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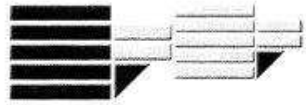

DEPARTMENT OF PHARMACEUTICAL SCIENCES

PhD Thesis in

"Methodologies for the Development of Molecules of Pharmacological Interest"

CHIM/09

POLYMERIC MATERIALS IN BIOMEDICAL, ANALYTICAL AND FOOD APPLICATIONS

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PREFACE

Polymers are an important part in everyday life; they are the basis not only for numerous natural materials, but also for most of the synthetic plastics that everyone encounters in their lives. Polymers consist of extremely large, chain-like molecules that are, in turn, made up of numerous smaller, repeating units called monomers.

Polymeric materials can be divided in two main types: synthetic and natural polymers.

Natural polymers include proteins (silk, collagen, keratin), carbohydrates (cellulose, starch, glycogen), DNA, RNA, rubber (hydrocarbon base) and silicones (alternating silicon and oxygen), as well as synthetic polymers include nylon, synthetic rubber, polyester, teflon, and so forth.

One of the reasons for the great popularity exhibited by polymers is their ease of processing. Polymer properties can be tailored to meet specific needs by varying the "atomic composition" of the repeat structure and by varying molecular weight. The flexibility can also be varied through the presence of side chain branching and according to the lengths and the polarities on the side chains. The degree of crystallinity can be controlled through the amount of orientation imparted to the polymers during processing, through copolymerization, by blending with other polymers, and via the incorporation (via covalent and non-covalent interactions) of an enormous range of compounds.

Below the main applications of polymeric materials are reported:

-Biological and Medical Materials

Polymeric materials have been used for many decades in biomedical applications such as drug delivery, implants, contact lenses, vascular grafts, dental materials, and select artificial organs. Their useful and

tunable mechanical properties have offered broad utility in the structural support or replacement of tissues or in controlled retention and release of drugs[1]. The development of polymers as bioactive pharmaceuticals has only more recently been exploited[2]. Long thought to be too heterogeneous with respect to molecular weight (polydispersity), composition, and structure to be useful therapeutically, polymers are now known to offer many specific advantages critical to treating human disease and have recently entered into medical practice. Indeed, the early studies of Duncan et al. in the late 1970s resulted in the first polymer-drug conjugates to be used as medical treatment[3].

The immunogenic responses and side effects of many drugs, especially protein drugs, are exacerbated by their hydrophobicity; therefore, drug toxicity can be reduced by increasing the drug solubility by conjugation of a hydrophilic polymer scaffold to the drug in question[4]. With conjugation to a polymer, drugs can also be protected from degradation, resulting in improved efficacy due to increased drug circulation times. In addition to the environmental protection afforded by polymers, the tunable and responsive properties of many polymeric scaffolds have also permitted improved routes for targeted drug delivery. The controlled release of drugs from polymer-drug conjugates, by variations in pH, temperature, enzyme concentration, or attachment of targeting ligands, can increase drug efficacy by increasing local drug concentration at the

[1] C. Fischbach, D.J. Mooney, Biomaterials 28 (2007) 2069.
[2] K.L. Kiick, Science 317 (2007) 1182-1183.
[3] R. Duncan, Nat. Rev. Cancer 6 (2006) 688-701.
[4] K.J. Watson, D.R. Anderson, S.T. Nguyen, Macromolecules 34 (2001) 3507-3509.

desired site of therapeutic need[5]. In a different therapeutic approach, toxic small molecules can be eliminated selectively from the body via their sequestration in polymeric scaffolds[6].

- Packaging Materials and Coatings

In the last decades, the use of polymers in food area, in particular as packaging materials, has increased enormously, due to their advantages over other traditional materials such as glass or tinplate.

A great advantage of polymers is the large variety of materials and compositions available, which make it possible to adopt the most convenient packaging design to the very specific needs of each product. Relevant characteristics of polymers are, for example, their low cost, lightness, thermosealability (which allows package closure), ease of printing, and microwaveability. They can also be conformed into an unlimited variety of sizes and shapes, and converters can easily modify them. The optical properties (brightness and transparency) can also be adapted to the specific requirements of the product. This property allows the consumer to see the packaged product, providing it with a nice appeal.

-Agricultural Fields

In agricultural field, polymers are also widely used for many applications. Although they were used, in the first time, just as structural materials for creating a climate beneficial to plant growth (inhert polymers), in the last decades functionalized polymers revolutionized the agricultural and food industry with new tools for the molecular treatment of diseases, rapid disease detection, enhancing the ability of plants to absorb nutrients etc..

[5] A.W. York, S.E. Kirkland, C.L. McCormick, Adv. Drug Delivery Rev. 60 (2008) 1018-1036.

[6] P.K. Dhal, S.R. Holmes-Farley, C.C. Huval, T.H. Jozefiak, Adv. Polym. Sci. 192 (2006) 9-15.

Smart polymeric materials and smart delivery systems helped the agricultural industry against viruses and other crop pathogens, while functionalized polymers were used to increase the efficiency of pesticides and herbicides, allowing to indirectly protect the environment through the use of filters or catalysts that reduce pollution and clean-up existing pollutants[7].

-Cosmetic Field

Polymers represent the second largest class of ingredients in cosmetics and personal care products. A diverse range of polymers are applied in this segment as film formers, fixatives, rheology modifiers, associative thickeners, emulsifiers, stimuli-responsive agents, conditioners, foam stabilizers and destabilizers, skin-feel beneficial agents, and antimicrobials.

Many cosmetic products are based on emulsions – small droplets of oil dispersed in water or small droplets of water dispersed in oil. Since oil and water don't mix, emulsifiers are added to produce the small droplets and to prevent the oil and water phases from separating. Emulsifiers work by changing the surface tension between the water and the oil, thus producing a homogeneous product with an even texture.

Preservatives are added to cosmetics to prevent the growth of microorganisms (eg, bacteria and fungi), which can spoil the product and possibly harm the user. Preservatives used in cosmetics can include parabens, benzyl alcohol and tetrasodium EDTA (ethylenediaminetetra-acetic acid).

[7] F.Puoci, F.Iemma, U.G. Spizzirri, G. Cirillo, M.Curcio, N.Picci American Journal of Agricultural and Biological Sciences 3 (2008) 299-314.

Thickening agents such as polymers are often added to cosmetics to change their consistency. Polymers can be synthetic (eg, polyethylene glycol) or derived from natural sources (eg, polysaccharides). Seaweeds are a common source of natural polysaccharides – carrageenans are extracted from red algae and alginates from brown algae. Cosmetics that are too thick can be diluted with solvents such as water or alcohol.

This PhD thesis is an exploration of the polymer science with particular attention to bridge the gap between organic synthesis and polymer chemistry.
In Particular the design and synthesis, by different techniques, of polymeric materials with potential applications in biomedical, analytical and food areas were performed.
For this purpose, the present work was divided into three main sections:

I. Synthesis of Molecularly Imprinted Polymers (MIP) and their application as sorbents in Solid Phase Extraction (SPE), selective traps of drugs and Drug Delivery Systems (DDS);

II. Synthesis of Thermo-Responsive Hydrogels by Reverse-Phase Suspension Polymerization and Molecular Imprinting Technique;

III. Synthesis of Polymeric Antioxidants by Free-Radical Grafting Procedure.

SECTION I

Design and Synthesis of Molecularly Imprinted Polymers for Pharmaceutical and Analytical Applications

1.1 Molecularly Imprinted Polymers: Developments in Mimicking Dynamic Natural Recognition Systems

Generations of scientists have been intrigued by the binding phenomena involved in interactions that occur between natural molecular species and over the years, numerous approaches have been used to mimic these interactions[1]. Complex formation between a host molecule and the guest involves recognition, which is the additive result of a number of binding forces (Figure 1).

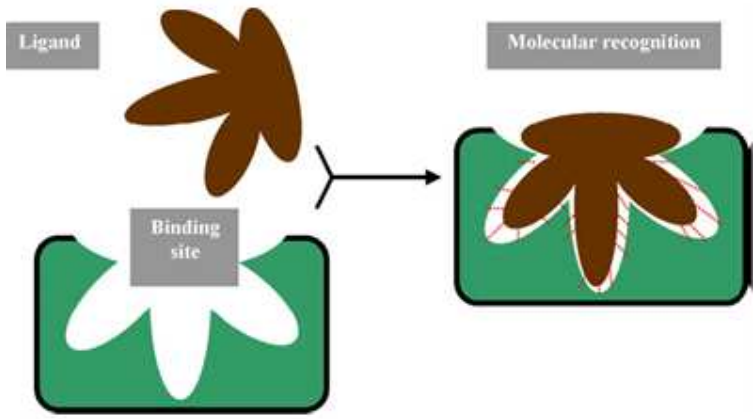

Figure 1. Example of molecular recognition within nature showing the mass of noncovalent interactions (…..) involved.

[1] A.L. Hillberg, M. Tabrizian, ITBM-RBM 29 (2008) 89-104.

Within biological systems, these are usually dynamic and are the result of a mass of non-covalent interactions, which act collectively to form a very stable system. Primarily, molecular imprinting aims to create artificial recognition cavities within synthetic polymers. It is a relatively simple concept, which involves the construction of sites of specific recognition, commonly within synthetic polymers. The template of choice is entrapped within a pre-polymerization complex, consisting of functional monomers with good functionality, which chemically interact with the template. Polymerization in the presence of crosslinker serves to freeze these template-monomer interactions and subsequent removal of the template results in the formation of a molecularly imprinted polymer matrix (Figure 2).

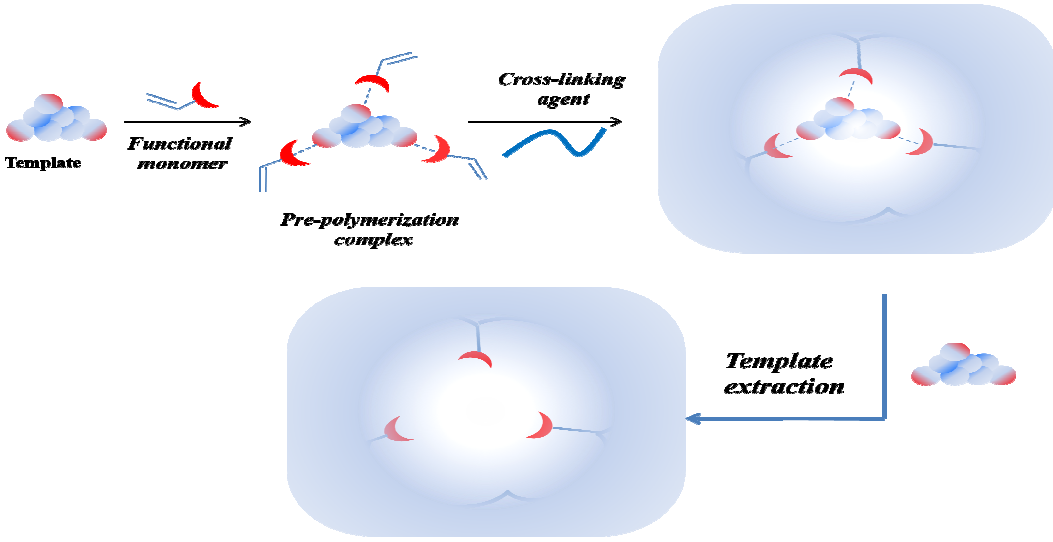

Figure 2. Schematic representation of molecular imprinting.

Enormous interest has also been shown in imprinted materials as they mime biological receptors for the screening of new substances with potential pharmacological activity or to specifically detect drugs in biological fluids in screening assays for drugs of abuse[2,3]. Such specificity is comparable with monoclonal antibodies used in immunoassay techniques[4]. Molecular imprinting is a well-developed tool in the analytical field, mainly for separating and quantifying very different substances, including drugs and bio-active molecules contained in relatively complex matrices[5]. Moreover, the information generated about polymer synthesis procedures and the properties outlined for optimum performance in separation-based technologies[6] may be a good starting point to create imprinted DDS.

1.2 Synthesis of MIP

MIPs can be synthesized following three different imprinting approaches[7], as follows:

1. The non-covalent procedure is the most widely used because it is relatively simple experimentally and the complexation step during the synthesis is achieved by mixing the template with an appropriate functional monomer, or monomers, in a suitable porogen (solvent)[8]. After

[2] C. Alvarez-Lorenzo, A. Concheiro, Journal of Chromatography B, 804 (2004) 231-245.
[3] O. Ramström, R. Ansell, Chirality 10 (1998) 195-209.
[4] K. Mosbach, O. Ramström, Biotechnol. 14 (1996) 163-170.
[5] P.K. Owens, L. Karlsson, E.S.M. Lutz, L.I. Andersson, Trends Anal. Chem. 18 (1999) 146-155.
[6] G. Wulff, Angew. Chem., Int. Ed. Engl. 34 (1995) 1812-1832.
[7] E. Caro, N. Masquè, R.M. Marcè, F. Borrull, P.A.G. Cormack, D.C. Sherrington, J. Chromatogr. A 963 (2002) 169-178.
[8] V.P. Joshi, S.K. Karode, M.G. Kulkarni, R.A. Mashelkar, Chem. Eng. Sci. 53 (1998) 2271-2284.

synthesis, the template is removed from the resultant polymer simply by washing it with a solvent or a mixture of solvents. Then, the rebinding step of the template by the MIP exploits non-covalent interactions.

2. All these features offer several advantages over the covalent protocol, in which formation of covalent bonds between the template and the functional monomer is necessary prior to polymerization. Furthermore, to remove the template from the polymer matrix after synthesis via the covalent protocol, it is necessary to cleave the covalent bonds. To this end, the polymer is then refluxed in a Soxhlet extraction or treated with reagents in solution[9].

3. The semi-covalent approach is a hybrid of the two previous methods. Thus, covalent bonds are established between the template and the functional monomers before polymerization, while, once the template has been removed from the polymer matrix, the subsequent re-binding of the analyte to the MIP exploits non-covalent interactions, as the non-covalent imprinting protocol.

The binding sites obtained by molecular imprinting show different characteristics, depending on the interactions established during polymerization. The average affinity of binding site prepared using bonding by non-covalent forces is generally weaker than those prepared using covalent methods because electrostatic, hydrogen bonding, π-π and hydrophobic interactions, between the template and the functional monomers, are used exclusively in forming the molecular assemblies[10]. Moreover, an excess of functional monomer relative to the template is usually required to favor template-functional monomer complex

[9] T. Ikegami, T. Mukawa, H. Nariai, T. Takeuchi, Anal. Chim. Acta 504 (2004) 131-135.
[10] C.C. Hwang, W.C. Lee, J. Chromatogr. A 962 (2002) 69-75.

formation and to maintain its integrity during polymerization. As a result, a fraction of the functional monomers is randomly incorporated into the polymer matrix to form non-selective binding sites.

However, when covalent bonds are established between the template and the functional monomer prior to polymerization, this gives rise to better defined and more homogeneous binding sites than the non-covalent approach, since the template-functional monomer interactions are far more stable and defined during the imprinting process.

Nevertheless, non covalent imprinting protocol is still the most widely used method to prepare MIP because of the advantages that it offers over the covalent approach from the point of view of synthesis.

In some polymers prepared by the non-covalent procedure, it has been observed that the binding of the template to the polymer can sometimes be so strong that it is difficult to remove the last traces of template, even after washing the polymer several times[11,12].

When the MIP is used, small amounts of residual template can be eluted. This bleeding is a problem mainly when the MIP has to be applied to extract trace levels of the target analyte.

To overcome this drawback, some authors have synthesized MIP using an analogue of the target molecule as a template (the template-analogue approach)[13]. In this way, if the MIP bleeds template, then the elution of the template does not interfere in the quantification of the target analyte. Andersson[12] was the first author to synthesize a MIP using a template

[11] P. Martin, G.R. Jones, F. Stringer, I.D. Wilson, Analyst (Cambridge, UK) 128 (2003) 345-352.

[12]L.I. Andersson, A. Paprica, T. Avirdsson, Chromatographia 46 (1997) 57-62.

[13] B. Dirion, F. Lanza, B. Sellergren, C. Chassaing, R. Venn, C. Berggren, Chromatographia 56 (2002) 237-243.

analogue. In this case, a MIP selective for sameridine was prepared using as a template a close structural analogue of sameridine. However, it should be pointed out that the use of template analogues is not always the solution, because sometimes is not possible to identify and to source a suitable analogue. For this reason, other methods, such as thermal annihilation, microwave-assisted extraction (MAE) and desorption of the template with supercritical fluids have also been developed to remove the template from the MIP[14].

It should also be mentioned that, as a control in each polymerization, a non-imprinted polymer (NIP) is also synthesised in the same way as the MIP but in absence of the template. To evaluate the imprinting effect, the selectivities of the NIP and MIP are then compared.

It is important to state that MIP can be obtained in different formats, depending on the preparation method followed[15]. To date, the most common polymerizations for preparing MIPs involve conventional solution, suspension, precipitation, multi-step swelling and emulsion core-shell. There are also other methods, such as aerosol or surface rearrangement of latex particles, but they are not used routinely.

When a MIP is obtained by conventional solution polymerization, the resultant polymer is a monolith, which has to be crushed before use, except when the MIP is prepared in situ. However, suspension polymerization (in fluorocarbons or water) and precipitation

[14] A. Ellwanger, C. Berggren, S. Bayoudh, C. Crecenzi, L. Karlsson, P.K. Owens, K. Ensing, P. Cormack, D. Sherrington, B. Sellergren, Analyst (Cambridge, UK) 126 (2001) 784-791.
[15] E. Turiel, A. Martìn-Esteban, Anal. Bioanal. Chem. 378 (2004) 1876-1881.

polymerization allow MIPs to be prepared in the form of spherical polymer particulates.

Conventional solution polymerization is the most common method because of its simplicity and universality. It does have some drawbacks as the processes of grinding and sieving not only are wasteful and time consuming, but also may produce irregularly sized particles.

In the last few years, some improvements in MIP synthesis have been made by developing combinatorial libraries, which allow us easily and quickly to check the best conditions for obtaining the optimal MIP. The development and implementation of these screening techniques is expected to accelerate the discovery of new MIP.

1.3 Applications of MIP

1.3.1 MIP as stationary phase in SPE technique

Molecularly Imprinted Solid Phase Extraction-MISPE (MISPE) is based on conventional SPE procedures, therefore conditioning, loading, clean-up and elution steps are performed as a matter of routine (Figure 3).

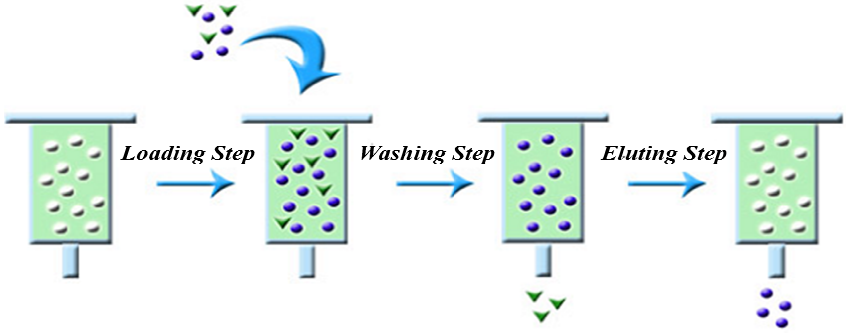

Figure 3. Schematic representation of SPE protocol

During the conditioning step, the cavities (binding sites) of the MIP are activated in order to maximize the interactions with the target analyte present in the sample. In the loading step, the sample medium has a direct influence on the recognition properties of the imprinted polymer. Thus, if the sample is percolated through the MIP in a low-polarity solvent, a selective loading step can be achieved, in which only the target analyte is selectively retained on the MIP while the sample matrix is non-retained[16]. However, when the analyte of interest is present in an aqueous medium, the analyte and other interfering compounds are retained nonspecifically on the polymer. Consequently, to achieve a selective extraction, a clean-up step with an organic solvent is introduced prior to the elution step[17]. This clean-up is more critical in MISPE procedures than in conventional SPE. The clean-up step must be optimized in terms of pH, nature, and volume of the washing solvent in order to exploit the MIP ability to be selective in recognizing the target molecule. Thus, the clean-up solvent should suppress the non-specific interactions without disrupting the selective interactions between the MIP and the target molecule. For this purpose, low-polarity organic solvents, such as dichloromethane, toluene or chloroform, are the most widely used[18]. However, good results have also been obtained with other solvents of high polarity, such as acetonitrile or methanol[19]; nevertheless, some authors have stated that recognition is often better when the porogen is used as the solvent

[16] T. Pap, V. Horvath, A. Tolokàn, G. Horvai, B. Sellergren, J. Chromatogr. A 973 (2002) 1-8.
[17] F. Chapius, V. Pichon, F. Lanza, B. Sellergren, M.C. Hennion, J. Chromatogr. A 999 (2003) 23-30.
[18] E. Caro, R.M. Marcè, P.A.G. Cormack, D.C. Sherrington, F. Borrull, J. Chromatogr. A 995 (2003) 233-240.
[19] F. Puoci, G.Cirillo, M.Curcio, F.Iemma, U.G.Spizzirri, N. Picci Anal. Chim. Acta, 593 (2007) 164-170.

because the environment established during the synthesis is reproduced (solvent memory effect)[20]. When water samples are percolated through MIPs, the clean-up step can therefore be problematic because the washing solvent used is usually non-polar, and that may give rise to miscibility problems. To avoid this drawback, the MIP can be dried (e.g., by drawing air through the polymer). If small amounts of water remain on the cartridge after applying the sample, the binding ability of the MIP may be influenced in the subsequent selective clean-up step. In that case, decreases in retention and selectivity may be observed.

Although a clean-up step seems to be essential to achieve a selective extraction, it can be avoided if the elution step is selective enough. In this case, although the analyte and the other components in the sample matrix are retained on the MIP by non-selective interactions during the loading step, a selective elution of the target analyte can be performed using an appropriate solvent. To attain high enrichment factors, it is necessary to use small volumes of solvent, but the interactions between the MIP and the analyte are sometimes so strong that the volume of eluting solvent has to be increased. To avoid this, mixtures of organic solvents or an organic solvent with water or with a modifier, such as acetic acid or pyridine, can be used. On the one hand, water disrupts the specific non-covalent interactions, and, on the other, the modifier competes with the binding sites to interact with the target molecule. Both represent effective ways of rapidly eluting the analyte. Definitively, on the basis of this experimental data, the advantages that MIP offer as selective sorbents have been

[20] I. Ferrer, F. Lanza, A. Tolokan, V. Horvath, B. Sellergren, G. Horvai, D. Barcelò, Anal. Chem. 72 (2000) 3934-3941.

demonstrated. The applicability of MIP in SPE procedures demonstrates the feasibility of using a MIP in several formats for extracting numerous templates from different samples.

1.3.2 MIP as basis of drug delivery systems

In the last few years, a number of significant advances have been made in the development of new technologies for optimizing drug delivery[21]. To maximize the efficacy and safety of medicines, drug delivery systems (DDS) must be capable of regulating the rate of release (delayed- or extended-release systems) and/or targeting the drug to a specific site. Efficient DDS should provide a desired rate of delivery of the therapeutic dose, at the most appropriate place in the body, in order to prolong the duration of pharmacological action and reduce the adverse effects, minimize the dosing frequency and enhance patient compliance. To control the moment at which delivery should begin and the drug release rate, the three following approaches have been developed[22]:

(a) *rate-programmed drug delivery*: drug diffusion from the system has to follow a specific rate profile;

(b) *activation-modulated drug delivery*: the release is activated by some physical, chemical or biochemical processes;

(c) *feedback-regulated drug delivery*: the rate of drug release is regulated by the concentration of a triggering agent, such as a biochemical substance, concentration of which is itself dependent on the drug concentration in the body.

[21] M.J. Rathbone, J. Hadgraft, M.S. Roberts, (Eds.) Modified-Release Drug Delivery Technology, Marcel Dekker, New York, 2003.
[22] Y.W. Chien, S. Lin, Clin. Pharmacokinet. 41 (2002) 1267-1271.

When the triggering agent is above a certain level, the release is activated. This induces a decrease in the level of the triggering agent and, finally, the drug release is stopped. The sensor embedded in the DDS tries to imitate the recognition role of enzymes, membrane receptors and antibodies in living organisms for regulation of chemical reactions and for maintenance of the homeostatic equilibrium.

Molecular imprinting technology can provide efficient polymer systems with the ability to recognize specific bioactive molecules and a sorption capacity dependent on the properties and template concentration of the surrounding medium; therefore, although imprinted DDS have not reached clinical application yet, this technology has an enormous potential for creating satisfactory dosage forms.

The following aspects should be taken into account.

• Compromise between rigidity and flexibility.

The structure of the imprinted cavities should be stable enough to maintain the conformation in the absence of the template, but somehow flexible enough to facilitate the attainment of a fast equilibrium between the release and re-uptake of the template in the cavity. This will be particularly important if the device is used as a diagnostic sensor or as a trap of toxic substances in the gastrointestinal tract. In this sense, non-covalent imprinting usually provides faster equilibrium kinetics than the covalent imprinting approach[23]. The mechanical properties of the polymer and the conformation of the imprinted cavities depend to a great extent on the proportion of the cross-linker. Mostly imprinted systems for analytical

[23] C.J. Allender, K.R. Brain, C.M. Heard, in: F.D. King, A.W. Oxford (Eds.), Progress in Medicinal Chemistry, vol. 36, Elsevier, Amsterdam, 1999, p. 235.

applications require around 25-90% of cross-linker agent[24]. These cross-linking levels increase the hydrophobicity of the network and prevent the polymer network from changing the conformation obtained during synthesis. As a consequence, the affinity for the template is not dependent on external variables and it is not foreseen that the device will have regulatory or switching capabilities. The lack of response capability to the alterations of the physico-chemical properties of the medium or to the presence of a specific substance limits their potential uses as *activation*- or *feedback-modulated* DDS. A high cross-linker proportion also considerably increases the stiffness of the network making it difficult to adapt the shape of the administration site and causing mechanical friction with the surrounding tissues (especially when administered topically, ocularly or as implants).

• High chemical stability.

MIP for drug delivery should be stable enough to resist enzymatic and chemical attack and mechanical stress. The device will enter into contact with biological fluids of complex composition and different pH, in which the enzymatic activity is intense. Ethylene glycol dimethacrylate (EGDMA) and related cross-linkers, which are the most usual ones, have been proved to provide stable networks in a wide range of pHs and temperatures under *in vitro* conditions[25]. However, additional research should be carried out to obtain information about its behaviour in vivo environments, where esterases and extreme pHs seem to be able to

[24] G. Wulff, Angew. Chem., Int. Ed. Engl. 34 (1995) 1812-1820.
[25] J. Svenson, I.A. Nicholls, Anal. Chim. Acta 435 (2001) 19-28.

catalyse its hydrolysis[26]. Additionally, it has to be taken into account that the adaptability of molecular imprinting technology for drug delivery also requires the consideration of *safety* and *toxicological* concerns. The device is going to enter into contact with sensitive tissues; therefore, it should not be toxic, neither should its components, residual monomers, impurities or possible products of degradation[27]. Therefore, to ensure biocompatibility it might be more appropriate to try to adapt the imprinting technique to already tested materials instead of creating a completely new polymeric system. On the other hand, most classical MIP are created in organic solvents to be used in these media, taking advantage of electrostatic and hydrogen bonding interactions. The presence of residual organic solvents may cause cellular damage and should be the object of a precise control. In consequence, hydrophilic polymer networks that can be synthesised and purified in water are preferable to those that require organic solvents. A hydrophilic surface also enhances biocompatibility and avoids adsorption of proteins and microorganisms[28]. Additionally, many drugs, peptides, oligonucleotides and sugars are also incompatible with organic media.

A wide range of cross-linked hydrogels have been proved to be useful as drug delivery platforms[29]. Molecular imprinting in water is still under development and difficulties arise due to the considerable weakness of electrostatic and hydrogen-bonding interactions in this polar medium,

[26] D.M. Yourtee, R.E. Smith, K.A. Russo, S. Burmaster, J.M. Cannon, J.D. Eick, E.L. Kostoryz, J. Biomed. Mat. Res. 57 (2001) 522-530.
[27] O. Aydin, G. Attila, A. Dogan, M.V. Aydin, N. Canacankatan, A. Kanik, Toxicol. Pathol. 30 (2002) 350-355.
[28] J.M. Anderson, Eur. J. Pharm. Biopharm. 40 (1994) 1-8.
[29] K.A. Davis, K.S. Anseth, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002) 385-398.

which decrease the affinity and selectivity of MIP for the ligand[30]. Nevertheless, hydrophobic and metal co-ordination interactions are proving to be very promising to enhance template and functional monomer association in water[31].

It is clear that the polymer composition and solvent are key parameters in the achievement of a good imprinting and that, in consequence, a compromise between functionality and biocompatibility is needed.

Depending on the specific application of the device, an adequate balance between the performance as imprinted systems, that determines the efficiency as drug delivery or biological sensor, and the safety when administered should be reached. Only for applications in which the physiological aspects play a less important role, it may be possible to prepare the networks considering mostly their performance as imprinted devices.

1.4 Synthesis of MIP for Biomedical and Analytical Applications

In the first section of this PhD work, several MIP were synthesized using different templates to test the different applications of the imprinted materials.

In particular, cholesterol (CHO), methotrexate (MTX) and 5-Fluorouracil (5-FU) imprinted polymers were synthesized and their suitability as sorbent in SPE, selective recognition system and device in controlled drug delivery, respectively, were determined.

[30] M. Komiyama, T. Takeuchi, T. Mukawa, H. Asanuma, Molecular Imprinting, Wiley–VCH, Weinheim, 2003, 119-125.
[31] S.A. Piletsky, H.S. Andersson, I.A. Nicholls, Macromolecules 32 (1999) 633-642.

Chapter 1

MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION FOR CHOLESTEROL DETERMINATION IN CHEESE PRODUCTS

1. Introduction

Low amounts of Cholesterol are essential to the human body for several biologic functions such as the manufacture of hormones[1]. However, high level of blood cholesterol increases the risk of heart diseases[2]. CHO, indeed, is involved in the atherosclerosis development and in heart degenerative processes and it is well-established that drastic lowering of blood cholesterol concentration is followed by a reduction of clinical events and mortality[3]. Cholesterol is also a constituent of animal foods such as eggs, meat and diary products. Determination of cholesterol content in food is of primary importance to select a diet for low intake of cholesterol. In the analytical practice, well known GC and HPLC methods are used for cholesterol measurement, but a very intensive pre-treatment of the samples is needed[4]. In the last few years a lot of research has gone into establishing rapid routine methods for fast determination of cholesterol. The most common and cheapest purification techniques are thin-layer chromatography (TLC) and Solid Phase Extraction (SPE)[5].

[1] N. Adanyi, M. Varadi, European Food Research and Technology, 218 (2003) 99-104.
[2] H. Okazaki, , F. Tazoe, , S. Okazaki, N. Isoo, K. Tsukamoto, M. Sekiya, N. Yahagi, S. Ishibashi, Journal of Lipid Research, 47 (2006) 1950-1958.
[3] Sellergren, B., Wieschemeyer, J., Boos, K.S., & Seidel, D. Chemistry of Materials, 10 (1998) 4037-4046.
[4]R.Z. Zhang, L. Li, S.T. Liu, R.M. Chen, P.F. Rao, Journal of Food Biochemistry 23 (1999) 351-361.
[5]E. Boselli, M.F. Caboni, G. Lerker, Zeitschrift fur Lebensmittel-Untersuchung und-Forschung Unters. Forsch., 205 (1997) 356-359.

This work reports on a novel approach based on Molecularly Imprinted Polymers (MIPs) for the clean-up and pre concentration of CHO from a complex food matrix and its HPLC detection. MIPs are synthetic materials with high recognition properties for a target molecule named template. These specific binding properties must be attributed to specific interactions between the template and the functional groups in the polymeric network[6]. As reported in literature[7], coupling MIPs and SPE is possible to combine the advantages of both molecular recognition and traditional separation methods. Molecularly Imprinted Solid Phase Extraction (MISPE) presents the high specificity, selectivity and sensitivity of molecular recognition mechanism and the high resolving power of separation methods.

In our study, CHO imprinted polymers were synthesized by non-covalent approach[8] using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as crosslinking agent. Although a recent article[9] reported a MISPE procedure for the detection of CHO using no water-miscible organic solvents, a very time consuming procedure in the pre-treatment of the sample is required and an expensive derivatizing agent for GC analysis of eluate is needed. On the contrary, we realized MIPs able to recognize CHO in water-organic solvents mixtures and developed a very straight-forward protocol, involving only crushing and filtration steps for the pre-treatment of the food samples, and

[6] G. Wulff, Angewandte Chemie - International Edition in English, 34 (1995) 1812-1832.
[7] E. Caro, R.M. Marcé, P.A.G. Cormack, D.C. Sherrington, F. Borrull, Anal. Chim. Acta, 552 (2005) 81-86.
[8] K. Mosbach, O. Ramstrom, Bio/Technology 14 (1996) 163-170.
[9] Y. Shi, J-H. Zhang, D. Shi, D., M. Jiang, Y.X. Zhu, S.R. Mei, Y.K. Zhou, K. Dai, B. Lu, Journal of Pharmaceutical and Biomedical Analysis, 42 (2006) 549–555.

the direct HPLC analysis of eluate solutions without any derivatization. After the evaluation of the recognition properties of the materials by performing binding experiments in aqueous media, MISPE cartridges were packed and their ability to selectively absorb cholesterol was studied by using two molecules similar to the template: in particular Progesterone (PROG) and Hydrocortisone (HY) were used for this purpose. Finally, the ability of the MISPE cartridges to selectively absorb CHO from food matrices was investigated. This procedure allows to concentrate CHO and, after its elution for the cartridges, to immediately analyze the concentrated eluate by HPLC without any drying and derivatization process.

2. Experimental Section

2.1 Reagents and standards

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2,2'-azoisobutyronitrile (AIBN), CHO, progesterone and hydrocortisone were obtained from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO). All solvents were reagent grade or HPLC-grade and used without further purification and they were provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

2.2 Preparation of molecularly imprinted polymers

The MIP stationary phase was prepared by bulk polymerization. Methacrylic acid was used as functional monomers to prepare the MIP by the non-covalent imprinting method. Briefly, template cholesterol, MAA, EGDMA and AIBN were dissolved in chloroform in a thick-walled glass tube. The tube was purged with nitrogen, sonicated for 10 min, and then

photopolymerized for 24 h with 360 nm light at 4 °C. After the photolysis, the tubes were incubated at 60°C for 24h (Schmidt, Belmont and Haupt, 2005). The resultant bulk rigid polymer was crushed, grounded into powder and sieved through a 63 nm stainless steel sieve. The sieved MIP material was collected and the very fine powder, suspended in the supernatant solution (acetone), was discarded. The resultant MIPs materials were soxhlet extracted with 200 ml of an acetic acid:tetrahydrofuran (1:1) mixture for at least 48 h, followed by 200 ml of tetrahydrofuran for another 48 h. The extracted MIPs materials were dried in an oven at 60 °C overnight. The washed MIPs materials were checked to be free of cholesterol and any other compound by HPLC analysis.

The formulations used for the preparation of the different matrices (MIP-1, MIP-2, MIP-3) are shown in Table 1. Blank polymers (to act as a control) were prepared under the same conditions without using the template.

2.3 Binding experiments

The binding experiments were performed in an acetonitrile: water mixture (7:3 v/v). The polymer particles (20 mg) were mixed with 1 ml cholesterol solution (0.2 mM) in a 1 ml eppendorf and sealed. Samples were shaken in a water bath for 6 h, centrifuged for 10 min (10000 rpm) and the cholesterol concentration in the liquid phase was measured by HPLC The amount of cholesterol bound to the polymer was obtained by comparing its concentration in the MIPs samples to the NIPs samples.

The same experiments were performed using progesterone and hydrocortisone solutions (Table 1). Experiments were repeated five times.

Sample	CHO	MAA	EGDMA	% Bound			A			ε	
	(mmol)	(mmol)	(mmol)	CHO	PROG	HY	CHO	PROG	HY	PROG	HY
MIP1	1	8	25	30±1	10±0.4	3±0.3	1.8	0.9	1.0	3.0	10.0
NIP1	-			17±0.7	11±0.8	3±0.2				1.5	5.7
MIP2	1	12	25	30±1	3±0.5	3±0.4	2.3	0.75	0.5	10.0	10.0
NIP2	-			13±0.5	4±1	6±0.7				3.3	2.2
MIP3	1	16	25	28±1	2±0.7	3±0.6	4.7	0.5	0.3	14.0	9.3
NIP3	-			6±0.5	2±0.3	2±1				3.0	3.0

All polymers were synthesized in 5.25 ml of chloroform using 0.045 g of AIBN.

Table 1: Polymers composition and percentage of bound analytes after 6 hours.

2.4 Molecularly imprinted solid-phase extraction conditions

The 500 mg amount of dry particles of polymer was packed into a 6.0 ml polypropylene SPE column. The column was attached with a stop cock and a reservoir at the bottom end and the top end, respectively. The polymer was rinsed with chloroform, acetonitrile and then with the loading solvent. CHO was dissolved in the loading solvent to final concentration of 0.2 mM. After conditioning, dry MISPE column was loaded with CHO standard solution. After column drying, washing solvent was passed through the cartridges and finally elution solvent were applied to perform the complete extraction of CHO. The loading, washing and eluting fractions were analysed by HPLC to detect the CHO amount. The MISPE protocol was optimized (Table 2) and the best conditions were: loading step: 2 ml of acetonitrile/water mixture (7/3 v/v); washing step: 7 ml of acetonitrile/water mixture (7/3 v/v); eluting step: 4 ml of hot

acetonitrile (50°C). In order to evaluate the selectivity of the MIPs, optimized protocol was also applied using PROG and HY solutions. Experiments were repeated five times.

Loading			**Washing**			**Eluting** CH_3CN (50°C)	
CH_3CN/H_20	*MIPs*	*NIPs*	CH_3CN/H_20	*MIPs*	*NIPs*	*MIPs*	*NIPs*
10/0	4 ± 2.4	8 ± 2.1	10/0	55 ± 3.1	89 ± 3.3	41 ± 3.4	3 ± 1.1
			9/1	48 ± 2.2	90 ± 3.7	48 ± 2.4	2 ± 0.7
			8/2	44 ± 3.2	91 ± 3.1	52 ± 1.7	1 ± 0.9
			7/3	41 ± 1.7	88 ± 2.9	55 ± 2.2	4 ± 1.3
9/1	3 ± 1.2	7 ± 1.1	9/1	50 ± 2.7	86 ± 2.1	47 ± 3.6	7 ± 1.2
			8/2	43 ± 2.4	87 ± 2.8	54 ± 3.3	6 ± 1.6
			7/3	40 ± 2.8	84 ± 2.6	57 ± 2.8	9 ± 2.1
8/2	1 ± 0.4	5 ± 2.7	8/2	37 ± 1.9	85 ± 2.2	62 ± 3.3	10 ± 1.8
			7/3	31 ± 1.4	81 ± 2.1	68 ± 2.8	14 ± 2.6
7/3	1 ± 0.7	3 ± 1.4	7/3	24 ± 1.1	80 ± 2.0	75 ± 1.7	17 ± 1.1

Table 2: % of collected CHO in the loading, washing and elution fractions

2.5 Cholesterol determination in Calabrian pecorino cheese

A Calabrian pecorino cheese sample and 200mL of a choroform/methanol mixture (2/1 v/v) mixture were mixed and maintained under magnetic stirring for 2 h. After filtration, the solvent phase was washed twice with 90mL of a KCl aqueous solution (0.9%) and 90mL of distilled water, respectively, filtered through anhydrous Na_2SO_4 and evaporated under vacuum. The residue was reconstitute to solution by adding acetonitrile and the sample was analysed by HPLC.

