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Knowledge Creation



5 chemistry

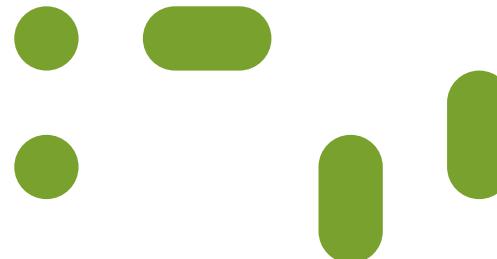
14 biological chemistry

32 biology

43 plant sciences

52 technology



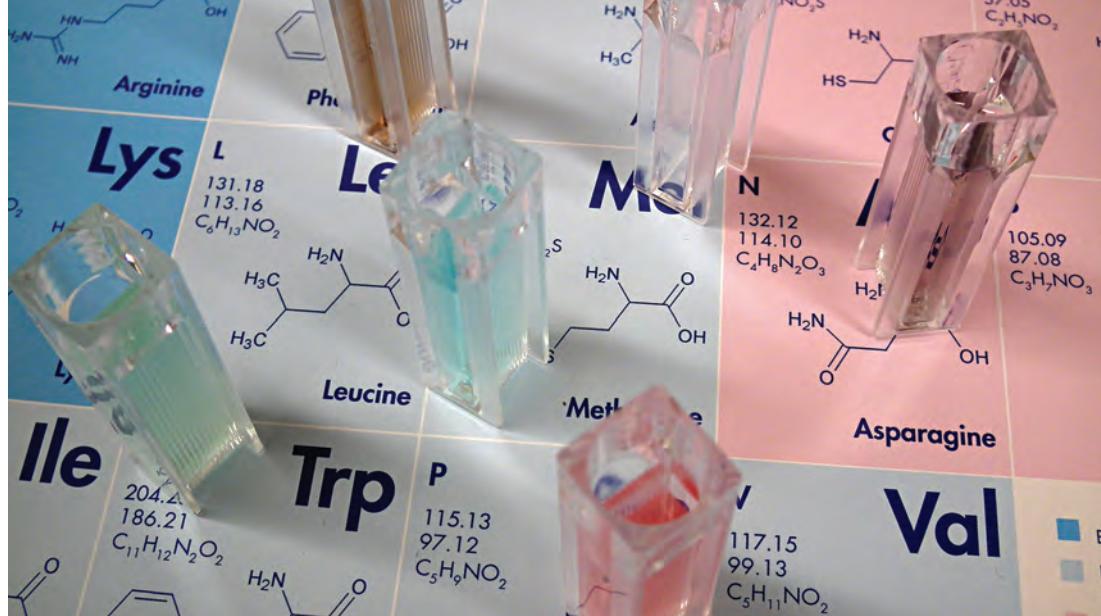


Instituto de Tecnologia Química e Biológica Universidade Nova de Lisboa

The *Instituto de Tecnologia Química e Biológica* (ITQB) is a research and advanced training institute of the *Universidade Nova de Lisboa*. Its mission is to develop high-quality research in chemistry and the life sciences, considering all levels of complexity and their potential applications, so as to contribute to the understanding of life's mechanisms. Its highly multidisciplinary nature makes ITQB a leading centre for advanced training of researchers in Portugal. Currently, ITQB hosts 62 independent laboratories grouped in five research divisions and consisting of a scientific staff of more than 350 researchers with different scientific interests and backgrounds. Researchers at ITQB benefit from outstanding facilities, equipment, and support services, some of which are unique in the country. Since 2001, the important contribution of ITQB in research and development has been highly enriched by the partnership with the *Instituto Gulbenkian de Ciência* and the *Instituto de Biologia Experimental e Tecnológica*. This scientific cluster was one of the first to be awarded the title of *Laboratório Associado* by the Portuguese Government in recognition of its scientific excellence as determined by international evaluation panels. ITQB's commitment to high quality scientific research includes a programme for raising public awareness of science.

Further information on ITQB's activities at www.itqb.unl.pt

The Bioinorganic Chemistry and Peptide Design Laboratory works on the design and synthesis of peptides and peptidomimetics capable of coordinating different metal ions and displaying different properties and functions.



Bioinorganic Chemistry and Peptide Design

Our group works in the field of inorganic chemistry at the interface between biological, catalytic and medicinal chemistry. We are particularly interested in designing peptides and peptidomimetics that contain binding sites with high specificity for metal ions. Following nature's lead and taking advantage of the advances in synthetic chemistry, we are generating different types of tridimensional structures that present attractive opportunities for the design of functional molecules. We are using these protein-like structures as starting frameworks for introducing metal binding sites and tailoring the metal ion properties to achieve both known and novel functions. The fact that these peptides and peptidomimetics are produced by chemical synthesis opens the door to the introduction of unnatural amino acids and chemical probes, expanding in this way the scope of potential functionalities.

Superoxide radicals are one of the most toxic reactive oxygen species and their damaging effects lead to a variety of detrimental health conditions including cardiovascular diseases, neurodegenerative disorders, and other types of age-related diseases. Inspired by the structure and function of natural superoxide dismutases, metalloenzymes that catalyze the disproportionation of superoxide radicals to molecular oxygen and hydrogen peroxide, we are currently developing metallopeptides for the removal of these toxic radicals. Our objective is to obtain better tools for identifying the role of these radicals in different biological processes and design better therapeutic agents for oxidative stress-related diseases.

Our group is also involved in the development of metallopeptide-based catalysts for asymmetric reduction and oxidation reactions. Recent years

have seen a tremendous demand for efficient and environmentally friendly industrial processes for the preparation of enantiomerically pure compounds. This challenge has driven the development of better and new catalysts. In this context, we are exploring the use of peptides and peptidomimetics to design a new generation of hybrid catalysts where the reactivity of a transition metal centre will be combined with the chiral environment of the peptidic-based scaffold.

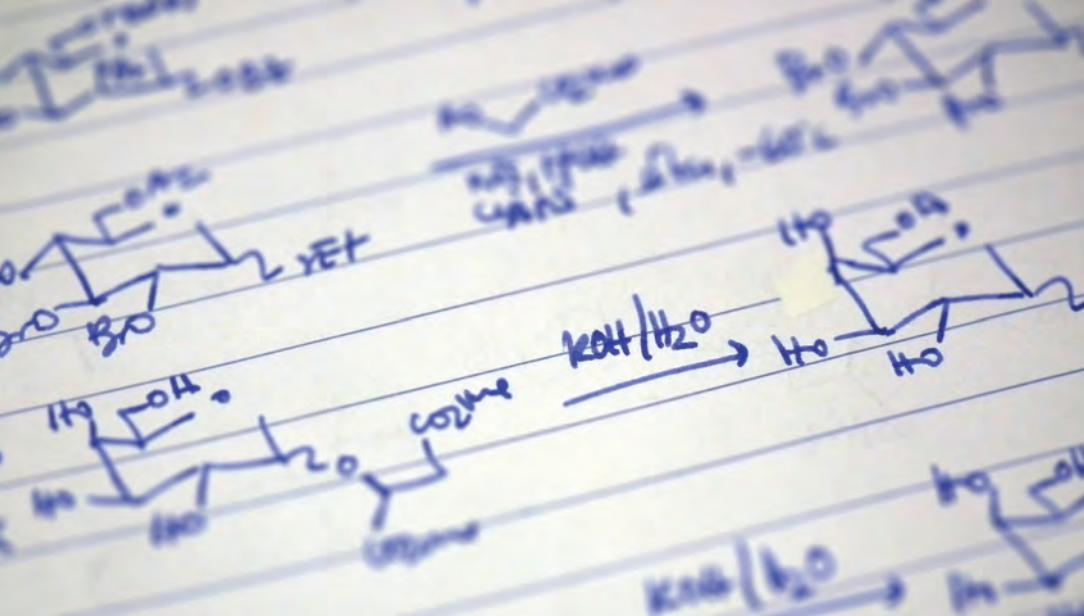
Iranzo O., Cabello C. and Pecoraro V. L. (2007). "Heterochromia in designed metallopeptides: Geometry-selective binding of Cd-II in a de novo peptide." *Angewandte Chemie-International Edition* 46(35): 6688-6691.

Iranzo O., Thulstrup P. W., Ryu S. B., Hemmingsen L. and Pecoraro V. L. (2007). "The application of Hg-199 NMR and Hg-199m perturbed angular correlation (PAC) spectroscopy to define the biological chemistry of Hg-II: A case study with designed two- and three-stranded coiled coils." *Chemistry - a European Journal* 13(33): 9178-9190.

Peacock A.F.A., O. Iranzo and V. L. Pecoraro (2009). "Harnessing nature ability to control metal ion coordination geometry using de novo design peptides". *Dalton Transactions* 2271-2280



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Bioorganic Chemistry is the interface between organic chemistry and biology. Our research uses the principles and techniques of organic chemistry in attempting to solve problems of relevance to biology. We can design synthetic derivatives of natural products that improve upon nature.

Bioorganic Chemistry



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Our group is interested in the synthesis of small molecules that can act as catalysts without the addition of metals – organocatalysts. This process can be utilised for the construction of enantiopure complex organic molecules, thus providing an alternative or a complement to organometallic and enzymatic catalysts. It is interesting to note that a simple aminoacid, proline, is an excellent organocatalyst for several transformations, emphasising the "greener" properties of organocatalysts compared with traditional ones. We prepared a range of structurally related organocatalysts with variations in the configuration of key differing functional groups and have drawn interesting conclusions regarding the most determinant chemical and spatial features for the enantio- and diastereoselectivity outcome of the reactions studied.

Another area of interest is carbohydrate chemistry. One of our aims is to gather together a collection of carbohydrate derived hypersolutes using solid supported synthesis, which will provide us with a wide range of new compounds to be tested by the Cell Physiology and NMR group for their ability to prevent protein aggregation and for protein thermostabilisation. We synthesised a series of chemical derivatives of mannose and glucose solutes, including disaccharides, some of which presented better thermostabilisation properties than the ones found in nature. In our studies, a new glucosyl donor was used as well as a much simpler glycosylation method that has afforded the desired alpha anomeric selectivity and avoided the need for using expensive reagents. The synthesis of new solutes, with more challenging structures, is being undertaken both in solution and in solid phase, requiring better glycosylation selectivities. Galactose derivatives are also being prepared.

Recently, we have also been collaborating with the Bacterial Signalling group in the synthesis of AI-2, a quorum sensing autoinducer, well known for its ability to mediate inter-species communication regulating important bacterial group behaviours such as biofilm formation, virulence, and antibiotic production. We have developed a new synthetic approach for synthesizing the precursor of AI-2, 4,5-dihydroxy-2,3-pentanedione (DPD). The new strategy will allow for the preparation of labelled DPD and a wide range of new analogues with a side group other than methyl. The labelled DPD constitute an important reagent for the ongoing elucidation of the biochemical fate of this molecule at the cellular level and the production of DPD. A quantum dot (QD) will be linked to DPD to produce fluorescent labelled AI-2 (AI-2-QD), which will be a valuable tool for the study of several aspects of AI-2 signalling.

Loureiro E. C., Maycock C. D. and Ventura M. R. (2009). "Synthesis of potassium (2R)-2-O-alpha-D-glucopyranosyl-(1 -> 6)-alpha-D-glucopyranosyl-2,3-dihydroxypropanoate: a natural compatible solute." *Carbohydrate Research* 344(15): 2073-2078.

Rodrigues M. V., Borges N., Henriques M., Lamosa P., Ventura R., Fernandes C., Empadinhas N., Maycock C., da Costa M. S. and Santos H. (2007). "Bifunctional CTP: Inositol-1-phosphate cytidylyltransferase/CDP-inositol: Inositol-1-phosphate transferase, the key enzyme for di-myoinositol-phosphate synthesis in several, (hyper)thermophiles." *Journal of Bacteriology* 189(15): 5405-5412.

Burke A. J., Maycock C. D. and Ventura M. R. (2006). "Stereoselective alkylation of tartrate derivatives. A concise route to (+)-O-methylpiscidic acid and natural analogues." *Organic & Biomolecular Chemistry* 4(12): 2361-2363.

As the name implies, our lab deals with surface/interface phenomena.



Colloids, Polymers and Surfaces

Within the broad field of colloid and surface chemistry, we largely concentrate on surfactant self-assembly, with or without the presence of polymers (or proteins), developing solutions of tuneable rheology, from gels to mesomorphic or smectic phases. Other studies involve pH definition near the colloid interfaces and the permeation through the soft interfaces developed.

From a biochemical/biomedical point of view, we have been working with polymeric matrices, namely hydrogels (entangled or cross-linked networks of polymer-based structures with swelling and entrapment capabilities) which show great potential for biomedical-oriented applications. Specifically, we have been developing matrices for drug delivery systems for the skin barrier, focusing on allergy, burn and pain treatments. These matrices are based upon cross-linked gels from chitosan and dextran (polysaccharides) due to their biocompatibility and biodegradability. After the drug is incorporated, studies of the stability of the formulation and the release properties of the drugs are modelled.

Another multidisciplinary topic is the extraction and identification of surface active agents of plant origin, including polyphenol families with antioxidant capabilities. Presently, most of these studies deal with the cork tree, coffee and some Portuguese varieties of beans.

From an environmental point of view, we have been working with the "green solvents" known as Room Temperature Ionic Liquids (RTIL) dispersed in bulky aqueous and non-aqueous solvents. Some of the RTIL's demonstrate unique abilities as surfactants, since they combine a highly advantageous character-

istic of being bulk solvents together with their capacity to be nano-dispersed either in water and/or some non-aqueous solvents. Thus, one can combine two reagents of completely different natures (hydrophilic and hydrophobic) into a macroscopically homogeneous solution, being micellar or micro-emulsion states. Very recent preliminary results suggest that in equimolar mixtures of SDS and long hydrocarbon chain RTIL's, both mixed micelle behaviour and catalytic effects are present. These phenomena are currently under further investigation.

Blesic M., Lopes A., Melo E., Petrovski Z., Plechkova N. V., Lopes J. N. C., Seddon K. R. and Rebelo L. P. N. (2008). "On the self-aggregation and fluorescence quenching aptitude of surfactant ionic liquids." *Journal of Physical Chemistry B* 112(29): 8645-8650.
Lopes A., Edwards K. and Feitosa E. (2008). "Extruded vesicles of dioctadecyldimethylammonium bromide and chloride investigated by light scattering and cryogenic transmission electron microscopy." *Journal of Colloid and Interface Science* 322(2): 582-588.

Lameiro M. H., Lopes A., Martins L. O., Alves P. M. and Melo E. (2006). "Incorporation of a model protein into chitosan-bile salt microparticles." *International Journal of Pharmaceutics* 312(1-2): 119-130.



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The Coordination and Supramolecular Chemistry group designs and synthesizes new molecules for the selective uptake of anions, neutral molecules or metal ions for environmental and medical applications.

Coordination and Supramolecular Chemistry



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The recognition of anionic substrates is a vigorous research field due to the importance of anions in biological, industrial, and environmental fields. In this area, the design and synthesis of molecular receptors for the selective binding of anions is crucial. In recent years our laboratory has contributed with the synthesis of new molecules having well-defined three-dimensional cavities capable of accommodating and selectively binding anionic or neutral substrates in aqueous solutions. The strength of the association of both partners is evaluated by the determination of the binding constant by spectroscopic or potentiometric techniques. The new molecules, which act as receptors, are used either for the detection and removal of anionic pollutants, or the separation of amino acids or chiral drugs. They should selectively encapsulate the substrate, forming supermolecules, with high binding affinity based on the best fit between the partners bound through several and cooperative weak interactions. For successful receptors, for which selectivity for a given substrate is achieved, analytical techniques for their detection or quantification in "real-life" samples will be developed. Small changes in the structure of such receptors can transform them into sensors and, when attached to solid matrices, they can be used in chromatographic separations.

Another important area of interest deals with compounds capable of uptaking certain metal ions and holding them so strongly that they resist liberation even in highly adverse situations. These compounds, known as chelators and chelate, when bound to some radioactive metal ions, are useful in medical applications such as in diagnosis or in tumour therapy. The chelate must be chemically stable under physiological conditions so as to avoid the release of toxic metal ions. Therefore, such chelates should present high thermody-

namic stability as well as strong kinetic inertness to dissociation. Moreover, when specific targeting is involved, the chelator must easily be functionalized with chemical groups or conjugated to biomolecules with high affinity and selectivity for specific molecular targets. As a general rule, a potential radiopharmaceutical must be prepared in quick and mild conditions and should be widely stable in physiological media and present good chemical properties in order to ensure efficient clearance from the body. Our laboratory has developed and studied some new macrocyclic chelators some of which exhibit very interesting properties for this field. These chelators may also prove useful in the removal of toxic metal ions from the body or from the environment.

Mateus P., Delgado R., Branda P. and Felix V. (2009). "Polyaza Cryptand Receptor Selective for Dihydrogen Phosphate." *Journal of Organic Chemistry* 74(22): 8638-8646.

Cruz C., Calisto V., Delgado R. and Felix V. (2009). "Design of Protonated Polyazamacrocycles Based on Phenanthroline Motifs for Selective Uptake of Aromatic Carboxylate Anions and Herbicides." *Chemistry-a European Journal* 15(13): 3277-3289.

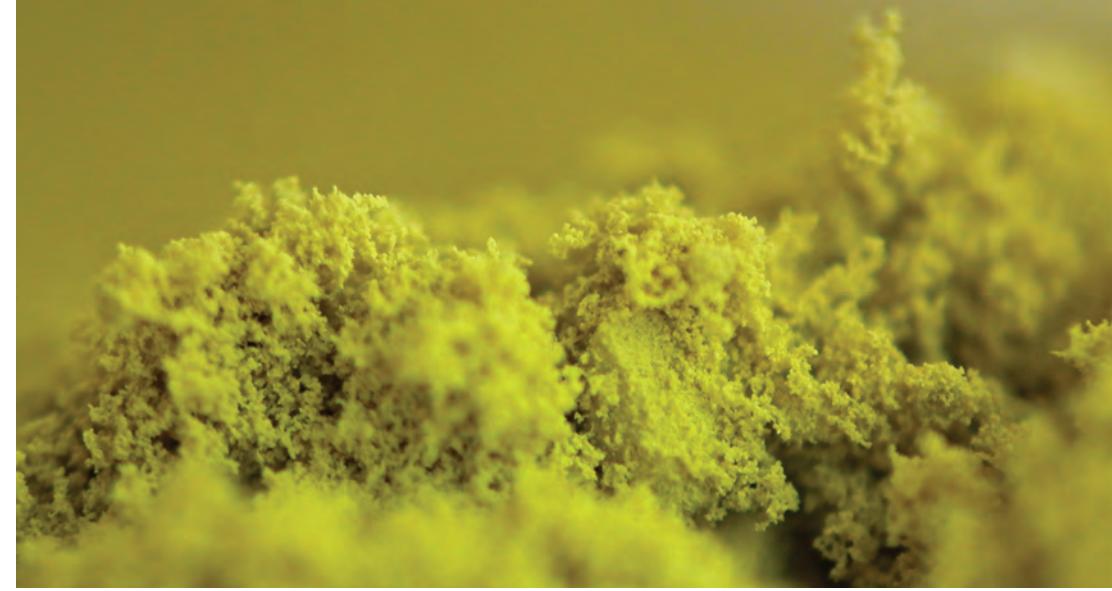
Delgado R., Felix V., Lima L. M. P. and Price D. W. (2007). "Metal complexes of cyclen and cyclam derivatives useful for medical applications: a discussion based on thermodynamic stability constants and structural data." *Dalton Transactions*(26): 2734-2745.

The Homogeneous Catalysis Group works on the synthesis of novel catalysts based on organometallic species. Our final goal is to develop sustainable, efficient and selective organic transformations.

Homogeneous Catalysis

The development of new catalytic systems allowing for rapid and selective transformations has had a significant impact on the chemical industry, both in fine and bulk chemical production. In this context, organometallic compounds have become an established tool for the synthesis of useful molecules.

Our research interests focus on the development of novel homogeneous catalytic systems that allow for efficient and environmentally benign synthetic transformations of simple molecules into useful building blocks for materials and medicinal compounds. Our research program deals with the synthesis of novel N-heterocyclic carbene (NHC) ligands tethered by a cyclopentadienyl unit and their coordination of transition metals. NHCs have become a key class of ligands in organometallic chemistry due to the application of their metal-based compounds in a vast number of catalytic reactions. Their structural versatility and functionalisation enables them to display an array of exploitable and tuneable properties. Recently, we discovered a straightforward route to unsubstituted and substituted-cyclopentadienyls functionalised N-heterocyclic carbenes (Cp-NHCs). The coordination of these novel ligands to transition metals such as iridium, ruthenium, rhodium and molybdenum shows the versatility of the new ligands. These novel metal complexes are effective catalysts in a wide set of reactions implying C-H activations. In particular, they have shown high catalytic activity toward transfer hydrogenation, β -alkylation of secondary alcohols with primary alcohols, and amination of primary alcohols. The design of the asymmetric version of these reactions is one of the most attractive topics under investigation in our group.



The reduction of a variety of functional groups using hydrogen as a reducing agent and high-valent metal oxo species as catalysts is another important subject in our laboratory. We have exploited catalysts normally used for oxidations, such as olefin epoxidation and olefin transfer. In 2005, we developed the reduction of carbonyl groups by using metal oxides as catalysts and silanes as reducing agents. We have recently extended the role of these catalysts to reductive processes using hydrogen, a cheaper and more convenient reducing agent than silane. These systems are capable of catalysing the selective hydrogenation of alkynes to alkanes-a challenging task in organic synthesis. Our latest discovery deals with the selective reduction of nitroarenes using a simple, inexpensive, clean and reusable catalytic system. This finding is remarkable since the selective reduction of a nitro group when other reducible functions are present is a difficult process, especially when hydrogen is used as reducing agent.

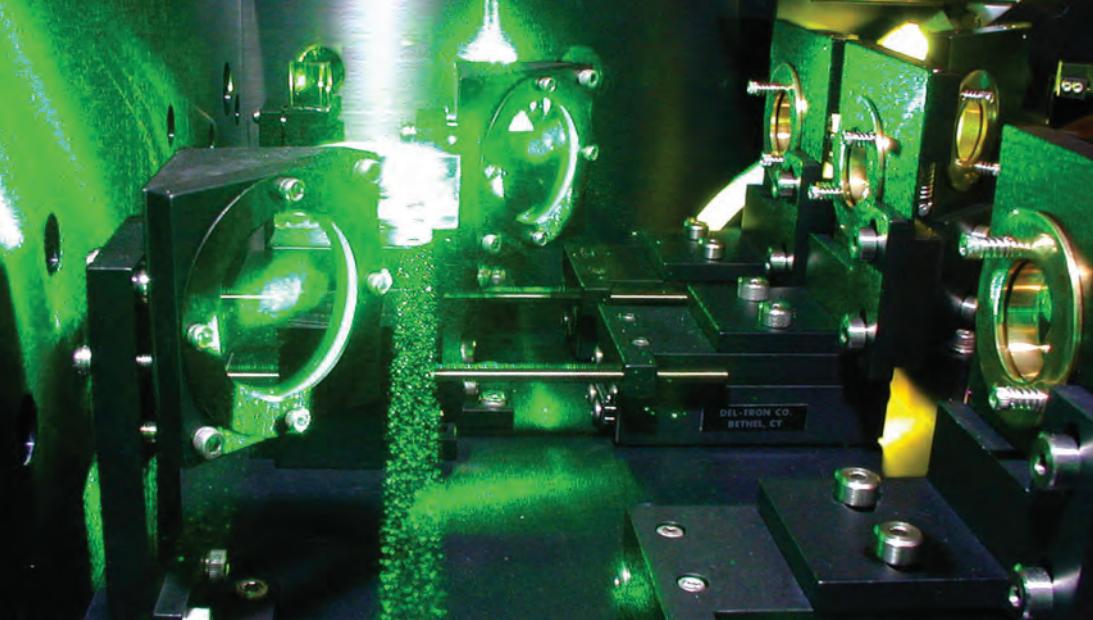
Reis P. M. and Royo B. (2009). "Chemoselective hydrogenation of nitroarenes and deoxygenation of pyridine N-oxides with H₂ catalyzed by MoO₂Cl₂." *Tetrahedron Letters* 50(8): 949-952.

Costa A. P. d., Viciano M., Sanau M., Merino S., Tejeda J., Peris E. and Royo B. (2008). "First Cp*-functionalized N-heterocyclic carbene and its coordination to iridium. study of the catalytic properties." *Organometallics* 27(6): 1305-1309.

Kandepi V., da Costa A. P., Peris E. and Royo B. (2009). "Molybdenum(II) Complexes Containing Cyclopentadienyl-Functionalized N-Heterocyclic Carbenes: Synthesis, Structure, and Application in Olefin Epoxidation." *Organometallics* 28(15): 4544-4549.



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We focus on the study of how small volumes, limited dimensions and the topology of the compartments in which biological reactions take place influence their kinetics and equilibrium.

Micro-heterogeneous Systems



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At the Laboratory of Micro-heterogeneous Systems of ITQB we investigate chemical equilibrium and the kinetics of bimolecular reactions in lipid mesophases. Specifically, we are interested in those mesophases that mimic cell structures such as the biological membrane or the cellular compartments. With synthetic lipid bilayers of selected composition, to which we add hydrophobic or amphiphilic reactants, we model reactions taking place in the biological membrane. Then, with submicron aqueous compartments delimited by aggregated lipids containing aquo-soluble reactants, we simulate reactions in cell compartments or at soft interfaces.

Models developed for large three-dimensional spaces containing an "infinite" number of reactant molecules and for which the effect of the wall of the reaction vessel is negligible can be expected to fail when applied to reactions in biological systems.

The vessel's geometry can alter the speed of a bimolecular reaction, while the number of available reactant molecules can influence not only the reaction's rate but also its yield.

We simulate the environment in which biological reactions take place, and follow the progression of reactant conversion over time in the sub-millisecond time scale in which many biological reactions occur.

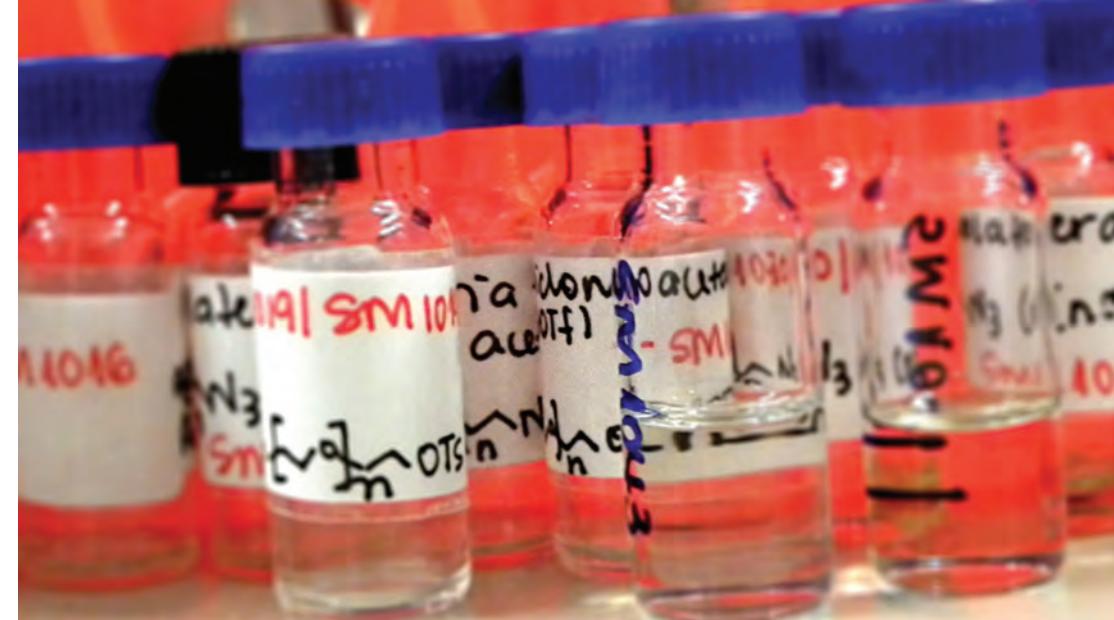
The aggregates of lipids forming bilayers or other targeted molecular constructs need to be characterised before dealing with them as microreactors. A large part of the Laboratory's work is devoted to the study of the structure

and topology of the lipid aggregates used as well as others of particular biological interest, namely those responsible for the creation of lipid inhomogeneity in biological membranes.

Souza S. L., Capitan M. J., Alvarez J., Funari S. S., Lameiro M. H. and Melo E. (2009). "Phase Behavior of Aqueous Dispersions of Mixtures of N-Palmitoyl Ceramide and Cholesterol: A Lipid System with Ceramide-Cholesterol Crystalline Lamellar Phases." *Journal of Physical Chemistry B* 113(5): 1367-1375.

Melo E. and Martins J. (2006). "Kinetics of bimolecular reactions in model bilayers and biological membranes. A critical review." *Biophysical Chemistry* 123(2-3): 77-94.

Targeted syntheses of stereochemically defined and isomerically pure natural products and compounds of biological interest are the main aims of the Organic Synthesis group.



