

BOOK OF PROCEEDINGS



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DI
CALORIMETRIA E ANALISI TERMICA

AICAT 2008

GICAT

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CALORIMETRIA E ANALISI TERMICA
Società Chimica Italiana

THE XXX NATIONAL CONGRESS ON CALORIMETRY, THERMAL ANALYSIS AND CHEMICAL THERMODYNAMICS

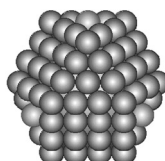
December 9th – 12th, 2008

Pisa – Italy



SOCIETÀ CHIMICA ITALIANA

Società Chimica Italiana
Divisione di Chimica Fisica



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FOREWORD

This volume collects the scientific papers presented at the XXX National Conference of Calorimetry and Thermal Analysis, AICAT2008, held in Pisa from the 9th to the 12th of December 2008.

The conferences of Calorimetry and Thermal Analysis are organized yearly by the Italian Association of Calorimetry and Thermal Analysis (AICAT) and by the Interdivisional Group of Calorimetry and Thermal Analysis (GICAT) of the Italian Chemical Society (SCI).

The AICAT Conference comes back to Pisa after exactly twenty years. Pisa indeed held the tenth edition and the AICAT2008 organizing committee is particularly grateful to both AICAT and GICAT for choosing Pisa again as location for the thirtieth.

Pisa is a city of deep cultural traditions, rich in study and knowledge, where an ancient and famous University, two other highly reputed academical institutions and prestigious research centres are located. Its atmosphere is that of a big open campus, permeated by the wide and varied culture derived from the exchange of knowledge and experience of scholars operating in the most diverse fields. We hope that this atmosphere can provide a propitious surrounding to the meeting and offer us a further occasion to share notions and experiences; in one word, to share culture.

In organizing AICAT2008 we chose to dedicate a special attention to those fields in which calorimetric methods are used in conjunction with other experimental techniques and that therefore represent an example of multidisciplinary approach to research. In particular, the program contains sessions devoted to the use of calorimetric methods for studies in the fields of solutions and mixtures, food, pharmaceuticals, proteins and other biological macromolecules, polymers and biomaterials, environmental safety and cultural heritage.

A session of the Conference is devoted to the memory of Prof. Sergio Cabani in occasion of the tenth anniversary of his passing. Prof. Cabani has been one of the AICAT founders and greatly contributed to the development of research in the field of thermodynamics and calorimetry, in Pisa as well as nationally and internationally.

We thank all those who helped us in organizing the Conference: the University of Pisa, the Faculty of Sciences and the Department of Chemistry and Industrial Chemistry gave support for the logistics and the publication of this volume; AICAT and the Division of Physical Chemistry of SCI provided some grants to promote the participation of young researchers; the National Interuniversity Consortium for Materials Science and Technology (INSTM) supported part of the scientific activities of the congress; the Chorus of the University of Pisa will perform a concert for the participants.

During the annual Conference the AICAT-SETARAM award and the Alberto Lucci award will be handed over. The AICAT-SETARAM award, at its third edition, recognizes an internationally famous scientist who has distinguished himself for his research in the field of calorimetry. This year the award was assigned to Prof. Jean Pierre Grolier from the University of Clermont Ferrand. Professor Grolier was selected as the 2008 AICAT-SETARAM Award recipient for his outstanding contribution to the study of thermal properties of organic mixtures and to the development of new techniques for calorimetric measurements at very high temperatures and pressures. The AICAT-LUCCI prize, which instead rewards, for his works in the field of calorimetry, a young and deserving researcher under 35 years of age, was assigned to Dr. Celia Duce. Remarkable were her contributions to the thermodynamic and

calorimetric studies of solvation and mixing properties, of molecular interactions in biological systems and the thermodynamic and kinetic studies on metallo-receptor systems for transport and activation of molecular oxygen.

The organizers, AICAT and GICAT thank SETARAM and the Lucci Family for sponsoring the two prizes.

The organizers also thank SETARAM, TA Instruments and Mettler Toledo for their financial contribution.

I strongly hope that the scientific proceedings of this meeting can fruitfully represent the state of the art in Italian calorimetry and provide a useful working tool for researchers in the field.

Maria Rosaria Tiné
Chair of the Conference

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PROGRAMME

Tuesday, December 9th

14.00 **Registration**

16.00 **Opening Ceremony**

16.30 **Setaram Award**

16.40 Setaram Award Lecture

Jean-Pierre Grolier

From Fluids to Polymers: Using Pressure for Controlling their Thermophysical Behavior (AL1)

17.40 **Lucci Award**

17.50 Lucci Award Lecture:

Celia Duce

Application of Calorimetry to Processes in Solution (AL2)

18.30 **Christmas Songs, University of Pisa Chorus**

19.30 **Welcome Party**

Wednesday, December 10th

SOLUTIONS, MIXTURES AND CHEMICAL THERMODYNAMICS LIFE SCIENCES, PHARMACEUTICALS AND FOOD (Life)

In honor of Prof. Sergio Cabani

9:00 **Presentation of the Memorial Symposium**

9.30 **Plenary Lecture**

Enrico Rizzarelli

The Chemistry of Metal Ions Binding to β -Amyloid: is there Sufficient Evidence to Elucidate a Role for Copper(II) and Zinc(II) in Alzheimer's Disease Metallo Biology? (PL1)

10.30 **Key Lecture**

Anthony E. Beezer

Microbial Microcalorimetry – Some Recent Results and Speculation (KL1)

11.00 **Coffee Break**

11.30 **Plenary Lecture**

Alberto Schiraldi

Water Activity: Experimental Approach and Data Treatment For Systems of Biological Interest (PL2)

