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"Environment, Health and Eco-friendly Processes"

XXIII Cycle

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POLYMERIC MATERIALS FOR BIOMEDICAL APPLICATIONS: SYNTHESIS AND CHARACTERISATION

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PREFACE

In the last years, the development of novel polymeric systems has aroused a growing and considerable interest. Many researchers, indeed, have explored strategies to create polymeric materials that can grant new potential functions for applications in different fields, such as biomedical, pharmaceutical, cosmetic, food (packaging materials and coatings).

The aim of this PhD research project was the preparation of new functional polymers to be employed in these fields, exploring different synthetic strategies.

The PhD thesis is divided into three main sections.

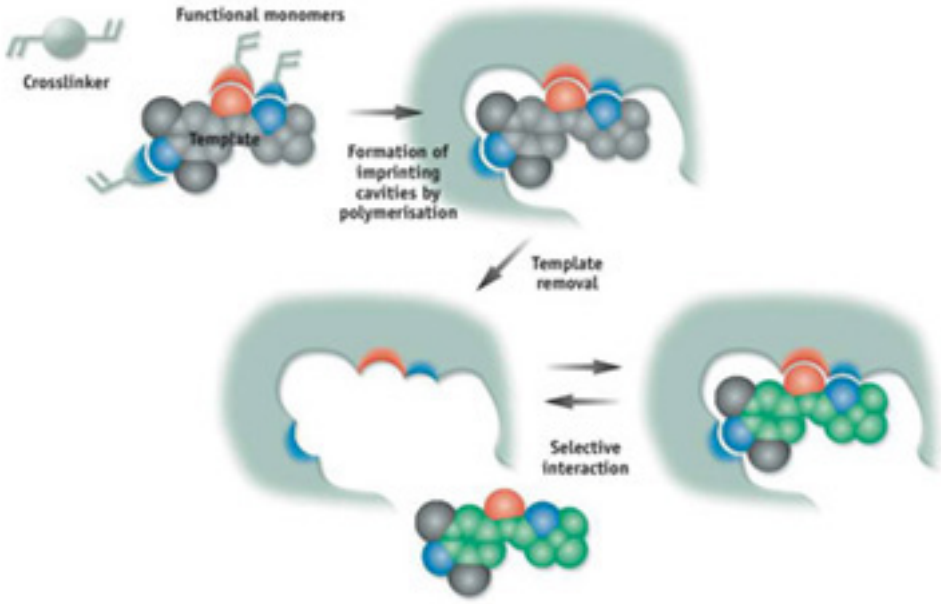
The first one is focused on the synthesis of molecularly imprinted polymers (MIPs) and the evaluation of their applicability as Drug Delivery Systems (DDS) and stationary phases in Solid Phase Extraction (SPE). For this purpose, molecular imprinting technique was adopted to obtain polymeric materials with high selective recognition properties for a target molecule named template.

The second section reports on the synthesis and characterisation of polymeric antioxidants. This class of materials is characterised by higher stability and slower degradation rate than compounds with low molecular weight. To this aim, novel and simple approaches were developed involving the use of water soluble redox initiators or one-pot reaction in presence of 2,2'-azoisobutyronitrile (AIBN) to covalently bind an antioxidant molecule onto a polymeric chain.

The last section of this PhD work was carried out, during a six months period, in the polymer research group at the Department of Pure and Applied Chemistry of University of Strathclyde. The aim of this study was the synthesis of branched polymers with biological activities via *"Strathclyde route"*. This facile, generic and cost effective route involves the conventional free radical polymerisation of vinyl monomers in the presence of chain transfer agents (CTAs) to inhibit cross-linking and gelation processes.

SECTION I

MOLECULARLY IMPRINTED POLYMERS



INTRODUCTION

Molecularly Imprinted Polymers (MIPs) are synthetic materials characterised by recognition sites able to selectively rebind a target molecule in preference to other closely related compounds[1].

MIPs are synthesised by polymerising functional and cross-linking monomers around a template molecule to obtain a highly cross-linked three-dimensional network polymer. The choice of the monomers depends on their ability to establish interactions with the functional groups of the template. Once polymerisation has taken place, the template is extracted to give a porous material containing binding sites that are complementary to the target molecule in terms of size, shape and functionalities (*Figure 1.*).

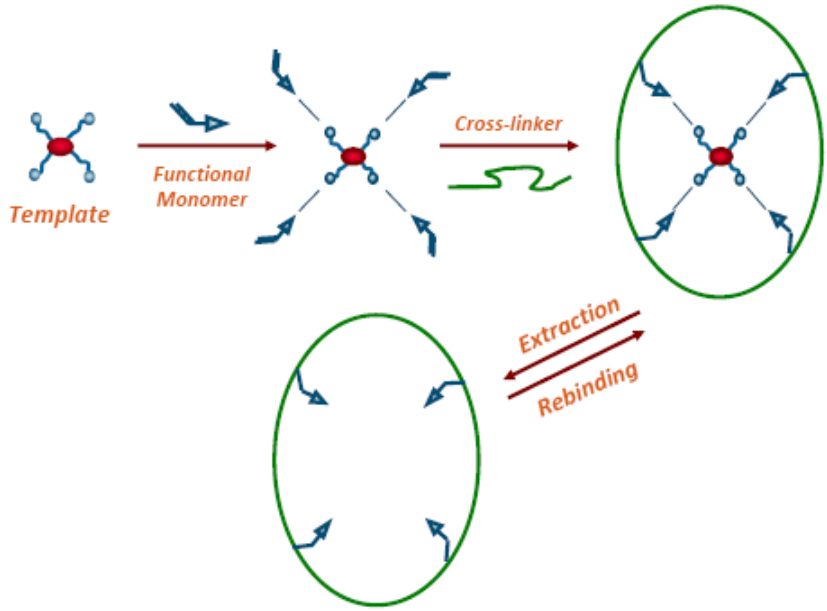


Figure 1. *Schematic representation of the imprinting process.*

The resulting MIPs are stable polymers with molecular recognition abilities and resistant to a wide range of conditions (pH, organic solvents, temperature, pressure).

[1] Turiel, E.; Martín-Esteban, A. *Anal. Chim. Acta* **2010**, *668*, 87.

The behaviour of imprinted polymers emulates the recognition and binding properties of natural biomolecules, such as antibodies and enzymes[2]. MIPs, indeed, can mimic the interactions established by natural receptors to selectively retain a target molecule (i.e. antibody-antigen) but without the associated stability limitations. Furthermore, MIPs synthesis is relatively cheap and easy, making them a clear alternative to the use of natural receptors.

Three different approaches to prepare MIPs have been reported: covalent, non-covalent and semi-covalent approaches. The covalent one[3], involves the formation of reversible covalent bonds between the template molecule and monomers before polymerisation. Then, the template is removed from the polymer structure by cleavage of the corresponding covalent bonds, which are re-formed upon rebinding of the analyte. The high stability of template-monomer interaction leads to a rather homogenous population of binding sites, minimising the existence of non-specific sites. However, the difficulty of designing an appropriate template-monomer complex in which covalent bond formation and cleavage are readily reversible under mild conditions makes this approach rather restrictive. An intermediate option is the semi-covalent approach[4,5]. In this case, the template is also covalently bound to a functional monomer, but the rebinding step is entirely non-covalent in nature. Finally, the non-covalent approach was introduced by Arshady and Mosbach[6], and is based on the formation of relatively weak non-covalent interactions (i.e. hydrogen bonding, ionic interactions) between template molecule and selected monomers before polymerisation. Today it represents the predominant method used, because it offers much more flexibility in terms of the functionalities on a template that can be targeted. The most obvious strength of this approach is that the experimental procedure is rather simple and it is generally assumed that a pre-polymerisation complex is formed between the template and functional

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[3] Wulff, G.; Sarchan, A.A. *Angew. Chem. Int. Ed. Engl.* **1972**, *11*, 341.
[4] Sellergren, B.; Andersson, L.I. *J. Org. Chem.* **1990**, *55*, 3381.
[5] Withcombe, M.J.; Rodriguez, M.E.; Villar, P.; Vulfson, E. *J. Am. Chem. Soc.* **1995**, *117*, 7105.
[6] Arshady, R.; Mosbach, K. *Makromol. Chem.* **1981**, *182*, 687.

monomers, which is then incorporated into the growing polymer network. The other great strength is the available wide range of functional monomers able to interact with almost any kind of template. However, there are some significant drawbacks derived from the fact that the template-monomer interactions are governed by an equilibrium process. Thus, in order to displace the equilibrium towards the formation of the template-monomer complex, a high amount of monomer is used. Consequently, the excess of free monomers is randomly incorporated to the polymeric matrix leading to the formation of non-selective binding sites which are indesiderable for most practical applications

MIPs were used for several different applications, such as chromatographic stationary phases[7], enantiomeric separation[8], solid-phase extraction (SPE)[9], and catalysis[10]; MIPs were also used as receptors[11], antibodies[12], enzyme mimics[13], and affinity and sensing materials[14]. In addition, in recent years, MIPs have been reported to be suitable as drug delivery systems (DDS)[15], and as base excipients for controlled release devices of several drugs[16].

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[8] Adbo, K.; Nicholls, I.A. *Anal. Chim. Acta* **2001**, *435*, 115.
[9] Puoci, F.; Curcio, M.; Cirillo, G.; Iemma, F.; Spizzirri, U.G.; Picci, N. *Food Chemistry* **2008**, *106*, 836.
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CHAPTER I

Molecularly Imprinted Polymers for α-Tocopherol Delivery

1. Introduction

In the last decade, many epidemiological, biological and clinical studies have proven that free-radical-induced oxidative damage to cell membranes, DNA, and proteins might play a causative role in several degenerative diseases, such as cancer, atherosclerosis, and other chronic diseases[1,2,3,4,5]. Reactive oxygen and nitrogen species (ROS/RNS) are essential to energy supply, detoxification, chemical signalling, and immune function[6]. These species are continuously produced in the human body, and their concentrations are controlled by endogenous enzymes (superoxide dismutase, glutathione peroxidase, and catalase). When there is an over-production of ROS and RNS, an exposure to external oxidant substances, or a failure in the defence mechanisms, biomolecular damage may occur[7]. Some compounds, such as *α*-tocopherol[8], L-ascorbic acid[9], and *β*-carotene[10], generally named antioxidants, might have beneficial effects in protecting human tissue against such damage.

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[1] Barnham, K.J.; Masters, C.L.; Bush, A.I. *Nature Rev. Drug Discov.* **2004**, *3*, 205.
[2] Cooke, M.S.; Evans, M.D.; Dizdaroglu, M.; Lunec, J. *FASEB J.* **2003**, *17*, 1195.
[3] Finkel, T.; Holbrook, N.J. *Nature* **2000**, *408*, 239.
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[9] Jayathilakan, K.; Sharma, G.K.; Radhakrishna, K.;Bawa, A.S. *Food Chem.* **2007**, *100*, 662.
[10] Bairati, I.; Meyer, F.; Jobin, E.; Gelinas, M.; Fortin, A.; Nabid, A.; Brochet, F.; Tetù, B. *Int. J. Cancer* **2006**, *119*, 2221.

Our interest focused on α-tocopherol (α-TP, *Figure 1.1*), a representative oil-soluble antioxidant of the vitamin E type[11]. The physiological relevance of α-TP, and the severe pathological consequences of its deficiency, impose a major challenge to the living organisms for sustaining an adequate supply of this compound to different tissues, particularly those highly sensitive to α-TP deficiency such as the brain and gonads[12]. α-TP deficiency could be overcome by dietary supplementation, and several studies have described the beneficial effect of oral supplementation with vitamin E on the prevention and treatment of cardiovascular diseases and cancer[13].

The goal of this work is the preparation of a new controlled drug delivery device for α-TP oral supplementation[14].

A controlled drug delivery system maximises the drug efficacy and safety and provides a suitable rate of delivery of the therapeutic dose, at the most appropriate site in the body. This would prolong the duration of the drug activity, to reduce side-effects, and minimise administration frequency, thus enhancing patient compliance[15,16].

Our approach for the realisation of α-TP delivery system is based on molecularly imprinted technology and the non-covalent approach was employed[17]. This method involves the pre-organisation of functional monomers around a template molecule to form a pre-polymerisation complex by hydrogen bonding, ionic, or hydrophobic interactions. The formed complex is subsequently radically copolymerised in a solution containing a high ratio of a suitable cross-linker to form, after the subsequent removal of the template, macroporous matrices having microcavities that retain specific orientation of

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[11] Qian, J.; Morley, S.; Wilson, K.; Nava, P.; Atkinson, J.; Manor, D. *J. Lipid Res.* **2005**, *46*, 2072.
[12] Mardones, P.; Rigotti, A. *J. Nutr. Biochem.* **2004**, *15*, 252.
[13] Morris, M.C.; Evans, D.A.; Tangney, C.C.; Bieinias, J.L.; Wilson, R.S.; Aggarwal, N.T.; Scherr, P.A. *Am. J. Clin. Nutr.* **2005**, *81*, 508.
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[15] Cunliffe, D.; Kirby, A.; Alexander, C. *Adv. Drug Del. Rev.* **2005**, *57*, 1836.
[16] Alvarez-Lorenzo, C.; Concheiro, A. *J. Chromatogr.* **2004**, *804*, 231.
[17] Caro, E.; Marce, R.M.; Cormack, P.A.G.; Sherrington, D.C.; Borrull, F. *Anal. Chim. Acta* **2006**, *562*, 145.

functional groups in a three-dimensional structure complementary to that of the template about which they were formed[18].

The aim of this study was the preparation of molecularly imprinted polymers able to selectively rebind and release *α*-TP in gastrointestinal simulating fluids. MIPs were synthesised using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as cross-linking agent[19]. The recognition properties of the synthesised materials were tested both in organic (*i.e.*, acetonitrile) and in aqueous media (*i.e.*, an ethanol/water 6/4, v/v mixture). Considerable differences in the recognition characteristics between imprinted and non-imprinted polymers have been observed. The selectivity of the polymeric device was also evaluated using a molecule structurally similar to the template, in particular 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (HMCA) was employed (*Figure 1.1*). Finally the release profile of *α*-TP in gastrointestinal simulating fluids was evaluated.

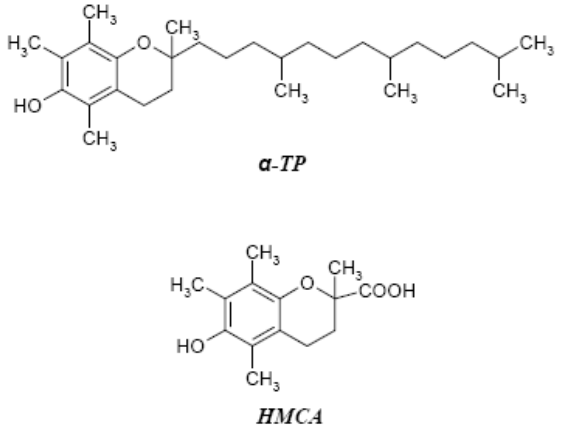

Figure 1.1 *Chemical structures of α-tocopherol (α-TP) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (HMCA).*

[18] Caro, E.; Marce, R.M.; Cormack, P.A.G.; Sherrington, D.C.; Borrull, F. *J. Sep. Sci.* **2006**, *29*, 1230.
[19] Puoci, F.; Cirillo, G.; Curcio, M.; Iemma, F.; Spizzirri, U.G.; Picci, N. *Anal. Chim. Acta* **2007**, *593*, 164.

2. Materials and methods

2.1 Materials

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2,2'-azoisobutyronitrile (AIBN), *α*-tocopherol (*α*-TP), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (HMCA) were obtained from Sigma Chemical Co. (St. Louis, MO).

All solvents were reagent grade or HPLC-grade, they were used without further purification, and they were provided by Fluka Chemie (Buchs, Switzerland).

2.2 HPLC analysis

The liquid chromatography consisted of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 292 nm. A 250×4 mm C-18 Hibar® column, particle size 10 μm (Merck, Darmstadt, Germany) was used. The mobile phase was methanol and the flow rate was 1.0 mL/min.