2.6 Molecularly imprinted solid-phase extraction of food sample extracts

25 grams of cheese were extracted with 150 ml of acetonitrile. The obtained solution was filtered and water was added to raise the loading solution concentration (acetonitrile/water 7/3 v/v). 8 ml of this solution were used to load the MISPE column. Two washing step are performed: 7 ml of an acetonitrile/water (7:3v/v) mixture and 5ml of an acetonitrile/water (9:1v/v) mixture. Finally, 4 ml of hot acetonitrile (50°C) was used as elution fraction. All the solutions were analysed by HPLC. Experiments were repeated five times.

2.7 Instrumentation

The liquid chromatography consisted of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 208 nm for cholesterol, at 268 nm for progesterone and hydrocortisone. A 25 x 0.4 mm C4 Kromasil column, particle size 5 μm (Teknocroma, Barcellona, Spain) was employed. The mobile phase was acetonitrile containing 0.5% of water for cholesterol, acetonitrile/water (7/3 v/v) mixture for progesterone and hydrocortisone. The flow rate was 1.0 ml/min.

The shaker and centrifugation systems consisted of a wrist action shaker (Burrell Scientific) and an ALC micro-centrifugette 4214 respectively.

2.8 Analytical Parameters

Calibration curve and the Detection and Quantitation limits (LOD and LOQ) were determined using CHO spiked Calabrian pecorino cheese. 0.050 grams of cheese were spiked with 0.03, 0.06, 0.10, 0.30, 0.75, 1.5, 7.5, 15, 30 mg of CHO. The samples was extracted with 150 ml of acetonitrile and then water was added to raise the right loading solution

composition (acetonitrile/water 7/3 v/v), extracted using the MISPE protocol and analyzed by HPLC. Detection and quantification limits were calculated as the concentration corresponding to a signal 3 and 10 times the standard deviation of the baseline noise (American Chemical Society, 1980).

Repeatability of the MISPE method was evaluated by performing five repetitive analyses. The intraday and interday precisions of the relative peak areas were calculated as RSDs for five measurements

3. Results and Discussion

3.1 Preparation of the imprinted polymers

MIPs for selective detection of cholesterol were synthesized by using methacrylic acid as functional monomers and ethylene glycol dimethacrylate as crosslinker[10].

In order to maximize the interactions between functional monomers and template in the prepolymerization complex, a photo-polymerization procedure was employed. The formation of the complex is a dynamic process and, when a template with poor functional groups like cholesterol is used, a low temperature is needed to reduce the kinetic energy of the system. In this case, indeed, an high temperature could drive the equilibrium away from the template-functional monomer complex toward the unassociated species[11].

[10] U.G. Spizzirri, N.A. Peppas, Chemical Materials 17 (2005) 6719-6727.
[11] S.H. Cheong, S. McNiven, A. Rachkov, R. Levi, K. Yano, I. Karube, Macromolecules 30 (1997) 1317-1322.

After UV irradiation at 4°C for 24 h, the performance of the initially formed polymer was improved by thermal stabilization at 60°C[12].

In literature, many different ratios of template and functional monomer were used[13].

Our purpose was the selective extraction of CHO from food matrices with different water percentages. Although some MIPs exhibit moderate recognition properties under aqueous conditions, current technology often could fail to generate MIPs for use in aqueous environment[14]. Thus, we synthesized polymers at various molar ratios of methacrylic acid (Table 1) to carry out MIPs with increased hydrophilicity and better imprinting efficiency due to reduction of non selective hydrophobic interactions.

3.2 Evaluation of the imprinting effect

The imprinting effect was initially evaluated by performing binding experiments in which amount of polymeric particles was mixed with both template and its analogues solutions. In table 1, the percentage of CHO, PROG and HY bound by imprinted and non-imprinted polymers after 6 hours incubation was shown.

For each polymers the binding percentage and the binding efficiency, α, were calculated. α was calculated as the ratio of percentage of bound template or analogues by MIPs and percentage of bound analytes by NIPs. As it is possible to note in Table 1, all the synthesized MIPs are able to rebind more template than the corresponding NIPs, confirming the

[12] Sellergren, B., & Shea, K.J.. Journal of Chromatography A, 635 (1993) 31-49.
[13] M. Kempe, K. Mosbach, Journal of Chromatography A, 694 (1995) 3-13.
[14] B. Dirion, Z. Cobb, E. Schillinger, L.I. Andersson, B. Sellergren, Journal of American Chemical Society, 125 (2003) 15101-15109

presence of imprinted cavities in their structure. Moreover, an increase of the amount of the functional monomer carries out to an increase of the α value for CHO: the most effective polymers were the MIP-3 ones, and a considerable reduction of the corresponding percentage of bound CHO of NIP-3 was observed. The reduction of non-specific interactions was ascribable to increased hydrophilicity of the polymers with the highest content of MAA. The binding percentages of the two analogues were much lower than that of CHO, confirming the selectivity of the imprinted cavities. The experiments were performed in the same condition used for cholesterol. For each polymer ε values were calculated as the ratio of percentage of bound template and percentage of bound analogues by MIPs.

3.3 Optimization of the molecularly imprinted solid-phase extraction procedure

The most effective MIPs (MIP-3) as sorbents for SPE of cholesterol were investigated, and a general procedure for a generic SPE (conditioning, loading, washing, eluting) was employed [15]. The cartridges were packed with 500mg of polymer and the loading and the washing steps were optimised. Different acetonitrile/water (10/0, 9/1, 8/2 and 7/3 v/v) mixtures were employed in loading, and washing step, and hot acetonitrile in the eluting one (Table 2). The best results were obtained when in the loading step a acetonitrile/water (7/3 v/v) mixture was employed. Both imprinted and non imprinted polymers retain all the CHO

[15] F. Puoci, C. Garreffa, F. Iemma, R. Muzzalupo, U.G. Spizzirri, N. Picci, Food Chemistry, 93 (2005) 349-353.

loaded. In order to eliminate the aspecific component of the interaction between cholesterol and polymeric matrices a washing step is needed, and it was performed using acetonitrile/water (7/3 v/v) mixture.

The optimized elution was obtained employing hot acetonitrile. In the eluting fraction of MIPs cartridges, 76% of loaded CHO was detected, while in NIPs cartridges CHO was only 20%.

The selectivity of the packing cartridges was evaluated by using PROG and HY solutions in the same condition tested for CHO (table 3).

For PROG solution, in the loading step, MIPs cartridges retains 95% of PROG, while NIPs cartridges the 81%. In the washing fraction of MIPs cartridges, 85% of PROG was detected, while in NIPs samples only 57% of the analyte was recovered. In all the two step, NIPs materials were found to retain much more PROG than MIPs ones, probably because of the presence of more non-specific interaction between the analyte and the polymeric matrices. In the eluting fractions, 10% and 24 % of PROG was detected for MIPs and NIPs cartridges respectively.

For HY solution, in the loading step, MIPs cartridges retains 30% of HY, while NIPs cartridges the 27%. In the washing fraction of MIPs cartridges, 25% of HY was detected, while in NIPs samples 20% of the analyte was recovered. In the eluting fractions, 5% and 7 % of HY was detected for MIPs and NIPs cartridges respectively.

The last two experiments clearly showed the high selectivity of the synthesized materials. In the elution fractions, indeed, 76% of loaded CHO was detected for MIPs cartridges, while for the same cartridges, this values was only 10% for PROG and 5% for HY.

3.4 Molecularly imprinted solid-phase extraction of food sample extracts

Food samples are complex chemical matrices and in chemical identification of their components, a intensive pretreatment of the samples is always required. The use of selective sorbents such MIPs can be very useful to obtain cleaner HPLC chromatograms[16].

In our experiments, Calabrian pecorino cheese was mixed with hot acetonitrile and crushed by stirring. After filtration, water was added to the acetonitrile to obtain the loading solution (acetonitrile/water (7/3 v/v)) and it was analyzed by HPLC (Figure 1).

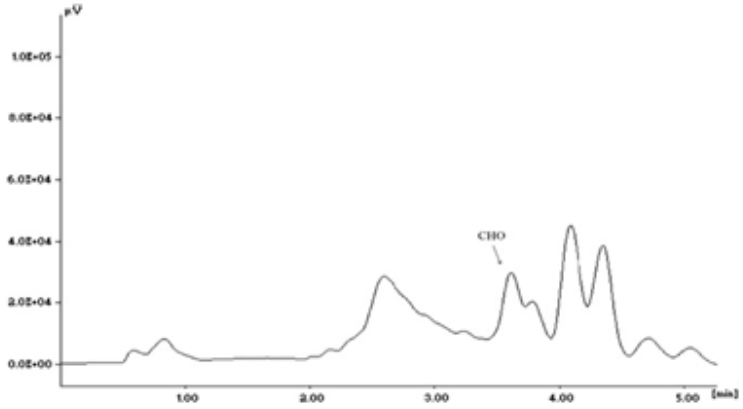

Figure 1. Chromatogram of the food extract.

Food extract solution was loaded to the polymeric cartridges and the complete retention of CHO by MISPE cartridges was raised (Figure 2a).

[16] E. Caro, R.M. Marcè, P.A.G. Cormack, D.C. Sherrington, F. Borrull, Anal. Chim. Acta 562 (2006) 145-151.

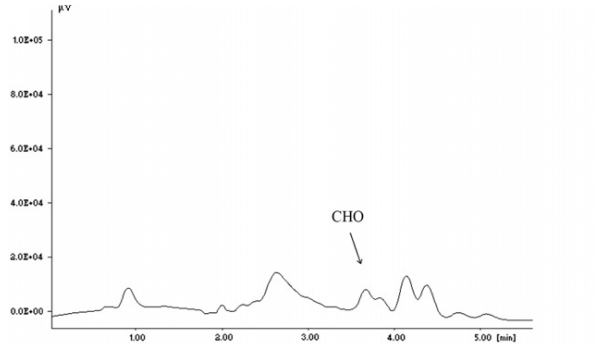

Figure 2a. Chromatogram of MISPE loading step

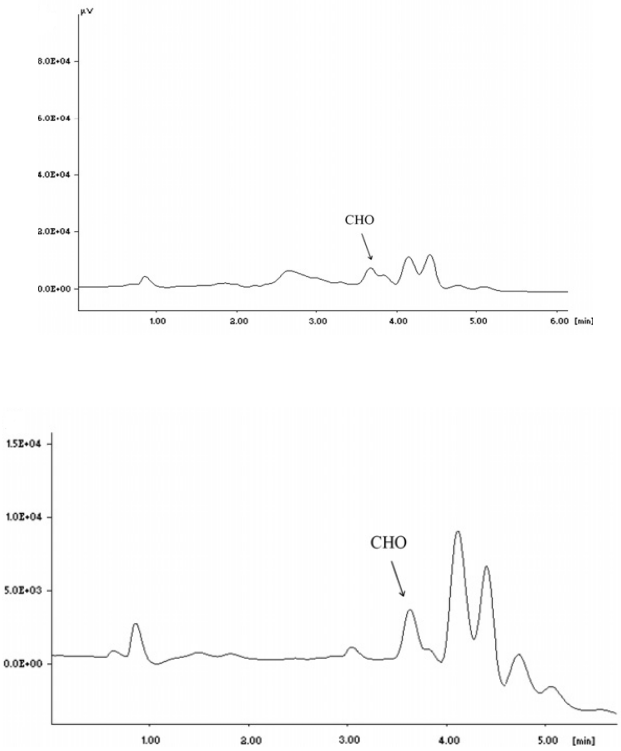

Figure 2 b,c. Chromatograms of washing steps

Two washing steps were performed: acetonitrile/water mixture (7/3 v/v) and acetonitrile/water mixture (9/1 v/v) respectively. In the washing fraction not so relevant peaks were observed at the retention time of CHO (Figure 2b,c).

Furthermore, a clean-up of the matrix was obtained as showed by the presence of several peaks referable to others compounds of the food sample. CHO was then selectively eluted with hot acetonitrile.

As it possible to note in Figure 3, a selective recovery of CHO was obtained: 80 % of loaded CHO, confirming that the compounds which co-eluted with CHO in the starting solution were completely removed during the MISPE procedure. The quantification of CHO content in cheese was performed by a methodology reported in literature[17] with small modifications.

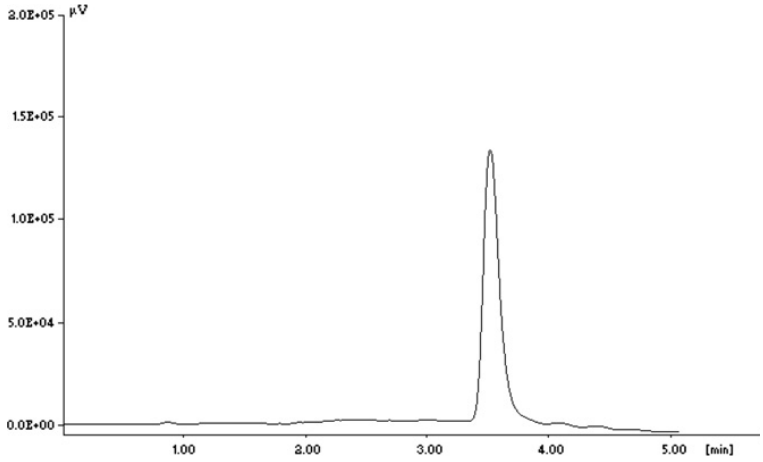

Figure 3. Chromatogram of MISPE eluting step.

[17] G. Contarini, M. Povolo, E. Bonfitto, S. Berardi, International Dairy Journal, 12, (2002) 573–578.

By performing the same experiments using NIPs cartridges, in the elution fraction no relevant amounts of CHO were detected.

Detection and Quantitation limits (LOD and LOQ) correspond to 5.94 x 10^{-7} mol l^{-1} and 1.84 x 10^{-6} mol l^{-1} respectively. The calibration curves were linear with correlation coefficients of $R^2 = 0.9982$.

The intraday precisions of the relative peak areas were between 2.3% and 3.5%; the interday precisions between 6.8% and 8.2%.

4. Conclusion

In this work, molecularly imprinted polymers have been synthesized using CHO as template, and the obtained materials were applied as SPE selective materials[18]. The imprinting effect and selectivity of the MIPs were evaluated by performing binding experiments in which CHO, PROG and HY solutions were employed. MIPs were found to be highly selective for CHO. After the optimization of the MISPE protocols using standard solutions of all the analytes, the application of MISPE in complex food samples was demonstrated.

These new sorbents based on Molecularly Imprinted Solid Phase Extraction (MISPE) are able to work in aqueous media and to clean/concentrate CHO in food matrices without any relevant pre-treatment of the sample. We used a cheese extract in acetonitrile to load the MIPs cartridges obtaining a selective purification and concentration of CHO in the elution step. In this fraction of MIPs cartridges, 80% of

[18] F. Puoci, M. Curcio, G. Cirillo, F. Iemma, U.G. Spizzirri, N. Picci, Food Chem. 106 (2008) 836-842.

cholesterol was detected and the correspondent chromatogram shows only a peak at the retention time of the template.

These relevant results showed that the method could be successfully applied for the determination of CHO in food samples.

Chapter 2

SELECTIVE RECOGNITION OF METHOTREXATE BY MOLECULARLY IMPRINTED POLYMERS

1. Introduction

Molecular imprinting are very useful technique to incorporate specific substrate recognition sites into polymers[1,2]. Molecular recognition characteristics of these polymers are attributed to complementary size, shape, and binding sites imparted to the polymers by the template molecules[3]. These specific binding properties must be attributed to specific interactions between the template and the functional groups in the polymeric network[4]. Usually, different kinds of template for the synthesis of MIPs were used, and our interest was focused on a antineoplastic drug widely used in clinical practice: (S)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzamido) pentanedioic acid, commonly named methotrexate (MTX).

Methotrexate is an antimetabolite that interferes with DNA replication and cell division by inhibiting the enzyme dihydrofolate reductase; it is commonly used in the treatment of various cancers, rheumatoid arthritis, and psoriasis[5]. However, since the cytotoxic effect of MTX is not selective for the cancer cells, it also affects the normal tissues that have a high rate of proliferation, including the hematopoietic cells of the bone

[1] R.A. Anderson, M.M. Ariffin, P.A.G. Cormack, E.I. Miller, Forensic Sci. Int. 174(2008) 40.
[2] F. Puoci, F. Iemma, N. Picci, Current Drug Deliv. 5 (2008) 85.
[3] K. Mosbach, Sci. Am. 295 (2006) 86
[4] F. Puoci, F. Iemma, G. Cirillo, N. Picci, P. Matricardi, F. Alhaique, Molecules 12 (2007) 805.
[5] R.J. Gibson, J.M. Bowen, D.M.K. Keefe, Cancer Treat. Rev. 34 (2008) 476.

marrow and the actively dividing cells of the gut mucosa[6]. Thus, the efficiency of this agent is often limited by severe side effects and toxic effects. For these reasons, in literature many studies reports on the development of sensitive methods for the monitoring of the concentration of MTX in human body[7].

Chen et al.[8] employed molecularly imprinted solid-phase extraction technique (MISPE) combined with electrochemical oxidation fluorimetry for the determination of methotrexate (MTX) in human serum and urine samples. However, because of the poor stability and solubility of MTX in aprotic and low polarity organic solvent, an analogue trimethoprim was employed as "dummy template" instead of MTX.

In this work, molecularly imprinted polymers were synthesized following the non-covalent approach by using MTX as template.

In the design of an imprinting protocol, particular attention was done in the choice of functional monomers, whose chemical groups, in order to obtain materials with good recognition properties, have to be complementary with those of the template. Functional monomers and template, indeed, are involved in formation of the prepolymerization complex, and the stability of this one is essential for the constitution of imprinted cavities. In our study, considering the functionalities of the template molecule, methacrylic acid (MAA), 2-(dimethylamino)ethyl methacrylate (MADAME) and a mixture of MAA and MADAME in different molar ratios were employed. The selectivity properties of

[6] L. Zhang, D. Russell, B.R. Conway, H. Batchelor, Crit. Rev. Ther. Drug 25 (2008) 259.
[7] V. Frenkel, Adv. Drug Deliver. Rev. 60 (2008) 1193.
[8] S. Chen, Z. Zhang, Spectrochim. Acta A 36 (2008) 70.

obtained materials were evaluated by using folic acid (FA), a MTX analogue molecule (Figure 1).

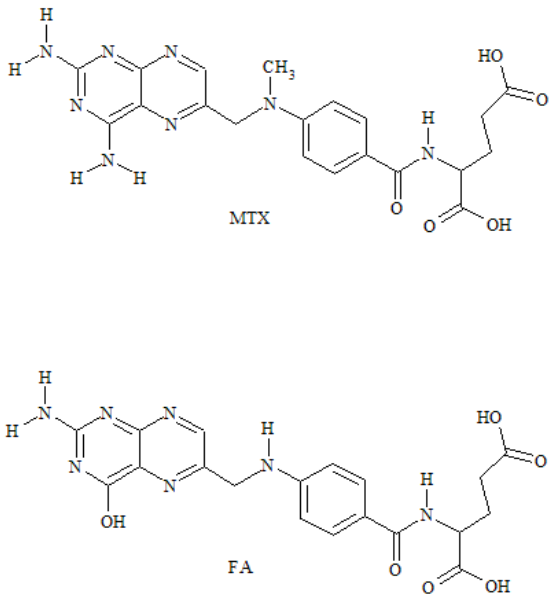

Figure 1. Chemical structure of methotrexate (MTX) and folic acid (FA).

2. Experimental Section

2.1 Materials

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2-(dimethylamino)ethyl methacrilate (MADAME), 2,2'-azoisobutyronitrile (AIBN), methotrexate (MTX), folic acid (FA), sodium hydrogen carbonate, hydrochloric acid (37% w/w), sodium hydrogen phospate, disodium hydrogen phosphate, dimethyl sulfoxide (DMSO) and acetonitrile were obtained from Aldrich. All solvents were reagent grade or HPLC-grade and used without further purification and they were

provided by Fluka Chemie. MAA and MADAME were purified before use by distillation under reduced pressure.

2.2 Instrumentation

The liquid chromatography consisted of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 290 nm. A 25 × 0.4 mm C4 Kromasil 100 column, particle size 5 μm (Teknocroma, Barcellona, Spain) was employed. The mobile phase was phosphate buffer 0.01 M, pH 7,4/acetonitrile (9/1, v/v) run isocratically at a flow rate of 1 mL/min and at room temperature.

2.3 Synthesis of methotrexate imprinted polymers

Molecularly Imprinted Polymers (MIPs) were prepared by bulk polymerization using methotrexate as template and according to the non-covalent imprinting approach. Briefly, MTX and functional monomer(s) were dissolved in 4.7 ml of DMSO in a thich-walled glass tube. The tube was sonicated for 6 min in a sonicating water bath and then EGDMA and AIBN (0.07g) were added. The polymerization mixture was bubbled with nitrogen, sonicated for 10 min and thermo-polymerized under a nitrogen atmosphere for 24 h at 60 °C. The resultant bulk rigid polymers were crushed, grounded into powder and sieved through a 63 nm stainless steel sieve. The sieved MIPs materials were collected and the very fine powder, suspended in the supernatant solution (acetone), was discarded. The obtained MIPs materials were extracted by Soxhlet apparatus with 200 ml of methanol for 48 h and successively they were dried under vacuum overnight at 40°C. The extracted imprinted polymers were boiled in 250 ml of a saturated solution of sodium hydrogen carbonate for 2 h

and then neutralized using HCl 0.1 N and distilled water. The resultant particles were dried under vacuum overnight at 40 °C. The washed MIPs materials were checked to be free of MTX and any other compound by HPLC analysis. Blank polymers, that act as a control, were synthesized under the same reaction conditions but in absence of the template. The molecular ratios of the different prepared polymers are shown in Table 2.

Sample	MTX (g)	MAA (g)	MADAME (g)	EGDMA (g)	MTX:MAA:MADAME: EGDMA
MIP1	0.14	0.43	-	3.10	1:16:0:50
NIP1	-				
MIP2	0.14	0.86	-	3.10	1:32:0:50
NIP2	-				
MIP3	0.14	-	0.79	3.10	1:0:16:50
NIP3	-				
MIP4	0.14	-	1.57	3.10	1:0:32:50
NIP4	-				
MIP5	0.14	0.43	0.79	3.10	1:16:16:50
NIP5	-				
MIP6	0.14	0.43	1.57	3.10	1:16:32:50
NIP6	-				

Table 2. Polymerization feeds composition.

2.4 Binding experiments

Evaluation of the capacity of the polymeric materials to recognize and bind MTX was performed by rebinding experiments in phosphate buffer solution (pH 7.4). Briefly, 50 mg of polymer particles were mixed with 1 ml MTX solution (0.05mM) in a 1 ml eppendorf and sealed. The

eppendorf were oscillated by a wrist action shaker (Burrell Scientific) in a water bath at 37 ± 0.5 °C for 24 h. Then the mixture was centrifuged for 10 min (10000 rpm) in an ALC® microcentrifugette® 4214 and the MTX concentration in the liquid phase was measured by HPLC. The amount of MTX bound to the polymer was obtained by comparing its concentration in the MIPs samples to the NIPs samples. The same experiments were also performed using folic acid solution and they were repeated five times.

3. Results and discussion

As before explained, the choice of the functional monomer(s) plays a key role in the preparation of an effective molecularly imprinted polymer. Because the non-covalent imprinting approach was employed, the interactions between functional monomers and template are based on H-bonds or ionic interactions[9]. In our study, the rebinding experiments were performed in water solution at pH 7.4, thus the ionization behaviour of both template and polymer structures strongly influences the performance of the imprinted polymers.

MTX is the prototype folate antagonist cytotoxic drug. Its molecule is made up of a heterocyclic portion (a 2,4-diaminosubstituted pterine ring) linked to a aminobenzoil portion, which is, in turn, an amide bonded to a glutamic acid unit[10]. Hence, MTX is a polyelectrolyte carrying two carboxyl groups, with dissociation constants (pKa) of 3.36 (α-carboxyl) and 4.70 (γ-carboxyl), and a number of potentially protonated nitrogen

[9] A. Beltran, E. Caro, R.M. Marcè, P.A.G. Cormack, D.C. Sherrington, F. Borrul, F.; Anal. Chim. Acta 597 (2007) 6.
[10] A.J. Hall, M. Quaglia, P. Manesiotis, E. De Lorenzi, B. Sellergren, Anal. Chem. 78 (2006) 8362.

functions, the most basic of which, presumably, the guanidinic N-1 on the pterine ring (pKa 5.71)[11].

It follows that, as shown in Figure 2, at pH of the rebinding media (7.4), the employed functional monomers establishes interactions with different parts of the template molecule. These interactions are due in part to hydrogen and partly to ionic bonds. Specifically, acidic monomer (MAA) bound to pteridine moiety, while basic monomer (MADAME) interacts with the carboxyl groups of glutamic acid.

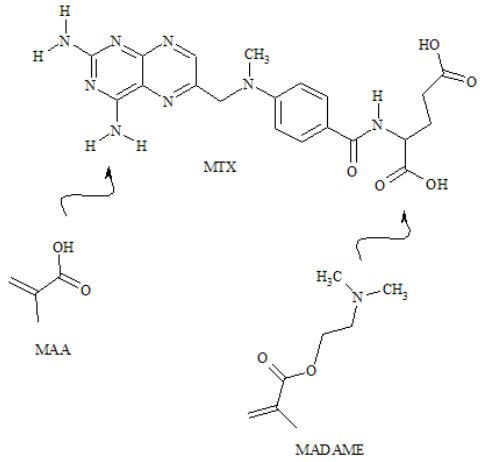

Figure 2. Interaction model between template and functional monomers.

The imprinting effect of synthesized materials was evaluated by binding experiments in which amounts of polymeric particles were incubated with a MTX solutions 0.05mM for 24 hours. These experiments were performed in buffered water solution at pH = 7.4. In Table 1 were reported MTX bound (%) and the relative α values.

[11] T.J. de Faria, A.M. de Campos, E. Lemos Senna, Macromol. Symp. 229 (2005) 228.

Polymers	% bound MTX	% bound FA	α_{MTX}	α_{FA}
MIP1	37 ± 1.2	7 ± 0.5	1.19	1.17
NIP1	31 ± 1.3	6 ± 0.6		
MIP2	14 ± 1.1	10 ± 0.7	0.93	0.91
NIP2	15 ± 0.9	11 ± 0.9		
MIP3	26 ± 1.1	36 ± 0.9	1.13	1.16
NIP3	23 ± 0.8	31 ± 0.7		
MIP4	64 ± 0.9	67 ± 1.0	1.08	1.06
NIP4	59 ± 0.7	63 ± 1.3		
MIP5	54 ± 0.7	31 ± 0.7	1.13	1.15
NIP5	48 ± 1.1	27 ± 0.8		
MIP6	80 ± 1.0	59 ± 1.3	1.51	1.20
NIP6	53 ± 1.3	49 ± 1.1		

Table 1. Bound (%) MTX and FA by imprinted and non-imprinted polymers.

Polymers in which only MAA was employed as functional monomer (MIP1 and MIP2) did not show any significant imprinting effect. In addition, by enhancing the amount of functional monomer (MIP2) in the polymeric feed, percentages of bound template significantly decreased. This behaviour is ascribable to the increasing of repulsive forces between ionized carboxylic groups of MAA and MTX in the rebinding media (pH 7.4) which overlay the attractive forces between functional monomer and pterine moiety of MTX. In order to avoid these negative interactions between carboxylic groups, MIP3 and MIP4 were synthesized by using a functional monomer presenting chemical groups able to coordinate the

glutammic acid moiety of MTX. For this purpose, a basic monomer such as MADAME was employed. As shown, also in this case the obtained resins present no significative imprinting effect. It was observed that, by increasing the amount of functional monomer, a considerable increasing of the no specific interactions template-polymeric matrices was raised.

Considering the results obtained by using single monomers, MIP5 and MIP6 were synthesized by employing both acidic and basic monomers in the polymeric feed and using two different stoichiometric ratios MADAME/MAA. In this way, each monomer interacts with complementary functionalities on template molecule (Figure 2), stabilizing the prepolymerization complex and minimizing the repulsive effects (due to the ionization of functional groups) in the rebinding media.

The best result was obtained by using the highest ratio MADAME/MAA (MIP6). As shown in table 1, indeed, the imprinting effect was improved, as highlighted by increasing of MTX bound (%) observed in moving from MIP5 to MIP6, and by unchanged value of aspecific bonds. In literature, different approaches were applied to make a quantitative determination of the imprinting effect[12]. The imprinting efficiency (α) is the easiest way to highlight the recognition properties in a MIP. In our work, α_{MTX} was determined as the ratio between the amount (%) of MTX bound by MIP and NIP, respectively[13]. By comparing α_{MTX} values (Table 1), is clear that MIP6 is the most effective polymer in selective recognition of MTX in a simulating physiological fluid. To evaluate cross-reactivity of imprinted polymers towards MTX analogue molecule, the same binding experiments were performed using FA, that differs from MTX in a

[12] L. Ye, K. Mosbach, Chem. Mater. 20 (2008) 859.
[13] M.A. Gore, R.N. Karmalkar, M.G. Kulkarni, J. Chromatogr. B 804 (2004) 211.

substituent on pterine moiety (-OH instead than $–NH_2$) and in the aminic group of the p-aminobenzoil portion (Figure 1). The most effective imprinted polymers in recognition of the template (MIP6), is characterized by a lower affinity towards the analogue FA. The binding percentages of MIP6 decrease of a 21% moving from MTX to FA, while they are unchanged in NIP6 case. As reported for MTX, α_{FA} was also determined as ratio between FA bound by MIP and NIP, respectively. In MIP6, a lower α_{FA} than α_{MTX} shows the chemical and spatial complementarity of MIP binding sites towards the template.

4. Conclusions

In this work[14], molecularly imprinted polymers selective for a useful antineoplastic drug, metotrexate, were synthesized. Methacrylic acid, 2-(dimethylamino)ethyl methacrilate and a mixture of them were tested as functional monomers in order to optimize the performance of imprinted materials. The specificity and the selectivity of imprinted polymers were tested by performing rebinding experiments in water at pH 7.4. The best results were obtained by employing both functional monomers in the polymeric feed. In this way, each monomer interacts with complementary functionalities on template molecule, stabilizing the prepolymerization complex and minimizing the repulsive effects (due to the ionization of functional groups) in the rebinding media.

[14] M. Curcio, O.I. Parisi, G. Cirillo, U.G. Spizzirri, F. Puoci, F. Iemma, N. Picci, E-polymers 78 (2009) 1-7.

Chapter 3

IMPRINTED HYDROPHILIC NANOSPHERES AS DRUG DELIVERY SYSTEMS FOR 5-FLUOROURACIL SUSTAINED RELEASE

1. Introduction

5-Fluorouracil (Figure 1) is a widely used antineoplastic agent for the treatment of many types of cancers, suh as colorectal, breast, head and neck malignancies[1]. Its endurance and longevity in cancer chemoterapy is due to the fact that 5-FU is the only anticancer agent on the market that shows synergism with the most other anticancer drugs in combination therapy[2].

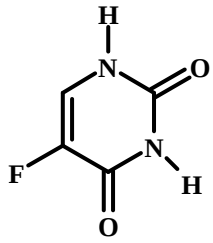

Figure 1a

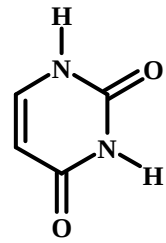

Figure 1b

Figure 1. Chemical structures of a) 5-FU and b) Uracil

[1] A. Di Paolo, D. Romano, F. Vannozzi, A. Falcone, E. Mini, L. Cionini, T. Ibrahim, D. Amadori, M. Del Tacca, Clin. Pharmacol. Ther. 72 (2002) 627-637.
[2] O.N. Al Safarjalani, R. Rais, J. Shi, R.F. Schinazi, F.N.M. Naguib, M.H. El Kouni Cancer Chemother. Pharmacol. 58 (2006) 692-698.

On the other hand, it is effective against cancers refractory to other treatments. Despite these advantages, the use of 5-FU is limited by its quick metabolism in the body, therefore the maintenance of high serum concentrations of this drug to improve its therapeutic activity is needed. The maintenance of these serum concentrations requires continuous administrations, but 5-FU shows severe toxic effects; consequently reaching and/or exceeding the toxic concentration must be avoided[3].

In order to improve therapeutic index and reduce toxic effects of this drug, numerous studies in literature are aimed at design of devices for the controlled release of 5-FU, many of them are based on polypeptidic and polysaccharidic systems[4]. In our previous study[5], irregular and not swellable microparticles of 5-FU imprinted polymers by employed bulk polymerization were prepared, and their application as devices for controlled release of this drug was demonstrated. In order to obtain a better control on particle size and shape, and so also on the 5-FU release profile, in this work molecularly imprinted hydrogel nanospheres have been synthesized applying straightforward method such as precipitation polymerization. Coupling the properties of hydrogels, nanospheres and MIP it is possible to obtain very useful systems to be applied in drug delivery field. Firstly, due to their significant water content, hydrogels possess a degree of flexibility very similar to natural tissue, which minimizes potential irritation to surrounding membranes and tissues[6].

[3] K.R. Johnson, K.K. Young, W. Fan, Clin. Cancer Res. 5 (1999) 2559-2565.
[4] E. Fournier, C. Passirani, N. Colin, P. Breton, S. Sagodira, J.P. Benoit, Eur. J. Pharm. Biopharm. 57 (2004) 189-197.
[5] F. Puoci, F. Iemma, G. Cirillo, N. Picci, P. Matricardi, F. Alhaique, Molecules 12 (2007) 805-814.
[6] M.E. Byrne, K. Park, N.A. Peppas, Adv. Drug Deliv. Rev. 54 (2002) 149-161.

Moreover, the high swelling properties of these materials improved their recognition characteristics, because of the enhanced accessibility of template to the imprinted cavities. Molecularly Imprinted Polymers (MIPs) are tailor-made materials with high selectivity for a target molecule named template. As before described, these synthetic molecularly selective receptors have broad application in many areas of science and the area of greatest potential, and probably an area of greatest challenge, is that of therapeutics and medical therapy. They have been applied usefully as excipients to modify drug release from solid dosage forms. Finally, advances in nanobiotechnology have resulted in the evolution of several novel colloidal carriers such as liposomes, polymeric micelles nanoparticles, and nanoemulsions to maximize tumor cell killing effect during the tumor growth phase, and to protect the surrounding healthy cells from unwanted exposure to the excess cytotoxic agent[7]. Polymeric nanoparticles are the most attractive colloidal carriers owing several merits such as ease of purification and sterilization, drug targeting possibility, and sustained release action[8]. In conclusion, the delivery of an anticancer drug by molecularly imprinted nanospherical hydrogels offers the possibility of maximising its efficacy and safety and providing a suitable rate of delivery of the therapeutic dose at the most appropriate site in the body, in order to prolong the duration of the pharmacological activity, to reduce the side effects and to minimize the administration frequency, and thus enhancing patient compliance[9]. The purpose of this

[7] A.K. Yadav, P. Mishra, S. Jain, P. Mishra, A.K. Mishra, G.P. Agrawal J. Drug Target. 16 (2008) 464-478.
[8] E. Allemann, R. Gurny, E. Doelker, Eur. J. Pharm. Biopharm. 39 (1993) 173–191.
[9] J.Z. Hilt, M.E. Byrne, Adv. Drug Del. Rev. 56 (2004) 1599-1660.

study was to investigate the possibility of employing these monodispersed imprinted nanoparticles as devices for the controlled/sustained release of 5-FU in biological fluids.

2. Experimental Section

2.1. Reagents and standards

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2,2-azoisobutyronitrile (AIBN), 5-fluorouracil (5-FU) and uracil (U) were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). All solvents were reagent grade or HPLC-grade and used without further purification and were provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

2.2. Synthesis of 5-FU spherical molecularly imprinted polymers

Spherical Molecularly Imprinted Polymers were prepared by precipitation polymerization using 5-fluorouracil as template, MAA as functional monomer and ethylene glycol dimethacrylate as crosslinking agent. General synthetic procedure was reported: template (1mmol) and MAA (8mmol) were dissolved in a mixture of acetonitrile (20 ml) and methanol (20 ml), in a 100 mL round bottom flask and then EGDMA (10mmol) and AIBN (50 mg) were added. The polymerization mixture was degassed in a sonicating water bath, purged with nitrogen for 10 min cooling with an ice-bath. The flask was then gently agitated (40 rpm) in an oil bath. The temperature was increased from room temperature to 60°C within 2 h, and then kept at 60 °C for 24 h. At the end of the reaction, the particles were filtered, washed with 100 ml of ethanol, 100 ml of acetone and then

with 100 ml of diethyl ether. The template was extracted by ‘‘Soxhlet apparatus’’ using methanol-acetic acid mixture (1:1 (v/v), 100ml) for at least 48h, followed by methanol for another 48h, and monitoring the drug concentration in the extraction solvent by HPLC. Particles were successively dried under vacuum overnight at 40 °C. Blank polymers, that act as a control, were also prepared when polymerization was carried out in the absence of 5-fluorouracil.

2.3. Water content of spherical polymers

Aliquots (40–50 mg) of the nanospheres dried to constant weight were placed in a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighted, and left to swell by immersing the filter plus support in a beaker containing the swelling media: phosphate buffer (pH 7.4, simulated biological fluids). At predetermined times (2 - 4 - 8 -10 - 15 - 20 - 24 h), the excess water was removed by percolation at atmospheric pressure. Then, the filter was placed in a properly sized centrifuge test tube by fixing it with the help of a bored silicone stopper, then centrifuged at 3500 rpm for 15 min and weighted. The filter tare was determined after centrifugation with only water. The weights recorded at the different times were averaged and used to give the water content percent (WR %) by the following Eq. (1):

$$WR\% = \frac{W_s - W_d}{W_d} \times 100 \qquad (1)$$

Where Ws and Wd are weights of swollen and dried spherical microparticles, respectively. Each experiment was carried out in triplicate.

2.4. Binding experiments

Binding experiments were performed both in organic (acetonitrile) and in aqueous media (phosphate buffer pH 7.4). Briefly, 50 mg of polymer particles were mixed with 5 ml 5-FU solution (0.3 mM) in a 10 ml conical centrifugation tube and sealed. The tubes were oscillated by a wrist action shaker (Burrell Scientific) in a water bath for 24 h. Then the mixture was centrifuged for 10 min (10000 rpm) and the 5-FU concentration in the liquid phase was measured by HPLC. The amount of 5-FU bound to the polymer was obtained by comparing its concentration in the imprinted samples to the non imprinted ones. The same experiments were performed using uracil solution. Experiments were repeated five times.