- 12.30 **Key Lecture**
Paolo Gianni
Interaction of surfactants with macromolecules. Calorimetric investigation of bile salts with human serum albumin (KL2)
- 15.00 **Key Lecture**
Elpidio Tombari
Heat capacity relaxation in pharmaceutical molecular glasses and its utility in understanding their stability against crystallization (KL3)
- 15.30 **Oral Presentations**
Andrea Melchior
Coordination of N-donors to Silver (I) in Different Solvents: Experiment and Theory (Life-O1)
- 15.50 Giuseppe Bellavia
Calorimetric Study of Myoglobin Embedded in Disaccharide-Water Matrixes (Life-O2)
- 16.10 Emma Chiavaro
The Use of DSC for Analysis and Characterization of Olive Oil (Life-O3)
- 16.30 Angiuli Marco
Calorimetric Authentication of Monocultivar Extra-Virgin Olive Oil (Life-O4)
- 16.50 **Coffee Break**
- 17.10 **AICAT Meeting**

Thursday, December 11th

POLYMERS AND BIOMATERIALS
(Poly)

- 9.15 **Plenary Lecture**
Attilio Cesaro
Structural relaxation and mobility in nanostructured soft-condensed biomatters: polymers and carbohydrates (PL3)
- 10.20 **Oral Presentations**
Giuseppe Lazzara
Melting, Thermal Degradation and Structural Characterization of Nanocomposites Based on Homopolymers or Block Copolymers (Poly-O1)
- 10.40 Michele Alderighi
AFM temperature study of PLLA-PEG-PLLA copolymer (Poly-O2)
- 11.00 **Coffee Break**

11.20 Oral Presentations

Marcella Ferri

Di-(2-Ethylhexyl)-Phthalate Leakage from Endotracheal Tubes after Application in High Risk Newborns: a Chemical-Physical Investigation (Poly-O3)

11.40

Anna Maria Piras

Polymeric Nanoconstructs for Controlled Release of Bioactive Agents (Poly-O4)

12.00

Sangram Keshari Samal

Thermal and Swelling Analysis of PVA/CNTs Membrane (Poly-O5)

12.20

Luigi Martino

Calorimetric Analysis of The Interaction of The Human La Protein With Rna Targets (Poly-O6)

12.40

Vincenzo Ierardi

*Controlled Phase Separation in Conjugated Polymer Blends Induced by Thermolithography (Poly-O7)*15.00 **Poster Session**17.00 **Coffee break**17.20-19.00 **Selected Poster Discussion****Friday, December 12th****ENVIRONMENTAL, SAFETY AND CULTURAL HERITAGE
(Heri)****9.15 Plenary Lecture**

Claudia Sorlini

*Viable Bacteria for the Biorecovery of Deteriorated Cultural Heritage Surfaces. The case of the Monumental Cemetery Frescoes of Pisa (PL4)***10.15 Oral Presentations**

Giuseppe Della Gatta

DSC Complemented by SEM, IR and NMR, an Effective Diagnostic Tool for Parchments' Damage Assessment and Preservation Monitoring (Heri-O1)

10.35

Luciano Lepori

On the Increase of Fossil CO₂ in the Atmosphere (Heri-O2)

10.55

Lucia Gigante

*Thermochemical Evaluation of The Reactivity of Some Organic Azides (Heri-O3)*11.15 **Coffee Break**

INORGANIC CHEMISTRY, CATALYSIS AND NEW INSTRUMENTS
(Inor)

11.40 Oral Presentations

Stefano Vecchio

Kinetic Study of Decomposition for Co(II)- and Ni(II)-Phenanthroline complexes Intercalated in γ -Zirconium Phosphate (Inor-O1)

12.00 Simona Delsante

Calorimetric Investigations of Reactive Metallic Systems Containing Sm, Mg, Zn and P (Inor-O2)

12.20 Jasbir Singh

Process Development using Calorimetry – breaking down barriers with real time, on-line calculation (Inor-O3)

12.40 Closing Ceremony

15.00 Guided visit of the Monumental Cemetery Frescoes of Pisa

20.00 Social Dinner

*The XXX National Congress on
Calorimetry, Thermal Analysis and Chemical Thermodynamics*

CONTRIBUTIONS

*The XXX National Congress on
Calorimetry, Thermal Analysis and Chemical Thermodynamics*

AWARD AND PLENARY LECTURES

FROM FLUIDS TO POLYMERS: USING PRESSURE FOR CONTROLLING THEIR THERMOPHYSICAL BEHAVIOR

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Introduction

The title could as well be "A journey under pressure from basic to industrial developments". This contribution reports most recent results obtained by the author over the last 40 years or so in the thermodynamics of gases, pure liquids, liquid mixtures and polymers. Scanning transitiometry is at the centre of several new developments to generate original unprecedented data over extended ranges of temperatures and pressures. The oscillation of temperature superimposed to the temperature control of reaction calorimetry (RC), on the one hand and the modulation of temperature superimposed to temperature differential scanning calorimetry (DSC) on the other hand is a major step forward to either properly monitor polymerization reactions or to unambiguously identify glass transitions from other overlapping phenomena. The combination of a weighing technique (vibrating-wire technique) with a pressure decay technique (pVT - technique) allows estimating simultaneously, *in situ* on the same polymer sample, the gas solubility and the concomitant polymer swelling. Selected examples serve to illustrate the importance of such techniques to produce key data and information for both fundamental and applied thermodynamics.

Experimental Developments

Scanning transitiometry

This now well established pVT -controlled scanning calorimetry, or simply scanning transitiometry [1], is particularly adapted to investigate transitions by scanning one the three thermodynamic variables. Scanning transitiometers allow the simultaneous determination of thermal and mechanical responses of investigated systems, perturbed by the variation of an independent thermodynamic variable while the other independent variable is kept automatically constant. Determination of thermodynamic derivatives over extended ranges of pressure and temperature, corresponding to four thermodynamic situations is thus possible by simultaneous recording of both the heat flow (thermal output) and the change of the dependable variable (mechanical output). Then, making use of the respective related Maxwell relations one readily obtains the main thermophysical properties as follows: *a)* scanning pressure under isothermal conditions yields the isobaric thermal expansivity α_p and the isothermal compressibility κ_T as functions of pressure at a given temperature; *b)* scanning volume under isothermal conditions yields the isochoric thermal pressure coefficient β_V and the isothermal compressibility κ_T as functions of volume at a given temperature; *c)* scanning temperature under isobaric conditions yields the isobaric heat capacity C_p and the isobaric thermal expansivity α_p ; *d)* scanning temperature under isochoric conditions yields the isochoric heat capacity C_V and the isochoric thermal pressure coefficient β_V . A scheme of the instrument (from BGR TECH, Warsaw, Poland) constructed according to the principle of scanning transitiometry is shown in Fig. 1. The actual operating ranges of scanning transitiometry are respectively $173\text{ K} < T < 673\text{ K}$ and $0.1\text{ MPa} < p < 200\text{ MPa}$ (or 400 MPa).