2.3 Synthesis of α-tocopherol imprinted polymers

α-TP imprinted polymers (MIPs) were prepared using MAA as functional monomer. Briefly, 0.43 g (1 mmol) of template *α*- tocopherol and 1.38 g (16 mmol) of MAA were dissolved in 5.25 mL of chloroform in a thick walled glass tube, and then 4.95 g (25 mmol) of EGDMA and 0.100 g (0.60 mmol) of AIBN were added. The tube was purged with nitrogen, sonicated for 10 min, and then photo-polymerised for 24 h with 360 nm light at 4°C. After the photolysis, the tubes were incubated at 60°C for 24 h. The resultant bulk rigid polymers were crushed, ground into powder, and strained through a 63 nm stainless steel sieve. The sieved MIP materials were collected and the very fine powder, suspended in the supernatant solution (acetone), was discarded. The resultant MIPs were placed in a Soxhlet and extracted with 200 mL of a tetrahydrofuran:acetic acid (8:2 v/v) mixture for at least 48 h, followed by 200 mL of tetrahydrofuran for others 48 h and then they were dried in an oven at 60°C overnight. The washed MIP materials were checked to be free of *α*-

tocopherol and any other compound by high-performance liquid chromatography (HPLC).

Non-imprinted polymers (NIPs), to act as a control, were also prepared when polymerisation was carried out in the absence of *α*-tocopherol.

2.4 Binding experiments

The binding efficiency of polymeric matrices toward *α*-TP was evaluated in the rebinding experiments, which were performed both in acetonitrile and in an ethanol:water mixture (6:4 v/v). Briefly, 50 mg of polymer particles were mixed with 1 mL of an *α*-TP solution (0.2 mM) in a 1 mL eppendorf and sealed. The eppendorf tubes 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 *α*-TP concentration in the liquid phase was measured by HPLC. The amount of *α*-TP bound to the polymer was obtained by comparing its concentration in the MIPs samples to the NIPs samples.

The same experiments were performed using HMCA solutions.

Experiments were repeated five times, and results were expressed as means (± SD).

2.5 Drug loading by the soaking procedure

Two grams of polymeric matrix were immersed in 20 mL of an *α*-TP solution (5.5 mM) in acetonitrile and soaked for 3 days at room temperature. During this time, the mixture was continuously stirred, and then the solvent was removed. Finally, the powder was dried under vacuum overnight at 40°C.

2.6 In vitro release studies

Release studies were done using the dissolution method described in the USP XXIV (apparatus 1-basket stirring element). To mimic the pH of the digestive tract, 0.1 N HCl (pH 1.0) was used as simulated gastric fluid, and after 2 h, disodium hydrogen phosphate (0.4 M) was added to adjust the pH value to

6.8 to simulate the intestinal fluid. To improve the solubility of released α-TP in the simulated fluid, each testing sample contained 0.1% of sodium dodecylsulfate (SDS).

The experiments were performed as follows: 30 mg of MIP and NIP particles loaded with α-TP were dispersed in flasks containing 10 mL of 0.1 N HCl and maintained at 37 ± 0.5°C in a water bath for 2 h under magnetic stirring (50 rpm). Disodium hydrogen phosphate (0.4 M, 5 mL) was then added to the samples. These conditions were maintained throughout the experiment. To characterise the drug release, 2 mL of samples were drawn from the dissolution medium at designated time intervals, and the same volume of simulated fluid was supplemented. α-TP was determined by HPLC analysis, and the amount of α-TP released from five samples of each formulation was used to characterise the drug release.

The percentage of released α-TP was calculated considering 100% the α-TP content in polymeric samples after drying procedure[20].

Experiments were repeated five times and were expressed as means (± SD).

3. Results and discussion

3.1 Synthesis of α-tocopherol imprinted polymers

For the preparation of molecularly imprinted polymers, generally three different approaches have been developed: the covalent, the semi-covalent, and the non-covalent approach.

The first one, pioneered by Wulff's group[21], involves the formation of a complex between functional monomers and template molecules via reversible covalent bond (such as boronate ester, ketal and acetal, or Schiff base) both prior to polymerisation and in the rebinding experiments.

[20] Pitarresi, G.; Pierro, G.; Giammona, G.; Iemma, F.; Muzzalupo, R.; Picci, N. *Biomaterials* **2004**, *25*, 4333.
[21] Wulff, G. *Chem. Rev.* **2002**, *102*, 1.

In the semi-covalent approach, covalent interactions are involved in MIP synthesis, while the subsequent rebinding phase is based on non-covalent interactions.

The non-covalent approach, pioneered and extensively developed by Mosbach and coworkers[22], instead, involves only non covalent interactions (such as hydrogen bonds, ionic interactions, hydrophobic interactions, and metalion chelating interactions) for both the molecular imprinting process and the subsequent rebinding. This approach is the most flexible because of the absence of complicated synthetic processes and the subsequent possibility of using a far greater variety of functional monomers.

For these reasons, we chose the non-covalent imprinting method for the preparation of bulk imprinted polymers for the sustained/controlled release of α-tocopherol. MAA was used as functional monomer and EGDMA as cross-linker.

The whole of the reaction conditions have to maximise the interactions between the template and the functional monomer and consequently to ensure strong and selective binding of the substrate to the polymeric matrices. Two main parameters must be considered. The first is the polymerisation temperature. The formation of the complex is a dynamic process and, when a template with few functional groups able to create hydrogen bonds is used, a low temperature is needed to reduce the kinetic energy of the system. In this case, indeed, a high temperature could drive the equilibrium away from the template-functional monomer complex toward the unassociated species, resulting in a decrease in the number of imprinted cavities and thus in the recognition properties of the final materials. Better results were obtained using photopolymerisation processes. Furthermore, in these conditions, a lesser degree of polymerisation was observed, so the performance of photopolymerised materials must be improved after high-temperature treatment of the initially formed polymer[23]. Based on

[22] Zhang, H.; Ye, L.; Mosbach, K. *J. Mol. Recognit.* **2006**, *19*, 248.
[23] Cheong, S.H.; McNiven, S.; Rachkov, A.; Levi, R.; Yano, K.; Karube, I. *Anal. Chem.* **1997**, *69*, 2017.

these consideration, MIPs were synthesised under UV irradiation at 4°C for 24 h and then with thermal stabilization at 60°C for another 24 h.

The second parameter to be considered is the nature of the porogenic agent. The inert solvent used in the polymerisation mixture may play a major role in determining the properties (surface area, internal pore volume, *etc*.) of the resulting polymer. Moreover, since polar solvents are more able to solvate polar molecules, this leads to the disruption of H-bonds between the template and the functional monomer[24,25]. Thus, our general procedure, employed to improve the recognition properties of molecularly imprinted polymers, was the choice of the least polar solvent in which the reagents were dissolved. In our case, we chose chloroform.

3.2 Recognition properties of polymers

Evaluation of the capacity of the matrices to recognise and bind the template was performed in acetonitrile (organic medium), and also in ethanol/water mixture (6/4 v/v).

Polymeric particles were incubated with an α-TP solution 0.2 mM for 24 hours. *Table 1.1* shows the percentage of α-TP bound by imprinted and non-imprinted polymers.

It should be noted that the synthesised materials have good recognition properties. The imprinted polymers, indeed, bind much more template than do their respective non-imprinted counterparts, confirming the presence of α-TP specific cavities. The higher amounts of bound α-TP in aqueous medium, compared with that in acetonitrile, are ascribable to the presence of more hydrophobically driven bonds, the extent of which depends on the hydrophobicity of the template and the surface of the material. But also in this case, MIPs samples bind much more α-TP than NIPs ones.

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[24] Whitcombe, M.J.; Rodriguez, M.E.; Villar, P.; Vulfson, E.N. *J. Am. Chem. Soc.* **1995**, *117*, 7105.

[25] Cheong, S.H:; McNiven, S.; Rachkov, A.; Levi, R.; Yano, K.; Karube, I. *Macromolecules* **1997**, *30*, 1317.

Table 1.1 *Percentage of bound α-TP and HMCA by MIP and NIP in organic (CH_3CN) and aqueous ($EtOH/H_2O$ 6/4 v/v mixture) media.*

POLYMER	Bound α-TP (%)		Bound HMCA (%)	
	CH_3CN	$EtOH/H_2O$	CH_3CN	$EtOH/H_2O$
MIP	27 ± 0.9	99 ± 0.7	9 ± 1.8	44 ± 0.6
NIP	1 ± 0.7	67 ± 1.2	14 ± 1.6	44 ± 1.1

Well imprinted polymers are asked to interact selectively with the template around which they are formed, compared with some structurally analogous molecules. The selectivity of the imprinted polymers was tested by performing the same binding experiments using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (HMCA) instead of *α*-TP (*Table 1.1*). The chemical difference between the two analytes drives the interactions with the polymeric matrices. The carboxylic group of HMCA makes this molecule much more hydrophilic than *α*-TP.

It should be noted that the polymers practically do not interact with HMCA in organic media, whereas in water solution the binding percentage of the analog is higher but considerably lower than that of the original template. Furthermore, in all the environments tested, the amounts of HMCA bound by the imprinted and the non-imprinted polymers are practically the same, and this clearly shows the non-specific nature of these interactions.

3.3 In vitro α-TP releasing properties

The possibility of employing the synthesised MIPs as devices for the controlled release of *α*-TP in gastrointestinal simulated fluids was investigated. In vitro release studies were performed by immersing aliquots of the microparticles loaded with *α*-TP at pH 1.0 (simulated gastric fluid) for 2 hours and then at pH 6.8 (simulated intestinal fluid) using the pH change method. To

improve the solubility of α-TP in aqueous media, SDS was added to the solutions.

Our hypothesis was that α-TP imprinted polymers have a better ability to control the drug release in compared with non-imprinted polymers due to the presence of specific binding sites in the polymeric network that are able to release the drug much more slowly. The experimental data confirm this hypothesis; the drug release from NIPs was indeed remarkably faster than that observed from MIPs. In particular, while in the first case the drug is completely released within 4 hours, only 50% of α-TP was released from MIP samples during the same period (*Figure 1.2*).

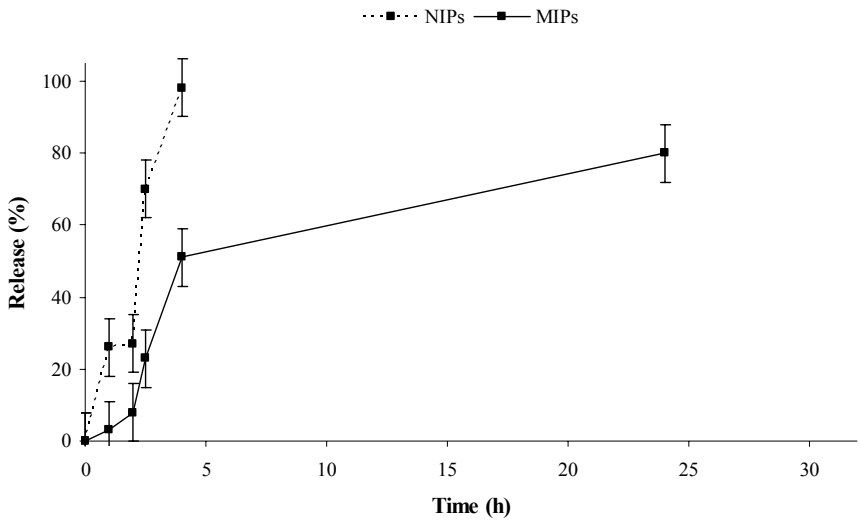

Figure 1.2 *Release profile of α-TP from MIPs and NIPs in gastrointestinal simulating fluids.*

After the pH changing, in intestinal simulating fluids, α-TP release from MIPs continues, and in 24 hours the percentage of α- TP released was about 80% (100% within 40 h). These remarkable differences depend on the different recognition properties of the two polymeric matrices. The non-imprinted polymers, indeed, do not have specific binding cavities for the drug, while the MIP samples, because of their specific structure, strongly bound α-TP by non-

covalent interactions in the cavities formed during the polymerisation procedure in the presence of the analyte.

This observation supports a model of retention mechanism, which assumes that the acid groups of the selective sites have stronger interaction with the drug than the non-selective sites. At low pH (1.0) values, the carboxylic groups are not ionised and there is a good interaction with the template. These results might help us to understand the behavior of these matrices when the pH increases. Under these conditions, that simulate the intestinal fluid, in the non-imprinted polymers the antioxidant is bound with non-covalent interactions on the surface of the matrices. At pH 6.8, the diffusion rate of the buffer on the polymer surface is fast, the carboxylic groups are ionised, and the drug is rapidly released. Instead, in the MIP case, the diffusion rate of the buffer into specific cavities of imprinted polymers is slower, and the functional groups are ionised more slowly, resulted in well controlled release.

For these reasons, the rate of the release was considerably different, and MIPs represent a very useful polymeric device for the selective and controlled release of the antioxidant agent in gastrointestinal fluids.

4. Conclusions

The aim of this work was the preparation of a specific oral delivery system for α-TP. To this end, we used molecular imprinting tecnique.

The polymerisation conditions involve the use of MAA and EGDMA as functional monomer and crosslinking agent, respectively. To improve the recognition properties of the synthesised materials, a photopolymerisation procedure was employed. Before evaluating of the α-TP release profile, the recognition and selectivity properties of the polymeric devices, both in organic and in aqueous media, were studied performing rebinding experiments. The percentage of α-TP bounded by the molecularly imprinted polymers was remarkably higher in both the environments tested compared with the non-imprinted ones, and MIPs also showed a high selectivity.

In vitro release tests were performed in gastrointestinal simulating fluids. MIPs samples was found to be able to release slowly the drug compared with NIPs, thus the synthesised polymeric matrices represent a very useful novel controlled drug dosage device.

CHAPTER II

Imprinted Hydrophilic Nanospheres as Drug Delivery Systems for 5-Fluorouracil Sustained Release

1. Introduction

5-Fluorouracil (5-FU; *Figure 2.1*) is a widely used antineoplastic agent for the treatment of many types of cancers, such 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].

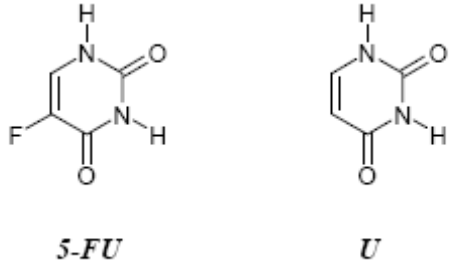

Figure 2.1 *Chemical structures of 5-fluorouracil (5-FU) and uracil (U).*

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

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

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 polymerisation 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 synthesised applying straightforward method such as precipitation polymerisation[6].

Coupling the properties of hydrogels, nanospheres and MIPs 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 minimises potential irritation to surrounding membranes and tissues[7]. 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 the template molecule[8,9] and, as reported in literature[10], these

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[3] Johnson, K.R.; Young, K.K.; Fan, W. *Clin. Cancer Re.* **1999**, *5*, 2559.
[4] Fournier, E.; Passirani, C.; Colin, N.; Breton, P.; Sagodira, S.; Benoit, J.P. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 189.
[5] Puoci, F.; Iemma, F.; Cirillo, G.; Picci, N.; Matricardi, P.; Alhaique, F. *Molecules* **2007**, *12*, 805.
[6] Cirillo, G.; Iemma, F.; Puoci, F.; Parisi, O.I.; Curcio, M.; Spizzirri, U.G.; Picci, N. *J. Drug Target.* **2009**, *17*, 72.
[7] Byrne, M.E.; Park, K.; Peppas, N.A. *Adv. Drug Deliv. Rev.* **2002**, *54*, 149.
[8] Puoci, F.; Curcio, M.; Cirillo, G.; Iemma, F.; Spizzirri, U.G.; Picci, N. *Food Chem.* **2008**, *106*, 836.
[9] Beltran, A.; Caro, E.; Marcé, R.M.; Cormack, P.A.G.; Sherrington, D.C.; Borrull, F. *Anal. Chim. Acta* **2007**, *597*, 6.
[10] Sellergren, B.; Allender, C.J. *Adv. Drug Deliv. Rev.* **2005**, *57*, 1733.