2.5. Drug Loading by the Soaking Procedure

Polymeric matrix (2.0 g) was immersed in a 5-FU solution in acetonitrile (20 mL, 5.5 mM) and soaked for 3 days at room temperature. During this time, the mixture was continuously stirred and then the solvent was removed by filtration. Finally the powder was dried under vacuum overnight at 40°C. The same experiments were performed using uracil solution.

2.6. In vitro release studies

Release studies were carried out using the dissolution method described in the USP XXIV (apparatus 1-basket stirring element). The samples were dispersed in flasks containing 10 mM phosphate buffer solution (pH 7.4 simulating biological fluids, 10 ml). Thus samples were drawn from dissolution medium at appropriate time intervals to determine the

amounts of drug released by HPLC. The amount of 5-fluorouracil released from six samples of each formulation was used to characterize drug release. The same experiments were performed using particles loaded with uracil. Experiments were repeated five times.

2.7. HPLC Analysis

A Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 266 nm were used. A 250 × 4 mm C-18 Hibar® column, 10 mm particle size (Merck, Darmstadt, Germany) was employed. The mobile phase was methanol/phosphate buffer 5mM, pH 6.8 (9/1, v/v) and the flow rate was 0.5 mL/min.

2.8. Scanning electron microscopy

Scanning electron microscopy (SEM) photographs were obtained with a Jeol JSMT 300 A; the surface of the samples was made conductive by deposition of a gold layer on the samples in a vacuum chamber.

2.9 Dimensional analysis

Approximate range in particle size was determined by measuring 300 particles per each sample with the use of an image processing and analysis system, a Leica DMRB equipped with a LEICAWild 3D stereomicroscope.

3. Results and discussion

3.1. Synthesis of imprinted nanospheres

5-Fluoruracil molecularly imprinted polymers were synthesized employing the non-covalent approach, in which the interactions between template molecule and polymeric matrices were based on H-bonds, the dominant interactions in biological systems. Furthermore, this approach is more convenient because of the easy access to a broad range of functional monomers from commercial sources. Conventionally, MIPs are synthesized by bulk polymerization methods in a porogenic solvent and block co-polymer particles with the desired size are obtained by grinding and sieving[10]. However, this method yields particles with limited control on particle size and shape. In literature, several attempts have been applied to produce monodisperse molecularly imprinted polymeric particles using methods such as suspension polymerization in water[11], dispersion polymerization[12], liquid perfluorocarbon[13], and via aqueous two-step swelling polymerization[14]. However, during the polymerization procedure, these techniques require water or highly polar organic solvents, which frequently decrease specific interactions between functional monomers and template molecules. Precipitation technique not only allows to avoid these disadvantages, but also to obtain monodispersed molecularly imprinted micro- and nanospheres, without

[10] E. Caro, R.M. Marcé, F. Borrull, P.A.G. Cormack, D.C. Sherrington, Trends Anal. Chem. 25 (2006) 143-154.
[11] J.P. Lai, X.Y. Lu, C.Y. Lu, H.F. Ju, X.W. He, Anal. Chim. Acta 442 (2001) 105-111.
[12] R. Say, E. Birlik, A. Ersoz, F. Yilmaz, T. Gedikbey, A. Denizli, Anal. Chim. Acta 480 (2003) 251-258.
[13] A.G. Mayes, K. Mosbach, Anal. Chem. 68 (1996) 769-774
[14] L. Piscopo, C. Prandi, M. Coppa, K. Sparnacci, M. Laus, A. Lagana, R. Curini, G. D'Ascenzo, Macromol. Chem. Phys. 203 (2002) 532-1538.

the integrity and stability of recognition sites are compromised[15]. Moreover, spherical shape should be advisable in order to avoid swelling anisotropic behaviour associated with other geometries. In our protocol, MAA as functional monomer and EGDMA as crosslinking agent were used. Spherical geometry and the practically monodispersion of prepared samples were confirmed by Scanning Electron Micrographs (Figure 2) and dimensional analysis.

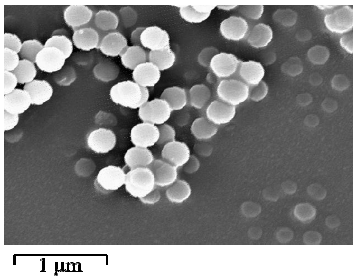

Figure 2. Scanning Electron Micrograph of MIP

Polymerization feeds composition and mean particle sizes (d_n) of the microspheres are shown in Table 1.

Polymers	**5-FU/MAA/EGDMA (mmol)**	**d_n (nm)**	**Polydispersivity**
SMIP	1.0 / 8.0 / 10.0	274	1.03
SNIP	-- / 8.0 /10.0	268	1.01

Table 1. Polymerization feed composition and mean particle sizes (d_n)of nanospheres as means ± S.D.

[15] S. Wei, A. Molinelli, B. Mizaikoff, Biosens. Bioelectr. 21 (2006) 1943-1951.

3.2. Evaluation of the imprinting effect: binding experiments in organic and in water media.

The imprinting effect of synthesized materials was evaluated by binding experiments in which amounts of polymeric particles were incubated with a 5-FU solutions 0.3 mM for 24 hours. These experiments were performed in organic (acetonitrile) and in water (buffered water solution at pH=7.4) media. As shown in Table 2, in both binding media, imprinted nanosphers were able to bind much more template than the respective non imprinted ones, confirming the presence of imprinted cavities in their structure.

Matrix	**% 5-FU bound**		**% Uracil bound**		**Water content (%)**
	CH_3CN	pH 7.4	CH_3CN	pH 7.4	pH 7.4
SMIP	32± 1.1	36±1.6	14± 1.4	16± 1.6	377 ± 0.3
SNIP	7± 1.3	10± 1.9	11± 1.1	14± 1.8	380 ± 0.5

Table 2. Percentages of bound 5-FU and Uracil by imprinted and non-imprinted polymers after 24 hours in acetonitrile and in buffered water solution at pH 7.4, and water content (%) of polymeric matrices at pH 7.4. Data are shown as means ± S.D.

In literature, different approaches were applied to make a quantitative determination of the imprinting effect[16]. The imprinting efficiency α is the easiest way to highlight the recognition properties in a MIP. In our work, $\alpha_{5\text{-FU}}$ was determined as the ratio between the amount (%) of 5-FU bound

[16] L. Ye, K. Mosbach, Chem. Mater. 20 (2008) 859-868.

by SMIP and SNIP[17]; these values (4.6 in CH_3CN and 3.6 in water media) clearly prove the specificity of the interaction between the template and the functional groups on the polymeric nanoparticles.

To evaluate cross-reactivity of imprinted polymers towards 5-fluorouracil analogue molecules, the same binding experiments were performed using uracil, that differs from 5-FU only in substituent at position 5 of the ring (Figure 1). As reported for 5-FU, α_U was also determined as ratio between U bound by SMIP and SNIP, respectively. The very low values (1.3 in CH_3CN and 1.1 in water media) show the high chemical an spatial complementarity of SMIP binding sites toward the template. It is considered that the selective interaction between the polymeric matrices and template is ascribable to fluorine atom that takes an active part in interaction with functional monomer. Finally, the selectivity of the synthesized polymeric nanospheres can be highlighted by introducing another coefficient (ε) which is a quantitative measure of the imparted selectivity within the imprinted nanospheres and were calculated to be 2.3 in both organic and water media, indicating that imprinted polymers had higher affinity (more than 2 times) for 5-FU comparing to U.

As reported, hydrogels possess a degree of flexibility very similar to natural tissue, but in MIPs structure a compromise between the rigidity and flexibility of the polymers is needed. The structure of the imprinted cavities in the MIPs should be stable enough to maintain the conformation in the absence of the template and it should also be flexible enough to facilitate the attainment of a fast equilibrium between the release and re-

[17] M.A. Gore, R.N. Karmalkar, M.G. KulKarni J. Chromatogr. B 804 (2004) 211-221.

uptake of the template in the cavity. In order to test swelling properties of the imprinted hydrogels, aliquots of nanospheres were immersed in swelling media: phosphate buffer at pH 7.4, simulating biological fluids. It can be observed from Figure 3 that the amount of water uptake by imprinted nanospheres increases with time and the maximum water uptake is obtained after 24 h (Table 2). These high swelling characteristics make the imprinted cavities of polimeric network easily accessible to the template; this is to advantage of recognition properties of imprinted nanospheres.

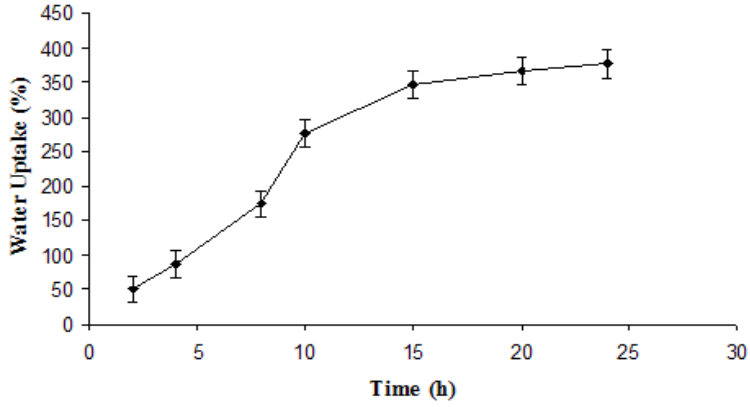

Figure 3. Swelling profile of SMIP

3.3. In vitro release studies

After evaluation of imprinting effect of synthesized materials, their application as devices for 5-FU delivery in plasma simulating fluids was verified. Different strategies were applied to determine the possibility to successfully employ an imprinted polymer to obtain a

sustained/controlled release of the selected drug[18]. In a first approach[19], the loading step is performed by binding experiments. After the incubation time, the supernatant is removed and the samples dried. In this way, different amounts of drug are bound by SMIP and SNIP according to the imprinting effect. In these conditions, because the releasing profile strongly depend on the loaded drug, SMIP particles are able to release an higher amount of drug than SNIP ones. In another approach, more useful to emphasize the differences in the releasing profiles from SMIP and SNIP, both the polymers are loaded with the same amount of drug by mixing them with a drug standard solution. After the incubation time, samples were dried and release experiments performed. The percentage of 5-FU released was calculated considering 100% the 5-FU content in dried samples[20]. In our work, the second approach is employed and the presence of imprinted cavities in spherical polymers makes the release of template more extended over time in comparison with non imprinted materials. As it is possible to observe in Figure 4, indeed, while the drug is completely released within five hours by SNIP, that do not have specific binding cavities, for SMIP samples even after 50 hours the release is not yet complete. Such behaviour is in accordance with results obtained from the binding experiments.

Moreover, monodispersed spherical particles allow to obtain isotropic release behaviour and so also a better control of 5-FU release profile in comparison with materials with irregular size and shape.

[18] C. Alvarez-Lorenzo, A. Concheiro, J. Chromatogr. B 804 (2004) 231–245 .
[19] B. Singh, N. Chauhan, J. Macromol. Sci. A Pure Appl. Chem. 45 (2008) 776–784.
[20] G. Pitarresi, P. Pierro, G. Giammona, F. Iemma, R. Muzzalupo, N. Picci Biomaterials 25 (2004) 4333–4343.

In order to further evaluate selectivity of SMIP, uracil release experiments in the same conditions tested with 5-FU were also performed. The data obtained from these experiments confirme results showed in binding tests: uracil release from imprinted polymers, indeed, that just in 2 hours was completed, was remarkably faster than that obtained when 5-FU was used (data not shown).

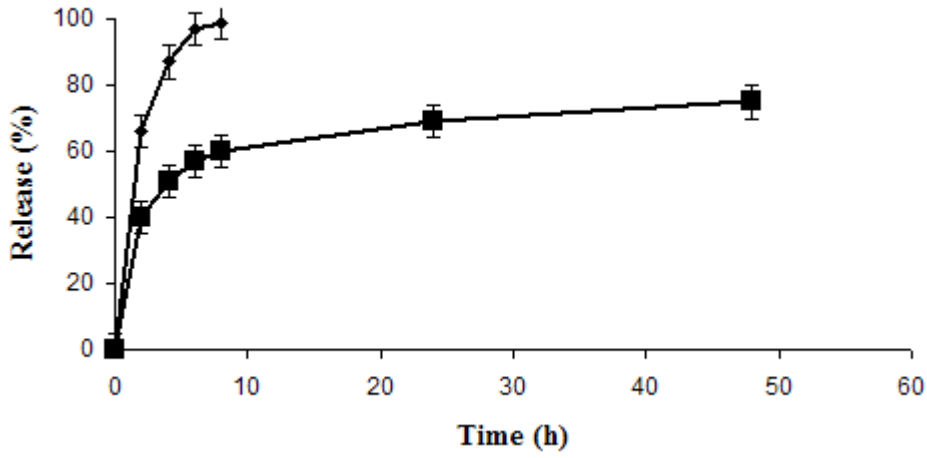

Figure 4. 5-FU release profile from SMIP (—■—) and SNIP (- -◆- -) in plasma simulating fluids.

4. Conclusions

In this work, 5-FU imprinted nanospheres with swelling characteristics were prepared using methacrylic acid and ethylene glicoldimethacrylate as functional monomer and crosslinker, respectively[21]. One pot

[21] G. Cirillo, F. Iemma, F. Puoci, O.I. Parisi, M. Curcio, U. Spizzirri, N. Picci, J. Drug Targ. 17 (2009) 72-77.

precipitation polymerization as synthetic technique to obtain the monodispersed particles was chosen. The recognition properties and selectivity of synthesized materials were demonstrated through binding experiments with template and its analogue, uracil.

The results obtained from the *in vitro* release studies in plasma simulating fluids indicated that these polymeric matrices are also suitable for a controlled/sustained delivery of the tested anticancer agent in biological fluids. Hydrogel characteristic of these materials contributes to make binding sites in the polymeric network easily accessible to the template, while spherical shape allows to obtain a better control on 5-FU release.

SECTION II

Preparation and Characterization of Thermo-Responsive Hydrogels as Drug Delivery Systems

2.1 Thermo-Responsive Hydrogels

Over the last two decades, the field of controlled drug delivery has been faced with two major challenges. One has been achieving sustained zero-order release of a therapeutic agent over a prolonged period of time. This goal has been met by a wide range of techniques, including osmotically driven pumps[1], matrices with controllable swelling[2], diffusion[3], or erosion rates[4], non-uniform drug loading profiles[5], and multi-layered matrices[6]. The second of these challenges is the controlled delivery of therapeutic molecules or proteins in a pulsatile or triggered fashion. Two different methodologies have been broadly investigated as possible solutions to these requirements. One is the fabrication of a delivery system that releases its payload at a predetermined time or in pulses of a predetermined sequence. The other is to develop a system that can respond to changes in the local environment. These systems have been shown to alter their rate of drug delivery in response to stimuli including the presence or absence of a specific molecule, magnetic fields, ultrasound, electric fields, temperature, light, and mechanical forces. Such

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[3] E.S. Lee, S.W. Kim, S.H. Kim, J.R. Cardinal, H. Jacobs, J. Membr. Sci. 7 (1980) 293-303.
[4] L. Yang, R. Fassihi, J. Control. Rel. 44 (1997) 135-140.
[5] P. Hildgen, J.N. McMullen, J. Control. Rel. 34 (1995) 263-271.
[6] Y. Qui, N. Chidambram, K. Flood, J Control. Rel. 51 (1998) 123-130.

systems are suitable for release of therapeutics that benefit from nonconstant plasma concentrations[7].

Several polymeric delivery systems undergo phase transitions and demonstrate marked swelling-deswelling changes in response to environmental changes including solvent composition ionic strength, temperature, electric fields, and light.

Among others, hydrogels belong to the most intensively investigated systems to be applied in biomedical field due to their unique properties such as the high water content and the possible control over the swelling kinetics[8].

Hydrogels that respond to temperature changes are the subject of the second section of this work. Their sensitivity to the thermal environment is the sole stimulus for their gelation with no other requirement for chemical or environmental treatment, and can be thus produced e.g. upon injection to the body, when temperature is increased from ambient to physiological. The phenomenon of transition from a solution to a gel is commonly referred to as sol-gel transition. Some hydrogels exhibit a separation from solution and solidification above a certain temperature. This threshold is defined as the lower critical solution temperature (LCST). Below the LCST, the polymers are soluble. Above the LCST, they become increasingly hydrophobic and insoluble, leading to gel formation.

[7] A.K. Anal, Recent Patents on Endocrine, Metabolic & Immune Drug Discovery 1 (2007) 83-90.

[8] N.A. Peppas, A.G. Mikos, Preparation methods and structure of hydrogels, in: N.A. Peppas (Ed.), Hydrogels in Medicine and Pharmacy, vol. 1, CRC Press, Boca Raton, Florida, 1986, pp. 1–25.

In contrast, hydrogels that are formed upon cooling of a polymer solution have an upper critical solution temperature (UCST)[9]. The sol-gel transition of thermosensitive hydrogels can be experimentally verified by a number of techniques such as spectroscopy[10], differential scanning calorimetry (DSC)[11] and rheology[12]. There are various mechanisms behind thermogelation in aqueous solutions, and for some polymers they are still a topic of debate. Many polymers show a decrease in solubility that is attributed by changes in the overall hydrophilicity of the polymer chains upon temperature change. When a polymer is dissolved in water, there are three types of interactions that take place: between polymer molecules, polymer and water and between water molecules. For polymers exhibiting an LCST, a temperature increase results in a negative free energy of the system which makes water–polymer association unfavorable, facilitating the other two types of interactions. This negative free energy (ΔG) is attributed to the higher entropy term (ΔS) with respect to the increase in the enthalpy term (ΔH) in the thermodynamic relation $\Delta G=\Delta H-T\Delta S$. The entropy increases due to water-water associations which are the governing interactions in the system. This phenomenon is the so-called hydrophobic effect[13] (Figure 1).

[9] N.A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Eur. J.Pharm.Biopharm. 50 (2000) 27–46.
[10] X. Yin, A.S. Hoffman, P.S. Stayton, Biomacromolecules 7 (2006) 1381–1385.
[11] Y.Y. Liu, Y.H. Shao, J. Lu , Biomaterials 27 (2006) 4016–4024.
[12] L. Li, H. Shan, C.Y. Yue, Y.C. Lam, K.C. Tam, X. Hu, Langmuir 18 (2002) 7291–7298.
[13] H.G. Schild, Prog. Polym. Sci. 17 (1992) 163–249.

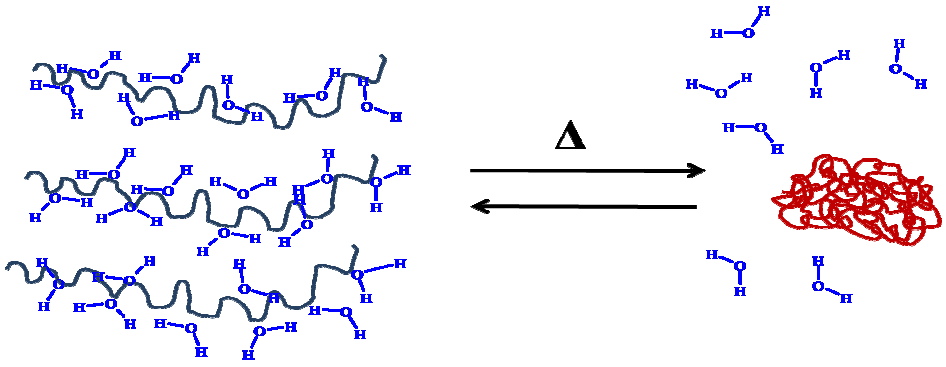

Figure 1. Swelling-deswelling mechanism of thermo-responsive hydrogels.

Hydrogels based on poly(N-isopropylacrylamide) (pNIPAAm) and its copolymers represent the most popular thermoreversible systems. Poly(N-isopropylacrylamide) is non-biodegradable and exhibits a sharp phase transition, with an LCST at about 32°C in pure water. Below the LCST, pNIPAAm assumes a flexible, extended coil conformation in aqueous solutions. A sudden temperature increase above the transition temperature of these gels resulted in the formation of a dense, shrunken layer on the gel surface, which hindered water permeation from inside the gel into the environment. Drug release from the pNIPPAm hydrogels at temperatures below 32°C was governed by diffusion, while above this temperature drug release was stopped completely, due to the 'skin layer' formation on the gel surface (on-off drug release regulation)[14]. Because of their non biodegradability and non sustained drug release under physiological

[14] R.A. Siegel, C.G. Pitt J. Control. Rel. 3 (1995) 173-188.

conditions, the biomedical and biological applications of such gels usually involve the chemical modification of pNIPAAm.

These modifications are usually performed to introduce functional groups that can increase the LCST towards body temperature[15], to improve the mechanical properties[16] or to interact with certain drugs[17].

Recently, much research has been done to associate biopolymers (proteins and polysaccharides) with NIPAAm in an attempt to prepare matrices with increased LCST. On the other hand, studies have been reported in the literature concerning stimuli-sensitivity in combination with molecularly imprinted polymers: they represent an important starting point for the development of new generations of intelligent and self-regulated drug delivery systems.

2.2 Thermo-Responsive Hydrogels Based on Natural Polymers

Many natural polymers have been shown to exhibit gelation upon temperature change. Researchers have used them alone or in combination with synthetic polymers to fabricate thermally responsive hydrogels with desired properties.

Liu et al.[18] have grafted methylcellulose with the synthetic N-isopropylacrylamide (NiPAAm), combining the thermogelling properties of both materials. It was possible to prepare fast reversibly thermogelling hydrogels by adjusting the ratios of the two components. They reported

[15] H. Feil, Y.H. Bae, J. Feijen , S.W. Kim, Macromolecules 25(1992) 5528-30.
[16] L.C. Dong, A.S. Hoffman, J. Control. Release 13 (1990) 21-31.
[17] T.G. Park Biomaterials 20 (1999) 517-21.
[18] W. Liu, B. Zhang, W.W. Lu, X. Li, D. Zhu, K. De Yao, Q. Wang, C. Zhao, C. Wang, Biomaterials 25 (2004) 3005- 3012.

that a low percentage of methylcellulose decreases the LCST as compared to pNiPAAm, but with a high MC ratio the LCST increases. They also found that addition of MC to NiPAAM polymers enhances the mechanical strength of the hydrogel with no syneresis.

Bhattarai et al.[19] incorporated poly(ethylene glycol) (PEG) into chitosan and were able to form a thermoreversible hydrogel with no additional crosslinking agents. Moreover, PEG grafting improved the solubility of chitosan in water, and the gelation was found to be possible in physiological pH values. The same group[20] evaluated the PEG-grafted chitosan for controlled drug release in vitro. Using albumin as a model protein, an initial burst release was observed, which was followed by a steady release from the hydrogel for about 3 days. After this time, the remaining albumin could not be released from the hydrogel until the gel matrix was dissolved in the media. When the PEG-grafted chitosan was crosslinked in situ with genipin, a crosslinking agent with low cytotoxicity, quasi-linear drug release was possible for up to 40 days; however the hydrogel lost its thermoreversibility at 37°C.

Gelatin is another biopolymer with thermoreversible properties. At temperatures below 25°C, an aqueous gelatin solution solidifies due to the formation of triple helices and a rigid three-dimensional network. When the temperature is raised above approximately 30 °C, the conformation

[19] N. Bhattarai, F.A. Matsen, M. Zhang, Macromol. Biosci. 5 (2005) 107–111.

[20] N. Bhattarai, H.R. Ramay, J. Gunn, F.A. Matsen, M. Zhang, J. Control. Release 103 (2005) 609–624.

changes from a helix to the more flexible coil, rendering the gel liquid again[21].

As the opposite thermal behavior is desired for biomedical applications, researchers have combined gelatin with other polymers, which show thermal gelation close to body temperature. Gelatin has the advantage of allowing for easy modification on the amino acid level; moreover, it is biodegradable and biocompatible. A binary-component hydrogel composed of gelatin and monomethoxy poly(ethylene glycol)–poly(D,L-lactide) (mPEG–DLLA) block copolymers was synthesized by Yang and Kao[22]. For most compositions of gelatin and mPEG-DLLA, the hydrogel was shown to flow at 37°C and gel at room temperature, however a 100 mg/mL gelatin solution underwent fast gelation at 37°C when mixed with 30% wt mPEG–DLLA. Different hydrogel compositions were also examined for drug release kinetics with gentamycin sulfate as the model drug. At room temperature, 5 days or longer was necessary for 50% drug release, and the release lasted up to 40 days. At 37°C, gelatin-mPEGDLLA showed an even slower release profile, however after 1 week the release was no longer detectable due to, degradation of the hydrogel matrices. Ohya and Matsuda[23] have grafted gelatin with NiPAAm in an effort to produce a thermoresponsive extracellular matrix analogue. Aqueous solutions showed a sol–gel transition at physiological temperature when the weight ratio of pNiPAAm to gelatin chains was

[21] C. Joly-Duhamel, D. Hellio, M. Djabourov, Biodiversity and physical chemistry, Langmuir 18 (2002) 7208-7217.
[22] H. Yang, W.J. Kao, Pharm. Res. 23 (2006) 205-214.
[23] S. Ohya, T. Matsuda, J. Biomater. Sci. Polym. Ed. 16 (2005) 809-827.

higher than 5.8. Smooth muscle cells were suspended in medium solutions of pNiPAAm/gelatin and subsequently incubated at 37°C. It was shown that a low hydrogel concentration (5% w/v) and a high pNiPAAm to gelatin ratio (P/G) supported the highest cell proliferation and extracellular matrix production. The authors suggested that this was due to increased hydrophobicity caused by higher pNiPAAm ratios, which would lead to the formation of large aggregates. As a result, a higher porosity with larger pore size occurs, which comprises a favorable cell environment.

2.3 Thermo-Responsive Molecularly Imprinted Polymers

The combination of stimuli-sensitivity and imprinting may have considerable practical advantages: the imprinting provides a high loading capacity of specific molecules, while the ability to respond to external stimuli contributes to modulate the affinity of the network for the target molecules, providing regulatory or switching capability of the loading/release processes[24].

From a theoretical point of view, it is also interesting to study the ability of the polymer network to memorize a specific conformation after a dramatic change in swelling degree. In this sense, Watanabe et al.[25] observed that NIPAAm (16 mmol)-acrylic acid (4 mmol) cross-linked (1mmol) polymers, synthesized in the presence of norephedrine hydrochloride or adrenaline hydrochloride, showed, after template

[24] C. Alvarez-Lorenzo, A. Concheiro Journal of Chromatography B, 804 (2004) 231–245.

[25] M. Watanabe, T. Akahoshi, Y. Tabata, D. Nakayama, J. Am. Chem. Soc. 120 (1998) 5577.

removal and in the collapsed state, an increase in swelling ratio with increasing imprint molecule concentration in water. Curiously, this template-induced swelling was observed only for the polymers prepared in dioxane but not for those prepared in water.

Tanaka and co-workers[26,27] proposed the creation of stimuli sensitive gels able to recognize and capture target molecules using polymer networks consisting of at least two species of monomers, each having a different role. One forms a complex with the template, and the other allows the polymers to swell and shrink reversibly in response to environmental changes. The gel is synthesized in the collapsed state and, after polymerization, washed in a swelling medium.

The imprinted cavities develop affinity for the template molecules when the functional monomers come into proximity, but when they are separated, the affinity diminishes. The proximity is controlled by the reversible phase transition of the gel that consequently controls the adsorption/ release of the template (Figure 2).

[26] T. Tanaka, C. Wang, V. Pande, A.Yu. Grosberg, A. English, S. Masamune, H. Gold, R. Levy, K. King, Faraday Discuss 102 (1996) 201.

[27] C. Alvarez-Lorenzo, O. Guney, T. Oya, Y. Sakai, M. Kobayashi, T. Enoki, Y. Takeoka, T. Ishibashi, K. Kuroda, K. Tanaka, G. Wang, A.Yu. Grosberg, S. Masamune, T. Tanaka, Macromolecules 33 (2000) 8693.

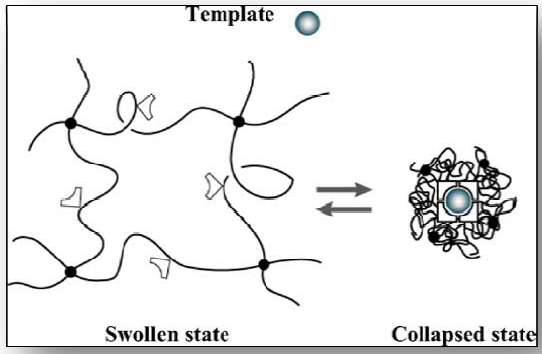

Figure 2. Diagram of the recognition process of a template by a stimuli sensitive imprinted hydrogel as proposed by Tanaka et al.

In order to obtain a system with the capacity to recognize calcium ions, imprinted and non-imprinted copolymers of NIPAAm and different methacrylic monomers with carboxyl groups, which form complexes with divalent ions in the relation 2:1, were prepared. The effect of temperature on the adsorption capacity of the imprinted copolymers prepared with different templates and in different organic solvents was compared with that of the non-imprinted ones. Successful imprinting was obtained with NIPAAm-lead dimethacrylate monomers in dioxane. After washing lead out and swelling in water at room temperature, the affinity for divalent ions disappeared. When the gels were shrunken by an increase in temperature, the affinity was recovered and the original relative position of the carboxylic groups was recalled. Control gels made using randomly distributed methacrylic acid experienced difficulty in forming pairs ("frustration") and their affinity for divalent ions decreased exponentially as a function of cross-linker concentration. In contrast, the topological

constraints were completely absent in the imprinted gels, showing that memorization had been achieved. The success of the imprinting can be attributed to the fact that the degree of dissociation between lead and two methacrylate molecules during polymerization is negligible, and therefore lead is responsible for fixing the juxtaposition of pairs of methacrylates[28]. These stimuli-sensitive imprinted gels are very weakly cross-linked (>2 mol%) systems and, therefore, the success of the imprinting strongly depends on the stability of them complexes template/functional monomers during polymerization and in the aqueous medium. However, if the interaction in water is too strong, it may be difficult to remove the template completely after polymerization to obtain the pure gel. To circumvent some of these drawbacks, functional monomers directly bonded to each other prior to polymerization, which we call "imprinters", were used[29]. An imprinter is a molecule that has three functional parts, two or more polymerizable double bonds, two or more functional groups and a link connecting the functional groups that is easily cleaved afterwards, such as a disulphide bond or a 1,2-glycol structure. The functional groups can be separated after polymerization to obtain pairs of ionic groups with the same charge. Since the members of each pair are close in the space, they can capture target molecules through multiple-point ionic interactions. An imprinted hydrogel for cationic divalent ions was obtained, without template, using a monomer with a disulphide bond

[28] C. Alvarez-Lorenzo, O. Guney, T. Oya, Y. Sakai, M. Kobayashi, T. Enoki, Y. Takeoka, T. Ishibashi, K. Kuroda, K. Tanaka, G. Wang, A.Yu. Grosberg, S. Masamune, T. Tanaka, J. Chem. Phys. 114 (2001) 2812.
[29] R. D'Oleo, C. Alvarez-Lorenzo, G. Sun, Macromolecules 34 (2001) 4965.

that, after polymerization, was oxidized and transformed in two sulfonic groups in contact each other. Thus, the hydrogel was suitable for the binding/release of calcium ions more efficient than the gels prepared with randomly distributed sulfonic groups[27].

2.4 Synthesis of Thermo-responsive Hydrogels

The following three chapters of this second section deals with the synthesis of thermo-sensitive hydrogels by employing two different polymerization techniques. In particular, thermo-responsive polymeric microspheres based on natural proteins (albumin and gelatin), synthesized by reverse-phase suspension polymerization, and thermo-sensitive microparticles by molecular imprinting method will be presented and their potential application in drug delivery field demonstrated.

Chapter 1

SYNTHESIS AND RELEASE PROFILE ANALYSIS OF THERMO-SENSITIVE ALBUMIN HYDROGELS

1. Introduction

Hydrogels consist of elastic networks that can uptake as much as 90–99% w/w of water in their interstitial space[1,2]. In a physical point of view, hydrogels resemble living tissues because they have high water content and a soft and rubbery consistency. Such systems have been especially focused in the biomedical area as they provide adequate semiwet three-dimensional environment for cells and tissue interaction and they can be combined, through covalent links or physical entrapments, with biological or therapeutic molecules. They can be also chemically controlled and designed to tailor their mechanical and functional properties. Therefore hydrogels have been proposed for a series of biomedical and biological applications, including tissue engineering, drug release systems, biological sensors, microarrays, imaging and actuators. Some kinds of hydrogels can change their shape and volume reversibly following changes in external physical and chemical conditions such as temperature, solvent composition, ionic strength, pH, electric field, and light. Recently, stimuli-responsive polymer hydrogels have been attracting the attention of many researchers and are playing a part in a variety of fields, such as

[1] C. Alvarez-Lorenzo, A. Concheiro J. Contr. Rel. 80 (2002) 247–257.
[2] N.A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Eur. J. Pharm. Biopharm. 50 (2000) 27-46.

chemical engineering, medicine and pharmacy, life sciences, biotechnology, etc.[3,4,5]

Among the family of temperature responsive hydrogels, poly(N-isopropylacrylamide) (PNIPAAm) hydrogel is one of the most widely studied. It exhibits a Lower Critical Solution Temperature (LCST) or transition temperature at 30°C in aqueous solution and shows an abrupt thermo-reversible change in volume as external temperature cycles around this critical temperature. PNIPAAm hydrogels are usually formed by the covalent crosslinking of PNIPAAm chains with a commercial crosslinking agent as N,N'-methylenebisacrylamide, to modifie physico-chemical properties of the materials (e.g. LCST, crosslinking degree, hydrophilic/hydrophobic balance, biodegradability, biocompatibility).

The hydrogels based on PNIPAAm exhibit negative thermal response which means that below its LCST, PNIPAAm chains hydrate to form an expanded structure with a large mesh size enabling the water diffusion, while above its LCST these chains dehydrate to form a shrunken structure with a small mesh size. The change in the hydration state, which causes the volume phase transition, reflects competing hydrogen bonding properties, where intra- and intermolecular hydrogen bonding of the polymer molecules are favoured compared to interaction with water molecules. Thermodynamics can explain this behaviour with a balance between entropic effects due to the dissolution process itself and to the ordered state of water molecules around the polymer. Enthalpic effects depend on the balance between intra- and intermolecular forces and on

[3] M. Prabaharan, J.F. Mano Macromol. Biosci. 6 (2006) 991–1008.
[4] I. Dimitrov, B. Trzebicka, A.H.E. Muller, A. Dworak, C.B. Tsvetanov, Progr. Pol. Sci. 32 (2007) 1275-1343.
[5] A. Kikuchi, T. Adv. Drug Del. Rev. 54 (2002) 53-77.

the solvation, e.g. hydrogen bonding and hydrophobic interactions. The transition is then accompanied by coil-to-globule transition[6].

PNIPAAm hydrogels are non-biodegradable, which may restrict their applications as biomaterials. The importance of the biodegradability for a biomaterial is self-evident due to the absence of a chronic foreign-body reaction, that is usually accompanied with the permanent presence of the non-biodegradable materials[7]. Furthermore, the devices obtained from biodegradable materials do not require additional surgery for their removal. So, there is a need to develop non-toxic, biodegradable hydrogels for the biomedical applications without losing their desirable properties, as temperature sensitivity. Hence, the bonding of the gel is designed to be labile and it can be degraded by enzyme or chemisorptions in the physiological phenomenon. The most of the binding is degraded in the organism by the way of hydrolysis producing the little member, showing a low toxicity.

In view of these aspects, a promising strategy for designing novel hydrogel drug delivery systems is to combine the merits of both bioresponsive and biodegradable hydrogels. Recently, great attention has been paid especially for biomedical applications to the development of stimuli-responsive polymeric gels with unique properties such as biocompatibility, biodegradability and biological functionality. They may be prepared by combining thermoresponsive polymers with natural based polymeric component, to form smart hydrogels. Polymeric systems composed of NIPAAm and acrylic acid were prepared by redox polymerization with peptide cross-linkers to create an enzimatically

[6] D. Schmaljohann, Adv. Drug Del. Rev. 58 (2006)1655–1670.
[7] E.J. Park, D.H. Na, K.C. Lee, Int. J. Pharm. 343 (2007) 281-283.

degradable matrix[8]. Peptide degradable crosslinkers were synthesized by the acrylation of the amine groups of lysine residues within peptide sequences potentially cleavable by matrix metalloproteinases. In addition, Yu et al. proposed the synthesis of an hydrogel based on P(NIPAAm-co-acrylic acid) and a polyaspartic acid derivatives (acryloyloxyethylamino polysuccinimide) as cross-linkers[9]. Thus, the covalent conjugation of a biodegradable macromolecule, as a protein (gelatin) or polysaccharides (chitosan, dextran, xyloglucan), to a thermo-responsive monomer represents a versatile strategy to produce intelligent biodegradable hydrogels, suitable for pharmaceutical and biomedical applications[10,11,12].
The albumin is a biodegradable protein, which is susceptible to enzymatic digestion in human body and considerable interest has been shown in the use of protein microspheres as starting materials for active drug targeting, as well as for producing a sustained and controlled rate of drug release and microspheres can be used in the treatment of many diseases that require controlled release of the drug into plasma, cells or organs. The albumin microspheres are suitable for drug delivery since they are biodegradable, biocompatible and relatively easy to prepare over a wide range of particle sizes. By virtue of their ability to interact with a wide variety of drugs and their simple and low cost preparation, albumin microspheres represent very interesting materials for therapeutic

[8] S. Kim, K.E. Healy, Biomacromolecules. 4 (2003) 1214-1223.
[9] Y.G. Yu, Z.Z. Li, H.I. Tian, S.S. Zhang, P.K. Ouyang, Coll. Pol. Sci. 285 (2007) 1553-1560.
[10] L. Jun, W. Bochu, W. Yazhou, Int. J. Pharm. 2 (2006) 513-519.
[11] L. Klouda, A.G. Mikos , Eur. J. Pharm. Biopharm. 68 (2008) 34-45.
[12] S. Ohya, T. Matsuda, J. Biomater. Sci. Polym. Ed. 16 (2005) 809–827.

applications[13]. A considerable number of strategies have been developed to obtain albumin microspheres; they can be achieved by thermal denaturation either by direct reaction between functional groups (usually carboxyl and amino goups) in the polypeptide side chains and also by chemically crosslinking agents as bifunctional carbonyl reagents[14]. By these techniques, hydrophobic materials, able to release the drug through an erosion mechanism, were prepared. In our previous works, the synthesis of bovine serum albumin (BSA) microspheres by radical copolymerization of methacrylate BSA (BSA-MA) with *N*,*N*-dimethylacrylamide[15] or methacrylic acid sodium salt[16] was reported. This polymerization technique allows to obtain albumin microparticles with a narrow size distribution, a spherical shape and an high water affinity. Furthemore, this proteic hydrogels were tested as carriers for water-soluble drugs in media simulating biological fluids.