Organic Synthesis

Most active pharmaceuticals are small synthetic molecules which have been and will continue to be the mainstay of the fight against a wide range of diseases and syndromes. The construction of multifunctional molecules requires an arsenal of reagents and strategies in order to meet emerging challenges. In addition to basic chemistry, we have the added sophistication of stereochemistry which increases the problem exponentially. Efficiency, sustainability and reduced dependence upon petroleum products are additional goals for the synthesis of small molecules in an isomerically pure form. Controlling all of these parameters is of fundamental importance for modern organic synthesis.

Natural product syntheses are a great challenge since the product gross structure and stereochemistry are rigorously defined. Any synthesis is a test of the viability of the strategy and of the compatibility of the reagents. The Organic Synthesis Group is dedicated to the synthesis of compounds which have a relatively complex three-dimensional structure not necessarily related to the gross structure. Recently, we have found that simple conjugate additions using nitrogen nucleophiles do not produce the result predicted by steric hindrance and that some other electronic effect is involved. Furthermore, the aziridines formed in these reactions are able to invert the stereochemical outcome of further reactions in conformationally restricted compounds. This has helped us achieve an important intermediate and stereocontrolled synthesis of some optically pure natural products. We are applying this technology to the synthesis of others. The reactivity of some aziridines, which are strained compounds, has also been the subject of study and the preparation of variously alfa-substituted cyclic enones has been made possible.

Quantum dots are nano-sized spheres of a semiconducting material which fluoresce over a wide range of irradiation frequencies. Depending on a variety of producible sizes, they can emit light of different colours. Lipophilic or hydrophilic QDs can be prepared using suitable ligands, loosely bound to the surface of the spheres. Bioconjugates QDs able to enter cells can be prepared for example, and their presence observed using a fluorescence microscope. We have prepared stable QDs having different terminations on the ligand molecules including sugar molecules capable of stabilising proteins and also those able to recognise binding sites on cell surfaces and hopefully be transfected through the cell wall. Functionalisation with antibodies for the study of parasitic diseases is also underway. Magnetic nanoparticles are also able to enter biological systems and we expect to be able to selectively magnetise living organisms for magnetic detection.

Barros M. T., Burke A. J., Lou J. D., Maycock C. D. and Wahnon J. R. (2004). "Highly stereoselective aldol reaction for the synthesis of gamma-lactones starting from tartaric acid." *Journal of Organic Chemistry* 69(23): 7847-7850.

Burke A. J., Maycock C. D. and Ventura M. R. (2006). "Stereoselective alkylation of tartrate derivatives. A concise route to (+)-*O*-methylpiscicidic acid and natural analogues." *Organic & Biomolecular Chemistry* 4(12): 2361-2363.

Barros M. T., Charmier M. A. J., Maycock C. D. and Michaud T. (2009). "Synthesis of gamma-lactones by desymmetrisation. A synthesis of (-)-muricatacin." *Tetrahedron* 65(1): 396-399.



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The Laboratory of Organometallic Chemistry is presently studying new metal derivatives of carbon monoxide (CO) to be used for the production of renewable energy and as a new class of drugs based on the therapeutic activity of CO.



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Organometallic Chemistry

Bridging Inorganic and Organic Chemistry, Organometallic Chemistry (OMC) has brought tremendous change in chemical synthesis by creating catalysts for many applications widely used in both large scale (refineries) and small scale (fine chemicals and pharmaceuticals) industries. Moreover, organometallic compounds have found applications in a variety of fields including biomedical and pharmaceutical areas like therapy and diagnostics.

At ITQB, we have been exploring for some years the synthesis and catalytic chemistry of organometallic oxides of Molybdenum and Rhenium a relatively underdeveloped area of OMC. These oxo-complexes, contain metal-oxygen ($M=O$) bonds like those involved in many enzymes. Such compounds are able to accelerate (catalyze) the oxidation of organic molecules using environmentally safer oxidants like hydrogen peroxide (H_2O_2) and alkylhydroperoxides ($ROOH$). In 2006 our work covered mainly mechanistic aspects of these reactions in an effort to unify the understanding of the wealth of experimental results obtained in previous years. In 2005 we disclosed a new type of reactivity of metal-oxo compounds, namely their ability to catalyze reduction reactions. These processes are based on the capacity of the $M=O$ bonds to break Si-H bonds, thereby transferring hydrogen atoms (reduction) to unsaturated organic molecules. Throughout 2006 we continued to explore these entirely new catalytic processes for the reduction of aldehydes, ketones, esters, amides, sulfoxides and N-oxides. Progress in this area includes the development of environmentally friendly processes in which such reductions are carried out using water as solvent. The development of organometallic compounds for therapeutic applications is the other main focus of our research. This has led to the formation of an independent company, Alfama

Inc., still operating in close collaboration with our research group within ITQB premises. The core of Alfama's proprietary technology is the development of CO releasing molecules for the treatment of inflammatory diseases. Besides, we are pursuing the search for anti-tumour molecules and the study of the interaction of organometallic molecules with the organism (e.g. with protein transporters) to improve their bio-compatibility and water solubility. Accordingly, encapsulation into soluble cyclic sugars (cyclodextrins) has received particular attention. It must be noted that very little is presently known about the behaviour of organometallic compounds *in vivo*.

Pereira C. C. L., Costa P. J., Calhorda M. J., Freire C., Rodrigues S. S., Herdtweck E. and Romao C. C. (2006). "Ring slippage vs charge transfer in the reductive chemistry of [$IndMo(CO)_2(\alpha\text{-diimine})$] $^+$ cations." *Organometallics* 25(22): 5223-5234.

Braga S. S., Paz F. A. A., Pillinger M., Seixas J. D., Romao C. C. and Goncalves I. S. (2006). "Structural studies of beta-cyclodextrin and permethylated beta-cyclodextrin inclusion compounds of cyclopentadienyl metal carbonyl complexes." *European Journal of Inorganic Chemistry*(8): 1662-1669.

Fernandes A. C. and Romao C. C. (2006). "A novel method for the reduction of sulfoxides and pyridine N-oxides with the system silane/ MoO_2Cl_2 ." *Tetrahedron* 62(41): 9650-9654.

This recent group at ITQB focuses on the study of chemical and biochemical processes at the single molecule level.

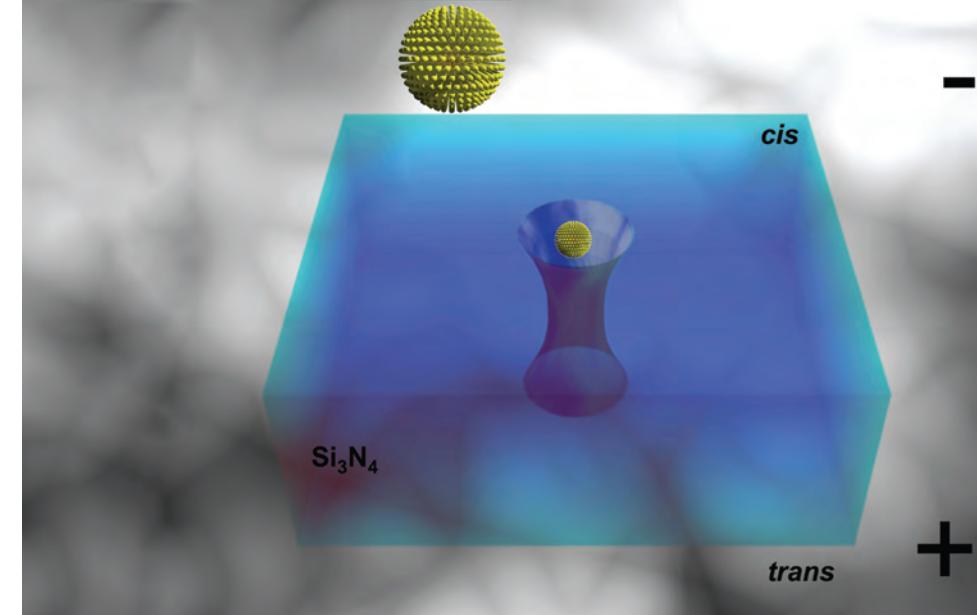
Observing a single molecule and its stochastic interactions with others provides a bottom up understanding of bulk chemistry.

Single Molecule Processes

Nanopores are emerging as very efficient single molecule sensors. They represent a promising technology in the field of single molecule unlabelled DNA sequencing. The mechanical robustness, nanometer range precision, and rigidity of synthetic nanopores promise easier integration into sensing technology as compared to membrane embedded nanopores. Synthetic nanopores (2 to 8 nm in diameter) produced out of Si₃N₄ membranes have found several applications in biophysics, such as the characterisation of DNA duplexes by electromechanical unzipping, the detection of DNA/protein complexes, and the characterisation of proteins and DNA.

We have utilized functionalized Si₃N₄ nanopore (3.2 to 6.5 nm in diameter, 30 and 50 nm in length) for the detection of negatively charged gold nanoparticles from 2.4 to 8.9 nm diameter. We have found that we can detect the interaction through ionic current alterations, whether the electric field driven nanoparticle threads through the pore or not. Our simulation work suggests that weak force interactions are essential to creating the initial nanoparticle nanopore interaction. Once a nanoparticle is engaged into the nanopore, Brownian motion theory fits our t_{off} data. We have also demonstrated how artificial nanopores are a viable single particle screening tool for assessing the size distribution of particles under 4 nm in diameter.

Gold nanoparticles coated with a self-assembled monolayer (SAM) of thiolated molecules are promising materials for many applications ranging from electronics to nanomedicine. The ligand shell is the key for determining the interfacial properties of the particles. These particles unfortunately are very often polydisperse in size, ligand shell composition, and probably morphol-



ogy. Hence the determination of their properties is always limited by bulk averaging. A method able to sort and characterise these particles sensitive to both their size and their interfacial (i.e. ligand shell) properties would help the field's progress. The nanopore detection scheme presented here is a first step in that direction as it characterises particles' size and ligand shell on a single particle basis.

Astier Y., Uzun O. and Stellacci F. (2009). "Electrophysiological Study of Single Gold Nanoparticle/alpha-Hemolysin Complex Formation: A Nanotool to Slow Down ssDNA Through the alpha-Hemolysin Nanopore." *Small* 5(11): 1273-1278.

Astier Y., Braha O. and Bayley H. (2006). "Toward single molecule DNA sequencing: Direct identification of ribonucleoside and deoxyribonucleoside 5'-monophosphates by using an engineered protein nanopore equipped with a molecular adapter." *Journal of the American Chemical Society* 128(5): 1705-1710.

Wu H. C., Astier Y., Maglia G., Mikhailova E. and Bayley H. (2007). "Protein nanopores with covalently attached molecular adapters." *Journal of the American Chemical Society* 129(51): 16142-16148.



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In the Bacterial Energy Metabolism Laboratory we investigate the metabolic pathways for energy production in microorganisms that are biotechnologically and environmentally important.

Bacterial Energy Metabolism



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Our lab is interested in the study of Energy Metabolism in bacteria that live by anaerobic respiration. We have been studying sulfate reducing bacteria, which are ancient organisms that existed long before the appearance of oxygen on Earth, and that are ubiquitously found in the environment and in animal guts. These bacteria are implicated in a range of environmental and health issues, and are important research targets in the areas of Bioremediation, Biological Hydrogen Production, Microbial Fuel Cells, Biocorrosion, Waste Treatment and Inflammatory Bowel Diseases.

The key players in energy metabolism are membrane-bound protein complexes. One of the important areas in our lab is the study of membrane complexes of sulfate reducing bacteria, which have a quite unique, but still poorly defined, respiratory chain. We isolated and characterised several of these complexes for the first time, which provided important insights into the mechanism of sulfate reduction. Ongoing studies focus on protein-protein interactions to investigate their physiological partners and elucidate the metabolic pathways.

Another area of research involves the study of soluble proteins that are directly involved in energy metabolism. The structure determination of the complex between the DsrAB sulfite reductase and the thiol protein DsrC (in collaboration with the MPC Lab), revealed the important role of this small protein. We are further investigating the link between DsrC and sulfate reduction, as well as trying to discover the physiological partners to the terminal reductases.

A third topic of interest in our lab is the study of hydrogenases, important targets in clean bioenergy production. We have been studying a [NiFeSe] hydrogenase that has a very high activity of H₂-production and shows tolerance to O₂ inactivation, making it an interesting target for biological H₂ production. We have established that this Hase is the principal one present in *D. vulgaris* when Se is available and shown that it is the first example of a bacterial lipoprotein lacking a standard signal peptide, and which is translocated by the Tat pathway. The recent determination of the crystal structure of this hydrogenase (in collaboration with IMAC lab) in the oxidized state, provided important insight into its oxygen tolerance. Ongoing studies focus on developing cell-based systems metabolically engineered for H₂ production, as well as hydrogenases with high activity and O₂ tolerance for practical applications.

Venceslau S. S., Lino R. R. and Pereira I. A. C. (2010). "The Qrc Membrane Complex, Related to the Alternative Complex III, Is a Menaquinone Reductase Involved in Sulfate Respiration." *Journal of Biological Chemistry* 285(30): 22772-22781.

Marques M. C., Coelho R., De Lacey A. L., Pereira I. A. C. and Matias P. M. (2010). "The Three-Dimensional Structure of NiFeSe Hydrogenase from *Desulfovibrio vulgaris* Hildenborough: A Hydrogenase without a Bridging Ligand in the Active Site in Its Oxidised, "as-Isolated" State." *Journal of Molecular Biology* 396(4): 893-907.

Oliveira T. F., Vonrhein C., Matias P. M., Venceslau S. S., Pereira I. A. C. and Archer M. (2008). "The Crystal Structure of *Desulfovibrio vulgaris* Dissimilatory Sulfite Reductase Bound to DsrC Provides Novel Insights into the Mechanism of Sulfate Respiration." *Journal of Biological Chemistry* 283(49): 34141-34149.

The Biological Energy Transduction Group addresses a fundamental process for all living organisms: energy conservation. A wide range of biochemical and biophysical techniques is used to investigate the mechanisms of energy transduction by membrane respiratory chains.



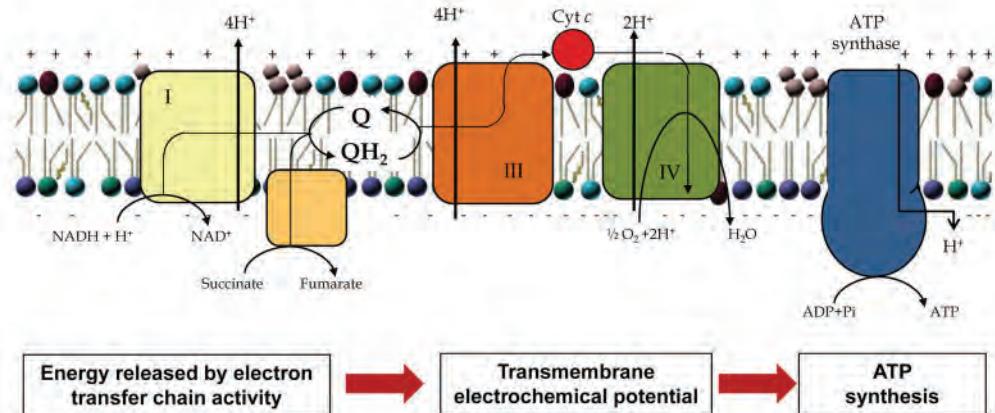
Metalloproteins and Bioenergetics Unit

Biological Energy Transduction

Life depends on constant energy transduction mechanisms. Non-photosynthetic organisms obtain energy by degradation of all food components, such as proteins, glycides and lipids which feed electrons to the respiratory chain. Here the electron transfer is coupled to the translocation of ions across the cytoplasmatic membrane and the energy thus released by the favourable electron transfer is transduced to the form of a transmembrane difference of electrochemical potential, upon which dissipation through the ATP synthase allows for the synthesis of ATP.

In our group we study the molecular mechanisms of electron transfer, ion translocation and their coupling. As model systems we use respiratory chains and their components. The research involves isolated proteins (wild type and recombinants) as well as reconstituted proteins or even membrane vesicles. Ours is a multidisciplinary approach using a wide range of biochemical and biophysical techniques. The group is part of the Metalloproteins and Bioenergetics Unit and has several collaborations within ITQB, as well as outside the institute.

Specifically, we have been investigating energy conservation by complex I from the thermohalophilic bacterium *Rhodothermus marinus*, namely studying its electron transfer kinetics and quinone reduction, proton and Na^+ translocation and the coupling mechanism of electron transfer to ion transport. The investigation may also in the future, contribute to more applied areas, such as health since Complex I malfunctioning has been implicated in several pathologies, namely neurodegenerative diseases like Parkinson and dystonia disorders.



We are also addressing protein-quinone interaction. For these studies we have elected type II NADH dehydrogenases as model systems. It is also our goal to recognize the structural elements/motives determinant for catalysis and substrate interaction. Taking advantage of working with NDH-II that are present in organisms expressing different types of quinones, we aim to elucidate the characteristics and specificity of the binding sites for the different types of quinones.

The catalytic and energy conservation mechanisms by the recently described alternative complex III are also investigated. Studies on oxygen reductases, the last enzyme of respiratory chains, are being performed in close collaboration within the Metalloprotein and Bioenergetic Unit.

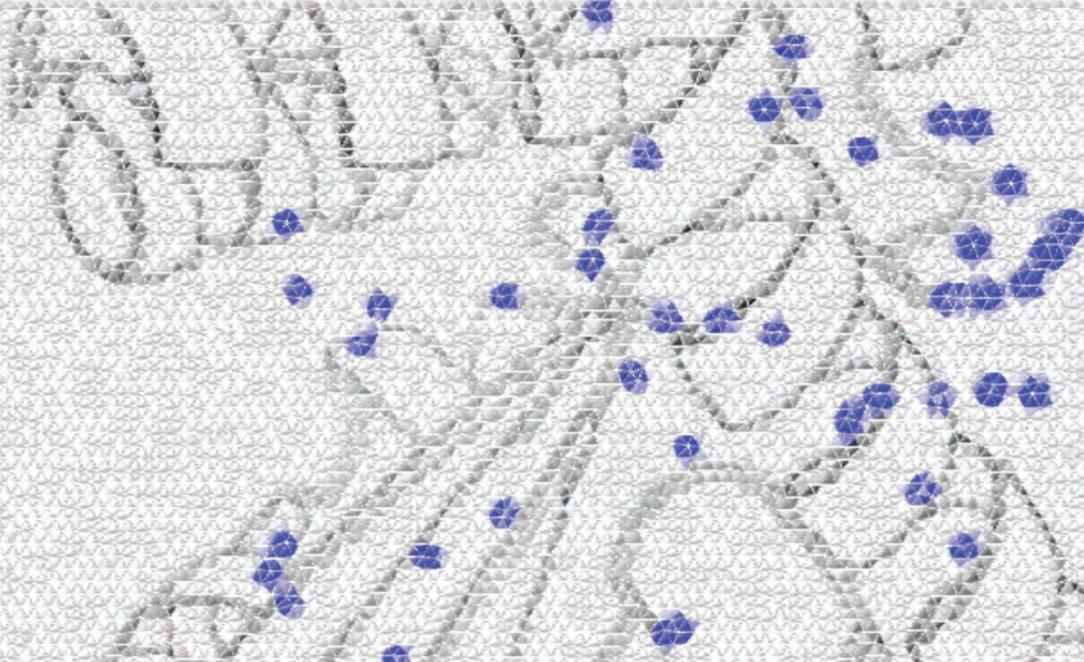
Batista A. P., Fernandes A. S., Louro R. O., Steuber J. and Pereira M. M. (2010). "Energy conservation by *Rhodothermus marinus* respiratory complex I." *Biochimica Et Biophysica Acta-Bioenergetics* 1797(4): 509-515.

Veríssimo A. F., Sousa F. L., Baptista A. M., Teixeira M. and Pereira M. M. (2008). "Thermodynamic Redox Behavior of the Heme Centers in A-Type Heme-Copper Oxygen Reductases: Comparison between the Two Subfamilies." *Biophysical Journal* 95(9): 4448-4455.

Refojo, P. N, Teixeira, M. and Pereira, M. M. (2010) "The alternative complex III of *Rhodothermus marinus* and its structural and functional association with *caa3* oxygen reductases." *Biochimica et Biophysica Acta*, 1797, 1477-1482.



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This lab specializes in the application of nuclear magnetic resonance (NMR) to biomolecular problems with an emphasis on the fields of structure determination, protein-protein interaction and the study of iron homeostasis.

Biomolecular NMR



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NMR is a very versatile technique that directly probes processes at the atomic level, whether in solution or in the solid state. Some of the tasks that can be achieved are the determination of the solution structure of small and medium sized proteins as well as the study of interactions of proteins with other biomolecules or pharmaceuticals.

We aim to develop fast and efficient methodologies for accelerated structure determination. We have implemented, and are in the process of optimising, many fast data acquisition techniques such as Projection Spectroscopy (APSY), fast scanning techniques (BEST, sofast-HSQC), Non Uniform Sampling (NUS) acquisition, and simultaneous acquisition experiments which can improve data collection times by as much as an order of magnitude compared to traditional methodologies. We are applying the above methods in a structural proteomics approach targeting hyperstable proteins.

Our second interest involves the study of ferrous transport mechanisms in pathogenic bacteria. While a significant amount of research has been done in the field of sequestering and transport system of iron, we are still discovering new proteins that seem to function as transporters. We are interested in the structural and functional characterisation of such proteins with an emphasis on pathogenic bacteria.

We are also actively collaborating with other groups on topics for which NMR can provide novel solutions. We have worked on protein-DNA interactions, protein-drug interactions and various structural projects.

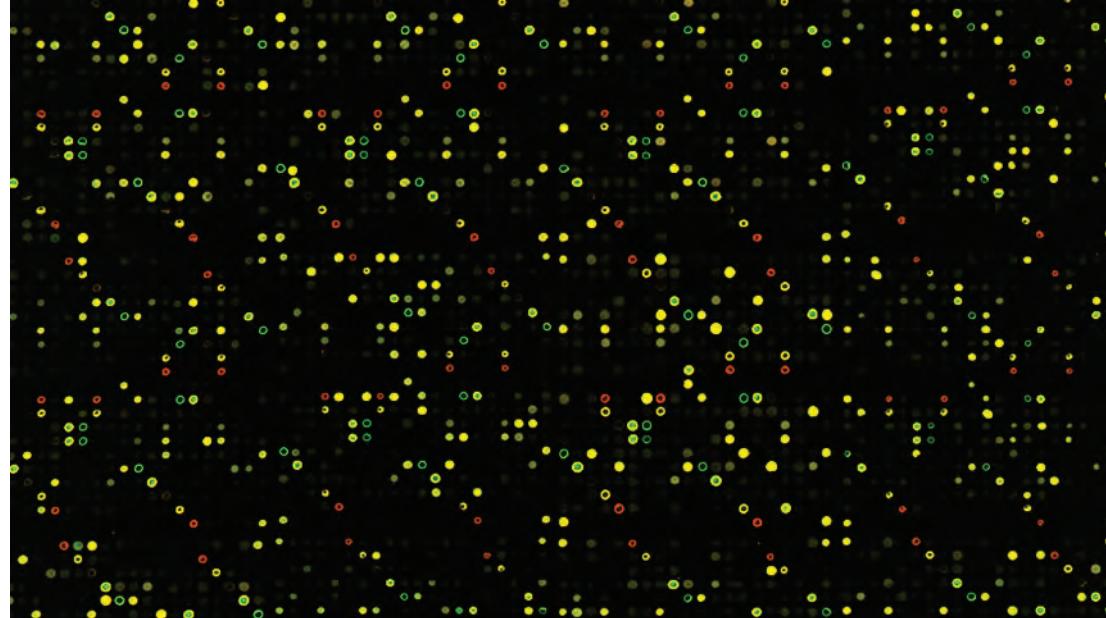
Our expertise in NMR is in the following fields: Solution structure determination, NMR of Large Proteins, Liquid and solid NMR, study of metalloproteins and the design and study of artificial proteins.

Bermel W., Bertini I., Felli I. C., Matzapetakis M., Pierattelli R., Theli E. C. and Turano P. (2007). "A method for C-alpha direct-detection in protonless NMR." *Journal of Magnetic Resonance* 188(2): 301-310.

Matzapetakis M., Turano P., Theil E. C. and Bertini I. (2007). "C-13-C-13 NOESY spectra of a 480 kDa protein: solution NMR of ferritin." *Journal of Biomolecular Nmr* 38(3): 237-242.

Collins T., Matzapetakis M. and Santos H. (2010). "Backbone and side chain ¹H, ¹⁵N and ¹³C assignments for a thiol-disulphide oxidoreductase from the Antarctic bacterium *Pseudoalteromonas haloplanktis TAC125*." *Biomolecular NMR Assignments*: 1-4.

The Genomics and Stress Laboratory works on the mechanisms involved in homeostasis control when yeast cells are exposed to different environmental cues. The function of Yap transcription factors in stress response is investigated.



Genomics and Stress

Metals are some of the most important environmental toxins causing acute and chronic health problems including cancer. The ubiquity of arsenic in the environment allowed for the evolution of very similar defence mechanisms in organisms ranging from bacteria to man. The genome-wide set of *S. cerevisiae* deletion strains provided an understanding of the mechanisms by which arsenic trioxide selectively kills acute promyelocytic leukemia cells. *S. cerevisiae* is an attractive biological model with powerful genetic and genomic approaches. Increased levels of arsenic in the environment is mediated by the expression of the ACR (Arsenic Compound Resistance) cluster that is composed of genes YAP8 (ACR1), ACR2 and ACR3. Yap8 is the key regulator of the expression of the arsenate-reductase Acr2 and the plasma membrane arsenite efflux transporter Acr3. Yap1 contributes to arsenic stress response by regulating the expression of a vacuolar arsenite detoxification pathway encoded by Ycf1. Yap1 also plays an important role in arsenic adaptation by controlling the redox homeostasis disturbed by inorganic arsenic compounds. To analyse whether Yap1 and Yap8 use similar mechanisms to transduce the stress signals to the basal transcription machinery, we are addressing the effect of mutations in specific subunits of the tail module of the mediator complex.

Furthermore, iron is an essential nutrient for almost every organism on earth, because it is utilised as a cofactor in key redox reactions of many central biochemical processes. The abnormal Fe accumulation, in either excessive or insufficient levels, underlies several human diseases including hereditary hemochromatosis, and Fe-deficiency anaemia. To give the cells iron homeostasis control, they possess not only the ability to regulate iron acquisition but

also to store iron once it is absorbed. The mechanisms of gene expression by which cells control iron overloading are being investigated.

Nitric oxide (NO) is a membrane-permeable free radical biologically produced by the NO synthase family of enzymes. Its exposure induces in cells a response at the transcriptional level, in which transcription factors are key elements. RNS can mediate chemical events similar to those involved in ROS detoxification. NO detoxification in prokaryotic and eukaryotic microorganisms is studied in order to obtain insights into the evolutionary conservation of the mechanisms involved. The identification of a common transcription factor to both stresses is investigated.

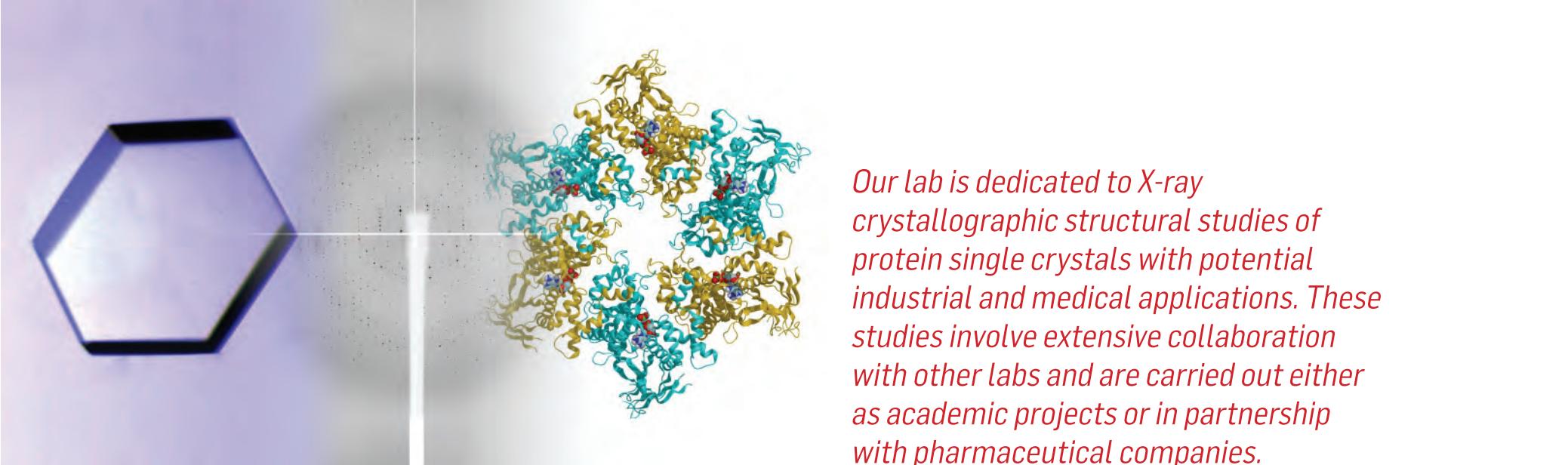
Pereira J., Pimentel C., Amaral C., Menezes R. A. and Rodrigues-Pousada C. (2009). "Yap4 PKA- and GSK3-dependent phosphorylation affects its stability but not its nuclear localization." *Yeast* 26(12): 641-653.