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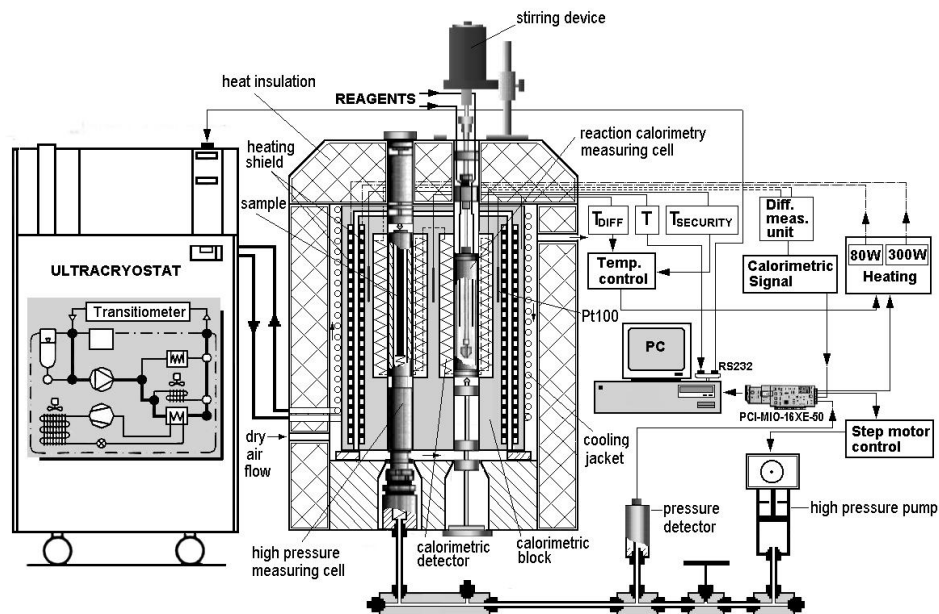


Figure1. Schematic view of a scanning transiometer showing the differential calorimetric detector and peripherals

Oscillating and Modulated Temperature Calorimetric Techniques

In the case of polymer synthesis, Reaction Calorimetry (RC) is an appropriate technique for on-line process monitoring, since polymerization reactions are highly exothermic. Viscosity increase and fouling at the reactor wall are typical features of many polymerizations. The overall heat transfer coefficient also changes drastically when viscosity increases and affects the accuracy of calorimetric measurements. Two methods were developed [2] for concomitant determination of heat of reaction ΔH_R and heat transfer coefficient k , namely intermediate jacket method and *Temperature Oscillating Calorimetry* (TOC). Reactions were performed with two different reaction calorimeters, *i.e.* a power compensation calorimeter with Peltier elements for controlling the temperature of intermediate thermostat and a Calvet type high sensitivity differential calorimeter, respectively. Comparison was made with data obtained using a classic isoperibolic RC. In addition, the combination of RC and simultaneous *in situ* Vis-spectroscopy for controlling the particles size and shape through the fine tuning of reaction parameters was advantageously realized [3]. Another major improvement to conventional DSC has been the modulation of temperature during the temperature scan to produce a new technique, *Modulated Temperature Differential Scanning Calorimetry* (MTDSC). This technique employs a modulated (usually a sinusoidal change) and the measured total heat flow signal can be separated, by a deconvolution method, into two components (as differential signals), the heat capacity-reversing contribution and the non kinetic-reversing contribution. MTDSC is now a well established technique which allows asserting no ambiguously the glass transition temperature T_g , since this transition and its characteristic temperature selectively appear, from other transitions, only on the reversing component [4].

The Vibrating Wire(VW)-pVT Technique for Gas Solubility and Polymer Swelling

The understanding of {gas-polymer} interactions is essential in many industrial applications of thermoplastic polymers. Simultaneous measurement of gas solubility and polymer swelling has been obtained using a concept that has the particularity to combine two techniques [5,6], a vibrating-wire (VW) sensor and a pressure decay *pVT*-technique. The vibrating-wire sensor VW is employed as a force sensor to weigh the polymer sample during the sorption: the buoyancy force exerted by the pressurized fluid on the polymer depends on the swollen volume, ΔV_{pol} , of the polymer due to the gas sorption. The combined VW-*pVT* apparatus is designed to measure sorption of gases and the concomitant volume change of polymers at pressures up to 100 MPa from room temperature to 473 K.

Selected Results

Pure Fluids and Liquid Mixtures

Scanning transitiometry has served to obtain thermophysical properties of pure liquids and liquid mixtures over extended temperature and pressure ranges [7-9]. A striking result is the single crossing point shown by isotherms of isobaric thermal expansivity α_p for a pure fluid; such single point being now recommended as a severe test of equations of state. Furthermore measurements of thermophysical properties have been made near and above the critical point [10].

Thermophysical Properties of Polymers

As for liquids, thermophysical properties have been determined over extended *T* and *p*-ranges for polymers of different crystallinities; good agreement with equation of state calculations is very satisfactory [11]. Interactions gas-polymer have been extensively investigated with all techniques described above. In particular, sorption of gas in different polymers and polymer foaming have been documented with respect to polymers either used for making tubing (pipelines) for the petroleum industry [12] or used to develop light thermal insulating foams [13]. Namely, the thermal energy of gas-polymer interactions and the thermophysical properties of gas saturated polymers have been determined. In this context, transitiometry and MTDSC have been used to investigate the influence of gas sorption on the glass transition temperatures [14]. Current investigations concern the influence of thermodynamic (*p* and *T*) parameters on the auto-assembling of molecular organizations at the interface between polymeric and crystal liquid moieties in di-block copolymers to form nanoscale structures [15] used as templates in microelectronics. The respective role played by the hydraulic fluid transmitting pressure, neutral Hg, inert N₂ or chemically active CO₂, to tailor such structures is abundantly documented [16-18].