In this paper, a novel class of biodegradable hydrogels with proteic structure and thermo-responsive behaviour was synthesized. In order to prepare thermo-sensitive microspheres, the present work describes the synthesis of materials by reverse phase suspension copolymerization of N-isopropylacrylamide (NIPAAm) and BSA-MA, as functional monomer and proteic crosslinker, respectively. This research proposes a new synthetic approach by radical polymerization of functionalized BSA and

[13] F. Iemma, U.G. Spizzirri, R. Muzzalupo, F. Puoci, S. Trombino, N. Picci , Coll. Polym. Sci. 283 (2004) 250-256.
[14] S. Sahin, H. Selek, G. Pronchel, M.T. Ercan, M. Sargon, A.A. Hincal, H.S. Kas, J. Contr. Rel. 82 (2002) 345-358.
[15] F. Iemma, U.G. Spizzirri, F. Puoci, R. Muzzalupo, S. Trombino, R. Cassano, S. Leta, N. Picci, Int. J. Pharm. 312 (2006) 151-157.
[16] F. Iemma, U.G. Spizzirri, F. Puoci, R. Muzzalupo, S. Trombino, N. Picci, Drug Del. 12 (2005) 179-184.

thermo-responsive monomer because of this synthetic procedure permits to modify polymeric network composition producing hydrogels with appropriate and mouldable physico-chemical properties.

The beads were characterized by Scanning Electronic Microscopy (SEM), Fourier Transform Infrared spectroscopy, particle size distribution, calorimetric and swelling analyses. The FT-IR spectra confirms the insertion of functional monomers and proteic crosslinker in the polymeric network. The functional monomer/crosslinker ratio in the polymerization feed strictly influences morphological and chemical properties of hydrogels and all samples showed high water affinity and a significant volume change in response to temperature variation across their LCST values.

Finally, to evaluate thermo-responsive microparticles as smart drug carrier, the hydrogels into a solution of Caffeine (CF) and Theophylline (TH) were soaked. Media release temperature, around materials LCST values, hydrogel crosslinking degree and chemical structure of entrapped drug strictly influence release profiles. Depending on ratio between functionalized protein and NIPAAm in the hydrogels, different release mechanisms were hypothesized.

2. Experimental Section

2.1 Reagents and standards

All the reagents were of analytical-reagent grade, and used without further purification unless otherwise stated. *n*-Hexane and chloroform, purchased from Carlo Erba Reagents (Milan, Italy), were purified by standard procedures. BSA fraction V (MW 68.000; pH 7.0±0.2; grade ≥98%) was from Roche Diagnostics GmbH. Methacrylic anhydride

(MA), 2,4,6-trinitrobenzensulphonic acid (TNBS), N-isopropylacrylamide (NIPAAm), sorbitan trioleate (Span 85), polyoxyethylene sorbitan trioleate (Tween 85), *N*,*N*,*N*',*N*'-tetramethylethylendiamine (TMEDA), ammonium persulfate, Caffeine (CF), Theophilline (TH) were provided from Sigma–Aldrich (Sigma Chemical Co, St. Louis, MO). Acetonitrile and water were from Carlo Erba Reagents (Milan, Italy) and all of HPLC grade. 2-propanol, ethanol, acetone and diethyl ether were from Carlo Erba Reagents (Milan, Italy) and all of analytical grade.

2.2 Derivatization of BSA

Functionalized BSA (BSA-MA), according to a procedure elsewhere reported, was prepared[13]. Derivatization of BSA with MA was carried out in distilled aqueous phase, under conditions of controlled pH and temperature (pH 7 and 0°C), using a suitable amount of MA and stirred for 1 hour at 0°C (Figure 1).

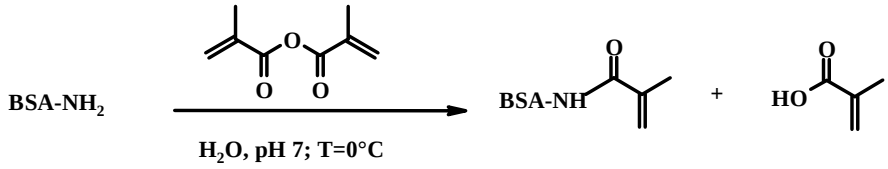

Figure 1. Schematic representation of BSA functionalization with MA

The aqueous solution obtained was introduced into dialysis tubes and dipped into a glass vessel containing distilled water at 20°C for 48 h with four changes of water. The resulting solution was frozen and dried with a freezing-drier apparatus to afford a vaporous solid. The derivatization degree (DD%) of BSA-MA in agreement with a procedure reported in

literature was determined[17].

2.3 Microspheres preparation (standard procedure)

Microspheres based on BSA-MA and NIPAAm were produced by radical copolymerization technique. Briefly a mixture of *n*-hexane and chloroform was placed in a round-bottomed cylindrical glass reaction vessel fitted with an anchor-type stirrer and thermostated at 30°C, then treated, after 30 minutes of N_2 bubbling, with a solution of BSA-MA, comonomer (NIPAAm) and ammonium persulfate in water as radical initiator. The density of the organic phase was adjusted by the addition of chloroform or *n*-hexane so that the aqueous phase sank slowly when stirring stopped. Under stirring at 1000 rpm, the mixture was treated with Span 85 and Tween 85, then after 10 min with TMEDA and stirring was continued for another 60 minutes. The Table 1 reports the experimental conditions of each polymerization reactions. The microparticles were filtered, washed with 50 ml portions of 2-propanol, ethanol, acetone and diethyl ether and dried overnight under vacuum at 40°C.

[17] S.L. Snyder, P.Z. Sobocinski, Anal. Biochem. 64 (1975) 284–288.

Dispersed Organic Phase		**Continous Organic Phase**	**Resin**				**WR(%)**	
BSA-MA (mg)	NIPAAm (mg)	$CHCl_3$/ n-hexane (ml/ml)	mg (conv %)	Code	LCST (°C)	Shape	25°C / 40°C	Sr
200	400	15/24	474 (79.0)	**I**	30.6	ISM	406 / 223	1.8
250	350	16/23	429 (71.4)	**II**	30.0	SM	365 / 222	1.6
300	300	17/23	411 (68.5)	**III**	31.1	SM	320 / 212	1.6
350	250	17/23	478 (79.6)	**IV**	32.0	SM	317 / 200	1.4
400	200	16/24	475 (79.2)	**V**	32.2	ISM	267 / 195	1.3

For all polymerizations, the amount of aqueous phase is 2.5 ml; initiator system is (NH4)2S2O8/TMEDA (100 mg/150 µl); surfactants are Span 85/Tween 85 (140 µl/30 µl). SM: Spherical micropartcles; ISM: Irregular and spherical microparticles. WR (%): water content percent; *Sr*: ratio between the swelling at 25 °C and 40 °C

Table 1. Copolymerization of BSA-MA with NIPAAm.

2.4 Water content of microspheres

The swelling characteristics were determined in order to check hydrophilic affinity of microparticles. Typically aliquots (40–50 mg) of the microparticles dried to constant weight were placed in a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighted, and left to swell by immersing the filter plus support in a beaker containing the swelling media (PBS solution, pH = 6.8, at 25°C and 40°C). At a predetermined time, the excess water was removed by percolation at atmospheric pressure. Then, the filter was placed in a properly sized centrifuge test tube by fixing it with the help of a bored silicone stopper, then centrifuged at 3500 rpm for 15 min and weighted. This operation was repeated at the different times (1, 4 and 24 hours). The filter tare was determined after centrifugation with only water. The weights recorded at the different times were averaged and used to give the water content

percent (WR %) by the following equation (1):

$$WR(\%) = \frac{W_s - W_d}{W_s} \times 100 \qquad \textbf{(1)}$$

Where W_s and W_d are weights of swollen and dried microparticles, respectively. Each experiment was carried out in quintuplicate and the results were in agreement within ± 4% standard error. The WR (%) for all prepared materials are reported on Table 1.

2.5 Thermo-behaviour of BSA–MA/NIPAAm hydrogels

The LCST property of the hydrogel samples was determined by using a DSC. The LCST value for all polymers are reported in Table 1. In a standard procedure the sample was immersed in distilled water at room temperature for at least 2 days to reach a swollen state. About 10 mg swollen sample was placed inside a hermetic aluminum pan, and then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25°C to 55 °C on the swollen hydrogel samples under a dry nitrogen atmosphere with a flow rate of 25 ml min^{-1} and heating rate 3°C min^{-1}.

2.6 Incorporation of drug into preformed microspheres

Incorporation of drugs (Caffeine and Theophilline) into microspheres was performed as follows: 200 mg of preformed empty microspheres (prepared as described above) were wetted with 2ml in a concentrated drug solution (10 mg/ml). After 3 days, under slow stirring at 37 °C, the microspheres were filtered and dried at reduced pressure in presence of

P_2O_5 to constant weight. The loading efficiency percent (LE, %) of all samples are determined by HPLC analysis of filtered solvent in according to equation (2):

$$LE\ (\%) = M_i \times \frac{C_i - C_0}{C_0}$$

Here C_i was the concentration of drug in solution before the loading study, C_0 the concentration of drug in solution after the loading study and M_i was the mass of drug available. The calculated LE (%) of different copolymers are listed on Table 2.

BSA-MA-co-NIPAAm	**LE (%)**	
	CF	**TH**
I	71±3	46±2
II	76±1	42±4
III	66±4	53±4
IV	67±2	51±5
V	69±1	73±3

Table 2. Microparticles loading efficiency percent (LE %) of CF and TH after 72 h at room temperature.

2.7 In vitro drug release at 25°C and 40°C from microparticles

In vitro drug release profiles were obtained by HPLC. Aliquots (10 mg) of drug-loaded microparticles were dispersed in flasks containing PBS solution (pH 6.8) and maintained at 25±0.1 and 40±0.1°C in a water bath.

Then at suitable time intervals, samples were filtered and the solutions were analysed by HPLC. Each experiment was carried out in triplicate and the results were in agreement within ±5% standard error. For caffeine and theophylline HPLC conditions were acetonitrile/water 6/4; 0.5 ml/min flow; UV detection at λ = 280 nm.

2.8 Instruments

The dialysis membranes of 6–27/32" Medicell International LTD (MWCO: 12-14000) were employed. The freeze drier Micro Modulyo, Edwards was utilized. The ultraviolet spectra with a U-2000 Hitachi spectrophotometer using 1 cm quartz cells were recorded. The FT-IR spectra were recorded as pellets in KBr in the range 4000–400 cm^{-1} using a Jasco FT-IR 4200 spectrophotometer (resolution 1 cm^{-1}). The particle size distribution was carried out using an image processing and analysis system, Leica DMRB equipped with a Leica Wild 3D stereomicroscope. This image processor calculates the particle area and converts it to an equivalent circle diameter. The scanning electron microscopy photographs were obtained with a Leo stereoscan 420; the sample surface was made conductive by the deposition of a layer of gold on the samples in a vacuum chamber. The X-ray diffraction analyses were performed using a diffractometer Philips PW 1729 X-ray generator. The experimental parameters were: Cu Kα radiation, tube setting 40 kV, 20 mA; angular speed 2° (2θ/min); range recorded 10-40° (2θ/min); time constant 1s, chart speed 2 cm/min. Calorimetric analyses were performed using a Netzsch DSC200 PC. The High-Pressure Liquid Chromatography (HPLC) analyses were carried out using a Jasco PU-2080 liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a

20 μl loop), a Jasco UV-2075 HPLC detector and Jasco-Borwin1 integrator. A reversed-phase C18 column (μBondapak, 10 μm of 250mm×4.6mm internal diameter obtained from Waters) was used.

3. Results and Discussion

Chemical groups susceptible to radical polymerization were introduced onto BSA by acylation with methacrylic anhydride (MA) in water at 0°C and neutral pH. Under mild reaction conditions only sterically accessible amino groups in the side chain of lysine react with acylation agent. The nucleophilic chemical groups in BSA that could react with MA are the thyolic groups of cysteine, hydroxilic groups of serine and tyrosine, and amino groups in the side chain of lysine residues. The former groups are involved in disulfide bridges, except cys-34, the latter are the least nucleophilic, and do not react in the mild experimental conditions. The amino groups of lysine, sterically accessible, react chiefly with acylant agent at controlled pH and temperature to produce water-soluble BSA-MA. If the reaction is carried out without pH and temperature control, denaturation of BSA was observed, and its water solubility is lost.

In this work BSA-MA with all the available amino groups acylated was prepared (DD% = 100%). The derivatization degree was determined by a spectrophotometric method, using 2,4,6-trinitrobenzensulphonic acid (TNBS) as the chromophore group. In this procedure, TNBS was employed as a reagent for measuring the free amino groups of BSA. The amino content is related to the increase in absorbance at 420 nm that is ascribable to the trinitrophenylsulfonic group bounded to BSA-MA, after a relatively short incubation period.

Thermo-responsive microspheres with proteic structure were synthesized

by reverse phase suspension copolymerization of BSA-MA with an a monomer showing a thermal behaviour, such as NIPAAm.

Varying crosslinker/comonomer ratio, hydrogels with different crosslinking degree were prepared (Table 1). The polymerization reaction, owing to steric and geometric constraints, involves only the methacrylic functions of BSA-MA which are accessible to the growing chains. The obtained microparticle structure is characterised by a network where the BSA chains are linked by hydrocarbon bridges. It can be presumed that in the copolymerization reaction the chains obtained consist of NIPAAm units randomly interrupted by methacrylic BSA-MA functions which are sterically and geometrically attainable (Figure 2).

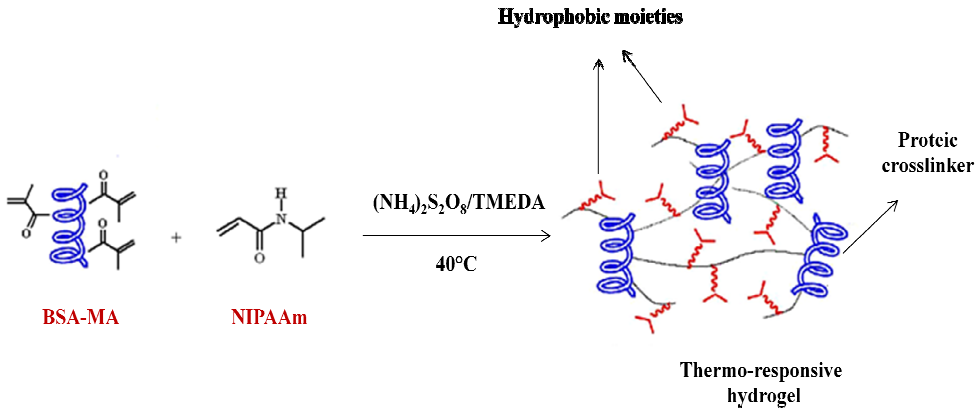

Figure 2. Synthesis of thermo-responsive hydrogels poly(BSA-MA-*co*-NIPAAm).

The reaction was started using TMEDA and ammonium persulfate as initiator system. Optimization of the polymerization method required several attempts. It was observed that hydrophilic/lipophilic balance (HLB) of surfactants is very important. Many tests were carried out to

determine the correct ratio of Span 85 (HLB=1.8) and Tween 85 (HLB=11). Finally, a system with a total HLB=3.4 was able to stabilize aqueous dispersed phase. The polymerizations produced materials with different shape; in particular the ratio (w/w) between BSA-MA and NIPAAm is significant for the preparation of spherical microparticles (Table 1). When BSA-MA/NIPAAm ratio in the copolymerization feed was in the range 0.7-1.4 spherical shape was observed (copolymers II, III and IV). A mixture of irregular and spherical particles was recorded outside this range (copolymers I and V).

The materials were characterized by FT-IR spectrophotometry, swelling behaviour, particle size distribution, morphological and calorimetric analyses. The FT-IR spectra of all samples show the disappearance of bands at 1307 and 934 cm^{-1} awardable to BSA-MA methacrylic groups and at 944 and 921 cm^{-1} ascribable to C–C double bond of NIPAAm. Furthermore, an absorption band at 617 cm^{-1} (a typical band of BSA-MA homopolymer) in all samples was observed. Investigation of the applicability of these hydrogels in controlled release was done by studying their swelling behaviour. The value of contained water percentage was determined in aqueous media (PBS solution pH = 6.8; 0.01 M) at 25°C and 40°C respectively. The data, reported in Table 1, illustrate the water uptake at different temperature, in grams per gram of dry copolymer, for each studied composition and the ratio between the swelling at 25°C and 40°C (*S*r) was reported for all samples. The prepared materials showed different water affinity at 25°C and 40°C due to pendant hydrophobic groups in the polymeric chains. In particular, at 40°C there is a considerable lowering of the water content, due to solvent diffusion outside the polymeric network and to resultant hydrophobic interactions

between hydrocarbon moiety on polymeric chains. When the temperature raises to 25°C, the water content is greater than that found at 40°C for all copolymers. Due to greater NIPAAm amount in the polymeric network sample I showed highest *S*r value. At both temperatures the water uptake decreases from copolymer I to V, according to network crosslinking degree and in contrast with the copolymer hydrophilicity. This finding can be explained assuming that the effect due to increased crosslinking degree is predominant respect to increased hydrophilic moieties in the network.

Using scanning electron microscopy the surface properties of the microparticles were evaluated, and spherical shape of microparticles was confirmed. In Figure 3 (A and B), the spherical shape of sample II is evident, while the Figure 3 (C) shows the high porosity of the outside surface of the microparticles. Similar results were obtained for all the spherical samples.

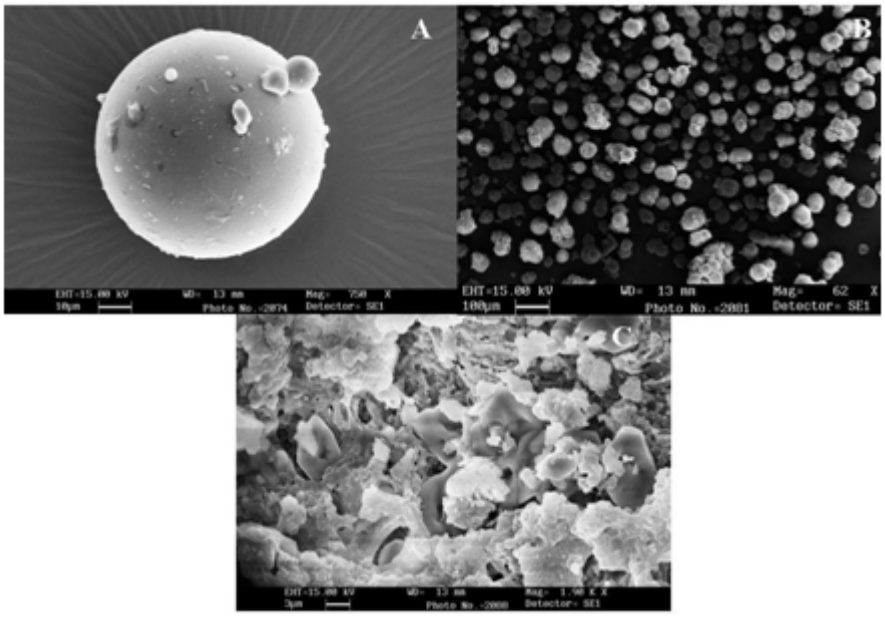

Figure 3. SEM micrographs for II (A and B) and II outside surface (C).

In our experiments the particle diameter was in the dimensional range 20-30 μm for III and IV and 40-50 μm for II and a narrow distributional profile for the samples II and III was recorded (Figure 4) The micropartcle diameters were strictly depending on the crosslinker amount in the polymeric networks; the values of mean particle diameter in general decreases as the crosslink density increases.

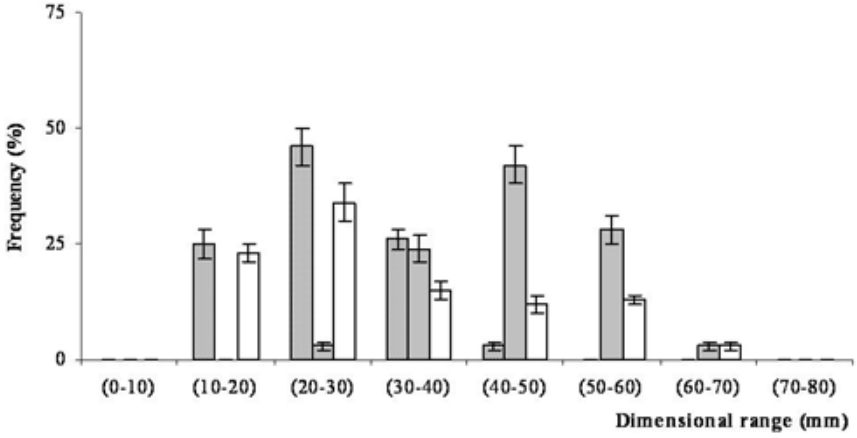

Figure 3. Size distribution profiles for II (■), III (▒) and IV (□).

Thermal analyses were performed from 25°C to 55°C on the swollen hydrogel samples and the range of LCST values was 30-32.2°C (Table I). The LCST values were strictly dependent on functional monomer/crosslinker ratio in the polymerization feed. The data indicate that all copolymers had a higher LCST than the pure PNIPAAm hydrogel, a polymer exhibiting LCST value equal to 30°C[18]. The increase in the LCST recorded in BSA-MA-co-NIPAAm copolymers can be attributed to the increased hydrophilic content respect to hydrophobic moiety, from I to V (Figure 5).

Thermo-responsive behaviour of PNIPAAm hydrogel is strongly influenced by polymer-water affinity. At temperature below its LCST, the hydrophilic groups (amide groups) in the side chains of the PNIPAAm hydrogel bond the water molecules through hydrogen bonds. However, as the external temperature increases, the copolymer-water hydrogen bonds

[18] H.G. Schild, Progr. Pol. Sci. 17 (2) (1992) 163-249.

are broken. When the temperature raises the LCST, the water molecules, rigidly structured around the polymer chains, gain more freedom degrees and they diffuse in their bulk phase. As a result, hydrogen bonds between solvent molecules in the continuous phase are formed; while, inside the polymeric network, hydrophobic interactions among the isopropyl groups become dominant.

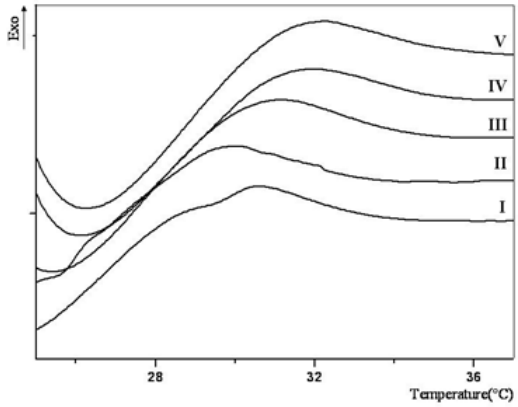

Figure 5. DSC thermograms of BSA-NIPAAm hydrogels

In order to estimate the ability of the matrices to release drug molecules, the beads were loaded with various drugs by soaking procedure. Theophilline and Caffeine were chosen as model drugs and the loading efficiency of all samples (LE %) was determined by HPLC analysis as reported in experimental part (Table 2). The CF was loaded on the polymeric beads with a LE (%) > 65 % for all hydrogels, whereas TH was poorly uptaken on the beads (LE (%) approximate to 50 % for all macromolecular systems except for V, where LE % = 73%). Different drug-polymer hydrophobic interactions explain these results, the interaction of CF, indeed, is particularly strong with the more

hydrophobic samples (copolymers I, II). On the contrary, these samples showed a weak interaction with the less hydrophobic drug, TH, while decresing the hydrophobicity of hydrogel (copolymer V), highest TH amount was uptaken. The determination of the drug dispersion state in all preformed hydrogels was performed by X-ray analysis. Analyzing X-ray diffraction patterns of pure drug, unloaded and drug-loaded hydrogels, it is evident that pure drugs are in the crystalline state; on the contrary, both the drug-unloaded and loaded microparticles are in the amorphous state (Figures 6 and 7). The results demonstrate that during the polymerization/crosslinking reaction no crystalline region was formed and that the drug is molecularly entrapped inside the network. Analogous results have been found for all samples.

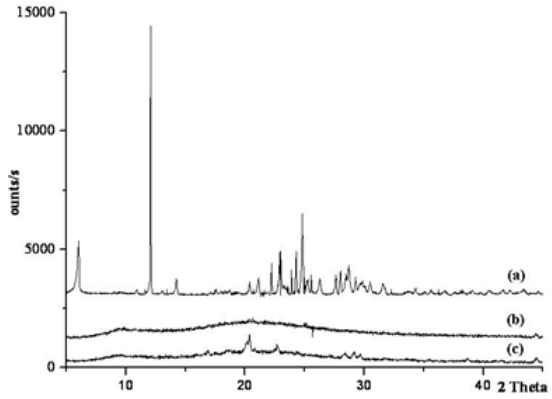

Figure 6. X-ray diffraction patterns of pure TH (a), TH-unloaded III microspheres (b) and TH-loaded microspheres samples (c). Analogous results have been found for all materials.

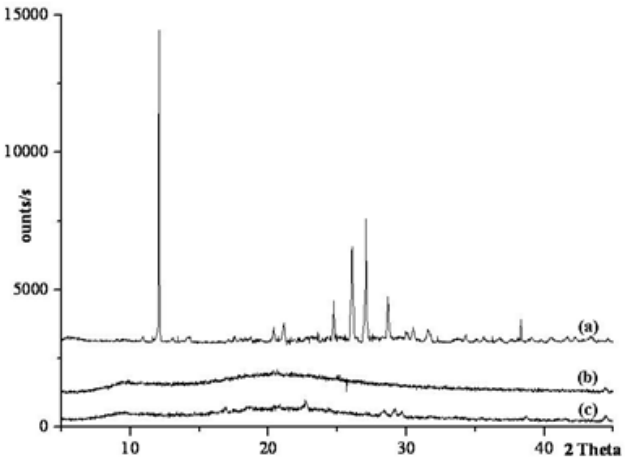

Figure 7. X-ray diffraction patterns of pure CF (a), CF-unloaded III microspheres (b) and CF-loaded microspheres samples (c). Analogous results have been found for all materials.

Drug release profile was determined by HPLC analysis. The drug release was expressed as drug delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time at 25°C and 40°C. All synthesized copolymers showed a thermo-responsive behaviour. Experimental data showed for copolymers I, II and III (functional monomer/crosslinker (w/w) ratio ≤ 1) a predominant drug release in the collapsed state (Figures 8 and 9), at temperature above the LCST, while the copolymers IV and V (functional monomer/crosslinker (w/w) ratio > 1) showed prominent drug release in the swollen state, below the LCST of the materials (Figures 10 and 11).

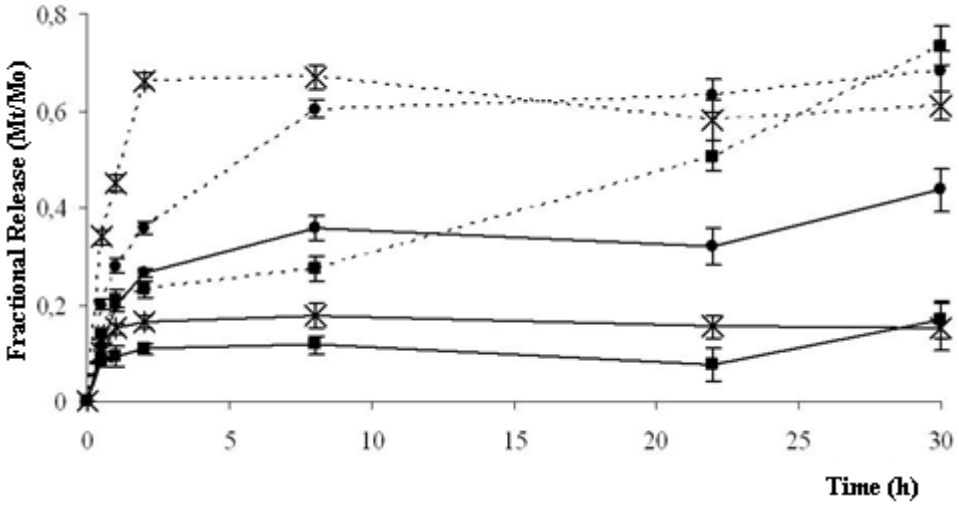

Figure 8. Drug release expressed as Theophylline delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time for beads I (■), II (∗), III (•) at 25°C (—) and 40°C (----).

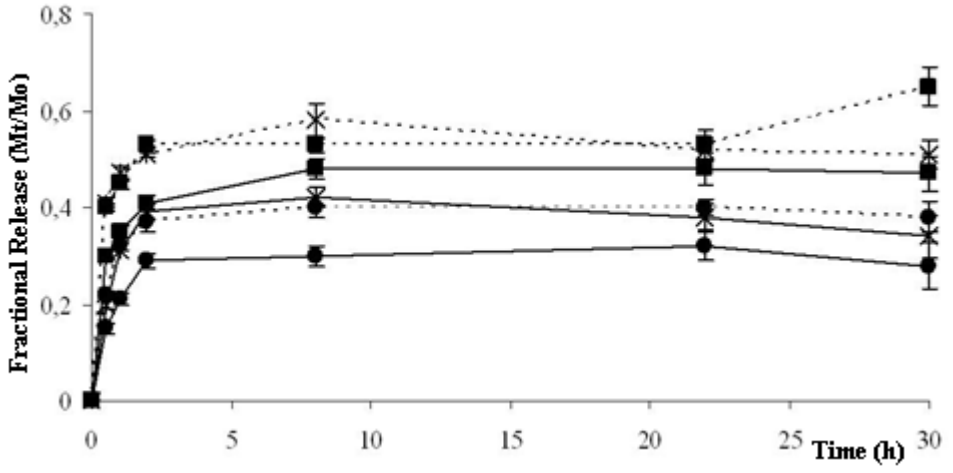

Figure 9. Drug release expressed as Caffeine delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time for beads I (■), II (∗), III (•) at 25°C (—) and 40°C (----).

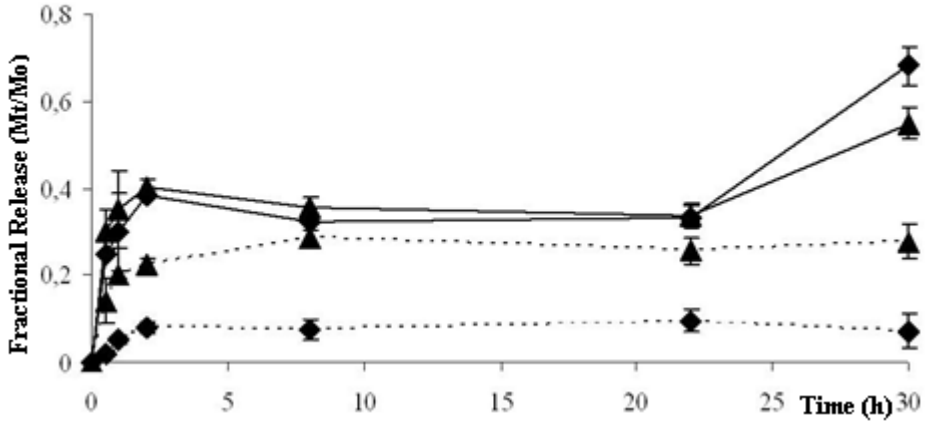

Figure 10. Drug release was expressed as Theophylline delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time for beads IV (♦) and V (▲)at 25°C (—) and 40°C (----).

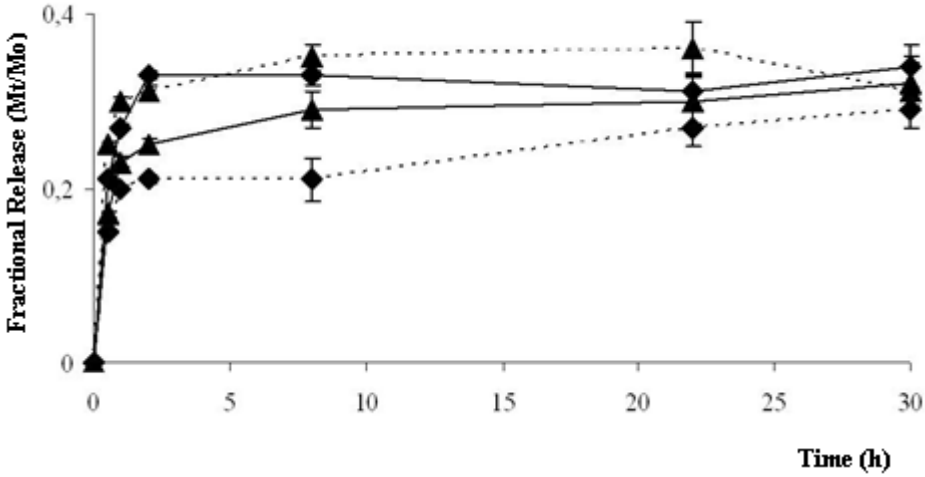

Figure 11. Drug release expressed as Caffeine delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time for beads IV (♦) and V (▲)at 25°C (—) and 40°C (----).

Drug-polymer interactions, beads crosslinking degree and temperature of release media determine release profiles. At 40°C squeezing-out effect controls the drug release. This effect is pronounced in the polymer I ($M_t/M_0 > 0.70$ after 30 h for TH), while it is irrelevant in V ($M_t/M_0 < 0.10$ after 30h for TH), where only a poor burst effect was observed. According to S_r values in Table 1, copolymers I, II and III, bearing more hydrophobic groups in the side chain and low density crosslink points, underwent a drastic volume transition phase. On the contrary, squeezing-out effect is less evident increasing crosslinking degree (copolymers IV and V), due to the constraints imposed by the crosslinks.

The data at 25°C suggest a release mechanism due to cooperation of different factor for all materials in the swollen state. In particular, it is important to consider the diffusion of drugs through a complex polymeric network containing proteic moieties.

Drug-polymer interactions were found to be important on TH and CF release profiles. A relationship between the release profiles and the substituent on xanthine ring at 7 position can be invoked. Drug release is strictly associated to hydration state of the copolymers; at 25°C evident differences between TH and CF were observed and more hydrophilic drug easily interacts with swelling media when density of crosslink points increased.

4. Conclusion

Thermo-responsive hydrogels were designed and synthesized by reverse suspension radical polymerization starting from bovine serum albumin derivative and temperature sensitive monomer (NIPAAm). Respect to the thermoresponsive hydrogels reported in literature the polymerization

technique proposed in this paper allows to synthesize systems with spherical shape suitable to prevent anisotropic swelling of materials in the media release. The mild and controlled reaction conditions in the functionalization of BSA with methacrylic anhydride permit to preserve water solubility of native protein. Thus, derivatized BSA was covalently inserted in a polymeric network by water/oil emulsion polymerization, using a water soluble radical initiator systems. Varying the feed composition, a novel class of temperature-responsive polymers with different physico-chemical properties was obtained. The insertion of a biomacromolecules in a crosslinked structure provides to a biocompatibile and biodegradable network, suitable as drug delivery systems. In this paper an extensive studies to achieve a spherical shape were performed and the functional monomer/crosslinker ratio was found to greatly influence the geometric and morphological properties of microparticles. On the other hand, materials with different hydrophobic/hydrophilic balance were synthesized; these ones showed changed affinity to aqueous media depending on swelling temperature. Negative thermo-responsive behaviour for all samples was observed. In order to test the materials as drug carriers, TH and CF were loaded on the microspheres and drug entrapment percent was determined. Depending on media temperature and loaded drug-bead interactions, drug release across the proteinous hydrogels takes place by modification of volume hydrogels.

Chapter 2

NEGATIVE THERMO-RESPONSIVE MICROSPHERES BASED ON GELATIN HYDROLYZATES AS DRUG DELIVERY DEVICE

1. Introduction

Hydrogels are composed of the three-dimensional network polymers and have properties of both solid and liquid. In the swollen state, hydrogels are soft and rubbery, and some hydrogels are similar to living tissue and possess excellent biocompatibility[1].

Among polymers that can form hydrogels, natural polymers are often preferred to synthetic materials because of their nontoxicity, low cost, free availability and biocompatibility. Besides, they can be copolymerized with some monomers to obtain copolymers with special functions, such as thermo-sensitive or pH-sensitive copolymers. This kind of systems are of great interest as drug carriers, food additives, and so on.

Thermo-sensitive hydrogels have attracted extensive interest due to their potential and promising applications in many fields. Among the family of temperature sensitive hydrogels, poly(N-isopropylacrylamide) (PNIPAAm) hydrogel is one of the most widely studied. It exhibits a lower critical solution temperature (LCST) at 32°C in aqueous solution and shows an abrupt thermo-reversible change in volume as external temperature cycles around this critical temperature. The LCST can be adjusted to the body human temperature by copolymerization or interpenetration with other monomers[2].

[1] Y. Lang, T. Jiang, S. Li, L. Zheng J. Applied Pol. Sci. 108 (2008) 3473–3479.
[2] C. Pan, Q. Long, D. Yu, Y. Rao, N. WU, X. LI, Front. Chem. China 3 (2008) 314–319.

For this purpose, and to improve the mechanical properties of the resultant materials, much research has been done to associate biopolymers (such as protein and polysaccharides) with thermo-sensitive macromolecules. Gelatin is a biopolymer with thermo-reversible properties[3]. At temperatures below 25°C, an aqueous gelatin solution solidifies due to the formation of triple helices and a rigid three-dimensional network. When the temperature is raised above approximately 30°C, the conformation changes from a helix to the more flexible coil, rendering the gel liquid again[4]. As the opposite thermal behavior is desired for biomedical applications, researchers have combined gelatin with other polymers, which show thermal gelation close to body temperature[5].

In addition to its good biological properties, such as non toxicity, non immunogenicity and the complete reabsorption in vivo, gelatin has the advantage of allowing for easy modification on the amino acid level. In literature, different methods to obtain gelatin thermosensitive hydrogels are reported and these materials are of great interest in drug delivery, tissue engineering, and so on.

Chun at al.[6] proposed the synthesis of an hydrogel-dispersed composite membrane based on poly(N-isopropylamide) (p(NIPAAm)) and crosslinked gelatin, and its thermally actuated transport characteristics of 4-acetamidophen were investigated in a diffusion cell. Lee et al.[7] studied

[3] L. Klouda, A.G. Mikos, Eur. J. Pharm. Biopharm. 68 (2008) 34–45.
[4] C. Joly-Duhamel, D. Hellio, M. Djabourov, Langmuir 18 (2002) 7208–7217.
[5] H. Yang, W.J. Kao, Pharm. Res. 23 (2006) 205–214.
[6] S. Chun, J. Kim, J. Control. Rel. 38 (1996) 39-47.
[7] W. Lee, S. Lee, J. Mater. Sci: Mater. Med. 18 (2007) 1089–1096.

the effect of gelatin on the release profile of anionic, cationic and neutral drugs from different organic hybrid gels, based on poly(N-isopropylacrylamide) and gelatin, crosslinked through a two-step process with genipin or glutaraldehyde. This paper reports on the synthesis of gelatin-based thermo-responsive microspheres in which hydrolyzed methacrylated gelatin (HGel-MA) was employed as pro-hydrophilic monomer, NIPAAm as thermo-responsive co-monomer and N,N'-methylenebisacrylamide (MEBA) as crosslinking agent. Our object was to design biodegradable thermo-responsive materials with spherical shape and characterized by LCST values close to body temperature. Varying the HGel-MA and NIPAAm amount in the polymerization feed, three different polymeric networks have been synthesized. The obtained microbeads were characterized by Scanning Electronic Microscopy (SEM), Fourier Transform Infrared spectroscopy, particle size distribution, calorimetric and swelling analyses. Finally, in order to verify the suitability of these materials as thermo-responsive devices for drug delivery, a commonly used antinflammatory drug, diclofenal sodium salt, was loaded on the polymeric structures and release profiles at 25 and 40°C evaluated; moreover, in order to test the thermo-responsive switching behavior pulsatile drug release experiments by temperature cycling were performed.