Menezes R. A., Amaral C., Batista-Nascimento L., Santos C., Ferreira R. B., Devaux F., Eleutherio E. C. A. and Rodrigues-Pousada C. (2008). "Contribution of Yap1 towards *Saccharomyces cerevisiae* adaptation to arsenic-mediated oxidative stress." *Biochemical Journal* 414: 301-311.

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Our lab is dedicated to X-ray crystallographic structural studies of protein single crystals with potential industrial and medical applications. These studies involve extensive collaboration with other labs and are carried out either as academic projects or in partnership with pharmaceutical companies.



Macromolecular Crystallography Unit Industry and Medicine Applied Crystallography



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Many proteins in nature have either industrial and/or medicinal applications. Knowledge of their 3D structure is essential to understanding their function at the atomic level, and can be used to control or improve their functional activity by the production of small molecules to act as substrates or ligands with specific purposes (e.g., drugs to fight disease) or by engineering selected mutants with enhanced biological activity. Our research programme is dedicated to doing just that: determining the 3D structure of selected proteins, and using that knowledge, in combination with other studies (biochemical, spectroscopic, etc.), to understand how these molecules work.

Human Polo-like kinase 1 (Plk-1) is a serine/threonine protein kinase and a key regulator of progression through mitosis. Plk-1 is overexpressed in highly proliferating cells and in many human tumors where this is often associated with poor prognosis. It has therefore been considered a very attractive target for cancer drug development. After many attempts, successful crystallisation and structure determination of the Plk-1 wild-type kinase domain was only possible in complex with selective DARPin 3H10. This work was carried out in collaboration with the pharmaceutical company Bayer Schering Pharma.

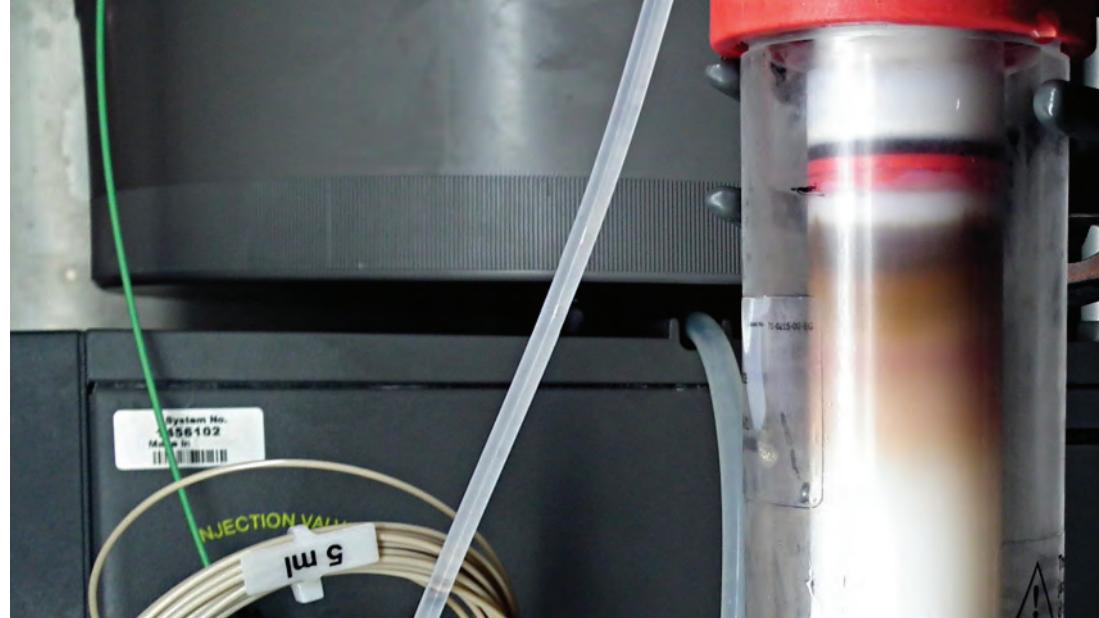
The 3D structure of [NiFeSe] hydrogenase from *D. vulgaris* Hildenborough in its oxidised, "as isolated", state provided a structural basis for the oxygen tolerance of [NiFeSe] hydrogenases; in the oxidised state there is an exogenous sulphur atom present in the active site, bound to the Ni atom and to the Se atom of the selenocysteine. This moves the selenocysteine side-chain conformation into a rotamer that shields the Ni atom from attack by O₂ molecules. When the enzyme is activated in production and consumption modes,

this sulphur atom can be released as either H₂S or HS and becomes located in a binding site near the active site, which in our structure was found occupied by a Cl⁻ ion. When the enzyme becomes quiescent, the sulphur species can become re-attached to the active site.

The 3D structure of mannosyl-3-phosphoglycerate synthase from *Thermus thermophilus* HB27 in its binary complex form, with bound GDP- α -D-mannose and Mg²⁺, shows a second metal binding site about 6 Å away from the mannose moiety. Kinetic and mutagenesis studies have shown this metal site to be co-catalytic. Additionally, the C1' atom in the mannopyranose ring is found within van der Waals contact distance of Asp167 in the DXD motif, suggesting it may act as a catalytic nucleophile, either in the formation of a glycosyl-enzyme intermediate according to the double-displacement S_N2 reaction mechanism, or in the stabilization of the oxocarbenium ion-like intermediate according to the D_N*A_{Nss} (S_Ni-like) reaction mechanism.

- Marques M. C., Coelho R., De Lacey A. L., Pereira I. A. C. and Matias P. M. (2010). "The Three-Dimensional Structure of NiFeSe Hydrogenase from *Desulfovibrio vulgaris* Hildenborough: A Hydrogenase without a Bridging Ligand in the Active Site in Its Oxidised, "as-Isolated" State." *Journal of Molecular Biology* 396(4): 893-907.
Bandeiras T. M., Hillig R. C., Matias P. M., Eberspaecher U., Fanganel J., Thomaz M., Miranda S., Crusius K., Puetter V., Amstutz P., Gulotti-Georgieva M., Binz H. K., Holz C., Schmitz A. A. P., Lang C., Donner P., Egner U., Carrondo M. A. and Muller-Tiemann B. (2008). "Structure of wild-type Plk-1 kinase domain in complex with a selective DARPin." *Acta Crystallographica Section D-Biological Crystallography* 64: 339-353.
Matias P. M., Gorynia S., Donner P. and Carrondo M. A. (2006). "Crystal structure of the human AAA(+) protein RuvBL1." *Journal of Biological Chemistry* 281(50): 38918-38929.

The Inorganic Biochemistry and NMR Laboratory utilises biophysical methods for the structural and functional characterisation of redox proteins that participate in the anaerobic bioenergetic metabolism of microorganisms.



Inorganic Biochemistry and NMR

This laboratory is currently engaged in the study of the molecular bases for the coupling exchange of electrons with exogenous solid substrates for energy conservation in several anaerobic organisms. Geological evidence from the beginning of life on Earth demonstrates that iron based anoxygenic photosynthesis is one of the most ancient forms of metabolism. Nowadays, extracellular metal respiration is fast becoming a hot-spot of biotechnological research on the development of novel bioremediation strategies for metal contaminated sites and for the development of microbial fuel cells for environmentally sustainable power generation.

These bioenergetic processes rely on the presence of complex networks of electron transfer proteins linking the extracellular solids and the membrane associated metabolism where energy transduction takes place and ATP is produced. A large fraction of the proteins that have been assigned to these bioenergetic networks are cytochromes but the structure and detailed functional mechanism of the majority of them remains unknown.

The lack of detailed knowledge regarding the energy metabolism of metal-respiring organisms has been identified as a fundamental barrier to the optimisation of applications in microbial fuel cell technology and bioremediation. In the IBN laboratory this issue is being tackled by integrating structural, thermodynamic and kinetic data on the proteins and enzymes of these bioenergetic networks in order to understand their functional properties at the molecular level. NMR spectroscopy is uniquely suited for collecting structural and dynamic information from the proteins under study because the size and complexity of biological macromolecules that can be studied in detail by this

technique has increased considerably in recent years. It is combined with other spectroscopic methods, fast transient kinetics methods and electrochemical methods to provide the experimental characterisation of the target proteins. This reductionist approach forms the basis for the progressive assembly of a systemic description of the complex network of redox proteins that ensures controlled electron exchange with the cell exterior in a way that is coupled with ATP generation.

Our ultimate goal is to apply this knowledge in the optimisation of microbial fuel cells capable of improving energy generation from renewable sources, and for the enhancement of bioremediation processes of sites contaminated with metals and radionuclides.

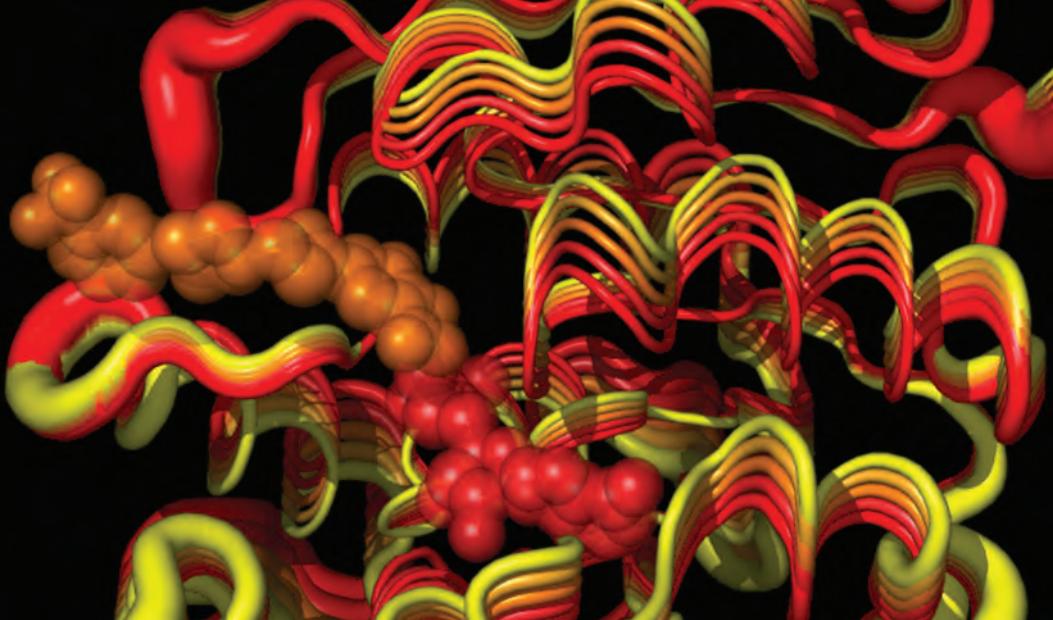
Paquete C. M. and Louro R. O. (2010). "Molecular details of multielectron transfer: the case of multiheme cytochromes from metal respiring organisms." *Dalton Transactions* 39(18): 4259-4266.

Fonseca B. M., Saraiva I. H., Paquete C. M., Soares C. M., Pacheco I., Salgueiro C. A. and Louro R. O. (2009). "The tetraheme cytochrome from *Shewanella oneidensis* MR-1 shows thermodynamic bias for functional specificity of the hemes." *Journal of Biological Inorganic Chemistry* 14(3): 375-385.

Pereira P. M., He Q., Valente F. M. A., Xavier A. V., Zhou J. Z., Pereira I. A. C. and Louro R. O. (2008). "Energy metabolism in *Desulfovibrio vulgaris* Hildenborough: insights from transcriptome analysis." *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 93(4): 347-362.



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X-ray crystallography allows for the determination of the three-dimensional structures of biological macromolecules. It enables the visualization of protein structures at the atomic level and enhances our understanding of protein function.



Macromolecular Crystallography Unit

Membrane Protein Crystallography



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Membrane proteins are key players in many cellular functions, such as energy production, transport of nutrients, signal transduction and intercellular communication. Our understanding of the functions of these proteins is highly circumscribed by the lack of structural information, due to the generally low abundance of membrane proteins and difficulty in obtaining crystals suitable for X-ray diffraction to allow for structure determination. Structural molecular biology plays a pivotal role in modern biology, both in the fundamental understanding of living things and in the design of new treatments for disease. Membrane proteins are challenging to study, but critical to understand because they represent 60 percent of drug targets.

The aim of our group is the structural characterization by X-ray Crystallography of membrane-bound proteins and complexes. The target proteins are involved in transport, metabolic or respiratory processes. Of particular interest are also proteins with biomedical relevance. We have close collaborations with other Portuguese laboratories and with various groups from the UK, Germany, Ireland and institutions in the U.S. The 3D structures of the target proteins are combined with biochemical data and biophysical experiments to shed light on their biological functions. Our objective is the comprehension of the structure-function relationship for these proteins.

We have also expanded our knowledge into the molecular biology field, so our Laboratory is now fully equipped to allow cloning, expression and purification of proteins, so we can go "from gene to structure". One project includes a structural genomics approach to studying membrane transport proteins from

Archaea, which are responsible for uptake of sugars, nucleosides and amino acids, as well as needed for efflux of antibiotics. Multidrug efflux proteins may be related with antibiotic resistance, and are thus potential targets for the development of new antimicrobial compounds. In particular, some archaeal transporters have homologues in Eukarya, and some are disease-related.

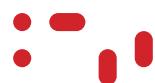
Other topic of interest is the determination of crystal structures of proteins isolated from extremophiles which grow at elevated temperatures (around 50 to 90°C). The identification of parameters involved in the thermostability of such proteins is an important issue with biotechnological implications for industry.

de Rosa M., de Sanctis D., Rosario A.L., Archer M., Rich A., Athanasiadis A., Carrondo M.A. (2010). *"Crystal structure of a junction between two Z-DNA helices"* **Proceedings of the National Academy of Sciences of the United States of America** 18 (107): 9088-9092.

Brito J. A. , Sousa F.L., Stelter M. , Bandeiras T.M. , Vonrhein C. , Pereira M.M. , Teixeira M. , Archer M. (2009). *"Structural and functional insights into Sulfide:quin one oxidoreductase"*, **Biochemistry** 48: 5613-5622.

Oliveira T. F., Vonrhein C., Matias P. M., Venceslau S. S., Pereira I. A. C. and Archer M. (2008). *"The Crystal Structure of Desulfovibrio vulgaris Dissimilatory Sulfite Reductase Bound to DsrC Provides Novel Insights into the Mechanism of Sulfate Respiration."* **Journal of Biological Chemistry** 283(49): 34141-34149.

The main research themes of the Laboratory are the study at the molecular level of the structure and functional mechanisms of soluble and membrane-bound metalloenzymes, namely those involved in oxygen and nitric oxide metabolisms.

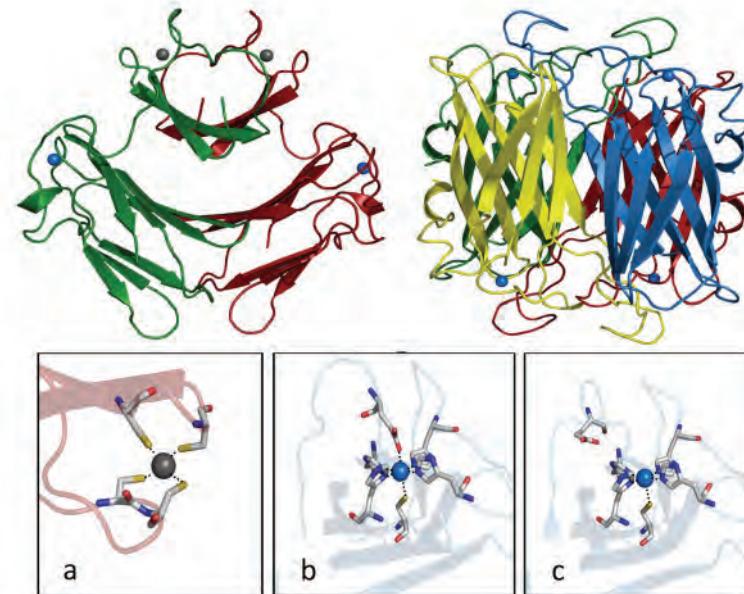


Metalloproteins and Bioenergetics Unit

Metalloenzymes and Molecular Bioenergetics

The appearance of oxygen in the atmosphere ca 2.5 Gy ago introduced a huge challenge for life, in what became known as the oxygen paradox: oxygen, when directly reduced to water by respiratory enzymes, namely the heme-copper oxygen reductases, permits energy conservation and, indeed, allows organisms to extract maximal energy from the reduced organic compounds. On the other hand, the reduction of oxygen occurring stepwise, in a non-controlled fashion, leads to the formation of reactive oxygen species, such as the superoxide anion or hydrogen peroxide.

In this laboratory we study metalloenzymes involved in both aspects of oxygen biochemistry. We aim at contributing to the understanding of the molecular mechanisms of membrane-bound heme-copper oxygen reductases, namely the processes whereby these enzymes couple oxygen reduction to the translocation of protons across the membrane. These studies have focused on one enzyme of each of the three types, A-C, in order to obtain a global understanding of the essential features of the mechanism. On the other hand, using wt and mutant enzymes, we have been closely studying the mechanism of superoxide reduction by the 1Fe and 2Fe superoxide reductases, in particular by coupling pulse radiolysis to very fast (microseconds) electronic absorption measurements. We proposed a catalytic mechanism that involved the formation of a ferric-hydroperoxo species, which, in some enzymes, decays through a ferric hydroxide to the final, ferric resting state. In other cases, the ferric hydroperoxo decays directly to the ferric resting state. The three dimensional studies of several of these enzymes have been determined, in different forms, in collaboration with the ITQB Macromolecular Crystallography Unit. Enzymes under more recent study are those endowed with hydrogen



peroxide reductase activity. These contain a di-iron site where the reaction takes place but the molecular mechanism remains to be established; this is one of our aims. Again, the 3D structure of one of these enzymes has already been obtained.

Finally, another major project is the study of the flavodiiron proteins, which have oxygen and/or nitric oxide reductase activities. Our present objectives are twofold: establishing the molecular mechanism of the reaction and understanding what dictates the preference for O₂ or NO.

These objectives, as well as other more specific ones, have been accomplished in collaboration with several groups at ITQB, in particular within the Metalloproteins and Bioenergetics Unit.

Rodrigues J. V., Victor B. L., Huber H., Saraiva L. M., Soares C. M., Cabelli D. E. and Teixeira M. (2008). "Superoxide reduction by *Nanoarchaeum equitans* neelaredoxin, an enzyme lacking the highly conserved glutamate iron ligand." *Journal of Biological Inorganic Chemistry* 13(2): 219-228.

Verissimo A. F., Sousa F. L., Baptista A. M., Teixeira M. and Pereira M. M. (2008). "Thermodynamic Redox Behavior of the Heme Centers in A-Type Heme-Copper Oxygen Reductases: Comparison between the Two Subfamilies." *Biophysical Journal* 95(9): 4448-4455.

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We want to contribute to the eco-efficient use of natural resources, the set-up of new bioremediation processes, and the production of bio-based products through the use of microorganisms and enzymes.

Microbial and Enzyme Technology



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Research in our laboratory involves the selection, characterisation, and engineering of promising microorganisms and enzymes for environmental and industrial applications.

For the last few years, we have been studying bacterial laccases, enzymes with a non-specific oxidation specificity (substrates are aromatics, such as polyphenols, methoxy-substituted phenols and diamines) that do not require the addition of expensive cofactors, and instead use readily available oxygen as electron acceptor.

We have undertaken a multidisciplinary approach to understanding the key structure-function determinants of laccases of bacterial origin. For example, through site directed mutagenesis we have examined how replacing key amino acid residues affects enzyme properties. By elucidating important aspects of the stability and catalytic mechanism of laccases, we have highlighted the limitation of the traditional rational approaches for enzyme design. Our method for improving enzyme properties is, therefore, to resort to directed evolution techniques, followed by robotic high-throughput screening.

Simultaneously, we have screened and characterised novel hyperthermophilic laccases; their superior stability compared with typically used enzymes offers new and interesting opportunities for biocatalysis.

On the technological side, we have focused on the biodegradation and biosynthesis of synthetic industrial colorants, namely the important azo and anthraquinonic dyes. Of the 10^6 tons of synthetic dyes produced every year,

over 10% is released into wastewater as stable organic pollutants. Resorting to biotechnology for the transformation of these dyes is both technically very attractive and quite promising.

We have recently begun investigating the enzymatic bioconversions of lignin, the recalcitrant biopolymer that gives wood its strength, using phenolic or non-phenolic model compounds. In the carbon cycle, degradation of lignin is a limiting step; clean technologies to unlock lignin, and hence cellulose, as renewable sources of chemicals and fuels may have great importance in the future.

Durao P., Bento I., Fernandes A. T., Melo E. P., Lindley P. F. and Martins L. O. (2006). "Perturbations of the T1 copper site in the CotA laccase from *Bacillus subtilis*: structural, biochemical, enzymatic and stability studies." *Journal of Biological Inorganic Chemistry* 11(4): 514-526.

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The Molecular Genetics of Microbial Resistance Laboratory mainly focuses on understanding the survival mechanisms of human pathogens related to oxidative and nitrosative stress imposed by the human immune system.



Molecular Genetics of Microbial Resistance

Reactive nitrogen and oxygen species are some of the principal weapons of the eukaryotic immune system for fighting prokaryotic pathogens and parasites. We have been analysing the mechanisms used by microbes to overcome the damage inflicted by those stresses. We study bacteria that have significant negative impact on human health and that demonstrate significant contributions to antibiotic resistance, like *Staphylococcus aureus* and *Helicobacter pylori*. Since iron and haem metabolisms are important for the functioning of all organisms we also address the study of iron/haem homeostasis in these pathogens, particularly when exposed to oxidative and nitrosative stress. Gene products involved in detoxification, regulation, assembly and repair of metalloproteins have already been identified and are currently under comprehensive study.

Our group also investigates sulphate reducing bacteria, in particular the *Desulfovibrio* genus, which are present in anaerobic environments and have a negative economic impact on industry due to their contribution to the corrosion of oil and gas pipelines. Nevertheless, these bacteria have potential for bioremediation applications since they are able to immobilise soluble forms of toxic metals like uranium, arsenate and chromate. In these organisms, we have been studying the mechanisms of oxygen tolerance and the iron and haem metabolism.

We have shown that carbon monoxide releasing compounds (CO-RMs) cause rapid death of pathogenic bacteria such as *Staphylococcus aureus*. We have begun analysing the mechanisms underpinning the bactericidal action of carbon monoxide. This knowledge will serve as a starting point for the devel-

opment of a novel type of therapeutic drug.

The group utilises and combines a wide range of methodologies, such as genomics, transcriptomics, microbiology, molecular genetics, biochemistry and host-pathogen interaction to address these areas of research.

Justino M. C., Almeida C. C., Teixeira M. and Saraiva L. M. (2007). "Escherichia coli di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters." *Journal of Biological Chemistry* 282(14): 10352-10359.

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Lobo S. A. L., Brindley A., Warren M. J. and Saraiva L. M. (2009). "Functional characterization of the early steps of tetrapyrrole biosynthesis and modification in *Desulfovibrio vulgaris Hildenborough*." *Biochemical Journal* 420: 317-325.



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Our research uses spectroscopic methods, especially Nuclear Magnetic Resonance (NMR) spectroscopy, to probe the molecular basis of interactions between molecules.

Molecular Interactions and NMR



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NMR spectroscopy is a versatile experimental tool. We mainly use it to study how small molecules bind to proteins, and this helps us to better understand the structural basis of biomolecular interactions. We carry out protein expression and purification to produce our own samples, and conduct complementary spectroscopic analyses. We also study proteins and ligands provided by our collaborators. Other research is based on improving and developing new methods for producing protein samples and conducting NMR experiments. Our favourite class of proteins is the abundant, EF-hand, calcium-binding proteins that are at the heart of many physiological signalling pathways. We also use NMR to measure the size of molecules and their complexes.

We work on short, focused projects. Recent topics include:

(1) Annexin A6 does not have a regular nucleotide-binding domain; we study its interactions with GTP analogs. There are many crystal structures of annexins but none feature nucleotide ligands. Our data will verify modelling studies of annexin A6/GTP complexes.

(2) DNA is a flexible molecule that adopts many alternative structures best captured by NMR spectroscopy. A rapid method for determining how short strands of DNA are folded was developed and based on measuring the DNA diffusion properties by NMR.

(3) There is no good assay to determine the contamination level of small molecules in protein samples. We have developed an assay based on diffusion NMR.

Future projects include:

(1) Studying the molecular basis of pH-sensing by two EF-hand proteins, calretinin and calbindin D28k, and searching for their pH-dependent, physiological targets.

(2) Exploiting the physical properties of lanthanides bound to proteins, and being the first to improve the protocols for introducing lanthanides into protein samples.

(3) Screening small molecules against membrane receptors in live cells by NMR. We must keep the cells alive and happy in a NMR-compatible medium.

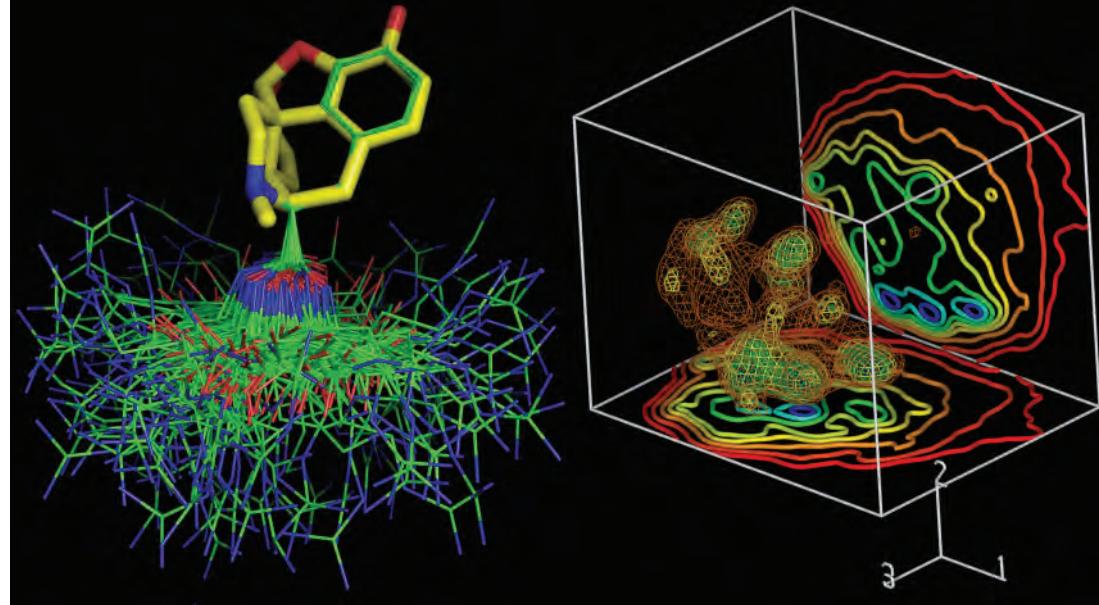
We are involved in extensive collaboration with researchers in Portugal, Spain, France, Hungary, Poland, Canada and the UK.

Ribeiro J. P., Palczewska M., Andre S., Canada F. J., Gabius H. J., Jimenez-Barbero J., Mellstrom B., Naranjo J. R., Scheffers D. J. and Groves P. (2010). "Diffusion nuclear magnetic resonance spectroscopy detects substoichiometric concentrations of small molecules in protein samples." *Analytical Biochemistry* 396(1): 117-123.

Feher K., Groves P., Batta G., Jimenez-Barbero J., Muhle-Goll C. and Kover K. E. (2008). "Competition Saturation Transfer Difference Experiments Improved with Isotope Editing and Filtering Schemes in NMR-Based Screening." *Journal of the American Chemical Society* 130(50): 17148-17153.

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*The Molecular Simulation Laboratory
develops and applies theoretical/
computational methods to the study of
the atomic-level determinants of the
behaviour of (bio)molecules.*



Molecular Simulation

Our laboratory places strong emphasis on developing novel biomolecule-oriented methods derived from physical principles, particularly Statistical Thermodynamics. This allows us to study biological processes that are difficult or impossible to address using standard methodologies. A major line of work deriving from such developments is the inclusion in simulation methods of experimentally important parameters that are essentially electrostatic, such as pH, ionic strength and the reduction potential of the solution. This has enabled a detailed study of the structural changes induced by pH and/or reduction potential on several peptides and proteins, such as kyotorphin (an analgesic neuropeptide), polylysine (a polymer displaying a helix-coil transition) and cytochrome c_3 (a protein involved in the respiration of sulfate reducing bacteria). A major extension to address biological membranes and nonequilibrium conditions is currently being undertaken.

Another subject is the study of peptide and protein folding/misfolding and the characterisation of their energy landscapes, a kind of topographic map displaying the conformational preferences of the molecule. We started by performing a critical study of energy landscapes in a decapeptide, identifying some problems with the usual approaches and proposing a robust method for identifying conformation classes. We are presently studying the prion protein, which is associated with Creutzfeldt-Jakob disease and other amyloid diseases, and whose misfolding is thought to be caused *in vivo* by the low pH of endosomes. Our study observed that pH decrease induces the loss of helical structure and the gain of new beta-structure, in agreement with experiments, identifying the regions where those changes take place. This approach will be applied to other cases of pH-induced misfolding.

