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Chimica Bioinorganica per Dottorandi

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Programma

Martedì 12 Febbraio 2019

CNR-Piazzale Aldo Moro 7- Aula Marconi 1 Piano

14.00- 15.00	Registrazione Workshop PAST, PRESENT AND FUTURE OF INORGANIC CHEMISTRY IN ITALY: A PATH DEFINED BY THE WINNERS OF THE NASINI PRIZE - Bioinorganic and Medicinal Chemistry
15.00- 15.20	**Silvio AIME**, University of Torino New routes in the design of MRI reporters
15.20- 15.40	**Mauro BOTTA**, University of Piemonte Orientale Enhancing the Sensitivity of Gd-based Nanoparticles as MRI Probes
15.40- 16.00	**Antonio ROSATO**, University of Firenze Bioinformatics of Metalloproteins.
16.00- 16.20	**Paola TURANO**, University of Firenze Integrated structural biology of iron biomineralization in the ferritin nanocage
17.40- 18:30	**Roberto PURRELLO**, University of Catania Relevance of chirality and conformational changes in bioinorganic chemistry

Mercoledì 13 Febbraio 2019

CNR-Piazzale Aldo Moro 7- Aula Bisogno 1 Piano

9.00-10.30	**Mauro BOTTA**, University of Piemonte Orientale Metal-based diagnostic probes in Magnetic Resonance Imaging: a Coordination Chemistry approach
10.30-11.00	Coffee Break
11.00-12.30	**Roberto FATTORUSSO**, University of Campania Luigi Vanvitelli NMR methodologies for the investigation of protein structures and functions
12.30-13.30	**Francesco Paolo FANIZZI**, University of Salento Metabolomics, a Powerful Tool for Bioinorganic Chemistry.
13.30-14.30	Lunch
14.30-15.30	**Giovanni NATILE**, University of Bari Forty years after the FDA-approval of Cisplatin: What we know about transport and mechanism of action
15.30-16.30	**Domenico OSELLA**, University of Piemonte Orientale Beyond cisplatin: the Pt(IV) antitumor prodrugs - Oltre il cisplatino: i profarmaci antitumorali a base di Pt(IV)
16.30-17.00	Coffee Break
17.00-18.30	**Enrico RIZZARELLI**, University of Catania Chemotype, Metallostasis and Diseases
18.30-19.00	Dibattito con studenti
20.30	Cena sociale

Giovedì 14 Febbraio 2019

CNR-Piazzale Aldo Moro 7- Aula Bisogno 1 Piano

9.00-10.30	**Giulietta SMULEVICH**, University of Florence Valuable insights into the heme protein structure-function relationships via resonance raman spectroscopy
10.30-11.00	Coffee Break
11.00-12.30	**Paolo ASCENZI**, University of Roma Tre Reactivity and enzymatic kinetic properties of hemoproteins: the case of heme-Albumin
12.30-13.30	**Cristina BOLZATI**, ICMATE-CNR When radiochemistry meets medicine: radiopharmaceuticals and molecular imaging
13.30-14.30	Lunch
14.30-16.00	**Antonio ROSATO**, University of Florence How to apply bioinformatics methods to metalloproteins
16.00-16.30	Coffee Break
16.30-18.00	**Mauro FASANO**, University of Insubria Proteomics approach to system biology: the case of multifactorial diseases
18.00-19.00	Dibattito con studenti

Venerdì 15 Febbraio 2019

CNR-Piazzale Aldo Moro 7- Aula Bisogno 1 Piano

9.00-10.30	**Stefano MANGANI**, University of Siena X-ray spectroscopy methods in Bioinorganic Chemistry
10.30-11.00	Coffee Break
11.00-12.30	**Giuseppe FALINI**, University of Bologna Molecular Recognition at Organic-Inorganic Interface in Biomineralization Processes
12.30-13.30	**Laura ZACCARO**, IBB-CNR New metal based nanoparticles coated with biomolecules for targeted therapy
13.30-14.00	Conclusioni

Relevance of chirality and conformational changes in bioinorganic chemistry

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Chirality, defined as “the property of an object of not being superimposable with its mirror image”, is widespread in the Universe where it is expressed at different levels: from subatomic to galactic. In chemistry, chirality has two relevant levels: the molecular and the supramolecular ones.
Involved in nature since the origin of life and fully exploited along the way of evolution, chirality has a plethora of roles in biological systems; from discrimination to regulation. The molecular bricks of life - amino acids and nucleotides – are chiral, as well as their polymeric forms. However – and very interestingly - the different molecularity of the chiral species means quite different roles in nature. The differences between these two levels are exemplified and actualized in nature, where chemical chirality finds a very effective representation of its functional and sophisticated relevance.
Molecular chirality of biological molecules is mainly related to the fixed disposition of four different groups around a stereogenic central atom. This peculiarity is fundamental to carry specific information (L- and not D-amino acids are absorbed by our organism, the smell and taste of L- and D-molecules are different) but limits their biological role. On the contrary, chiral biopolymers – which have complex spatial relationships between the monomeric constituents - are flexible and their conformational chirality can be modulated with deep consequences on the biological meaning.
During my talk, I will present some examples of the latter topic; from DNA and miRNA to proteasome.

Metal-based diagnostic probes in Magnetic Resonance Imaging: a Coordination Chemistry approach

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After the initial experiments in the early '70s, the decade of the 1980s is characterized by the advent and remarkable growth of MRI, which has evolved rapidly into one of the most powerful techniques in clinical diagnostic and biomedical research. This is due to many favourable properties, among which the following stand out: i) lack of ionizing radiation for image acquisition; ii) non-invasiveness with a high patient acceptability; iii) excellent delineation of anatomical structures; iv) superb temporal and spatial resolution (mm scale); v) possibility of application to virtually any body district.[1] The traditional imaging procedures have been combined with the use of dedicated contrast media, to help enhance the visualization of morphology and physiology. The synergistic action and the combination of technical progress with the development of new contrast agents (CAs) have been very important factors for the emergence of modern clinical radiology. In this context, MRI has not evolved along a different path and, since the advent in early '80, this imaging modality has been improved by the use of exogenous agents to increase the signal intensity and diagnostic confidence and reduce overall cost. About one third of all routine clinical MRI procedures use intravenously introduced magnetic agents to alter image contrast.[2] Contrast agents on the market and most on those in clinical or pre-clinical trials focus upon changes of nuclear magnetic relaxation times ($T_{1,2}$). In general, the class of CAs most largely utilized is represented by complexes of the Gd(III) ion that are capable to markedly affect the proton relaxation rates in the region where they distribute. This choice is due to the combination of high magnetic moment and favourable properties in terms of electronic relaxation of this f^7 lanthanide ion (seven unpaired electrons in an S ground state). Contrast-enhanced MRI is used annually in approximately 30 million procedures with over 300 million

