Ultrafast mixing techniques are necessary to elucidate the mechanisms of high turnover enzymes like chlorite dismutase

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Chlorite dismutase is mostly found in chlorate respiring bacteria and catalyses the conversion of the toxic compound chlorite (ClO_2^-) to chloride (Cl^-) and molecular oxygen (O_2). The turnover rate of chlorite dismutase is circa 5000 s^{-1} which makes it difficult to investigate the reaction mechanism in detail using convential pre-steady state kinetic methods since one single turnover has a half-life time of circa 140 µs. Until now investigators have used peroxides like peracetic acid and H_2O_2 as alternative substrates to measure Compound I formation, under the presumption that this is the seminar intermediate in the reaction. The general hypothesis is that chlorite dismutase from e.g. *Dechloromonas aromatica* converts chlorite via a heterolytic cleavage to form a Compound I intermediate and a hypochlorite anion (ClO^-). Via a rebound step the ClO^- reacts with the Compound I species to form the O-O bond [1]. However, this hypothesis is based on the results with peracetic acid and there is no real evidence that chlorite reacts in a similar way.
Ultrafast microsecond timescale mixing techniques like the Nanospec and MHQ can be used to establish the mechanism of chlorite dismutase with its natural substrate chlorite. Both techniques rely on a special four-jet tangential mixer in which the substrate and enzyme are mixed in <1 µs. The reaction mixture is forced through an orifice (20-100 µm) in a platinum inlay to create a jet perpendicular to the plane of the channels.

The jet can be frozen on a cold plate at a particular distance that corresponds to a certain reaction time in a Microsecond Hyper Quench device (MHQ, dead time 80µs) [2] . The obtained frozen powder can be analysed with low temperature spectroscopy techniques like UV-vis, EPR and resonance Raman. Alternatively an optical observation cell can be attached directly to the mixer to create a nanosecond continuous flow spectrophotometer (Nanospec, dead time 4µs) [2]. Time resolved spectra can be taken at different distances from the mixer and analysed with singular value decomposition (SVD).

Using the above techniques a unique triplet state amino acid bi-radical species (Compound T) was discovered for *Azospira oryzae* chlorite dismutase with chlorite as substrate. Based on the results we believe that the previously reported mechanism for chlorite dismutase should be revised [3]. We propose that after binding of the chlorite molecule it is cleaved heterolytically in 97 µs (9°C) to form a Compound T species which subsequently decays within one millisecond to the resting state ferric enzyme.).

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A multi-technique approach for the study of the interactions between 1-anthracenyl-3-butyl functionalized Ag(I)/Au(I) NHC bis-carbenes and selected biosubstrates

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Auranofin (AF), introduced in 1985 for the clinical treatment of rheumatoid arthritis, was found cytotoxic against some selected cancer cell lines.[1] Since then, extensive studies have explored the possible applications of gold complexes as anticancer agents.[2] N-heterocyclic carbenes (NHCs) ligands turned out of particular interest for medicinally relevant gold complexes.[3] Also, Ag(I)-NHCs have been tested and showed an antitumor activity comparable to and, in some cases, higher than that of Au(I)-NHCs.[4] We will first describe some recent results on the activity of the Ag(I)-NHC-anthracenyl butyl imidazole bis-carbene fluorescent complex shown in Figure 1.

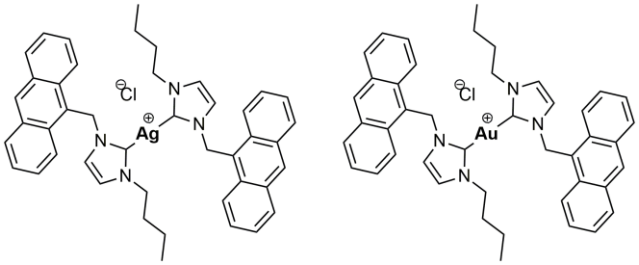

Figure 1. Molecular structure of the 1-anthracenyl-3-butyl functionalized Ag(I)/Au(I) NHC bis-carbenes.

We analysed the mechanistic details of its interaction with relevant biosubstrates such as duplex DNA, RNA, DNA G-quadruplexes (G4s), i-motif and serum albumin. To this aim, we did spectrophotometric and spectrofluorometric titrations, melting experiments, viscometric tests, FRET melting assays and gel electrophoresis experiments. The Ag(I)-NHC complex selectively interacts with DNA and not with RNAs and differently binds to G4s depending on their hybrid/parallel/antiparallel geometry. The solubility of the Au(I)-NHC is lower and prevents some experiments. However, some data on the gold counterpart enable us to discuss the different reactivity of the metal centres. Regarding their anticancer potential, MTT assays and bright field microscopy images let us to observe a high cytotoxic activity towards selected cancer cell lines.