2. Experimental section

2.1 Materials

Gelatin (Ph Eur, Bloom 160), methacrylic anhydride (MA), potassium hydroxide (KOH), N-isopropylacrylamide (NIPAAm), N,N'-methylenebisacrylamide (MEBA), sorbitan trioleate (Span 85),

polyoxyethylene sorbitan trioleate (Tween 85), *N*,*N*,*N*',*N*'-tetramethylethylendiamine (TMEDA), ammonium persulfate, sodium hydrogen phospate, disodium hydrogen phosphate and diclofenac sodium salt were provided from Sigma–Aldrich (Sigma Chemical Co, St. Louis, MO). Acetonitrile and water were from Carlo Erba Reagents (Milan, Italy) and all of HPLC grade. 2-propanol, ethanol, acetone and diethyl ether were from Carlo Erba Reagents (Milan, Italy) and all of analytical grade. *n*-hexane and chloroform, purchased from Carlo Erba Reagents (Milan, Italy), were purified by standard procedures.

2.2 Synthesis of Methacrylated Gelatin Hydrolyzate

Methacrylated gelatin hydrolyzates (HGel-MA) were prepared according to the literature with some modifications[8].

A reaction mixture containing 40 g of gelatin were taken up in 60 g of water and, after the addition of 1.6 g of sodium hydroxide, the solution was heated for 16 h to 130°C. Then, after cooling to room temperature, 3ml of methacrylic anhydride were added to the reaction mixture. The pH value of the reaction mixture was kept at 10 by addition of dilute sodium hydroxide. After a reaction time of 5 h, the mixture was cooled to room temperature and adjusted with dilute hydrochloride acid to a pH value of 7 (Figure 1).

[8] L.-H. Lin, K.-M. Chen, Colloids and Surfaces A: Physicochem. Eng. Aspects 272 (2006) 8–14.

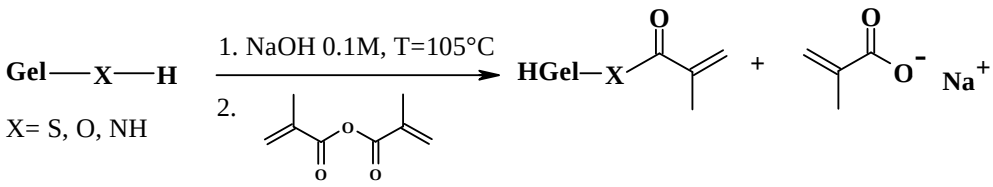

Figure 1. Schematic representation of HGel-MA synthesis

The obtained functional hydrolyzate was precipitated by adding the polymeric solutions to an excess volume of acetone under agitation at room temperature. The suspensions were filtered by sintered glass filter funnel (Pyrex, Ø30 mm; porosity 3) and washed with diethyl ether and the recovered gelatine hydrolizate was dried in a vacuum oven at 40 °C.

2.3 Microspheres preparation (standard procedure)

Microspheres based on HGel-MA, NIPAAm and MEBA were produced by radical copolymerization technique. Briefly a mixture of *n*-hexane and chloroform was placed in a round-bottomed cylindrical glass reaction vessel fitted with an anchor-type stirrer and thermostated at 30°C, then treated, after 30 minutes of N_2 bubbling, with an aqueous solution of HGL-MA, the comonomer (NIPAAm), the crosslinker MEBA and ammonium persulfate as radical initiator. The density of the organic phase was adjusted by the addition of chloroform or *n*-hexane so that the aqueous phase sank slowly when stirring stopped. Under stirring at 1000 rpm, the mixture was treated with Span 85 and Tween 85, then after 10 min with TMEDA and stirring was continued for another 60 minutes. The table 1 reports the experimental conditions of each polymerization reactions.. The microparticles were filtered, washed with 50 ml portions

of 2-propanol, ethanol, acetone and diethyl ether and dried overnight under vacuum at 40°C.

Aqueous dispersed Phase			Organic continuous Phase	Hydrogel	
HGel-MA mg	**NIPAAm Mg**	**MEBA mg**	**$CHCl_3$/ n-hexane ml/ml**	**mg (conv.%)**	**Code**
200	300	100	19/22	450	HG-1
300	300	120	19/22	400	HG-2
300	400	120	19/22	425	HG-3

For all polymerizations, the amount of aqueous phase is 2.5 ml; initiator system is $(NH_4)_2S_2O_8$/TMEDA (100 mg/150 µl); surfactants are Span 85/Tween 85 (150 µl/40 µl).

Table 1. Polymerization conditions for the synthesis of HG-1-HG-3

2.4 Water content of HG microspheres

The swelling characteristics were determined in order to check hydrophilic affinity of microparticles. Typically, aliquots (40–50 mg) of the microparticles dried to constant weight were placed in a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighted, and left to swell by immersing the filter plus support in a beaker containing the swelling media (PBS solution, pH 7, at 25°C and 40°C). After 24h, the excess water was removed by percolation at atmospheric pressure. Then, the filter was placed in a properly sized centrifuge test tube by fixing it with the help of a bored silicone stopper, then centrifuged at 3500 rpm for 15 min and weighted. The filter tare was determined after centrifugation with only water. The weights recorded at the different times were averaged and used to give the water content percent (WR %) by the following equation (1):

$$WR(\%) = \frac{W_s - W_d}{W_s} \times 100 \quad \textbf{(1)}$$

Where W_s and W_d are weights of swollen and dried microparticles, respectively. Each experiment was carried out in quintuplicate and the results were in agreement within ± 4% standard error. The WR (%) for all prepared materials are reported on Table 2.

2.5 Thermo-behaviour of HG microspheres

The LCST property of the hydrogel samples was determined by using a DSC. The LCST value for all polymers are reported in Table 2. In a standard procedure the sample was immersed in distilled water at room temperature for at least 2 days to reach a swollen state. About 10 mg swollen sample was placed inside a hermetic aluminum pan, and then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25°C to 55 °C on the swollen hydrogel samples under a dry nitrogen atmosphere with a flow rate of 25 ml min^{-1} and heating rate 3°C min^{-1}.

2.6 Incorporation of drug into preformed microspheres

Incorporation of Diclofenac sodium salt into microspheres was performed as follows: 200 mg of preformed empty microspheres were wetted with 2.0 ml in a concentrated drug solution (10 mg/ml). After 3 days, under slow stirring at 37°C, the microspheres were filtered and dried at reduced pressure in presence of P_2O_5 to constant weight. The loading efficiency percent (LE %) of all samples are determined by HPLC analysis of filtered solvent in according to equation (2):

$$LE(\%) = \frac{C_i - C_0}{C_i} \times 100 \qquad \textbf{(2)}$$

Here C_i was the concentration of drug in solution before the loading study, C_0 the concentration of drug in solution after the loading study. The values of calculated LE (%) and the drug loaded percent (DL%) in each matrix were listed on Table 2, according to equation 3:

$$DL(\%) = \frac{\text{Amount of drug in the beads}}{\text{Amount of beads}} \times 100 \qquad \textbf{(3)}$$

Hydrogel	Calorimetric analysis	Swelling behavior			LE %	LD%
	LCST °C	WR_{25}	WR_{40}	S_r		
HG-1	35.4	200±0.9	129±0.7	1.55	98.76	
HG-2	36.5	267±0.6	128±0.8	2.08	98.45	
HG-3	36.9	229±0.7	128±0.4	1.78	98.46	

Table 2. Calorimetric analyses, water uptake experiments, LE and LD percentages of HG hydrogels

2.7 In vitro drug release at 25°C and 40°C from microparticles

In vitro drug release profiles were obtained by HPLC. Aliquots (10 mg) of drug-loaded microparticles were dispersed in flasks containing PBS solution (pH 7) and maintained at 25.0±0.1 and 40.0±0.1°C in a water bath., The samples, at suitable time intervals, were filtered and the

solutions were analysed by HPLC. Each experiment was carried out in quintuplicate and the results were in agreement within ±5% standard error. The HPLC conditions were acetonitrile/phosphate buffer 0.0001M 7/3 (v/v); 0.5 ml/min flow; UV detection at 284 nm.

2.8 In vitro pulsatile drug release from 25°C to 40°C

Oscillatory drug release profile of the obtained materials was investigated by immersing the gel beads in a solution at pH 7.0 (0.001 M, phosphate saline buffer), and alternating the temperature between 25 and 40°C at suitable time intervals. At each time, the samples were filtered and the solutions were analysed by HPLC. Each experiment was carried out in triplicate and the results were in agreement vithin ±5% standard error. The release period was extended over several cycles until no further drug was released. Two different experiments were performed: the first starting from 25°C and the second from 40°C. The larger temperature difference was used to help increase the speed of collapse, since DC is a small molecule.

2.9 Statistical analysis

All of the experiments were done in triplicate. One-way analysis of variance was performed to assess the significance of the differences among data. Tukey–Kramer post-test was used to compare the means of different treatment data. $P < 0.05$ was considered statistically significant.

2.10 Instruments

The FT-IR spectra were recorded as pellets in KBr in the range 4000–400 cm^{-1} using a Jasco FT-IR 4200 spectrophotometer (resolution 1 cm^{-1}). The

scanning electron microscopy photographs were obtained with a Leo stereoscan 420; the sample surface was made conductive by the deposition of a layer of gold on the samples in a vacuum chamber. Calorimetric analyses were performed using a Netzsch DSC200 PC. The High-Pressure Liquid Chromatography (HPLC) analyses were carried out using a Jasco PU-2080 liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 μl loop), a Jasco UV-2075 HPLC detector and Jasco-Borwin1 integrator. A reversed-phase C18 column (μBondapak, 10 μm of 250mm×4.6mm internal diameter obtained from Waters) was used.

3. Results and Discussion

3.1 Synthesis of HG microspheres

Chemical groups susceptible to radical polymerization were introduced onto hydrolized gelatin by acylation with methacrylic anhydride. Comparing to the native protein, the hydrolyzates are characterized not only by an enhanced water solubility, but also by a greater number of nucleophilic groups disposable for the functionalization. In addition to the thiolic groups of cysteine, hydroxilic groups of serine and tyrosine, and the amino groups in the side chain of lysine residues, indeed, the terminal amino groups, deriving from the alkaline hydrolysis of the peptide bonds, represent reactive sites toward the derivatization with MA.

Our goal was to obtain proteic functional monomers to be directly copolymerized with NIPAAm and MEBA, to synthesize useful spherical polymeric materials that summarize the thermo-responsive behaviour of PNIPAAm with the biocompatibility and the hydrophilic characteristics of the gelatine (Figure 2).

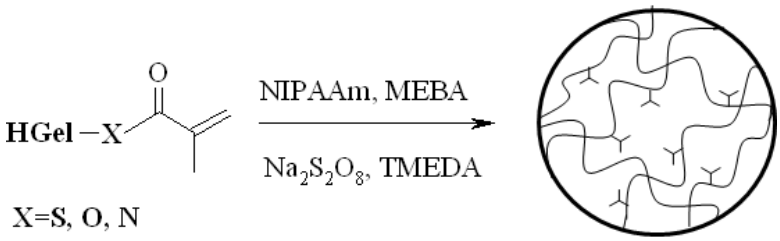

Figure 2. Copolymerization of HGel-MA monomers with NIPAAm and MEBA

On the other hand, the choice to obtain hydrogels characterized by spherical shape was dictated to the fact that this kind of materials are ideal vehicles for many controlled delivery applications, due to their ability to encapsulate a variety of drugs, biocompatibility, high bioavailability and sustained drug release characteristics[9].

It can be presumed that, in the copolymerization reaction, the obtained chains consist of NIPAAm units randomly interrupted by MEBA moieties and the methacrylic functions of HGel-MA sterically and geometrically attainable. The optimization of the polymerization method was performed and it was observed that hydrophilic/lipophilic balance (HLB) of surfactants represents an important parameter to produce a water-in-oil emulsion consisting of water drops uniformly dispersed in the organic phase ($CHCl_3$/*n*-hexane) when stirring was stopped. Generally, water-in-oil emulsions are stabilised by surfactants in concentrations of 0.5–1.5% by weight in to water and literature data report that the best results were obtained using surfactant mixtures with different values of HLB (26). In

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particular, the volume ratio of the surfactant mixture strictly depends on the dispersed phase. In our experiments, different tests were carried out, allowing to determine the correct ratio for Span85 (HLB=1.8) and Tween85 (HLB=11). A system with a total HLB = 3.7 was eventually found to be able in stabilizing the aqueous phase dispersed in the organic one. Varying the amount of HGel-MA and the molar ratio NIPAAm/MEBA in the polymerization feed, three different hydrogels were prepared, as reported in Table 1. In particular, NIPAAm/MEBA molar ratio was 5.0 for HG-1 and HG-3 and 3.7 for HG-2, while the amount of hydrophilic crosslinker (HGel- MA) was 33.3% (w/w) for HG-1 and was increased to 36.6% and 41.1% for HG-3 and HG-2, respectively. In the polymerization protocols proposed in this paper, we found that the change of both the crosslinking degree and the hydrophilic/hydrophobic balance of the polymeric networks seems to greatly influence the water-beads affinity and consequently the swelling/shrinking transition temperatures of the thermo-sensitive microspheres.

3.2 Characterization of gelatine microspheres

The obtained materials were characterized by FT-IR spectrophotometry, swelling behaviour, morphological and calorimetric analyses.

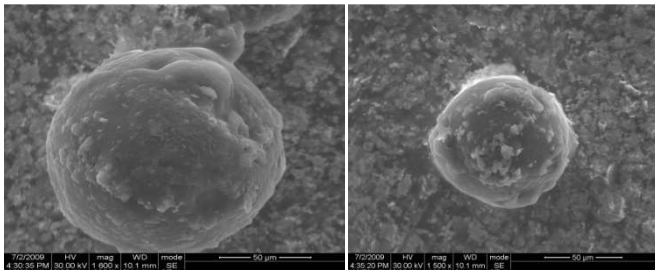
The FT-IR spectra of all samples shown the disappearance of bands at 944 and 921 cm^{-1} ascribable to C–C double bond of both NIPAAm and HGel-MA and the appearance of the typical absorption bands of gelatine. In particular, the peaks at 3450 cm^{-1} and 3423 cm^{-1} due to N-H stretching of secondary amide, C=O stretching at 1680 cm^{-1} and 1640 cm^{-1} , N-H bending between 1550 cm^{-1} and 1500 cm^{-1}, N-H out of plane wagging at

670 cm^{-1} and C-H stretching at 2922 cm^{-1} and 2850 cm^{-1} are visible in the spectra of three samples.

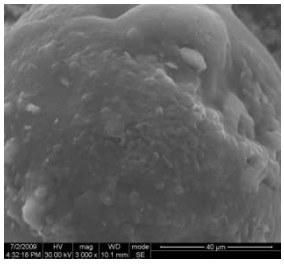
Investigation of the applicability of these hydrogels in controlled release was done by studying their swelling behaviour. The values of contained water percentages were determined in aqueous media (PBS solution pH=7; 0.01M) at 25°C and 40°C, respectively. The data, reported in Table 1, illustrate the water uptake at different temperature, in grams per gram of dry copolymer, for each studied composition, and the ratio between the swelling at 25°C and 40°C (*S*r) was reported for all samples. The prepared materials showed different water affinity at 25°C and 40°C due to pendant hydrophobic groups in the polymeric chains.

In particular, at 40°C a considerable lowering of the water content was observed, due to the predominance of the hydrophobic interactions between hydrocarbon moiety on polymeric chains that causes the solvent diffusion outside the polymeric network. When the temperature raises to 25°C, the interactions between the water molecules and the hydrophilic moieties of microspheres prevail with a consequent enhancing of swelling degree. Using scanning electron microscopy, the surface properties of the microparticles were evaluated, and spherical shape of microparticles was confirmed. In Figure 3 (A and B), the spherical shape of sample II is evident, while the Figure 3 (C) shows the high porosity of the outside surface of the microparticles. Similar results were obtained for all the spherical samples. The shape and the morphology of the prepared microparticles suggest their potential use as drug delivery systems. The spherical shape, indeed, allows to eliminate the anisotropic swelling normally associated with others geometries, while the presence of

micropores could facilitate the drug diffusion through the polymeric network.



(A) (B)



(C)

Figure 3. Scanning electron micrographs of HG-2. Spherical shape (A) (B) and porous surface (C).

Finally, the transition temperature of the obtained hydrogels was determined by performing thermal analyses from 25°C to 55°C on the swollen hydrogels. As shown in Table 2, all samples shown a higher LCST than the pure PNIPAAm hydrogel. This is due to the presence of gelatine moieties that increase the hydrophilic content in the polymeric structure.

Moreover, from HG-1 to HG-3 an increasing trend of the transition temperature was observed as a result of the enhancing HGel and MEBA content in the polymerization feed. The LCST values, indeed, were strictly dependent on functional monomer/crosslinker ratio in the

polymerization feed. At temperature below its LCST, the hydrophilic groups (amide groups) in the side chains of the PNIPAAm hydrogel bond the water molecules through hydrogen bonds. However, as the external temperature increases, the copolymer-water hydrogen bonds are broken. When the temperature raises the LCST, the water molecules, rigidly structured around the polymer chains, gain more freedom degrees and they diffuse in their bulk phase. As a result, hydrogen bonds between solvent molecules in the continuous phase are formed, while, inside the polymeric network, hydrophobic interactions among the isopropyl groups become dominant. Thus, a reduction of the HG amount in the polymeric structure results in a decrease of LCST values, while, as the hydrophilic moieties increased, the strength of hydrogen bonds between the water molecules and the hydrogels, and so also the transition temperature, enhanced.

3.3 In vitro release studies

Thermally responsive drug delivery systems have attracted ever-increasing attention because they can control the release of drug in response to changes in body temperature and therefore act as self-regulating systems.

In order to estimate the potential application of prepared matrices as drug delivery devices, the beads were loaded with one of the most commonly used anti-inflammatory drug, Diclofenac sodium salt (DC), by soaking procedure and the loading efficiency of all samples (LE %) was determined by HPLC analysis (Table 2). The DC was loaded on the beads with a LE (%) > 98% for all grafted microbeads. DC release experiments were carried out in PBS solution (pH 7.0, 10^{-3} M) at 25 and 40°C and the

amount of drug released was expressed as drug delivered (M_t) related to the effectively entrapped total mass (M_0), as a function of time.

As reported in Figure 4, a different release rate was recorded. In each experimental time and for all samples, indeed, the amount of drug molecules that moves from the polymeric beads to the surrounding media was higher at 40°C than 25°C, and a shape rise at the first 30 minutes was noted at 40°C, comparing to a slow increase at 25°C. This jump in the release profile was ascribed to rapid collapse of the hydrogel from swollen to collapsed state.

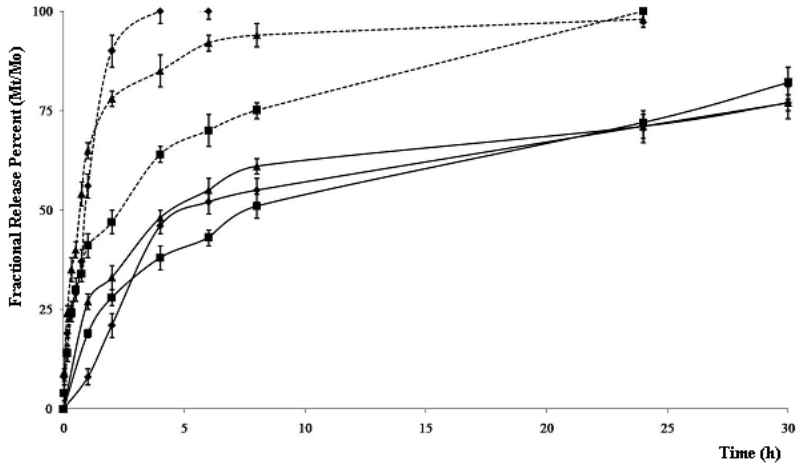


Figure 5. Drug release expressed as percent of DDA delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time for beads HG-1(◆), HG-2 (▲) and HG-3 (■) at 25°C (solid lines) and 40°C (dashed lines) and at pH 7.0 (PBS solution 10^{-3} M).

Since the microparticles have a well-defined geometry and a narrow dimensional distribution, we determined the mechanism of drug release (Fickian or non-Fickian). In particular, the kinetics of DC release at 25°C,

under LCST value, were analyzed by the semi-empirical equation (4) for $M_t/M_0 \leq 0.6$.(20)

$$\frac{M_t}{M_0} = Kt^n \qquad \textbf{(4)}$$

Where M_t/M_0 is the drug fraction released at time t, K and n are a constant and the kinetic exponent of drug release, respectively. Although the use of this equation requires detailed statistical analysis, the calculated exponent, *n*, gives an indication of the release kinetics. If n = 0.43, the drug diffuses and releases out of the polymer matrix following a Fickian diffusion. For n > 0.43, an anomalous or non-Fickian type drug diffusion occurs. If n = 0.85, a completely non-Fickian or Case II release kinetics is operative. The intermediary values ranging between 0.43 and 0.85 are attributed to anomalous type diffusive transport. The least-squares estimations of the fractional release data along with the estimated correlation coefficient values, r, are presented in Table 3. The empirical transport equation (4) represents an extension of the short time solutions for Fickian and non-Fickian diffusional releasevfrom a thin film. In theory, this equation should only be applicable to the first 60% of fractional release from thin slabs, for which the assumption of one-dimensional diffusion under perfect sink conditions is valid. In practice, however, the equation has been applied to systems of different geometries, to systems where one dimensional diffusion cannot be assumed, and to systems where perfect sink boundary conditions are not maintained. In the experiments at 25°C, the exponents n was 0.38 and

0.41 for copolymers HG-1 and HG-2, indicating a predominantly diffusive mechanism in the release of the drug, while n value for HG-3 indicates an anomalous release profile. For this devices, at temperature below the LCST, the diffusion plays a major role as it occurs through the available space between macromolecular chains, regarded as the "pore".
A more informative analysis can be obtained by fitting the data with the model proposed by Peppas and Sahlin (21). The equation for this model is:

$$\frac{M_t}{M_0} = K_1 \cdot t^{1/2} + K_2 \cdot t \qquad \textbf{(5)}$$

with $M_t/M_0 \leq 0.95$. In this equation, the first term is the Fickian contribution and the second term is the Case II relaxational contribution. Table 3 reports K_1 and K_2 values according to equation 5. For all investigated samples the term $K_1t^{1/2}$ is greater (about 15-20 times) than the term K_2t, indicating that the predominant mechanism, at 25°C, for DC release is the Fickian diffusion through the swollen microparticles. Thus, the drug release was determined by two factors, the swelling rate of polymer and the diffusivity of the drug through the network. Because at the same temperature there are no marked differences of the diffusivity of drug in each polymer, the swelling rate of the polymer was the dominating factor. In swelling-controlled (and in general swellable) controlled release systems the dissolution medium surrounding the controlled release device may enter the polymer at a rate that controls the drug release and under certain experimental conditions zero-order release

can be achieved. The prevailing molecular mechanism is a coupling of diffusion and macromolecular relaxation as a result of which the drug diffuses outward with a kinetic behavior that is dependent on the relative ratio of diffusion and relaxation

When the hydrogels are placed in an aqueous solution at 40°C, above their LCST, the release behaviours were complex and not only diffusional effects of drug through the polymer network has to be considered, but also the squeezing effect of swelled polymer contributed to the apparent release rate. The n values of HG-1 and HG-3 samples (0.66 and 0.58, respectively) indicate a pronounced non-Fickian diffusion transport, while HG-2 (n = 0.41) seems to follow a Fickian trend of release profile. Fitting the experimental data using the Peppas-Sahlin equation, more information can be obtained; in particular, at 40°C and for HG-1 and HG-3 samples, the ratio between K_1 and K_2 values are considerably lower than that recorded at 25°C, as a result of the minor diffusional contribute to the release profile. On the contrary, the hydrogel HG-2 showed a K_1/K_2 a greater contribution due to K_2 confrontable to the fitting at 25°C as a consequence of the role of the diffusional component in the release mechanism at both this temperature.

$$M_t / M_0 = Kt^n \quad (4)$$

Sample	K 10^3 (min^{-n})		N		R	
	25°C	40°C	25°C	40°C	25°C	40°C
HG-1	21.87±4.72	3.36±1.41	0.38±0.08	0.66±0.11	0.85	0.93
HG-2	20.47±1.36	6.69±1.08	0.41±0.02	0.41±0.03	0.98	0.97
HG-3	31.22±2.59	5.99±0.70	0.27±0.03	0.58±0.03	0.93	0.99

$$M_t / M_0 = K_1 \cdot t^{1/2} + K_2 \cdot t \quad (5)$$

Sample	K_1 10^3 ($min^{-1/2}$)		K_2 10^3 (min^{-1})		R	
	25°C	40°C	25°C	40°C	25°C	40°C
HG-1	22.83±2.99	2.88±0.70	-1.61±0.65	0.52±0.08	0.93	0.99
HG-2	20.19±0.83	5.70±0.21	-1.01±0.18	-0.11±0.01	0.99	0.99
HG-3	29.01±0.88	9.18±0.37	-2.82±0.19	0.22±0.02	0.99	0.98

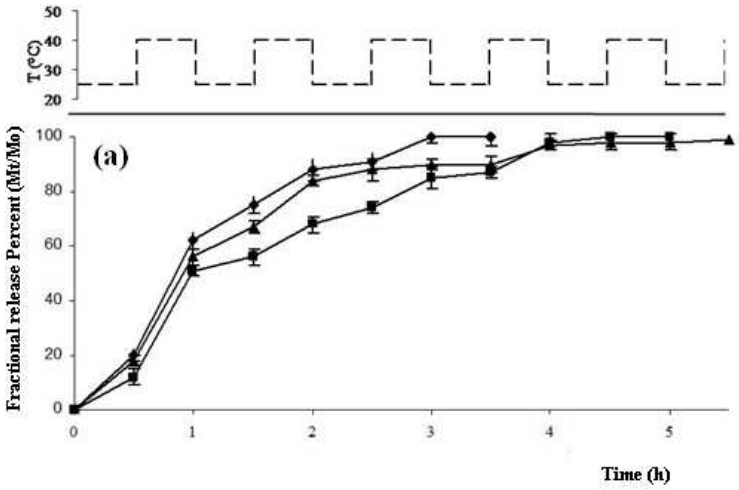
Table 3. Release kinetics parameters of different formulations.

3.4 Pulsatile drug release experiments

Pulsatile devices may have many applications in areas of medicine where a constant rate of drug release does not match the physiological requirements of the body. To demonstrate reversible on/off-switching of DC release, the microspheres were repeatedly heated above and cooled below their LCST in *in vitro* pulsatile release experiments. In these conditions, the release profiles during 5 hours were found to have very good sustaining efficacy and the effect of temperature cycling on drug release may reflect a response rate to various environments. The

experiments started placing the samples both in a swelling (25°C) and in a collapsed state (40°C) and recording the amount of DC released, expressed as M_t/M_0 percent, in the surrounding environment after each temperature change. Figure 6 (a) and 6 (b) shows the DC release profile and release rate from GM hydrogels as a function of temperature cycling at fixed pH value (PBS solution 10^{-3} M, pH 7.0).

Generally, for all samples it can be observed an higher DC amount released at 40 versus 25°C. This behaviour is due to the strong hydrophobic interactions between the isopropyl groups of NIPAAm as the temperature reached the LCST, so a significant amount of aqueous drug solution was dispelled from the collapsed hydrogels. As a consequence, exposing the DC-loaded samples to a temperature of 40°C, a significant burst effect was observed for all samples, with M_t/M_0 percent values ranged from 30.0 to 61.0 after an half hour. These percentages decreases to values between 9 and 25 when the microspheres are placed to a temperature of 25°C. These results clearly showed that the HG systems allow to obtain an effective modulation of the drug release rate.



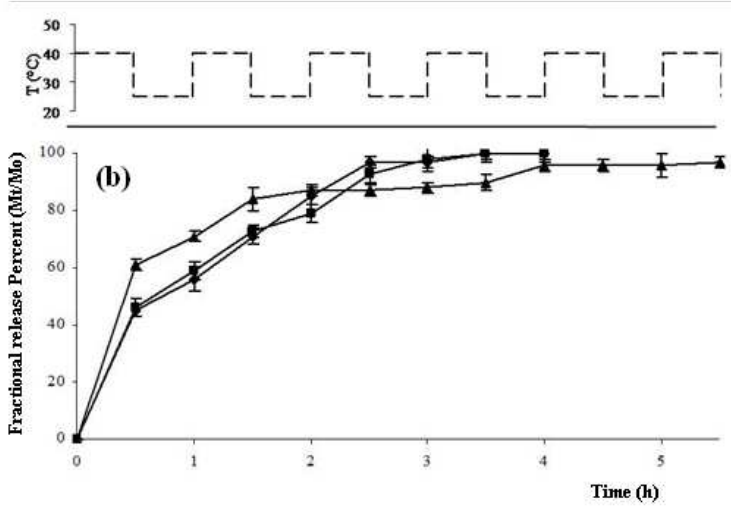


Figure 6. Repeated on/off switching of crosslinked grafted membrane. The temperature in the off and on-situation is 25 and 40°C, respectively.

4. Conclusions

In this work, methacrylated gelatin hydrolizates were employed as pro-hydrophilic monomers in the synthesis of thermo-responsive microspheres by free-radical suspension polymerization and using NIPAAm and MEBA as thermo-responsive functional monomer and crosslinking agent, respectively. Varying the HGel-MA and NIPAAm amounts in the feed composition, hydrogels with different hydrophobic/hydrophilic balance were synthesized. The spherical shape and the high degree of porosity of the obtained materials were confirmed by SEM analyses, while the negative thermo-responsive behaviour by water uptake experiments at temperatures across the LCST was checked. Moreover, since the gelatine moieties in the networks increase the strength of the interactions between the water molecules and polymers, a

considerably enhancing of the transition temperatures to values close to the body temperature was recorded. The potential application of these materials as drug carriers was demonstrated by performing DC release experiments at temperature above and below the LCST. Depending on temperature of the surrounding environment, drug release across the hydrogels takes place by abrupt modification of volume hydrogels and by diffusion of the therapeutic through the polymeric network. In order to estimate the diffusional contribute on the drug delivery, semi-empirical equations were employed, showing the enhanced role of the diffusional component in the release mechanism at temperature below the LCST.

Finally, the reversible on/off-switching behavior of our microspheres was demonstrated by performing *in vitro* pulsatile release experiments.

Chapter 3

SYNTHESIS OF THERMO-RESPONSIVE MOLECULARLY IMPRINTED POLYMERS

1. Introduction

Molecular imprinting is a method for the preparation of ligand-selective cavities in a synthetic polymer matrix[1]. The technique has attracted significant interest from a large number of areas within chemistry and analytical sciences due to the relative ease with which these polymers may be prepared, the range of template structures which are amenable to use and the apparent mechanical and chemical stability of the types of synthesized polymers. Briefly, the technique may be described as follows: a template molecule is allowed to form reversible interactions (covalent or non-covalent) with suitable functionalized monomers. Polymerization in the presence of a cross-linking monomer, affords a highly cross-linked macroporous polymer containing cavities with sites of varying complementarity to the template. Removal of the template by competitive extraction procedures renders a material with the capacity to selectively re-adsorb the template. One of the objectives in molecular recognition is to achieve stimulus-responsive recognition behavior[2]. The intelligent-imprinted gels are able to memorize their binding conformation and can be switched on and off by external stimuli, such as pH, temperature, light[3,4]. Temperature is the most widely utilized triggering signal for a

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variety of triggered or pulsatile drug delivery systems. The use of temperature as a signal has been justified by the fact that the body temperature often deviates from the physiological temperature (37°C) in the presence of pathogens or pyrogens[5].

Poly(N-isopropylacrylamide) (PNIPAAm) has been the focus of significant attention because of its thermo-responsive properties[6,7,8]. Several studies have demonstrated that aqueous solutions of PNIPAAm exhibit a lower critical solution temperature (LCST) around 32°C. Dehydration of polymer chains upon heating causes a coil-to-globule transition and produces temperature reversible phase separation in solutions and changes in the hydrophobic nature of surfaces modified with PNIPAAm. Recent studies have exploited these phenomena for applications in drug delivery[9,10], separation[11,12],energy transduction[13] and catalysis[14]. When a molecular recognition host and a thermo-sensitive polymer are integrated in the same material, a novel function can be achieved in the combined compound which is different from the component materials.

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In this work, thermo-responsive molecularly imprinted polymers were synthesized by using caffeine as model template, methacrylic acid as functional monomer able to interact with caffeine, NIPAAm as thermo-sensitive monomer and ethylene glycol dimethacrylate as crosslinking agent.

The thermo-responsive behavior of the obtained materials was tested in water uptake experiments at 25 and 60°C, while the imprinting effect and the selectivity of MIP were evaluated by performing binding experiments with the template and its analogue, theophilline (TH), respectively, at 25 and 60°C.

2. Experimental Section

2.1. Reagents and standards

NIPAAm, methacrylic acid (MAA), Ethylene glycol dimethacrylate (EGDMA), 2,2-azoisobutyronitrile (AIBN), Caffeine (CF), Theophylline (TH) were obtained from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO). All solvents were reagent grade or HPLC-grade and used without further purification and were provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

2.2 Synthesis of Thermo-responsive Molecularly Imprinted polymers (TMIP)

TMIP were prepared by bulk polymerization using caffeine as template and according to the non-covalent imprinting approach. Briefly, CF (1 mmol), NIPAAm (8 mmol) and MAA (16 mmol), were dissolved in 5 ml of a methanol/acetonitrile mixture (5:5v/v) in a thich-walled glass tube. The tube was sonicated for 6 min in a sonicating water bath and then

EGDMA (8 mmol) and AIBN (100 mg) were added. The polymerization mixture was bubbled with nitrogen, sonicated for 10 min and thermo-polymerized under a nitrogen atmosphere for 24 h at 60°C. The resultant bulk rigid polymer was crushed, grounded into powder and sieved through a 63 μm stainless steel sieve. The sieved microparticles were collected and the very fine powder, suspended in the supernatant solution (acetone), was discarded. Then, obtained TMIP materials were extracted by Soxhlet apparatus with 200 ml of methanol for 48 h and successively they were dried under vacuum overnight at 40°C. The polymers were checked to be free of CF and any other compound by HPLC analysis. Blank polymers (Thermo-responsive Non Imprinted Polymers-TNIP), that act as a control, were synthesized under the same reaction conditions but in absence of the template.

2.3 Water content of TMIP

The swelling characteristics were determined in order to check the hydrophilic affinity of microparticles. Typically aliquots (40–50 mg) of the microparticles dried to constant weight were placed in a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighted, and left to swell by immersing the filter plus support in a beaker containing the swelling media (PBS solution, pH 7), at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C). After 24h, the excess water was removed by percolation at atmospheric pressure. Then, the filter was placed in a properly sized centrifuge test tube by fixing it with the help of a bored silicone stopper, then centrifuged at 3500 rpm for 15 min and weighted. The filter tare was determined after centrifugation with only water. The weights recorded at the different times were averaged and used to give the

water content percent (WR %) by the following equation (1):

$$WR(\%) = \frac{W_s - W_d}{W_s} \times 100 \quad \textbf{(1)}$$

Where W_s and W_d are weights of swollen and dried microparticles, respectively. Each experiment was carried out in quintuplicate and the results were in agreement within ± 4% standard error.

2.4 Thermo-behaviour of TMIP

The LCST property of the hydrogel samples was determined by using a DSC. In a standard procedure the sample was immersed in distilled water at room temperature for at least 2 days to reach a swollen state. About 10 mg swollen sample was placed inside an hermetic aluminum pan, and then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25°C to 55 °C on the swollen hydrogel samples under a dry nitrogen atmosphere with a flow rate of 25 ml min^{-1} and heating rate 3°C min^{-1}.

2.5 Binding experiments

Evaluation of the capacity of the polymeric materials to recognize and bind CF at different temperatures was performed by rebinding experiments in phosphate buffer solution (pH 7.4). Briefly, 50 mg of polymer particles were mixed with 1.5 ml of CF solution (0.1 mM) in a 1,5 ml eppendorf and sealed. The eppendorf were oscillated by a wrist action shaker (Burrell Scientific) in a water bath at 25 and 60°C ± 0.5°C for 24 h. Then, the mixture was centrifuged for 10 min (10000 rpm) in an

ALC® microcentrifugette® 4214 and the CF concentration in the liquid phase was measured by HPLC. The amount of CF bound to the polymer was obtained by comparing its concentration in the TMIP samples to the TNIP samples. The binding experiments were also performed using TH solution in the same conditions and they were repeated five times.

2.6 Instrumentation

The liquid chromatography consisted of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 290 nm. A 25 × 0.4 mm C4 Kromasil 100 column, particle size 5 μm (Teknocroma, Barcellona, Spain) was employed. The mobile phase was a methanol/water mixture (8/2, v/v) run isocratically at a flow rate of 1 mL/min and at room temperature, wavelength 280 nm.

3. Results and Discussion

A great number of synthetic, naturally occurring, and semisynthetic polymers display discrete, rapid, and reversible phase transformations as a result of conformational changes in response to temperature[15,16,17].

Combining the properties of a thermo sensitive polymer with molecular imprinting techniques may provide a promising strategy for ensuring the system responds more rapidly to an external temperature change.

In this work, thermo-responsive molecularly imprinted polymers with low crosslinking density were synthesized using two species of monomers,

[15] L.E. Bromberg, E.S. Ron, Adv. Drug Deliv. Rev. 31(1998) 197.
[16] H.G. Schild, Prog. Polym. Sci. 17 (1992) 163.
[17] Y.Y. Lang, S.M. Li, W.S. Pan, L.Y. Zheng,. J. Drug Deliv. Sci. Technol. 16 (2006) 65.

MAA and NIPAAm, having a different role. MAA forms a complex with the template exploiting the H-bonds, while NIPAAm allows the polymers to swell and shrink reversibly in response to environmental changes.

On the other hand, the low cross-linked characteristics of the imprinted materials facilitate the reversible swelling and shrinking in response to the temperature variation and, at the same time, increase the number of binding sites available to the target molecule.

The thermo-responsive behavior of TMIP and TNIP was evaluated by performing water uptake experiments at different temperature. The values of contained water percentages were determined in aqueous media (PBS solution pH 7; 0.01M) at eight different temperature (25, 30, 35, 40, 45, 50, 55 and 60°C). Figure 1 shows the effect of temperature on swelling ratio of polymer gels. When the temperature increased to lower critical solution temperature (LCST) of polymer gels (almost 38°C), the swelling ratio of polymer gels drastically decreased. Such results show the polymer gels have good temperature sensitivity and swelling capability.