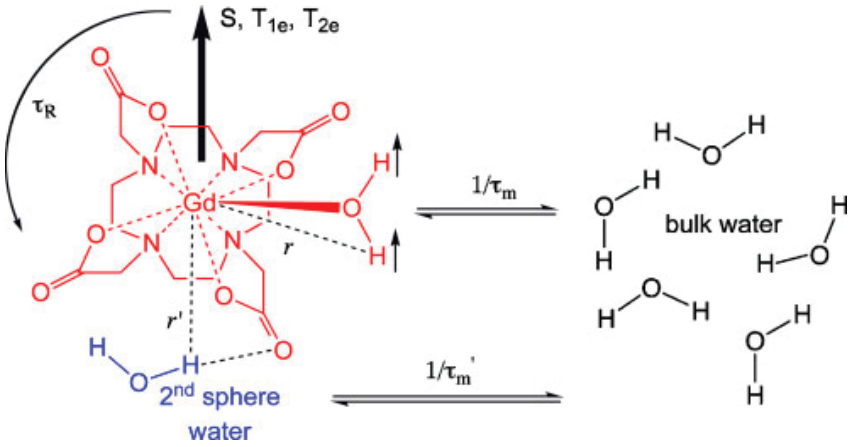

***Figure 1**: Factors influencing solvent water relaxation. The metal complex has an inner sphere (IS) of nitrogen and oxygen atoms from the ligand and a coordinated water molecule. There is a distinct second hydration sphere (SS) with Gd–H distance r', and water molecules from both spheres undergo exchange with bulk water at rates $1/\tau_m$ and $1/\tau'_m$ for first- and second-sphere exchange*

patients having been dosed so far.[3]

In this lecture, we want to illustrate the state of the art of clinically approved contrast agents, their mechanism of action, and factors influencing their safety. The lesson begins with a presentation of the basics of paramagnetic relaxation and its dependence on a number of molecular parameters, such as the hydration state of the molecule, its rotational dynamics and the molecular size.[4] These molecular parameters can be tuned and optimized to create contrast agents with much higher efficiency than conventional Gd-DTPA. This is followed by an illustration of the techniques of investigation and models of interpretation.[5] From there we describe different mechanisms of generating MR image contrast such as relaxation, chemical exchange saturation transfer, and direct detection and the types of molecules that are effective for these purposes.[6] Next we describe efforts to make safer contrast agents either by increasing relaxivity, increasing resistance to metal ion release, or by moving to gadolinium(III)-free alternatives. Finally, we survey approaches to make contrast agents more specific for pathology either by direct biochemical targeting or by the design of responsive or activatable contrast agents.[7]

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NMR methodologies for the investigation of protein

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NMR spectroscopy is a powerful technique for structural studies of chemical compounds and biomolecules such as DNA and proteins. Since the NMR signal sensitively reflects the chemical environment and the dynamics of a nuclear spin, NMR experiments provide a wealth of structural and dynamic information about the molecule of interest at atomic resolution. In the past several decades, progress in solution NMR has significantly contributed to the elucidation of three-dimensional structures, the understanding of conformational motions, and the underlying functional properties of biomacromolecules1,2,3. This presentation discusses recent methodological development of NMR, their applications and some of the remaining challenges. Furthermore, the principles enabling NMR to provide information on the nature of molecular interactions will be surveyed and, on this basis, current NMR-based strategies that allow to identify weak-binding compounds and aid their development into potent, drug-like inhibitors for use as lead compounds in drug discovery4,5 will be discussed.

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Metabolomics, a Powerful Tool for Bioinorganic Chemistry

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Metabolomics is the latest of the "omic" sciences but its use increased in last twenty years together with the application field. Thanks to recent advances in analytical technologies and statistical capabilities Metabolomics is currently used to investigate biological substrates looking for metabolic profile alterations, diseases markers and drug effects. [1] The most frequently used analytical techniques in metabolomics are NMR and GC or HPLC-MS. In particular, NMR spectroscopy, able to detect multiple (10's to 100's) metabolites at once without separation, has shown great potential for assessing tumor response to anticancer agents and providing insights into the mechanism of action and resistance of drugs. [2] Only in last few years, NMR based metabolomics has been extended to investigate the cell metabolic alterations induced by metal based antitumor drugs. These studies, actually focused in particular on platinum [3] and ruthenium [4] complexes, open new prospective in the development of new metal-based drugs and in the understanding of their action mechanism. NMR based metabolomics resulted a powerful tool for detecting variations in a range of intracellular compounds upon in vitro metal based drugs exposure. Nevertheless this approach also offers the possibility of identifying markers for in vivo monitoring of tumor responsiveness to treatments. [2]

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Forty years after the FDA-approval of Cisplatin: What we know about transport and mechanism of action

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Cisplatin, a potent anticancer drug targeting the DNA of cancerous cells, was introduced in the clinic in 1978. Unfortunately, cisplatin-based treatments are plagued by resistance mechanisms, which greatly limit the efficacy of the drug.

One of these resistance mechanisms is the enzyme-based repair of the platinum lesions [1]. The high mobility group box 1 (HMGB1) protein binds to the distorted DNA [2], inhibiting the repair of the cisplatin–DNA damage in vitro [3, 4] and in cell [5], thus sensitizing cells to drug treatment [6]. Understanding the details of the HMGB1 recognition of cisplatin-DNA adducts in vivo is key for a strategy aiming to increase the efficacy of platinum-based drugs. Recently, several HMGB1 isoforms bearing extensive acetylation and phosphorylation were identified in vivo and found to be able to bind cisplatin-DNA adducts with high affinity [7].

Another resistance mechanism is the reduced cellular uptake of the drug. The uptake and efficacy of the three clinically approved platinum anticancer drugs (cisplatin, carboplatin, and oxaliplatin) rely at least in part upon copper transporters.[8, 9] CTR1, the major copper influx transporter, is a plasma membrane permease that appears to favor internalization of cisplatin and its derivatives. [10] Copper efflux pumps ATP7A and ATP7B (whose mutations cause Menkes and Wilson diseases, respectively) are membrane-bound ATPases, localized in the trans-Golgi network, that appear to mediate cisplatin efflux and/or sequestration into vesicular compartments, thus influencing cellular drug resistance.[11, 12] Finally, the copper chaperone ATOX1, which transports copper from CTR1 to ATP7A and ATP7B in the cytoplasm, is overexpressed in some cisplatin-resistant cells.[13, 14]

Several studies indicate that the copper transport machinery is also involved in the cellular response (sensitivity or resistance) to Pt anticancer drugs [15].