A recent research line in our laboratory is the structural characterisation of peptide dendrimers, tree-like synthetic molecules formed by alternating functional amino acids with branching diamino acids. There is a large variety of applications for peptide dendrimers, including in the area of biomedicine, but the understanding of their structure/function relationship has so far been hindered by an inability to experimentally determine their structures. We are currently investigating the conformational preferences of several dendrimers, including those mimicking the cobalamin binding of B_{12} -dependent enzymes.

Machuqueiro M. and Baptista A. M. (2007). "The pH-dependent conformational states of kyotorphin: A constant-pH molecular dynamics study." *Biophysical Journal* 92(6): 1836-1845.

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At the Mössbauer Spectroscopy Lab at ITQB this technique is used together with EPR and DFT to study iron proteins. The main goal is to characterise intermediates in catalytic reactions to understand their enzymatic mechanisms.

Mössbauer Spectroscopy



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Iron is the most abundant transition metal in biological systems. Iron-containing proteins play the most frequent roles in all three life domains, including transcriptional regulation, sensing, and an enormous number of catalytic activities in anabolic or catabolic processes, oxidative stress responses, DNA protection, and oxygen transport. Furthermore, iron metabolism is very tightly controlled; e.g. iron excess or deficiencies lead to severe diseases, such as anemia or hemochromatosis, and mutations of iron enzymes may cause a plethora of other diseases. Therefore, a detailed study of iron-containing proteins continues to be fundamental in the life sciences, strongly related to biomedicine due to its importance for human health, including the combat of invading pathogens.

The biophysical technique best suited to the study of iron proteins is ^{57}Fe Mössbauer spectroscopy (ME), which is based on the recoilless absorption of gamma rays by ^{57}Fe nuclei. This technique is one of the most sensitive methods for determining the chemical characteristics of metal sites and has served as the basis for the discovery of many novel iron centres, as well as the determination of their enzymatic mechanisms.

We are interested in the flavodiiron proteins (FDPs) which are involved in the protection against oxidative and/or nitrosative stress. FDPs from different micro organisms show distinct specificities for NO or O₂ reductase activity (NOR or O₂R activity, respectively); some merely show NOR or O₂R activity, and others both NOR and O₂R. However, no significant structural differences in the active site environment were found to explain this behaviour. These enzymes are present both in prokaryotes and lower eukaryotes, such as

pathogenic protozoa, and play a decisive role in the detoxification of those two small molecules; they are also believed to play an important role in host-pathogen interactions.

Our research focuses on the characterisation of trapped intermediates in catalytic reactions of these novel prokaryotic enzymes by Mössbauer and Electron Paramagnetic Resonance spectroscopies together with theoretical studies based on Density Functional Theory. The main goal is to determine their catalytic mechanisms and to address the question of specificity for each one of the substrates. These studies are performed both *in vitro* (purified proteins) and in whole cells.

de Oliveira F. T., Chanda A., Banerjee D., Shan X. P., Mondal S., Que L., Bominaar E. L., Munck E. and Collins T. J. (2007). "Chemical and spectroscopic evidence for an Fe-V-Oxo complex." *Science* 315(5813): 835-838.

Ghosh A., de Oliveira F. T., Yano T., Nishioka T., Beach E. S., Kinoshita I., Munck E., Ryabov A. D., Horwitz C. P. and Collins T. J. (2005). "Catalytically active mu-oxodiiron(IV) oxidants from iron(III) and dioxygen." *Journal of the American Chemical Society* 127(8): 2505-2513.

Chanda A., de Oliveira F. T., Collins T. J., Munck E. and Bominaar E. L. (2008). "Density Functional Theory Study of the Structural, Electronic, and Magnetic Properties of a mu-oxo Bridged Dinuclear Fe-IV Complex Based on a Tetra-Amido Macrocyclic Ligand." *Inorganic Chemistry* 47(20): 9372-9379.

The laboratory investigates the biology and biophysics of protein folding, an essential cellular process through which proteins acquire a functional conformation. This process is affected in neurodegenerative and metabolic diseases currently under investigation.

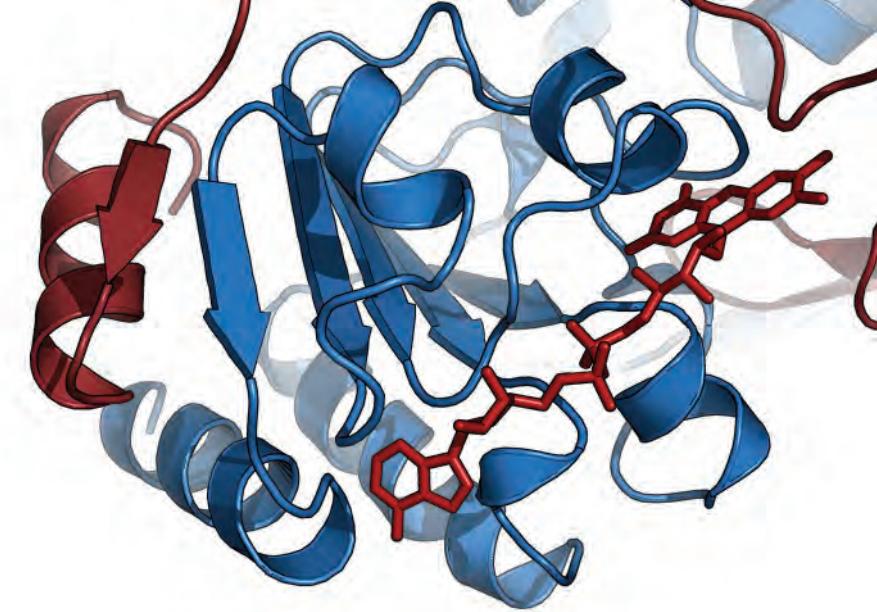


Protein Biochemistry Folding and Stability

Protein folding is a fundamental biological process through which proteins acquire the specific structure that determines their function. Despite considerable knowledge of the physical interactions controlling protein structure, the mechanisms of folding and the rules determining how a linear polypeptide wraps itself into a unique fold are still unknown. Moreover, while the molecular determinants underlying protein misfolding, structural inter-conversions, and aggregation in the form of amyloid are unclear, these defects in protein folding are the hallmark of protein misfolding diseases. Our work addresses many of the open questions in the field.

One interest of the lab involves the mechanisms of protein misfolding in neurodegeneration. We seek to understand how mutations and cellular factors trigger protein misfolding and disease. In this respect, the chemical biology of metal ions such as calcium, zinc and copper - important chemical modulators in the nervous system - play a key role. For example, we are addressing the effect of zinc bursts on the toxic aggregation pathways involving SOD1, the protein affected in amyotrophic lateral sclerosis. Also, in recent years we have analysed the effects of mutations in the protein frataxin, defective in Friedreich's ataxia. More recently, our finding that S100B, one of the most abundant proteins in the brain, is intrinsically amyloidogenic suggests that its self-assembly can affect the A-beta amyloid pathways, thus contributing also to Alzheimer's disease pathology.

Protein misfolding is also central to metabolic diseases. We concentrate on fatty acid oxidation deficiencies such as MADD to understand how folding changes modulate biological activity and how housekeeping molecular chap-



erones process these variants. Recently we have established the molecular basis of the therapeutic intake of vitamin B2 by MADD patients. We now hope to correlate molecular data with physiology by looking into more complex systems, such as patient's fibroblasts and *Drosophila*.

Overall, our experimental drive is to go from molecules, to cells, to organisms and for this purpose we combine our core expertise in protein biophysics and biochemistry with cell biology and molecular methods. Through basic research, our work therefore impacts on different areas, from basic protein science to biotechnology and biomedicine.

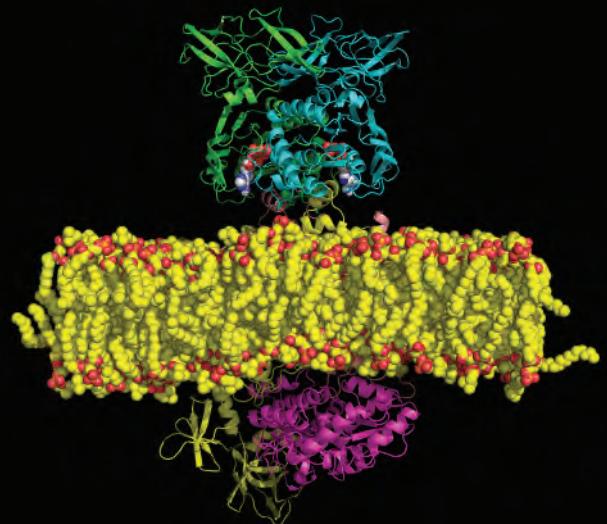
Protein Folding and Metal Ions: Mechanisms, Biology and Disease Editor(s): Gomes and Wittung-Stafshede (2010). CRC Press, Taylor and Francis (USA)

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The Protein Modelling Laboratory works on molecular modelling of proteins using physical methods. Our topics of interest range from basic research in modelling methodologies to applications with biotechnological and biomedical interest.

Protein Modelling



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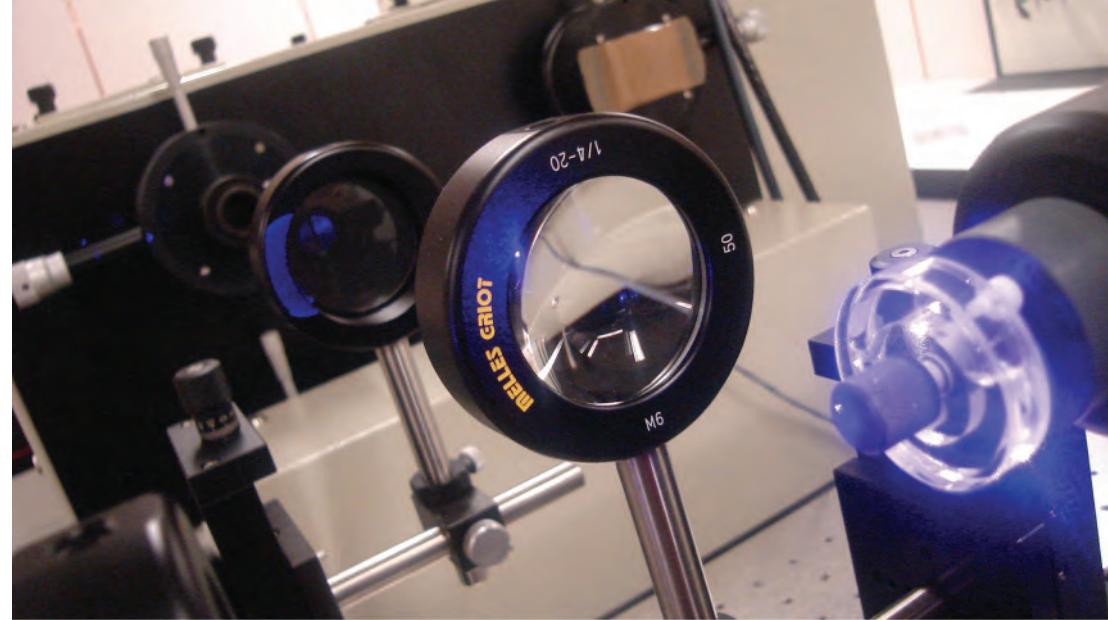
Modelling redox proteins and chains is one of our research interests, and one of the most relevant examples is our study of hydrogenases, which are enzymes that catalyse the reversible molecular hydrogen cleavage into electrons and protons. We study molecular hydrogen permeation towards the internal active site, using molecular dynamics simulations, as well as proton transfer within the protein, using continuum electrostatic and Monte Carlo calculations. In collaboration with other groups at ITQB, we are trying to understand the mechanism of laccases at the molecular level, as well as their engineering. Studying enzymes in non-aqueous solvents is another important area in our laboratory, with focus on the molecular mechanisms of enzyme hydration, enantioselectivity, imprinting and catalysis. The non-aqueous solvents studied include ionic liquids, and we were the first to simulate protein molecules within this media, clarifying the molecular reasons for stability. ABC transporters constitute a new topic for our laboratory. Our first research on the subject was on the NBD1-NBD association in CTFR, the chloride transporter involved in cystic fibrosis, one of the most common genetic diseases. We built structural models for this association, which is responsible for ATP hydrolysis, and used them to understand the effect of mutations on disease causing genotypes. To study how these molecular machines work, we turn to prokaryotic ABC transporters and in particular NBD dimers. We recently published a paper on the conformational changes involved in ATP hydrolysis, and their consequences on substrate translocation. Other studies on complete transporters are on the way.

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Micaelo, NM, Soares, CM (2008) "Protein structure and dynamics in ionic liquids. Insights from molecular dynamics simulation studies". *Journal of Physical Chemistry B*, 112, 2566-2572

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Research in the Laboratory for Raman Spectroscopy of Metalloproteins focuses on the structural and functional characterisation of redox proteins that perform diverse functions in cells, including electron transport, detoxification and enzymatic catalysis.



Raman Spectroscopy of Metalloproteins

We are interested in the biophysico-chemical aspects of functioning of various metalloproteins. In particular, we are trying to understand how different proteins fine-tune the reactivity of their metal cofactors and, additionally, how the metal centres help to define the functional features, dynamics and stability of protein molecules. By using resonance Raman (RR) spectroscopy to address structural properties of the metal centres in heme, non-heme iron, iron-sulfur, blue copper and cobalt proteins, we learn about their respective functional features.

A special emphasis in our research is given to investigations of the redox processes of membrane proteins under conditions that reproduce some basic features of a cell (or mitochondrial) membrane. Respiratory chain electron transfer (ET) complexes are incorporated into the phospholipid membrane, therefore exerting their function in a hydrophobic environment under the influence of membrane potential. In this respect, immobilisation of these heme proteins on biocompatible metal supports that function as electrodes represents a powerful experimental alternative, allowing for the application of electrochemistry coupled to surface-enhanced resonance Raman (SERR) spectroscopy. These methods provide insights into structural and redox properties of the protein, as well as the kinetic and thermodynamic parameters of the heterogeneous ET. The molecular basis of the functioning of several oxygen reductases has been established using this approach, allowing for the determination of the parameters that control inter- and intra-protein ET in these complex enzymes.

We use SERR spectroscopy to address the enzymatic processes in bioelectronic devices. It can simultaneously probe the active site structure and conformational dynamics of the immobilized enzyme concomitant to the ET, a determinant for the functioning of these devices. To this end, we have contributed to the characterisation of immobilized nitrite reductases, peroxidases and cytochromes P450, used in biosensors for the detection of nitrite, hydrogen peroxide and other substrates.

The high sensitivity and specificity of RR spectroscopy help us to gain important insights into the physiological processes that involve heme group interactions: upon its release from hemoglobin in mammals with inflammatory diseases; in the sensing of external stimuli by chemotaxis proteins that enable bacteria to respond to environmental conditions; with antibiotics in proteins that help pathogens to deal with the host's immune system.

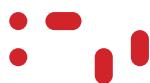
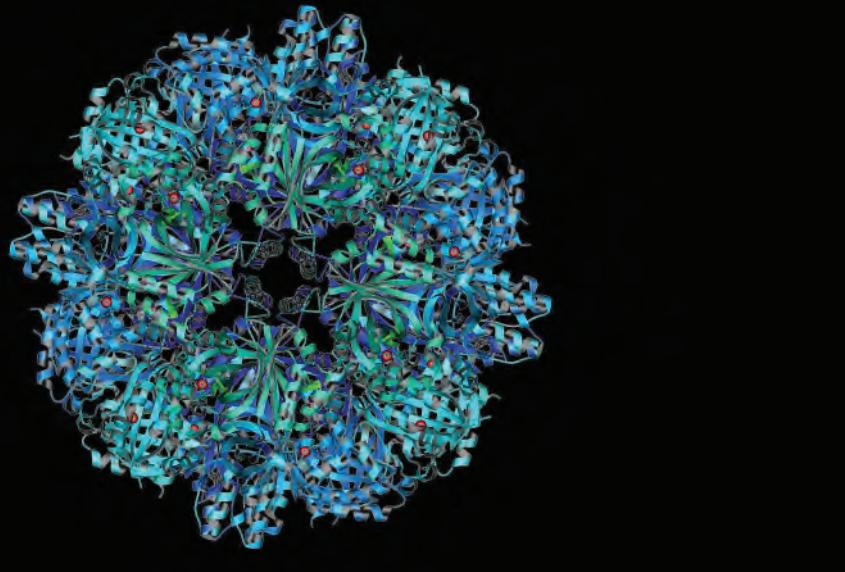


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Todorovic, S., Pereira M., Bandeiras, T., Teixeira, M., Hildebrandt, P., and Murgida, D. H. (2005) "Midpoint potentials of Hemes *a* and *a3* in the Quinol Oxidase from *Acidianus amoenus* are Inverted" **Journal of the American Chemical Society** 127 13561-13566

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Macromolecular Crystallography Unit Structural Biology



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The Structural Biology Laboratory (SBL) seeks to understand biochemical processes at the atomic level, mainly based on the determination of biological macromolecules 3D structures using X-rays diffraction analysis. The interpretation of these structures provides clues as to how biomolecules may interact, and has enabled the rationalisation of applications in diverse fields of the life sciences. SBL collaborates with other research groups to study key biological macromolecules, including enzymes as well as complexes between proteins and nucleic-acids. SBL is involved in several research projects, utilising the 3D structure , e.g.:

- the study of the structures of sulphur oxygenase reductases from *Acidianus ambivalens* and *Halothiobacillus napolitanus*, in collaboration with the University of Darmstadt, Germany. Immediate goals are to increase the resolution of their structures, and to capture relevant reaction intermediates analogues. These enzymes are involved in the sulphur geo-bio-cycle being responsible for the insertion of inorganic sulphur into the life-cycle. They work as self-compartmentalising reactors at the nano scale, being prototypes of proto-organelles in archaea.
- the determination of native and site-directed mutants of UDP-glucose dehydrogenases (UGDs) from *Burkholderia cepacia* and *Sphingomonas elodea*, in a collaboration with Universidade Técnica de Lisboa (IST). These studies belong to a research programme researching two bacterial extracellular polysaccharides (EPS), namely cepacian and gellan, with relevance in Medicine and Biotechnology. The EPS cepacian seems to play a role in the persistence and virulence in cystic fibrosis lung disease, whereas gellan is one of the most

The Structural Biology Laboratory works on the 3D structural determination of biological macromolecules aiming to understand biological processes at the atomic and molecular levels.

commercially important bacterial exopolysaccharides.

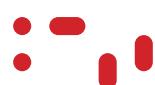
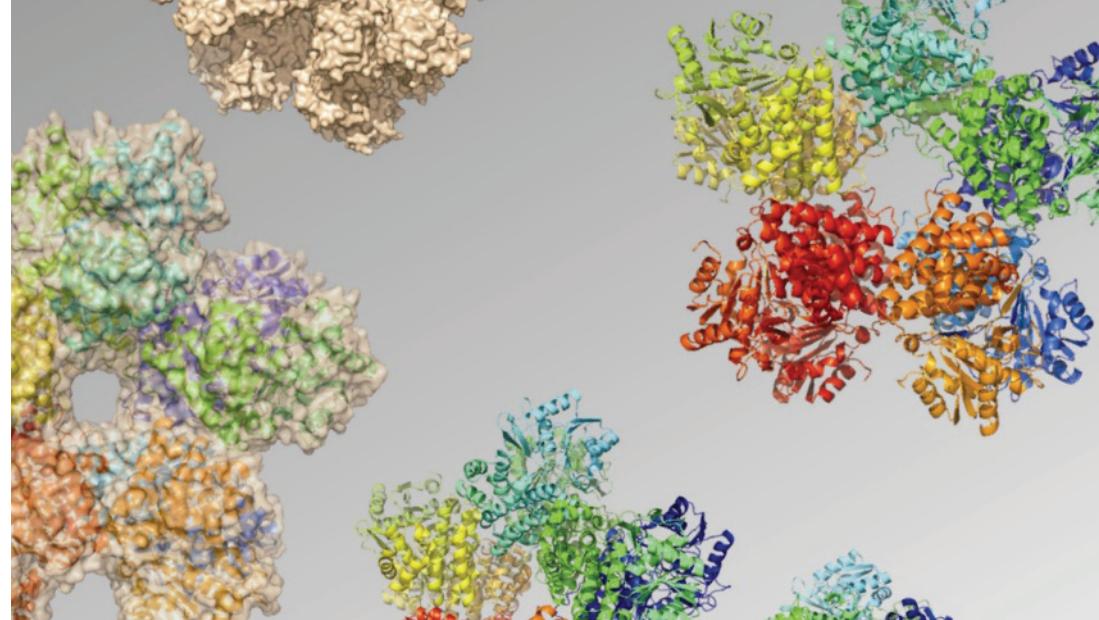
- the characterization of type I (oxygen-insensitive) nitroreductase from *Rhodobacter capsulatus* in collaboration with the University of Saragoza, Spain, and ESRF, Grenoble, France. Microorganisms such as *Rhodobacter capsulatus* are able to degrade and remove nitroaromatics compounds. These highly toxic materials are widely spread throughout nature mainly as a result of human activities, namely paints or dyes, explosives, and pharmacology industries. This nitroreductase is thus an enzyme with potential applications in bioremediation, and also in cancer therapy.

Urich T., Gomes C. M., Kletzin A. and Frazao C. (2006). "X-ray structure of a self-compartmentalizing sulfur cycle metalloenzyme." *Science* 311(5763): 996-1000.

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Andrade J., Karmali A., Carrondo M. A. and Frazao C. (2007). "Structure of amidase from *Pseudomonas aeruginosa* showing a trapped acyl transfer reaction intermediate state." *Journal of Biological Chemistry* 282(27): 19598-19605.

The Structural Genomic Group uses a structural genomic approach employing X-ray crystallography to study proteins that are involved in the innate immune response and different prokaryote proteins that are targets for health and biotechnological applications.



Macromolecular Crystallography Unit Structural Genomics

The Laboratory interests are focused on NOD-like receptors (NLRs) and their role in pathogen recognition. NLRs comprise a family of cytosolic proteins that have been implicated as ancient cellular sentinels mediating protective immune responses elicited by intracellular pathogens or endogenous danger signals. Our aim is to understand the interactions and modes of binding within signal transduction and regulation of NOD2 activation in the innate immune response. Our work focuses primarily on strategies to produce protein complexes involved in innate immunity employing co-expression and domain truncation methods to determine the molecular and structural basis of NLR interactions. A number of other proteins are been studied that play a pivotal role in NOD2 functions, these will provide the platform to further elucidate and study the interactions in innate immune signalling. One of these is Grim-19, a novel protein involved in cell apoptosis. Our aim is to clarify the functional mechanism of this apoptotic protein and its molecular basis.

Additionally, the Laboratory research is also focused in understanding the molecular basis of enzymatic mechanisms and on the elucidation of physiological roles and functions of glycoside hydrolases and metallo-proteins. In particular, we are interested in the mechanism of cleavage of glycosidic bonds and in the mechanism of dioxygen reduction to water by multi-copper oxidases and in several protein targets of the bacteria *Deinococcus radiodurans* which is extremely resistant to ionizing radiation, desiccation, as well as to several other stress conditions.

The Laboratory is also involved in the preparatory phase of the EU Integrated Structural Biology Infrastructure, INSTRUCT, one of the six biomedical infra-

structures that are part of ESFRI program. This infrastructure builds on the continuation of the integrated project SPINE2-Complexes. Our Laboratory was one of the partners of this large European consortium.

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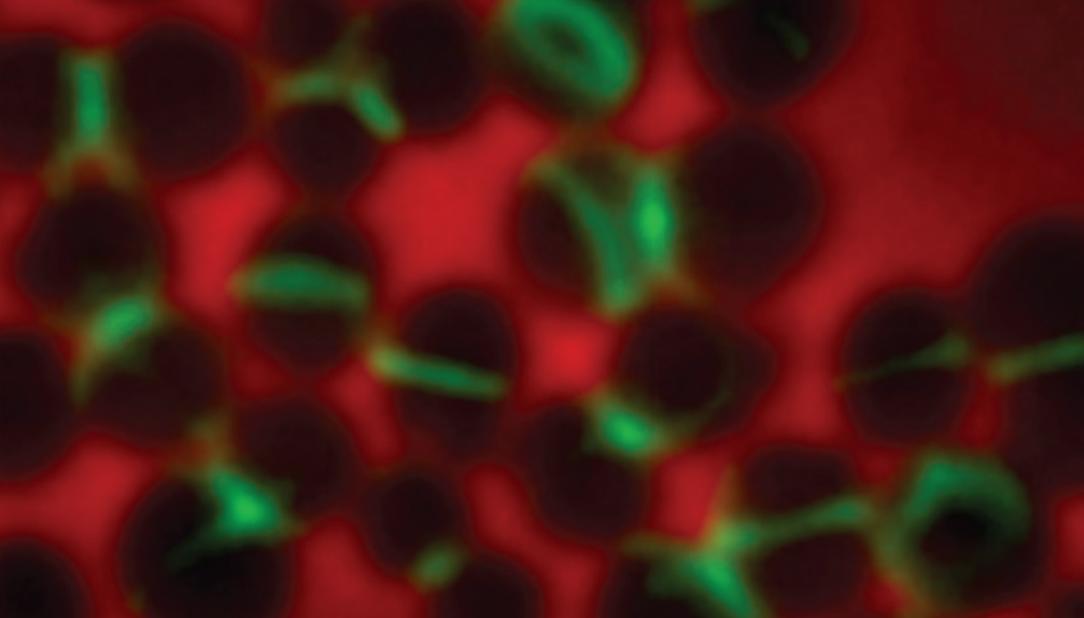
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*In the Bacterial Cell Biology Laboratory we use the Gram positive pathogen *Staphylococcus aureus* to study the mechanisms of cell division and of antibiotic resistance to cell wall targeting antibiotics.*



Bacterial Cell Biology



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Bacterial cells have revealed a surprising degree of protein organisation. Many essential cellular processes are performed by higher order protein complexes, which are precisely regulated in time and space. An example of such processes is cell division, which has received detailed study in very few model organisms.

Staphylococcus aureus is a Gram positive pathogen and the most common cause of antibiotic resistant hospital-acquired infections. Besides its clinical relevance, *S. aureus* is also a very interesting model for studying cell division because it has a different shape and mode of division from the traditional, widely used, model organisms *Escherichia coli* and *Bacillus subtilis*: it has round (coccoid) shaped cells and, more interestingly, divides in three consecutive perpendicular division planes over three division cycles, similar to the first divisions of a fertilized egg. For a bacterial cell to divide it has to double its mass, replicate its genome and synthesise a septum between two daughter cells. It is this last process that is inhibited by a large number of antibiotics, such as beta-lactams or glycopeptides, which target cell wall synthesis.

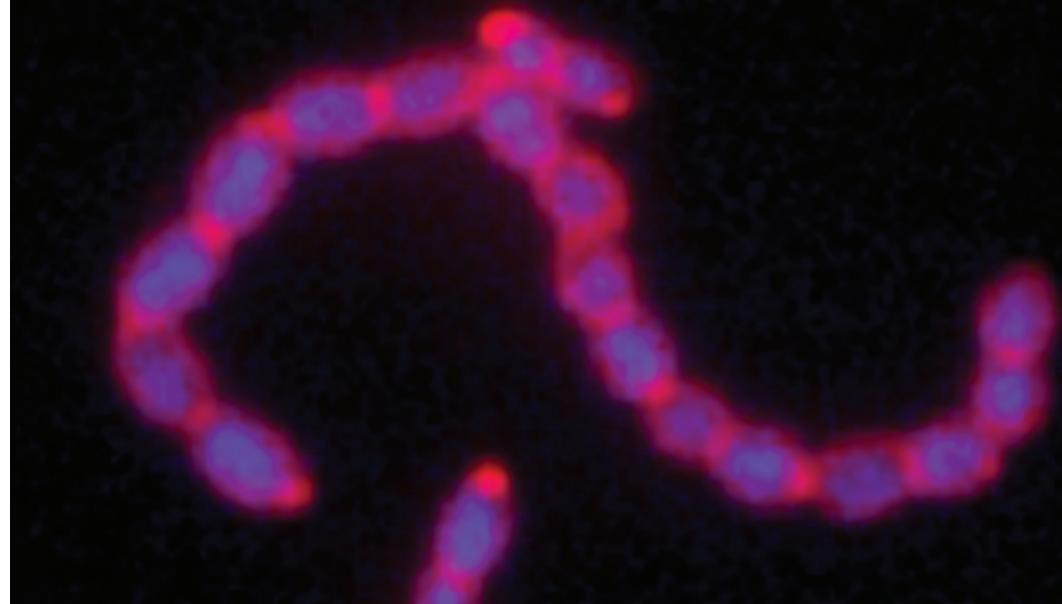
In the Bacterial Cell Biology Laboratory, which started in early 2006, the aim has been to understand, at a molecular level, the organisation and the temporal and spatial regulation of two fundamental steps of cell division - the segregation of the bacterial chromosome and the synthesis of the division septum, as well as to integrate this information into a better understanding of antibiotic resistance mechanisms in *S. aureus*.

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Zapun A., Vernet T. and Pinho M. G. (2008). "The different shapes of cocci." *Fems Microbiology Reviews* 32(2): 345-360.