Conclusion

Newly developed experimental techniques, thermal methods and coupled techniques, permit to determine fluids as well as polymers properties in various conditions in terms of temperature and pressure, in presence or in absence of gas sorption. Typically, the obtained data constitute essential information to, for example, control the choice of polymers for industrial applications, in the petroleum industry, polymer foaming or microelectronics. In this respect, scanning transitiometry appears as a universal thermodynamic technique.

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APPLICATIONS OF CALORIMETRY TO PROCESSES IN SOLUTION

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This contribution is a summary of applications of calorimetric techniques to a few research subjects I have dealt with, in particular Isothermal Titration Calorimetry (ITC) and Differential Scanning Calorimetry (DSC) in solution.

A TAM heat-flow calorimeter equipped with a titration cell has been employed to obtain partial molar enthalpies and excess enthalpies, H^E 's, of mixtures of perfluoroalkanes + alkanes and ethers by means of a newly designed calorimetric technique and calculation procedure. [1, 2] This procedure was demonstrated capable to successfully replace the mix-flow technique, and to obtain precise enthalpies of solution at infinite dilution in addition to H^E . Moreover, Vapour-Liquid Equilibria (VLE), Liquid-Liquid Equilibria (LLE), and density measurements were performed on the same binary systems in order to fully characterise their thermodynamic behaviour (G^E , V^E). [3,4] Solute-solvent and solute-solute interactions have been examined by calculating and discussing solvation properties, in terms of Scaled Particle Theory, and Kirkwood-Buff integrals. The results were considered also in the framework of group additivity scheme and DISQUAC, an extended quasichemical group contribution theory. [1-5] ITC has been also used to investigate the self-assembly process of some self-complementary ionic oligopeptides in synergistic combination with viscosimetry, fluorescence, Atomic Force Microscopy (AFM) and Molecular Dynamics (MD) simulations. The interest on these systems lies in the capability of self-complementary ionic oligopeptides to spontaneously assemble by forming fibrils or stable macroscopic membranes. Moreover, their biocompatibility and biodegradability can be exploited in biotechnology applications (e.g. drug carriers, drug delivery systems, tissue engineering scaffolds). [6-7]

A DSC study on the conformation stability of α -crystallin in solution is going on. α -Crystallin is a protein of the vertebrate lens responsible for transparency, refractive power and structural stability. It is a multimer of about 800 kDa formed by two subunits, αA and αB , of approximately 20 kDa each. α -Crystallin subunits belong to the small heat shock protein (sHsp) family, a class of molecular chaperones of small monomer size (12–40 kDa) that can be induced by heat and other stress conditions. It has been shown that α -Crystallin exerts its chaperone action by preventing protein aggregation, by refolding the target protein into a functional conformation, or by preserving enzyme activity. [8]

Calorimetric data, in particular glass transition temperatures of (meth)acrylic polymers, have been used as a benchmark for a QSPR/QSAR method based on Recursive Neural Network developed in collaboration with the Department of Computer Science of the University of Pisa. Recursive Neural Networks deal with prediction tasks in a structured domain where compounds can be naturally represented. This approach allows for combining the flexibility and advantages of neural networks with the representational power of structured domains. [9-10]

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METAL IONS AND ALZHEIMER DISEASE

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Alzheimer's disease (AD) is characterized by the insurgence of extra cellular senile plaques and intracellular neurofibrillar tangles in the brain [1]. Plaques are mainly constituted of amyloid peptides (A β) containing 39-42 amino acidic residues generated proteolytically by a membrane protein (APP) [2]. A β forms both soluble oligomers and insoluble amyloid fibrils characterized by β -sheet conformations [3]. However, the genesis of A β and its derivatives is still being disputed. Some authors claim that oligomers inhibit neuronal vitality ten times as much as fibrils in neuroblastoma cells, whilst others maintain that A β fibrillar aggregates, rather than its amorphous aggregates, are mainly responsible for neuronal death [4]. The degree and the typology of aggregation would account for the toxicity level, thus suggesting that A β aggregation is the main event in AD pathology.

Consequently, the factors involved in A β aggregation have been extensively investigated. In this framework, the *Amyloid Cascade Hypothesis* [5], according to which AD would originate after an excess build-up of A β in the central nervous system (CNS), is the most widely accepted hypothesis. However, recently it has been shown that the increase of A β levels cannot solely cause AD, though A β central role is still thought to be out of discussion. If A β high levels were the only cause of the formation of toxic aggregates, it would be difficult to explain why amyloid deposits are found only in the brain since APP and A β form also in compartments other than the brain. Connecting plaques to the presence of A β (1-42) only is somewhat surprising since A β is a normal constituent of the cerebrospinal fluid (CSF). In addition, whilst the formation of amyloid plaques increases with age there are no evidences for a parallel increase of A β . Thus, changes depending on age very likely play a key role in the vents causing A β build-up in cerebral areas specifically involved in AD. On the other hand, the levels of the block *d* metal ions and of their transport and deposit proteins have been shown to increase significantly with ageing [6]. Mn, Fe, Cu and Zn levels in small animals have been reported to increase with ageing, and have been shown to vary within the various compartments of the brain. Hence, changes in the level and distribution of metal ions with ageing may play a role in AD insurgence. Recently, A β interaction with metal ions (specifically copper and zinc) has been hypothesized to initiate AD; this new hypothesis has been termed *The Metal Hypothesis of Alzheimer* [7].