In the presentation, the most recent mechanistic insights into the cross-talk of cisplatin with damaged-DNA recognition proteins and Cu-trafficking proteins will be discussed [16-18].

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Beyond cisplatin: the Pt(IV) antitumor prodrugs

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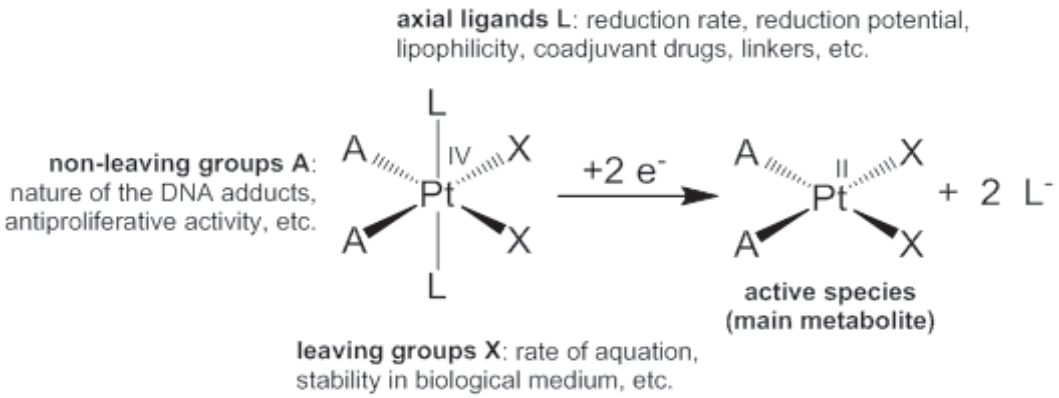
The serendipitous discovery of the anticancer activity of cisplatin by Rosenberg prompted decades of extensive research aimed at the development of novel therapeutics based on metal complexes. Platinum complexes are the most successful class of metal-based antiproliferative agents.
Seven platinum complexes have been approved for use in humans. Three (Cisplatin, Carboplatin and Oxaliplatin), approved by the FDA and EMEA, are in worldwide use. In addition, Nedaplatin (Japan), Lobaplatin (China), Heptaplatin (Korea), and, very recently, Miriplatin (Japan), were approved in Asian Countries for treating several carcinoma.
Such platinum drugs are employed in 50% of chemotherapeutic regimens administered in the clinic against solid tumors.
Their side effects, low selectivity and bioavailability, and the failure to administer these drugs orally (they are administered intravenously only) are mainly ascribed to their high reactivity with biological nucleophiles prior to reaching the tumor site.
A strategy that gained popularity recently is the use Pt(IV) complexes as prodrugs. Pt(IV) complexes are prepared by oxidative addition of the square-planar Pt(II) complexes to yield octahedral counterparts that retain the original Pt(II) equatorial coordination sphere. Most often, the oxidation is performed using hydrogen peroxide resulting in two hydroxidos in axial position, which can be modified to tether a variety of ligands, generally via their carboxylic functions, designed to improve the pharmacological properties of the complex [1, 2].
Because Pt(IV) complexes have a low-spin, d6 octahedral geometry, they are more inert to substitution than Pt(II) counterparts, can be administered orally, and show reduced. This was demonstrated by Satraplatin, cis,trans,cis-[Pt(NH3)2(OAc)2Cl2], that completed phase III clinical trials by oral administration. Once inside the cell, the Pt(IV) complexes undergo a two electron reduction regenerating the original square-planar Pt(II) drug and releasing the two axial ligands (Figure 1). This activation by reduction of the Pt(IV) complexes is believed to take place mainly inside the hypoxic and then reducing tumor microenvironment. The use of Pt(IV) complexes as prodrugs was pioneered by Johnson Matthey in the 1990s and has gained attention ever since.



The axial ligands can be relatively innocent spectators (devoid of significantly biological activity – such as halidos, hydroxidos, or acetatos), or they can be designed to achieve specific goals. For instance, the axial ligands can be very lipophilic moieties, that enhance passive uptake of the conjugate (synergistic cellular accumulation), or they can be cancer-targeting cargos (such as folates), or subcellular targeting agents (such as properly designed peptides). The axial ligands can possess two terminal functionalities (as succinic acid or β-alanine) used to tether the prodrug to a delivery system such as inorganic or polymeric nanoparticles (NP). Finally, they can be bioactive moieties such as auxiliary drugs, enzyme inhibitors, epigenetic modifiers, and antimetabolites, that work in synergy with the Pt(II) cytotoxic metabolite to improve the overall pharmacological properties.
These conjugates are particular promising as most of the antitumor treatments (including those involving platinum drugs) consist of two or more drugs that act additively or synergistically (combination therapy). The administration of two or more single drugs can suffer from drawbacks such as different bioavailability, pharmacokinetics and metabolism, and possible drug-drug interactions. The preparation of a single agent containing different chemically bonded drugs in a single conjugate (combo) as a Pt(VI) prodrug may represent a more effective strategy to address this task [3, 4].
In this talk, the term "multi-action" Pt(IV) conjugates is used to describe prodrugs that release inside the cancer cell bioactive ligand/s in addition to the cytotoxic platinum moiety.

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Chemotype, Metallostasis and Diseases

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Today it is of common knowledge in cosmology how our sun was formed from an explosion of a supernova and how the interstellar dust that did not collapse into the sun formed, over time, the earth and the other planets of our solar system. Our planet was in fact formed more than 4.5Ga (billions of years ago). and within its first 500 million years the earth rapidly differentiated: the heavier Fe and Ni collapsed into the center, creating the core and the magnetic field of our planet, while an early fast "outgassing" created more than 85% of a primitive atmosphere in which N_2, CO_2, and H_2O were probably already present.

In a few million years the surface of the earth rapidly cooled down, giving rise to heavy rains that ultimately formed our oceans. The original environment of Earth shortly after it cooled was a reduced surface and aqueous solution with an atmosphere of CO_2, CO, CH_4, N_2 (possibly NH_3 and HCN), H_2S and H_2O. Before cells formed weathering activity had removed much of the CO_2 as $CaCO_3$. The sea at 3.5 Ga was a reducing medium of something like today's Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and some HPO_4^{2-} ionic solution of fixed pH but with no O_2 or SO_4^{2-}. This solution inevitably contained H_2S, making it reducing, and a limited number of d block metal ions, plenty of Fe^{2+}, some Ni^{2+} and Mn^{2+} but little Zn^{2+}, no Cu^+(Cu^{2+}) and little Mo but probably some W. The conditions that were created at this time are believed to be sufficient to sustain the origin of life; in order to allow life, it had to use those elementswhich were available in catalysts and in assisting organisation of its chemistry.