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 maximise tumor cell killing effect during the tumor growth phase, and to protect the surrounding healthy cells from unwanted exposure to the excess cytotoxic agent[11]. Polymeric nanoparticles are the most attractive colloidal carriers owing several merits such as ease of purification and sterilisation, drug targeting possibility, and sustained release action[12].

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 minimise the administration frequency, and thus enhancing patient compliance[13].

The purpose of this 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. Materials and methods

2.1 Materials

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).

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[11] Yadav, A.K.; Mishra, P.; Jain, S.; Mishra, P.; Mishra, A.K.; Agrawal, G.P. *J. Drug Target* **2008**, *16*, 464.
[12] Allemann, E.; Gurny, R.; Doelker, E. *Eur. J. Pharm. Biopharm.* **1993**, *39*, 173.
[13] Hilt, J.Z.; Byrne, M.E. *Adv. Drug Deliv. Rev.* **2004**, *56*, 1599.

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 Synthesis of 5-FU spherical molecularly imprinted polymers

Spherical molecularly imprinted polymers (SMIP) were prepared by precipitation polymerisation using 5-fluorouracil as template, MAA as functional monomer and ethylene glycol dimethacrylate as crosslinking agent.

General synthetic procedure was reported: template (1 mmol) and MAA (8 mmol) were dissolved in a mixture of acetonitrile (20 mL) and methanol (20 mL), in a 100 mL round bottom flask and then EGDMA (10 mmol) and AIBN (50 mg) were added. The polymerisation 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 48 h, followed by methanol for another 48 h, and monitoring the drug concentration in the extraction solvent by HPLC. Particles were successively dried under vacuum overnight at 40 °C.

Spherical non-imprinted polymers (SNIP), that act as a control, were also prepared when polymerisation 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 equation (1) :

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

where W_s and W_d 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 of a 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 released drug by HPLC. The amount of 5-fluorouracil released from six samples of each formulation was used to characterise the 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 a Jasco UVDEC-100-V detector set at 266 nm[14] was used. A 250 × 4 mm C-18 Hibar® column, 10 μm particle size (Merck, Darmstadt, Germany) was employed. The mobile phase was methanol/phosphate buffer 5 mM, 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.

[14] Fournier, E.; Passirani, C.; Colin, N.; Breton, P.; Sagodira, S.; Benoit, J.P. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 189.

3. Results and discussion

3.1 Synthesis of imprinted nanospheres

5-Fluoruracil molecularly imprinted polymers were synthesised employing the non-covalent approach[15], 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 synthesised by bulk polymerisation methods in a porogenic solvent and block co-polymer particles with the desired size are obtained by grinding and sieving[16,17]. 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 polymerisation in water[18], dispersion polymerisation[19], liquid perfluorocarbon[20], and via aqueous two-step swelling polymerisation[21]. However, during the polymerisation 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 the integrity and stability of recognition sites are compromised[22]. Moreover, spherical shape

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[15] Puoci, F.; Cirillo, G.; Curcio, M.; Iemma, F.; Spizzirri, U.G.; Picci, N. *Anal. Chim. Acta* **2007**, *593*, 164.
[16] Caro, E.; Marcé, R.M.; Borrull, F.; Cormack, P.A.G.; Sherrington, D.C. *Trends Anal. Chem.* **2006**, *25*, 143.
[17] Puoci, F.; Cirillo, G.; Curcio, M.; Iemma, F.; Parisi, O.I.; Castiglione, M.; Picci, N. *Drug Deliv.* **2008**, *15*, 253.
[18] Lai, J.P.; Lu, X.Y.; Lu, C.Y.; Ju, H.F.; He, X.W. *Anal. Chim. Acta* **2001**, *442*, 05.
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[20] Mayes, A.G.; Mosbach, K. *anal. Chem.* **1996**, *68*, 769.
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[22] Wei, S.; Molinelli, A.; Mizaikoff, B. *Biosens. Bioelectr.* **2006**, *21*, 1943.

should be advisable in order to avoid swelling anisotropic behaviour associated with other geometries[23].

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 2.2*) and dimensional analysis. Polymerisation feeds composition and mean particle sizes (d_n) of the microspheres are shown in *Table 2.1*.

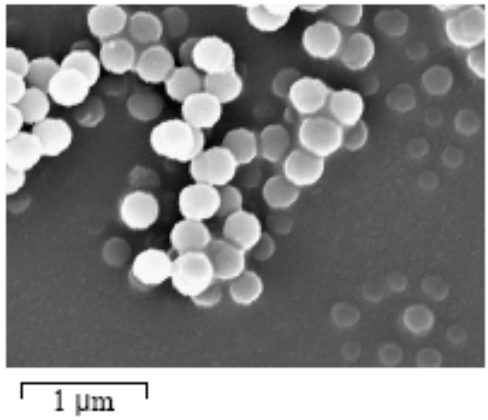

Figure 2.2 *Scanning electron micrograph of SMIP.*

Table 2.1 *Polymerisation feed composition and mean particle sizes (d_n) of nanospheres as means ± SD.*

POLYMER	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

[23] Puoci, F.; Iemma, F.; Spizzirri, U.G.; Cirillo, G.; Castiglione, M.; Picci, N. *Macromol. Ind. J.* **2007**, *3*, 64.

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

The imprinting effect of synthesised materials was evaluated by binding experiments in which amounts of polymeric particles were incubated with a 5-FU solution (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 2.2*, 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.

Table 2.2 *Percentages of bound 5-FU and U and water content (%) at pH 7.4. Data are shown as means ± SD.*

MATRIX	% 5-FU bound		% U 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

In literature, different approaches were applied to make a quantitative determination of the imprinting effect[24]. 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 by SMIP and SNIP[25]; 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 2.1*). As reported for 5-FU, α_U was also determined as ratio between U

[24] Ye, L.; Mosbach, K. *Chem. Mater.* **2008**, *20*, 859.
[25] Gore, M.A.; Karmalkar, R.N.; Kulkarni, M.G. *J. Chromatogr. B* **2004**, *804*, 211.

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 and spatial complementarity of SMIP binding sites toward the template, while the affinity for the analogue is very low. 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 the functional monomer.

Finally, the selectivity of the synthesised polymeric nanospheres can be highlighted by introducing another coefficient (ε) which is a quantitative measure of the imparted selectivity within the imprinted nanospheres and was calculated as the ratio between the amount (%) of 5-FU and U bound by SMIP. This value was found 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-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 2.3* that the amount of water uptake by imprinted nanospheres increases with time and the maximum water uptake is obtained after 24 h (*Table 2.2*). These high swelling characteristics make the imprinted cavities of polymeric network easily accessible to the template; this is to advantage of recognition properties of imprinted nanospheres.

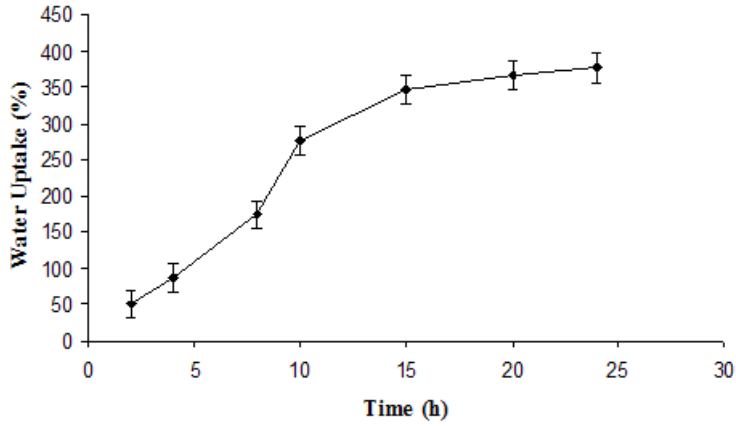

Figure 2.3 *Swelling rate profile of SMIP.*

3.3 In vitro release studies

After evaluation of imprinting effect of synthesised 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[26].

In a first approach[27], 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 depends on the loaded drug, SMIP particles are able to release a higher amount of drug than SNIP ones.

In another approach, more useful to emphasise 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

[26] Alvarez-Lorenzo, C.; Concheiro, A. *J. Chromatogr. B* **2004**, *804*, 231.
[27] Singh, B.; Chauhan, N. *J. Macromol. Sci. A Pure Appl. Chem.* **2008**, *45*, 776.

percentage of 5-FU released was calculated considering 100% the 5-FU content in dried samples[28].

In our work, the second approach is employed and the presence of imprinted cavities in spherical polymers makes the release of the template more extended over time in comparison with non-imprinted materials. As it is possible to observe in *Figure 2.4*, 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.

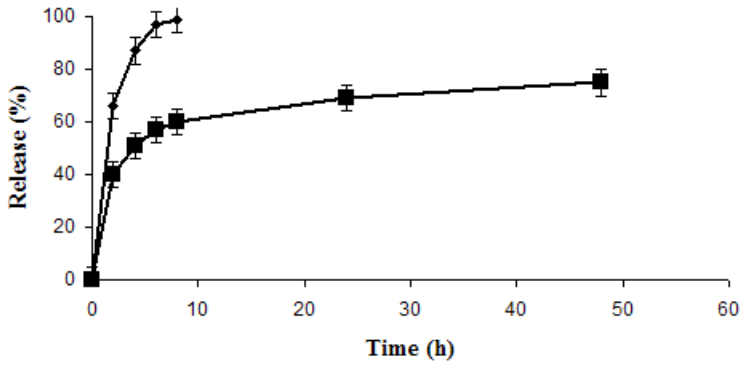

Figure 2.4 *5-FU release profile from SMIP (—■—) and SNIP (- - ◆ - -) in plasma simulating fluids.*

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 the results showed in binding tests: uracil release from imprinted polymers, indeed, that just in 2 hours was

[28] Pitarresi, G.; Pierro, P.; Giammona, G.; Iemma, F.; Muzzalupo, R.; Picci, N. *Biomaterials* **2004**, *25*, 4333.

completed, was remarkably faster than that obtained when 5-FU was used (data not shown).

4. Conclusions

In this work, 5-FU imprinted nanospheres with swelling characteristics were prepared using methacrylic acid and ethylene glycol dimethacrylate as functional monomer and crosslinker, respectively.

One-pot precipitation polymerisation, as synthetic technique to obtain the monodispersed particles, was chosen. The recognition properties and selectivity of synthesised 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.

CHAPTER III

Molecularly Imprinted Polymers for Selective Extraction and Sustained Release of Glycyrrhizic Acid

1. Introduction

Glycirrhiza glabra (liquorice) roots are extensively used in herbal medicines for their emollient, antitussive, anti-inflammatory, antiviral and gastroprotective properties[1,2].

The product called "liquorice" in confectionery manufacturing (i.e. flakes or pastilles of pure liquorice) is obtained by treating dried *G. glabra* roots with boiling water, which is then evaporated to obtain a semi-solid extract[3].

The main active compound of *G. glabra* is glycyrrhizin or glycyrrhizic acid (GL), also known as (3*β*, 20*β*) - 20 - Carboxy - 11 - Oxo - 30 - Norlean - 12 - En-3-yl-2-*O*-*β*-17- Glucopyranuronosyl- *α*- D-Glucopyranosiduronic acid. This molecule is present in the root and consists of an aglycon (a pentacyclic triterpenic structure) bound to two glucuronic acid molecules[4]. GL has been demonstrated to possess anti-inflammatory, antiallergenic, antihepatotoxic, antiulcer and antiviral properties[5,6] and is currently suggested as a possible chemopreventive drug[7]. Furthermore, GL is one of the leading natural compounds for clinical trials in recent studies on chronic viral hepatitis and human immunodeficiency virus (HIV) infections[8]. Chronic consumption of GL

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[1] Sabbioni, C.; Ferranti, A.; Bugamelli, F.; Cantelli Forte, G.; Raggi, M.A. *Phytochem. Analysis* **2006**, *17*, 25.
[2] Chung, J.G.; Chang, H.L.; Lin, W.C.; Wang, H.H.; Yeh, C.C.; Hung, C.F.; Li, Y.C. *Food Chem. Toxicol.* **2000**, *38*, 163.
[3] Fenwick, G.R.; Lutomski, J.; Nieman, C. *Food Chem.* **1990**, *38*, 119.
[4] Andersen, F.A. *Int. J. Toxicol.* **2007**, *26*, 79.
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prevents the development of hepatic carcinoma from C hepatitis and the antiviral activity of GL against SARS associated corona virus has been demonstrated in vitro[9]. Finally, GL finds application in inhibiting unwanted effects of contraceptive formulations, such as alterations in blood coagulation and thrombosis[10].

Based on these considerations, the determination, the selective extraction of GL from liquorice roots and the development of a Drug Delivery System (DDS) for this compound have gained great interest.

Several papers report on analytical methods for the determination of GL with different techniques such as HPLC, capillary electrophoresis, gas chromatography, and high-performance thin layer chromatography[11,12]. In all these techniques, different kind of sample preparation methods is required: plant extracts are, indeed, complex mixtures with a large variety of chemical compounds.

Among the extraction technique, Solid-Phase Extraction based on Molecularly Imprinted Polymers (MISPE) is an efficient approach for purification of analytes from complex matrices and preconcentration of the samples, and it is gaining considerable interest in environmental, clinical, and food analysis[13,14,15].

Moreover, in recent years several studies are focused on the development of drug delivery systems based on polymeric materials and the importance of this research field is growing as ever more complex drugs and

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[9] Nassiri-Asl, M.; Hosseinzadeh, H. *Phytoter. Res.* **2008**, *22*, 709.
[10] Stähli, B.E.; Breitenstein, A.; Akhmedov, A.; Camici, G.G.; Shojaati, K.; Bogdanov, N.; Steffel, J.; Ringli, D.; Lüscher, T.F.; Tanner, F.C. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2007**, *27*, 2769.
[11] Sabbioni, C.; Mandrioli, R.; Ferranti, A.; Bugamelli, F.; Saracino, M.A.; Cantelli Forti, G.; Fanali, S.; Raggi, M.A. *J. Chromatogr. A* **2005**, *1081*, 65.
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[14] Zhang, H.; Ye, L.; Mosbach, K. *J. Mol. Recognit.* **2006**, *19*, 248.
[15] Benito-Peña, E.; Urraca, J.L.; Sellergren, B.; Moreno-Bondi, M.C. *J. Chromatogr. A* **2008**, *1208*, 62.

biopharmaceuticals are being developed, many of which cannot be administered without a controlled dosage system.

A controlled drug delivery system offers the possibility to maximise the drug efficacy and safety and to provide 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 minimise the administration frequency thus enhancing patient compliance[16].

Among the different kind of polymeric materials applied in this research fields[17] (erodible devices, implants, etc.), a particular attention have been devoted to molecularly imprinted polymers (MIPs)[18].

In this study, glycyrrhizic acid imprinted polymers were synthesised by the non-covalent imprinting approach, and a screening of the functional monomers (acidic, neutral or basic) was performed. After the evaluation of the imprinting efficiency, the best materials were tested as stationary phase in a SPE protocol for the selective extraction of GL from liquorice roots[19] and as releasing device[20] in gastrointestinal simulating fluids.

2. Materials and methods

2.1 Materials

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2-(dimethylamino)ethyl methacrilate (DMAEMA), 2-hydroxyethyl metacrylate (HEMA), 2,2'-azoisobutyronitrile (AIBN), glycyrrhizic acid (GL) and glycyrrhetic acid (GA) were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO).

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[16] Cunliffe, D.; Kirby, A.; Alexander, C. *Adv. Drug Deliv. Rev.* **2005**, *57*, 1836.
[17] Langer, R.; Peppas, N.A. *AIChE J.* **2003**, *49*, 2990.
[18] Caro, E.; Marce, R.M.; Cormack, P.A.G.; Sherrington, D.C.; Borrull, F. *J. Sep. Sci.* **2006**, *29*, 1230.
[19] Cirillo, G.; Curcio, M.; Parisi, O.I.; Puoci, F.; Iemma, F.; Spizzirri, U.G.; Restuccia, D.; Picci, N. *Food Chem.* **2011**, *125*, 1058.
[20] Cirillo, G.; Parisi, O.I.; Curcio, M.; Puoci, F.; Iemma, F.; Spizzirri, U.G.; Picci, N. *J. Pharm. Pharmacol.* **2010**, *62*, 577.