Since the mid nineties, the interaction of A β (1-40) and A β (1-42) with Cu²⁺ and Zn²⁺ and the simultaneous increase of the *d* block metal ion concentration in the amyloid plaques of AD patients were thought to induce AD [8]. Recent results indicate that the dyshomeostasis of transition metal ions in the brain is associated with AD; in fact, *i.* endogenous synaptic Zn²⁺ contributes to the cerebral deposition of the amyloid peptide [9] whilst the suppression of Zn-T3, the Zn²⁺ specific transporter, leads to a reduction of plaque formation in animal models [10]; *ii.* amyloid plaques and cognitive deficit show up in animals that utilize water containing Cu²⁺ traces [11]. In contrast, high Cu²⁺ levels in the diet reduce A β concentration *in vivo* and inhibit A β aggregation *in vitro* [12]. Similarly to Cu²⁺,

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Zn²⁺ has a double effect on A β as it also shows a protective role against it [13]. Contrasting results have also been reported for *i.* the stoichiometry; *ii.* the affinity; *iii.* the coordination modes of metal ions; *iv.* the conformation changes induced by them; *v.* the coordination environments in both monomers and oligomers [14]. The need to have a reliable speciation for the species formed by A β with transition metal ions, their thermodynamic stability and coordination characteristics is borne out by findings showing that the protective or toxic action of metal ions depends not only on their concentration but also on the metal/peptide ratio [15]. The different species formed would be responsible for different morphologies with different toxicity, relating to the specific coordination geometry.

A β (1-42) N-terminus region, comprising the 1-16 amino acidic residues, has been shown to be the site of interaction with metal ions; even though A β (1-16) is more soluble than the parent 1-42 peptide, the study of the stoichiometry, the affinity and the coordination characteristics of its complexes with transition metal ions has been limited to metal/peptide ratios 1:1 due to the occurrence of a precipitate with larger metal/peptide ratios. A β (1-16) conjugation with polyethylene glycol (PEG) has made it possible to overcome solubility limitations and, consequently, to resort to potentiometry for an accurate thermodynamic study, that, in turn, has led to a reliable speciation of both Cu²⁺ and Zn²⁺ complexes (and their stability constants) as a function of both pH and metal/ligand ratios [16]. We have been able for the first time *i.* to obtain the stability constant values for each species; *ii.* to show that A β may bind four Cu²⁺ equivalents; the different contributions of each single species may explain the variability encountered in the literature. K_D values for Zn²⁺ complexes with A β (1-40) as well as A β (1-28) and A β (1-16) have been reported to range from 100 nmol/L to 300 μ mol/L while those for Cu²⁺ vary from 0.1 nmol/L to 10 μ mol/L. Though different reasons have been invoked to explain such variability, the binding sites are not well established yet. High and low affinity binding sites have been reported neglecting that the same binding site may lead to different complex species (protonated, neutral and deprotonated complexes) that may form (although to a different extent) within the same pH interval [17].

The combination of the thermodynamic and the spectroscopic results has made it possible to show *i.* the formation of macrochelates (at pH<7 and with sub-stoichiometric ratios) involving the histidine and the carboxylic residues; *ii.* the formation of species (at physiological pH and with increasing copper and zinc equivalents) in which the metal ion is anchored to histidine residues and also to deprotonated peptide nitrogens; *iii.* the lack of coordination of the hydroxo group of Tyr; *iv.* copper and zinc preferential coordination to the N-terminus (1-16 residues) and to the C-terminus (13 and 14). A β has also been shown to form mixed metal complexes with Cu²⁺ and Zn²⁺, in analogy with similar findings reported for the prion protein [18].

Recent findings indicate that copper may also induce redox processes in addition to the aggregation of amyloid peptides [19]. *In vitro* studies have shown that Cu²⁺ may form oxygen reactive species in the presence of A β via Fenton reactions [20]. Thus, a strategy based on the chelation of metal ions has been proposed for AD treatment [21]. Clioquinol has been reported not only to decrease protein aggregation in transgenic mice [22] as done by other lipophilic ligands (e.g. DP-109 [23]) but also to slow down the cognitive deficit [24]. More recently, multifunctional ligands able to bind metal ions as well as to exert antioxidant activity and cross the brain blood barrier have been reported [25]. Carnosine (β -alanyl-L-histidine), a naturally occurring dipeptide, and its analogues have an antioxidant activity and can chelate Cu²⁺ and Zn²⁺ and inhibit the non-enzymatic glycosilation of proteins [26]. These peptides have been suggested to play a role in AD and other neurodegenerative disorders [27]. Recently, we have shown that carnosine prevents the *up-regulation* of NO-synthase that may be induced by an oxidative stress [28]. We have also found a direct correlation between

cellular protection and carnosine scavenging activity towards NO and shown through cell free experiments that such an activity is due to the formation of stable adduct with NO [29]. These results indicate that this dipeptide and its analogues may be used as multifunctional agents to prevent and/or treat neurodegenerative disorders [30], provided that a way is found to block the degradation activity of the specific carnosinase present in the brain. To this end we have synthesized carnosine (and its analogues) glycoconjugates. These compounds show chelating [31], ROS, RNS scavenging [32] and antiaggregating activity [33]; interestingly, such conjugates are resistant to carnosinase [34].

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WATER ACTIVITY: EXPERIMENTAL APPROACH AND DATA TREATMENT FOR SYSTEMS OF BIOLOGICAL INTEREST

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Introduction

When looking for “Water Activity” in any data bank, one is directly addressed to references dealing with food products, their preservation from microbial spoilage and extent of their shelf life. Although such practical applications are of great relevance, they are rather misleading when the interest of the searcher concerns the thermodynamic meaning of water activity, a_w , in systems of biological interest that contain water soluble or water compatible substances.

Nobody can nowadays cast doubts about the direct effects of water activity on the stability of bio-macromolecules, namely proteins, carbohydrates and nucleic acids, etc.. However, whether such a role has to be described in a phenomenological way, according to classic Thermodynamics, or by singling out links between water activity and structure of biopolymers, is an issue of discussion.

Use of Thermal Analysis and Calorimetry

Various methods have been so far proposed to determine a_w , although all of them demand a calibration with respect to the assessed a_w values of saturated salt solutions. Most of these methods have been devised for isothermal conditions and can be easily applied only at ambient temperature. The time span required to attain equilibrium conditions can be reasonably short when dealing with very soluble substances at low concentration, but can become much longer (several days) when dealing with systems of large viscosity. This is a critical point of many experimental determinations of a_w that may lead to poorly reliable data. Another crucial issue concerns the physical meaning of a_w values relevant to heterogeneous systems where two or more aqueous phases coexist because of the thermodynamic incompatibility of different solutes (e.g. proteins and high molecular mass carbohydrates), or in the presence of cell membranes that keep apart aqueous compartments. In heterogeneous systems any a_w gradient induces a water flow that tends to level off the gradient: such a process may however be rather lasting because of the high viscosity of some phase where the molecular mobility is very low.