The basic principles involved in the bioselection of elements are governed by four fundamental rules related to:

- (i) the abundance of the element,
- (ii) its efficiency,
- (iii) its basic fitness for a given task and
- (iv) the evolutionary pressure.

Water soluble ferrous iron was present during prebiotic times and was the form used in the first stage of life. Without going into detail, iron became the source of redox catalysts and magnesium the catalyst for use of ATP.

At the same time, copper was in the water-insoluble Cu(I) state, in the form of highly insoluble sulphides, and then not available for life. The early chemistry of life used iron (II).

About 10^9 years ago, an evolution of dioxygen into the earth's atmosphere began to develop, due to the metabolism of a prokaryote (Cyanobacteria). A lag of 200–300 millions years is estimated to have been required between the first production of O_2 and the appearance of a significant O_2 concentration in the atmosphere, because the O_2 produced was initially consumed by the oxidation of ferrous ions in the oceans.

The advent of O_2 was a catastrophic event for most living organisms and can be considered as the first general irreversible pollution of the earth.

Iron was oxidized and transformed into the insoluble iron(III) state. Iron hydroxides precipitate and the bioavailability of iron was lost. On the contrary, the oxidation of insoluble Cu_2S and ZnS led to soluble $CuSO_4$ and $ZnSO_4$.(Figure 1)

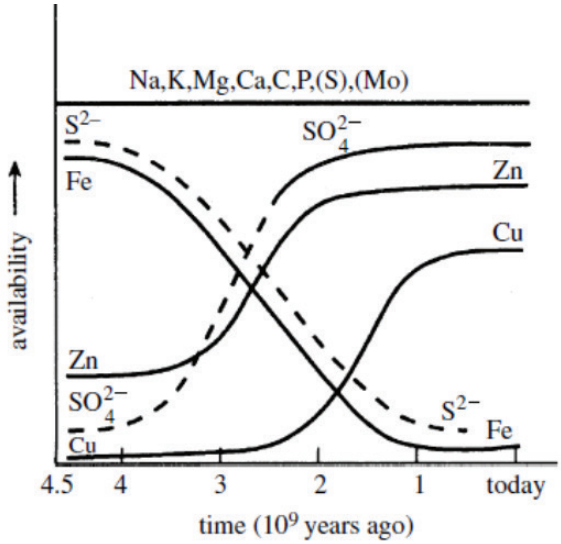

***Figure 1**: Temporal evolution of metal ion concentration.*

This chemistry change of reduction to reduction-with-oxidation in cells is the main line of chemical evolution and it is decided by oxidative equilibria in the environment that has a fast rate on the timescale of millions of years. It increased the presence of certain metal ions, some possibly useful, but poisonous at first to simple cells, including Cu^{2+}, Zn^{2+} and MoO_4^{2-}. Organisms, with a reductive cytoplasm, then had to live in this changing, poisonous, environment which they created.

The drastic changes in O_2 and metal bioavailability prototypically exemplified by Zn^{2+} and Cu^{+} constrained early life forms to avoid, adapt, detoxify or use in different ways these elements, and the reason is the paradoxical dichotomy between the toxicity and energetic advantages of O_2 mediated processes. Consequently, different evolutionary strategies have been used to fine-tune Zn and Cu cellular homeostasis.

Cells have evolved specific and complex trafficking and storage systems that participate in Cu and Zn binding and compartmentalization, to ensure both the delivery and detoxification of any metal excess.

Cellular copper homeostasis is ensured by some copper binding proteins that control concentrations, binding interactions and location of single metal species that determine copper metallome. Prominent modulators of copper homeostasis are: i) import (high-affinity copper transporter 1, CTR1 and export (ATPase) Cu^{+} transporters across membranes; ii) trafficking components, including small molecules (GSH) and chaperones (CCS, Atox1, COX17/Sco1-Sco2, COX17) that escort Cu^{+} inside cell ; iii) insertase agents, CCS and Sco1/Sco2 (that insert Cu^{+} into apo-SOD1 and cytochrome c oxidase, respectively), Atox1 (that transfers Cu^{+} to ATP7A and ATP7B); iv) storage molecules (metallothionines); and v) metal transcription factors (Atox1, p53, MTF-1, Sp1).

To maintain the zinc homeostasis, a number of proteins are involved in the metal cellular influx and efflux process between extracellular and intracellular compartments, thus, tuning the zinc concentration and distribution. Ten members of the ZnT family (SLC30A) export zinc from the cytosol, whereas 14 members of the zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like protein (ZIP) family (SLC39A) import zinc into the cytosol. Together these transporting and sensing zinc proteins control cellular zinc and its traffic through the plasma membrane and between

cytosol and various cellular compartments. The expression of zinc transporters is regulated transcriptionally, translationally, and at the protein level through heterodimer formation, ubiquitination, phosphorylation, and proteolysis all of which are conducted in a cell-specific and tissue-specific or a differentiation and developmentally coordinated manner. These metallostasis regulators can partially remedy copper and zinc ion dyshomeostasis, that is responsible for a broad range of human diseases.

Two subsets of total copper and zinc can be distinguished: the static, tightly bound and the dynamic, relatively weakly bound (labile or exchangeable) pools. Changes to the labile copper pool may be assessed by the use of fluorescent small-molecule indicators that can equilibrate with kinetically accessible copper and zinc in the cytosol, enabling one to distinguish labile from total metal ion, the latter of which is typically measured using direct techniques, including X-ray fluorescence microscopy (XFM) or mass spectrometry imaging.

Augmentation or restriction of the labile metal ion pools alters the function of a number of enzymes, some through direct binding to zinc and copper, leading to the notion that they may serve as a cellular signal that modulates the activity of proteins. d-block metal ion signaling has emerged as an exciting new field of study in living systems. In this expanded paradigm, copper and zinc not only serve as static structural and metabolic cofactors, but also are mobilized by cells to dynamically regulate enzymatic activity and communicate information both within cells and between cells. Indeed, recent advances in the observation of d-block metal ion signaling across multiple organ systems in both healthy and diseased states highlight its importance in mammalian biology.

Alterations to metallostasis are implicated in cancer and several neurodegenerative diseases; but what has become clear with the evolution of techniques used to investigate metals, is that this involvement is more complex than changes in overall levels and more often involves the above complex machinery including specific metal transporters, storage proteins and metal chaperones: Incorrect metal distribution, compartmentalisation and metal protein interactions have been implicated in diseases. Therefore, bulk measurement of metal content in biofluids and tissue samples reveal only the ''tip of the iceberg'', with most of the important changes occurring on a microscopic and biochemical level. Each of the major proteins implicated in these disorders interacts with biological d-block metal ions. Changes in metal distribution, cellular deficiencies, oxidative stress or sequestration all present abnormalities that can be corrected also in animal models by small molecules.