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We study how bacteria synthesise a major component of their cell surface, the peptidoglycan, whilst simultaneously preventing the infected host from detecting this inflammatory macromolecule that could trigger an innate immune response.



Bacterial Cell Surfaces and Pathogenesis

The main interest of the laboratory of Bacterial Cell Surfaces and Pathogenesis is to understand how bacteria synthesise their peptidoglycan, a large macromolecule from their cell wall that surrounds and protects bacteria. The synthesis of peptidoglycan, which is involved in determining the bacterial shape and which serves as an attachment site for extracellular proteins, is the target of different families of antibiotics and it seems to play an important role in the detection of bacterial infection by different host immune systems.

In the last few years an increasing number of reports have shown that peptidoglycan (or its small components) is able to induce an inflammatory response in different hosts. In order to understand how the infected host senses this macromolecule, we must learn how bacteria try to disguise it and how this molecule is synthesised, organised, modified and degraded during the regular bacterial cell cycle.

The current research programme at the laboratory of Bacterial Cell Surfaces and Pathogenesis has the following aims:

- understanding how the different chemical composition and the structure of the PGN found among bacteria determine their recognition by the host
- determining whether the regular metabolism of the PGN, which occurs as bacteria divide into two daughter cells, might interfere with the accessibility of the PGN to the host PGN detectors

• investigating whether PGN is hidden from the infected host by molecules that are found in different bacteria covalently attached to PGN, such as bacterial capsular polysaccharides

• identifying particular strategies that bacteria may have found to hide or eliminate the synthesis of the inflammatory PGN macromolecule, namely in obligatory intracellular bacterial pathogens such as *Chlamydia trachomatis*

We expect that understanding the role of PGN in the trigger of inflammatory processes will allow us to devise strategies to modulate the inflammatory response of a given host during bacterial infection.

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We study a process called Quorum Sensing which enables bacteria to synchronise their behaviour and act in group to regulate important processes such as virulence, biofilm formation and antibiotics production.



Bacterial Signalling



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Bacteria use small chemical molecules called autoinducers to communicate with one another by a process called quorum sensing. This process enables a population of bacteria to regulate behaviours which are only productive when many bacteria act in concert, in similarity with what happens with multi-cellular organisms. Behaviours regulated by quorum sensing include the production of virulence factors, the formation of biofilms, and the synthesis of secondary metabolites like antibiotics. Importantly, these processes are often crucial for successful bacterial-host relationships whether symbiotic or pathogenic. In the Bacterial Signalling Laboratory we combine biochemical and genetic approaches from molecules to circuits in order to understand how the molecular mechanisms underlying quorum sensing regulate bacterial behaviours.

Most Quorum Sensing systems studied to date rely on autoinducer signals which are species-specific and therefore promote intra-species cell-cell communication. However, in nature, most bacteria live in poly-microbial species communities and thus it is expected that inter-species signal interactions between the community members are important. We therefore focus on Quorum Sensing systems which can promote bacterial inter-species communication. One of these systems relies on one autoinducer termed AI-2 which is produced and detected by a wide variety of bacterial species. Our research studies new chemical molecules that are used as inter-species signals, the network components involved in detecting the signals and processing information inside individual cells, and, finally, the characterisation of the behaviour of the bacterial community in multi-species bacterial consortia. Our ultimate goal is to understand how bacteria use inter-species cell-cell

communication to coordinate population-wide behaviours in consortia and in microbial-host interactions. These studies can potentially lead to the development of new therapies to control functions regulated by quorum sensing, such as virulence, whilst also developing biotechnological applications for controlling industrial scale production of beneficial bacterial products, like antibiotics or recombinant proteins.

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Research at the Cell Physiology & NMR Lab focuses on beneficial microbes, i.e. microorganisms that either promote human health or well-being or are sources of new metabolites and enzymes with potential applications in biotechnology.



Cell Physiology & NMR

Our research has two ultimate goals: i) understanding the molecular basis of adaptation to thermophily in marine microorganisms adapted to growth at temperatures near 100°C; and ii) understanding the principles that govern cell biology so that metabolic engineering strategies for the production of desirable end-products can be efficiently implemented (Systems Metabolic Engineering). This research group started in 1986 working on a topic that, at the time, was very challenging: NMR methods for studying the metabolism of living cells in a non-invasive way. It was this practice of examining whole cells, instead of breaking them apart, which led us to the two research lines that are active today: the role of low-molecular organic compounds in the thermo-protection of cellular components of microbes adapted to hot environments, and the metabolic engineering of industrial bacteria.

Hyper/thermophiles isolated from seawater accumulate organic solutes (thermolytes) not only in response to an increase in salinity, but also in response to heat stress. These solutes are different from those used by mesophilic microorganisms for osmoadaptation. Our team characterised a number of thermolytes and demonstrated their superior efficacy in the stabilisation of different biomaterials such as proteins. Therefore, we gathered evidence supporting a link between solute accumulation by (hyper)thermophiles and structural protection against heat damage. We continue our efforts to screen for novel thermolytes and to answer the following questions: What novel pathways and enzymes are used in the synthesis of thermolytes? What is the total molecular and regulatory network, from sensing the stress to synthesising the solute? What are the molecular mechanisms underlying protein protection by thermolytes? Are they effective in the cell milieu?

We believe that the answers will allow for the design of useful tailor-made stabilisers and lead to new knowledge about the physiology supporting life at high temperatures.

In another line, we use *in vivo* NMR coupled with ¹³C-labelling to measure on line the dynamics of intracellular metabolites to provide useful guidelines for metabolic engineering strategies. The team collaborates with American and EU groups with expertise in mathematical modelling for the integration of the data at the pan-organisational level. Target organisms include: *Lactococcus lactis* for the production of nutraceuticals and *Corynebacterium glutamicum* for the industrial production of reagents from renewable resources.

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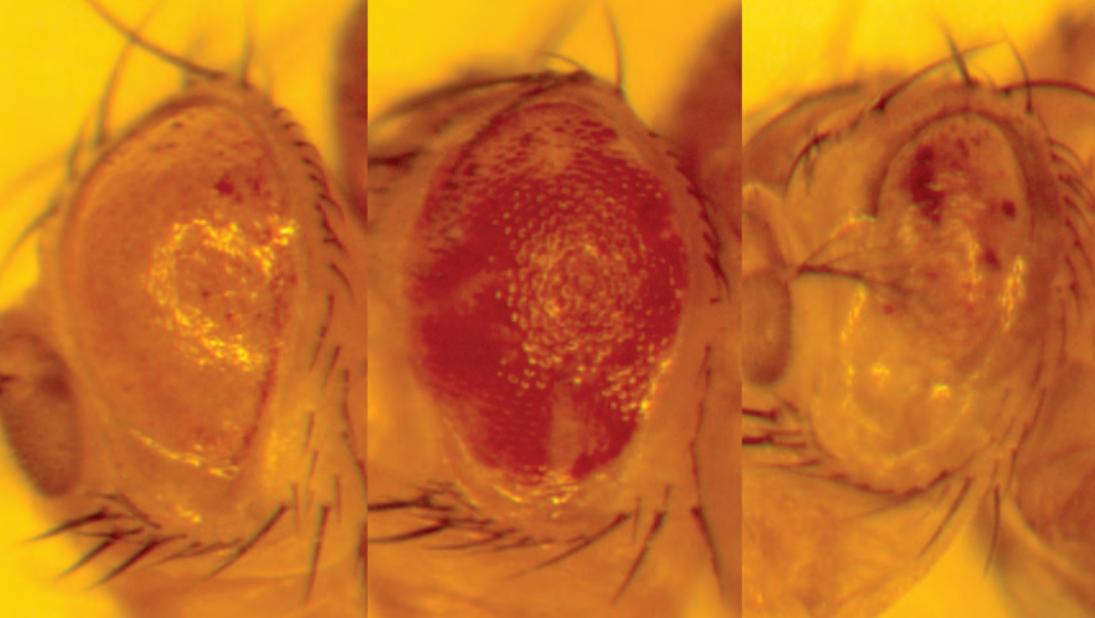
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We use *Drosophila* as a model system for studying the molecular and cellular signalling mechanisms involved in the degeneration of photoreceptors, the cells that sense light in the visual system.

Cell Signalling in *Drosophila*



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The endoplasmic reticulum (ER) is the cell organelle where secreted and membrane proteins are synthesized and folded. This process requires the recruitment of ribosomes, translocation of the nascent peptides into the lumen of the ER, and a variety of post-translational modifications and folding events. When the folding capacity of the ER is impaired, the presence of misfolded proteins in the ER causes stress to the cell ("ER stress") and activates a cellular response, the Unfolded Protein Response (UPR), to restore homeostasis in the ER. The UPR is mediated by several signalling pathways, which sense stress in the ER and activate a variety of cellular responses, such as translational attenuation to reduce protein synthesis and prevent further accumulation of unfolded proteins and the transcriptional upregulation of genes encoding ER chaperones and enzymes, to increase the folding capacity of the ER. However, in situations where ER stress is severe or prolonged, or when the cellular responses induced by UPR are insufficient to overcome the origin of ER stress, cells can undergo programmed cell death (Apoptosis).

Retinitis pigmentosa (RP) is a major cause of human blindness. In this disease, the photoreceptor cells in the eye progressively degenerate over time. About 30% of autosomal dominant RP cases are caused by mutations in Rhodopsin, the light sensitive protein of photoreceptors. In *Drosophila*, equivalent mutations in *ninaE* (the gene encoding Rhodopsin 1) also cause dominant degeneration of the retina and most of these mutations produce misfolded forms of Rhodopsin 1, which are not properly processed and accumulate in the ER.

We have shown that one of the branches of the UPR, the IRE1/Xbp1 signalling pathway, has a protective role against *ninaE* induced photoreceptor degeneration.

We are currently using the tools of modern genetics, cell biology and imaging to investigate the genes downstream from the IRE1/Xbp1 signalling pathway that regulate photoreceptor degeneration.

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Our studies focus on the control of gene expression. We have studied RNA degradation and characterised enzymes that mediate decay. Other interests are stress and microbial growth. This work has many applications in biotechnology and health.



Control of Gene Expression

Our research has concentrated on elucidating factors determinant for the control of gene expression. Biological processes cannot be fully understood without a deep understanding of RNA metabolism. In 2006 and 2009 three Nobel prizes were dedicated to research in the field of RNA. Our laboratory has focused on the study of RNA degradation mechanisms and the characterisation of enzymes mediating RNA decay in microorganisms. Namely we have studied RNase II family ribonucleases in the maturation, degradation, and quality control of mRNAs and functional non-coding small RNAs. Our studies have been applied to areas of biotechnological interest and health. We have extended our research to eukaryotes to further understand the role of RNases in global regulation. Another area of interest is stress, bacterial cell growth and survival.

The continuous breakdown and resynthesis of prokaryotic mRNA allows for the fast production of new kinds of proteins and best explains the rapid adaptation of micro-organisms to a changing environment. In this way mRNA levels can regulate protein synthesis and cellular growth. However, the inherent instability of prokaryotic mRNA has been one of the main obstacles to the profitable production of proteins of interest in industrial micro-organisms. Prokaryotic mRNAs differ in their susceptibility to degradation by endonucleases and exonucleases that may be due to differences in their sequence and structure. The analysis of mRNA degradation has been difficult in all systems and, despite numerous studies, the process of mRNA degradation is still poorly understood. Recent results appear to show that the similarities between mRNA decay in the pro- and eukaryotic systems are greater than were generally believed. It is important to study RNA metabolism in different

systems to unveil universally conserved features. Future work will involve the identification and study of the action of more RNases, relating them to the decay of RNAs. In close collaboration with international partners, the team of C. Arraiano, utilising state-of-the-art technologies such as *in vitro* systems, functional genomics and RNomics, will continue to contribute to the knowledge of the regulating mechanism of gene expression.

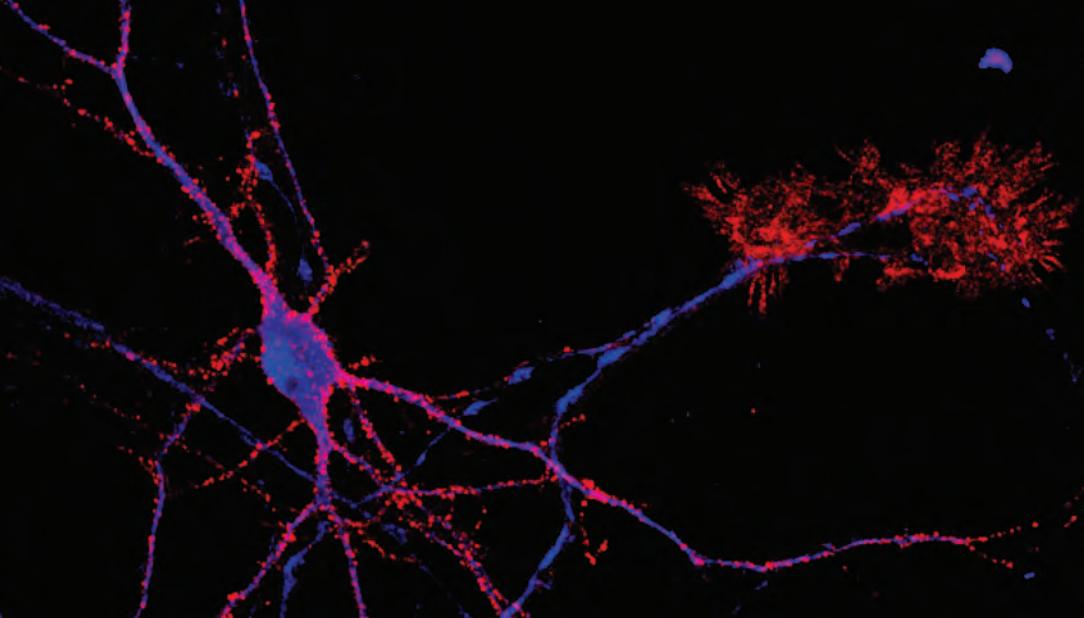
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Most mammalian proteins contain oligosaccharides covalently linked. We are studying the glycosylation of neuronal tissue.



Glycobiology



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Most mammalian proteins contain covalently linked oligosaccharides. The initial step in N-glycosylation occurs in the endoplasmic reticulum and consists of the transfer of a precursor oligosaccharide to the nascent polypeptide chain. Processing this precursor oligosaccharide by several glycosidases and glycosyltransferases in the endoplasmic reticulum and Golgi apparatus results in the final oligosaccharide chain. O-glycosylation occurs in the Golgi and consists of the sequential addition of monosaccharide residues to the protein. Peripheral fucosyltransferases are late acting glycosyltransferases that synthesise the carbohydrate adhesion determinants of the Lewis type.

Protein glycosylation depends on several factors including the three-dimensional structure of the protein and the set of glycosidases and glycosyltransferases of the host cell. Oligosaccharides have been shown to be important for several processes including protein folding, intracellular transport, cell-cell interactions and signalling.

The following carbohydrate structures have been found in neuronal tissue: the fucosylated determinant Lewis X, peripheral alpha2-linked fucose, polysialylation, the HNK-1 epitope, bisecting GlcNAc and O-mannosylation among others. We have previously obtained evidence that supports the importance of the determinant Lewis X in neurite outgrowth. Furthermore, it has been found in synaptic sites of glutamatergic neurons. We are currently studying the Lewis X determinant in neuronal tissue with the aim of elucidating its regulation (e.g., interplay with the cell adhesion molecule L1) and functional role, and identifying possible lectin receptors. On the other hand, we have studied the substrate specificity of fucosyltransferase 9, which is predominantly

expressed in the brain, as well as other fucosyltransferases, and used them to produce fucosylated compounds *in vitro* in order to study their biological effect on our cellular systems.

Another line of research in the laboratory consists of the analysis of glycoproteins from the plasma and cerebrospinal fluid of patients with the neurodegenerative disease Amyotrophic Lateral Sclerosis in order to identify possible biomarkers. Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of the motor neurons. The identification of validated biomarkers for the disease would prove helpful for the diagnosis and the testing of potential therapeutic compounds.

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The Infection Biology Laboratory aims to understand the mechanisms by which bacterial pathogens manipulate animal host cells. This basic knowledge might help develop strategies to combat infectious diseases.

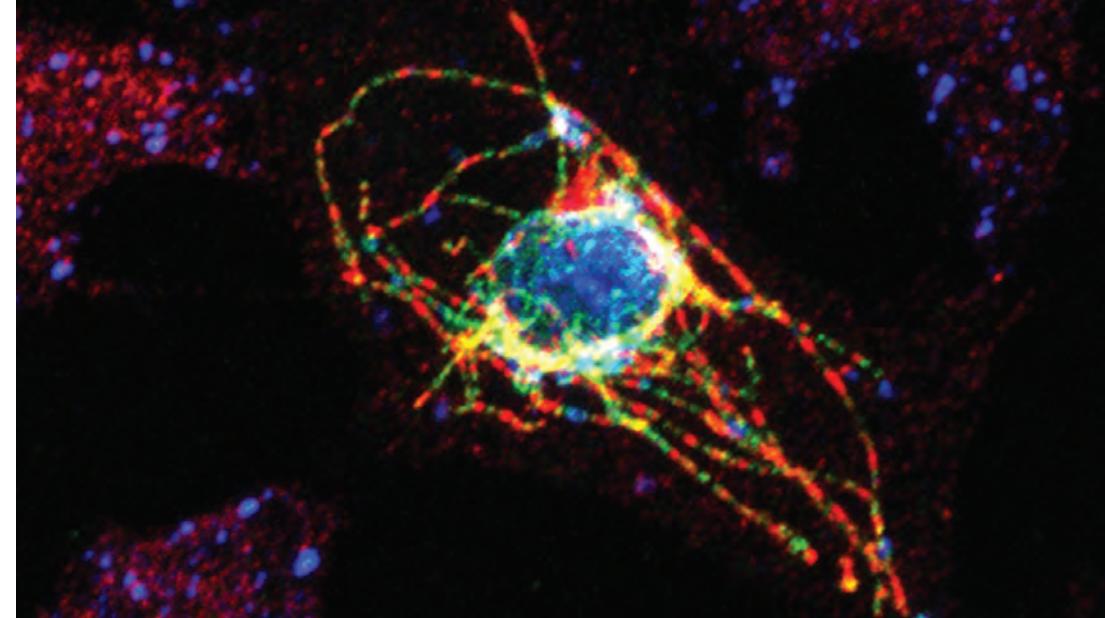
Infection Biology

We focus on *Chlamydia trachomatis* and *Salmonella enterica* as experimental models for studying molecular and cellular mechanisms underlying host-pathogen interactions.

C. trachomatis serovars are human pathogens causing ocular and genital infections. About 80 million people are affected by *Chlamydia* ocular infections and > 90 million new *Chlamydia* genital infections occur each year. Chlamydial infections can lead to chronic conditions such as blindness or infertility. *C. trachomatis* are obligate intracellular bacteria, and there are no established methods to genetically manipulate them.

S. enterica serovars infect a wide range of animals and cause gastrointestinal and systemic diseases in humans. Worldwide, it is estimated that over half a million deaths occur each year due to typhoid fever (caused mainly by serovar Typhi), and over 3 million deaths due to non-typhoidal *Salmonella* infections. *S. enterica* are facultative intracellular bacteria, and serovar Typhimurium is an excellent model for studying host-pathogen interactions. This is due to the availability of sophisticated genetic tools combined with established tissue culture and animal models of infection.

Like several other Gram-negative pathogenic bacteria, *Chlamydia* and *Salmonella* employ a type III secretion system to inject effector proteins into their eukaryotic host cells. The effector proteins modulate several host cell functions to benefit the bacteria. Both *Chlamydia* and *Salmonella* use effector proteins to invade non-phagocytic cells, to replicate intracellularly within membrane-bound vacuoles, and to modulate host immune responses.



We study the function of type III secretion effectors of *Chlamydia* and *Salmonella*. Although some *Salmonella* effectors are well studied, we focus on those which remain to be characterised. Much less is known about the function of Chlamydial effectors. To study these proteins, we are exploring the ability of type III secretion systems to recognise substrates from other bacteria. We are using *Yersinia* as a heterologous system for screening for type III secretion signals within hypothetical proteins of *C. trachomatis*. We will generate antibodies against the candidates found, to then test by immunofluorescence microscopy whether they are secreted in infected cells. We will then choose proteins for further analysis of their molecular functions, for example, to find host cell targets by performing unbiased protein-protein interaction screens.

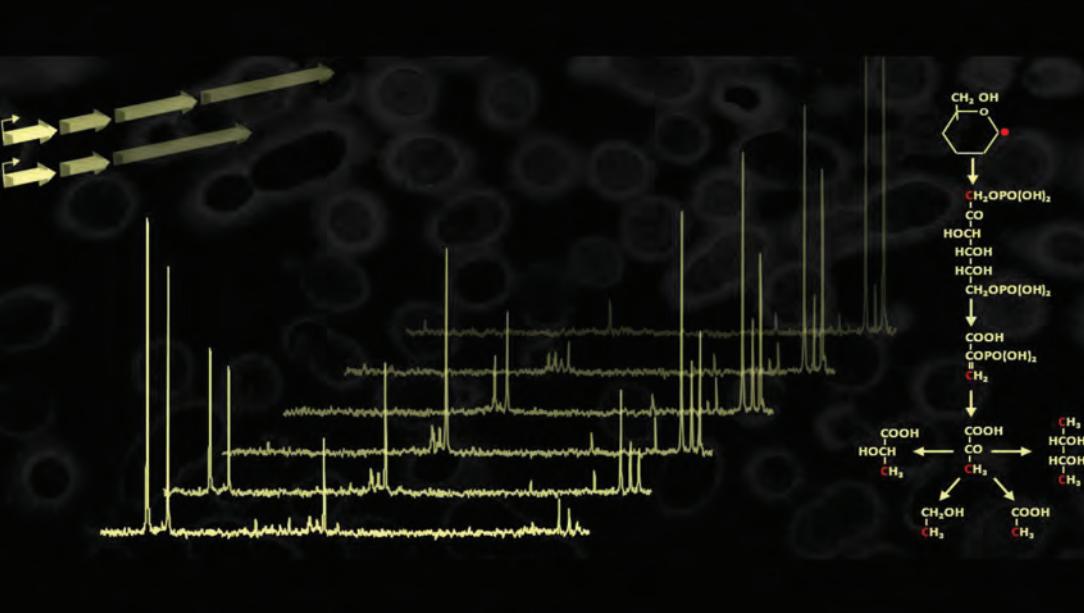
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*The Lactic Acid Bacteria & *in vivo* NMR group studies the regulation of metabolism in bacteria using NMR and molecular techniques. Our research spans mechanisms of virulence in pathogens to metabolic engineering of industrial bacteria.*

Lactic Acid Bacteria & *in vivo* NMR



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Our research interests focus on metabolic and transcriptional regulation in Gram-positive bacteria, in particular, the elucidation of mechanisms involved in sugar uptake and degradation. This subject is especially relevant when studying fermentative organisms, in which sugar metabolism is central to physiology. To expand our understanding of the metabolic and regulatory networks, we use as models the human pathogen *Streptococcus pneumoniae* and the food-grade *Lactococcus lactis*. We are convinced that these data are useful in (i) the identification of virulence and pathogenic factors (*S. pneumoniae*) and (ii) in tailoring microbial metabolism for the production of health-promoting compounds (*L. lactis*), and therefore will generate fundamental and applied knowledge.

In collaboration with H. Santos (Cell Physiology & NMR, ITQB), we have pursued a line of research initiated about a decade ago aimed at characterising central metabolism in the model organism *L. lactis*. Studying the regulation of the glycolytic pathway, we have succeeded in assigning the function of several unknown genes and identified new metabolic pathways. Our *in vivo* NMR time-series data are used worldwide for metabolic modelling and allow us and others to direct metabolic engineering towards the production of health-promoting compounds.

The knowledge of *in vivo* physiology and metabolism of *S. pneumoniae* is limited, even though pneumococcal pathogenesis also relies on the efficient acquisition and assimilation of nutrients. Being a strictly fermentative organism, the ability to take up and metabolise sugars is of chief importance. In the human body *S. pneumoniae* encounters great variation in sugar composition

and availability. Therefore, mechanisms involved in carbon catabolite control are of utmost importance for fitness, survival and virulence in the host. The metabolic pathways and mechanisms involved remain, however, largely elusive. We are addressing questions such as which sugars and pathways are used during colonisation, and whether the involved players are essential for persistence. We want to understand how pneumococcus copes with changes in sugar nature and availability and how these mechanisms relate to virulence and pathogenesis. Presently we are pursuing these goals using molecular biology tools and global approaches such as *in vivo* NMR for metabolite profiling and proteome analysis. In addition, we use transcriptomics and animal models through international collaborations.

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*We are interested in the developmental biology of the bacterium *Bacillus subtilis*. We study cell division and morphogenesis, and the molecular mechanisms that trigger and govern cell differentiation.*



Microbial Development

Bacterial spores can withstand extremes of physical and chemical parameters that would promptly kill other cells. Spores can also remain viable for periods of time in excess of millions of years, and are arguably the most resistant cellular structure found on this planet.

Spore resilience is in part determined by its structural architecture, in which a copy of the bacterial genome is encased in a series of concentric structural layers that act as highly efficient protective shields.

At the onset of spore differentiation, the rod-shaped cell, which normally divides at midcell, undergoes a polar division. This creates a larger cell (the mother cell) and a small cell (the prospective spore or forespore). The mother cell then engulfs the forespore in a phagocytic-like process that converts it into a cell within a cell. The spore protective layers are assembled around the engulfed forespore.

Each of the dissimilar progeny cells formed through asymmetric division receives a copy of the chromosome, and deploys specific programs of gene expression, orchestrated by a cascade of cell type-specific transcriptional regulators. Their activation relies on intercellular signalling pathways that operate following completion of key stages in spore development, ensuring that gene expression in the mother cell and forespore compartments is coordinated, and kept in close register with the course of morphogenesis.

We are interested on the molecular mechanisms by which these signalling pathways enforce proper timing and cell-specificity of developmental gene

expression and ultimately, how proteins are targeted to their correct subcellular addresses and what are their interactions during assembly of the various spore structures.

We are also interested on the switch of the division site to one of the cell poles, and how chromosome segregation is harmonized with asymmetric division. Precise control over the cell's shape is essential for spore development, and we are also interested in the function and dynamics of the cytoskeleton, and its role during cell differentiation.

Spores of *B. subtilis*, a non-pathogenic species, have many applications in biomedicine and biotechnology, which we also explore. On the other hand, in pathogenic species such as *Clostridium difficile*, *B. cereus* or *B. anthracis*, the spore is often the infectious vehicle. Other projects deal with the link between spore formation and pathogenesis in these important human pathogens.

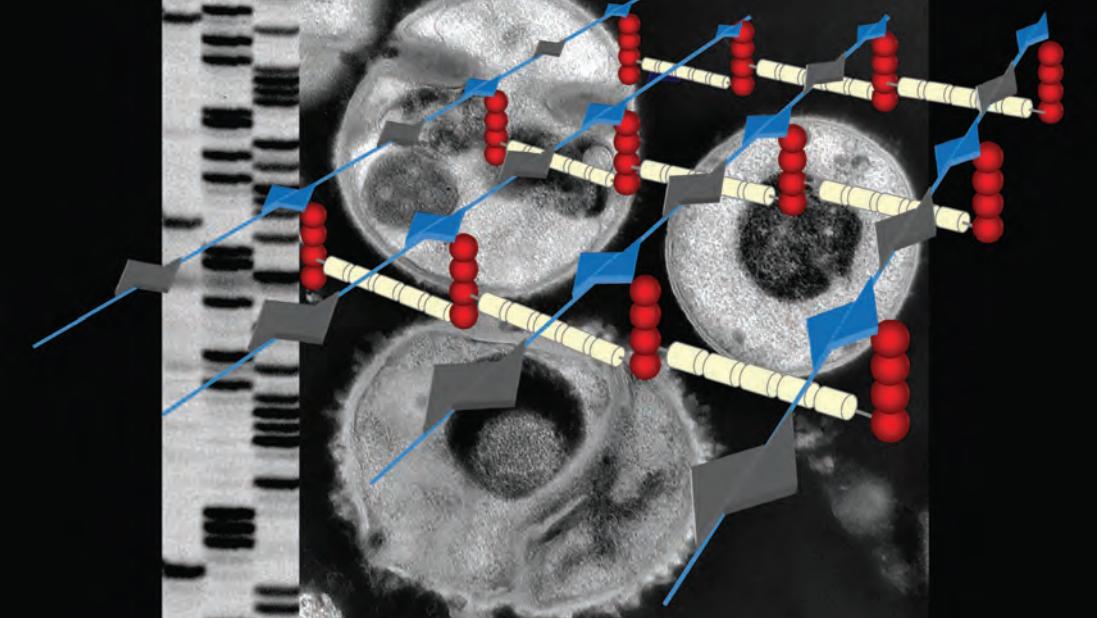


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The long-range interest of this laboratory is in the epidemiology, genetics, evolutionary and biochemical mechanisms of antibiotic resistant pathogens, specifically, *staphylococci*, *Streptococcus pneumoniae*, and *enterococci*.