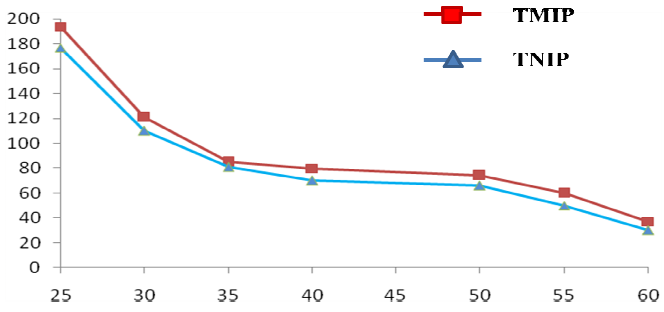

Figure 1. Effect of temperature on swelling ratio of polymer gels.

Thermal analyses were performed from 25°C to 55°C on the swollen

hydrogel samples obtaining, for both TMIP and TNIP, LCST values at 37.7°C. The LCST values were strictly dependent on the hydrophobic/hydrophilic ratio in the polymeric structure. The increase in the LCST recorded in the synthesized hydrogels, compared to PNIPAAm, can be attributed to the increased hydrophilic content respect to hydrophobic moiety due to the presence of MAA and EGDMA.

The imprinting effect of the synthesized TMIP was evaluated by performing binding experiments in which amounts of polymeric particles were incubated with a CF solutions 0.1 mM for 24 hours. The experiments were performed in buffered water solution (pH 7.0) at 25 and 60°C.

As shown in Table 1, the recognition properties of the imprinted microparticles are visible only at 60°C, while at 25°C no imprinting effect was observed.

Sample	**% CF bound**		**% TH bound**	
	25°C	**60°C**	**25°C**	**60°C**
TMIP	**0**	**27**	**0**	**2**
TNIP	**0**	**6**	**0**	**4**

Table 1. Bound (%) CF and TH by imprinted and non-imprinted polymers.

This is due to the fact that 60°C is the temperature at which the polymeric structure, and so also the binding sites, have been formed. Thus, only at this value a considerable increase of the affinity for caffeine can occur because the pendant groups of the imprinted cavities can assume the

specific complementary orientation towards the template. On the other hand, when the temperature is below the LCST and the polymer is in the swollen state, the affinity diminishes because the typical complementarity in size and shape of the imprinted cavities is lost.

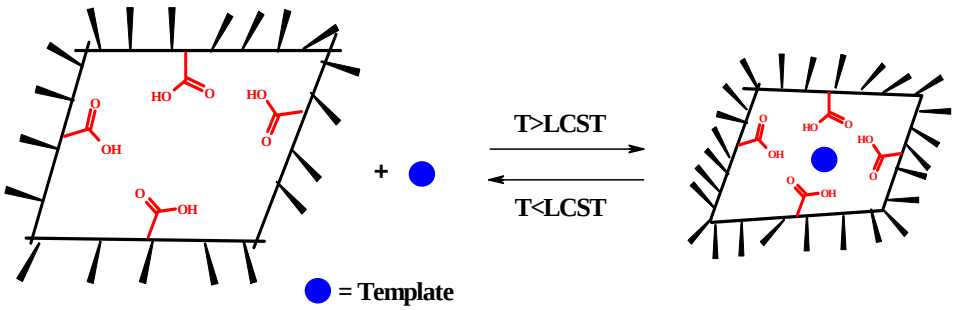

Figure 2. Schematic representation of the adsorption and release process of CF by the imprinted polymeric gels.

To evaluate cross-reactivity of imprinted polymers towards CF analogue molecule, the same binding experiments were performed using TH. The low bound percentages by TMIP shown the good selectivity properties of the imprinted materials.

4. Conclusions

In this preliminary study, lightly cross-linked thermo-responsive molecularly imprinted polymers were synthesized by using CF as model template, MAA as functional monomer able to interact with CF, NIPAAm as thermo-responsive co-monomer and EGDMA as cross-linking agent. The thermo-responsive behavior of the synthesized polymers was evaluated by performing water uptake experiments at

different temperature, while the imprinting effect was checked only at the polymerization temperature at which the complementarity of binding sites toward the template molecules was maintained. The good temperature sensitivity and high selectivity of gels make these systems useful as start materials in controlled drug release or in separation field.

SECTION III

Synthesis of Antioxidant Polymers by Free Radical Grafting Procedure

3.1 Radical Graft Functional Modification of Macromolecular Systems

Generally, some materials which have excellent bulk physical and chemical properties often do not possess suitable surface properties required for specific applications.

For this reason, surface-modification techniques that can transform these materials into valuable finished products became an important part of surface science and technology[1]. Surface modification of materials with functional polymers is an important research area in polymer science due to the wide applications of polymers in the fields of adhesion, biomaterials, protective coatings, friction and wear, composites, microelectronics, and thin-film technology[2,3].

To achieve materials with desired surface properties for specific applications, several procedures were applied.

Basically the traditional procedures consist to functionalize the materials by drastic chemical conditions (high temperature, extreme pH conditions, etc). Furthermore, these processes were often performed in organic solvent, and this reducing the potential use of these modified materials (for example, reduced biocompatibility due to presence of organic solvents). One of the most promising method to functionalize the polymeric materials under mild and biocompatible conditions is the

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[1] E. Ruckenstein, Z.F. Li, Advances in Colloid and Interface Science 113 (2005) 43– 63

[2] J.P. Fischer, U. Becker, S.P. Halasz, K.F. Muck, H. Puschner, S. Rosinger, J Polym Sci, 63 (1979) 443.

[3] Y. Ikada, Adv Polym Sci 57 (1984) 103.

grafting procedure. Graft polymerization is a well-known method for the modification of chemical and physical structure to tailor properties for specific application. This method is of particular interest to achieve specifically designed polymer properties by connecting different types of polymers having the desired properties in the same polymer chain[4]. The advantages in the use grafting reaction consist in the functionalize the materials without affecting its bulk properties, elimination of the need to redesign the bulk material to achieve a target surface performance and a considerably reduction of the cost.

Thus, the modification is achieved by grafting suitable macromolecular chains on the surface of materials through covalent bonding[5]. The key advantage of these techniques is that the surface of the materials can be modified or tailored to acquire very distinctive properties through the choice of different grafting monomers, while maintaining the substrate properties. It also ensures an easy and controllable introduction of graft chains with a high density and exact localization onto the surface with a considerable reduction of the reaction cost. Compared with the physically coated polymer chains, the covalent attachment of the grafted chains onto a material surface avoids their desorption and maintains a long-term chemical stability of the introduced chains. Surface grafting commonly includes two steps: surface activation and graft polymerization. Because of the absence of chemically reactive functional groups on most substrate surfaces, a surface activation process is needed to create reactive sites on them that can generate further grafting processes. Practically, one can

[4] L. Nurmi, S. Holappa, H. Mikkonen, J. Seppälä, European Polymer Journal 43 (2007) 1372–1382.
[5] S. Liu, G. Sun, Carbohydrate Polymers 71 (2008) 614–625.

generate reactive groups through chemical reactions, UV, high-energy electrons, γ-irradiation, plasma treatment, ozone exposure, etc[6,7,8,9]. The grafting methods can also be generally divided into two classes, i.e., grafting-to and grafting from processes. In the case of grafting-to method, preformed polymer chains carrying reactive groups at the end or side chains are covalently coupled to the surface. The grafting-from method utilizes the active species existing on the material surfaces to initiate the polymerization of monomers from the surface toward the outside bulk phase. In literature, many studies show that biopolymers, such as chitosan, cellulose and starch, can be chemically modified to possess new functional groups. One of the chemical methods is to graft vinyl functional monomers onto glucose rings employing radical polymerization systems.

Many different radical graft polymerization processes have been developed by using initiators such as peroxides and hydroperoxides[10], diazo compounds[11], or redox pairs[12]. Most of the processes were conducted in solutions containing radical initiators and functional vinyl monomers, but radical initiators can directly react with the monomers in the solution, producing a large amount of homopolymer byproducts instead of grafted products. Thus, grafting efficiencies were lower in

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[7] S.J. Yamakawa, J. Appl. Polym. Sci. 20 (1976) 3057.
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[11] A. Hebeish, N.Y. Abou-Zeid, A.I. Waly, E.A. El-Alfy, Angewandte Makromolekulare Chemie, 86 (1980) 47–63.
[12] S.A. Abdel-Hafiz, Polymers and Polymer Composites 1 (1995) 41–47.

general, which is a concern to applications in many areas. Another concern of using the radical graft polymerization is the production of long chains of the functional monomers, which may alter surface properties of the fibers and affect applications as textile materials.

On the basis of these considerations, Liu et al. carried out the radical graft functional modification of cellulose with allyl monomers using as initiator system potassium persulfate.

In the first step, cellulose macroradicals were generated by potassium persulfate on cotton cellulose fibers under solvent-free condition, and coupling reactions occurred between sulfate and cellulose radicals. Then, in the presence of allyl monomer, the cellulosic macroradicals initiated a graft polymerization and the grafted cotton cellulose structures were confirmed.

Graft polymerization was also selected as polymerization method to obtain thermo- and pH-responsive hydrogels based on chitosan. For this purpose, NIPAAm was grafted onto chitosan by ^{60}Co γ-radiation. The grafting percentage and grafting efficiency can be controlled by appropriate selection of grafting conditions, such as monomer concentration and total irradiation dose.

Moreover, 2-hydroxyethyl acrylate was successfully grafted onto chitosan up to 300% in homogeneous phase under inert atmosphere by using ammonium persulfate as the initiator. It was possible to control the grafting parameters by varying the reaction conditions such as concentration of the initiator and the monomer, reaction duration and temperature.

3.2 Bioactive Polymers by Graft Reaction

The possibility to graft antioxidant moieties in a macromolecular structure, by radical procedure, represents an interesting innovation that significantly improves the performance of the biomacromolecules, opening new applications in the biomedical and pharmaceutical fields. Polymeric antioxidants are a particular class of systems characterized by higher stability and slower degradation rate than compounds with low molecular weight.

They could be applied in those fields in which the employment of a single molecule with antioxidant activity is prohibitive[13]; for example, they can be used in hemodialysis application, in particular, by their introduction in dialysis membranes. Hemodialysis patients, indeed, are exposed to oxidative stress, which contributes to cardiovascular disease and accelerated atherosclerosis, the major causes of mortality in these patients. Another field of application of this kind of material could be cosmetic formulations, to avoid the oxidation of their components, but it can also be used as a preservative agent in food packaging.

Generally, to obtain this kind of materials, three main strategies, involving several different steps, are used. The first one is based on the functionalization of a molecule with antioxidant properties by the insertion of polymerizable groups and its subsequent polymerization or copolymerization[14]. The second strategy involves the derivatization of a preformed polymeric structure with an antioxidant[15].

[13] F. Iemma, S. Trombino, F. Puoci, G. Cirillo, U.G. Spizzirri, R. Muzzalupo, N. Picci Macromol. Biosci. 5 (2005) 1049–1056.
[14] C. Ortiz, B. Vazquez, J.S. Roman, J. Biomed. Mater. Res. 45 (1999) 184–191.
[15] D. Atkinson, R. Lehrle, Eur. Polym. J. 28 (1992) 1569–1575.

Finally, the third approach is the grafting of a synthesized monomeric antioxidant onto a polymeric chain via melt processing with free radical initiators[16]. These strategies show some limitations. The synthesis of a monomeric antioxidant requires the purification of reaction products, whereas in the derivatization process, especially if the macromolecular system consists of a cross-linked polymer, a difficult optimization of the reaction conditions is often needed.

Our challenge was to obtain the covalent insertion of antioxidant molecules onto macromolecular systems by employing free radical grafting reaction in which a water soluble redox pair as initiator system was used. Following this procedure, the copolymerizazion of ferulic acid with methacrylic acid and the functionalization of chitosan and gelatin backbones with gallic acid and cathechin and were carried out.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid), one of the most abundant phenolic acids in plants, is rarely found in the free form, is usually found as ester cross-links with polysaccharides in the cell wall, such as arabinoxylans in grasses, pectin in spinach and sugar beet and xyloglucans in bamboo. It also can cross-link with proteins. The cross-linking property of ferulic acid with both polysaccharides and proteins suggests that it can be used in the preparation of complex materials to be used in biomedical, food and cosmetic applications.

Gallic acid is a natural phenolic antioxidant extractable from plants, especially green tea[17]. It is widely used in food, drugs, and cosmetics to prevent rancidity induced by lipid peroxidation and spoilage.

[16] S. Al-Malaika, N. Suharty, Polym. Degrad. Stab. 49 (1995) 77–89.
[17] Z. Lu, G. Nie, P.S. Belton, H. Tang, B. Zhao Neurochem. Int. 48 (2006) 263–274.

Catechins are one of main classes of flavonoids present in tea, wine, chocolate, fruits, etc. They are potentially beneficial to human health as they are strong antioxidants, anti-carcinogens, anti-inflammatory agents, and inhibitors of platelet aggregation in *in vivo* and *in vitro* studies[18]. However, several low-molecular-weight flavonoids have been shown to act as pro-oxidants and generate reactive oxygen species, such as hydrogen peroxide; in addition, the activities of low-molecular-weight flavonoids generally persist for limited short periods in vivo while a relatively high-molecular weight fraction of extracted plant polyphenols has been reported to exhibit enhanced physiological properties and a relatively longer circulation time in vivo[19].

The antioxidant activity of catechin was amplified by conjugation with amine-terminated polyhedral oligomeric silsesquioxane, using horseradish peroxidase as catalyst, while gelatin-catechin conjugate was synthesized by the laccase catalyzed oxidation of catechin in the presence of protein[20]. Enzymatic synthesis of macromolecule-oxidant conjugate carried out to materials with a good scavenging activity against superoxide anion radicals but the synthetic procedure appears complex and expensive for industrial application.

The synthetic strategy proposed in this wok appears suitable from an industrial point of view. It was planned an one step polymerization, without preventive functionalization of the reactive, in aqueous media; the initiator system allows to avoid high temperature preserving both

[18] S.F. Asad, S. Singh, A. Ahmad, S.M. Hadi, Med. Sci. Res. 26 (1998) 723-728.
[19] M. Kurisawa, J.E. Chung, H. Uyama, S. Kobayashi Biomacromolecules 4 (2003) 1394-1399.
[20] E. Mendis, N. Rajapakse, S.K. Kim, J. Agric. Food Chem. 53 (2005) 581-587.

biomacromolecules and antioxidant; finally, the synthesized copolymers were easily purified without organic solvent.

Chapter 1

SYNTHESIS OF COPOLYMER WITH ANTIOXIDANT PROPERTIES BY SINGLE-STEP FREE RADICAL POLYMERIZATION

1. Introduction

It is well known that active oxygen and free radicals are involved in the pathogenesis of several human diseases, including cancer, aging and atherosclerosis[1].

Oxidative stress, indeed, can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues, resulting in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and even lead to cell death induced by DNA fragmentation and lipid peroxidation[2,3].

Active oxygen and free radicals, such as superoxide anion ($^{\cdot}O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), are constantly formed in the human body by normal activities such as immunological defences and metabolic reactions. Their excess is opposed by a balanced system of antioxidant defences, including antioxidant compounds and enzymes. Upsetting this balance causes oxidative stress, which can lead to cell injury and death[4]. Therefore, much attention has been focussed on the use of antioxidants to inhibit lipid peroxidation, or to protect against the damage of free radicals[5].

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[2] J. Nordberg, E.S.J. Arner Free Radical Bio. Med. 31 (2001) 1287–1312.
[3] O.I. Aruoma Food Chem. Toxicol. 32 (1994) 671–683.
[4] B. Halliwell, J.M.C. Gutteridge, O.I. Aruoma Anal. Biochem. 165 (1987) 215–219.
[5] G. Vendemiale, I. Grattagliano, E. Altomare Int. J. Clin. Lab. Res. 29 (1999) 49–55.

(E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid, commonly named ferulic acid, as well as phenolic acid derivatives, is a good antioxidant since it forms a resonancestabilized phenoxy radical. Ferulic acid showed high scavenging activity for hydrogen peroxide, superoxide, hydroxyl radical and nitrogen dioxide free radicals.

It is also known to play an important role for the antifungal activity[6] and could potentially serve as effective alternatives to conventional antifungal agents which are frequently perceived to present hazard to human health and environment[7].

Due to its important properties, the goal of this study was to evaluate the biological activity of a ferulic acid polymeric derivative.

Antifungal polymers, due to their high stability, could be very useful in all the environments susceptible of contamination by pathogenic fungi which have strong abilities to survive on different surfaces[8]. Contaminated materials are often associated with increased risk of infections. Medically important fungi, such as *Candida*, *Aspergillus*, *Fusarium*, *Mucor*, and *Paecilomyces* spores, survived on hospital fabrics from one day to several weeks. In response to these challenges, much effort has been devoted to the development of infection resistant materials for hospital, medical, pharmaceutical, bioprotective, and related hygienic applications[9].

[6] B.K. Sarma, U.P. Singh, World J Microbiol Biotech 19 *(*2003) 123.
[7] F. Bisogno, L. Mascoti, C. Sanchez, F. Garibotto, F. Giannini, M. Kurina-Sanz, R.J. Enriz, J. Agric Food Chem 2007, 55, 10635.
[8] B. Narasimhan, D. Belsare, D. Pharande, V. Mourya, A. Dhake, Eur. J. Med. Chem. 39 (2004) 827.
[9] E.S. Abdou, S.S. Elkholy, M.Z. Elsabee, E. Mohamed, J. Appl. Polym. Sci. 108 (2008) 2290.

In this work, ferulic acid was copolymerized with methacrylic acid in order to obtain a useful material with both antioxidant and antifungal properties. The employed synthetic strategy is an one-step radical polymerization based on the use of water soluble redox initiatiors, that allows to obtain the copolymer through the direct polymerization of ferulic and methacrylic acid.

Because of its ability to inhibit the autoxidation of oils, ferulic acid has been largely used as a food preservative. It also constitutes the active ingredient in many skin lotions and sunscreens designed for photoprotection[10]. For these reasons, a polymeric device based on this compound could be very interesting from an industrial point of view.

2. Experimental Section

2.1 Materials

Ferulic acid (FA), methacrylic acid (MAA), N,N-dimethylformamide (DMF), hydrogen peroxide (H_2O_2), ascorbic acid (AA), linoleic acid, disodium hydrogen phosphate, sodium hydrogen phosphate, Tween 20, 2,2′-azobis(2-methyl)propionamidine dihydrochloride (AAPH), ammonium thiocyanate, ferrous chloride, hydrochloric acid (37% w/w), ethanol, 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), sulphuric acid (96% w/w), trisodium phosphate, ammonium molybdate, methanol, Folin-Ciocalteu reagent, sodium carbonate, ethyl acetate and acetic acid were obtained from Sigma-Aldrich. All solvents were reagent grade or HPLC-grade. MAA was purified before use by distillation under reduced pressure.

[10] H. Priefert, J. Rabenhorst, A. Steinbuchel Appl. Microbiol. Biot. 56 (2001) 296-314.

2.2 Synthesis of MAA-FA copolymer (PMAA-FA)

The polymerization of methacrylic acid with ferulic acid by ascorbic acid-hydrogen peroxide redox initiators was carried out as follows: in a 10 ml glass tube, 0.50 g of FA were dissolved in 5.50 ml of DMF and then MAA (3.50 g) and 2 ml of distilled water containing 10 mM ascorbic acid and 7 mM hydrogen peroxide were added. The mixture was maintained at 25 °C for 3 h under atmospheric air.

The obtained polymer was precipitated by adding the polymeric solutions to an excess volume of diethyl ether (5:1), under agitation at room temperature. The suspensions were filtered by sintered glass filter funnel (Pyrex, Ø30 mm; porosity 3) and washed with diethyl ether and the recovered polymer was dried in a vacuum oven at 40 °C. They were then further purified by dissolution in water and precipitation in diethyl ether (5:1) for three times.

The copolymer was checked to be free of unreacted FA and any other compounds by HPLC analysis after each purification step. In Figure 1 the chromatogram of a 0.1mM FA standard solution in ethanol is reported. Retention time: 3.34 min.

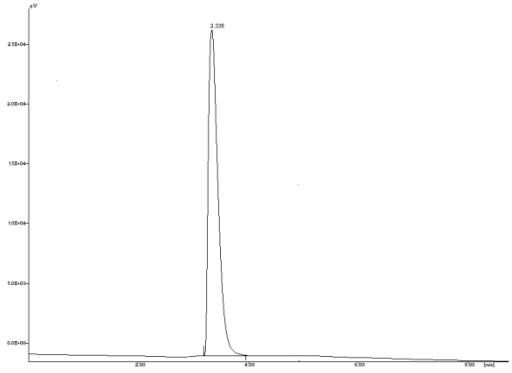


Figure 1. HPLC chromatogram of the FA standard.

Blank polymer (PMAA) was prepared under the same conditions without using FA.

2.3 Instrumentation

The liquid chromatography consisted of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 240 nm. A 250mm×4mm C-18 Hibar® Column, particle size 5μm (Merck, Darmstadt, Germany) was employed. As reprted in literature[11], the mobile phase was methanol at a flow rate of 0.5 mL min−1 and at room temperature. IR spectra were recorded as films or KBr pellets on a Jasco FT-IR 4200. UV-Vis absorption spectra were obtained with a Jasco V-530 UV/Vis spectrometer.

2.4 Evaluation of the antioxidant activity

2.4.1 Determination of scavenging effect on DPPH radicals

Synthesized PMAA-FA was allowed to react with a stable free radical, 2,2'-diphenyl-1- picrylhydrazyl radical (DPPH), with the aim of evaluating the free radical scavenging properties of these materials[12]. 100 mg of PMAA-FA were dissolved in 12.5 ml of distilled water in a volumetric flask (25 ml) and then 12.5 ml of ethanol solution of DPPH (200 μM) were added obtaining a solution of DPPH with a final concentration of 100 μM. The sample was incubated in a water bath at 25 °C and, after 30 minutes, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The same reaction conditions were applied for the blank polymer in order to evaluate the interference of

[11] S.V.S. Chakravartula, N. Guttarla *Nat Prod Res*. *12* **(2007)** 1073-1077.
[12] A. Ardestani, R. Yazdanparast *Food Chem.* 104 *(***2007)** 21–29.

polymeric material on DPPH assay. The scavenging activity of the tested polymeric materials was measured as the decrease in absorbance of the DPPH and it was expressed as percent inhibition of DPPH radicals calculated according the following equation 1:

$$inhibition\% = \frac{A_0 - A_1}{A_0} \times 100 \qquad (1)$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any polymers, and A_1 is the absorbance of polymeric samples.

A calibration curve was recorded by using five different ferulic acid standard solutions. 0.5 ml of each solution were added to DPPH system to obtain the final concentrations of 2.0; 6.0; 12.0; 24.0; 42.0 μM respectively. DPPH assay was performed and percent inhibition of DPPH radicals was calculated to record the calibration curve. The correlation coefficient (R^2), slope and intercept of the regression equation were obtained by the method of least square.

2.4.2 Linoleic acid emulsion system–thiocyanate assay

The antioxidant properties of synthesized PMAA-FA was evaluated through measurement of percent inhibition of peroxidation in linoleic acid system by using the thiocyanate method[13] with some modification. A linoleic acid emulsion (0.2 M, pH 7) was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier and 50 ml phosphate buffer (0.2M, pH 7.0), then the obtained mixture was homogenized. Subsequently, 2.5 ml of the prepared emulsion were mixed to 100 mg of

[13] C. Dufour, M. Loonis, O. Dangles Free Radic Biol Med. 43 (2007) 241-252.

PMAA-FA and AAPH (final concentration 25 mM) was added to start the peroxidation of linoleic acid. Then the reaction mixture was incubated at 40°C for 3 days to accelerate the oxidation process and after this period, the degree of oxidation was measured by ferric thiocyanate method as following. Subsequent to the incubation, the sample was centrifuged and, in 10 ml volumetric flask, 0.2 ml of sample solution, 0.2 ml of an aqueous solution of ammonium thiocyanate (30%), 0.2 ml of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl) and ethanol (75% v/v) to 10 mL were added in sequence. After 3 min stirring, the absorbance was measured at 500 nm to determinate the peroxide content. Linoleic assay was performed in the same conditions using 100 mg of the blank polymer to established eventual interferences of polymeric material.

Ferulic acid was employed to record a calibration curve. 0.1 ml of five different ferulic acid standard solutions were added to 25 ml linoleic system to raise the final concentrations of 0.12; 0.36; 0.60; 0.84; 1.08 mM respectively. The peroxidation protocol was applied and, after peroxide content measurement, the calibration curve was recorded. Percent inhibition of linoleic acid peroxidation was calculated according to equation 1.

2.4.3 Evaluation of disposable phenolic groups by Folin-Ciocalteu procedure

Amount of total phenolic groups was determined using Folin-Ciocalteu reagent procedure, according to the literature with some modifications[14].

[14] Y. Pan, J. Zhu, H. Wang, X. Zhang, Y. Zhang, C. He, X. Ji, H. Li Food Chem. 103 (2007) 913–918.

PMAA-FA (100 mg) was dissolved in distilled water (6ml) in a volumetric flask. Folin-Ciocalteu reagent (1ml) was added and the contents of flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2%) were added, and then the mixture was allowed to stand for 2 h with intermittent shaking.

The absorbance was measured at 760 nm against a control prepared using the blank polymer under the same reaction conditions. The amount of total phenolic groups in polymeric materials was expressed as ferulic acid equivalent concentration by using an equation that was obtained from a ferulic acid calibration curve. This one was recorded by employing five different ferulic acid standard solutions. 0.5 ml of each solution were added to the Folin-Ciocalteu system to raise the final concentration of 0.03; 0.06; 0.09; 0.12; 0.15 mM respectively. After 2 h, the absorbance of the solutions was measured to record the calibration curve and the correlation coefficient (R^2), slope and intercept of the regression equation obtained were calculated by the method of least square.

2.4.4 Determination of total antioxidant activity

The total antioxidant activity of polymeric materials was evaluated according to the method reported in literature[15]. Briefly, 100 mg of PMAA-FA were mixed with 1.2 ml of reagent solution (0.6 M sulphuric acid, 28 M sodium phosphate and 4 M ammonium molybdate) and 0.3 ml of methanol, then the reaction mixture was incubated at 95°C for 150 min. After cooling to room temperature, the absorbance of the mixture was measured at 695 nm against a control prepared using blank polymer

[15] P. Prieto, M. Pineda, M. Aguilar Anal Biochem. 269 (1999) 337–341.

in the same reaction. The total antioxidant activity of polymeric materials was expressed as ferulic acid equivalent concentration.

By using five different ferulic acid standard solutions, a calibration curve was recorded. 0.2 ml of each solution were mixed with 0.8 ml of reagent solution to obtain the final concentration of 1.0; 1.5; 2.0; 2.5; 3.0 mM respectively. After 150 min incubation, the solutions were analyzed by UV-Vis spectrophotometer and the correlation coefficient (R^2), slope and intercept of the regression equation obtained by the method of least square were calculated.

2.4.5 Evaluation of the scavenging activity on hydroxyl radical

PMAA-FA (75 mg) were incubated with 0.5 ml deoxyribose (3.75 mM), 0.5 ml H_2O_2 (1 mM), 0.5 ml $FeCl_3$(100 mM), 0.5 ml EDTA (100 mM) and 0.5 ml ascorbic acid (100 mM) in 2.5 ml potassium phosphate buffer (20 mM, pH7.4) for 60 min at 37°C [28]. Then to 1 ml amount of sample, 1 ml of TBA (1% w/v) and 1 ml of TCA (2% w/v) were added and the tubes were heated in a boiling water bath for 15 min. The content was cooled and the absorbance of the mixture was read at 535 nm against control reagent without polymer.

The antioxidant activity was expressed as a percentage of scavenging activity on hydroxyl radical according to equation (1).

$$inhibition\% = \frac{A_0 - A_1}{A_0} \times 100 \qquad (1)$$

The same reaction conditions were applied for the blank polymer PMAA in order to evaluate the interference of polymeric material on deoxyribose assay[18]. All samples were assayed in triplicate and data expressed as means (± SEM).

2.5 Evaluation of the antifungal activity

The synthesized polymeric material was screened in vitro for their antifungal activity against *Aspergilllus niger*. Antifungal assays were performed as follows[16]: PMAA-FA was dissolved in distilled water at a concentration of 100 mg ml^{-1}. Then, the solution was added to sterilized potato dextrose agar (PDA) to give a final concentration of 10 mg ml^{-1}. After the mixture was cooled, the mycelium of fungi was transferred to this test plate and incubated at 29 °C for 3 days. The antifungal index (A.I.) was calculated according to equation (2):

$$A.I.(\%) = (1 - \frac{D_t}{D_c}) \times 100 \qquad (2)$$

where D_t is the diameter of the growth zone in the test plate and D_c is the diameter of growth zone in the control plate (without antifungal agent).

The same reaction conditions were applied for the blank polymer PMAA in order to evaluate the interference of polymeric material.

Each experiment was performed three times, and the data were averaged.

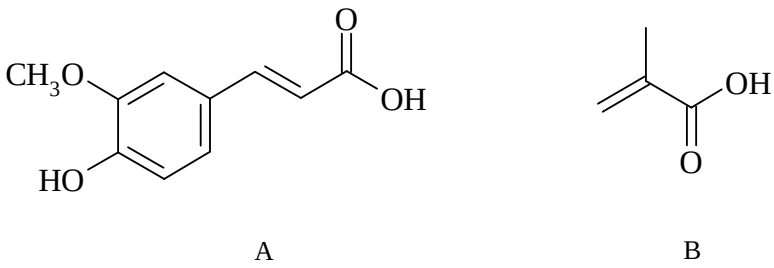
3. Results and Discussion

3.1 Synthesis of antioxidant PMAA-FA

PMAA-FA were synthesized by direct employing of ferulic acid as co-monomer (without any other derivatization reactions) and ascorbic acid-H_2O_2 as redox initiators at room temperature.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (Figure 2) has received much attention in the study of medicine.

[16] Z. Zhong, R. Chen, R. Xing, X. Chen, S. Liu, Z. Guo, X. Ji, L. Wang P. Li, Carbohydr Polym 342 *(*2007) 2390.



A B

Figure 2. Structure of ferulic acid (A) and methacrylic acid (B).

In recent years, there have been an increasing number of reports on the physiological functions of ferulic acid and its derivatives in human. Free ferulic acid is a good antioxidant since it forms a resonancestabilized phenoxy radical. It showed high scavenging activity for hydrogen peroxide, superoxide, hydroxyl radical and nitrogen dioxide free radicals. In addition, many applications of ferulic acid in the food and cosmetic industry have also been discovered[17]. It is usually found as ester cross-links with polysaccharides in the cell wall and also with proteins[18]. The cross-linking property of ferulic acid with both polysaccharides and proteins suggests that it can be used in the preparation of complex gels in food applications.

The choice of methacrylic acid as comonomer is related to broad application field of methacrylate polymers in biomedicine and biotechnology. Acrylate and methacrylate polymers have been applied in

[17] S. Ou, K.-C. Kwok J Sci Food Agr. 84 (2004) 1261–1269.
[18] M.C. Figueroa-Espinoza, M.H. Morel, A. Surget, M. Asther, S. Moukha, J.C. Sigoillot, X. Rouau Food Hydrocolloid. 13 (1999) 65–71.

drug delivery systems, contact lenses, food technology, quality control systems and synthetic membranes for biosensors[19].

In a radical polymerization process, another parameter to be considered is the initiator system. Conventional initiators including azo compounds, peroxides and thermal iniferters require relatively high polymerization temperature to ensure the rapid decomposition of initiator. By employing a redox initiation system is possible to perform polymerization processes at lower temperatures, with all the polymer chains initiated almost instantaneously because of the reduction of the induction time[20]. Furthermore, the lower polymerization temperature reduces the risks of antioxidant degradation.

In this work, AA/H_2O_2 redox pair was employed in order to avoid the generation of any kind of toxic reaction products.

The ratio 1/7 w/w between FA and MAA in the prepolymerization feed represents the optimal value to obtain the polymer with the highest antioxidant efficiency. An higher amount of the ferulic acid, indeed, carries out to the formation of oligomers hard to be purified by the conventional purification technique.

On the basis of chemical structure of ferulic acid, characterized by a carbon-carbon double bond in styrenic position and a phenolic group, a polymerization mechanism could be hypothesized. It is known, indeed, that styrenic group of cinnamic acid can undergo free radical polymerization in a wide range of conditions[21]. Phenolic group compatibility with this kind of polymerization was also proved in

[19] J.P. Hervás Pérez, E. López-Cabarcos, B. López-Ruiz Biomol Eng. 23 (2006) 233–245.
[20] R.W. Simms, M.F. Cunningham Macromolecules 40 (2007) 860-866.
[21] H. Esen, S.H. Küsefoğlu, J. Appl. Polym. Sci. 89 (2002) 3882–3888.

different research work[22]: monomers with active functional groups (phenolic groups) as side substituents, indeed, were used for the preparation of chelating[23] or grafted polymeric systems using free radical initiators. On the other side, phenolic group could be directly involved in polymerization process, it is indeed reported that phenolic radical undergoes in dimerization processes by reaction between hydroxyl radical and aromatic ring[24].

Based on these considerations, our hypothesized polymerization mechanism involves reaction of both styrenic carbon carbon double bond and phenolic oxygen for ferulic acid insertion in polymeric chain.

3.2 Characterization of PMAA-FA

PMAA-FA and the respective control polymer were characterised by Fourier Transform IR spectrophotometry and UV analysis.

By comparing two IR spectra, it is clear that in the spectra of the PMAA-FA (trace B) new peak, which is absent in the spectrum of PMAA (trace A), can be ascertained (Figure 3).

[22] T.H. Kim, D.R. Oh, Polym. Degrad. Stabil. 84 (2004) 499-503.
[23] S. Nanjundan, C.S.J. Selvamalar, R. Jayakumar, Eur. Polym. J. 40 (2004) 2313–2321.
[24] E. Larsen, M.F. Andreasen, L.P. Christensen, J. Agr. Food Chem. 49 (2001) 3471-3475.

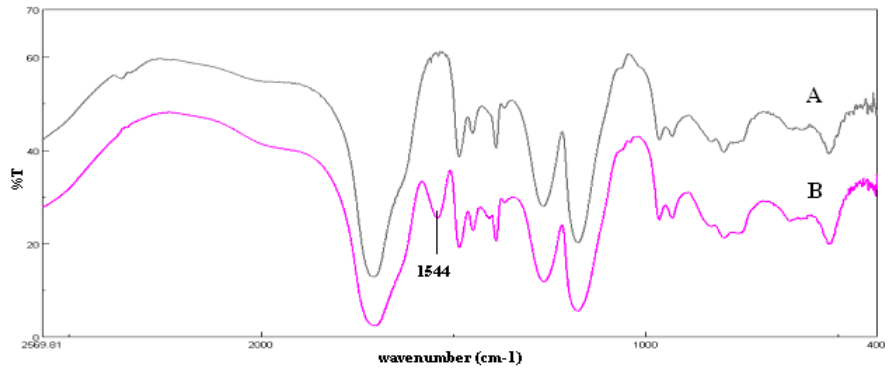


Figure 3. FT-IR spectra of PMAA (A) and PMAA-FA (B).

The new peak at 1544 cm^{-1}, indeed, is awardable to carbon to carbon stretching within the aromatic ring of ferulic acid.

The incorporation of FA in the polymeric structure is also clear in comparing the UV spectra of FA (25 μM) and PMAA-FA in ethanol (4 mg/ml) (Figure 4).

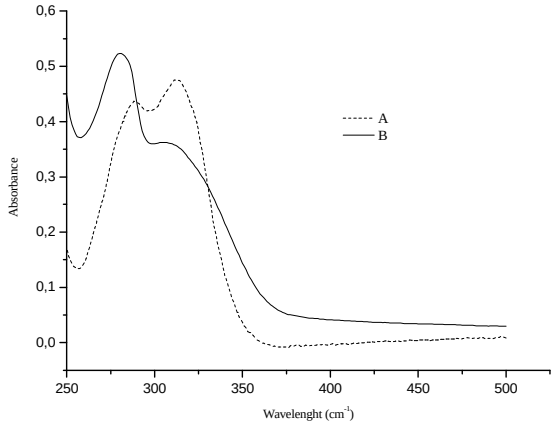


Figure 4. UV spectra of FA (A) and PMAA-FA (B).

Firstly, in PMAA-FA spectrum, the presence of absorption peaks in the aromatic region is related to the presence of FA in the sample. In addition, the wavelength of the aromatic peaks is shorter in PMAA-FA than in FA. This effect, together with the inversion in the magnitude of the two peaks, are ascribable to the formation of a covalent bond between FA and the polymeric chain.

3.3 Measurement of antioxidant activity

3.3.1 Determination of scavenging effect on DPPH radicals

The DPPH radical is a stable organic free radical with an absorption maximum band around 515–528 nm and thus, it is a useful reagent for evaluation of antioxidant activity of compounds.

In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., ferulic acid, hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1,1-diphenyl-2- picrylhydrazine by their hydrogen donating capabilities. Polymer scavenger ability were evaluated in term of DPPH reduction using ferulic acid as reference compound and data are expressed as inhibition (%).

Antioxidant polymer showed high scavenging activity and blank polymer did not significantly interfere with the scavenger process (Table 1).

3.3.2 Linoleic acid emulsion system–thiocyanate assay

The antioxidant activity of synthesized material was measured using ferric thiocyanate test which determines the amount of peroxide produced at the initial stage of lipid peroxidation.

Linoleic acid, an unsaturated fatty acid, is usually used as a model compound in lipid oxidation and antioxidation-related assays in which carbon-centered, peroxyl radicals and hydroperoxides, etc., are involved in the oxidation process. During the linoleic acid oxidation, peroxides are formed. These compounds oxidize Fe^{2+} to Fe^{3+}. The later Fe^{3+} ions form complex with SCN^{-}, which have maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation, while lower absorbance indicates a higher level of antioxidant activity.

In Table 1, the effects of polymeric particles (100 mg) on linoleic acid peroxidation compared to ferulic acid after 72 h are shown. Ferulic acid-containing polymer are very effective in peroxidation process inhibition.

Polymer	**Inhibition (%)**		
	DPPH	*Linoleic acid Peroxidation*	*Hydroxyl Radicals*
PMAA-FA	80 ± 1.1	50± 1.3	95±1.2
PMAA	30 ± 1.2	7 ± 0.9	19±1.1

Table 1. Inhibition percentages of DPPH radical and linoleic acid peroxidation by PMAA-FA and PMAA. Inhibition percentage = [1-(absorbance of sample)/(absorbance of control)] x 100. Results are means ± SD of three parallel measurements.

The lowest inhibition value obtained from the experiments performed with blank polymer, clearly shown that absorbance reduction is ascribable at ferulic acid moieties in the polymeric structure.