Metal dyshomeostasis and an oxidizing cellular environment are hallmarks of cancer cells. As a result, substantial research has been dedicated to understanding and manipulating copper in cancer to prevent cancer progression.

Delivery of copper to cancer cells, takes advantage of the oxidizing environment in cancer cells, which is less equipped to buffer increases in copper, to cause cell death.

Efforts to rigorously identify the molecular mechanisms of copper signaling in other tissues, especially the brain, will advance the field of copper signaling by connecting intriguing observations about copper-related phenomena, such as learning and memory [3], to molecular targets that may be subjected to pharmacological and/or genetic manipulation, against, for instance, Alzheimer's diseases.

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Valuable insights into the heme protein structure-function relationships via resonance Raman spectroscopy

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Raman spectroscopy is a powerful technique, which provides information on bond strength, angle and geometry of the molecule of interest. Resonance Raman (RR) spectroscopy is of particular interest in the analysis of biomolecules containing a chromophore, as it permits the intensification of the Raman bands belonging to the chromophore. In fact, the coincidence of the incident laser excitation wavelength with the electronic transition energy of the chromophore (resonance effect) leads to a considerable intensity enhancement of the Raman vibrational modes of the chromophore of interest (about 102–106 fold). The enhancement mechanism of Raman scattering by heme proteins has been reported in many reviews [1,2]. RR spectroscopy has a number of characteristics that have been advantageous in its application in the field of heme proteins: (i) it requires only very small quantities of protein (samples are normally~40μL, 30–50 mM); (ii) it can be used easily for proteins in aqueous media, hence reflecting physiological conditions, since the water Raman spectrum is very weak; (iii) it is able to enhance only the vibrational modes of a chosen chromophore in proteins with multiple chromophores. In particular, excitation in the Soret and Q bands leads to the intensification of the heme vibrational modes, whereas by excitation in the CT bands, intensification of the Fe–ligand stretching modes is obtained. RR has been demonstrated to be an extremely informative technique in probing heme protein active site structures [3,4]. In particular, in combination with site-directed mutagenesis, its marked sensitivity to small structural changes in the heme pocket has enabled it to give considerable insight into the roles of active site amino acids and provided important information on protein function, flexibility and stability [5-7]. A general introduction to the technique, with particular attention to the information that RR can provide on the study of heme proteins will be given. Moreover, examples will be provided showing the application of resonance Raman microscopy on protein single crystals to highlight not only the artifacts induced by the crystallization process [8], or the conformational alteration induced by cooling [9-10], but also our recent results showing that the combined spectroscopic/crystallographic approach is a powerful new weapon in the structural biologist's armamentarium.

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Reactivity and (pseudo-)enzymatic properties of hemoproteins: the case of heme-albumin

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Human serum albumin (HSA), the most prominent plasma protein (~7.5×10−4 M), (i) controls the plasma oncotic pressure and normalizes fluid distribution between the body compartments, (ii) acts either as a depot or as a carrier of endogenous and exogenous compounds, (iii) increases the solubility of hydrophobic compounds in plasma, (iv) affects drug pharmacokinetics, (v) inactivates highly reactive compounds by affecting their lifetime, and (vi) displays (pseudo-)enzymatic properties.
HSA is assembled by three structurally-similar domains (indicated as I, II, and III), which are built by 10 α-helices that are packed in the subdomains A and B containing 6 and 4 α-helices, respectively. HSA displays nine main ligand binding sites, the most relevant clefts are those recognizing the fatty acids (FAs; FA1 to FA9). In particular, the FA1 site (positioned in subdomain IB) is the third major binding region of HSA, hosting endogenous and exogenous ligands such as the heme. In fact, under physiological and pathological conditions, HSA plays a pivotal role in heme scavenging, acquiring globin-like properties.
Ferrous HSA-heme-Fe (HSA-heme-Fe(II)) binds reversibly NO and CO, but is quickly oxidized by O2. Moreover, HSA-heme-Fe(II) catalyzes the conversion of NO2− to NO, and nitrosylated HSA-heme-Fe(II) (HSA-heme-Fe(II)-NO) facilitates peroxynitrite scavenging and O2 detoxification. Furthermore, ferric HSA-heme-Fe (HSA-heme-Fe(III)) accelerates the conversion of peroxynitrite to NO3−, displays catalase and peroxidase activities, and undergoes reductive nitrosylation.
The allosteric modulation of the ligand binding and (pseudo-)enzymatic properties of HSA-heme-Fe(II) and HSA-heme-Fe(III) reflects the functional link between the FA1, FA2, and FA7 sites. Thus, ligand (e.g., drug) binding to the FA2, and FA7 sites shifts allosterically the penta-to-six coordination equilibrium of the heme-Fe atom from the highly reactive penta-coordinated derivative towards the low reactive species showing the six-coordinated metal center. Of note, the allosteric modulation of heme-Fe-based properties of HSA-heme-Fe(II) and HSA-hemeFe(III) by drugs, represents a relevant issue in the pharmacological therapy management.
The presentation will deal with the molecular mechanisms at the root of the allosteric modulation of the heme-based reactivity of HSA-heme-Fe by drugs.

When radiochemistry meets medicine: radiopharmaceuticals and molecular imaging

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The term 'radiopharmaceuticals' denotes a class of drugs containing a radionuclide routinely used in Nuclear Medicine (NM) practices for diagnosis and/or therapy. They are mainly small organic or inorganic compounds with definite chemical structure, or more rarely are macromolecules such as proteins, monoclonal antibodies or their fragments, that are not stoichiometrically labeled with a radionuclide.[1,2]

According to World Nuclear Association more than 10,000 hospitals worldwide use radioisotopes in medicine for several disorders, and approximately 90% of the procedures are for diagnosis to give information about the function of specific organs or tissues and to evaluate biochemical-metabolic processes.[1] In developed countries, the occurrence of diagnostic nuclear medicine is 1.9% per year, and the frequency of therapy with radioisotopes is about one-tenth of this. It is estimated that the use of radiopharmaceuticals in diagnosis is growing at over 10% per year.[3]

Positron Emission Tomography (PET) and Single-Photon Emission Computed Tomography (SPECT) are the two main methodologies currently used in diagnostic imaging. The non-invasive nature of these technologies, together with the ability to provide, through the radiotracer accumulation, a rapid evaluation of organ/tissues functioning from out-site the body, make PET and SPECT two powerful diagnostic tools. Radioactive probes, actually offer significant advantages over existing less sensitive diagnostic methods (***e.g*** MRI, XRay CT etc) as regards imaging of specific molecular targets, in particular those present at low concentrations. The benefits of using such diagnostic methods, are that they enable the early detection of disease, facilitate expedient delivery of therapies, allow for selection of the most appropriate treatments, and are a way to assess early the efficacy of a therapy. These unique capabilities improve patient outcomes and safety, and can reduce the hospital stays thereby helping to diminish the high cost of modern healthcare.