All solvents were reagent grade or HPLC-grade, used without further purification and were provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

Monomers were purified before use by distillation under reduced pressure.

2.2 Synthesis of glycyrrhizic acid imprinted polymers

Three different GL imprinted polymers (MIPs) were prepared using MAA, DMAEMA or HEMA as functional monomers. Briefly, 0.5 mmol of template glycyrrhizic acid and 16.0 mmol of functional monomer were dissolved in 5 mL of anhydrous DMF in a thick-walled glass tube, and then 25.0 mmol of EGDMA and 0.100 g of AIBN were added. The tube was purged with nitrogen, sonicated for 10 min, thermo-polymerised 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 μm stainless steel sieve. The sieved MIP materials were collected and the very fine powder, suspended in the supernatant solution (acetone), was discarded. The resultant imprinted polymers were soxhlet extracted with 200 mL of an ethanol/acetic acid (8/2 v/v) mixture for at least 48 h, followed by 200 mL of ethanol for others 48 h and then dried in an oven at 60°C overnight. The washed MIP materials were checked to be free of GL and any other compound by HPLC analysis of the washing media.

Non-imprinted polymers (NIPs), to act as a control, were also prepared when polymerisation was carried out in the absence of GL.

The formulations used for the preparation of the different matrices are shown in *Table 3.1*.

Table 3.1 *Polymer compositions.*

POLYMER	GL (mmol)	MAA (mmol)	DMAEMA (mmol)	HEMA (mmol)	EGDMA (mmol)
MIP1	0.5	16.0	---	---	25.0
NIP1	---	16.0	---	---	25.0
MIP2	0.5	---	16.0	---	25.0
NIP2	---	---	16.0	---	25.0
MIP3	0.5	---	---	16.0	25.0
NIP3	---	---	---	16.0	25.0

2.3 Binding experiments

The binding efficiency of polymeric matrices towards GL was evaluated by performing rebinding experiments in ethanol and in an ethanol/water (6/4 v/v) mixture. Briefly, 50 mg of polymer particles were mixed with 1 mL of a GL solution (0.2 mM) in a 1 mL eppendorf and sealed. The eppendorf tubes 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 GL concentration in the liquid phase was measured by HPLC. The amount of GL bound to the polymer was obtained by comparing its concentration in the MIP samples to the NIP samples.

The same experiments were performed using GA solutions.

Specific imprinting parameters α and ε were determined. α value corresponds to the ratio between the amount (%) of analyte (GL or GA) bound by MIP and NIP, while ε to the ratio between the amount (%) of GL and GA bound by MIP.

2.4 Molecularly imprinted solid-phase extraction conditions

The 500 mg amount of dry particles of MIP and NIP 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, ethanol and then with the loading solvent.

GL was dissolved in the loading solvent to a final concentration of 0.2 mM. After conditioning, dry MISPE column was loaded with the GL standard solution. After loading, vacuum was applied through the cartridges for 5 min in order to remove residual solvent. Washing solvent was then passed through the cartridges and finally, after column drying, elution solvent was applied to perform the complete extraction of GL. The loading, washing and eluting fractions were analysed by HPLC to detect the GL amount.

The MISPE protocol was optimised and the best conditions were:

- loading step: 2 mL of ethanol, flow rate ~ 0.2 mL/min;
- washing step: 8 mL of ethanol/water (9/1 v/v) mixture, flow rate ~ 0.2 mL/min;
- eluting step: 2 mL of hot ethanol/water (5/5 v/v) mixture, flow rate ~ 0.2 mL/min.

In order to evaluate the selectivity of the MIP, optimised protocol was also applied using GA solutions.

Each measurement was carried out in triplicate and the data expressed as means (± SD).

2.5 Extraction procedure

A liquorice sample was ground to a fine powder and a sample (200 mg) transferred to a 50 mL round bottom flask together with 20 mL of ethanol:water (5/5, v/v). The mixture was maintained by thermostat at 60°C for 25 min with stirring and then centrifuged for 10 min at 3000 rpm[21].

The supernatant was filtered through a paper filter and an aliquot of the filtrate, suitably diluted with mobile phase, was subjected to HPLC analysis.

[21] Sabbioni, C.; Ferranti, A.; Bugamelli, F.; Cantelli Forte, G.; Raggi, M.A. *Phytochem. Analysis* **2006**, *17*, 25.

2.6 Molecularly imprinted solid-phase extraction of vegetable sample extracts

The extract was evaporated to dryness and then reconstituted in solution by adding ethanol. 2 mL of this solution were used to load the MISPE column. The washing solvent consists of 8 mL of an ethanol/water (9:1 v/v) mixture, while 2 mL of a hot ethanol/water (5/5 v/v) mixture were used as elution fraction. All the solutions, suitably diluted with mobile phase, were subjected to HPLC analysis.

Each measurement was carried out in triplicate and the data expressed as means (± SD).

2.7 Drug loading by the soaking procedure

Polymeric matrix (2.0 g) was immersed in a GL solution in ethanol (40 mL, 0.2 mM) and soaked for 24 hours at room temperature. During this time, the mixture was continuously stirred and then the solvent was removed under vacuum. Finally the powder was dried under vacuum overnight at 40°C.

The same experiments were performed using a GA solution (GL analogue molecule).

2.8 In vitro release studies

Release studies were carried out using the dissolution method described in the USP XXIV (apparatus 1-basket stirring element). To mimic the pH of the digestive tract, 0.1 N HCl (pH 1.0) was used as simulated gastric fluid and after two hours, disodium hydrogen phosphate (0.4 M) was added to adjust the pH value to 6.8 to simulate the intestinal fluid.

The experiments were performed as follows: 30 mg of MIP and NIP particles loaded with GL were dispersed in flasks containing 10 mL of 0.1 N HCl and maintained at 37 ± 0.5°C in a water bath for 2 h under magnetic stirring (50 rpm). Disodium hydrogen phosphate (0.4 M, 5 mL) was then added to the samples. These conditions were maintained throughout the experiment. To characterise the drug release, 2 mL of samples were drawn from the dissolution medium at designated time intervals and the same volume of simulated fluid was

supplemented. GL was determined by HPLC analysis and the amount of GL released from five samples of each formulation was used to characterise the drug release profile. The percentage of released GL was calculated considering 100% the GL content in polymeric samples after drying procedure.

2.9 HPLC analysis

The liquid chromatography consists of a Jasco Model (Tokyo, Japan) consisting of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 254 nm. A Tracer Excel 120 ODS-A column particle size 5μm, 15 x 0.4 cm (Barcelona, Spain) was employed. The mobile phase was a methanol/acetonitrile/water/glacial acetic acid mixture (35/35/30/1, by volume) and the flow rate was 1.0 mL/min[22].

2.10 Analytical parameters

In order to evaluate intermediate precision (inter-day precision) and repeatability (intra-day precision), assays were performed by extracting and injecting the same sample six times on the same day and six times over different days. The percentage relative standard deviations (RSD %) of the data thus obtained were calculated.

Accuracy was evaluated by means of recovery assays carried out by adding standard solutions of the analytes to the samples. The added amounts of analytes corresponded to concentrations of 5, 10 and 20 μg/mL. The mean recoveries of the added analytes were then calculated.

2.11 Statistical analysis

All of the experiments were done in triplicate and data expressed as means ± SD.

One-way analysis of variance was performed to assess the significance of the differences among data in *Table 3.2*. Tukey-Kramer post-test was used to

[22] Sabbioni, C.; Ferranti, A.; Bugamelli, F.; Cantelli Forti, G.; Raggi, M.A. *Phytochem. Anal.* **2006**, *17*, 25.

compare the means of different treatment data. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Synthesis and characterisation of imprinted polymers

For the synthesis of GL imprinted polymers, we chose the non-covalent approach[23] because of the simplicity of the process and because of the wide range of functional monomers, acidic, basic or neutral, that can be used[24]. This method is the most widely used to synthesise MIPs because of the fast kinetic of binding and the absence of toxic reaction products[25].

This approach is preferred over the covalent one because in physiological application, especially in drug delivery field, the latter approach is not the most appropriate as there are only a limited number of readily reversible covalent linkages that are known. In addition, the cleavage of the monomer-template complex can lead to the formation of toxic species with possible serious safety issues[26].

When considering the chemical structure of GL template, it is clear that the functionalities suitable to interact with the functional monomer are mainly the carboxylic ones. In literature, it is reported that these kinds of template can be successfully imprinted by using acidic, basic or neutral monomers[27]. Thus, in our study, a screening of different functional monomers was performed.

Three different MIPs were, indeed, synthesised employing MAA (MIP1 and NIP1) as acidic, DMAEMA (MIP2 and NIP2) as basic and HEMA (MIP3 and NIP3) as neutral functional monomer (*Table 3.1*). In order to evaluate the effect of the different monomer to the recognition properties of the resulting

[23] Alexander, C.; Andersson, H.S.; Andersson, L.I. Ansell, R.J.; Kirsch, N.; Nicholls, I.A.; O'Mahony, J.; Whitcombe, M.J. *J. Mol. Recognit.* **2006**, *19*, 106.
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[26] Liu, J.Q.; Wulff, G. *J. Am. Chem. Soc.* **2008**, *130*, 8044.
[27] Beltran, A.; Marcé, R.M.; Cormack, P.A.G.; Borrull, F. *J. Chromatogr. A* **2009**, 1216*, 2248.*

materials, all the polymeric matrices were prepared using the same molar ratio between template, functional monomer and crosslinker, which is the ratio commonly used in several research works involving MIPs[28].

Table 3.2. *Percentage of bound analytes after 24 h incubation in ethanol and ethanol-water (6:4 v/v) mixture with GL and GA concentration 0.2 mM.*

Polymer	% Bound GL		% Bound GA		α GL		α GA		ε	
	EtOH	EtOH/H_2O	EtOH	EtOH/H_2O	EtOH	EtOH/H_2O	EtOH	EtOH/H_2O	EtOH	EtOH/H_2O
MIP1	40±1.3	38±0.9	18±1.1	32±0.9	3.07	6.33	0.94	0.97	2.02	1.25
NIP1	13±0.6	6±0.7	19±0.6	33±0.6						
MIP2	73±0.8	90±1.1	1±0.5	16±1.4	4.86	1.50	1.00	1.13	73.00	4.56
NIP2	15±1.0	60±1.3	1±0.8	14±1.2						
MIP3	27±1.4	17±1.2	20±1.2	25±0.9	2.45	5.00	1.05	1.04	1.35	1.08
NIP3	11±0.8	3±0.9	19±0.9	24±0.4						

The imprinting effect of synthesised materials was evaluated by binding experiments in which amounts of polymeric particles were incubated with a GL solution (0.2 mM) for 24 hours in water and in an ethanol/water (6/4 v/v) mixture. The obtained results are collected in *Table 3.2*, and a good imprinting efficiency was observed for all the matrices as shown by the higher amount of template bound by the imprinted cavities in the MIP structure. NIPs, because of the absence of these cavities with high chemical and spatial complementarity to the template, showed lower interactions with the template.

[28] Puoci, F.; Curcio, M.; Cirillo, G.; Iemma, F.; Spizzirri, U.G.; Picci, N. *Food Chem.* **2008**, *106*, 836.

To evaluate cross-reactivity of imprinted polymers towards GL analogue molecules, the same binding experiments were performed using GA, which differs from GL because of the absence of the two sugar moieties (*Figure 3.1*).

The imprinting effect was characterised by the introduction of two specific parameters, α and ε, which are reported in *Table 3.2*. α value is a quantitative determination of the recognition properties of the synthesised material and is determined as the ratio between the amount (%) of analyte (GL or GA) bound by MIP and NIP[29], while ε value, which is a quantitative measure of the imparted selectivity within the imprinted microparticles, is calculated as the ratio between the amount (%) of GL and GA bound by MIP.

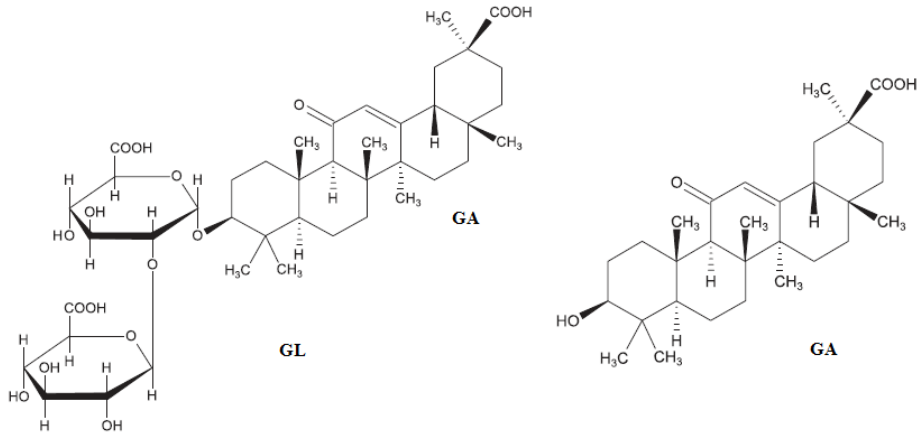

Figure 3.1. *Chemical structures of glycyrrhizic acid (GL) and glycyrrhetic acid (GA).*

In this work, for all the polymers, high α_{GL} values were recorded in all the tested environments, proving the specificity of the interaction between the template and the functional groups on the polymeric microparticles. This specificity was confirmed by the recorded α_{GA} values, which are lower than the corresponding α_{GL} values.

[29] Cirillo, G.; Iemma, F.; Puoci,F.; Parisi, O.I.; Curcio, M.; Spizzirri, U.G.; Picci, N. *J. Drug Target.* **2009**, *17*, 72.

In addition, all the obtained ε values are higher than 1.00, confirming that the imprinted cavities are characterised by stronger interaction with the template around which they are formed and lower affinity for the analogue.

The three synthesised polymers show different behaviour in ethanol and in ethanol/water mixture. Polymers from series 1 and 3, which are characterised by acidic and neutral functional monomer, respectively, bound more analytes in ethanol than in ethanol/water mixture, while the opposite behaviour is observed with polymers from series 2. This effect is ascribable to the presence of a basic functional monomer in MIP2 and NIP2 which strongly interact with the carboxylic functionalities on GL and GA in water media, as proved by the higher GL and GA (%) bound by these polymers. In this case, high non-specific hydrophobic interaction between polymeric matrices and analytes were observed (*Table 3.2*). Comparing MIP1 and MIP3, higher imprinting effect was recorded from MIP1, as a result of the higher capacity of MAA to form hydrogen bond comparing to HEMA.

3.2 Molecularly imprinted solid-phase extraction procedure and its optimisation

After the evaluation of MIP efficiency, MISPE cartridges were packed with MIP and the corresponding NIP, and their performance as sorbents for GL SPE was compared.

The cartridges were packed with 500 mg of polymer and the chemical and flow variables, such as composite and volumes of elution solution, sample acidity and loading flow rate were optimised to achieve good sensitivity and precision of this method. It should be considered that the employed solvents (water and ethanol) were chosen for the required eco- and bio-compatibility of the process, while the acidification of the elution solutions perturbs the formation of the hydrogen bonds between hydroxyl and carboxyl groups of GL and those of the specific functional monomer.

By considering the loading step, it was found that the complete retention of GL (0.2 mM) on MIP and NIP cartridges was achieve by using ethanol at a flow rate of 0.2 mL/min, while water and ethanol/water mixture were not

effective. This behavior is the same when considering polymers from series 1 and 3, while an almost complete retention of the analyte by polymer from series 2 is raised in all the tested environments.