3.3.3 Evaluation of scavenging activity on hydroxyl radicals

Hydroxyl radical exhibits very high reactivity and tends to react with a wide range of molecules found in living cells. They can interact with the purine and pyrimidine bases of DNA. They can also abstract hydrogen atoms from biological molecules (e.g. thiol compounds), leading to the formation of sulphur radicals able to combine with oxygen to generate oxysulphur radicals, a number of which damage biological molecules.[16] Due to the high reactivity, the radicals have a very short biological half-life. Thus, an effective scavenger must be present at a very high concentration or possess very high reactivity towards these radicals. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is the Fenton reaction where a transition metal is involved as a prooxidant in the catalyzed decomposition of superoxide and hydrogen peroxide. These radicals are intermediary products of cellular respiration, phagocytic outburst and purine metabolism. Hydroxyl radical can be generated in situ by decomposition of hydrogen peroxide by high redox potential EDTA–Fe^{2+} complex, and in the presence of deoxyribose substrate, it forms TBARS which can be measured. Antioxidant activity is detected by decreased TBARS formation, which can come about by donation of hydrogen or electron from the antioxidant to the radical or by direct reaction with it. Consequently, the ability of PMAA-FA to scavenge hydroxyl radical was

evaluated by the Fenton-mediated deoxyribose assay and good antioxidant properties were found, as shown in Table 1.

3.3.4 Evaluation of disposable phenolic groups by Folin-Ciocalteu procedure

Since the antioxidant activity of PMAA-FA is derived from phenolic groups, it is useful express the antioxidant potential in terms of phenolic content.

The Folin–Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of disposable phenolic groups present in polymer chain. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin–Ciocalteu reactant. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valence. Disposable phenolic groups in the samples were expressed as mg equivalent of ferulic acid and this value was 2.77 mg/g of dry polymer. Control experiments were also performed with blank polymer, and no activity was recovered.

3.3.5 Determination of total antioxidant activity

The assay is based on the reduction of Mo(VI) to Mo(V) by ferulic acid and subsequent formation of a green phosphate/Mo(V) complex at acid pH. The total antioxidant activity was measured and compared with that of ferulic acid and the control, which contained no antioxidant component. The high absorbance values indicated that the sample possessed significant antioxidant activity. According to the results,

synthesized materials had significant antioxidant activities, and ferulic acid equivalent concentration were found to be 0.59 mM for 1g of PMAA-FA. Control experiments were also performed with blank polymer, and no activity was recovered.

3.4 Determination of antifungal activity

Aspergillus niger is less likely to cause human disease than some other *Aspergillus* species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis, can occur. Aspergillosis is particularly frequent among horticultural workers who inhale peat dust, which can be rich in *Aspergillus* spores. *A. niger* is one of the most common causes of otomycosis (fungal ear infections) which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympani membrane.

PMAA-FA showed antifungal activity, as it inhibited the mycelial growth of *A. niger* on PDA. The growth declined with the increase of polymer concentrations and growth was completely inhibited at a concentration of 10 mg/ml (A.I. 97 ± 1.4 %). No antifungal activity was found in PMAA treated samples.

4. Conclusions

In this work, MAA-FA copolymer were successfully synthesized by employing redox water soluble initiators. IR and UV spectra performed on the copolymer testified the success of the procedure.

Antioxidant properties of obtained materials were evaluated by different assays[25]. In particular, determination of scavenging activity on DPPH and hydroxyl radicals, linoleic acid emulsion system–thiocyanate assay, determination of disposable phenolic groups in polymeric matrices, and determination of total antioxidant capacity were performed. Moreover, antifungal properties against *A. niger* was verified[26]. The good obtained results, together with its biocompatibility and improved stability, clearly shows the utility of the proposed polymeric device in hospital, medical, pharmaceutical, bioprotective, and related hygienic applications. By using PMAA-FA it is possible to raise a good protection against free radical damages and fungal contamination.

[25] F. Puoci, F. Iemma, M. Curcio, O.I. Parisi, G. Cirillo, U.G. Spizzirri, N. Picci, J. Agric. Food Chem. 56 (2008) 10646-10650.
[26] F. Iemma, F. Puoci, M. Curcio, O.I. Parisi, G. Cirillo, U.G. Spizzirri, N. Picci J. Appl. Polym. Sci. 115 (2010) 784-789.

Chapter 2

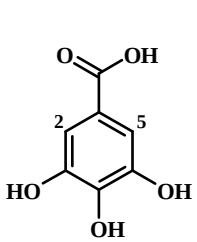
NOVEL ANTIOXIDANT POLYMERS BY GRAFTING OF GALLIC ACID AND CATECHIN ON GELATIN

1. Introduction

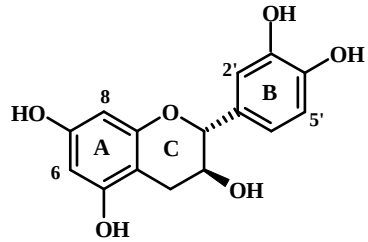
Natural proteins, such as gelatin and albumin are widely employed in industry due to their biocompatibility, biodegradation, non-toxicity and non-immunogenicity. Gelatin is a mixture of high molecular weight and water-soluble proteins extensively used in food, adhesives and pharmaceutical fields. Because of the various potential uses of gelatin, it is useful to investigate its modification to develop new materials with improved properties. It is well know that proteins are major targets for photo-oxidation within cells, due to their high abundance, the presence of endogenous chromophores within the protein structure (amino acid side-chains), their ability to bind exogenous chromophoric materials, and their rapid rates of reaction with other excited state species. In particular, Met, Trp, Tyr, Cys, and His side-chain residues have been shown to be the most vulnerable to modification by photooxidation[1,2,3]. The residues in the side chains of gelatin able to undergo oxidative modifications represent suitable target groups to prepare composite materials showing both protein and grafted molecule characteristics. The graft copolymerization of gelatin with various monomers is an effective method to improve the properties of the gelatin. Among the monomers

[1] P.E. Morgan, D.I. Pattison, C.L. Hawkins, M.J. Davies Free Radical Bio. Med. 45 (2008) 1279–1289.
[2] V.V. Agon, W.A. Bubb, A. Wright, C.L. Hawkins, M.J. Davies Free Radical Bio. Med. 40 (2006) 698–710.
[3] M.J. Davies, R.J.W. Truscott J. Photochem. Photobiol. B: Biology 63 (2001) 114–125.

grafted upon gelatin for its modification, the most widely used are methyl methacrylate, acrylonitrile, and ethyl acrylate, polyvinylpirrolidone[4,5]. Grafting reaction was generally carried out by chemical means and the initiators were redox systems, such as potassium persulfate, ceric ammonium nitrate and ammonium persulfate, producing free radical species after warming at 40°C [6,7]. In the present work our challenge was to synthesize novel proteic antioxidants by a simple method, involving an one-step reaction, to obtain copolymers through the simple conjugation of gelatin with an antioxidant molecule in the presence of water-soluble redox initiatiors able to generate free radical species at room temperature.
The addition of antioxidant molecules on the proteic side chains took place by radical reaction of gelatin, such as biomacromolecule, Gallic acid (GA) and Catechin (CA) (Figure 1), as antioxidants, and using the hydrogen peroxide (HPO)/ascorbic acid (AA) redox pair, as initiator system[8].



GA



CA

Figure 1. Chemical structures of Gallic Acid (**GA**) and Catechin (**CA**).

[4] J. Stejskal, D. Strakova, P. Kratochvil, J. Appl. Pol. Sci. 36 (1988) 215-227.
[5] T. Mikołajczyk, A. Krasínska J. Thermal Anal. Calor. 63 (2001) 815-822.
[6] Liu, S.; Sun, G. Carbohyd. Polym. 71 (2008) 614-625.
[7] A. Mishra, J.H.V.A. Clark, S. Daswal Pol. Adv. Tech. 19 (2008) 99-104.
[8] R.W. Simms, M.F. Cunningham, Macromolecules 40 (2007) 860-866.

In this way, we synthesized two antioxidant polymers, labelled **I** and **II**, where GA and CA, respectively, were conjugated with the macromolecule.

The materials was exhaustively characterized by UV-Vis, fluorescence and calorimetric analyses to demonstrate the chemical bond between antioxidant and proteic structure.

Antioxidant activity of conjugated polymers was tested and both functionalized macromolecules were able to interact with free radical species and to minimize the oxidative damage. The materials combine the characteristics of amminoacidic structure (biocompatibility, biodegradability, high molecular weight) with antioxidant properties of CA and GA. These novel polymeric systems could be widely used in food and pharmaceutical fields.

2. Experimental section

2.1 Materials

Gelatin (Ph Eur, Bloom 160), (+)-Catechin hydrate (CA), Gallic acid (GA), hydrogen peroxide (H_2O_2), ascorbic acid (AA), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), Folin-Ciocalteu reagent, sodium carbonate, sulphuric acid (96% w/w), trisodium phosphate, ammonium molybdate, β-Carotene, linoleic acid, Tween 20, deoxyribose, $FeCl_3$, ethylenediaminetetraacetic acid disodium salt (EDTA), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), hydrochloric acid (37% w/w) were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). Acetonitrile, methanol, water, ethanol, chloroform, were HPLC-grade and provided by Carlo Erba reagents (Milan, Italy).

2.2 Instrumentation

The dialysis membranes of 6–27/32" Medicell International LTD (MWCO: 12-14000 Dalton) were employed. Freeze drier Micro Modulyo, Edwards was employed. Spectrofluorimetric grade solvents was used for the photophysical investigations in solution, at room temperature. A Perkin Elmer Lambda 900 spectrophotometer was employed to obtain the absorption spectra, while the corrected emission spectra, all confirmed by excitation ones, were recorded with a Perkin Elmer LS 50B spectrofluorimeter, equipped with Hamamatsu R928 photomultiplier tube. The calorimetric analyses were performed using a Netzsch DSC200 PC. The high-Pressure Liquid Chromatography (HPLC) analyses were carried out using a Jasco PU-2089 Plus liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 μl loop), a Jasco UV-2075 HPLC detector and Jasco-Borwin integrator. A reversed-phase C18 column (μBondapak, 10 μm of 250mm×4.6mm internal diameter obtained from Waters) was used. As reported in literature[9], HPLC conditions for the antioxidants were:

- GA: mobile phase was methanol/water/ orthophosphoric acid (20/79.9/0.1) at a flow rate of 1.0 mL min^{-1} at 260 nm.
- CA: mobile phase was acetonitrile/water/ orthophosphoric acid (20/79.9/0.1) at a flow rate of 0.5 mL min^{-1} at 230 nm. Molecular weight distributions of gelatin were analyzed by GPC. Gelatin solutions were prepared at 0.5 mg/mL in PBS buffer pH 7.5. The GPC system was composed of: μBondagel E-125 and E-500 GPC columns (Millipore, Water Associates) connected in series; a Jasco PU-2080 Plus liquid

[9] H. Wang, K. Helliwell, X. You Food Chem. 68 (2000) 115-121.

chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 μl loop); and Jasco UV-2075 HPLC detector and Jasco-Borwin integrator. The mobile phase was PBS buffer pH 7.5 at a flow rate of 0.8 mL/min. The eluent fractions were monitored by UV-detection (220 nm).

2.3 Synthesis of conjugate polymers

The polymers **(I)** and **(II)** were synthesized following the general procedure: in a 50 ml glass flask, 0.5 g of gelatin were dissolved in 50 ml of H_2O, then 1.0 ml H_2O_2 5.0 M (5.0 mmol) and 0.25 g of ascorbic acid (1.4.mmol) were added and the mixture was maintained at 25°C under atmospheric air. After 2h, 0.35 mmol of antioxidant were added to solution. The solution of gelatin and antioxidant, after 24h, was introduced into dialysis tubes and dipped into a glass vessel containing distilled water at 20°C for 48 h with eight changes of water. The resulting solution was frozen and dried with a freeze drier to afford a vaporous solid. Purified **(I)** and **(II)** were checked to be free of unreacted antioxidant and any other compounds by HPLC analysis after the purification step. Blank gelatin, that act as a control, was prepared when grafting process was carried out in the absence of CA and GA.

The reaction conditions were optimized and the reported protocol is related to the most effective polymer. A lower amount of antioxidant in the reaction feed is not enough to obtain a material with significant antioxidant properties because of the inefficiency of the conjugation process. On the other hand, an higher amount carries out to a material with no improvement in antioxidant property.

2.4 Calorimetric analysis of gelatin-antioxidant conjugates

Calorimetric analysis of the samples was carried out using a DSC. In a standard procedure about 6.0 mg of dried sample were placed inside a hermetic aluminum pan, and then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25°C to 400°C under a dry nitrogen atmosphere with a flow rate of 25 ml min^{-1} and heating rate 5°C min^{-1}. DSC analysis of CA requires preliminarily warming of the sample at 100°C for 2 hours.

2.5 Evaluation of the Antioxidant Activity

2.5.1 Determination of scavenging effect on DPPH radicals

In a standard procedure grafted polymer was allowed to react with a stable free radical, 2,2'-diphenyl-1- picrylhydrazyl radical (DPPH), with the aim of evaluating its free radical scavenging properties[10]. 50.0 mg of functionalized protein were dissolved in 12.5 ml of distilled water in a volumetric flask (25 ml), and then 12.5 ml of an ethanol solution of DPPH (200 μM) were added to obtain a solution of DPPH, with a final concentration of 100 μM. The sample was incubated in a water bath at 25°C and, after 30 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The same reaction conditions were applied for the blank polymer to evaluate the interference of polymeric material on DPPH assay. The scavenging activity of the tested polymeric materials was measured as the decrease in absorbance of the DPPH, and it was expressed as percent inhibition of DPPH radicals, calculated according to the equation (1)

[10] A. Ardestani, R. Yazdanparast, Food Chem. 104 (2007) 21–29.

$$Inhibition\,\% = \frac{A_0 - A_1}{A_0} \times 100 \qquad (1)$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any polymer, and A_1 is the absorbance of polymeric sample. Each measurement was carried out in quintuplicate, data were expressed as means (± SEM), and analysed using One-Way Analysis of Variance (ANOVA).

2.5.2 Evaluation of disposable phenolic equivalents by Folin-Ciocalteu procedure

The amount of total phenolic equivalents was determined using Folin-Ciocalteu reagent procedure, according to the literature with some modifications[11]. Grafted protein (20 mg) dissolved in distilled water (6.0 ml) and Folin-Ciocalteu reagent (1.0 ml) were added in a volumetric flask, mixing thoroughly. After 3 min, 3.0 ml of Na_2CO_3 (7.5 % w/w) were added, and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm against a control prepared using the blank polymer under the same assay conditions. Measurement was carried out in triplicate and data expressed as means (± SEM).

The amount of total phenolic equivalents in polymeric materials was expressed as antioxidant equivalent concentration by using an equation that was obtained from an antioxidant calibration curve, recorded by

[11] Y. Pan, J. Zhu, H. Wang, X. Zhang, Y. Zhang, C. He, X. Ji, H. Li, Food Chem. 103 (2007) 913–918.

employing five different antioxidant standard solutions. Half a millilitre of each solution was added to the Folin-Ciocalteu system to raise the final concentrations of 8.0, 16.0, 24.0, 32.0, and 40.0 □M, respectively. After 2 h, the absorbance of the solutions was measured to record the calibration curve, and the correlation coefficient (R^2), slope, and intercept of the regression equation obtained were calculated by the method of least-squares.

2.5.3 Determination of total antioxidant activity

The total antioxidant activity of polymeric materials was evaluated according to the method reported in the literature[12].

Briefly, 10.0 mg of grafted polymer were dissolved in 0.3 ml of distilled water and then mixed with 1.2 ml of reagent solution (0.6 M H_2SO_4, 28.0 M Na_3PO_4, and 4.0 M $(NH_4)_2MoO_4$). The reaction mixture was incubated at 95°C for 150 min and after cooling to room temperature, the absorbance of the mixture was measured at 695 nm against a control prepared using blank polymer in the same assay conditions. Measurement was carried out in triplicate and data expressed as means (± SEM).

The total antioxidant activity of polymeric materials was expressed as antioxidant equivalent concentration. By using five different antioxidant standard solutions, a calibration curve was recorded. A volume of 0.3 ml of each solution was mixed with 1.2 ml of phosphomolybdate reagent solution to obtain the final concentrations of 8.0, 16.0, 24.0, 32.0, and 40.0 μM, respectively. After 150 min of incubation, the solutions were analyzed by using a UV-Vis spectrophotometer, and the correlation

[12] P. Prieto, M. Pineda, M. Aguilar, Anal. Biochem. 269 (1999) 337–341.

coefficient (R^2), slope, and intercept of the regression equation obtained by the method of least-squares were calculated.

2.5.4 β-Carotene bleaching test

The antioxidant properties of synthesized conjugates were evaluated through measurement of percent inhibition of peroxidation in linoleic acid system by using the β-carotene bleaching test[13]. Briefly, 1.0 ml of β-carotene solution (0.2 mg/ml in chloroform) was added to 0.02 ml of linoleic acid and 0.2 ml of Tween 20. The mixture was then evaporated at 40°C for 10 min in a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 ml of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion. The emulsion (5.0 ml) was transferred to different test tubes containing 50.0 mg of antioxidant-gelatin conjugate dispersed in 0.2 ml of 70% ethanol, and 0.2 ml of 70% ethanol in 5ml of the above emulsion was used as a control. The tubes were then gently shaken and placed in a water bath at 45°C for 60 min. The absorbance of the filtered samples and control was measured at 470 nm against a blank, consisting of an emulsion without β- carotene. The measurement was carried out at the initial time (t = 0) and successively at 60 min. The same reaction conditions were applied for the blank gelatin in order to evaluate the interference of the ungrafted protein on β-Carotene bleaching test.

The antioxidant activity ($A_{ox}A$) was measured in terms of successful bleaching of β-carotene using the following equation (2):

[13] F. Conforti, G. Statti, D. Uzunov, F. Menichini Pharm. Bull. 29 (2006) 2056-2064.

$$A_{ox}A = 1 - \frac{A_0 - A_{60}}{A_0^o - A_{60}^o} \qquad (2)$$

where A_0 and A_0^o are the absorbance values measured at the initial incubation time for sample and control, respectively, while A_{60} and A_{60}^o are the absorbance values measure in the sample and control respectively at t = 60 min. Each measurement was carried out in quintuplicate, data were expressed as means (± SEM), and analysed using ANOVA.

2.5.5 Scavenging activity on hydroxyl radical

20 mg of grafted protein were dispersed in 0.5 ml of 95% ethanol and incubated with 0.5 ml deoxyribose (3.75 mM), 0.5 ml H_2O_2 (1.0 mM), 0.5 ml $FeCl_3$ (100 mM), 0.5 ml EDTA (100 mM) and 0.5 ml ascorbic acid (100 mM) in 2.0 ml potassium phosphate buffer (20 mM, pH = 7.4) for 60 min at 37 °C[14].[33] The samples were filtered and to 1.0 ml amount of filtrate, 1.0 ml of TBA (1% w/v) and 1.0 ml of TCA (2% w/v) were added and the tubes were heated in a boiling water bath for 15 min. The content was cooled and the absorbance of the mixture was read at 535 nm against reagent blank without conjugate.

The antioxidant activity was expressed as a percentage of scavenging activity on hydroxyl radical, according to equation (1). Each measurement was carried out in quintuplicate, data were expressed as means (± SEM), and analysed using ANOVA.

[14] Y. Pan, J. Zhu, H. Wang, X. Zhang, Y. Zhang, C. He, X. Ji, H. Li, Food Chem. 103 (2007) 913–918.

3. Results and discussion

3.1 Synthesis of antioxidant-gelatin conjugates

Since gelatin is a natural polymer, commonly used for pharmaceutical and biomedical applications, because of its biodegradability and biocompatibility in physiological environments, it was chosen as polymer backbone to be functionalized with CA and GA to obtain biomacromolecules showing raised antioxidant properties. The employed synthetic strategy involved the use of the ascorbic acid/hydrogen peroxide redox pair, a biocompatible and water-soluble system, as radical initiators: hydroxyl radical, that initiates the reaction, was formed by the oxidation of AA by H_2O_2 within ascorbate radical. Comparing to conventional initiator systems (i.e. azo compounds and peroxides), which require relatively high reaction temperature to ensure their rapid decomposition, the employed redox pair shows several advantages. First of all, this kind of system does not generate toxic reaction products; moreover, it is possible to perform the reaction processes at room temperatures, to avoid antioxidant degradation[15]. On the other hand, in order to activate gelatin toward radical reactions, and thus to promote the insertion of antioxidant molecules (avoiding self reactions), radical initiators should preferably react with the macromolecule before adding GA and CA.

A possible mechanism to bind antioxidant molecules to proteic side chains is proposed in Figure 2. The hydroxyl radicals, generated by the interaction between redox pair components, attack the sensible residues in the side chains of protein, producing radical species on the aminoacidic

[15] M. Kitagawa, Y. Tokiwa, Carbohydr. Polym. 64 (2006) 218–223.

structure. These ones react with the antioxidant molecules inducing an antioxidant-gelatin covalent bond.

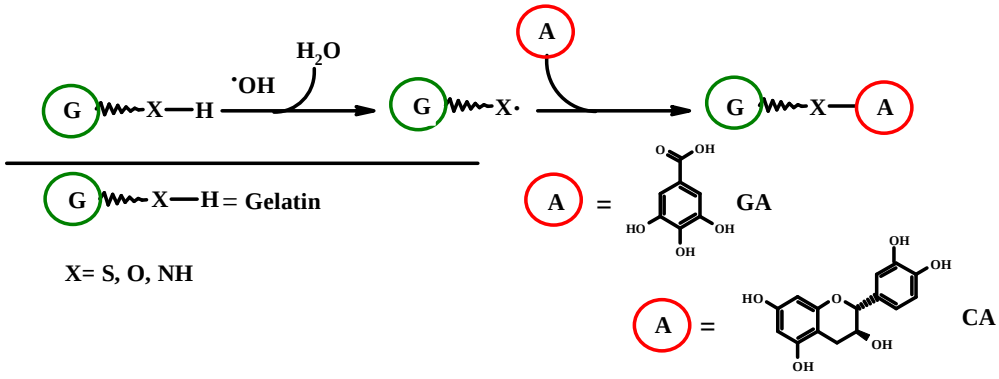


Figure 2. Grafting of gelatin with antioxidant molecules.

Literature data suggests that on the phenolic ring free radical species attack at the orto- and para-positions relatively to the hydroxyl group[16,17]. This findings support the hypothesis that the binding sites involved in the antioxidant-protein conjugation are the positions 2 and 5 for GA and 2', 5' (B ring) and 6, 8 (A ring) for CA (Figure 1). The heteroatom-centered radicals in the side chains of protein preferentially react in some of the above mentioned positions. This synthetic strategy permit to realize two biomacromolecules with antioxidant activity, where gelatin was functionalized with GA and CA, labeled **(I)** and **(II)** respectively. In the reaction feed the amount of antioxidant was 0.7 mmol per grams of protein for both conjugate systems; this value represents the optimum to

[16] S. Kobayashi, H. Higashimura, Prog. Polym. Sci. 28 (2003) 1015–1048.
[17] H. Uyama, N. Maruichi, H. Tonami, S. Kobayashi Biomacromolecules 3 (2002) 187-193.

obtain a material with the highest efficiency. To remove unreacted antioxidant, physically incorporated in the proteic structure, the conjugates underwent dialysis process (MWCO: 12-14000 Dalton) and washing media were analyzed by HPLC. Finally, the solution of grafted protein was lyophilized, to obtain a porous material extensively characterized by UV-Vis, fluorescence and calorimetric analyses. To test antioxidant properties of conjugates a control polymer was prepared carrying on grafting process in the absence of CA and GA.

3.2 Characterization of antioxidant-gelatin conjugates

The grafting procedure allows to obtain water soluble gelatin conjugates, because when the grafting reaction is carried out in large amount of solvent (water) and functional molecules (in our case GA and CA), the possibility to have inter molecular crosslinking is statistically very low compared to possibility for the small functional molecules to react (graft) with the reaction site of Protein. The absence of both inter- or intra-molecular crosslinking involving only the gelatin was confirmed by GPC analyses, that show no relevant changes in the molecular weight distribution of native, blank and conjugate gelatin (Figure 3).

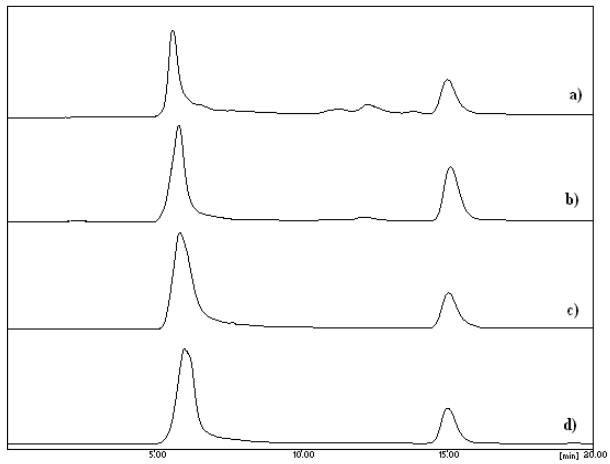


Figure 3. Gel permeation chromatograms of native (a), blank (b), (**I**) (c) and (**II**) (d) gelatin.

The incorporation of antioxidant moieties in the proteic structure was proved by UV-Vis, fluorescence and calorimetric analyses of gelatin conjugates, control polymer and antioxidants. UV-Vis spectra of antioxidants (10 μM) and conjugate polymers (1.5 mg/mL) clearly shows covalent bond formation between antioxidant moieties and protein. In the spectrum of conjugate **(I)**, the presence of two absorption peaks in the aromatic region (227 and 272 nm) is related to the presence of GA covalently bonded to the aminoacidic chains. In addition, in the free antioxidant the wavelength of the aromatic peaks is lower (211 and 258 nm) than in grafted protein, as depicted in Figure 4a. The same results appear in the UV-Vis spectrum of conjugate **(II)**, where it is evident the displacement of the adsorption bands to higher wavelength (230 and 275 nm) respect to free

CA (204 and 229 nm), as showed in Figure 4b.

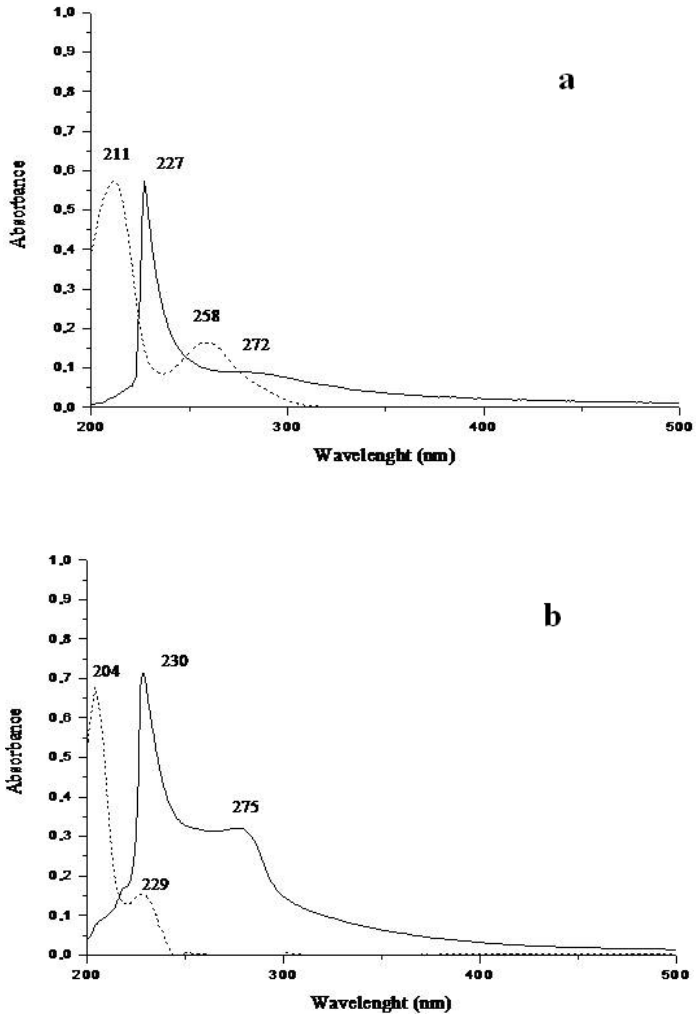


Figure 4. UV-Vis spectra: a) GA (- - -) and **(I)** (—); b) CA (- - -) and **(II)** (—)

The shift of spectral band position at higher wavelengths is ascribable to the extension of the conjugation due to the formation of the covalent bonds between heteroatom in the protein side-chains and antioxidant

aromatic ring. The emission spectra of antioxidants and conjugate polymers also confirm the covalent functionalization of the protein. In the spectra of conjugates (**I**) and (**II**), bathochromic shifts of the emission peaks of GA (from 354 to 416 nm) and CA (from 318 to 409 nm), are detected (Figure 5).

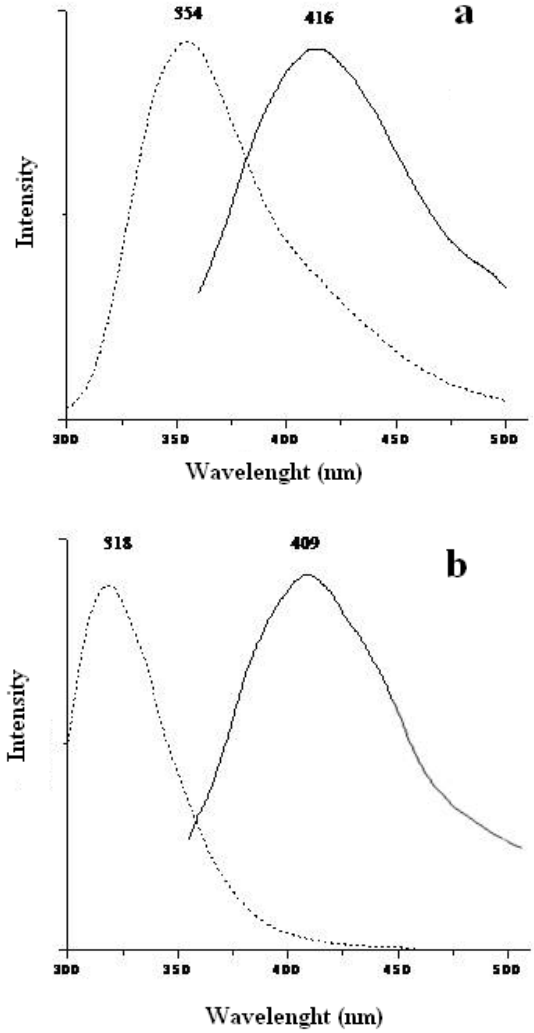


Figure 5. Emission spectra: a) GA (- - -) and **(I)** (—); b) CA (- - -) and **(II)** (—)

These spectral red shifts are due to the covalent conjugation, because no emission peak is detected in the same wavelength range for blank gelatin.
Thermal characterization of prepared conjugates was also performed by recording of DSC thermograms of dried grafted protein (a), blank gelatin (b) and pure antioxidants (c), as depicted in Figures 5 and 6, for **(I)** and **(II)**, respectively.
As far as DSC of gelatin is concerned, a broad endothermic peak, located around 60–180°C, have been assigned to the glass transition of α-amino acid blocks in the peptide chains; ΔH_t associated to this transition was -242.1 J per grams of grafted protein (Figures 6b and 7b). The calorimetric analysis of pure GA shows a narrow melting endotherm at 266.5°C, corresponding to the melting point of antioxidant molecule (Figure 6c), while for pure CA a melting endotherm at 155.8°C was displayed (Figure 7c). Since the grafting of GA produces structural modification onto the proteic chains, in the DSC thermogram of conjugate **(I)** (Figure 6a) marked differences appear. The calorimetric analysis displays the absence of melting endotherm of GA, while, the ΔH_t value associated to the protein glass transition was -304.2 J per grams of grafted protein, probably as consequence of more rigidity of the aminoacidic chain. This discrepancy suggests that glass transition conjugate **(I)** needs 25% more heat respect to unmodified protein. The same results were observed in the DSC thermogram of conjugate **(II)** (Figure 7a), where the endotherm due to melting of CA disappears, while the endotherm at 40-180°C shows a ΔH_t equal to -285.5 J per grams of grafted protein; in this case to produce glass transition in the conjugate **(II)** about 18% more heat is necessary respect to unmodified polymer. Thus, the conjugation of gelatin with GA and CA causes an increasing in the thermal stability of the native protein.

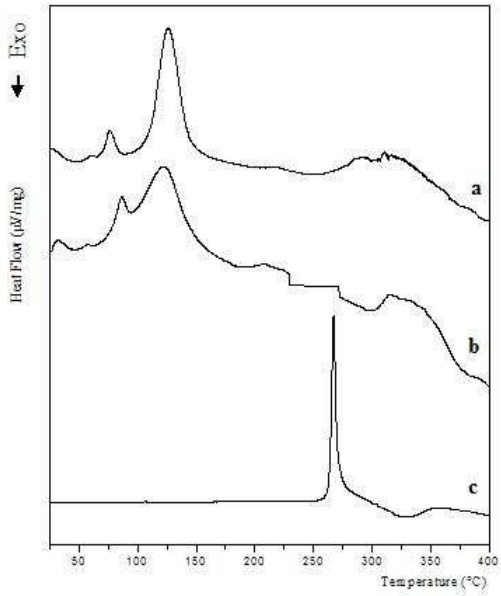


Figure 6. Calorimetric analyses of **(I)** (**a**), blank gelatin (**b**) and GA (**c**).

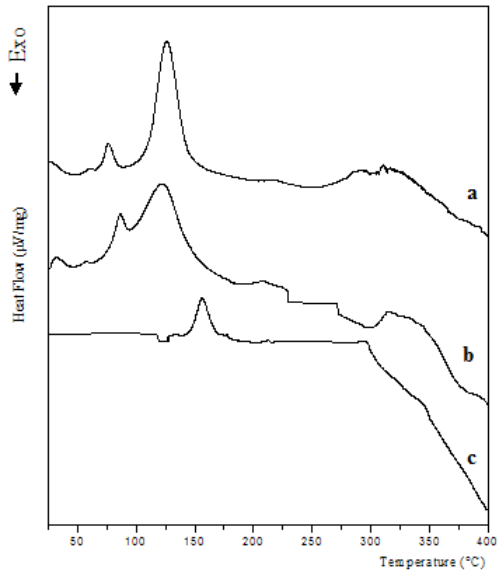


Figure 7. Calorimetric analyses of **(II)** (**a**), blank gelatin (**b**) and CA (**c**).

3.3 Measurement of Antioxidant Activity

3.3.1 Determination of scavenging effect on DPPH radicals

The DPPH radical is a stable organic free radical with an absorption maximum band around 515-528 nm and, thus, it is a useful reagent for evaluation of the antioxidant activity of compounds. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen-donating ability of the antioxidants. It has been documented that cysteine, glutathione, ascorbic acid, tocopherols, and polyhydroxy aromatic compounds (e.g., ferulic acid, hydroquinone, catechin, gallic acid) reduce and decolorize 1,1-diphenyl-2-picrylhydrazine (DPPH) by their hydrogen-donating capabilities.

Polymers scavenger ability was evaluated in terms of DPPH reduction using GA and CA, as reference compounds, and data are expressed as inhibition (percent). Antioxidant polymers showed high scavenging activity, and the blank polymer did not significantly interferes with the scavenger process (Table 1). In particular, in our experiments a DPPH reduction of 66% was recorded for conjugate **(I)**, while **(II)** fully inhibits radical formation (98%) ($p < 0.01$, one-way ANOVA analysis).

3.3.2 Determination of scavenging activity on hydroxyl radical

Hydroxyl radical exhibits very high reactivity and tends to react with a wide range of molecules found in living cells. They can interact with the purine and pyrimidine bases of DNA. They can also abstract hydrogen atoms from biological molecules (e.g. thiol compounds), leading to the formation of sulphur radicals able to combine with oxygen to generate

oxysulphur radicals, a number of which damage biological molecules[18]. Due to the high reactivity, the radicals have a very short biological half-life. Thus, an effective scavenger must be present at a very high concentration or possess very high reactivity towards these radicals. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is the Fenton reaction, where a transition metal is involved as a prooxidant in the catalyzed decomposition of superoxide and hydrogen peroxide. These radicals are intermediary products of cellular respiration, phagocytic outburst and purine metabolism. Hydroxyl radical can be generated in situ by decomposition of hydrogen peroxide by high redox potential EDTA–Fe^{2+} complex, and in the presence of deoxyribose substrate, it forms thiobarbituric acid-reactive substances (TBARS) which can be measured. Antioxidant activity is detected by decreased TBARS formation, which can come about by donation of hydrogen or electron from the antioxidant to the radical or by direct reaction with it.

Consequently, the ability of gelatin-antioxidants conjugates to scavenge hydroxyl radical was evaluated by the Fenton-mediated deoxyribose assay[19]. Good antioxidant properties were found, with an inhibition values of 64 % for conjugate **(I)** and 76% for **(II)**, as reported in Table 1 ($p < 0.01$, one-way ANOVA analysis).

[18] B. Halliwell, J.M.C. Gutteridge, O.I. Aruoma, Biochem. 165 (1987) 215-219.
[19] O.I. Aruoma, Methods in Enzymology 1994, 233, 57-66.

3.3.3 β-Carotene bleaching test

(I) and **(II)** exhibited significant antioxidant capacity in the β-carotene–linoleic acid test system. In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant, which results in a reduction in absorbance of the test solution with reaction time. This is due to the oxidation of linoleic acid that generates free radicals (lipid hydroperoxides, conjugated dienes and volatile by-products) that attack the highly unsaturated β-carotene molecules, resulting in bleaching of its characteristic yellow colour in ethanolic solution. When this reaction occurs, the β-carotene molecule looses its conjugation and, as a consequence, the characteristic orange colour disappears. The presence of antioxidant avoids the destruction of the β-carotene conjugate system and the orange colour is maintained because oxidation products were scavenged and bleaching was prevented. The analysis of scavenging activity of **(I)** and **(II)** displays an excellent capacity of both polymer to inhibit linoneic acid peroxidation. As reported in the Table 1, the gelatin grafted with GA and CA show a very high efficiency compared to blank polymer ($p < 0.01$, one-way ANOVA analysis).

3.3.4 Evaluation of disposable phenolic equivalent by Folin-Ciocalteu procedure

Because the antioxidant activity of gelatin-antioxidant conjugates derived from phenolic groups, it is useful to express the antioxidant potential in terms of phenolic content.[33] The Folin-Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of disposable phenolic groups present in the polymer chain. Phenolic compounds undergo a complex

redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reactant.

The colour development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complex to form chromogens in which the metals have lower valence. Disposable phenolic groups in the samples were expressed as milligram equivalents of antioxidant moieties bounded. The values were 0.7 mg per grams of dry polymer for **(I)** and 0.9 for **(II)** (Table 1).