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Microscale thermophoresis for characterizing metals, small molecules, and biomolecules interactions

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Microscale thermophoresis (MST) is a relatively recent biophysical technique, introduced to the scientific realm in the last decade. The term thermophoresis denotes the movements of particles under the effect of a temperature gradient in microscale dimensions. This phenomenon is known as a thermodiffusion, thermal diffusion, Ludwig-Soret effect, or Soret effect. MST is sensitive to minor changes in molecular size, hydration entropy, and effective charges upon interactions. Therefore, it is capable to investigate all matters starting from atomic levels to supramolecular interactions [1]. MST sensing mechanism is optically for the fluorescent partners in free solution within ~ 50 µm of heated spot diameter. MST has gained popularity due to its simplicity, less time consumption, very low sample volume, and the binding event occurs in free solution without the need for immobilization procedures for one reacting partner [2]. Indeed, MST is dedicated to biomolecular interaction mainly due to the fluorescence properties however; MST is capable to measure the binding events of low binding affinity systems. Our research group tracked the most featured applications for MST in life science such as drug discovery and drug design, biosensor development, and cell biology [3]. Furthermore, metal-chelate interactions were reported for the first time by our group using label-free MST for investigating deferiprone with different selected essential metal ions including Fe^{3+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2}. The MST measurements showed different binding affinity between deferiprone and divalent essential metal ions and the presented results were in agreement with the previous reports[4].

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Predicting nonequilibrium reactivity through Reactive Mode Composition Factor analysis: Rebounds, dissociations, and bifurcations

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The selective functionalization of C–H bonds is one of the Grails of synthetic chemistry, and the prediction of reactivity and selectivity usually rely on transition state theory (TST). In this talk, I will present scenarios where TST breaks down, and strategies to make predictions in such cases.

The main scenario is the C–H bond cleavage from an organic substrate by high-valent iron-oxo oxidants. After the C-H scission step, these reactions can follow either (a) fast hydroxylation, or (b) diffusion of the nascent radical out of the solvent cage. These diverging routes are known as the *OH-rebound* and *dissociation* mechanisms. Although enzymes can control selectivity through steric effects, biomimetic complexes in solution tend to follow different preferences for these reactive channels. The diversity of outcomes shows no evident trend and, intriguingly, DFT calculations suggest that in many cases both competing reaction channels are equally accessible, and even barrierless. What controls the selectivity here?

We developed the theory of reactive mode composition factor (RMCF) analysis, that allows quantifying the kinetic energy distribution (KED) at the reactive mode of any given transition state (TS) calculated using electronic structure methods.[1] With RMCF analysis at hand, we found that the selectivity of C-H activations leading to either rebound or dissociation can be predicted by decoding the KED, that 'dissects' the motion signatures of the relevant TS.[2] Further, we predict that the H/D primary kinetic isotope effect can serve as an experimental probe for these mechanisms.

Given its predictive power, the RMCF analysis can be employed even in complex processes involving nonequilibrium reactivity, as I will illustrate with its recent application to bifurcating reactions, which feature a single TS leading to two different products. Albeit TST is unsuitable in this scenario, the outcome of such reactions can be reliable predicted by RMCF analysis.[3]

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Oxygen-sensitive metalloprotein structure determination by cryo-Electron Microscopy

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Metalloproteins are involved in key cell processes, such as photosynthesis, respiration, and oxygen transport and it's estimated that approximately one third of all protein activities depends on transition metals.[1] However, the presence of transition metals, notably iron as a component of [Fe-S] clusters, often makes these proteins sensitive to oxygen-induced degradation. Consequently, their study usually requires strict anaerobic conditions. Although X-ray crystallography has been the method of choice to solve macromolecular structures for many years, recently cryo-electron microscopy (cry-EM) has also become an increasingly powerful structure-solving technique. [2] It now offers a way to solve structures at resolutions suitable for mechanistic studies without some of the X-ray crystallography limitations, because the amount of sample needed is much smaller and no crystal is required. We have used our previous experience with cryo-crystallography to develop a method to prepare cryo-EM grids in an anaerobic chamber. Then we have applied it to solve the structures of apoferritin, a gold-standard protein for cryo-EM and image analysis,[3] and 3[Fe_4S_4]-PFOR, responsible for the reversible ferredoxin-dependent oxidative decarboxylation of pyruvate in anaerobic microorganisms,[4] at 2.4 Å and 2.9 Å resolution respectively. The maps are of similar quality to the ones obtained under air, hence validating our method as an improvement in the structural investigation of oxygen-sensitive metalloproteins by cryo-EM as well as of that of their complexes. [5]