Nowadays, molecular imaging is a hot topic in radiopharmaceutical applications in particular in oncology, because it is increasingly perceived as a tool to support go/no-go decisions during the development of new oncology drugs. Imaging can provide additional information about, target validation; tumor targeting; whole-body target expression and drug distribution; pharmacokinetic features such as central nervous system penetration; and pharmaco-dynamic effects.

This unique application, demand for designing and developing of high sensitive and specific imaging agent, namely 'target specific' radiopharmaceuticals (usually radiolabeled peptides, drug or other molecules) able to detect and identify his own molecular target by specific mechanisms as receptor binding, biochemical or enzymatic interactions. Needless to say their design and development require an interdisciplinary process involving different fields of natural science and medicine.

Radiolabeling can be performed using a variety of radionuclides, which are preferably matched to the compound on the basis of size and half-life.[4] Many of the useful radionuclides are metals, spanning all parts of the periodic table, and most relevant compounds are coordination complexes. Among these, technetium-99m for its reach chemistry, optimal nuclear properties and low cost easy availability, is the most common radioisotope in NM being used in different chemical forms in more than 85% of the nuclear medicine procedure for: cardiac, bone, lung, kidney, liver and brain imag-

ing; tumor and metastatic lesions imaging; preoperative SPECT imaging and RadioGuided Surgery. Hence, inorganic chemistry and organometallic chemistry play a key role in both the design and development of metal based radiopharmaceuticals and the development of efficient technologies to firmly incorporate a radionuclide into a target specific molecule leaving unaltered the biological property of the native molecule is the heart of the modern radiopharmaceutical research.[4-9]

This presentation briefly introduces the most relevant radionuclides and their utility in the radiopharmaceutical and NM fields.

An overview on the different approaches to design high performance perfusion and target specific imaging agents, which includes the description of the mostly apply labeling procedures, and a snapshot of the current research in radiopharmaceutical field are also provided.

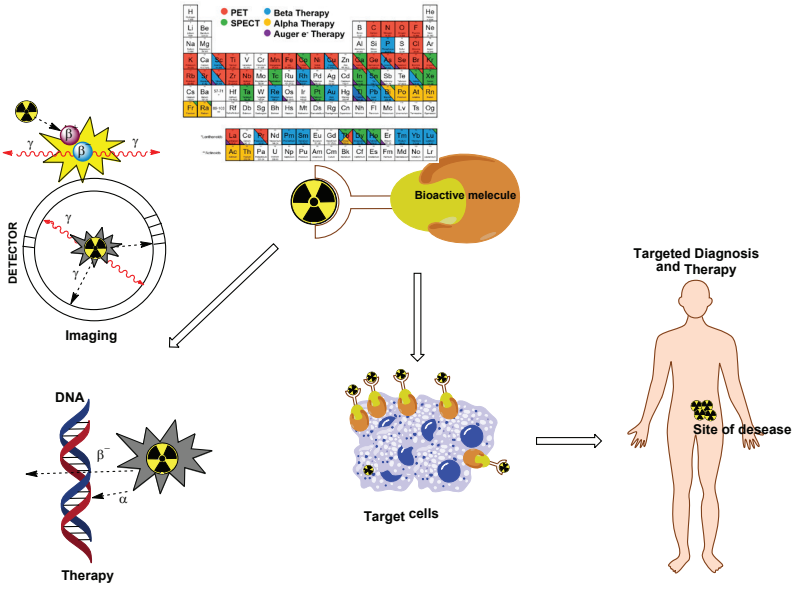

***Figure 1**: Radiometals and their Nuclear Medicine applications*

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How to apply bioinformatics methods to metalloproteins

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Metalloproteins are essential to life and widespread in organisms. Dysregulation of the intracellular concentration and distribution of metal ions is associated with important diseases such as cancer and neurodegenerative disorders.
Here we will address the application of bioinformatics methods to the prediction of metal-binding sites in proteins (i.e. the identification of metalloproteins in proteomes), the determination of metalloprotein functional networks, and the analysis of the structure-function relationship in metal sites. In addition, we will discuss the MetalPDB database of metal sites in biological macromolecules (http://metalweb.cerm.unifi.it/) [1].
The tools for the above tasks include:
• The MetalPredator server for the identification of iron-sulfur cluster binding proteins from protein sequence(s) (http://metalweb.cerm.unifi.it/tools/metalpredator/) [2]
• The CheckMyMetal server for the correct identification of metal ions in crystallographic protein structures (https://csgid.org/metal_sites) [3]
• The RDGB tool for the identification of conserved pathways in organisms [4]
• The MetalS2 [5] and MetalS3 [6] servers for the comparison and the database search of metal-binding sites of metalloproteins
Along with the above tools, we will discuss the use of bioinformatics methods not specifically designed to the case of metalloproteins, to identify any relevant caveats and limitations.

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Proteomics approach to system biology: the case of multifactorial diseases