The subsequent washing step was performed to minimise the non-specific interactions between polymeric matrices and template. It was found that the three matrices work in different ways. Neither water and ethanol, as well as ethanol/water mixtures were found able to remove GL from MIP2 and NIP2, and even acidifying the solutions no GL was detected in the washing solvents. This effect clearly proves that these polymers cannot be applied as sorbents in GL SPE protocols. On the contrary, good results were obtained with polymers 1 and 3. It should be considered that, in this step, the employed solvent is required to remove the template from NIP, while it should be retained by MIP. The results show that this effect was obtained by using an ethanol/water (9/1 v/v) mixture in series 3 and HCl (0.01 M) in series 1. In particular, in the washing fraction of MIP3, almost 19% of GL was recovered, while the template was almost completely removed from the corresponding NIP3 (*Table 3.3*). When considering the MIP1, the amount (%) of washed GL is almost 15%, while this value is increased to 97% in NIP1 case.

The elution of GL was successfully obtained by using water in MIP3 and PBS (10^{-3}M, pH 7) in MIP1.

Table 3.3. *Percentages of collected GL in the optimised loading, washing and eluting steps. Data are shown as means ± RSD.*

POLYMER	LOADING	WASHING	ELUTING
MIP1	6 ± 1.1	15 ± 1.2	79 ± 0.6
NIP1	5 ± 1.4	93 ± 1.5	2 ± 0.3
MIP2	3 ± 0.9	7 ± 0.6	3 ± 1.0
NIP2	5 ± 1.0	6 ± 1.3	2 ± 1.4
MIP3	1 ± 0.8	19 ± 1.4	81 ± 0.9
NIP3	3 ± 0.7	95 ± 1.1	2 ± 1.0

At last, the selectivity of the packing cartridges was evaluated by using GA solution in the optimised condition tested for GL.

In the loading step, MIP1 cartridges retain only 23% of GA, NIP1 cartridges the 19%, while for MIP3 and NIP3 these values are 31% and 35%, respectively.

In the washing fraction of all the MIP and NIP cartridges, all the retained GA was recovered, showing the high selectivity of the synthesised materials for the original template.

Based on these results, MIP1 and MIP3 are very useful as stationary phases for SPE procedures because, besides the adequate selectivity and affinity properties in the recognition of the analyte, a low level of template bleeding is raised. MIP2 is not effective because of the high bleeding effect.

3.3 Molecularly imprinted solid-phase extraction of liquorice roots

The previous SPE methods have been applied to a liquorice extract for the selective extraction of GL.

First of all, a performing extraction protocol must be applied to the liquorice roots crashed sample. In the development of such a kind of protocol, particular attention should be done to the the chemical characteristic of the analytes. As reported in the European Pharmacopoeia, 2005, GL is usually > 4% in liquorice root and 3-5% in ethanolic extract. In literature, different solvents were tested for the quantitative extraction of GL from liquorice roots, including water, ethanol, water/ethanol mixtures, ammonium hydroxide and water. A recent work deals with the comparison of these methods and the conclusion of this study is that the optimum extraction solvent is an ethanol/water (1/1 v/v) mixture, and in this study this procedure was adopted for the preparation of the liquorice extract to be loaded on MISPE cartridges. In particular, 200 mg of grounded liquorice sample was extracted with the ethanol/water mixture, dried and then reconstituted to solution by adding ethanol. An aliquot of this solution, suitably diluted with mobile phase, was subjected to HPLC analysis (*Figure 3.2*).

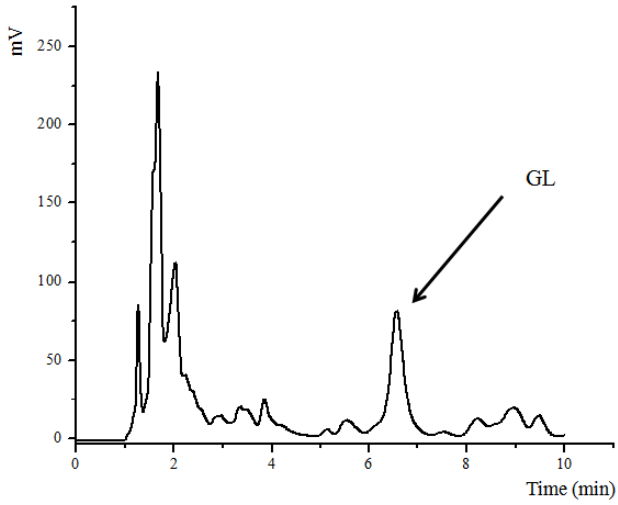

Figure3. 2. *Chromatogram of the liquorice roots extract.*

Firstly, MIP3 and NIP3 cartridges were tested. Two millilitres of the prepared loading solution were employed to load the cartridges which almost completely absorb GL (*Figure 3.3a*). Thus, in order to obtain a satisfactory clean-up of the template, the washing solvent was applied on the cartridges and the GL amount in the washing fractions determined. In the washing chromatogram of MIP cartridges (*Figure 3.3b*), not so relevant peaks were observed at the retention time of GL, confirming the selective interaction between polymers and template. The GL amount in these fractions represents about the 20 % of the loaded one. Furthemore, an important clean-up of the extracted mixture was obtained, as showed by the presence of several peaks referable to the others compounds of the sample.

Finally, the selective elution of GL was obtained by using hot ethanol/water (5/5 v/v) mixture, obtaining a satisfactory recovery of pure GL in MIP cartridges (about the 80% of the loaded one), confirming that the washing step was able to completely remove all the compounds which co-eluted with the template in the starting extract (*Figure 3.4*).

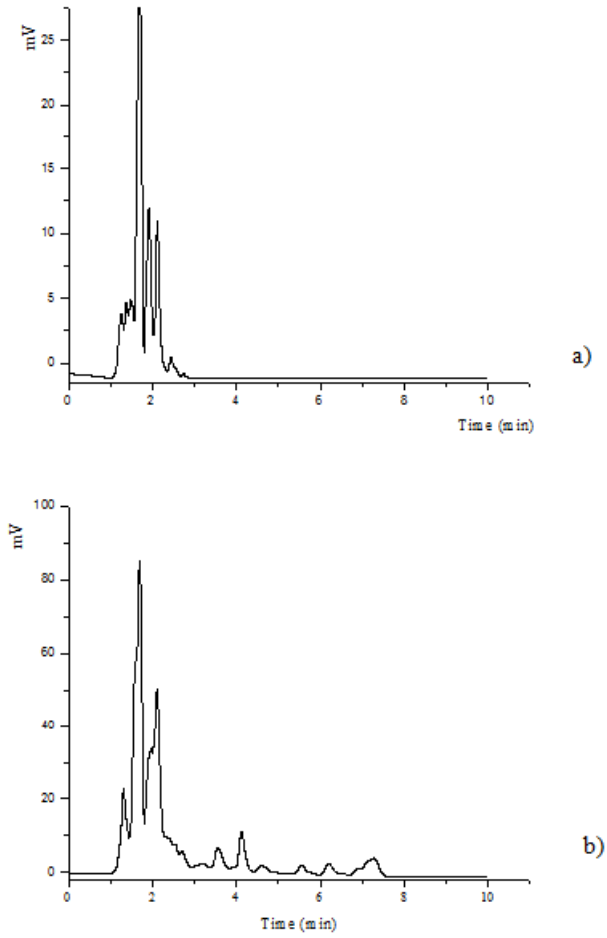

Figure 3.3 *Chromatograms of MISPE loading (a) and washing steps (b).*

In the SPE protocol performed on NIP3 cartridges, the loaded GL was almost completely washed out in the washing step and no GL was detected in the eluting fraction.

When MIP1 and NIP1 were employed, the loading step is characterised by the complete retention of GL on the cartridges, but the HCl solution employed in the washing step was not able to clean the cartridges from the compounds which co-eluted with GL in the original extract, which was not found pure in the eluting chromatograms.

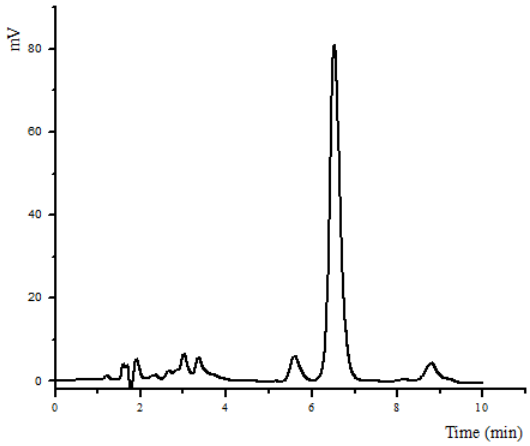

Figure 3.4 *Chromatogram of MISPE eluting step.*

3.4 Accuracy and precision

The precision of the method was then evaluated: intermediate precision values were obtained by repeating the SPE extraction of the same sample six times over different days; the SD% thus calculated was 3.9%.

Accuracy was assessed spiking the extract with three different levels of the analyte. The recovery values were between 93 and 107%, thus indicating the satisfactory accuracy of the method.

3.5 In vitro release studies

After the characterisation of the synthesised MIPs in terms of imprinting effect, their applicability as devices for the controlled release of GL in gastrointestinal simulated fluids was tested. Our hypothesis was that MIPs have a better ability in controlling drug release in comparison to the corresponding NIPs because of the presence of specific imprinted cavities in the polymeric structure, which are able to slowly release the template.

For the development of such a kind of drug delivery system, the most promising polymer is MIP1, as shown by the highest α_{GL} value recorded in water media, but also MIP3 was employed for the development of the release studies. MIP2 was not tested in this direction because of the high non-specific hydrophobically driven interactions between polymeric matrices and template,

as shown by the higher value of bound GL (%) by NIP2 and by the low α_{GL} value recorded in water media (only 1.50, while the others α_{GL} were 6.33 and 5.00 for MIP1 and MIP3, respectively).

In vitro release studies were performed by immersing aliquots of the microparticles loaded with GL at pH 1.0 (simulated gastric fluid) for 2 hours and then at pH 6.8 (simulated intestinal fluid) using the pH change method.

For both the tested polymers, the experimental data confirm that MIPs are more effective in controlling the template release because of the presence of the specific functional groups in the imprinted cavities which strongly interact with GL.

By comparing the release profile showed in *Figures 3.5 and 3.6* for MIP1 and MIP3, respectively, a better control in the drug release was observed with MIP1, because, as reported in the characterisation of the imprinting efficiency, MAA is much able to form interactions with the template than HEMA.

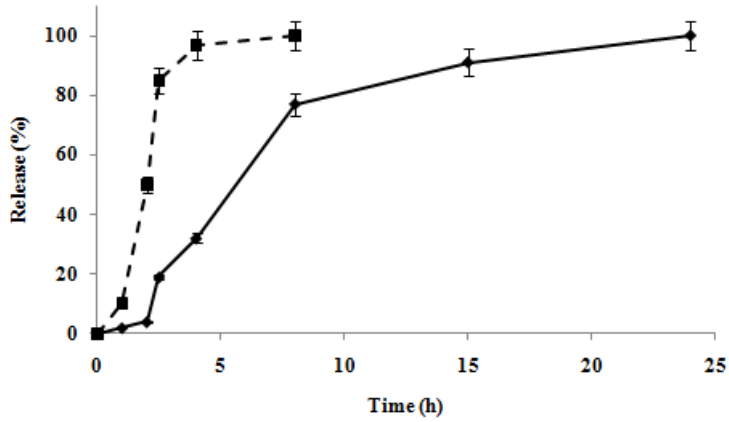

Figure 3.5. *Gastrointestinal release profile of glycyrrhizic acid from MIP1 (-♦-) and NIP1 (-■-).*

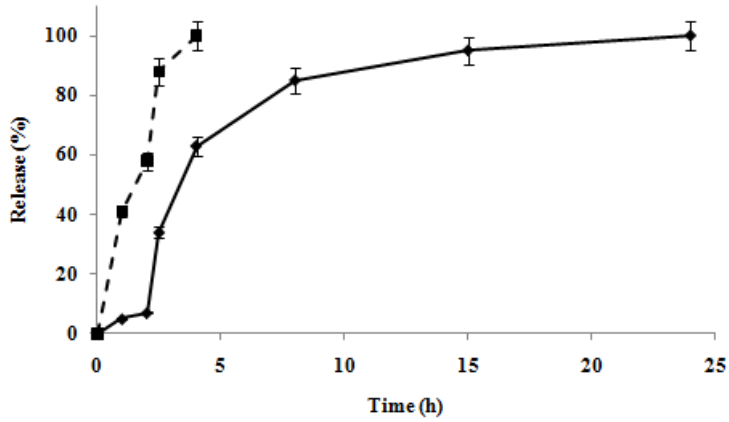

Figure 3.6. *Gastrointestinal release profile of glycyrrhizic acid from MIP3 (-♦-) and NIP3 (-■-).*

By considering the release profiles at acidic pH, the drug is highly released within 2 hours in both NIP1 and NIP3 cases (the amount of GL released is 50% and 58%, respectively), because the interaction between polymeric matrices and GL is ascribable to non-specific interaction, while at this time, only 19% and 34% of GL was released from MIP1 and MIP3 samples, respectively (*Figures 3.5 and 3.6*).

After pH change, GL release from MIPs continued and the release was completed in almost 15 hours for both MIP1 and MIP3, but the release was slower for MIP1. The acid groups of the selective sites have, indeed, stronger interaction with the template than the alcoholic ones.

Considering the release from polymers of series 1, at low pH (1.0) values the carboxylic groups are not ionised and so there is a good interaction with the template. These results might help to understand also the behaviour of these matrices when the pH increases to simulate the intestinal fluid. At pH 6.8 the diffusion rate of the buffer, on the polymeric surface of NIP is fast; the carboxylic groups of both the polymer and the template are ionised, and the drug is rapidly released. Instead, with MIPs, the diffusion rate of the buffer into specific cavities of imprinted polymers is slower and the functional groups are ionised more slowly, obtaining a good control of release.

In polymers of series 3, the same effect is observed but the ionisation process involves only the functionalities on the template molecule, and the faster release after the pH change is ascribable to the lower efficiency of the alcoholic functionalities of HEMA in interacting with the template.

These observations show that MIP1 can be considered as a very useful polymeric device to be applied for the selective release of GL in gastrointestinal simulating fluids.

4. Conclusions

In thi work, three different MIPs for the selective recognition of GL were synthesised by employing acidic, neutral and basic functional monomers.

All the synthesised polymers were characterised by the evaluation of the imprinting efficiency; a good imprinting effect was recorded for all the materials, even if the DMAEMA-containing matrices were characterised by a higher non-specific interaction with the template, which reduces their imprinting efficiency.

In the aim to develop a molecularly imprinted solid-phase extraction protocol for the selective extraction of GL from liquorice roots, MIPs were applied as sorbents in SPE. It was found that DMAEMA-containing materials are not effective, while good results were obtained with HEMA and MAA containing matrices. When the SPE protocol was employed by using liquorice extract, by using HEMA-MIP a selective extraction of 80% of pure GL is raised. On the contrary, MAA-containing materials were not effective. Thus, GL-MISPE protocol using HEMA-MIP offers excellent recovery of the template and is suited to routine use for GL isolation from liquorice.

About the possibility to employ one of the synthesised polymeric materials as releasing device for GL, the most promising matrix was found to be the MAA-containing MIP.

In vitro release studies were carried out using the GL loaded MIPs, and the results show that MIP1 was the most effective material in controlling the GL release over time. The release from non-imprinted polymers was almost

completed in acidic pH conditions, while the release from both MIP1 and MIP3 was completed in 15 hours with different kinetics of ionisation referable to the stronger interaction between MAA and GL compared to HEMA.

CHAPTER IV

Surface Modifications of Molecularly Imprinted Polymers for Improved Template Recognition in Water Media

1. Introduction

The main characteristic of polymeric materials designed for working in physiological environments is firstly the compatibility with water and the others components of the biological system.

When considering MIPs, their use in aqueous solutions shows two main drawbacks: non-specific interactions between small molecules and polymeric matrices and the predominance of hydrophobically driven bonds[1]; furthermore, biological sample components, such as proteins and lipids, could be strongly adsorbed on the polymeric surfaces with a subsequent reduction of the recognition properties[2].