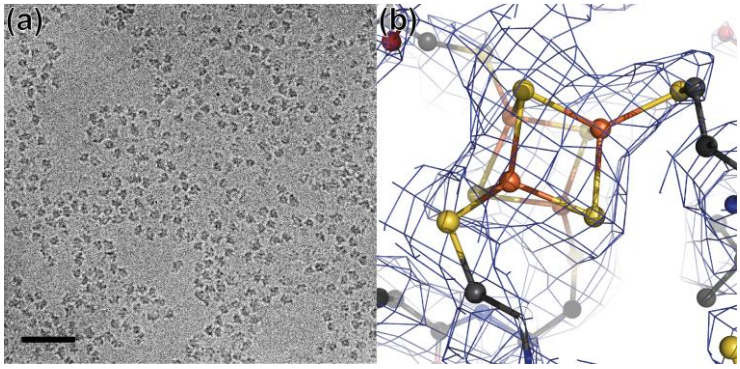

Figure: (a) Example of a PFOR collected micrograph (scale bar: 50 nm). (b) Close view of the proximal [Fe_4S_4] clusters of one PFOR monomer in the reconstructed cryo-EM density map. The density mesh is colored blue and contoured at 5 σ.

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X-ray Spectroscopic Evidence for a Physical Copper(III) Oxidation State in Organocopper Systems

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The Cu(III) oxidation state is commonly invoked in model and real biological systems. These typically unstable complexes are formally low-spin, magnetically silent $3d^8$ systems and characterization is generally limited to X-ray crystallography, electrochemistry and X-ray absorption spectroscopy (XAS). Of these techniques, XAS has been the go-to technique for identifying high valent Cu, with energy shifts of +1.0-1.5 eV being observed in both the pre-edge and rising edge features of Cu(II)/Cu(III) pairs.[1] Over recent years, hot debate has ensued over the "inverted ligand field" description of formally Cu(III) systems, and the appropriateness of the implied $3d^{10}$ electronic configuration.[2] This has led to the pursuit for further evidence in support of the $3d^8$ electronic configuration of formally Cu(III) systems. Herein, a series of organocopper complexes[3] in oxidation states ranging from +I to +III, are probed *via* Cu K-edge XAS and Cu Kβ valence-to-core X-ray emission spectroscopy (XES) and the $3d^8$ electronic configuration is supported. This experimental determination of Cu oxidation states provides a framework for the investigation of transient copper-based species in biological systems and elucidation of crucial intermediates in reaction pathways.

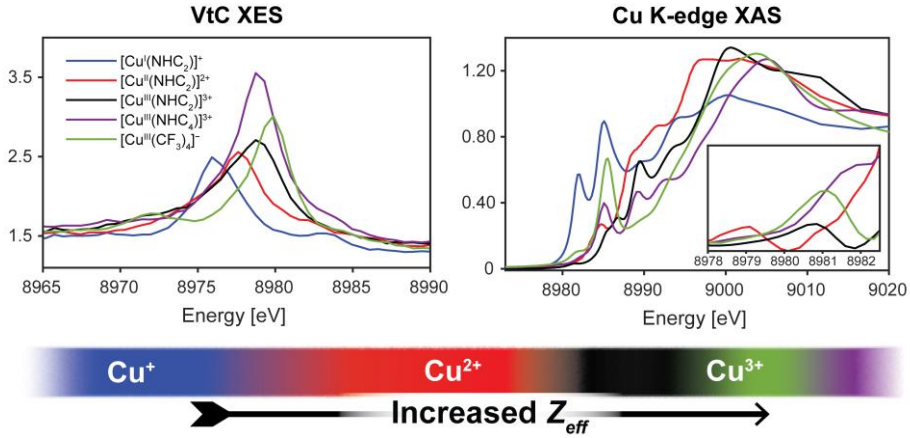

Figure 1 Experimental VtC XES (left) and Cu K-edge XAS (inset: 1st derivative) of organocopper complexes in +I, +II and +III oxidation states.