Molecular Genetics



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The emergence and spread of antibiotic resistant clones of *Staphylococcus* spp., *Streptococcus pneumoniae* and enterococci pose a public health threat worldwide. The phenomenon also presents fascinating issues for basic science, for example: the evolutionary origin of resistance genes; the mechanism of antibiotic resistance; and the question of what combination of determinants provides the epidemic "success" of these pathogens. Our laboratory is actively involved in both the biological and public health related aspects of this problem.

Methicillin-resistant *Staphylococcus aureus* (MRSA) are a major cause of nosocomial infections worldwide and have emerged recently in the healthy community, posing a public health concern. We aim to characterise the molecular epidemiology of MRSA in hospitals and in the community and to track the evolutionary origin and spread of the β -lactam resistance gene. Other staphylococcal species are under study as they are thought to be important reservoirs and key players in the evolution of β -lactam resistance determinants.

Moreover, we investigate the molecular mechanisms leading to β -lactam resistance in MRSA, namely through the study of the biosynthetic steps of peptidoglycan, a major cell wall component and target of these antibiotics. Our approaches include the biochemical characterisation of cell wall mutants, studies of gene expression regulation and protein interactions.

Enterococci have emerged as the leading cause of nosocomial infections in many countries. Portugal has one of the highest European rates of invasive vancomycin-resistant *E. faecium* (>20%). Our research focuses on the population structure, resistance mechanisms and epidemic genetic markers of

isolates circulating in Portuguese hospitals. In addition we are planning to compare Portuguese/European vancomycin-resistant isolates with those of New York/USA.

S. pneumoniae remains a leading cause of morbidity and mortality worldwide causing a wide range of infectious diseases. Its sole ecological niche is the human nasopharynx and children of preschool age their major reservoir. Our laboratory is engaged in extensive studies aimed at better understanding the nasopharyngeal ecosystem and how it is affected by interventions such as antibiotic use and vaccines.

Our research is carried out in collaboration with Portuguese and foreign scientists worldwide through CEM/NET and other initiatives and is supported by the European Community, the Fundação Para a Ciência e Tecnologia and the Fundação Calouste Gulbenkian.

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D&SB research focuses on: plant biodiversity, conservation and screening of bioactive metabolites; biomedical applications of plant secondary metabolites; new and non-toxic strategies for controlling and fighting pathogenic fungi; and DNA damage and repair mechanisms in plants.



Disease and Stress Biology

Our biodiversity and conservation studies centre on *in vitro* vegetative propagation of endemic Portuguese plant species, some of which under risk of extinction. The novel bioactive metabolites (including polyphenols) present in these plant species are being scrutinised and chemically characterised. In biomedical applications, the plant secondary metabolites selected are being evaluated for antioxidant, antiproliferative and/or antimicrobial activities. For that purpose we utilise several models, such as human neuroblastoma, colorectal and cancer cells, yeast, human and also plant fungal pathogens. For example, for neurodegeneration cell models, neuroblastoma cells treated with rotenone and yeasts overexpressing alpha-synuclein are used for selection of natural products potentially useful in the treatment and/or prevention of Parkinson's disease. To access the bioavailability of these natural products, particularly of antioxidant compounds from edible plants, like berry fruits, *in vitro* digestion and human intervention studies are underway.

The search for and development of novel and non-toxic fungicides active against human and plant pathogens allow for the development of new strategies to control and fight pathogenic fungi. To that end human and plant cell interactions with pathogenic fungi are being analysed, in particular, host-induced changes in human fungal pathogen exoglycomes. In plants, molecular interactions of grapevine with pathogenic fungi responsible for grapevine powdery mildew and wood diseases are also being studied. Genetically transformed grapevine, rose and other plants, constitutively expressing antifungal proteins (e.g. blad) are being evaluated, and specific bioelectronic methodologies capable of detecting and treating asymptomatic grapevine plants infected with recalcitrant fungal wood diseases are under development.

Finally, for an additional understanding of fungal interactions, the challenging of *Aspergillus fumigatus* and *A. alternata* by other fungi is being examined.

Taking advantage of novel and promising natural products, in the near future such strategies may provide treatments with low toxicity levels. A number of patent applications have either been granted, are under examination or are under preparation.

For the understanding of genome integrity maintenance in plants the role of chromosome cohesion during DNA repair is under study and meiotic chromosome mis-segregation mutants are being characterised.

Tavares L., Carrilho D., Tyagi M., Barata D., Serra A. T., Duarte C. M. M., Duarte R. O., Feliciano R. P., Bronze M. R., Chicau P., Espírito-Santo M. D., Ferreira R. B. and dos Santos C. N. (2010). "Antioxidant Capacity of Macaronesian Traditional Medicinal Plants." *Molecules* 15(4): 2576-2592.

Monteiro S., Freitas R., Rajasekhar B. T., Teixeira A. R. and Ferreira R. B. (2010). "The Unique Biosynthetic Route from *Lupinus beta*-Conglutin Gene to Blad." *PLoS ONE* 5(1):e8542.

Ferreira R. B., Monteiro S., Freitas R., Santos C. N., Chen Z., Batista L. M., Duarte J., Borges A. and Teixeira A. R. (2007). "The role of plant defence proteins in fungal pathogenesis." *Molecular Plant Pathology* 8(5): 677-700.



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In the Forest Biotech Lab we study aspects of plant development and growth underlying traits of interest in economically important forest tree species. Genomic tools are used to elucidate gene function and regulation.

Forest Biotech



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Plant embryogenesis can be induced *in vitro* from somatic cells in a process called somatic embryogenesis. This process is of great interest worldwide for large scale propagation and rapid production of genetically improved and uniform seedlings. In our lab we have established a somatic embryogenesis system for maritime pine (*Pinus pinaster*) and several lines of research have been developed based on this system. We have been studying how artificial conditions provided during the *in vitro* culture steps, namely the supply of plant growth regulators in the culture medium, may affect somatic embryos and derived plants at the level of DNA sequence and methylation.

Because of the experimental accessibility of somatic embryos, we have been using somatic embryogenesis in combination with plant cell transformation to investigate the role of genes that may regulate unique characteristics of early embryo development in Gymnosperms (in which pine is included). One gene coding for a GTPase of the Rab family plus another one for a lipid transferase, which we identified through the use of transcriptomic approaches along embryo development, are now being functionally characterised by overexpression and down-regulation strategies. Additionally, we are utilising the transformation of embryogenic cell lines as a tool for gene function validation within an integrated project addressing strategies to fight the pine nematode in Portugal.

Another main area of research focuses on aspects of plant development related to secondary growth resulting in the radial expansion of woody stems. Two lateral meristems (regions of indeterminate cell division) are responsible for secondary growth in gymnosperms and dicotyledonous species. The

vascular cambium produces the cells needed for wood formation and the cork cambium or phellogen produces the cells for cork formation. We have several ongoing projects aimed at the identification and characterisation of regulatory networks underlying vascular development and phellogen activity. Transcription factors of the class III HD-Zip (homeodomain-leucine zipper) and GRAS families are being targeted. These studies utilise model species such as poplar and *Arabidopsis* but we are also involved in national and international working groups for developing genomic tools in species such as cork oak and maritime pine.

Marum L., Loureiro J., Rodriguez E., Santos C., Oliveira M. M. and Miguel C. (2009). "Flow cytometric and morphological analyses of *Pinus pinaster* somatic embryogenesis." *Journal of Biotechnology* 143(4): 288-295.

Marum L., Rocheta M., Maroco J., Oliveira M. M. and Miguel C. (2009). "Analysis of genetic stability at SSR loci during somatic embryogenesis in maritime pine (*Pinus pinaster*)."
Plant Cell Reports 28(4): 673-682.

Goncalves S., Cairney J., Rodriguez M. P., Canovas F., Oliveira M. and Miguel C. (2007). "*PpRab1*, a Rab GTPase from maritime pine is differentially expressed during embryogenesis." *Molecular Genetics and Genomics* 278(3): 273-282.

At GPlantS we utilise a number of different genomic approaches to study the effect of environmental factors on the regulation of gene expression and plant development, with special emphasis on salt, drought and temperature stresses.



Genomics of Plant Stress (GPlantS)

Plant growth and yield depend greatly on the environment, which plays a critical role in the expression of plant genetic potential. Environmental (abiotic) stress is a major cause of losses in plant productivity worldwide, with resulting economical instability. Our team is particularly interested in the study of the environmental impact on plant growth regulation and on the adaptation strategies that permit some plants to survive the stress.

We are utilising a genomics approach to explain plant stress responses, from the regulation of chromatin structure and epigenetic modifications, to the study of post-translational protein modifications (e.g. SUMOylation) and functional characterization of new players in the transcriptional network regulated by stress.

Our main focus is rice, due to its worldwide importance as a food crop and because Portugal is the main rice consumer/capita in the EU, producing 60% of its internal needs. Rice production in Portugal is mainly affected by salinity and cold (in the Tejo/Sado and Mondego riverbeds, respectively). In close collaboration with rice associations and the National Rice Breeding Program, we aim to bring to market a Portuguese variety, using old genotypes with good grain quality and improving blast resistance and plant architecture by marker-assisted strategies. We have already identified a number of new genes/alleles and uncovered some of their roles in stress adaptation, thus establishing a link between specific environmental factors and particular developmental patterns. It is the case, for instance, of OsHOS1 gene that, when silenced, impairs root curling in response to mechanical stimulation (in contrast to wild type varieties). We have also found a cross-talk between biotic and abiotic



stress at the level of the transcription factors that regulate OsRMC₁, and a link between cold stress responses and light conditions through a putative phytochrome interacting factor (OsPIF).

We have also shown that both heat shock and salinity trigger the transition from hetero- to euchromatin (also occurring after induced hypomethylation), indicating a rapid ribosomal chromatin plasticity in response to stress challenge. Additionally, while supported by the *Arabidopsis* model in which we study the defense machinery to oxidative stress and express heterologous genes, we also study woody species (Rosaceae fruiting trees and cork oak) and the emerging biodiesel plant *Jatropha curcas*, contributing to the unveiling of the molecular mechanisms underlying their responses to abiotic stress conditions.

Saibo N. J. M., Lourenco T. and Oliveira M. M. (2009). "Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses." *Annals of Botany* 103(4): 609-623.

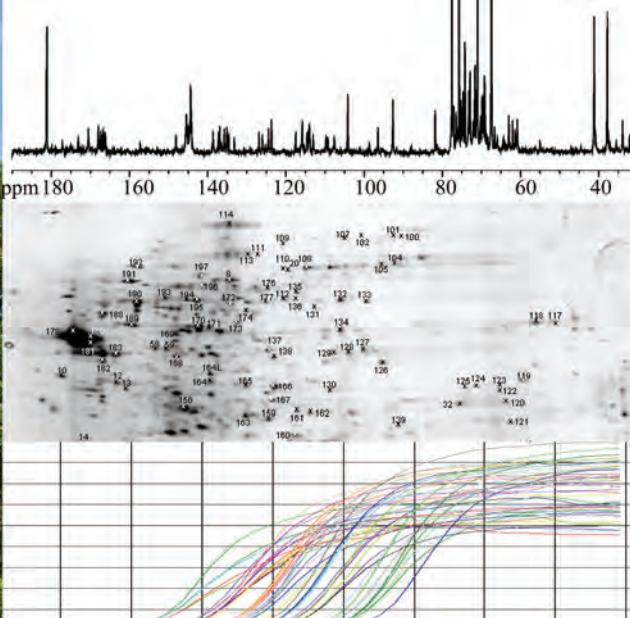
Santos A. M., Oliver M. J., Sanchez A. M., Payton P. R., Gomes J. P., Miguel C. and Oliveira M. M. (2009). "An integrated strategy to identify key genes in almond adventitious shoot regeneration." *Journal of Experimental Botany* 60(14): 4159-4173.

Batista R., Saibo N., Lourenco T. and Oliveira M. M. (2008). "Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion." *Proceedings of the National Academy of Sciences of the United States of America* 105(9): 3640-3645.



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The Plant Biochemistry Laboratory applies proteomics and metabolomics to study plant development and stress response. Cellular processes of model plants and molecular plasticity of plant genetic resources are areas of research.

Plant Biochemistry



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We are mainly interested in researching drought, temperature and mineral stresses, because these abiotic stresses are often interrelated and greatly affect plant productivity and are expected to become more serious still in the near future. We study plant reaction to the stresses through the changes expressed in the protein patterns (obtained after separation by 2-D electrophoresis) and in the metabolite profiles (determined by NMR and HPLC techniques). Proteins and metabolites are screened during extreme conditions, in order to detect and identify those associated with plant tolerance and survival. For instance, in the model plant *Arabidopsis*, responses to severe drought are compared with those of *Thellungiella*, a close relative tolerant to stress. The same type of study is performed with crops, for example the beet plant (*Beta*), for which cultivated forms versus their wild relatives are compared. Beets are important plant systems to study not only drought but also salinity, because beet wild relatives live near the sea and therefore constitute a good system for understanding the mechanisms that allow the plant to tolerate high salt levels. Salinity is a cause of rising concern worldwide, as it is often associated with drought and also results from improper irrigation procedures in agriculture.

Our research on abiotic stress also focuses on legume plants, which comprise important crops, such as lupin (*Lupinus*), faba bean (*Vicia faba*), common bean (*Phaseolus vulgaris*) and chick pea (*Cicer arietinum*), as well as model plants such as *Medicago* and *Lotus*. Our investigation of this group of plants is not only directed at the alterations in the vegetative organs, but also at seeds, due to their high physiological and economic value. Seed proteins, small soluble metabolites, minerals and anti-nutrition and allergenic factors are analysed

during seed development and in relation to the stresses that are applied to the plant. Special attention is being devoted to iron (Fe) deficiency, because legume seeds are important sources of protein and Fe for human nutrition, and knowledge of the mechanisms of Fe storage needs to be improved.

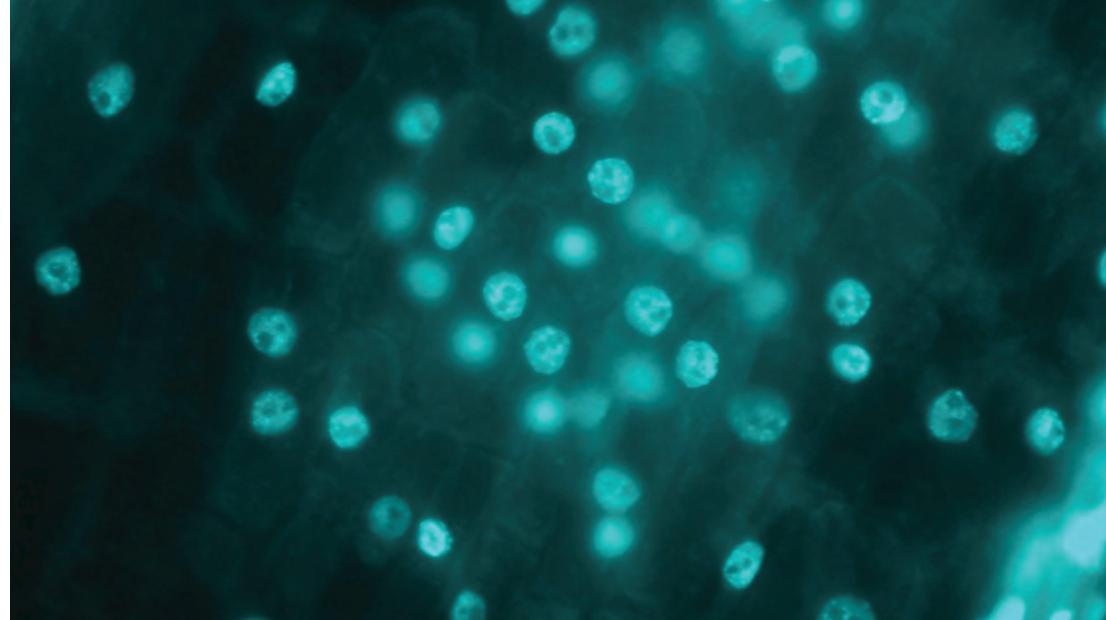
Considering the high commercial value of cork and the scarcity of information about its biosynthesis, we study the biology of this material by identifying proteins implicated in suberisation in the cork oak (*Quercus suber*) stem, as well as in the potato (*Solanum tuberosum*) tuber, used as a model system.

Pinheiro C., de Carvalho M. H. C., Bartels D., Ricardo C. P. and Chaves M. M. (2008). "Dehydrins in *Lupinus albus*: pattern of protein accumulation in response to drought." *Functional Plant Biology* 35(1): 85-91.

Antonio C., Pinheiro C., Chaves M. M., Ricardo C. P., Ortuno M. F. and Thomas-Oates J. (2008). "Analysis of carbohydrates in *Lupinus albus* stems on imposition of water deficit, using porous graphitic carbon liquid chromatography-electrospray ionization mass spectrometry." *Journal of Chromatography A* 1187(1-2): 111-118.

Chaves I., Pinheiro C., Paiva J. A. P., Planchon S., Sergeant K., Renaut J., Graca J. A., Costa G., Coelho A. V. and Ricardo C. P. P. (2009). "Proteomic evaluation of wound-healing processes in potato (*Solanum tuberosum L.*) tuber tissue." *Proteomics* 9(17): 4154-4175.

Our laboratory works on several aspects of the biology of the plant cell, including the functional organisation of the cell nucleus and protein processing within the plant secretory pathway.



Plant Cell Biology

The main objective of our current research is to integrate the fundamental and applied aspects of molecular farming - the large scale production of recombinant proteins in plants - by determining which processes influence recombinant protein expression, accumulation and stability. We are particularly interested in understanding how transgene expression is influenced by higher-order chromatin structure, for example how integration and epigenetic modifications influence the expression and stability of transgenes. We are also studying the accumulation of recombinant products in the plant cell and how it is influenced by protein processing, transport and deposition in various tissues. We are mainly using the model plants *Medicago truncatula* and *Arabidopsis thaliana* (both whole plants and suspension cell cultures), and BY-2 tobacco cells. For our studies we use transgenes encoding pharmaceutical and feed additive proteins, which adds an applied dimension to this research and enhances the importance of the fundamental cell biology questions we are addressing.

We are also investigating some fundamental processes involved in the organisation and function of the cell nucleus such as Chromatin Dynamics. Our studies focus on the organisation of genomes from different plant species including *Medicago truncatula*. We are also analysing chromatin dynamics and cellular differentiation in *Arabidopsis*.

Gonzalez-Melendi P., Pires A. S. and Abranches R. (2009). "Cell-line-dependent sorting of recombinant phytase in cell cultures of *Medicago truncatula*." *Functional Plant Biology* 36(5): 431-441.

Pires A. S., Cabral M. G., Fevereiro M. P. S., Stoger E. and Abranches R. (2008). "High levels of stable phytase accumulate in the culture medium of transgenic *Medicago truncatula* cell suspension cultures." *Biotechnology Journal* 3(7): 916-923.

Abranches R., Arcalis E., Marcel S., Altmann F., Ribeiro-Pedro M., Rodriguez J. and Stoger E. (2008). "Functional specialization of *Medicago truncatula* leaves and seeds does not affect the subcellular localization of a recombinant protein." *Planta* 227(3): 649-658.



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We aim to develop molecular strategies to support plant selection and breeding programs, to apply biotechnology to the development of business strategies and to train researchers in plant biotechnology and plant molecular biology.

Plant Cell Biotechnology



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Humanity depends on primary production for its survival. To guarantee the production and productivity of food and renewable materials we need varieties of crops, commodities and forest trees adjusted to the ever changing edafo-climatic conditions. Traditionally, this aim has been pursued by phenotyping and selecting in the fields and by mixing observable characteristics through controlled breeding.

Our laboratory uses and develops molecular tools to speedily identify desired genotypes in selection or breeding programs and to alter specific genotypes to cope with specific challenges: disease or environmental stress. To select a new trait, or to identify a modification, we must understand the characteristics of the varieties from which we start. To understand how different crops respond to specific environmental conditions, we characterise the physiological and molecular responses of the varieties under study. We also look at how the allelic variants of genes influencing a specific characteristic are spread through populations.

We utilise genomic – DNA arrays, qRT-PCR, genotyping (SNPs and SSRs) and southern and northern blots - and proteomic – 2D electrophoresis and mass spectrometry - tools to identify genes and alleles involved in plant adaptation and to understand the regulations of their expression either at the transcriptional or at the post-transcriptional level.

We employ *in vitro* culture and DNA recombinant technologies - cloning, sequencing and genetic transformation - to modify the expression of genes or to introduce new genes in specific crops.

We use quantitative genetic tools – QTLs - to understand the influence of genes in the expression of complex quantitative traits.

We are specially interested in legumes and their adaptation to environmental conditions. Taking advantage of *Medicago truncatula* as a model plant, we apply our knowledge to the study of traditional grain legumes like lathyrus and beans. Portuguese maize varieties utilised in the production of maize bread (brôa) is another concern. We are developing molecular markers to carry out association studies related to wood and cork qualities in forest species (eucalyptus, maritime pine and cork tree) as well as anthocyanin accumulation in grapevines.

Finally, mainly through the cooperation with CiB (Center for Biotechnology Information), we develop science communication strategies to explain biotechnology to the public.

Trindade I., Capitao C., Dalmay T., Fevereiro M. P. and dos Santos D. M. (2010). "miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*." *Planta* 231(3): 705-716.

Confalonieri M., Cammareri M., Biazzi E., Pecchia P., Fevereiro M. P. S., Balestrazzi A., Tava A. and Conicella C. (2009). "Enhanced triterpene saponin biosynthesis and root nodulation in transgenic barrel medic (*Medicago truncatula* Gaertn.) expressing a novel beta-amyrin synthase (*AsOXA1*) gene." *Plant Biotechnology Journal* 7(2): 172-182.

Patto M. C. V. and Rubiales D. (2009). "Identification and characterization of partial resistance to rust in a germplasm collection of *Lathyrus sativus* L." *Plant Breeding* 128(5): 495-500.

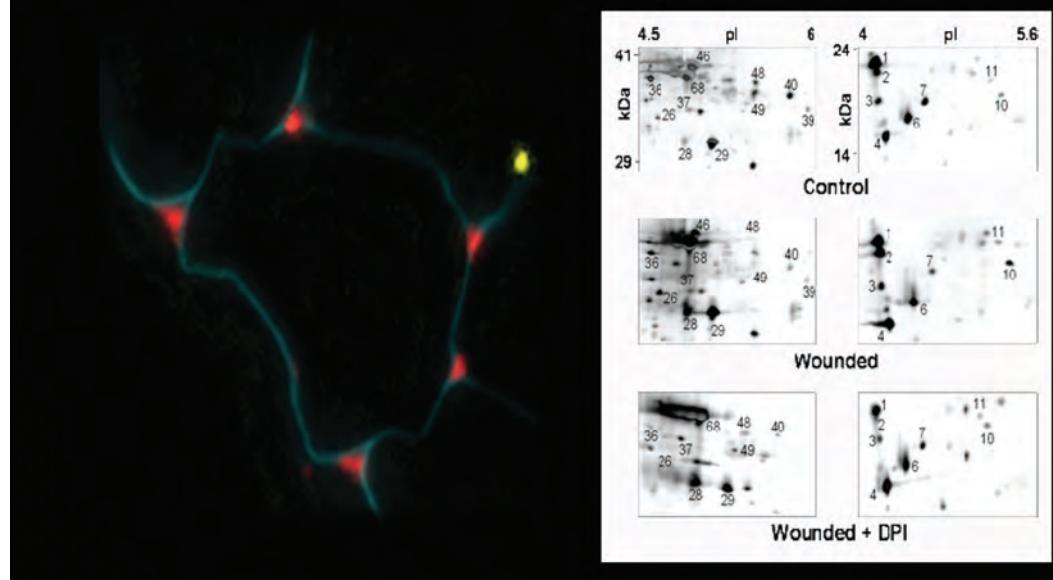
We study extracellular plant matrices using a range of biophysical, biochemical and molecular techniques. Current topics include how adaptive changes in extracellular proteomes are regulated by reactive oxygen signal events and the impact of structural proteins on cell wall development and modification.

Plant Cell Wall

The plant cell wall is a natural and developmentally dynamic nanostructure consisting of cellulose, cross-linking glycans, pectins and structural proteins. The variable composition of the wall matrix in a plant species is determined within a complex interaction of developmental or stress cues with wall precursor biosynthesis and cell wall modification in situ, and reflects local structural, chemical and mechanical requirements. We have investigated how the structural protein, extensin, and its cross-linking into a three-dimensional network, can alter cell wall properties.

Recently, we demonstrated that extensin cross-linking by endogenous extensin peroxidases causes primary cell wall dehydration, leading to the formation of a thinner and denser matrix, with significant consequences to wall polymer spacing and thermomobility. The results suggest that extensin network formation can effect localised changes in primary wall rigidity during the formation of cell-cell contacts and the regulation of cell elongation.

The plant cell wall and apoplast-cell membrane interface also hosts many proteins with functions ranging from matrix modification to signal transduction from environmental cues for the mobilization of adaptive responses. We have characterized the early changes in the cell wall proteome in wounded Medicago. A robust data set of 90 apoplastic proteins was identified, a third of which was shown to be wound-responsive. By using pharmacological inhibitors of ROS production, we were able to identify those wound-responsive proteins mobilised by ROS signals originating within the first 3 min after wounding. The results obtained with this reproducible system form the basis of on-going work to identify early-activated components of



ROS signalling pathways in stressed *Medicago*.

A new area of research concerns the comparative exploration of Ascomycota secretomes for enzyme mixtures appropriate for the biodegradation of lignocellulosic substrates, including those destined for the production of bioethanol. In conjunction with the laboratory of Physiology of Environmentally Conditioned Microbiota, we have recently used both Gel-based MALDI-TOF/TOF and 1D LC-MS to explore the secretome of *Neurospora sitophila*. The data has revealed a surprising complexity in secreted proteins, ranging from abundant endo and exocelluloses, to minor constituents consisting of other glycolytic hydrolases and enzymes active against aromatic wall compounds.

Soares N. C., Francisco R., Vielba J. M., Ricardo C. P. and Jackson P. A. (2009). "Associating Wound-Related Changes in the Apoplast Proteome of *Medicago* with Early Steps in the RIDS Signal-Transduction Pathway." *Journal of Proteome Research* 8(5): 2298-2309.

Soares N. C., Francisco R., Ricardo C. P. and Jackson P. A. (2007). "Proteomics of ionically bound and soluble extracellular proteins in *Medicago truncatula* leaves." *Proteomics* 7(12): 2070-2082.

Ribeiro J. M., Pereira C. S., Soares N. C., Vieira A. M., Feijo J. A. and Jackson P. A. (2006). "The contribution of extensin network formation to rapid, hydrogen peroxide-mediated increases in grapevine callus wall resistance to fungal lytic enzymes." *Journal of Experimental Botany* 57(9): 2025-2035.



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This group seeks to uncover mechanisms of development and evolution of plant morphological traits using a combination of genetics and cell and molecular biology in model plants.

Plant Developmental Genetics



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Research focuses on plant development and evolution using *Antirrhinum majus* and its close relatives as models. A particular theme that can be advantageously addressed using *Antirrhinum* is floral symmetry. Most plants species belong in one of two categories according to the symmetry of their flowers: actinomorphic or zygomorphic. Actinomorphic, or radially symmetrical flowers, have two or more planes of mirror symmetry whereas zygomorphic, or bilaterally symmetrical flowers, have merely one. Bilateral symmetry can usefully be seen as a form of asymmetry relative to a dorsoventral axis of floral development. Hence, zygomorphic flowers are also known as dorsoventrally asymmetrical.

The dorsoventrally asymmetrical condition is thought to have arisen from radial symmetry several times during evolution. To understand how such transitions may have occurred, genetic interactions underlying dorsoventral asymmetry of flowers are being dissected in *Antirrhinum majus*, a species with bilaterally symmetrical flowers. Three genes acting in dorsal regions of the flower, CYC, DICH and RAD, determine its overall asymmetry. Mutant plants in which both CYC and DICH or RAD are inactive have completely ventralised radially symmetrical flowers. One role of the dorsal genes is to inhibit a determinant of ventral identity, DIV. In contrast to cyc, dich or rad, mutant plants for null div have flowers in which ventral regions have lateral identity. A particular aim of the research is to analyse these gene interactions at a detailed molecular level (collaboration with the group of Manuela Costa at the Universidade do Minho).