Literature reports some interesting calorimetric methods to determine water activity and enthalpy of mixing (differential heat of sorption) as functions of composition at high water activities. All of them demand a peculiar arrangement of vessels that allows exchange of water with a reference saturated salt solution. Although correct in principle, these methods demand special operative skills and very special equipments. A recently proposed experimental approach allows determination of a_w by means of a standard thermobalance equipped with Knudsen cells and operating under a high dynamic vacuum. The method allows investigations in a wide temperature range and provide instrumental outputs that can directly be reported in a moisture-vs- a_w plot. Knudsen effusion through an orifice implies a flux that is proportional to the pressure drop across the orifice; when the external pressure is

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much smaller than the pressure within the cell, p_{int} , the flux becomes proportional to p_{int} and can be determined as the mass loss rate. In dehydration processes p_{int} is the partial pressure of water:

$$J_w = K \cdot p_{\text{int}} = \frac{dm_w}{dt}$$

With a couple of runs, one for the system to study and one for pure water, one can easily determine a_w :

$$a_w = \frac{p_w}{p_w^*} = \frac{\left(\frac{dm_w}{dt}\right)}{\left(\frac{dm_w}{dt}\right)^*}$$

This method allows a much easier investigation of systems of biological interest, but may also be used to determine thermodynamic parameters, like solubilities, activity coefficients, etc. of many aqueous solutions. The peculiar instrument used by our group allows the simultaneous determination of the heat flow related to the process that takes place. If pure water (or any other pure compound) is examined, the ratio between heat flux and mass loss rate corresponds the vaporization enthalpy at the temperature considered:

$$\frac{dQ/dt}{dm/dt} = \frac{dQ}{dm} = \Delta_{\text{vap}} H$$

Treatment of a_w data to estimate thermodynamic properties

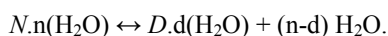
When dealing with subsaturated solutions of a salt or a simple sugar, the methods allows the simultaneous evaluation of a_w and solute concentration. These data may be processed via the Gibbs-Duhem expression,

$$X_w d\mu_w + X_{\text{salt}} d\mu_{\text{salt}} = 0$$

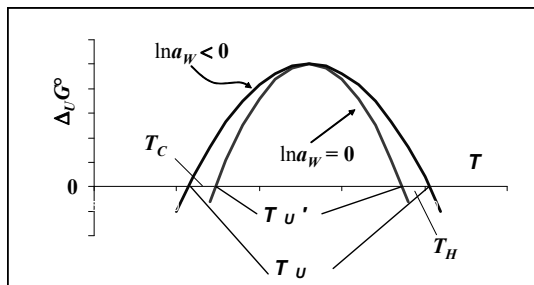
so as to draw an expression for the relationship between the solute concentration and its thermodynamic activity, referred to the state of unit molality. As for carbohydrates, it is of some interest to compare the a_w values observed for a solution of given simple sugar and those observed for a solution of its polymer with the same solute/solvent mass ratio. The scope of such a comparison is to verify that these carbohydrate polymers do not affect a_w more than the corresponding monomers, their action being mainly related to the increase of viscosity.

The effect of a_w on the conformational transitions of proteins

Were the unfolding transition of proteins not related to the solvation extent of the molecule, the prediction of the cold denaturation through thermodynamic parameters relevant to the heat denaturation would be untenable. The protein folding too is related to the solvation since it takes place in an aqueous environment the properties of which at determine the final “native” structure of the molecule. It is indeed possible to highlight the role of a_w in the unfolding process simply taking into account that the simple two-state model does actually apply to solvated species, namely:



This details allows one to rewrite the equations that lead to the prediction of two unfolding temperatures, T_U , for heat; T_H , and cold, T_C , denaturation, respectively, that directly depend on a_w .



The interaction between water and hydrocolloids

Biological systems, including most food products, contain polymers that severely affect the overall physical properties even at concentrations as low as 0.5 mass %. These substances, currently dubbed hydrocolloids, can trap large amounts of water but do not have a large effect on the value of

a_w . Unfortunately, some researchers erroneously reported opposite conclusions, based on measurements largely affected by the high viscosity of these systems. The determination of a_w requires the achievement of a thermodynamic equilibrium between condensed and vapor phase. When the viscosity of the condensed phase is large, as in many biological systems, the migration of the solvent can be very slow and the attainment of the true equilibrium conditions can require a very long time either for adsorption or for desorption processes. A large viscosity implies a small molecular mobility. This means that even at the molecular level one can detect, e.g. with NMR relaxometry, a poor mobility of water molecules. As a result, one can record an apparent water activity that is smaller than the actual a_w . A simplist treatment allows to predict a rough phenomenological correlation between apparent and actual water activity

$$a_w = a_w^{\text{app}} \frac{\eta_w}{\eta_w^*}$$

where η and “*” stand for viscosity and pure component, respectively.

In aqueous solutions the shape of the polymer molecules affects the solvating surface available for the interaction with water molecules which are linked to binding sites: however, there is an excess of “empty” binding sites with respect to the solvating water molecules. This excess mainly produces intra-molecular effects, like bridging binding sites of a given macromolecule which are close to each other because of the secondary or tertiary conformation of the polymer chain. Intermolecular interaction can also take place when the solute concentration becomes large enough.