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Swimming of bacteria near surface in the presence of chemoattractants and chemorepellents in microfluidic channel.

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Bacteria can travel long distances thanks to flagellar appendages that allow them to move. When a motile bacterium approaches a surface, it undergoes hydrodynamic interactions that result in a directed circular motion of its trajectory that confines it to the surface. However, the trajectory of a bacterium is marked by reorientations and stops that are the result of a change in the direction of rotation of the bundle of flagella. This change of direction occurs periodically, about every 1s, for 100ms for the bacterium *E. coli.* The random reorientation of the bacteria near a surface allows the trapped bacteria to abruptly extract to the volume and reorient to explore their environment.

Bacterial chemotaxis, which takes advantage of a frequency bias in reorientation, allows the relocation of a bacterial population. We study the effects of known chemorepellent and chemoattractant substances such as Ni^{2+} and Mg^{2+} cations, on bacterial relocation. For this purpose, we conduct experiments in dark field video microscopy by varying the sources of chemical agents in microfluidic channels

My thesis project and the tools that will be developed will lead to a better understanding of the phenomena involved when approaching a bacterium to a reactive surface. The main physico-chemical laws that prevail between an individual bacterium and the reactive surface will be specified. This work should also establish a documented reference of the behavior of a population of bacteria near reactive surfaces.

X-ray Absorption Spectroscopy of [NiFe] Hydrogenase Under Electrochemical Control

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X-ray absorption spectroscopy (XAS) is a powerful technique, capable of providing structural and electronic information in an element specific manner. This makes it particularly useful for studying metalloproteins, as it provides key details about the nature of metal atoms that allow them to perform their function. However, biological applications of XAS are hindered by the effects of radiation damage and photoreduction, leading to sample degradation and, in the case of metalloenzymes, unwanted changes in metal oxidation state.[1] This means the sample composition can change during data acquisition, limiting reproducibility. Therefore, development of methods to mitigate photodamage are important, particularly given the current drive towards ambient temperature structural biology.

One important class of metalloenzyme, [NiFe] hydrogenase, catalyses hydrogen oxidation and proton reduction.[2] This makes them inspirational for development of catalysts for green energy technologies. During catalysis, [NiFe] hydrogenase utilises Ni(I), (II) and (III) oxidation states, all of which have been characterised using infrared spectroscopy.[3] Detailed information on electronic structure under physiologically relevant conditions is lacking, owing to the difficulty maintaining the enzyme in a single catalytic state. We have combined X-ray spectroscopy with electrochemistry, allowing us to access specific catalytic states, and importantly, protect against photoreductive damage at room temperature.

Using a specially designed electrochemical flow cell, a series of HERFD-XAS experiments were performed on [NiFe] hydrogenase 1 from E. coli, in solution at room temperature, at a range of applied potentials. The data was successfully acquired from a sample exposed to x-rays over 48 hours of almost continuous exposure, without signs of radiation damage in the resulting spectra.

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Accurate Spin-State Energetics of Iron Complexes: a Local Coupled Cluster Approach

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Spin-state energetics of transition metal complexes remain one of the most challenging targets for electronic structure methods. DFT may fail when it comes to providing the relative energies of transition metal complexes with different electronic configurations.[1] Breakthrough developments in electronic structure methods bring highly accurate and reliable wave function theory methods at the forefront of current research. The domain-based local pair natural orbital approach to coupled cluster theory, DLPNO-CCSD(T),[2] offers a highly efficient way to extend the applicability of the "gold standard" coupled cluster theory to large systems.

Herein, we investigate the spin-state energetics of a varied set of twelve iron complexes using specific operational protocols that we show to be essential in applications of the DLPNO-CCSD(T) approach.[3] Specifically, we apply a DLPNO-CCSD(T) protocol that involves both complete PNO space extrapolation and Fe-centered complete basis set limit extrapolation. The results are compared with CASPT2/CC, which was proposed[4] as the most accurate method for the exceptionally hard problem of spin-state energetics in iron complexes. Overall, even for the demanding case of iron complexes DLPNO-CCSD(T) is able to accurately and systematically reproduce CASPT2/CC and canonical CCSD(T) spin-state energetics while retaining its practical benefits of ease-of-use, efficiency, and scalability. The methodology developed here has the potential to become a new computational standard in the quantum chemical simulation of multi-state transition metal systems.[5]

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