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Proteomics, together with modern transcriptomics approaches such as next generation sequencing, is a quasi-global post-genomic approach. In particular, proteomics aims at the protein-level description of the response of a system to a perturbation, such as drug treatment, exposure to xenobiotics or more generally a disease. Biological systems are complex systems, where this response results from nonlinear regulation of protein levels. In this context, a new vision has been developed, where biology is considered at the level of complex nonlinear system, i,e, systems biology. Thus, the cell/tissue response to a perturbation is an emergent property of the system, rather than a resultant.
The analysis of emergent properties should allow researchers to propose an underlying mechanism that leads to the observed phenotype. In classical biology, one starts from a hypothesis, designs the experiments and analyzes results to confirm or falsify the starting hypothesis. Such a reductionist method, though, does not apply very well to complex systems, with rare exceptions where there is a single strong causative effect (so-called molecular diseases). In the field of multifactorial diseases (neurodegeneration, cancer, metabolic diseases, etc.) holism should be preferred to reductionism. This means collecting observations from the system and then induce a hypothesis proposal. Although inductive reasoning was strongly criticized by epistemologists such as Bertrand Russell and Karl Popper, we have now statistical tools and bioinformatics assets that allow us to assign an expectation value to any mechanistic hypothesis we propose [1].
Proteomics was born basically as a top-down approach. Briefly, proteins were separated by a high-resolution, high capacity technique, usually two-dimensional electrophoresis, and quantified by gel densitometry. Proteins showing significant variations were enzymatically digested and resulting peptides identified by mass spectrometry. Indeed, technological growth in ion sources and mass analyzers was the driving force in proteomics. The availability of nano-electrospray ionization sources coupled with ultra-high-performance chromatographic columns, together with very-high resolution mass analyzers, opened the door to the quantitative analysis of very complex mixtures. Consequently, a bottom-up approach can be attempted. In shotgun proteomics the whole proteome is fragmented into peptides and analyzed as a whole. Bioinformatics tools identify a peptide sequence and associate it to a protein sequence. Monte-Carlo simulations on a decoy database provide a false discovery rate for each identification, both at the peptide and at the protein level. Interactomics is shedding new light on protein complexes truly involved in biochemical pathways and how their alteration can lead to dysfunctionality (in disease pathogenesis, for example). Terminomics is revealing the function of new discovered proteoforms and attributing a novel role to proteolysis [2].
Modern proteomics lead to large datasets that are sparse in nature. In other words, only a subset of observed features correlate with the phenotype. This problem may be addressed to as the feature selection problem. There are several methods to sparsify a dataset, i.e., to eliminate features that do not correlate with our perturbation. Some methods are based on univariate tests, whereas others are based on building a multivariate classificator, either supervised or unsupervised [3].
Eventually, the signature built in this way is the starting point for a functional analysis. Instruments based on the Fisher's exact test lead to an over-representation analysis where any biochemical

pathway present in a pathway database is compared to a list of proteins arising from a feature selection procedure as briefly indicated above. Additionally, graph theory applied to proteomics data is the starting point to build and analyze complex protein networks, both based on functional or physical interactions [2].

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X-ray spectroscopy methods in Bioinorganic Chemistry

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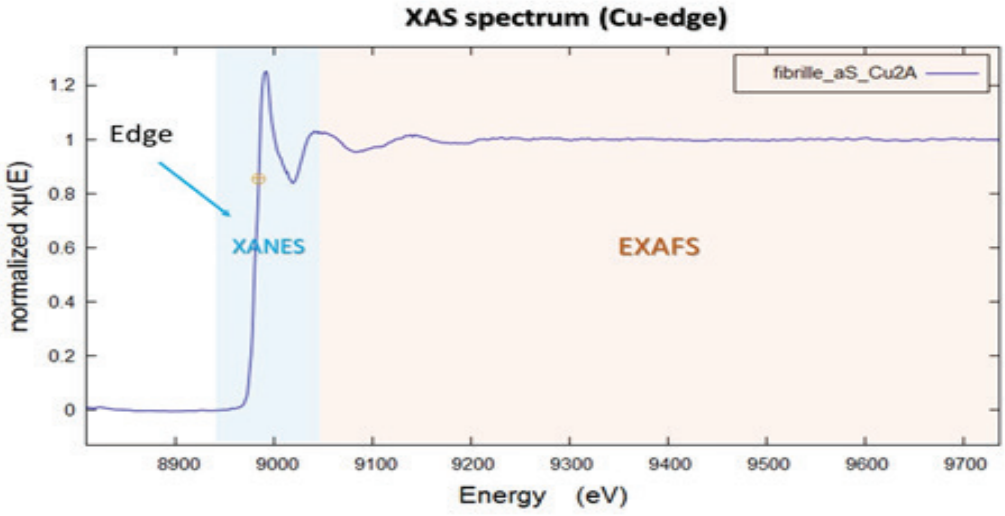
Metal ions are involved in most important biological process like photosynthesis, cellular respiration, gene expression and regulation, global nitrogen, hydrogen and carbon cycles, biosynthesis of natural products [1-2]. Thanks to their peculiar chemical properties, metal ions are cofactors of enzyme and proteins deputed to accomplish the most difficult chemical reactions occurring in living systems. On the other hand, every organism might become exposed to toxic concentrations of metal ions in the environment.

Understanding the details of the chemistry of metal ions in organisms and their beneficial and detrimental effects is of paramount importance in order to exploit the power of metal ion chemistry to design and develop new compounds for many applications, such small molecular activation, devices for sustainable energy production, new pharmaceuticals, environmental monitoring, bioremediation.

To achieve these goals, we need multiple disciplinary approaches to characterize the structures of metal-binding sites in proteins and nucleic acids and their regulation or reaction mechanisms, and then apply insights gained from the study to harness power of the native systems or design artificial mimetics.

Figure 1: example of a whole XAS spectrum showing the edge, near-edge (XANES) and EXAFS (Extended



X-ray Absorption Fine Structure) regions.

Among the available techniques able to provide deep insights into metallo-biochemistry, X-ray absorption spectroscopy (XAS) offers unique capability to describe structural and electronic properties of metal ions bound to proteins and enzymes as well as in any other matrix of biological origin. XAS has the unique ability to probe the local environment (up to ~ 5.0 Å in typical biological sam-

ples) of any element having its X-ray absorption edge within the energy range available at modern synchrotron beamlines (i.e. ~ 3.0 – 35.0 keV) [3-4]. The technique is elemental specific as it involves the excitation of a core electron of a given atom species to continuum and studies the near-edge structure (XANES) and the modulation of the X-ray absorption coefficient at energies beyond the edge (extended spectrum: EXAFS; Figure 1). XAS gives the possibility to study multiple elements present in the same sample independently of the physical state of the sample.

XAS is also perfectly suited to be coupled to other structural techniques like NMR or X-ray crystallography in order to obtain a deeper and more accurate view of the system under study. Numerous examples are present in the literature [5-7].

Recent advances in beamline design have allowed the availability of micro/nano X-ray beams (1µm – 20 nm) that allow performing scanning X-ray microscopy on cells and tissues, coupling direct imaging with X-ray fluorescence recording of the different elements distribution in cells. In this way, images showing, not only the element distribution, but also the coordination environment of a given element [8-9].

The advent of X-ray free electron lasers introduces the exciting possibility to study chemical reactions at the femtosecond scale by time-resolved X-ray spectroscopy [10].