This picture is substantially modified when different hydrophilic polymers are present in the same solution. Because of the difference between the respective excluded volumes, separated aqueous phases are formed which are dispersed within one another. A huge inter-phase region is also formed which is the “ideal” residence for amphiphilic molecules. Gel phases can stem from each separated phase and interpenetrated gels can become the peculiar physical character of the system.

a_w and glass transition

The phenomenological correlation between a_w and viscosity is the main reason for another pseudo relationship, namely, between a_w and the so called glass transition. It is well known

that the mean temperature of this transition, T_g , is strongly affected by the moisture content of a given polymer system, i.e. on increasing the water content the observed T_g decreases. This is not surprising as long as the glass transition is indeed a process that implies a large viscosity drop (on heating). The proposed correlation is depicted in the 3D plot (T_g , a_w , $m_w/m_{\text{dry matter}}$) reported below: the reader should notice that the a_w axis corresponds to data collected at room temperature (namely, isothermal records): such a plot indeed obeys to practical rather than scientific scopes. One may single out the a_w value that corresponds to $T_g = 25^\circ\text{C}$. This is the a_w threshold that should not be trespassed to preserve the system from microbial or enzymatic spoilage when it is stored at room temperature.

STRUCTURAL RELAXATION AND MOBILITY IN NANOSTRUCTURED SOFT-CONDENSED BIOMATTERS: POLYMERS AND CARBOHYDRATES

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1. Premise

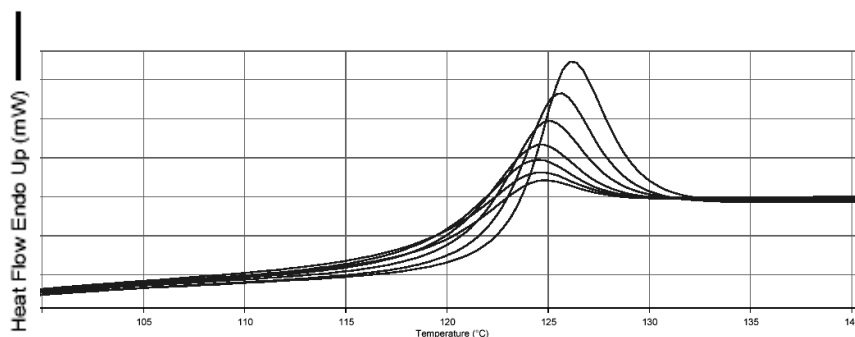
The aim of this paper is to highlight the “thermal” and “dynamical” properties of polymer and carbohydrate-based soft-condensed matter that is often organized in terms of nanostructured clusters. The application of the systems presented is specifically foreseen in advanced nano-encapsulation of high value bio-pharmaceutics in protected delivery. A further aim is, therefore, the generalization of the central role of the solvent on the heterogeneous perturbation and on the phenomenology of the glass and (pseudo-) first order transitions [1-7]. Indeed, the central issue is on the experimental and computational evidence that the glassy state slow-down is related to the growth of distinct relaxing domains (spatial heterogeneity, especially evidenced by experiments with polymers and biopolymers) [8-11]. Whether each of these spatially heterogeneous domains relaxes exponentially or not is also a matter of considerable current interest [12-13].

2. Glass transition and structural relaxation

Thus, the first section concerns the well known calorimetric phenomenology of the glass transition, that is analyzed first with the intention of providing a tutorial approach to its determination and speculation. A “standard” system such as polyethyleneterephthalate, with glass transitions and crystallization data collected from student lab, is presented [14].

3. Structure and mobility in a sugar matrix

After this outline of concepts, the presentation is developed by studying a simple, yet complex system [15], that is discussed in terms of thermodynamic (i.e. calorimetric, at frequency of the order of Hz) [16-20], spectroscopic (anelastic scattering, order of GHz) time-scale relaxation [20-23], and conformational viewpoint [24].



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Figure 1. Glass transition and physical aging of the sugar matrix

The features of the glass transition phenomenon are reported for this carbohydrate system which gives structural polymorphs that may be relevant in the bioprotection [15,25,26]. Physical eliciting these crystalline polymorphic structures shows a “polyamorphism” can be speculatively associated to these forms on the basis of original calorimetric data [27]. The role of water mobility at the origin of this duality is presented. Then, the relaxation phenomenon from medium to low moisture is studied by scattering methods at the Gigahertz frequencies to disclose the water dominated structural mobility [23].

4. Soft-condensed biomatter encapsulation

Finally, a look at the physical processing of polysaccharidic capsules used in nano-biopharmaceutics from the view-point of water removal by freeze-drying or evaporation. In particular, this part focusses on a model system of a great interest in advanced biosystems by investigating: i) the state of water in a nanostructured gelling phase in relation with drug loading and delivery, ii) water immobilization and removal during processing, iii) the local mobility of residual water in the sugar matrix in relation with the issue of life biopreservation and bench-life stability of nanobiopharmaceutics.

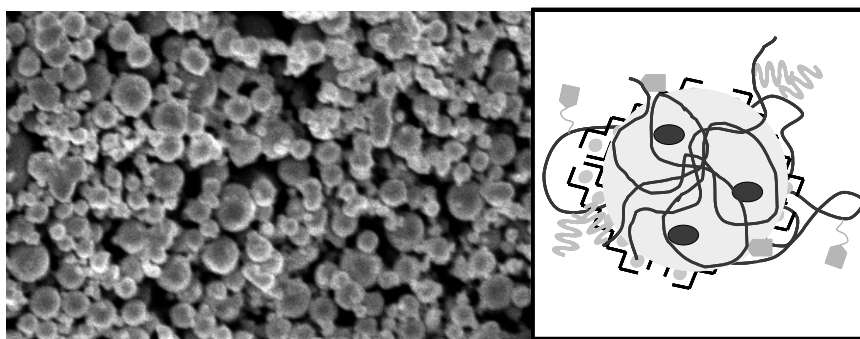


Figure 2. SEM view and schematic molecular sketch of the nanoparticle structure

5. Conclusions

Some final comments underline that the emphasis of the presentation is on the implementation of “classical” thermodynamic methods with physics and pharmaceutics. Indeed, part of the suggestive bibliography is intended to provide hints at some unvaluable classical reports as well as at recent debate and new findings in the glassy state story.

Acknowledgement

The present results have been achieved in collaboration with several groups and coworkers, as given in the authors’ list of references (part 2), carried out in part within the EU Project FP6 NanoBioPharmaceutics (NMP 026723-2).

