractive strategy to protect the cardiovascular system.

Luminescent Eu-complex loaded polymer nanoparticles as bright probes for cellular imaging

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Lanthanides have emerged over the last years as attractive probes for bioimaging.[1] Their exceptionally long lifetime as well as their large Stokes shift can be used to overcome autofluorescence issues in biological samples. However, they are strongly limited by their low extinction coefficients providing a limited brightness. Here we propose an approach to design probes with superior brightness by encapsulating large amounts of a lanthanide complex inside polymer nanoparticles (NPs).[2] We previously described charge-controlled nanoprecipitation for making ultrasmall polymer NPs with efficient encapsulation of high amounts of hydrophobic fluorescent dyes.[3,4]

Using this approach, we assembled three series of poly(methyl methacrylate) based NPs with different sizes (10, 20 and 30 nm) encapsulating up to 40 wt% of an Eu3+ complex (>200 complexes per particle). The resulting NPs were characterized with respect to their size, absorbance and luminescence properties. The polymer matrix provides an efficient protection of the encapsulated Eu3+ complex, increasing its lifetime and reaching quantum yields of up to 25%.

The resulting particles are bright enough to be imaged at the single particle level and are readily internalized by cells with excellent stability for cellular imaging.

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Synthesis and characterization of new organic nanoantennas for FRET-based molecular detection

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Dye-loaded polymeric nanoparticles have proved themselves as a powerful bioimaging tool due to their high brightness and capacity to bear multiple functional groups[1]. Per contra when the nanoparticles are loaded with high concentration of fluorophores, aggregation caused quenching (ACQ) phenomenon arises, thus limiting the brightness of these systems. This problem was solved by our group by employing cationic dyes functionalized with hydrophobic groups paired with bulky hydrophobic counterions. Such strategy leads to an effective spacing of the fluorophores inside the nanoparticles decreasing the quenching; moreover it generates a supramolecular organization which features a cooperative behavior of the dyes[2], [3]. Such collective behavior of our system was employed for preparing light-harvesting nanoantenna particles that enable Fluorescence Resonance Energy Transfer (FRET) from thousands of rhodamine B derivative dyes to few cyanine 5 derivative dyes; leading to >1000-fold amplification of the acceptor intensity[4].

The aim of the current work is to obtain super-bright nanoantenna particles operating in different spectral regions. Too this end, two rhodamine derivatives have been tested so far: green-emitting octadecyl rhodamine 110 ester and yellow-emitting octadecyl rhodamine 6G ester were paired with various hydrophobic bulky counterions and nanoprecipitated to obtain dye-loaded nanoparticles. A complete photophysical characterization of the as-obtained nanoantenna particles was performed: absorption and emission spectra, quantum yield, single-particle brightness and ON/OFF switching behavior were investigated. Then, FRET nanoparticles, containing a hydrophobic cyanine 5 derivative as acceptor, were prepared and their performance was assessed. Finally new nanoantenna systems were tested in a FRET assays for detection of nucleic acids.

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Role of endocytosis in EGFR-targeted therapy resistance in Glioblastoma

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Epidermal Growth Factor Receptor (EGFR) trafficking dysregulation is involved in tumor progression and targeted-therapy resistance. EGFR has been described as a major therapeutic target in glioblastoma (GBM), since its overexpression drives GBM cell invasion and tumor progression. However, clinical trials were disappointing, and we are still missing molecular basis to explain these poor results. So is important to identify potential new therapy resistance mechanisms, focusing mainly in EGFR trafficking.

We started to work on known regulators of EGFR trafficking, such as α5β1 integrin and dynamin-2. Integrin is known to be regulator of EGFR oncogenic activity during tumor progression by affecting its trafficking, mainly the recycling. Therefore, we seek to evaluate the impact of EGFR trafficking modulation in GBM towards target-therapy response.

Material and Methods: we used GBM cell line (U87) overexpressing or down expressing α5 integrin subunit. EGFR was inhibited by clinically approved tyrosine kinase inhibitors (TKIs) (gefitinib, erlotinib, lapatinib). To further evaluate the role of endocytosis in response to targeted therapies, we inhibit dynamin using chemical inhibitors and siRNA. Cell proliferation was evaluated by crystal violet and cell motility by spheroid dissemination assays. Confocal microscopy confirmed integrin, EGFR and endosomes markers localization. Protein co-localization in endosomal structures was confirmed by confocal image analysis and dSTORM super-resolution. Dynamic studies were performed to study EGFR trafficking, for example by using EGF-Alexa 488.

Results and Discussions: TKIs provoked a massive re-localization of EGFR inside of vesicular structures. EGFR was found after treatment in early endosomes (co-localization with Rab5 and EEA1 markers) and also with integrin in the same vesicle. Dynamic studies confirmed once again the impact of TKIs in EGFR endocytosis. By colocalization studies and super-resolution dSTORM imaging, we clearly showed that in intracellular vesicles integrin and EGFR are in close proximity, suggesting a potential interaction that can influence receptor activity and trafficking leading to therapy resistance. When evaluated the impact of α5β1 integrin in response to therapy, it was observed that loss of integrin α5 expression increased U87 cell sensitivity to TKIs in spheroid cell evasion assay. Perhaps α5β1 integrin regulates EGFR by modulating its trafficking. Currently, we are modulating dynamin expression and activity to evaluate the impact on therapy response.

Conclusion: we showed in GBM cells that endocytosis may play a relevant role in cell response to targeted ttherapy treatment. Modulators of EGFR endosomal trafficking such as α5β1 integrin or dynamin may predict responsiveness towards EGFR targeted therapies.

Total Synthesis of Isoprekinamycin key-precursor via a Fukuyama Cross-Coupling Reaction

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Isoprekinamycin (IPK), made up of a 6−5−6−6 ring system, has an in vitro cytotoxicity against cancer cells with a mode of action different from classical anticancer agents in current clinical use.[1-3] Despite the importance of this natural product, to date there is only one study reporting the total synthesis of IPK, through a Suzuki cross-coupling reaction as a key step. Besides the low overall yields, the reported synthetic route involved the use of dangerous reagents and harsh conditions, which will be unfavorable in the synthesis of IPK analogs and scale up.

Herein, we proposed a concise and mild synthetic route to obtain the key intermediate of IPK by using a novel catalyst for Fukuyama cross-coupling reaction.

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Production of Antibodies Against Model G Protein Coupled Receptors Using Recombinant Semliki Forest Virus Particles as Immunogens

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G Protein Coupled Receptors (GPCRs) superfamily comprises more than 800 members encoded by the human genome. Those proteins stand at the crossroads of numerous key biological function, making them highly interesting therapeutic targets. Antibodies represent an interesting tool for GPCR study and functional regulation, offering a good alternative to small molecule drugs. But GPCR-specific antibodies remain rare, mainly because of the technical issues encountered when it comes to GPCR overexpression and purification.

Semliki Forest Virus (SFV) recombinant viral particles have the ability to induce a high-level expression of functional membrane proteins and receptors in infected cells, and have proved their efficiency in generating immune responses to a broad range of target species through different vaccinal studies.

In the present study, recombinant SFV particles coding for the Adenosine A2A Receptor (AA_2AR) have been used as immunogens to generate specific mice and camelid antibodies. The analysis of the serum samples collected throughout the immunization process reveal the emergence of a receptor-specific immune response.

Functionally-active purified receptors reconstituted into lipidic discs (nanodiscs) are to be used for screening and characterization of the antibody candidates.