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Molecular Recognition at Organic-Inorganic Interface in Biomineralization Processes

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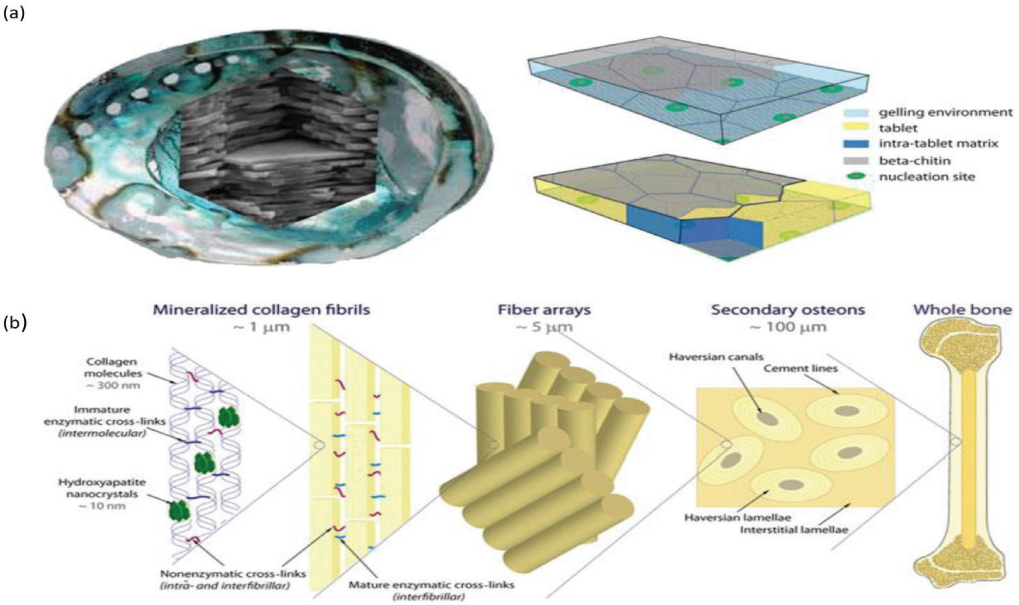
Solid state bio-inorganic chemistry is an integral aspect of biology as the aqueous phase organic and inorganic reactions of conventional biochemistry. More than 70 biominerals have been identified in organisms, the most common being calcium carbonate and phosphate salts that are used in conjunction with organic macromolecules such as collagen and chitin to give structural support to shells and bones. Salts of silicon, strontium, barium and iron are also common. The biominerals are formed by cellular processes to produce organized structures in cells ranging from bacteria, algae and protozoa to the osteoblasts of bone. The biominerals may be in membrane-bound vesicles within cells, in the mucilaginous layers of cell walls in bacteria, or impregnated in macromolecules in extracellular space. In all these structures, the inorganic crystals are deposited in orderly arrays in association with a matrix of organic macromolecules. There is a growing body of information on the structure, composition and synthesis of the macromolecules, but the key and exciting question is to identify the molecular processes that produce minerals of precise form with uniform particle size, novel crystal morphology and specific crystallographic orientation. How do the proteins and polysaccharides of the substrate interact to regulate solid-state chemical reactions?

Because the interactions resulting in controlled crystallization are tailored to biological function, the underlying molecular processes must have a specificity analogous to those involved in the reactions of aqueous-phase biological molecules.

The influence of organic macromolecules is important in the regulation of nucleation and growth



of the biomineral and in the resulting specificity in crystal morphology and particle aggregations, but the molecular interactions inherent in these processes are not well understood. Current ideas focus about the role of organic macromolecules in initiating the nucleation and growth of inorganic solids in biology [1]. This presentation will give a brief overview on the current knowledge on the nucleation, growth and organization of crystals within living organisms, either inorganic or organic crystalline structures.

Three examples will be presented as representative cases of study (Fig. 1). The biomineralization in coral will be focused on the role of the intra-skeletal organic molecules on the crystallization of calcium carbonate [2]. The structure of the nacre will be illustrated pinpointing the role of the diverse organic component present in proximity of the mineral phase [3]. The bone formation will be presented paying attention of how the organic matrix in involved in the definition of bone hierarchical structure [4]. Finally, current trends and future perspectives of biological crystallization linked to its potential applications in fields such as biomedicine and materials science will be illustrated [5]

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New metal based nanoparticles coated with biomolecules for targeted therapy

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Recent advances in nanotechnology and biotechnology have contributed to the development of engineered nanoscale materials as innovative prototypes to be used for biomedical applications and optimized therapy. Due to their unique features, including a large surface area, structural properties, and a long circulation time in blood compared with small molecules, a plethora of nanomaterials has been developed, with the potential to revolutionize the diagnosis and treatment of several diseases, in particular by improving the sensitivity and recognition ability of imaging contrast agents and by selectively directing bioactive agents to biological targets. Potential advantages of engineered therapeutic nanoparticles are the ability to: revert unfavorable physicochemical properties of bioactive molecules to desirable biopharmacologic profiles; improve delivery of therapeutics across biological barriers and compartments; control release of bioactive agents; enhance therapeutic efficacy by selective delivery of therapeutics to biological targets; and perform theranostic functions by combining multimodal imaging and simultaneous diagnosis and therapy into multifunctional nanoplatforms. (Lee DE et la 2012)

Over the last few decades, many original nanosystems have been developed based on various components from metals to proteins, including carbon, silica oxides, metal oxides, nanocrystals, lipids, polymers, dendrimers, and quantum dots, as well as newly developed materials. (Bae KH et al 2011; Kim BY et al. 2010).

Focusing on cancer, promising nanosystems have been designed to overcome the lack of specificity of conventional chemotherapeutic agents, as well as for early detection of precancerous and malignant lesions. Nanoparticles accumulate preferentially in the neoplastic tissues as a result of the enhanced permeability and retention (EPR) phenomenon

(Maed H et al. 2000). The EPR is based on the nanometer size range of the nanoparticles and two fundamental characteristics of the neoplastic tissues, namely, the leaky vasculature and impaired lymphatic drainage (passive targeting).

Since passive targeting does not necessarily assure the internalisation of nanoparticles by the targeted cell, therefore nanoparticles are modified with molecular targeting ligands for active targeting. In this respect, polymeric nanoparticles allow for versatile modification possibilities, and can act as functional platforms for the assembly of well defined multifunctional structures. Active drug targeting involves the use of a variety of biological molecules including functional peptides, nucleic acids, and small ligand compounds able to bind to specific tumor biomarkers involved with tumor molecular profiling behaviour and its clinical outcome facilitating the specific targeting of a carrier to the malignant and surrounding cells. (Akhter S et al. 2011)

Nanothecnologies have also provided opportunities for the development of theranostic agents for detecting and treating cancer. Advance in the development of metallic nanoparticles (MNP) has significantly impacted the development of systems both as therapeutic and diagnostic agents. MNPs are attractive options for theranostic application due to their stability on which desired multiple functionalities can be fabricated.

In particular the lesson will summarize the properties, advantages, disadvantages and characteristics of metal nanomaterials and focus on application along with potential side effects and the future

perspective of some novel systems in the field of targeted therapy. (Depalo N et al 2017; Valente G et al. 2016).

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