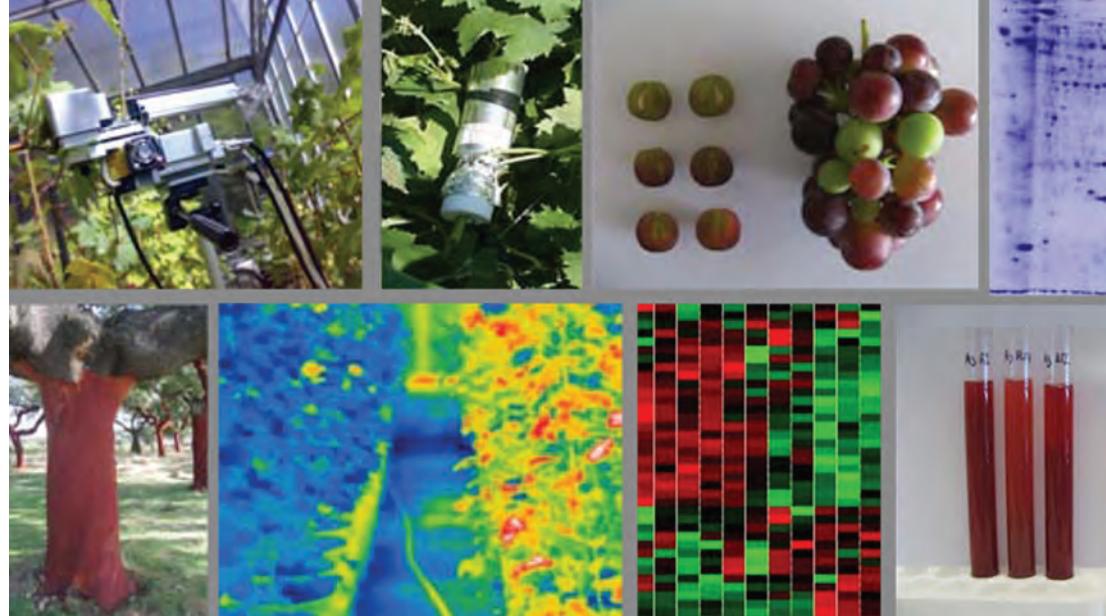
Our group is also interested in understanding how such genetic interactions might have evolved through comparative studies of flower development in *Antirrhinum* and its close relative *Linaria*. The latter flower's blooms have a spur at the base of the ventral petal. Otherwise their corolla dorsoventral patterns are identical. With the aim of investigating mechanisms underlying these similarities and differences, steps are being undertaken to develop *Linaria maroccanna* as a model for genetic and molecular analysis. This has involved isolating an active copy of a transposable element that might be of use as a tool for mutagenesis and functional gene analysis. A major target for such analysis is DIV, the determinant of ventral petal identity.

Almeida J., Rocheta M. and Galego L. (1997). "Genetic control of flower shape in *Antirrhinum majus*." *Development* 124(7): 1387-1392.

Galego L. and Almeida J. (2002). "Role of DIVARICATA in the control of dorsoventral asymmetry in *Antirrhinum* flowers." *Genes & Development* 16(7): 880-891.

Galego L. and Almeida J. (2007). "Molecular genetic basis of flower colour variegation in *Linaria*." *Genetical Research* 89(3): 129-134.

Our goal is to better understand the physiological and molecular mechanisms underlying plant responses to environmental stresses as well as the differences among genotypes regarding their capacity to utilise external resources.



Plant Molecular Ecophysiology

The growth and development of plants is largely dependent upon external conditions. Plants generally fail to achieve their optimal growth rates due to limitations by one or more essential factors, e.g., light, temperature, available water, etc.

This research group studies the interaction between plants and the environment. The analysis of plant responses to stressful conditions from the molecular level up to the intact plant contributes to a fully integrated understanding of plant-environment interaction. We have expertise in the study of the mechanisms contributing to the determination of photosynthesis, transpiration, plant growth, yield and the quality of fruit when subjected to various abiotic stresses. More efficient and sustainable utilisation of resources such as water by plants has become a research priority in order to cope with exploding demographics and climate change. This necessitates further knowledge of plant adaptability on marginal lands and in sub-optimal environments. At present, most of our research concentrates on grapevines but we also have projects on chickpea, *Lupinus spp*, and *Quercus spp*.

We have demonstrated that large fluxes of water are not essential for optimal grapevine performance. Moderate water deficits, induced by irrigation below evapotranspiration (deficit irrigation), may possibly control sink-source relationships without negatively affecting berry quality. The analysis of mild water deficits on grape berry quality (e.g. flavonoid compounds like anthocyanins and other non-flavonoid polyphenols like stilbenes) is under investigation. Furthermore, gene expression analysis at proteomic and transcriptomic

levels has been performed under mild drought stress.

In a more targeted approach, genes related to grape berry quality traits have been cloned.

In the case of Mediterranean-type forest ecosystems, we are particularly interested in assessing how external drivers (such as season, temperature, soil water content) affect nitrogen and carbon cycles.

Chaves M. M., Flexas J. and Pinheiro C. (2009). "Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell." *Annals of Botany* 103(4): 551-560.

Costa e Silva F., Shvaleva A., Broetto F., Ortuno M. F., Rodrigues M. L., Almeida M. H., Chaves M. M. and Pereira J. S. (2009). "Acclimation to short-term low temperatures in two *Eucalyptus globulus* clones with contrasting drought resistance." *Tree Physiology* 29(1): 77-86.

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This group utilises chromatographic methods to analyse volatile and non-volatile components of food and plant extracts providing important data for food and pharmaceutical industries.

Analytical Chemistry

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Food product components may play an important role in human health even at low concentrations; it is known, for example, that phenolic compounds help to prevent some diseases and also slow degenerative processes. Phenolic compounds are present in a great variety of food such as fruits and related products (e.g. fruit juices, wines, olive oil, vinegars) but other sources such as industrial wastes and plants have been studied in order to develop dietary supplements, nutraceuticals. The optimisation of selective and sensitive methodologies of chemical analysis in order to identify and quantify these components is crucial.

To be active these compounds, or their metabolites, must reach their targets after ingestion. Great progress in understanding the mechanisms of human absorption, distribution, metabolism and excretion of phenolic compounds has been made but many questions remain.

With a few exceptions, food products are complex chemical systems with a large number of different components occurring with large variations in concentration. The chemical analysis of foods thus requires high performance separation methods and sensitive detection techniques. Methods validation and careful quality control of data are also required while particular attention must be paid to methodologies of sample preparation since phenolic compounds and their metabolites in biological samples (e.g. plasma, urine) are present in the lowest concentrations.

The importance of phenolic compounds is not restricted to health issues. Anthocyanins, for example, are responsible for some organoleptic properties such as colour, taste and flavour, and may influence consumers' preferences.

The characteristic and pleasant aromas of some foods are due to the so called "key aroma compounds" (e.g. terpene and norisoprenoids).

We have implemented different methodologies of sample preparation in order to identify and quantify the volatile compounds (gas phase chromatography-mass spectroscopy) and to detect the compounds that are responsible for characteristic flavours (gas chromatography-mass spectrometry-olfactometry).

We mostly study the volatile and non-volatile compounds of food products to characterise raw materials with several goals such as, determining changes in the chemical composition during processing and storage, identifying compounds which determine either foods' biological properties or their organoleptic characteristics. Bioavailability studies are performed in collaboration.

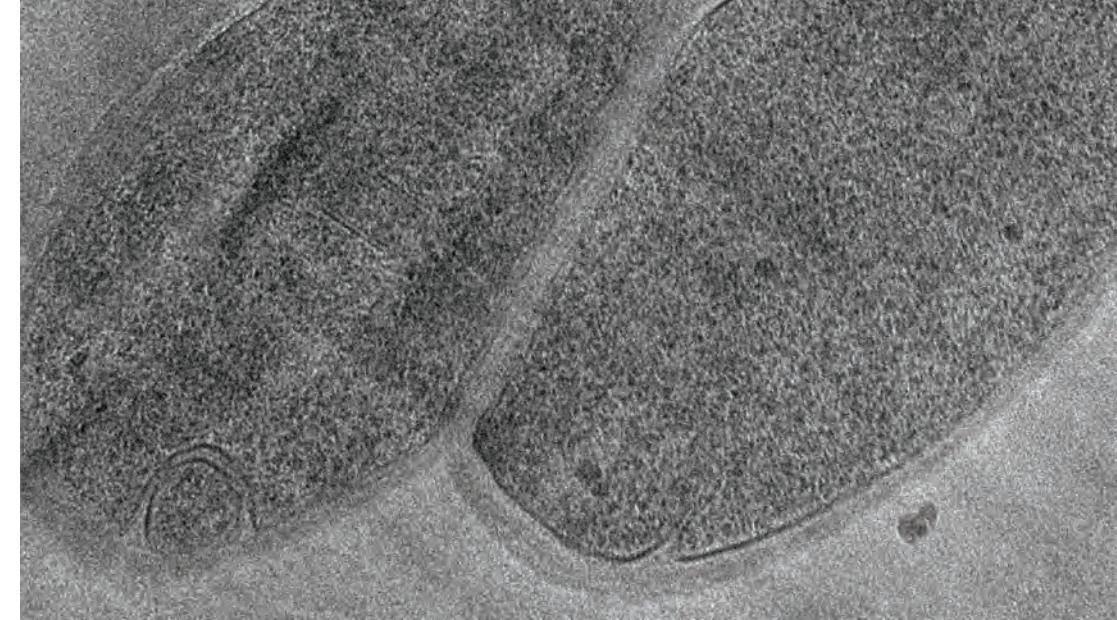
Plant extracts are also studied with emphasis on the characterisation of the phenolic fraction and for the identification of trends in phenolic composition.

Feliciano R. P., Antunes C., Ramos A., Serra A. T., Figueira M. E., Duarte C. M. M., Carvalho A. d. and Bronze M. R. (2010). "Characterization of traditional and exotic apple varieties from Portugal. Part 1 - Nutritional, phytochemical and sensory evaluation." *Journal of Functional Foods* 2(1): 35-45.

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Bravo M. N., Feliciano R., Silva S., Coelho A. V., Boas L. V. and Bronze M. R. (2008). "Analysis of trans-resveratrol: Comparison of methods and contents in Muscatel fortified wines from Setubal region in Portugal." *Journal of Food Composition and Analysis* 21(8): 634-643.

The SAVE Lab works on antibiotic resistance and virulence mechanisms of Enterococcus. Areas include studies of the role of particular genes both on the bacteria themselves and on the relationship with the infected host.



Stress by Antibiotics and Virulence of Enterococci

Enterococcus are human commensal bacteria which have emerged in parallel with the use of antibiotics, as major nosocomial opportunistic pathogens. Understanding their virulence is as essential as uncovering the mechanisms behind their ability to survive antibiotics and other stresses, if we want to find new ways to fight infections caused by multi-resistant strains of enterococci.

Antibiotics are molecules used by microorganisms to communicate. Thus, it is essential to understand the response of the bacterial cell to antibiotic concentrations below their bactericidal/bacteriostatic activity. We have found that vancomycin, a cell-wall active antibiotic, induces changes in the expression of genes with functions ranging from stress response, cell-wall integrity, cell-division, regulation, transport to those no function has been attributed (the hypothetical proteins). Further analysis is carried out on each category of gene involved in the response to vancomycin, which could be also involved in responses to other antibiotics with similar behaviour, i.e. cell-wall active and not entering the cell. A similar transcriptomic approach is being used to study other stresses namely metal ions (often involved in the regulation of virulent behaviours in other bacteria), molecules involved in cell-cell communication (by quorum-sensing mechanisms and others) and biocides. We are trying to identify proteins involved in the response and resistance to biocides and understand the potential for cross-resistance between biocide use and antibiotic resistance emergence.

Analysis of the results obtained from those studies has provided us with possible targets for further studies regarding their contribution to the virulence of enterococci. Often these studies involve mutant construction and testing in

virulence models. In collaboration with groups from other institutions we are using insect models, mice and animal cell cultures.

A new area of investigation deals with hypothetical proteins. Conserved among organisms or specific to enterococci, they constitute nearly 30% of all sequenced genomes so far. We want to ascertain the role of some of these genes by resorting to protein structure (in collaboration with the Membrane Chrystallography Lab) and other molecular biology and biochemical approaches.

In parallel, we continue studying environmental enterococcal isolates. These investigations concern the dissemination of strain types and genes involved in antibiotic resistance and virulence.

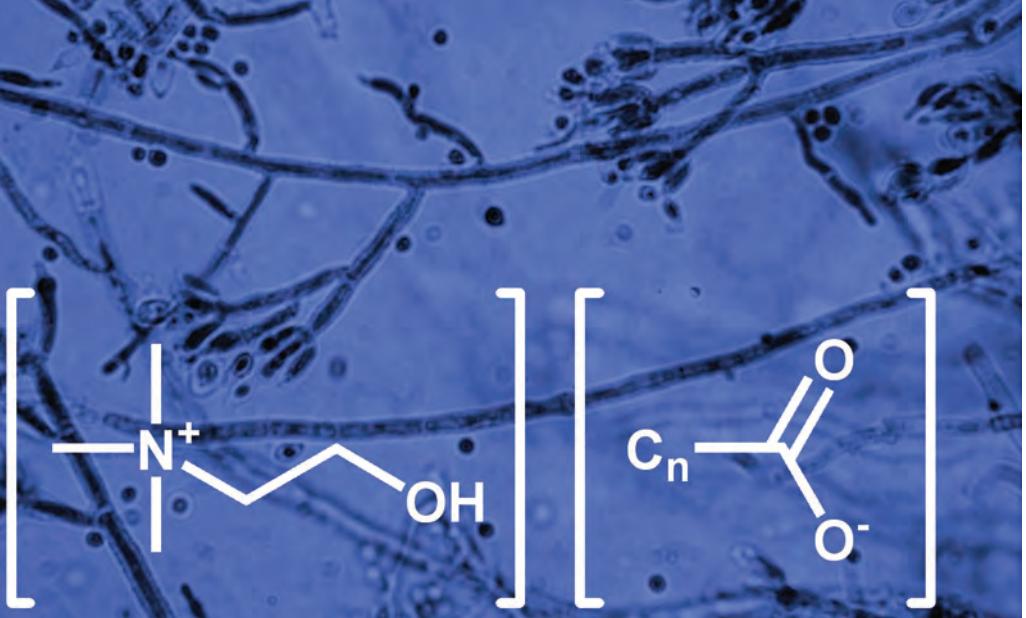
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This group aims to expand the biotechnological potential of filamentous fungi. Research ranges from fundamental studies of fungal biology to applications in bioremediation and biocatalysis, while also highlighting the potential interest of ionic liquids.

Applied and Environmental Mycology



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Fungi are ubiquitous in all ecosystems and vital for their function. There are more than 70,000 species of fungi described and more than 1.5 million estimated to exist. They segregate a broad range of hydrolytic enzymes that can break down complex biopolymers and produce chemically and structurally complex compounds of high industrial interest.

The Applied and Environmental Mycology group aims to expand the biotechnological potential of filamentous fungi. Our research plan was set in order to tackle real-world global challenges. Current advances in fungal biology, namely the increasing number of available sequenced organisms, favour the use of functional genomics to study the response of fungi to natural and anthropogenic stresses. A better understanding of fungal biology may lead to the identification of novel species, functions and biomolecules for a variety of biotechnological applications, especially within biodegradation and bioremediation. We have recently demonstrated e.g. that a broad range of common Ascomycota fungi are able to degrade pentachlorophenol, a persistent organic pollutant which is globally recognised as a major environmental concern. Efforts to describe the degradation pathways are underway in order to define fungi's potential for bioremediation as well as the environmental fate of the toxin and its inherent risks.

Ionic liquids, i.e. molten salts, are classified as "green" alternative solvents, offering unexpected opportunities at the interface with the life sciences. However, in order to move these solvents beyond their current status as an academic curiosity, their environmental, health, and safety impacts must be further investigated. Our team is addressing this multidisciplinary subject,

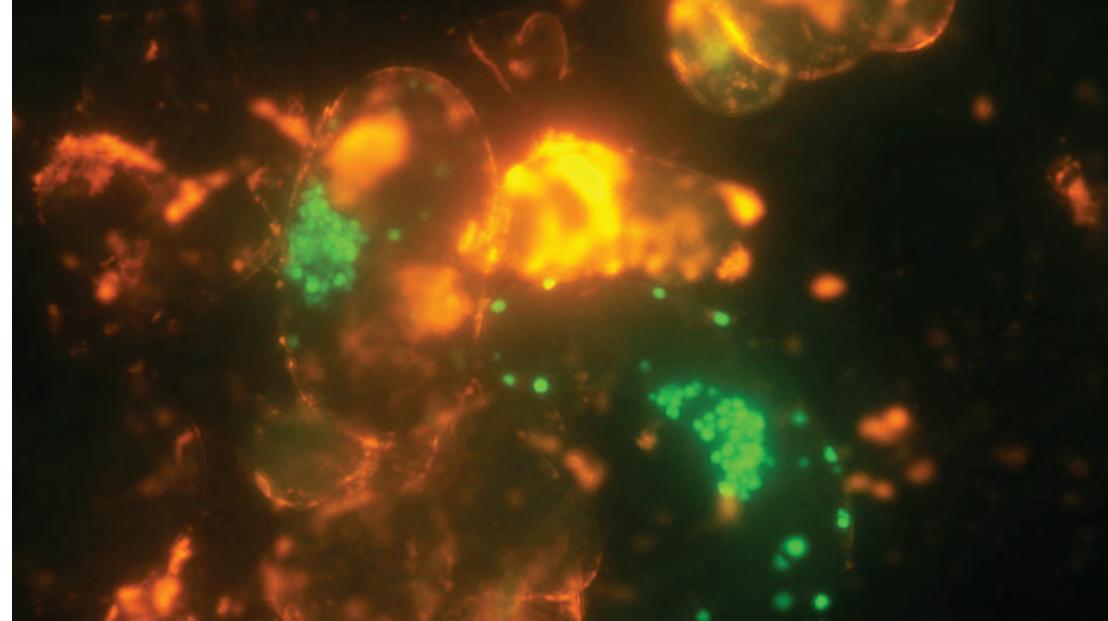
which links microbiology to Green Chemistry, aiming to understand ionic liquids' impact on fungal metabolism, highlighting their biotechnological potential. Initial observations have demonstrated that Ascomycota fungi show much higher tolerance to ionic liquids than any other microorganism so far studied. Furthermore, guided by the paradigm that the choice of an ionic liquid as catalyst can alter the outcome of a given chemical reaction, we have studied their ability to alter the metabolic profile in fungi. Surprisingly, fungal cultures respond to specific ionic liquids by changing their cell biochemistry, resulting in an altered pattern of secondary metabolites. We are currently investigating the potential use of novel biocompatible ionic liquids in processes aimed at biopolymer extraction and dissolution and fungal biodegradation.

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This multidisciplinary research team is committed to developing novel biomolecular tools such as nanoparticles ($\text{CdSe}@\text{ZnS}$ quantum dots) and biosensors for practical applications like disease diagnosis and bioprocess monitoring.



Biomolecular Diagnostics

Ours is a multidisciplinary research group specialised in the development of biosensing tools for molecular detection and quantification, as in applications for veterinary diagnosis and/or plant studies. One of our main concerns is the development of nanoparticles ($\text{CdSe}@\text{ZnS}$ quantum dots), including their chemical functionalisation and bioconjugation with chosen molecules. The photoluminescence properties of the nanoparticles are strongly size dependent. These highly stable fluorescent nanoparticles can be tuned for specific fluorescence emission during synthesis. Shaping the size and external molecule envelope of the nanoparticles can define specific properties. This allows for the use of varying quantum dots simultaneously in any assay, an advantage as compared to standard fluorophores in cellular studies, microscopic imaging and clinical diagnostics.

We use quantum dots for the identification of morphological and structural cell changes, e.g. in studies of *Babesia spp* infection in bovine erythrocytes or in studies of *Fusarium spp* invasion in plant cultures, providing an unexplored perspective of the phenomena, with capabilities superior to those of standard techniques. Upon binding with biotarget molecules, the event can be detected from individual nanoparticles or nanoparticle clusters by monitoring nanoparticle property change response.

We have also been involved in the development of devices and specific instruments for biosensing applications. At this moment we are working on the development of a hybrid biosensor (optical and electrochemical) for cell diagnostic and monitoring. Following our previous development of an impedance spectroscopy microfluidic sensor for the evaluation of infected cells, a

system based on a microfluidic chip, with electrode arrays surrounding the channels deployed onto a waveguide, is under investigation. This hybrid sensing structure will be adopted for the analysis of complex biological samples (e.g. whole blood, fermentation media or *in vitro* cell cultures), in order to obtain a wealth of information from the studied cells as they pass through the microchannel.

These techniques are currently used in our laboratory for e.g. the study of a veterinary parasitic disease, *Babesia spp*, which is transmitted by ticks and infects erythrocytes. Despite this parasite's wide distribution throughout subtropical regions, it has yet to be adequately studied.

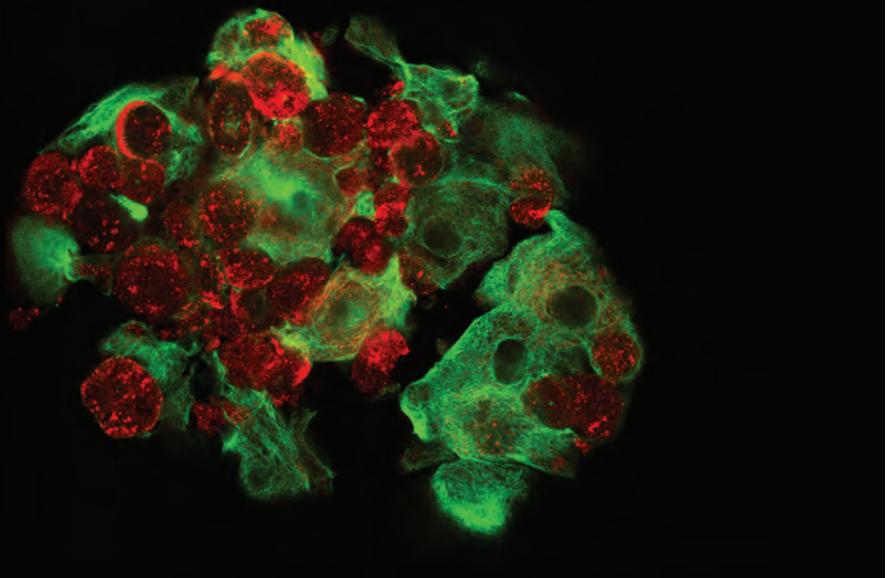
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Kuttel C., Nascimento E., Demierre N., Silva T., Braschler T., Renaud P. and Oliva A. G. (2007). "Label-free detection of *Babesia bovis* infected red blood cells using impedance spectroscopy on a microfabricated flow cytometer." *Acta Tropica* 102(1): 63-68.



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As befits a technological area, the Cell Bioprocess Laboratory is integrated into the Animal Cell Technology Unit where a number of competences have to be appropriately balanced: (i) the more analytical approaches of molecular biology, biochemistry and the physiology of cells, viruses or tissues; and (ii) the more synthetic physico-mathematical tools required for process integration and optimisation. Both approaches provide keys for understanding complex phenomena like viral infection kinetics and multi-protein particle assembly, disassembly and reassembly phenomena.

The Cell Bioprocesses Laboratory carries out research and development on complex biopharmaceuticals namely for the production, purification and storage of vaccines (e.g. virus like particles-VLP's and marker vaccines), recombinant proteins (e.g. MAbs) and viral vectors (adenovirus, retrovirus, lentivirus and baculovirus). In particular we seek to understand cell physiology and metabolism to improve the efficiency of bioprocesses.

We are also interested in developing methodologies for pre-clinical research and cell therapy applications, namely in 3-D cell models, bioreactor technology and novel cell cryopreservation strategies. These approaches are used for primary cultures of brain cells (rat and mice), hepatocytes (rat and human) and more recently, stem cells (human and rat, adult and embryonic). A research niche headed by Dr Helena L.A. Vieira, a senior post-doc in the lab, focuses on applying these cell models in a more fundamental perspective to study apoptosis and preconditioning events, more specifically the effect of CO in neuroprotection. All projects are performed in collaboration either with internal or international research laboratories and companies.

Our research centres on the development of bioprocesses for complex biopharmaceuticals, namely vaccines, recombinant proteins and viral vectors for gene therapy. Current efforts also include the development of tools and methodologies for cell therapy applications and pre-clinical research.

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Our primary research activity centres on the development and improvement of animal cell lines for the manufacturing of complex biopharmaceuticals, as recombinant proteins and recombinant virus for vaccines and gene therapy.

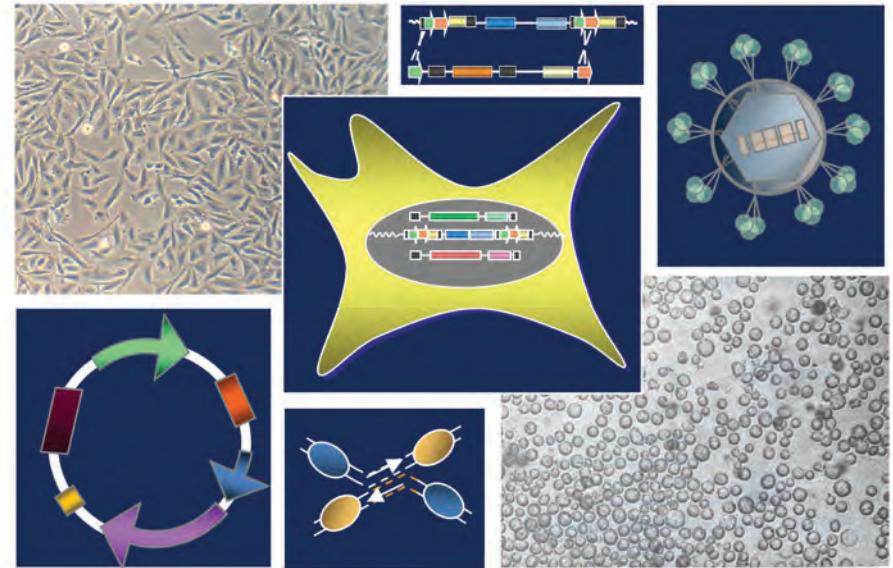
Animal Cell Technology Unit Cell Line Development and Molecular Biotechnology

The Cell Line Development and Molecular Biotechnology Laboratory is integrated into the Animal Cell Technology Unit (<http://tca.itqb.unl.pt>) where a number of knowledge competences have to be appropriately balanced.

The principal research interests of our laboratory focus on establishing and improving animal cell lines to be used in the manufacture of complex biopharmaceuticals, as recombinant virus to be used in vaccination or gene therapies.

The work involves the design of expression genetic cassettes, requiring the use of molecular biology tools, and the establishment of new cell lines by clone selection and screening, requiring, as well, the use of cell culture, biochemistry and physiology among other techniques to characterise and study cells. Additionally, we are interested in the genetic enhancement of the cells per se, in order to improve both product efficacy and cell robustness (improved growth, survivability, metabolism, productivity, etc.).

In this context the laboratory is involved in several projects, such as: i) the development of recombinase cassette exchange systems for faster establishment of high producing cell lines; ii) the establishment of robust mammalian cell lines and improving retroviral vectors to be used in gene therapy; iii) the development of alternative cell lines for the manufacture of non-human adenovirus for gene therapy; and iv) the improvement of human cell lines for vaccine production by silencing immunogenic host cell proteins that are incorporated in the viral particles reducing their efficacy. All projects are carried out in collaboration either with internal or foreign research laboratories.

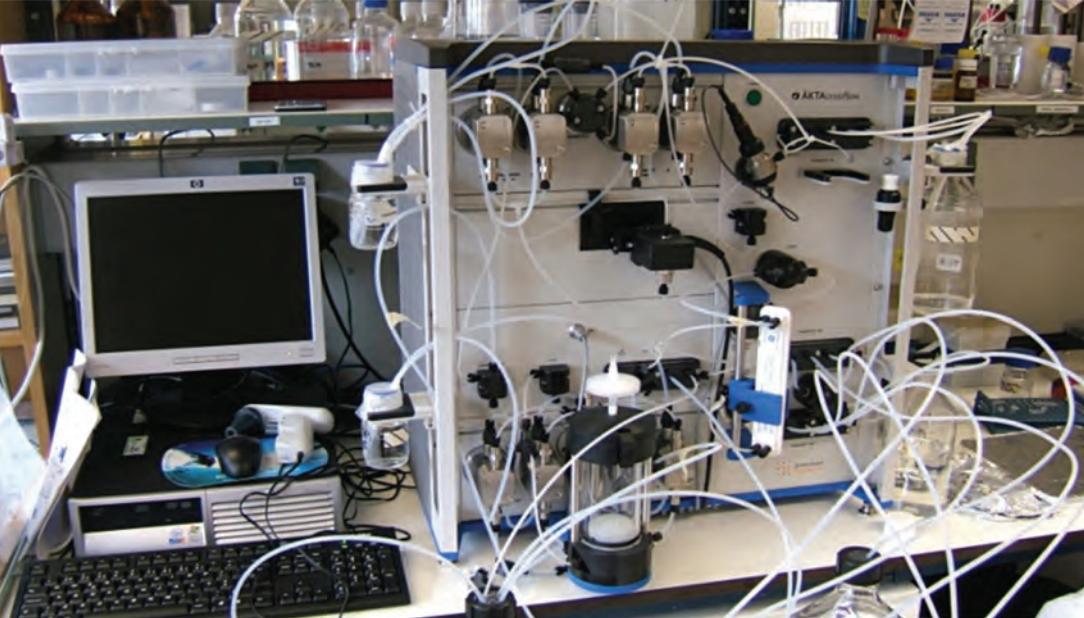


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Within the Animal Cell Technology Unit, the Engineering Cellular Applications Laboratory deals with integrating upstream and downstream processes and physico-mathematical tools particularly geared towards applications for the production of complex and novel biopharmaceuticals. Simplicity, feasibility, transferability and economics are criteria used to design processes that may lead to industrial opportunities. Having started with posttranslationally modified protein biopharmaceuticals in the early nineties, such criteria, design and process applications have been extended to vaccines, gene and cell therapy, drawing from the expertise and research achievements of the other labs in the ACTU. Over the last five years, downstream technologies and modelling for biological and chemical process control have constituted new targets of research.