Recent developments in the field of molecularly imprinted polymers are based on the preparation of new kind of MIPs to be used in biological fluids or environmental waters[3,4].

The first possibility is the employment of a hydrophilic co-monomer which does not interact with the template molecule in the pre-polymerisation complex formation, but able, at the same time, to confer high water compatibility to the final system. For this purpose, 2-hydroxyethyl methacrylate (HEMA) was widely used, but a considerable modification of the recognition properties of the polymeric matrices was often observed because of the formation of hydrogen bonds between various functionalities of the template molecule and hydroxyl group of HEMA[5].

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[1] Boos, K.S.; Fleischer, C.T. *J. Anal. Che.* **2001**, *371*, 16.
[2] Bures, P.; Huang, Y.; Oral, E.; Peppas, N.A. *J. Contr. Rel.* **2001**, *72*, 25.
[3] Sambe, H.; Hoshina, K.; Haginaka, J. *J. Chromatogr. A* **2007**, *1152*, 130.
[4] Cobb, Z.; Sellergren, B.; Andersson, L.I. *Analyst* **2007**, *132*, 1262.
[5] Tunc, Y.; Hasirci, N.; Yesilada, A.; Ulubayram, K. *Polymer* **2006**, *47*, 6931.

Another approach was the modification of a pre-formed MIP surface by a second polymerisation step in which hydrophilic monomers were used to form a polymeric water-compatible coating around imprinted microparticles. In this case, the widely used monomers were glycerol monomethacrylate (GMMA) and glycerol dimethacrylate (GDMA)[6,7].

Our approach is to employ a monomer that less interferes in the pre-polymerisation complex formation, but able, at the same time, to impart water compatibility to the system after a post-polymerisation straightforward modification[8]. Epoxy group is useful for this purpose because its oxygen atom, bound to two carbons, has lower capacity to form hydrogen bonds than a free hydroxyl group. Furthermore, a hydrophilic behaviour to the matrices can be induced by epoxy rings opening with water compatible reactants.

In this study[9], new restricted access materials using molecular imprinting technique were synthesised by employing glycidyl methacrylate (GMA) as pro-hydrophilic co-monomer and caffeine (*Figure 4.1*) as model template. On the basis of literature data[10], in our synthetic protocol, methacrylic acid (MAA) and divinyl benzene (DVB) were also chosen as functional monomer and crosslinking agent, respectively. Different molecular ratios of functional monomer to template were investigated in order to improve the performance of the imprinted polymers.

The hydrophilic layer around polymeric microparticles was created by opening GMA epoxide ring with hydroxyl and aminic reactants. In particular, perchloric acid, ammonia, lactic acid, glycine and triethylene glycol were chosen for this purpose. The polymeric matrices treated with perchloric acid and ammonia were further modified by phosphorylation and methylation procedures,

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[6] Sanbe, H.; Haginaka, J. *Analyst* **2003**, *128*, 593.
[7] Haginaka, J.; Takehira, H.; Hosoya, K.; Tanaka, N. *J. Chromatogr. A* **1999**, *849*, 331.
[8] Puoci, F.; Iemma, F.; Cirillo, G.; Curcio, M.; Parisi, O.I.; Spizzirri, U.G.; Picci, N. *Eur. Polym. J.* **2009**, *45*, 1634.
[9] Parisi, O.I.; Cirillo, G.; Curcio, M.; Puoci, F.; Iemma, F.; Spizzirri, U.G.; Picci, N. *J. Polym. Res.* **2010**, *17*, 355.
[10] Villamena, F.A.; De La Cruz, A.A. *J. Appl. Polym. Sci.* **2001**, *82*, 195.

respectively. Different amounts of GMA were added to the pre-polymerisation mixture to improve the water compatibility of the polymeric matrices.

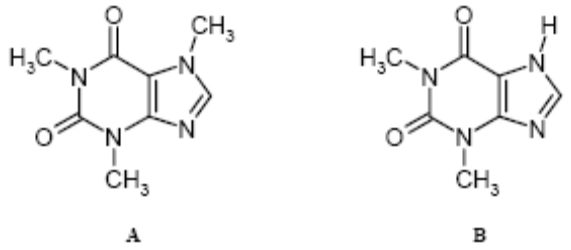

Figure 4.1. *Chemical structures of caffeine (**A**) and theophylline (**B**).*

Imprinting efficiency of all the obtained materials was evaluated by binding experiments using template caffeine and its analogue theophylline (*Figure 4.1*). Restricted access materials-molecularly imprinted polymers (RAM-MIPs) were also characterised by water absorption measurement and protein binding experiments in order to verify the effective formation of the hydrophilic layer around the matrices.

2. Materials and methods

2.1 Materials

Divinyl benzene (DVB), methacrylic acid (MAA), glycidyl methacrylate (GMA), 2,2'-azoisobutyronitrile (AIBN), caffeine (CAFF), theophylline (THEO), perchloric acid, glycine, phosphoric acid (water solution 85% w/w), lactic acid, methyl iodide, triethylene glycol, ammonia (water solution 30% w/w), human serum albumin (HSA) were purchased from Aldrich.

All solvents were reagent grade or HPLC-grade and used without further purification and they were provided by Fluka Chemie.

2.2 HPLC analyses

HPLC was performed using a Jasco Model (Tokyo, Japan) consisting of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 280 nm. A 250 ×

4 mm C-18 Hibar® column, particle size 10 μm (Merck, Darmstadt, Germany) was employed. The mobile phase was an 80:20 (v/v) mixture of sodium phosphate (0.05 M)/methanol and the flow was maintained at 1.0 mL/min[11].

2.3 Synthesis of caffeine molecularly imprinted polymers

Caffeine imprinted polymers were prepared by bulk polymerisation.

General synthetic procedure was reported: template caffeine and MAA were dissolved in chloroform (5.5 mL) in a thick-walled glass tube and then DVB, GMA and AIBN (50 mg) were added. The tube was purged with nitrogen, sonicated for 10 min, and polymerised for 24 h at 60°C. The resultant bulk rigid polymer was crushed, grounded into powder and sieved through a 63 nm stainless steel sieve. The sieved MIP materials were collected and the very fine powders, suspended in the supernatant solution (acetone), were discarded. The template was extracted by ‘‘Soxhlet apparatus’’ using 200 mL of an acetic acid:methanol (1/10) mixture for at least 48 h, followed by 200 mL of methanol for another 48 h and monitoring the template concentration in the extraction solvent by HPLC. Particles were successively dried under vacuum overnight at 40°C.

The formulations used for the preparation of the different matrices are shown in *Table 4.1*.

Blank polymers, to act as a control, were also prepared in absence of caffeine.

2.4 Epoxide rings opening

A general procedure is reported: in a 50 mL round bottom flask, 500 mg of polymeric microparticles were added to 15 mL of a solution containing the nucleophilic agent and stirred (100 rpm) for 24 h.

At the end of the reaction, the particles were filtered, washed with water to pH 7.0, 100 mL of ethanol, 100 mL of acetone, 100 mL of diethyl ether and then dried under vacuum overnight at 40°C.

[11] Farrington, K.; Magner, E.; Regan, F. *Anal. Chim. Acta* **2006**, *566*, 60.

All the polymers containing GMA were treated with the reported reactants (*Table 4.2*). In the same table the reaction conditions were reported and RAM codes assigned.

Table 4.1. *Polymerisation feed composition and bound (%) analytes by imprinted and non-imprinted polymers in acetonitrile after 24 h incubation.*
Data are shown as means ± SD.

Polymer	CAFF (mmol)	MAA (mmol)	GMA (mmol)	DVB (mmol)	% Bound			
					CH_3CN		H_2O (pH 7.0)	
					CAFF	THEO	CAFF	THEO
MIP1	1	8	--	25	20 ± 1.3	22 ± 1.3	97 ± 1.6	93 ± 1.1
NIP1	--				10 ± 1.5	21 ± 1.7	96 ± 1.8	90 ± 1.0
MIP2	1	8	8	25	18 ± 1.2	20 ± 1.1	97 ± 1.9	90 ± 1.1
NIP2	--				8 ± 1.1	21 ± 1.5	96 ± 1.1	92 ± 1.2
MIP3	1	8	12	25	19 ± 1.7	23 ± 1.8	98 ± 1.6	95 ± 1.5
NIP3	--				9 ± 1.5	22 ± 1.5	96 ± 1.7	93 ± 1.2
MIP4	1	8	16	25	17 ± 1.4	19 ± 1.6	97 ± 1.2	94 ± 1.3
NIP4	--				9 ± 1.6	22 ± 1.9	98 ± 1.7	94 ± 1.3
MIP5	1	16	--	25	30 ± 1.1	23 ± 1.4	97 ± 1.8	90 ± 1.7
NIP5	--				9 ± 1.0	28 ± 1.1	97 ± 1.9	90 ± 1.4
MIP6	1	16	8	25	27 ± 1.5	22 ± 1.7	98 ± 1.5	92 ± 0.9
NIP6	--				8 ± 1.4	23 ± 1.5	98 ± 1.6	93 ± 1.3
MIP7	1	16	12	25	26 ± 1.2	22 ± 1.4	95 ± 1.4	91 ± 1.6
NIP7	--				8 ± 1.5	23 ± 1.8	95 ± 1.5	91 ± 1.4
MIP8	1	16	16	25	28 ± 1.4	20 ± 1.6	96 ± 1.3	92 ± 1.5
NIP8	--				7 ± 1.3	24 ± 1.5	94 ± 1.5	93 ± 1.7

2.5 Phosphorilation procedure

500 mg of RAM(A) polymers were added to 50 mL of phosphoric acid (85 w/w) and heated at 120°C for 12 h. After cooling, the particles were filtered, washed with distilled water to pH 7.0, then with 100 mL of ethanol, acetone, diethyl ether and successively dried under vacuum overnight at 40°C. RAM(F) code was assigned to the obtained microparticles.

Table 4.2. *Epoxide group opening conditions and RAM code assignment.*

RAM code	REACTANT	TEMPERATURE
(A)	Perchloric acid solution in water (10% w/w)	r.t.
(B)	Perchloric acid solution in triethylene glycol (10% w/w)	r.t.
(C)	Perchloric acid solution in lactic acid (10% w/w)	r.t.
(D)	Ammonia solution in water (30% w/w)	60°C
(E)	Glycine solution in water at pH 10.0 (4.5 mol l^{-1})	60°C
(F)	Phosphoric acid (85% w/w) [1]	120°C
(G)	Methyl iodide [2]	r.t.

[1] reaction carried out on RAM(A)
[2] reaction carried out on RAM(D)

2.6 Methylation procedure

500 mg of RAM(D) polymers were added to 15 mL of acetone and then 2.5 mL of methyl iodide were added at room temperature. After 8 h, particles were filtered, washed with distilled water to pH 7.0, then with 100 mL of ethanol, acetone and diethyl ether and successively dried under vacuum overnight at 40°C.

RAM(G) code was assigned to the obtained microparticles.

2.7 Water absorption measurement

The swelling characteristics of polymers were determined in order to check increased hydrophilic affinity of RAM polymers obtained from MIP8 and NIP8 compared to unmodified materials (MIP5 and NIP5).

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: phosphate buffer at pH 7.0. At a predetermined time (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 were averaged and used to give the water content percentage (WR %) by the following equation (1) :

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

where W_s and W_d are weights of swollen and dried microparticles, respectively.

Each experiment was carried out in triplicate.

2.8 Binding experiments

The binding experiments were performed both in organic (acetonitrile) and in aqueous media (PBS, pH 7.4).

Briefly, 50 mg of polymer particles were mixed with 1 mL of a CAFF solution (0.2 mM) in a 1 mL eppendorf and sealed. Samples were shaken in a water bath at 37 ± 0.5°C for 24 h, centrifuged for 10 min (10000 rpm) and the CAFF concentration in the liquid phase was measured by HPLC.

The amount of CAFF bound to the polymer was obtained by comparing its concentration in the imprinted samples to the non-imprinted samples.

The same experiments were performed using a THEO solution.

Experiments were repeated five times.

2.9 Protein binding

The 150 mg amount of dry particles of MIP5, NIP5, MIP8, NIP8 and RAM polymers obtained from MIP8 and NIP8, was packed into a 6.0 mL polypropylene SPE column. The column was attached with a stop cock and a reservoir at the bottom and the top end, respectively.

Before use, the columns were preconditioned by successive washings with water, HCl (0.07 M), water, methanol/water (50/50 v/v) mixture, water, and finally 25 mM phosphate buffer (pH 7.4).

The adsorption test was performed by loading the cartridge with 2.0 mL of an HSA standard solution (1.2 mg/mL) in 25 mM phosphate buffer at pH 7.4.

The amount of adsorbed protein after loading step was calculated by UV-Vis spectrophotometer at 290 nm.

Experiments were repeated five times.

2.10 Regeneration properties

In order to test the reusability of the polymeric microparticles, template absorption-desorption cycle was repeated five times using the same microparticles.

Template removal was obtained by ‘‘Soxhlet apparatus’’ using 200 mL of an acetic acid:methanol (1:1v/v) mixture for at least 48 h, followed by 200 mL of methanol for another 48 h.

3. Results and discussion

3.1 Synthesis of imprinted polymers

The goal of this study was the synthesis of MIPs able to work in physiological environments, and to interact with the selected template by miming the dominant interactions in biological systems: the H-bonds. For this reason, the selected imprinting approach was the non-covalent one[12].

[12] Beltran, A.; Caro, E.; Marcè, R.M.; Cormack, P.A.G.; Sherrington, D.C.; Borrull, F. *Anal. Chim. Acta* **2007**, *597*, 6.

The preliminary step was the preparation of conventional MIPs able to selectively recognise the model template (caffeine) (*Figure 4.2*).

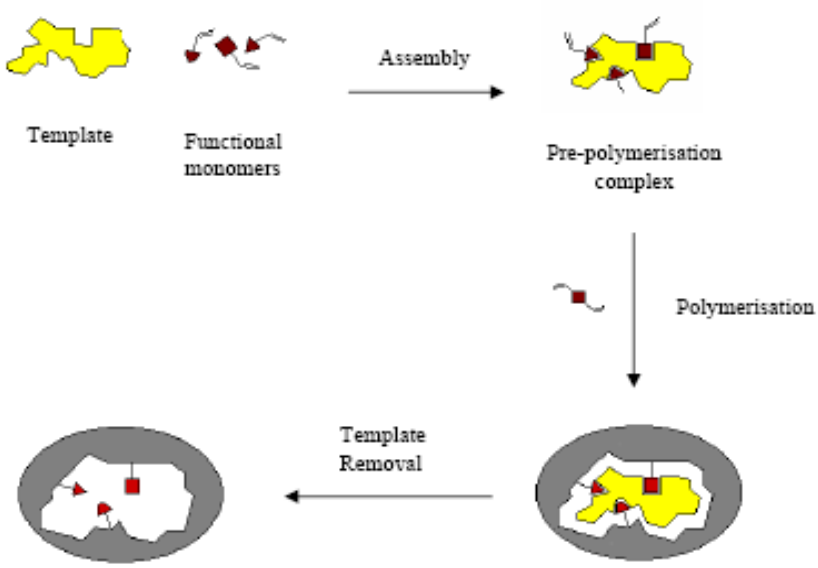

Figure 4.2. *Conventional molecularly imprinted polymers.*

MIPs were synthesised by bulk polymerisation using MAA and DVB as functional monomer and crosslinking agent, respectively, and two different ratios of template to functional monomer were tested (1/8 and 1/16 for MIP1 and MIP5, respectively). The imprinting effect of synthesised polymers was tested by binding experiments in organic (acetonitrile) and water (PBS pH 7.4, 0.01 M) media. Particularly, the recognition properties were evaluated by using template solutions, while the selectivity by mixing polymeric microparticles with the analogue teophylline (THEO).

In literature, different approaches were applied to make a quantitative determination of the imprinting effect[13]. Among them, the imprinting efficiency α is the easiest way to highlight the recognition properties in a MIP.

[13] Ye, L.; Mosbach, K. *Chem. Mater.* **2008**, *20*, 859.