Sample	**Inhibition (%)**			**Disposable Phenolic equivalent** *(mg/g polymer)*	**Total Antioxidant Activity** *(mg/g polymer)*
	DPPH Radicals	*Hydroxyl radicals*	*Linoneic acid peroxidation*		
Blank gelatin	**36±2%**	**28±2%**	**43±3%**	/	/
(I)	**66±3%**	**64±3%**	**98±1%**	**0.7±0.1**	**0.5±0.1**
(II)	**98±3%**	**76±3%**	**99±2%**	**0.9±.0.3**	**0.4±0.1**

Table 1. Antioxidant activity of gelatin-antioxidant conjugates

3.3.5 Determination of total antioxidant activity

The assay is based on the reduction of Mo(VI) to Mo(V) by antioxidant species and subsequent formation of a green phosphate-Mo(V) complex at acid pH. The total antioxidant activity was measured and compared with that of free antioxidant and the control, which contained no antioxidant component. The high absorbance values indicated that the samples possessed significant antioxidant activity. According to the results, synthesized materials had significant antioxidant activities, and

the antioxidant equivalent concentrations were found to be 0.4 and 0.5 mg per grams of protein, for conjugates **(I)** and **(II)** respectively, as showed in Table 1.

4. Conclusions

This paper reports on a novel synthetic strategy to realize antioxidant–protein conjugates by grafting reaction employing a hydrogen peroxide-ascorbic acid pair as radical initiator system[20]. In this way two new antioxidant polymers were synthesized, choosing gelatin as biomacromolecule and Gallic acid and Catechin, as antioxidant species. Literature data clearly show that the grafting of gelatin with various chemical species is an effective method to improve the properties of the protein. Covalent bond between protein and antioxidant was confirmed by calorimetric, UV-Vis and fluorescence analyses, while the antioxidant activity of gelatin-antioxidant conjugates was compared to a control, treated in the absence of antioxidant molecule.

The ability of synthesized materials to inhibit 2,2'-diphenyl-1-picrylhydrazyl, hydroxyl radicals and linoneic acid peroxidation was determined and to well characterize antioxidant properties of grafted macromolecules disposable phenolic equivalents and total antioxidant activity were calculated.

The results of all antioxidant assays confirmed that grafting reaction produced a protein bearing antioxidant moieties covalently bounded to the gelatin side-chains and clearly showed that the synthetic strategy allows to improve the properties of the natural polymer, introducing in the

[20] U.G. Spizzirri, F. Iemma, F. Puoci, G. Cirillo, M. Curcio, O.I. Parisi, N. Picci, Biomacromolecules 10 (2009) 1923-1930.

protein new particular features for specific applications in pharmaceutical, cosmetic and food industry.

Chapter 3

COVALENT INSERTION OF ANTIOXIDANT MOLECULES ON CHITOSAN BY FREE RADICALS GRAFTING PROCEDURE

1. Introduction

Chitosan is a copolymer of N-acetyl-d-glucosamine and d-glucosamine obtained by alkaline N-deacetylation of chitin. The sugar backbone consists of β-1,4-linked glucosamine[1]. It has been known as a bioactive molecule. Several bioactivities such as antitumor activity[2] immunoenhancing effects[3], wound healing effects[4], antifungal and antimicrobial properties[5], and antioxidant activity[6] of chitosan have been reported.

These characteristics, togheter with several unique properties such as nontoxicity, biocompatibility, and biodegradability offer chitosan good potentials for biomedical applications and in food industry as edible coatings for fruit and vegetables[7] packaging films[8] and waste water purification[9].

[1] S.Y. Chae, M. Jang, J. Nah J. Control. Release, 102 (2004) 383–394.
[2] H.O. Pae, W.G. Seo, N.Y. Kim, G.S. Oh, G.E. Kim, Y.H. Kim, H.J. Kwak, Y.G. Yun, C.D. Jun, H.T. Chung Leukemia Res. 2001, 25, 339– 346.
[3] M. Maeda, H. Murakami, H. Ohta, M. Tajima, Biosci. Biotech. Biochem. 56 (1992) 427–431.
[4] C. Porporatto, I.D. Bianco, C.M. Riera, S.G. Correa, Biochem. Bioph. Res. Co. 304 (2003) 266–272.
[5] S. Hirano, Biotechnol. Annual Rev. 2 (1996) 237– 258.
[6] M. Yen, J. Yang, J. Mau Carbohyd. Polym. 74 (2008) 840–844.
[7] H.J. Park, Trends Food Sci. Tech. 10 (1999) 254–260.
[8] C. Caner, P.J. Vergano, J.L. Wiles, J. Food Sci. 63 (1998) 1049–1053.
[9] D. Knorr, Food Technol., 45 (1991) 114–122.

It is well known that for some specific polymeric products, especially medical equipment and food packaging, sterilization via radiation is needed with a potential risk of degradation, i.e., chain scission and/or crosslinking, resulting in discoloration, cracking of the surface, stiffening, and loss of mechanical properties[10]. These serious drawbacks could be controlled by performing chemical modifications of the polymeric backbone.

Specifically for chitosan, in order to improve the polymer processability, chemical and enzymatic modification reactions were designed. However, chemical modifications are generally not preferred for food applications because of the formation of potential detrimental products[11].

In addition, several research works report on the applicability of antioxidants as additives for polymers, as they stabilize the polymer from resin extrusion to the molded pieces production. During processing, the antioxidant retards thermal or/and oxidative degradation[12]. On the other hand, antioxidants with low molecular weight are less effective owing to their poor thermal stability. To overcome this limitation, an useful approach is the covalent linkage of these molecules on a polymeric matrix, enhancing their stability and reducing the effects of migration and blooming. These ones can cause antioxidants to be easily removed from the host polymer by mechanical rubbing-off, volatilization or leaching[13].

[10] M.S. Jahan, K.S. McKinny, Nucl. Instrum. Meth. B 151 (1999) 207-212.
[11] S.R. Kanatt, R. Chander, A. Sharma, Food Chem. 106 (2008) 521-528.
[12] W. Pasanphan, G.R. Buettner, S. Chirachanchai J. Appl. Polym. Sci. 109 (2008) 38–46.
[13] T.W. Tseng, Y. Tsai, J.S. Lee Polym. Degrad. Stabil. 58 (1997) 241-245.

In recent years, several synthetic strategies[14] are proposed in order to obtain macromolecular systems, consisting of antioxidants-polymer conjugates, that, combining the advantages of both the components, show higher stability and slower degradation rate that molecules with low molecular weight, but preserve the unique properties of antioxidant molecules. In literature, many studies about the synthesis of chitosan-antioxidant conjugates are reported, but multi-step organic synthesis are required[15,16].

This work reports on a rapid and eco-friendly procedure to perform the covalent insertion of antioxidant molecules on chitosan by employing free-radical grafting procedure.

Our synthetic strategy is based on the use of H_2O_2/ascorbic acid redox pair to functionalize, in a single-step, chitosan with (2R)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-chroman-4-one ((+)-catechin-CA) and 3,4,5-trihydroxy benzoic acid (gallic acid-GA). The use of this redox system allows to perform the chemical functionalization of chitosan without the generation of toxic compounds and with high reaction yields.

The conjugates were characterized by DSC, UV and FT-IR analyses, then their antioxidant properties by performing different antioxidant assays were tested.

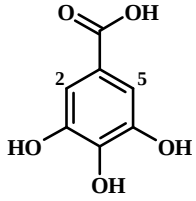
[14] S. Menichetti, C. Viglianisi, F. Liguori, C. Cogliati, L. Boragno, P. Stagnaro, S. Losio, M.C. Sacchi J. Polym. Sci. Pol. Chem. 46 (2008) 6393-6406.
[15] W. Pasanphan, S. Chirachanchai, Carbohyd. Polym. 72 (2008) 169–177.
[16] L. Wu, H.D. Embree, B.M. Balgley, P.J. Smith, G.F. Payne, Environ. Sci. Technol. 36 (2002) 3446-3454.

2. Experimental Section

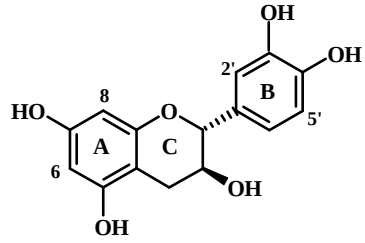
2.1 Materials

Gallic acid, (+)-catechin (**Figure 1**), chitosan from crab shells (MW = 95 kDa, 85% deacetylation), hydrogen peroxide (H_2O_2), ascorbic acid (AA), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), Folin-Ciocalteu reagent, sodium carbonate, sulphuric acid (96% w/w), trisodium phosphate, ammonium molybdate, β-Carotene, linoleic acid, Tween 20, deoxyribose, $FeCl_3$, ethylenediaminetetraacetic acid disodium salt (EDTA), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), hydrochloric acid (37% w/w) were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA).

Ethanol, chloroform were HPLC-grade and provided by Fluka Chemika-Biochemika (Buchs, Switzerland).



GA



CA

Figure 1. Chemical structures of gallic acid (GA) and (+)-catechin (CA).

2.2 Synthesis of Chitosan Conjugates

The synthesis of both catechin-*grafted*-chitosan (CA-*g*-CA) and gallic acid-*grafted*-chitosan (GA-*g*-CA) was performed as follows: in a 25 ml glass tube, chitosan (0.5g) was dissolved in 10 ml of acetic acid water

solution (2% v/v). Then, 1ml of H_2O_2 1.0 M containing 0.054 g of ascorbic acid were added. Finally, after 30 min., 0.35 mmol of antioxidant molecule were introduced in the reaction flask and the mixture was maintained at 25°C for 24 h under atmospheric air. The obtained polymer solution was introduced into dialysis tubes (MWCO: 12-14000 Dalton) and dipped into a glass vessel containing distilled water at 20° C for 48 h with eight changes of water. The copolymer was checked to be free of unreacted antioxidants and any other compounds by HPLC analysis after purification step.

The resulting solution was frozen and dried with "freezing-drying apparatus" to afford a vaporous solid. Blank chitosan (BCH), that act as a control, was prepared in the same conditions but in the absence of antioxidant agents.

2.3 Instrumentation

The liquid chromatography consisted of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 230 nm. A 250mm×4mm C-18 Hibar® Column, particle size 5μm, pore size 80 Å (Merck, Darmstadt, Germany), was employed. As reported in literature[17], the mobile phase adopted for the detection of CA and GA was methanol/water/orthophosphoric acid (20/79.9/0.1) and the flow rate was 1.0 ml/min. The column was operated at 30°C. The sample injection volume was 20 μl. IR spectra were recorded as films or KBr pellets on a Jasco FT-IR 4200. Freeze drier Micro Modulyo, Edwards was employed. UV-Vis absorption spectra were obtained with a Jasco V-530 UV/Vis spectrometer. Calorimetric

[17] H. Wang, K. Helliwell, X. You, *Food Chem. 68* (**2000)** 115-121.

analyses were performed using a Netzsch DSC200 PC. In a standard procedure about 6.0 mg of sample was placed inside a hermetic aluminum pan, and then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25°C to 400°C under a dry nitrogen atmosphere with a flow rate of 25 ml min^{-1} and heating rate 5°C min^{-1}.

2.4 Determination of Scavenging Effect on DPPH Radicals

In order to evaluate the free radical scavenging properties of synthesized GA-*g*-CH and CA-*g*-CH, their reactivity towards a stable free radical, 2,2'-diphenyl-1- picrylhydrazyl radical (DPPH), was evaluated[18]. For this purpose, 20 mg of each polymer were dissolved in 1 ml of distilled water in a volumetric flask (25 ml) and then 4 ml of ethanol and 5ml of ethanol solution of DPPH (200 μM) were added, obtaining a solution of DPPH with a final concentration of 100 μM. The sample was incubated in a water bath at 25 °C and, after 30 minutes, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The same reaction conditions were applied on the BCH in order to evaluate the interference of polymeric material on DPPH assay. The scavenging activity of the tested polymeric materials was measured as the decrease in absorbance of the DPPH and it was expressed as percent inhibition of DPPH radicals calculated according the following equation 1:

$$inhibition\% = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any polymers, and A_1 is the absorbance of

[18] A. Ardestani, R. Yazdanparast, Food Chem. 104 (2007) 21–29.

polymeric samples. Each measurement was carried out in triplicate and data expressed as means (± SEM).

2.5 β-Carotene-Linoleic Acid Assay

The antioxidant properties of synthesized functional polymers was evaluated through measurement of percent inhibition of peroxidation in linoleic acid system by using the the β-carotene bleaching test[19]. Briefly, 1ml of β-carotene solution (0.2 mg/ml in chloroform) was added to 0.02 ml of linoleic acid and 0.2 ml of Tween 20. The mixture was then evaporated at 40 °C for 10 min in a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 ml of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion. The emulsion (5 ml) was transferred to different test tubes containing 50 mg of antioxidants-*grafted*-chitosan dispersed in 0.2 ml of 70% ethanol, and 0.2 ml of 70% ethanol in 5ml of the above emulsion was used as a control. The tubes were then gently shaken and placed in a water bath at 45 °C for 60 min. The absorbance of the filtered samples and control was measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at the initial time (t = 0) and successively at 60 min. The same reaction conditions were applied by using BCH.

The antioxidant activity ($A_{ox}A$) was measured in terms of successful bleaching of β-carotene using the following equation (2):

[19] I. Amin, M.M. Zamaliah, W.F. Chin, Food Chem. 87 (2004) 581–586.

$$A_{ox}A = (1 - \frac{A_0 - A_{60}}{A_0^o - A_{60}^o}) \qquad (2)$$

where A_0 and A_0° are the absorbance values measured at the initial incubation time for samples and control, respectively, while A_{60} and A_{60}^{o} are the absorbance values measured in the samples and in control, respectively, at *t*=60 min. All samples were assayed in triplicate and data expressed as means (± SEM).

2.6 Evaluation of Disposable Phenolic Groups by Folin-Ciocalteu Procedure

Amount of total phenolic equivalents was determined using Folin-Ciocalteu reagent procedure, according to the literature with some modifications[20] *(23)*.

20 mg of GA-*g*-CH and CA-*g*-CH were dissolved in distilled water (6ml) in a volumetric flask. Folin-Ciocalteu reagent (1ml) was added and the contents of flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2%) were added, and then the mixture was allowed to stand for 2 h with intermittent shaking.

The absorbance was measured at 760 nm against a control prepared using the blank polymer under the same reaction conditions. The amount of total phenolic groups in each polymeric materials was expressed as gallic acid and catechin equivalent concentrations, respectively, by using the equations obtained from the calibration curves of each antioxidant. These ones was recorded by employing five different gallic acid and catechin standard solutions. 0.5 ml of each solution were added to the Folin-

[20] Y. Pan, J. Zhu, H. Wang, X. Zhang, Y. Zhang, C. He, X. Ji, H. Li Food Chem. 2007, 103, 913–918.

Ciocalteu system to raise the final concentration of 8.0, 16.0, 24.0, 32.0, and 40.0 μM, respectively. After 2 h, the absorbance of the solutions was measured to record the calibration curve and the correlation coefficient (R^2), slope and intercept of the regression equation obtained were calculated by the method of least square.

2.7 Determination of Total Antioxidant Activity

The total antioxidant activity of polymeric materials was evaluated according to the method reported in literature[21] *(24)*. Briefly, 100 mg of GA-*g*-CH and CA-*g*-CH were mixed with 2.4 ml of reagent solution (0.6 M sulphuric acid, 28 M sodium phosphate and 4 M ammonium molybdate) and 0.6 ml of methanol, then the reaction mixture was incubated at 95°C for 150 min. After cooling to room temperature, the absorbance of the mixture was measured at 695 nm against a control prepared using blank polymer in the same reaction. The total antioxidant activity of each polymeric material was expressed as equivalent concentration of the respective antioxidant molecule.

By using five different GA and CA standard solutions, a calibration curve was recorded. 0.3 ml of each solution were mixed with 1.2 ml of reagent solution to obtain the final concentration of 8.0, 16.0, 24.0, 32.0, and 40.0 μM, respectively. After 150 min incubation, the solutions were analyzed by UV-Vis spectrophotometer and the correlation coefficient (R^2), slope and intercept of the regression equation obtained by the method of least square were calculated.

[21] P. Prieto, M. Pineda, M. Aguilar, Anal. Biochem. 269 (1999) 337–341.

2.8 Determination of Scavenging Effect on Hydroxyl Radical (OH·)

The scavenging effect on hydroxyl radical was evaluated according to the literature[22]. Briefly, 20 mg of CA-*g*-CH and GA-*g*-CH were dispersed in 0.5 ml of 95% ethanol and incubated with 0.5 ml deoxyribose (3.75 mM), 0.5 ml H_2O_2 (1 mM), 0.5 ml $FeCl_3$(100 mM), 0.5 ml EDTA (100 mM) and 0.5 ml ascorbic acid (100 mM) in 2.0 ml potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C. Then samples were filtered and to 1 ml omount of filtrate, 1 ml of TBA (1% w/v) and 1 ml of TCA (2% w/v) were added and the tubes were heated in a boiling water bath for 15 min. The content was cooled and the absorbance of the mixture was read at 535 nm against reagent blank without extract. The antioxidant activity was expressed as a percentage of scavenging activity on hydroxyl radical according to equation (1). All samples were assayed in triplicate and data expressed as means (± SEM).

3. Results and Discussion

3.1 Synthesis of Antioxidants-Chitosan Conjugates

Chitosan was chosen as a polymeric backbone to synthetize two different biomacrolecules-based antioxidant containing the antioxidative groups of catechin and gallic acid, respectively.

The conjugation of the antioxidant moieties on the chitosan chains was performed by free radicals-induced grafting reaction. A biocompatible and water-soluble system, ascorbic acid/hydrogen peroxide pair, was chosen as redox initiator system. The interaction mechanism between the two component of redox pair involves the oxidation of AA by H_2O_2 at

[22] B. Halliwell, J.M.C. Gutteridge, O.I. Aruoma Anal. Biochem. 165 (1987) 215–219.

room temperature with the formation of ascorbate and hydroxyl radicals, that initiate the reaction[23].

Comparing to conventional initiator systems (i.e. azo compounds and peroxides), which require relatively high reaction temperature to ensure their rapid decomposition, redox initiators shows several advantages. First of all, this kind of systems do not generate toxic reaction products; moreover, it is possible to perform the reaction processes at lower temperatures, reducing the risks of antioxidant degradation. The best reaction conditions involve a first step designed for the chitosan activation toward radical reactions, and a second step for the insertion of the antioxidant molecules on the preformed macroradical.

In Figure 2 a possible mechanism of antioxidants insertion onto chitosan is proposed. The hydroxyl radicals, generated by the interaction between redox pair components, attack H-atoms in α-methylene (CH_2) or hydroxyl groups (OH) of the hydroxymethylene group of the chitosan (STEP 1)[24]. In addition, the reactive amino group in chitosan is important in several of the structural modifications targeted because the deprotonated amino group acts as a powerful nucleophile (pKa 6.3) readily reacting with electrophilic reagents[25]. Even in free radical initiated copolymerization, NH_2 groups of chitosan involve in macroradical formation[26]. At those sites, the insertion of the antioxidant molecules can occur (STEP2).

[23] M. Kitagawa, Y. Tokiwa Carbohyd. Polym. 64 (2006) 218–223.
[24] G.A. Mun, Z.S. Nurkeeva, S.A. Dergunov, I.K. Nam T.P. Maimakov, E.M. Shaikhutdinov, S.C. Lee, K. Park, React. Funct. Polym. 68 (2008) 389–395.
[25] T. Chen, G. Kumar, M.T. Harris, P.J. Smith, G. Payne Biotechnol. Bioeng. 70 (2000) 564–573.
[26] K.V.H. Prashanth, R.N. Tharanathan, Carbohyd. Polym. 54 (2003) 343–351.

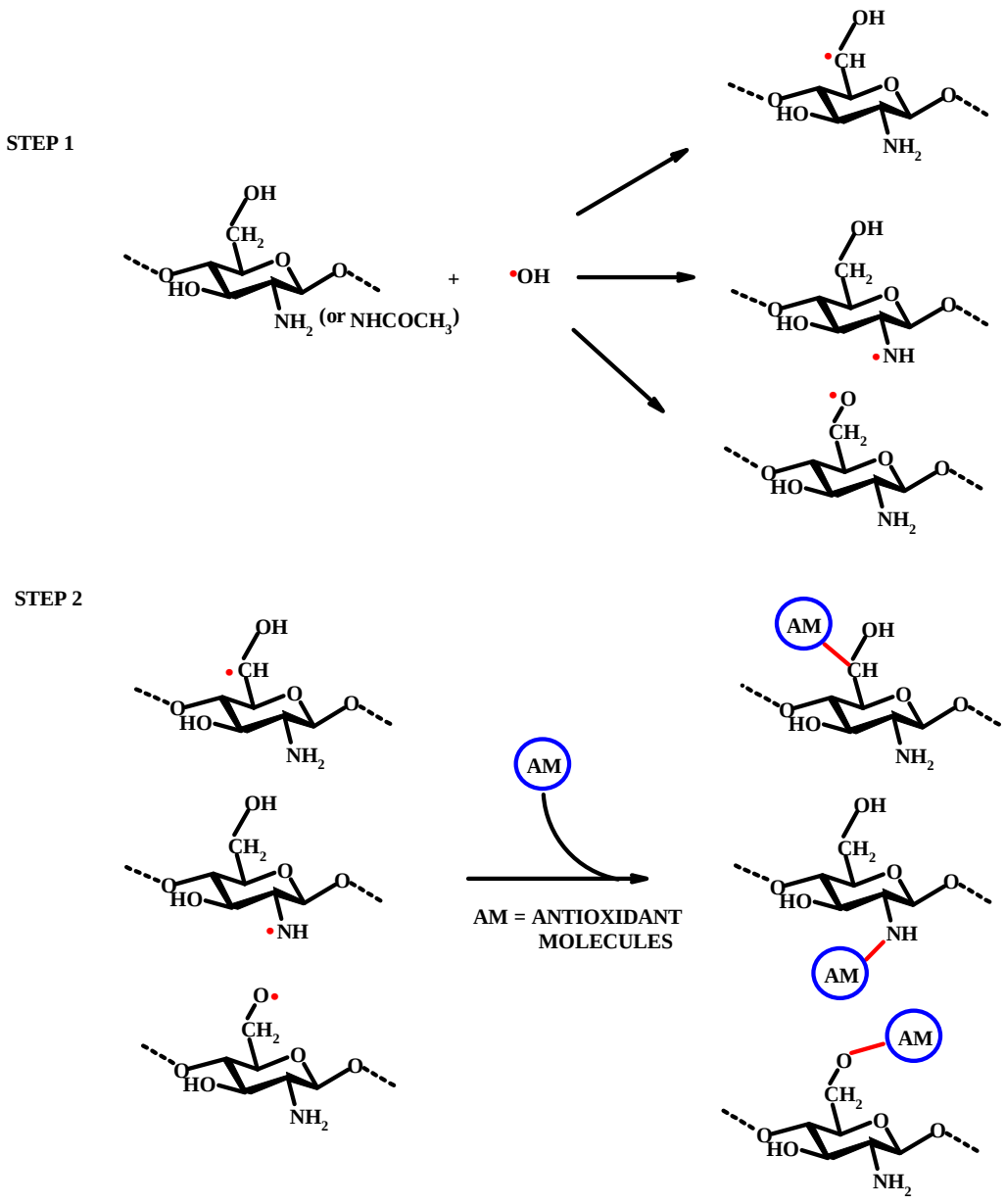


Figure 2. Insertion of antioxidant molecules in chitosan backbone.

On the other hand, in literature, many research works report on the reactivity of phenolic compounds toward this kind of reaction: monomers with active functional groups (phenolic groups) as side substituents, indeed, were used for the preparation grafted polymeric systems[27] using free radical initiators. However, phenolic group could be directly involved in polymerization process; it is reported, indeed, that phenolic radical undergoes in dimerization processes by reaction between hydroxyl radical and aromatic ring in the orto- or para-position relatively to the hydroxyl group[28,29].

On the basis of these data, it can be reasonably hypotesized that the insertion of antioxidants on the chitosan chains occurs in position 2 and 5 of the aromatic ring of gallic acid and in position 2', 5' (B ring) and 6, 8 (A ring) for catechin (Figure 1), respectively. In the reaction feed the amount of antioxidant was 0.7 mmol/g of chitosan for both conjugate systems; this value represents the optimum to obtain a material with the highest efficiency. The yield of grafting procedure was 70% for each conjugate.

3.2 Characterization of Antioxidant-Chitosan Conjugates

In order to verify the covalent insertion of CA and GA into the chitosan chains, the functionalized materials and the respective control polymers were characterized by Fourier Transform IR spectrophotometry, UV and DSC analyses. IR-spectra of both GA-*g*-CH and CA-*g*-CH shown the appearence of new peaks at 1558 cm^{-1} and at 1538 cm^{-1}, respectively,

[27] S. Nanjundan, C.S.J. Selvamalar, R. Jayakumar, Eur. Polym. J. 40 (2004) 2313–2321
[28] S. Kobayashi, H. Higashimura, Prog. Polym. Sci. 28 (2003) 1015–1048.
[29] Uyama, H.; Maruichi, N.; Tonami, H.; Kobayashi, S. Peroxidase-Catalyzed Oxidative Polymerization of Bisphenols. Biomacromolecules 3 (2002) 187-193

awardable to carbon to carbon stretching within the aromatic ring of gallic acid and catechin; moreover, in the GA-*g*-CH IR spectrum, a new peak at 1771 cm^{-1} ascribable to carbon to oxigen stretching within the carbonilic group of gallic acid appeared. A further confirmation of antioxidants insertion in the biopolymer was obtained by comparing UV spectra of each antioxidant molecule (10 μM) and the respective chitosan-conjugates in water (0.6 mg/ml).

As depicted in Figure 3 and in Figure 4, both the UV spectra of GA-*g*-CH and CA-*g*-CH shown the presence of absorption peaks in the aromatic region, that can be related to the presence of GA and CA in the samples. In addition, the absorption is shiftet at higher wavelenghts as a consequence of the extention of the conjugation due to the formation of the covalent bonds between chitosan reactive groups and antioxidant aromatic ring.

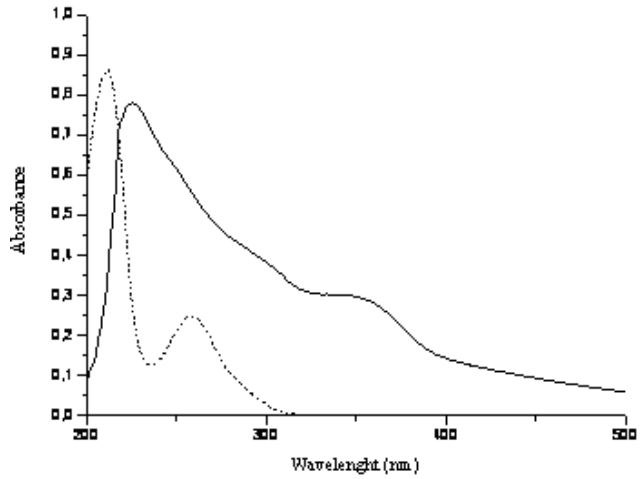


Figure 3. UV-spectrum of gallic acid (---) and GA-g-CH (──)

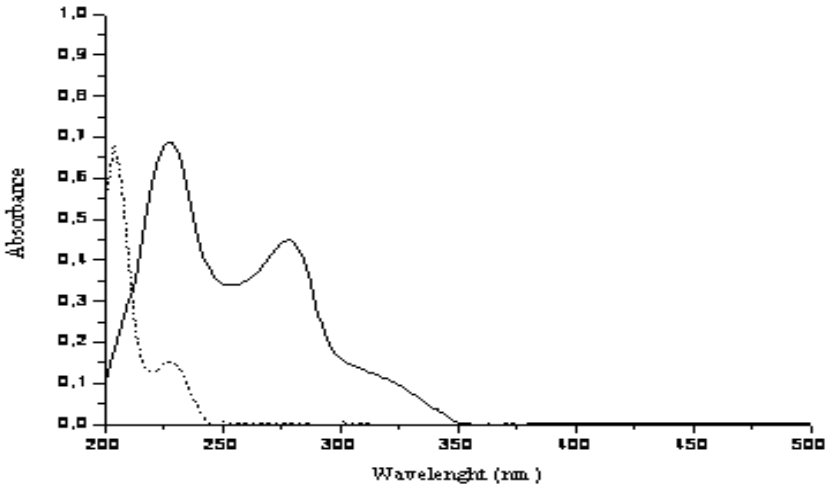


Figure 4. UV-spectrum of catechin (---) and CA-g-CH (──)

Finally, DSC analyses of pure antioxidants, BCH and each chitosan conjugate were performed (Figures 5 and 6).

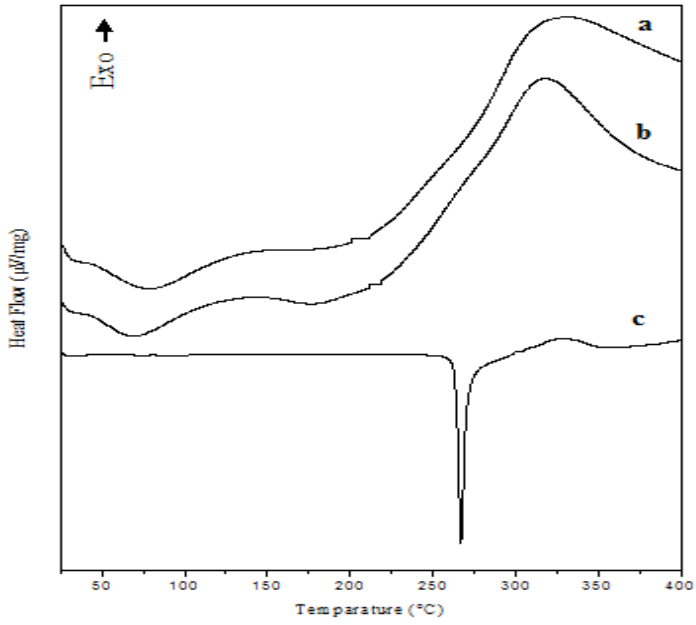


Figure 5. DSC of GA (c), BCH (b) and GA-g-CH (a).

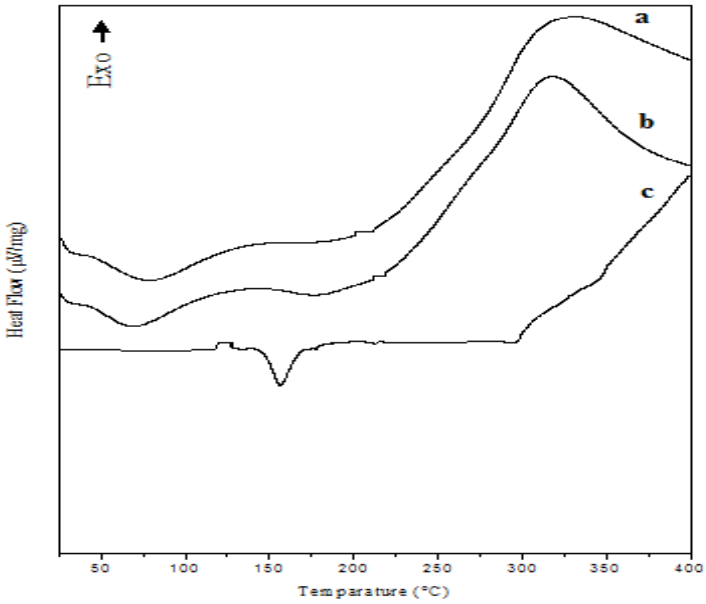


Figure 6. DSC of CA (c), BCH (b) and CA-g-CH (a).

The calorimetric analysis of pure GA shows a sharp melting endotherm at 266.5°C, corresponding to the melting point of antioxidant molecule (Figure 5c), while for pure CA a melting endotherm at 155.8°C was displayed (Figure 6c). As far as DSC of BCH is concerned by (Figures 5b and 6b), a broad endotherm, located around 39–151°C, are clearly visible and have been assigned to the glass transition of polysaccharidic chain; ΔH_t associated to this transition was -195 J/g. DSC thermograms of GA-*g*-CH (Figure 5a) displays the disappearance of melting endotherm of GA and an ΔH_t value (-241 J/g), associated to the polysaccharidic gel transition, higher than that observed in BCH. Similar results were observed in the DSC thermogram of CA-CH conjugate (Figure 6a). Then different thermal behaviours between

BCH and these conjugated systems were observed and can be ascribable to the covalent doping of CH with antioxidant compounds.

3.3 Determination of Scavenging Effect on DPPH Radicals

The DPPH radical is a stable organic free radical with an absorption maximum band around 515-528 nm and thus, it is a useful reagent for evaluation of antioxidant activity of compounds.

In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., ferulic acid, hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1,1-diphenyl-2-picrylhydrazine by their hydrogen donating capabilities. Polymers scavenger ability were evaluated in term of DPPH reduction using, for each synthesized polymer, GA and CA as reference compounds and data are expressed as inhibition (%). As reported in Table 1, in our operating conditions, both chitosan conjugates can totally inhibit DPPH.

SAMPLE	% INHIBITION		
	Linoleic acid peroxidation	**DPPH radical**	**Hydroxyl radical**
BCH	23± 1.2	14± 1.1	17± 1.4
GA-*g*-CH	85± 0.9	92± 1.3	60± 1.1
CA-*g*-CH	98± 0.8	98± 1.1	95± 0.9

Table 1. Inhibition percentages of linoleic acid peroxidation, DPPH radical and Hydroxyl radical by BCH, GA-*g*-CH and CA-*g*-CH.

3.4 β-Carotene-Linoleic Acid Assay

In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant, which results in a reduction in absorbance of the test solution with reaction time. This is due to the oxidation of linoleic acid that generates free radicals (lipid hydroperoxides, conjugated dienes and volatile byproducts) that attacks the highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. When this reaction occurs, the β-carotene molecule loses its conjugation and, as a consequence, the characteristic orange colour disappears. The presence of antioxidant avoids the destruction of the β-carotene conjugate system and the orange colour is maintained. Also in this case, good antioxidant activities for GA-*g*-CH and CA-*g*-CH were recorded, with inhibition percentages of lipidic peroxidation equal to 85% and 98%, respectively (Table 1).

3.5 Evaluation of Disposable Phenolic Groups by Folin-Ciocalteu Procedure

Since the antioxidant activity of GA-*g*-CH and CA-*g*-CH is derived from phenolic groups in the polymeric backbone, it is useful express the antioxidant potential in terms of phenolic content. The Folin–Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of disposable phenolic groups present in polymer chain. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin–Ciocalteu reactant. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valence.

For each biopolymer, disposable phenolic groups were expressed as mg equivalent of the respective functionalizing antioxidant. Particularly, for GA-CH and CA-CH conjugates these values were 4mg/g and 7mg/g of dry polymers, respectively. These different values could be due to the presence, in catechin, of a number of free-radicals reactive sites greater than that existing in gallic acid molecule.

3.6 Determination of Total Antioxidant Activity

The assay is based on the reduction of Mo(VI) to Mo(V) by ferulic acid and subsequent formation of a green phosphate/Mo(V) complex at acid pH. The total antioxidant activity was measured and compared with that of antioxidants and the control chitosan, which contained no antioxidant component. The high absorbance values indicated that the sample possessed significant antioxidant activity.

Synthesized materials had significant antioxidant activities, and gallic acid and catechin mg equivalents in the respective functionalized polymers were found to be 3mg and 5 mg for 1g of dry functional polymers.

3.7 Hydroxyl Radical (OH·) Scavenging Activity

The deoxyribose test has been considered the most suitable means for detecting scavenging properties towards the OH radical.

Hydroxyl radical exhibits very high reactivity and tends to react with a wide range of molecules found in living cells. They can interact with the purine and pyrimidine bases of DNA. They can also abstract hydrogen atoms from biological molecules (e.g. thiol compounds), leading to the formation of sulphur radicals able to combine with oxygen to generate oxysulphur radicals, a number of which damage biological molecule. Due to the high reactivity, the radicals have a very short biological half-life. Thus, an effective scavenger must be present at a very high concentration or possess very high reactivity toward these radicals. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is the Fenton reaction where a transition metal is involved as a prooxidant in the catalyzed decomposition of superoxide and hydrogen peroxide. These radicals are intermediary products of cellular respiration, phagocytic outburst and purine metabolism. Hydroxyl radical can be generated in situ by decomposition of hydrogen peroxide by high redox potential EDTA–Fe^{2+} complex, and in the presence of deoxyribose substrate, it forms thiobarbituric acid-reactive substances (TBARS) which can be measured. Antioxidant activity is detected by decreased TBARS formation, which can come about by donation of

hydrogen or electron from the antioxidant to the radical or by direct reaction with it. Consequently, the ability of the syntesized polymers to scavenge hydroxyl radical was evaluated by the Fenton-mediated deoxyribose assay.

Also this test confirmed the good antioxidant properties of functional materials compared to blank chitosan, with the inhibition percentages of hydroxyl radical by CA-*g*-CH and GA-*g*-CH equal to 95% and 60%, respectively, while the value for BCH was 17% (Table 1).

4. Conclusions

A novel solvent-free synthetic procedure based on the use of water-soluble redox initiators was proposed to covalently bind two antioxidant molecules, catechin and gallic acid, onto chitosan, one of the most widely used natural biopolymer[30]. The rapid reaction kinetics, toghether with the absence of toxic reaction products, make this procedure very useful to exalt the biological properties of chitosan.

Moreover, the high reaction yields, mild reaction conditions, simple setup and workup procedure are additional merits of our protocol.

The covalent insertion of gallic acid and catechin in the polymeric chain was confirmed by UV and FT-IR analyses, while the enhanced thermal stability of the functional materials was demonstred by DSC thermograms.

Finally, antioxidant properties of GA-*g*-CH and CA-*g*-CH were evaluated by performing five different assays. Particularly, determination of

[30] M. Curcio, F. Puoci, F. Iemma, O.I. Parisi, G. Cirillo, U.G. Spizzirri, N. Picci J. Agric. Food Chem. 57 (2009) 5933-5938.

scavenging activity on DPPH radicals and hydroxyl radical, β-carotene-linoleic acid assay, determination of disposable phenolic groups in polymeric matrices and determination of total antioxidant capacity were performed. Good antioxidant properties were recorded in all the tested conditions, confirming that the antioxidant activity of chitosan were strenghtened after its functionalization with the antioxidant molecules.

The obtained results shown the applicability of these materials in food industry as food preservative or nutritional supplements.

CONCLUSIONS

Macromolecular science has had a major impact on the way we live. It is difficult to find an aspect of our lives that is not affected by polymers. Just 50 years ago, materials we now take for granted were non-existent. With further advances in the understanding of polymers, and with new applications being researched, there is no reason to believe that the interest for these materials will stop any time soon. Polymers already have a range of applications that far exceeds that of any other class of material available to man. Current applications extend from adhesives, coatings, foams, and packaging materials to textile and industrial fibers, composites, electronic and biomedical devices.

In the wide view of polymer chemistry, this research contributes to the development of innovative functional polymers with potential applications in biomedical, analytical and food areas. For this purpose, Molecularly Imprinted Polymers, Thermo-Responsive Hydrogels and Polymeric Antioxidants have been synthesized and their applicability as drug delivery devices, stationary phases in analytical separations and additive in pharmaceutical and food industry demonstrated. The design and development of this kind of materials is a challenge that can be expected to yield a new generation of macromolecular systems that can profoundly impact these research fields.