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VIALE BACTERIA FOR THE BIORECOVERY OF DETERIORATED CULTURAL HERITAGE SURFACES THE CASE OF THE MONUMENTAL CEMETERY FRESCOS OF PISA

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The interest for the cultural heritage conservation in the world is increasing, but the traditional techniques of restoration (chemical and physical–mechanical) are not considered optimal. New conservation strategies are under investigation. Among these, the most important are represented by microbial biotechnologies.

Microorganisms are generally considered biodeterioration agents. Indeed they are often responsible of alteration of works of art, but they can be also used as means of conservation. In fact, during the last ten years a new technology, based on the use of microorganisms, has been set up to remove undesired materials from the surfaces of altered works of art.

The application of this technology to stone monuments and frescoes gave very interesting outcomes with better results than traditional methods.

Until now viable cells of bacteria have been applied to remove many kind of alterations and undesired materials from the surfaces. “Sulfatation” of calcareous stones, resulting in black, brown and grey crusts [1], and “nitration”, resulting in disaggregation and pulverization, are caused by atmospheric pollutants on stones and, in the case of “nitration”, also by the capillary rise of water rich in nitrates in the walls. Residues of synthetic polymers used in conservation treatments and layers of organic patinas, e.g. for frescoes detachment from the walls, are also biologically removed.

The procedure used for this application consists on: (a) the selection of appropriate bacterial strains that must be effective in the alteration materials removal and safe for the works of art, operators and the environment; (b) the selection of the delivery system that immobilises the bacteria and can be easily applied to surfaces, including vertical surfaces and vaults; (c) to set up the procedure of delivery system application and to define the duration of the treatment; (d) monitoring of environmental parameters during the application; (e) the removal of the bacteria after the treatment and long-term monitoring of the treatment effects.

In this presentation particular attention will be devoted to the case study of the frescoes of the Monumental Cemetery of Pisa [2]. On the walls of this cemetery there are 1,500 square meters of frescoes painted during 300 years (1300-1600) by several artists as Buonamico Buffalmacco, Taddeo Gaddi, Spinello Aretino, Benozzo Gozzoli. More than twenty years ago, because of their poor state of conservation, these frescoes have been detached using the “strappo” technique and stored in warehouse waiting the funds for restoration.

The technique of “strappo” consists in the following procedure: the fresco is covered by a layer of glue (1-3 mm) on which a large gauze is pasted; subsequently another layer of glue is spread on the gauze, for a better adhesion of the gauze to the fresco. When the glue is dried, the fresco is torn up from the wall, and spread on a frame and stored.

After twenty years, it had been possible to proceed to the restoration interventions, and proteolytic enzymes were used in order to solve the glue and free the surface of frescoes from

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the gauze; but in some frescoes neither enzymes (proteases e collagenases) nor physical and chemical techniques were effective. The gauze remained hardly pasted to the fresco.

For this reason we tried the application of living bacterial cells to 20 square meters of the Spinello Aretino fresco named “Conversione di S. Efsio e battaglia”. The preliminary investigations had showed that the organic material present was animal glue while the pigments used to paint the fresco were inorganic. Aerobic heterotrophic bacteria, effective in the biodegradation of specific organic compounds used on the frescoes, have been tested. Among many bacterial cultures, a strain of *Pseudomonas stutzeri* has been selected. This strain was very good at degrading animal glue. The identification of this strain has been performed by the 16S rRNA gene sequencing.

As regard the delivery system, strips of hydrophilic cotton were laid on the gauze covering the fresco; a suspension of 10^6 UFC/ml bacterial cells has been used to soak the cotton. During the application, temperature, humidity and microbial population by cultural, biochemical and bioluminescence techniques were monitored; in fact environmental conditions affect the activity of microorganisms and, as a consequence, the time necessary for the treatment.

The duration varies according to the chemical composition of the material and the thickness of the layer to remove. In the case of the Spinello Aretino’s fresco after 12 hours the glue was solved and it has been possible to separate the gauze from the paint surface without any damage to the fresco [Fig.1].



Fig. 1. Removal of cotton strips soaked with a *Pseudomonas stutzeri* suspension

The surface was cleaned with care. The residual glue has been removed by the application of a protease type XIX from *Aspergillus sojae*. At the end of the treatment it was possible to observe the fresco.

This kind of bioapplication is suggested when the glue used for the “strappo” has been treated with formaldehyde as antibacterial agent that causes the cross-linking of the proteins. In this condition the common protease are less (or not effective) than bacteria to remove the organic patinas. The advantage offered by viable cells of bacteria is that they are able to produce inducible enzymes able to attack and degrade the undesirable compounds that they come in contact with.

On the basis of the experiences acquired, it is possible to claim that: in comparison to traditional methods (chemical and mechanical treatment), the biological treatment is non-invasive, extremely selective, and friendly for the environment; in addition, it does not require equipment [3]. Moreover, viable cells of bacteria are able to produce inducible enzymes when

they come in contact with the molecules to be broken down [Fig.2]; these enzymes are not available on the market [4].

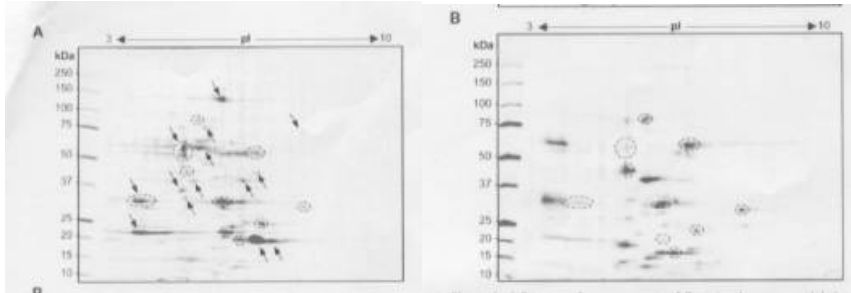


Fig. 2. Proteins produced by *Ps. stutzeri* grown on (A) the original glue and (B) glucose. (2.D maps of supernatant)

Companies, with competence in the field of the biotechnologies and in the microbial biomass production, have shown a great interest in the production and marketing of these bioformulates. We think that this biotechnology offers new employment opportunities for the young microbiologists working in the field of the cultural heritage conservation.

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