In our work, α_{CAFF} and α_{THEO} were determined as the ratio between the amount (%) of CAFF and THEO bound by MIP and NIP[14], respectively. The selectivity of MIP can be highlighted by introducing another coefficient (ε), which is a quantitative measure of the imparted selectivity within the imprinted polymers and was calculated as the ratio between the amount (%) of template CAFF and THEO bound by MIP.

As shown in *Table 4.1*, in organic media, the most effective polymer was MIP5; with a higher amount of functional monomer in the pre-polymerisation complex, it is indeed possible to obtain more stable imprinted cavities (α_{CAFF} 3.4, α_{THEO} 0.8, ε 1.3).

In water media, no imprinting effect was recorded, due to the presence of much emphasised hydrophobic interactions between analytes and polymeric microparticles.

In the second step of this work, in order to improve the imprinting effect of polymers in water media, new kinds of imprinted materials with increased hydrophilic characteristics were prepared. The adopted synthetic strategy involved the use of a pro-hydrophilic co-monomer, GMA, inserted in the polymerisation feed after the formation of the pre-polymerisation complex. In this way it is possible to avoid that GMA interferes with the H-bonds formed between template and functional monomer and thus with the formation of imprinted cavities in the final materials (*Figure 4.3*).

To optimise the water compatibility of polymers, different amount of GMA (8.0; 12.0 and 16.0 mmol) were added to the polymerisation feeds of MIP1, MIP5 and of the corresponding non-imprinted polymers. Binding experiments in organic media were performed by using the resulting polymers, in order to confirm that GMA did not significantly interact with the template in the pre-polymerisation step and that the interactions between template and matrices are exclusively ascribable to MAA (*Table 4.1*). Also in this case, no imprinting effect was raised in water media due to the high hydrophobic characteristics of the materials.

[14] Gore, M.A.; Karmalkar, R.N.; KulKarni, M.G. *J. Chromatogr B* **2004**, *804*, 211.

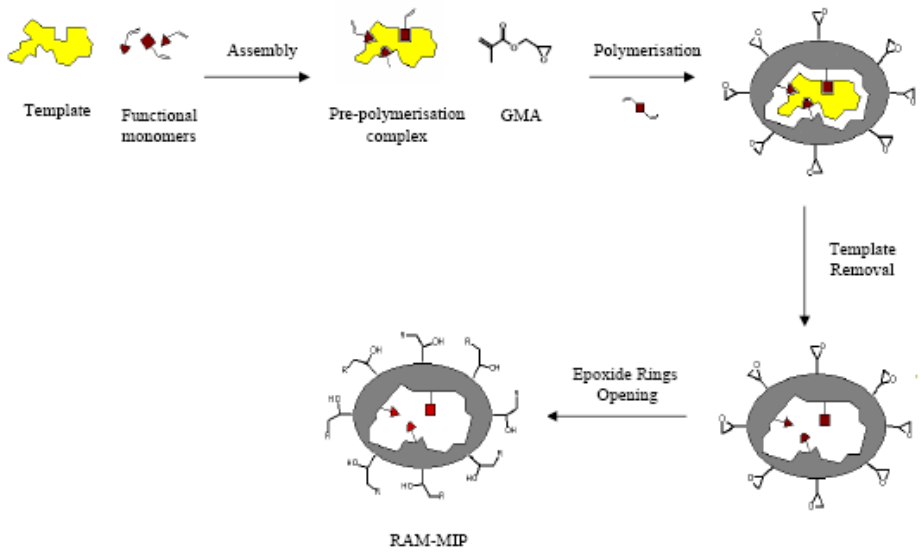

Figure 4.3. *Restricted access materials-molecularly imprinted polymers.*

3.2 Surface modification

In order to obtain the external hydrophilic layer around polymeric microparticles, GMA epoxide ring was opened by using several reactants and different hydrophilic layers around the polymeric microparticles can be obtained. In particular, perchloric acid, ammonia, lactic acid, glycine and triethylene glycol were chosen for epoxide ring opening reactions. In perchloric acid and ammonia cases, a further modification was performed by phosphorylation and methylation procedures, respectively. Reaction conditions and RAM code assignment are shown in *Table 4.2*.

The surface modifications are asked to do not damage the imprinted cavities in the polymeric structure, and thus, to do not negatively interfere with the recognition properties. For these reasons, binding experiments were carried out in organic media in the same conditions tested for the unmodified materials. In *Table 4.3*, data obtained from RAM-MIP8 and the corresponding RAM-NIP8 are shown. It was observed that binding percentages did not significantly change moving from unmodified MIP8 to RAM materials, except for RAM(F). In this

case, the phosphorylation process is responsive for matrix degeneration and thus a poor imprinting effect is observed.

3.3 Hydrophilic properties of RAM-MIPs

After the preliminary characterisation in organic media, hydrophilic properties of different polymers of RAM-MIP8 and RAM-NIP8 series by binding experiments in water media (*Table 4.3*) and water absorption measurement (*Table 4.5*) were evaluated.

Table 4.3. *Percentage of bound CAFF and THEO by RAM polymers obtained from MIP8 and NIP8 after 24 hours in acetonitrile and water. Data are shown as means ± SD.*

POLYMER	% Bound			
	CH_3CN		H_2O	
	CAFF	**THEO**	**CAFF**	**THEO**
RAM(A)-MIP8	27 ± 1.1	20 ± 1.2	80 ± 1.2	48 ± 1.3
RAM(A)-NIP8	7 ± 1.5	24 ± 1.1	46 ± 1.4	50 ± 1.1
RAM(B)-MIP8	28 ± 1.3	40 ± 1.3	77 ± 1.1	56 ± 1.2
RAM(B)-NIP8	10 ± 1.4	42 ± 1.5	53 ± 1.2	46 ± 1.3
RAM(C)-MIP8	26 ± 1.2	42 ± 1.4	83 ± 1.3	70 ± 1.5
RAM(C)-NIP8	9 ± 1.4	46 ± 1.1	70 ± 1.5	67 ± 1.3
RAM(D)-MIP8	30 ± 1.0	78 ± 1.3	64 ± 1.4	49 ± 1.4
RAM(D)-NIP8	10 ± 1.1	80 ± 1.2	57 ± 1.2	43 ± 1.0
RAM(E)-MIP8	27 ± 1.4	56 ± 1.5	82 ± 1.4	60 ± 1,4
RAM(E)-NIP8	9 ± 1.5	50 ± 1.1	70 ± 1.6	53 ± 1.1
RAM(F)-MIP8	33 ± 1.3	88 ± 1.2	94 ± 1.5	77 ± 1.0
RAM(F)-NIP8	25 ± 1.1	85 ± 1.3	88 ± 1.3	75 ± 1.3
RAM(G)-MIP8	35 ± 1.1	51 ± 1.0	63 ± 1.1	55 ± 1.1
RAM(G)-NIP8	13 ± 1.2	45 ± 1.1	54 ± 1.3	56 ± 1.2

The best imprinting effect was raised in RAM(A)-MIP8, in which hydroxyl groups obtained by reaction of GMA with perchloric acid make polymeric surface more hydrophilic, reducing the hydrophobic non-specific interactions between matrices and analytes without interfering with the

recognition properties of the imprinted cavities. The α and ε values in water clearly show the high imprinting efficiency recorded in these materials (*Table 4.4*).

Table 4.4. *α and ε values in aqueous media by RAM polymers obtained from MIP8 and NIP8.*

		RAM (A)	RAM (B)	RAM (C)	RAM (D)	RAM (E)	RAM (F)	RAM (G)
α	***CAFF***	1.8	1.5	1.2	1.1	1.2	1.1	1.2
	THEO	1.0	1.2	1.0	1.0	1.1	1.0	1.0
	ε	1.7	1.4	1.2	1.3	1.4	1.2	1.2

Furthermore, the increased water compatibility of RAM(A) polymers, confirmed by water absorption measurements, improved their recognition characteristics, because of the enhanced accessibility of template to the imprinted cavities.

Similar results were obtained by insertion of triethylene glycol on the polymeric surface (RAM(B)-MIP8), but in this case the efficiency is reduced compared to RAM(A)-MIP8 (*Table 4.4*). These results are consistent with the observed higher value of hydrophobic interactions and lower water regain data.

The presence in the external layer of carboxyl groups arising from lactic acid and glycine, carried out to materials (RAM(C)-MIP8 and RAM(E)-MIP8, respectively) in which lower water absorption properties corresponded to a lower specificity in template recognition; the binding percentage of RAM(C)-NIP8 and RAM(E)-NIP8 were indeed more relevant (*Table 4.3*).

No improvement in the polymer performance was raised by epoxide opening reaction with ammonia (RAM(D)-MIP8 and RAM(D)-NIP8), and neither by the subsequent methylation of the inserted aminic groups (RAM(G)-MIP8 and RAM(G)-NIP8). The two kinds of materials showed comparable water absorption values, but no imprinting effect was observed because of the

negative interference of the amminic groups on the recognition properties of the materials (*Table 4.4*).

In RAM(F)-MIP8 and RAM(F)-NIP8 cases, phosphorylation process of RAM(A)-MIP8 and RAM(A)-NIP8 carried out to un-effective matrices, probably due to the degradation of the polymeric structures as shown by the high water uptake values.

RAM polymers with lower MAA content (RAM-MIP4) showed no imprinting efficiency because of the poor amount of functional monomer, as for MIP1, although their hydrophilic properties were comparable with RAM-MIP8 polymers.

By performing the same epoxide ring reactions on the polymers with lower GMA content (MIP2, MIP3, MIP6, MIP7 and the corresponding non-imprinted polymers), no improvement in the polymer performance was raised. The obtained RAM polymers showed, indeed, lower hydrophilic characteristics, and thus no imprinting effect was recorded in water media.

Finally, a further increase in GMA content carried out to materials with very low recognition properties both in organic and in water media.

3.4 Protein binding

Water compatible materials are asked to slowly interact with proteins due to the presence of the hydrophilic external layer which avoids destructive protein deposition on the polymeric surfaces, while allows the interactions with smaller template.

Thus, to prove the usefulness of the hydrophilic materials, non-specific hydrophobic adsorption of protein on the different matrices was estimated by injecting 2.0 mL of an HSA standard solution (1.2 mg/mL) on SPE columns packed with MIP8 and with all the polymers of RAM-MIP8 series.

According to the results in *Table 4.5*, all the RAM-MIP8 series showed lower protein absorption than MIP8. This is a further evidence of the most hydrophilic character of RAM series that makes this kind of materials less susceptible to fouling by proteins.

Table 4.5. *Hydrophilic properties of polymers: water content (%) and percentage of bound HSA. Data are shown as means ± SD.*

POLYMER	Water Content (%)	Bound HAS (%)
MIP8	3 ± 1.3	87 ± 1.9
RAM(A)-MIP8	189 ± 2.1	4 ± 1.5
RAM(B)-MIP8	176 ± 1.9	8 ± 1.2
RAM(C)-MIP8	147 ± 2.0	1 ± 0.6
RAM(D)-MIP8	140 ± 1.8	18 ± 1.9
RAM(E)-MIP8	142 ± 2.2	4 ± 1.2
RAM(F)-MIP8	220 ± 2.1	1 ± 0.7
RAM(G)-MIP8	136 ± 1.7	25 ± 1.1

RAM(D)-MIP8 and RAM(G)-MIP8, due to the presence of free amminic groups on their surface, were the RAM-MIP8 polymers showing the most relevant interactions with HSA.

3.5 Regeneration properties

In order to obtain the reusability of the synthesised materials, the absorption-desorption cycle was repeated five times by using the same MIP. The absorption capacity of all the polymeric materials for the template did not significantly change during the repeated absorption-desorption operations.

4. Conclusions

New Restricted Access Materials-Molecularly Imprinted Polymers (RAM-MIPs) able to work in physiological environment were successfully prepared by using GMA as pro-hydrophilic co-monomer.

Conventional MIPs, without GMA, were prepared as control materials and a screening of different ratio of functional monomer to template was performed. After this preliminary work, RAM-MIPs were obtained by GMA epoxide ring opening reactions. In this way, a hydrophilic external layer around polymeric microparticles was formed, and different reaction conditions were tested for this purpose.

The best reaction conditions involved the use of perchloric acid, while triethylene glycol, glycine and lactic acid showed lower efficiency. On the other hand, the introduction of amminic or phosphoric groups in the external layer was ineffective.

In perchloric acid case, the formed hydroxyl groups make polymeric surface more hydrophilic, reducing the hydrophobic non-specific interactions between matrices and analytes without interfering with the recognition properties of the imprinted cavities.

The increased water compatibility of RAM polymers was confirmed by the increased water absorption capacity and by protein binding experiments, in which a very relevant reduction of destructive protein deposition on the polymeric surfaces was observed.

Finally, absorption-desorption cycles confirm the regeneration properties of the proposed RAM-MIPs.

CHAPTER V

New Restricted Access Materials combined to Molecularly Imprinted Polymers for Selective Recognition/Release in Water Media

1. Introduction

Most of the developed imprinting protocols can be successfully used to produce molecularly imprinted polymers (MIPs) for recognition of a large range of guest molecules predominantly in organic solvent-based media, while they often fail to generate MIPs for use in pure aqueous environments[1]. This depends on the non-specific hydrophobically driven bonds between template and surface of materials. In addition, biological sample components, such as proteins and lipids, are strongly adsorbed to the polymeric surfaces, negatively interfering with their recognition properties[2]. Thus, in order to obtain MIPs able to work in aqueous media, such as biological fluids or environmental waters, a considerable reduction of these non-specific interactions is required[3].

For this purpose, different methodologies were developed[4,5].

Our approach was to use a monomer that less interferes in the pre-polymerisation complex formation, but able, at the same time, after a post-polymerisation straightforward modification, to impart water compatibility to the system. Glycidyl methacrylate (GMA) is useful for this purpose because its oxygen atom, bounded to two carbons, has lower capacity to form hydrogen bonds than a free hydroxy group. Furthermore, the epoxide ring opening induces a hydrophilic behaviour to the imprinted particles (*Figures 5.1 and 5.2*).

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[1] Puoci, F.; Curcio, M.; Cirillo, G.; Iemma, F.; Spizzirri, U.G.; Picci, N. *Food Chem.* **2008**, *106*, 836.
[2] Boos, K.S.; Fleischer, C.T. *J. Anal. Chem.* **2001**, *371*, 16.
[3] Bures, P.; Huang, Y.; Oral, E.; Peppas , N.A. *J. Contr. Rel.* **2001**, *72*, 25.
[4] Mullett, W.M.; Pawliszyn, J. *J. Sep. Sci.* **2003**, *26*, 251.
[5] Sambe, H.; Hoshina, K.; Haginaka, J. *J. Chromatogr. A* **2007**, *1152*, 130.

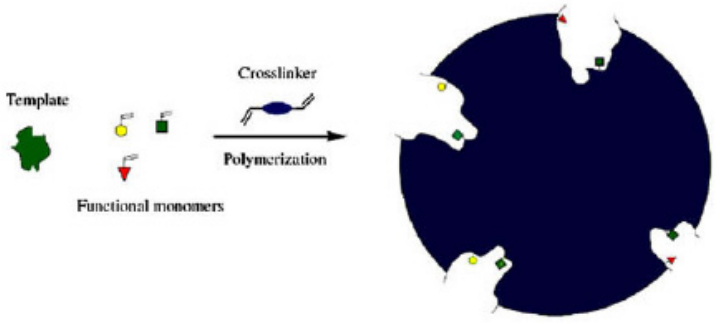


Figure 5.1. *Traditional molecularly imprinted polymers.*

In this work, the possibility to employ restricted access materials-molecularly imprinted polymers (RAM-MIPs) in the recognition and controlled/sustained release of a common anti-inflammatory drug, such as p-acetaminophenol (paracetamol, *Figure 5.3*), was investigated[6]. In the synthetic protocol, methacrylic acid (MAA) was chosen as functional monomer, divinyl benzene (DVB) as crosslinking agent and GMA as pro-hydrophilic co-monomer in one-pot polymerisation procedure.