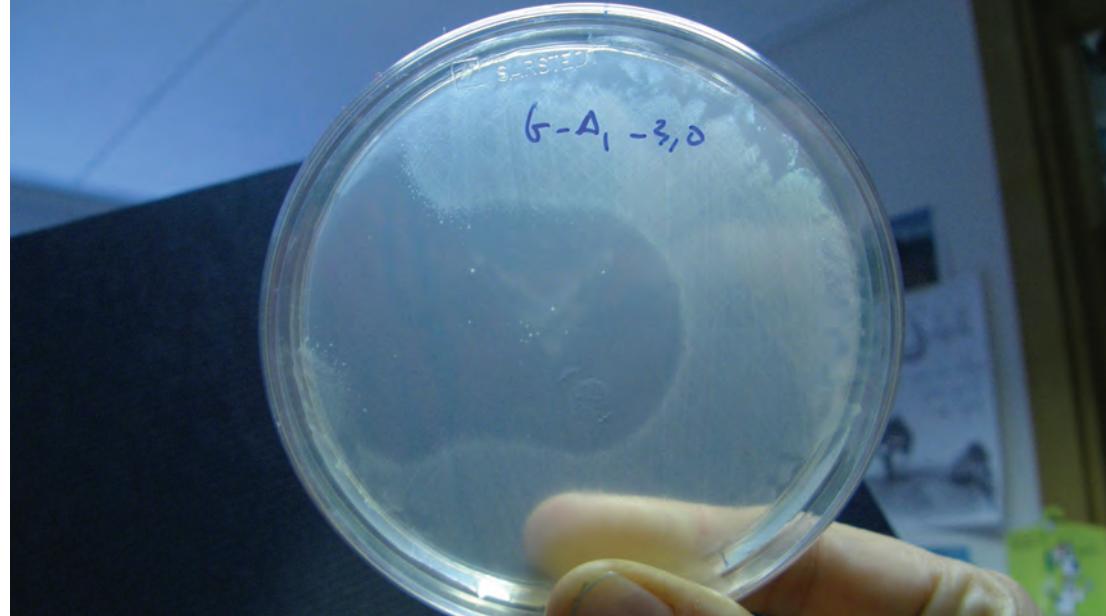
Our research focuses on the integrative development of bioprocesses for complex biopharmaceuticals, namely vaccines, recombinant proteins and viral vectors for gene therapy.

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Carinhas N., Bernal V., Monteiro F., Carrondo M. J. T., Oliveira R. and Alves P. M. (2010). "Improving baculovirus production at high cell density through manipulation of energy metabolism." *Metabolic Engineering* 12(1): 39-52.

*This laboratory has been developing know-how on *Lactobacillus* species from fermented olives and expanding it into new insights into functional foods, new pharmaceuticals and biocides against plant disease.*



Food Microbial Technology

Eating should be not only pleasurable but may also aid in the prevention of disease. The nutritional and beneficial properties of fermented foods are well documented, as is their use as vehicles of probiotic bacteria. Besides dairy products, fermented vegetables such as olives are also suspected to carry probiotics, while containing many functional compounds - fibres, vitamins, fatty acids, anti-oxidizers. The search for new phytochemicals and bioactive peptides from fermented foods is currently a popular trend in functional food development.

We have been studying novel applications for lactic acid bacteriocins, namely against *Helicobacter pillyor* - a human pathogen with multiple resistances to conventional antibiotics. In addition to antimicrobial activity, other bioactive peptides produced by *Lactobacillus sp.* include cytomodulatory, anti-carcinogenic, antihypertensive and immune stimulating activities. Studies are required to understand the mechanisms by which they exert specific health benefits. Many aroma compounds are the result of aminoacid metabolic pathways from lactic acid bacteria and the knowledge and stimulation of aroma production will be crucial for the characterisation and quality improvement of traditional fermented foods.

Such foods often include aromatic herbs for flavouring (table olives), and other active principles from wild plants have specific enzymatic (e.g. cinnarase for cheese-making), and therapeutic activities (e.g. extract of *Genista tenera*). These food ingredients and plant extracts need to be deeply studied in regard to their antimicrobial, anti-oxidant and alpha-glucosidase inhibitory activity. This research will focus on identifying active anti-diabetic compounds from

wild plant extracts, and on their chemical improvement to enhance biological activity.

Plant disease control by synthetic chemicals is likely to become more restricted in the near future. Studies of the biocide activity of a wide range of phytochemicals and microbial metabolites have been reported. The search for new biologically active substances is a relevant issue for this lab, based on preliminary results on fungal and Gram-negative bacteria inhibition.

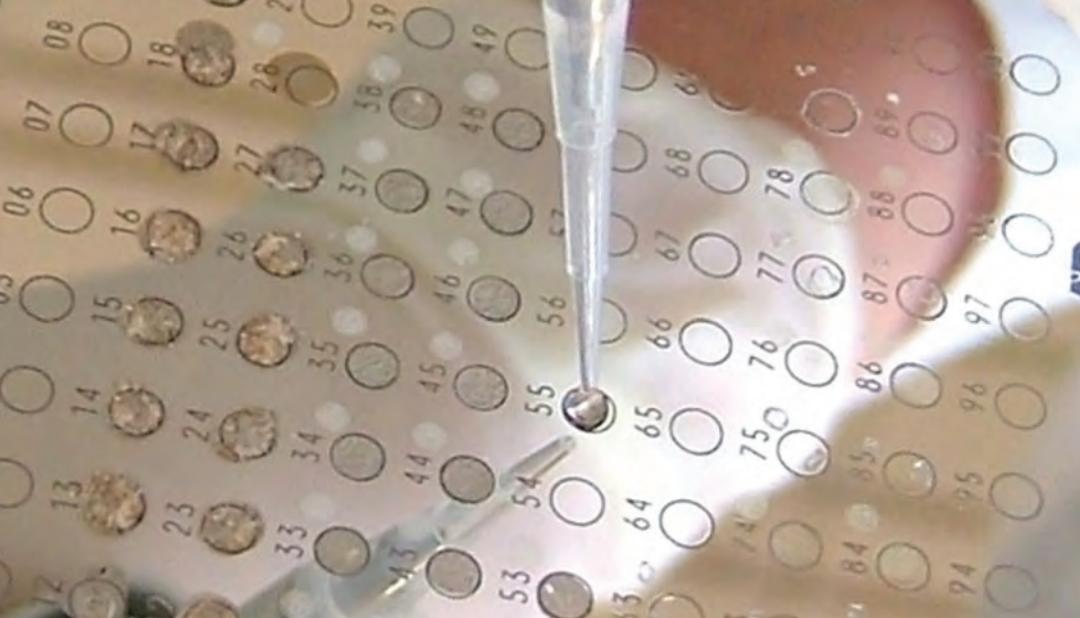
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We work to develop MS methodologies and implement them in the study of biological and chemical systems, namely those with biotechnological and biomedical applications. Some recent successful studies follow.

Mass Spectrometry



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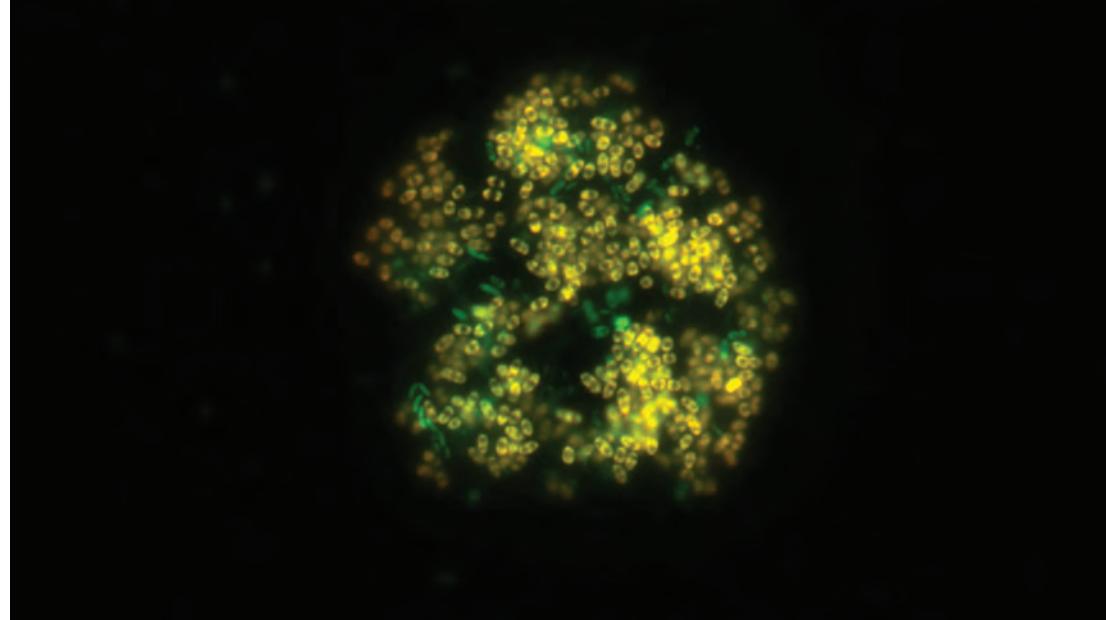
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André Almeida
Investigador auxiliar IICT
Romana Santos
Investigador auxiliar FMD-UL

The characterisation of sea urchin's adhesive secretions and the starfish's nerve proteome may be utilised in the biomimicry of biocompatible water-resistant glues, contributing to a greater understanding of echinoderms impressive regeneration abilities, with likely biomedical applications. By optimising an efficient solubilisation buffer we managed to overcome the sea-urchin's adhesive insolubility, which enabled researchers to visualise 13 protein bands by SDS-PAGE. A homology-database search identified seven of these proteins, while the remaining 6 seem to be either novel or highly modified. We have also identified for the first time several starfish radial nerve cord proteins related to the functioning of the echinoderm's nervous system and visual perception. This discovery should allow us to paint a clearer picture of the starfish and to demonstrate similarities with higher animals' synapse mechanisms.

Studies of protein complexes characterisation have included the optimisation of a disassembly procedure and of a MS method for determining proteins' molecular mass in viral particles. Comparative protein expression profiles in rabbit muscle in animals suffering food restriction identified the relevance of actin in preserving muscle structure and sensitivity of both myosin light chain and α -crystallin protein. Differential proteomic studies have demonstrated that an α -amylase isoform is overexpressed in mice saliva from animals fed on tannin rich diets. Marked differences between parotid salivary protein profiles of goats and sheep can be used as a starting point for investigating a potential relationship between physiological functions and different feeding behaviour of two domesticated species. A study of the changes in hepatome proteome induced by hepatitis delta virus replication has identified the proteins involved in virus replication and cellular defence against viral infection.

MALDI-TOF data used for the structural characterisation of enolase glycation (PTM related with amyloid diseases and diabetes) have been correlated with thermal stability and enzyme activity results. These are examples of established and fruitful collaborations with institutions participating in the National Mass Spectrometry Network, the UNL, other academic institutions and Laboratórios do Estado without omitting the LAO and international collaborations.

This laboratory studies isolated microbial strains and populations not only in natural environments but mostly in man-made ones such as food products, polluted water and/or microbial/host pairs.



Microbiology of Man-made Environments

The growth, survival and biochemical activity of microorganisms in these environments result from stress reactions in response to changes in physical and chemical conditions of the particular microenvironment. These micro-organisms grow under the constraints imposed by spatial heterogeneity and the in situ cell-to-cell ecological interactions, often occurring at interfaces. The data are integrated into microbial and molecular biology operations intended to evaluate which members of the microbial communities are present, as well as their dynamics and biochemical activity along the production/growth process. Case studies have included traditional food products, where data from molecular biology and biochemical methods, for example the use of C-sources by the population as a whole, have led to new and more efficient methods for evaluating the quality of food products. Our group has also studied the microbial populations responsible for pollutant removal using bioreactors for the treatment of wastewater. We have also utilised total DNA, denaturing gradient gel electrophoresis (DGGE), cloning and sequencing, analytical chemistry, biochemistry, microscopy, fluorescence in situ hybridization (FISH) and other strategies in order to identify the members of populations responsible for optimal operation/production processes. We have also applied our expertise in microbiology to studies of specific microbial groups with other applications, such as agricultural ones. For example, a study of a collection of rhizobial strains that nodulate annual medics has recently begun intending to devise new strategies for identifying strains with potential agricultural applications.

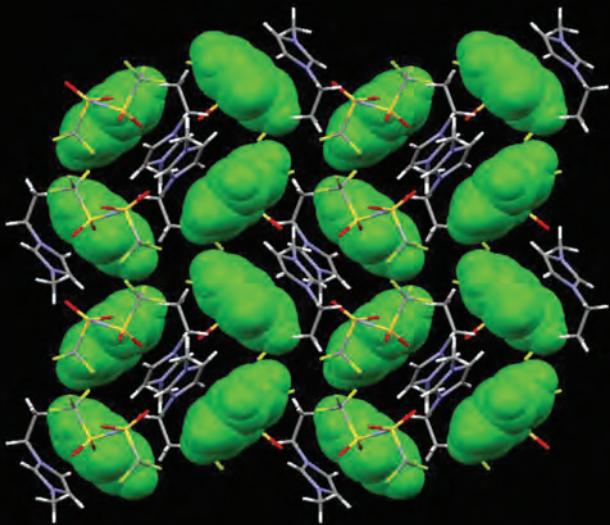
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The molecular thermodynamics of liquids and liquid solutions, in particular, studies of Ionic Liquids and ionic liquid-containing systems constitute the main activity of this laboratory. Other research topics include isotope effects, polymer solutions, and metastable liquids.

• Molecular Thermodynamics



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Recent years have witnessed the growing importance of ionic liquids – excellent solvents, which are liquid salts at or close to room temperature, and have been frequently studied as part of the important field of green chemistry. Their study has impacted a wide range of distinct areas, from chemistry to physics, from the environmental to the life sciences. Generally non-flammable and non-volatile, these liquids are easily manipulated in order to engineer distinct chemical functionalities, while offering extraordinary solvent quality and easy recovery. It is these characteristics which have driven the recent development of these salts as alternative clean media for chemical and enzymatic reactions, novel composites, separation and extraction processes, fuel cells, nuclear fuel reprocessing, additives for lubricants, and, more recently, biotechnology and pharmaceutical applications.

After launching our research into the Ionic Liquids field in 2001, a significant majority of the research of the group has been directed to the study of this novel type of fluid. Since then, some key and pioneering experimental work has been performed both in respect to the thermodynamic characterisation of these innovative compounds at a broad range of pressures and temperatures as well as to their solution behaviour. Molecular simulation began in 2006.

One of the main objectives of our current work is to chart solid and precise background data on the thermodynamic properties of this new class of compounds and their mixtures with other solutes/solvents, and to understand their structure and interactions at a molecular level, which will provide predictive tools for their behaviour. Other objectives include mapping phase diagrams with conventional solvents, polymers or inorganic salts using both

laser scattering techniques and common turbidity measurements. The group also focuses on the determination of the isotope effect of solutes and solvents, e.g. water, which impact the liquid-liquid phase diagrams and/or metastable regimes at deep absolute negative pressures. Another challenging and rewarding area of research is located at the interface with the life sciences. Namely, we are highly interested in knowing how toxic and how biodegradable these novel liquid salts are by using selected fungi as model microorganisms. As the fungi come into contact with ionic liquids, one can observe changes both in the fungi activity itself as well as in the generated metabolites; new compounds, including some with potential medical applications, may result.

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This laboratory uses clean technologies for the isolation and development of health promoting products. High-pressure methodologies are applied for the extraction of bioactive compounds and preparation of new delivery systems.



• Nutraceuticals and Delivery

As the name suggests, this laboratory encompasses two different areas of research: nutraceuticals, related to the extraction of natural bioactive ingredients and the formulation of functional products with health benefits; and controlled delivery which is associated with the development of adequate systems for overcoming barriers to drugs' usefulness, involving particle formation (micro and nano-scale), and the incorporation of the active principles in biocompatible and biodegradable matrixes.

Our main activities include:

- The isolation of functional ingredients from several botanical sources. Efficient clean processes are optimised for the extraction of natural high added-value products (antioxidants, anti-inflammatory, anti-cancer compounds, anti-fungal and anti-microbial agents) from solid or liquid materials.
- The chemical and biological characterisation of functional products such as in-depth sample analysis, the determination of antioxidant capacity (e.g. total phenolics content, ORAC and HORAC values, inhibition of AAPH-induced LDL oxidation) and the determination of (physiological) anti-inflammatory activity. The biological properties of the active components are assessed at the cellular level in appropriate cell lines searching for protection responses to environmental stresses and cell death signals.
- The formulation of new products by incorporating high value natural extracts, with potential applications in the food, nutraceutical and cosmeceutical industries. The optimisation of final formulations in terms of stability/

shelf-life, solubility/bioavailability and compatibility with target end products.

- The encapsulation and micronisation of active compounds using non-conventional techniques. Supercritical fluid technology has been shown to be a viable option with relevant advantages like the use of mild conditions for pharmaceutical processing, the minimisation of organic solvents and the use of environmentally benign non-toxic materials, and the production of smaller particles with controllable morphology and narrow size distribution. Also, the unique solvent tuneability of supercritical fluids, from gas-like to liquid-like properties, has been demonstrated to offer the intriguing possibility of precise control over processing conditions. Several forms using lipids and/or (biocompatible and/or biodegradable) polymers as carrier materials have been successfully prepared.

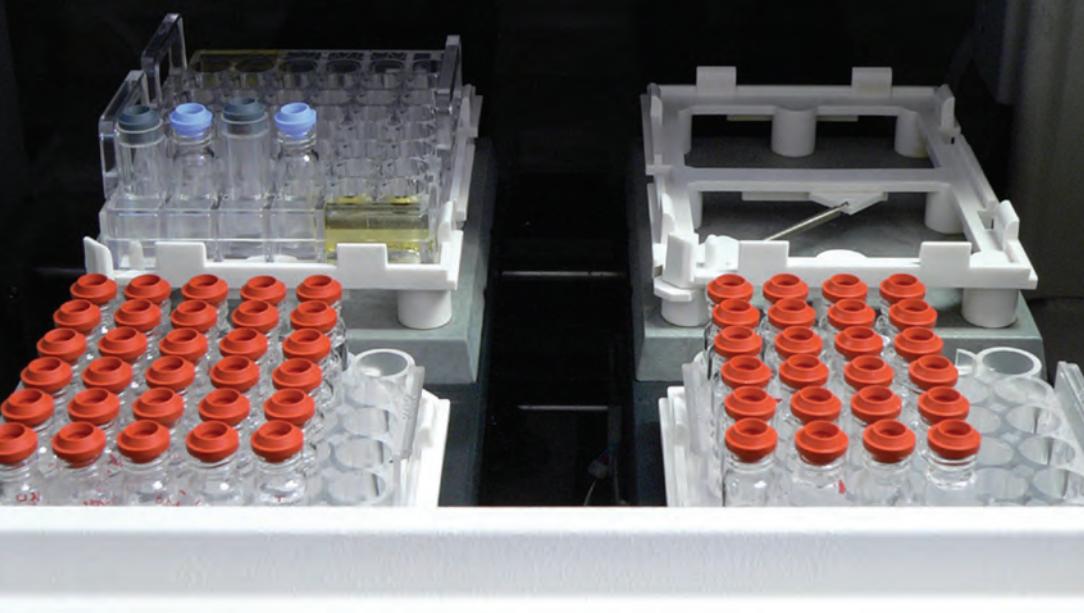


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The PABA group develops in vitro models for studying pharmacokinetics and metabolism. These models are applied to dietary supplements or prospective drugs.

Pharmacokinetics and Biopharmaceutical Analysis



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Our work includes the assessment, modulation and modelling of the pharmacokinetics of prospective drugs and prodrugs. We are particularly interested in intestinal membrane transport and metabolism and in drug-drug interactions.

Among the in vitro systems used for the study of drug intestinal absorption, the Caco-2 cell model is the most popular. Although the model provides significant information regarding active transport and efflux prediction, it lacks modulation of pre-absorption metabolism. Differentiated Caco-2 cell monolayers express most drug-metabolising enzymes and transporters but fail to express some relevant enzymes at the same relative level that they can be encountered in the intestine. Our laboratory is currently developing, in collaboration with the Animal Cell Technology Group, an improved Caco-2 model able to express those enzymes.

The issue of drug-drug interactions has generated significant concern within the pharmaceutical industry and among US and European regulatory authorities, since co-administration of different drugs or dietary supplements can affect their therapeutic outcome. Such interactions may be due to metabolising or transport enzyme inhibition/induction and affect pharmacokinetics or pharmacodynamic properties of the drug. We are currently studying the effect that some dietary supplement formulations may have on the metabolism of prescription drugs.

Since the discovery of fullerenes, various biological activities of their derivatives have been claimed, mostly related to fullerene free radical quenching ability. Surprisingly, these compounds have not yet found their way to clinical

use, probably due to their usually low water solubility which interferes with formulation and biological testing. Extremely low water solubility of this class of compounds has precluded activity tests in physiologically related media. We are studying ways to overcome this problem and investigating the antioxidant potential, toxicity and proliferative effects as well as the ability of fullerenes and fullerene conjugates to cross biological membranes.

In the field of analytical development we are particularly interested in in-capillary reactions. Electro-mediated microanalysis has been frequently used to study enzymatic reactions; however, correlation with off-line methodologies is difficult because the fluid dynamics inside the capillary have not been thoroughly characterised. We are now engaged in the evaluation of in-capillary reactions for the study of enzyme kinetics, taking into consideration stacking and/or diffusion effects.

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This lab strives to deepen our understanding of the microbial phenomena sustaining the well-being of humans and of the environment.

Physiology of Environmentally Conditioned Microbiota

Our research follows the guidelines established by IBET and ITQB-UNL priorities, whilst keeping EVNINIA objectives in mind.

Two areas of investigation are underway:

- 1) The study of the formation in wine of biogenic amines (BA) by lactic acid bacteria (LAB). We hope to select regional LAB isolates able to perform malo-lactic fermentation in wine without the formation of BA or other unfavourable compounds. Selected isolates will later be immobilised.
- 2) The study of fungal communities. a) Studies of the fungal dynamics in a homogeneous community utilise the cork colonising community as the study model. We intend to characterise the total fungal population present in cork throughout the cork stopper manufacturing process so as to understand the relationship between the fungal population, especially concerning the evolution of the forest endogenous population and its importance in determining the resident populations at manufacturing plant locations. The taxonomic characterisation of the fungal species is being used to solve species inter and intra genetic variety. This work is being carried out in cooperation with Amorim & Irmão. b) We also target the potential of fungi for applied and environmental mycology. Most species amongst the cork colonising community are able to tolerate and degrade high concentrations of a model polychlorohalogenates pollutant, pentachlorophenol. This research is being performed in collaboration with the Applied and Environmental Mycology Laboratory.



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This laboratory uses computational and mathematical methods to analyse and predict the response of biological systems like plant leaves or microbial biofilms to dynamical variations in environmental conditions.

Systems Biodynamics



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Our research centres on computational and mathematical studies of the responses of multicellular systems, e.g. plant leaves or microbial biofilms, when exposed to multiple, dynamic environmental stimuli. More than specialising on a determined biological process or organism, or on one specific computational tool, we approach any one biological problem from an array of different perspectives, bringing together diverse computational tools for simulation and data analysis in order to tailor problem specific solutions.

One line of research is rooted in the time-keeping systems generating endogenous, circadian (24h) rhythms in plants. A variety of mathematical models with different degrees of abstraction facilitates the development of experimentally testable hypotheses regarding the coupling of rhythmic gene transcription with plant carbon metabolism and leaf water fluxes. A principal question addresses which structural classes of intra- and intercellular regulatory networks are capable of maintaining the organisms' synchrony with the environment, despite multiple and contradictory simultaneous stimuli.

Another line of research has emerged from the development of an integrative, web-based computational infrastructure for the management and analysis of heterogeneous data from phototrophic microbial biofilms. We intend to integrate this database with computational data analysis modules and various types of quantitative biofilm models. Of specific interest is the spatio-temporal development of biofilm biomass, in particular, its spatial heterogeneity and the stochastic occurrence of large detachment events.

The universal character of the statistical and computational methodology employed enables us to participate in various interdisciplinary projects and collaborations. Given our focus on the organism/environment interface, computational and analytical support serves as a catalyst both for the discovery of knowledge and innovation in both fundamental and applied environmental plant- and microbiology.

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Index (Labs)

A

Analytical Chemistry, 52
Applied and Environmental Mycology, 54

B

Bacterial Cell Biology, 32
Bacterial Cell Surfaces and Pathogenesis, 33
Bacterial Energy Metabolism, 14
Bacterial Signalling, 34
Bioinorganic Chemistry and Peptide Design, 5
Biological Energy Transduction, 15
Biomolecular Diagnostics, 55
Biomolecular NMR, 16
Bioorganic Chemistry, 6

C

Cell Bioprocesses, 56
Cell Line Development and Molecular Biotechnology, 57
Cell Physiology and NMR, 35
Cell Signalling in Drosophila, 36
Colloids, Polymers and Surfaces, 7
Control of Gene Expression, 37
Coordination and Supramolecular Chemistry, 8

D

Disease and Stress Biology, 43

E

Engineering Cellular Applications, 58

F

Food Microbial Technology, 59
Forest Biotech, 44

G

Genomics and Stress, 17
Genomics of Plant Stress (GPlantS), 45
Glycobiology, 38

H

Homogeneous Catalysis, 9

I

Industry and Medicine Applied Crystallography, 18
Infection Biology, 39
Inorganic Biochemistry and NMR, 19

L

Lactic Acid Bacteria & *In Vivo* NMR, 40

M

Mass Spectrometry, 60
Membrane Protein Crystallography, 20
Metalloenzymes and Molecular Bioenergetics, 21
Microbial & Enzyme Technology, 22
Microbial Development, 41
Microbiology of Man-made Environments, 61
Micro-heterogeneous Systems, 10
Molecular Genetics, 42
Molecular Genetics of Microbial Resistance, 23
Molecular Interactions and NMR, 24
Molecular Simulation, 25

Molecular Thermodynamics, 62
Mössbauer Spectroscopy, 26

N

Nutraceuticals and Delivery, 63

O

Organic Synthesis, 11
Organometallic Chemistry, 12

P

Pharmacokinetics and Biopharmaceutical Analysis, 64
Physiology of Environmentally Conditioned Microbiota, 65
Plant Biochemistry, 46
Plant Cell Biology, 47
Plant Cell Biotechnology, 48
Plant Cell Wall, 49
Plant Developmental Genetics, 50
Plant Molecular Ecophysiology, 51
Protein Biochemistry Folding & Stability, 27
Protein Modelling, 28

R

Raman Spectroscopy of Metalloproteins, 29

S

Single Molecule Processes, 13
Stress by Antibiotics and Virulence of Enterococci, 53
Structural Biology, 30
Structural Genomics, 31
Systems Biodynamics, 66

Index (Head of Lab)

A

- Abranches, Rita, 47
Almeida, Jorge, 50
Alves, Paula M., 56
Archer, Margarida, 20
Arraiano, Cecília, 37
Astier, Yann, 13

B

- Baptista, António M., 25
Boas, Luís Filipe Vilas, 52
Bohn, Andreas, 66
Bronze, Maria Rosário, 52

C

- Carrondo, Manuel J. T., 58
Carrondo, Maria Arménia, 31
Chaves, Manuela, 51
Coelho, Ana, 60
Coroadinha, Ana Sofia, 57
Costa, Júlia, 38
Crespo, Teresa, 61

D

- de Lencastre, Hermínia, 42
Delgado, Rita, 8
Domingos, Pedro, 36
Duarte, Catarina, 63

F

- Ferreira, Ricardo, 43
Fevereiro, Pedro, 48
Filipe, Sérgio R., 33
Frazão, Carlos, 30

G

- Gomes, Cláudio M., 27
Groves, Patrick, 24

H

- Henriques, Adriano O., 41

I

- Iranzo, Olga, 5

J

- Jackson, Phil, 49

L

- Lopes, António, 7
Lopes, Maria de Fatima Silva, 53
Louro, Ricardo O., 19

M

- Martins, Lígia O., 22
Matias, Pedro, 18
Matzapetakis, Manolis, 16
Maycock, Christopher, 11
Melo, Eurico, 10
Miguel, Célia, 44
Mota, Jaime, 39

N

- Neves, Ana Rute, 40

O

- Oliva, Abel Gonzalez, 55
Oliveira, Filipe Tiago de, 26
Oliveira, Margarida, 45

P

- Pereira, Cristina Silva, 54
Pereira, Inês A. Cardoso, 14
Pereira, Manuela M., 15
Peres, Cidália, 59
Pinho, Mariana G., 32

R

- Rebelo, Luís Paulo, 62
Ricardo, Cândido Pinto, 46
Rodrigues-Pousada, Claudina, 17
Romão, Carlos, 12
Romão, Maria Vitória San, 65
Royo, Beatriz, 9

S

- Santos, Helena, 35
Saraiva, Lígia M., 23
Simplício, Ana Luísa, 64
Soares, Cláudio M., 28

T

- Teixeira, Miguel, 21
Todorovic, Smilja, 29

V

- Ventura, Rita, 6

X

- Xavier, Karina, 34

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