The recognition properties of RAM-MIPs were compared to MIPs, synthesised without GMA insertion, both in organic and in water media. Binding experiments were also performed to determine the imprinting properties of RAM-MIPs before epoxide ring opening. Finally, the release profile of p-acetaminophenol from RAM-MIPs in gastrointestinal simulating fluids was evaluated.

[6] Puoci, F.; Iemma, F.; Cirillo,G.; Curcio, M.; Parisi, O.I.; Spizzirri, U.G.; Picci, N. *Eur. Polym. J.* **2009**, *45*, 1634.

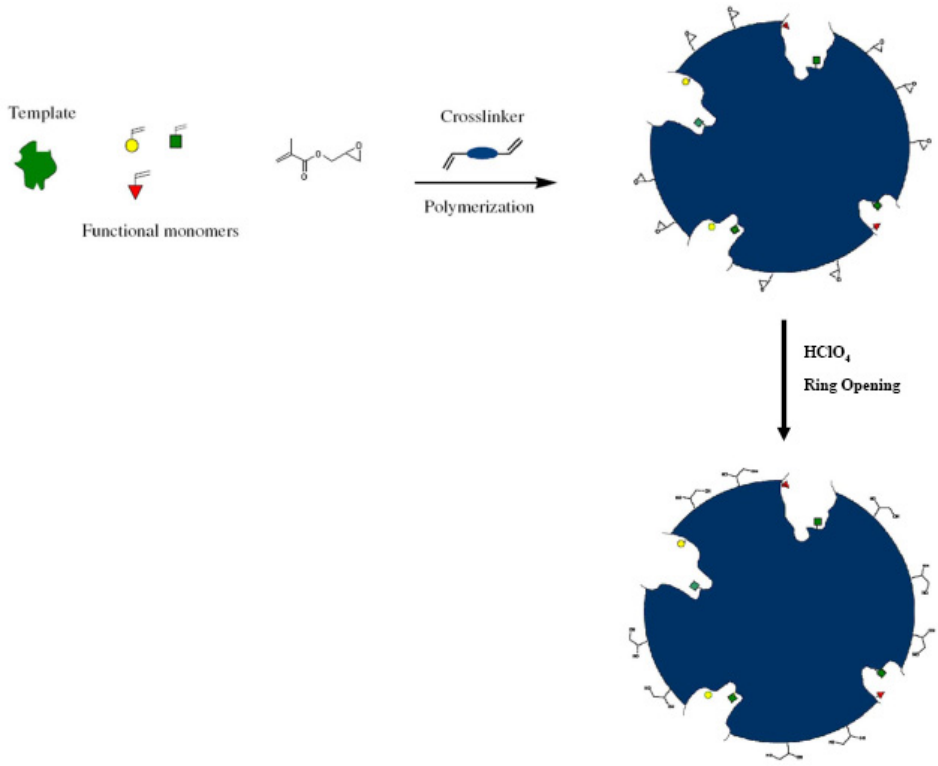


Figure 5.2. *Restricted access materials-molecularly imprinted polymers.*

2. Materials and methods

2.1 Materials

Divinyl benzene (DVB), methacrylic acid (MAA), glycidyl methacrylate (GMA), 2,2'-azoisobutyronitrile (AIBN), perchloric acid, p-acetaminophenol (AMP), p-aminophenol (AP), N-phenylacetamide (PA), diflunisal (DF) were purchased from Aldrich. Bovine serum albumin (BSA) fraction V (MW 68,000; pH 7.0 ± 0.2; grade ≥ 98%) was from Roche Diagnostics GmbH.

All solvents were reagent grade or HPLC-grade and used without further purification and they were provided by Fluka Chemie.

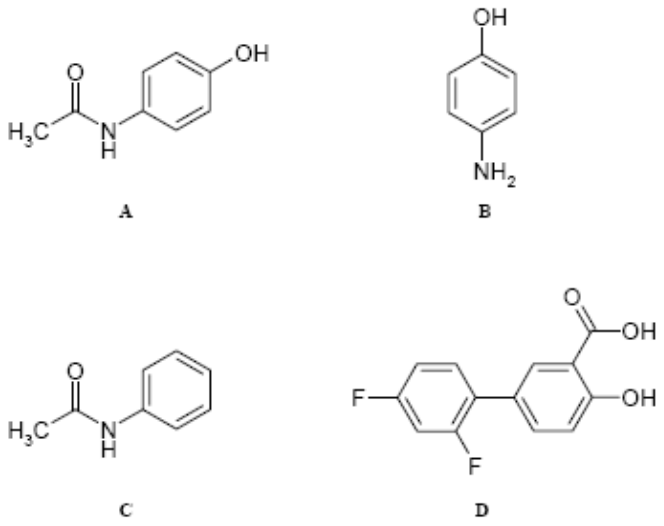


Figure 5.3. *Chemical structures of p-acetaminophenol (A), p-aminophenol (B), N-phenylacetamine (C), diflunisal (D).*

2.2 Instrumentation

HPLC was performed using a Jasco Model (Tokyo, Japan) consisting of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 254 nm. A 250 x 4 mm C-18 Hibar® column, particle size 10 μm (Merck, Darmstadt, Germany) was employed. The mobile phase was acetonitrile/phosphate buffer 5 mM, pH 4.5 (3/7, v/v) and the flow rate was 0.5 mL/min.

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. 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.

2.3 Synthesis of p-acetaminophenol spherical molecularly imprinted polymers

The spherical imprinted polymers were prepared by precipitation polymerisation using p-acetaminophenol as template, MAA as functional monomer, DVB as crosslinking agent and GMA as co-monomer.

General synthetic procedure was reported: template and MAA were dissolved in a mixture of acetonitrile (33 mL) and toluene (11 mL), in a 100 mL round bottom flask and then DVB, GMA and AIBN (50 mg) were added. The polymerisation 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 100 mL of diethyl ether. The template was extracted by ‘‘Soxhlet apparatus” using ethanol and monitoring the drug concentration in the extraction solvent by HPLC. Particles were successively dried under vacuum overnight at 40°C.

The formulations used for the preparation of the different matrices are shown in *Table 5.1*.

Non-imprinted polymers (NIPs), to act as a control, were also prepared when polymerisation was carried out in the absence of p-acetaminophenol.

Table 5.1. *Polymerisation feeds composition and mean particle sizes (d_n) of microspheres expressed as means ± SD.*

POLYMER	AMP/MAA/GMA/DVB (mmol)	d_n (µm)
MIP-1	1.0/4.0/--/24.0	2.46 ± 0.09
NIP-1	--/4.0/--/24.0	2.61 ± 0.11
MIP-2	1.0/4.0/2.0/24.0	2.54 ± 0.14
NIP-2	--/4.0/2.0/24.0	2.87 ± 0.13
MIP-3	1.0/4.0/4.0/24.0	2.49 ± 0.12
NIP-3	--/4.0/4.0/24.0	2.66 ± 0.10

2.4 Epoxide ring opening

In a 100 mL round bottom flask, 400 mg of MIP-3 and NIP-3 were added to 25 mL of a perchloric acid water solution (10% v/v). The flask was then agitated (200 rpm) for 24 h at room temperature. At the end of the reaction, the particles were filtered, washed with 100 mL of ethanol, 100 mL of acetone, 100 mL of diethyl ether and then dried under vacuum overnight at 40°C.

2.5 Water content of spherical polymer

The swelling characteristics of polymers were determined in order to check increased hydrophilic affinity of RAM-MIP-3 and RAM-NIP-3 compared to MIP-3 and NIP-3.

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: HCl 0.1 N (pH 1, simulated gastric fluid) and phosphate buffer (pH 6.8, simulated intestinal fluid). At a predetermined time (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 were averaged and used to give the water content percent (WR %) by the following equation (1):

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

where W_s and W_d are weights of swollen and dried spherical microparticles, respectively.

Each experiment was carried out in triplicate.

2.6 Binding experiments

Binding experiments were performed both in organic (acetonitrile) and in water media.

Briefly, 50 mg of polymer particles were mixed with 1 mL of an AMP solution (0.2 mM) in a 1 mL eppendorf and sealed. Samples were shaken in a water bath at 37 ± 0.5°C for 24 h, centrifuged for 10 min (10,000 rpm) and the AMP concentration in the liquid phase was measured by HPLC. The amount of AMP bound to the polymer was obtained by comparing its concentration in the imprinted samples to the non-imprinted samples.

The same experiments were performed using AP, PA (analogues) and DF (*Figure 5.3*) solutions.

Experiments were repeated five times.

2.7 Protein binding

Two hundred milligrams of dry particles of MIP-3, NIP-3, RAM-MIP-3 and RAM-NIP-3 were packed into a 6.0 mL polypropylene SPE column. The columns were attached with a stop cock and a reservoir at the bottom end and the top end, respectively.

Before use, the columns were preconditioned by successive washings with water, HCl (0.07 M), water, methanol/water (50/50 v/v), water, and finally 25 mM phosphate buffer (pH 7.4).

The adsorption test was performed by loading the cartridge with 2.0 mL of a BSA standard solution (1.2 mg/mL) in 25 mM phosphate buffer at pH 7.4.

The amount of adsorbed protein after loading step was calculated by UV-Vis spectrophotometer at 290 nm.

Experiments were repeated five times.

2.8 Drug loading by soaking procedure

Two grams of RAM-MIP-3 and RAM-NIP-3 particles were immersed in 20 mL of an AMP solution (5.5 mM) and soaked for 3 days at room temperature

with stirring. Then the solvent was evaporated under vacuum and the powders dried overnight at 40°C.

2.9 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 0.1 N HCl (pH 1.0, simulating the gastric fluid) and maintained at 37 ± 0.5°C in a water bath for 2 h with stirring (50 rpm). Sodium phosphate was then added to the samples to adjust pH to 6.8 (simulating intestinal fluid). These conditions were maintained throughout the experiment. Samples were drawn from dissolution medium at appropriate time intervals to determine the amounts of released drug by HPLC. The amount of AMP released from six samples of each formulation was used to characterise drug release.

Experiments were repeated five times.

3. Results and discussion

3.1 Synthesis of imprinted polymers

For the synthesis of p-acetaminophenol molecular imprinting polymers, the employed imprinting approach was the non-covalent one, in which the interactions between template molecule and polymeric matrices were based on H-bonds, the dominant interactions in biological systems[7]. Furthermore, this approach is more convenient because of the easy access to a broad range of functional monomers from commercial sources[8].

To synthesise MIPs, different procedure were proposed and the most widely used is the bulk copolymerisation. Moreover, this technique shows some limitations: first, it carries out to polymers that have to be ground and sieved to obtain imprinted particles with irregular shape; second, typically less than 50%

[7] Cirillo, G.; Iemma, F.; Puoci, F.; Parisi, O.I.; Curcio, M.; Spizzirri, U.G.; Picci, N. *J. Drug Target.* **2009**, *17*, 72.
[8] Alexander, C.; Andersson, H.S.; Andersson, L.I.; Ansell, L.J.; Kirsch, N.; Nicholls, I.A.; O'Mahony, J.; Whitcombe, M.J. *J. Mol. Recognit.* **2006**, *19*, 106.

of the ground polymer is recovered as useable particles; finally, during the grinding process some recognition sites are destroyed[9].

For these reasons, in this work the chosen synthetic procedure was the precipitation polymerisation, which allows to obtain spherical and practically monodispersed polymeric particles without any ground process. In this case, the integrity of the recognition sites is preserved improving the imprinting effect, and spherical shaped microparticles offer a better control on drug release profile.

In our protocol, MAA was used as functional monomer and DVB as crosslinker. GMA was added after the formation of the pre-polymerisation complex between template and functional monomer. In this way, it is possible to avoid the interference of this co-monomer with the H-bonds formed between template and functional monomer and thus with the creation of imprinted cavities in the final materials. Spherical geometry and the practically monodispersion of prepared samples were confirmed by Scanning Electron Micrographs (*Figure 5.4a*) and dimensional analysis.

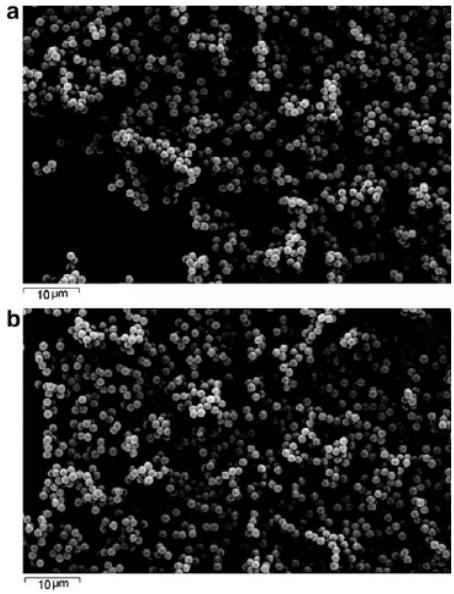


Figure 5.4. Scanning electron micrographs of MIP-3 (a) and RAM-MIP-3 (b).

[9] Oxelbark, J.; Legido-Quigley, C.; Aureliano, C.S.A.; Titirici, M.M.; Schillinger, E.; Sellergren, B.; Courtois, J.; Irgum, K.; Dambies, L.; Cormack, P.A.G.; Sherrington, D.C.; De Lorenzi, E. *J. Chromatogr. A* **2007**, *1160*, 215.

Polymerisation feeds composition and mean particle sizes (d_n) of microspheres are shown in *Table 5.1*.

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

The imprinting effect of the synthesised materials was evaluated by binding experiments in which amounts of polymeric particles were incubated with a p-acetaminophenol solution 0.2 mM for 24 hours. These preliminary experiments were performed in organic media (acetonitrile).

As it is possible to note in *Table 5.2*, the synthesised materials showed good recognition properties: MIP-1, MIP-2 and MIP-3, indeed, were able to bind much more template than the respective non-imprinted ones, confirming the presence of imprinted cavities in their structure.

Table 5.2. *Percentage of bound AMP, AP and PA by the imprinted and non-imprinted polymers after 24 h in acetonitrile. Data are shown as means ± SD.*

POLYMER	% bound AMP	α_{AMP}	% bound AP	α_{AP}	% bound PA	α_{PA}
MIP-1	42.6 ± 1.4	2.1	32.7 ± 1.1	1.2	20.3 ± 1.7	1.0
NIP-1	20.2 ± 1.1		27.9 ± 1.2		20.6 ± 1.0	
MIP-2	44.6 ± 1.3	1.6	34.3 ± 1.2	1.1	22.8 ± 1.6	1.0
NIP-2	27.3 ± 1.1		29.3 ± 1.5		23.3 ± 1.4	
MIP-3	48.8 ± 1.7	2.8	38.5 ± 1.5	1.4	24.5 ± 1.8	1.1
NIP-3	17.4 ± 1.0		27.7 ± 1.2		21.3 ± 1.6	
RAM-MIP-3	43.1 ± 1.9	2.8	33.2 ± 1.3	1.0	15.7 ± 1.4	1.2
RAM-NIP-3	15.6 ± 1.5		33.1 ± 1.7		13.3 ± 1.2	

In literature, different approaches were applied to make a quantitative determination of the imprinting effect[10]. The imprinting efficiency α is the

[10] Ye, L.; Mosbach, K. *Chem. Mater.* **2008**, *20*, 859.

easiest way to highlight the recognition properties in a MIP. In our work, α_{AMP} was determined as the ratio between the amounts (%) of AMP bound by MIP and NIP[11]; these values (*Table 5.2*) clearly prove the specificity of the interaction between the template and the functional groups on the polymeric particles.

To evaluate cross-reactivity of imprinted polymers towards AMP analogues molecules, the same binding experiments using p-aminophenol (AP) and N-phenylacetamide (PA) were performed and good selectivity properties were found in all the imprinted materials. As reported for AMP, α_{AP} and α_{PA} were also determined as ratio between AP and PA bound by MIP and NIP, respectively. The very low values (*Table 5.2*) show the high chemical and spatial complementarity of MIP binding sites toward the template, while the affinity for the analogue is very low. Furthermore, the percentages of bound template by MIP-1, MIP-2 and MIP-3 were very similar, confirming the theory that GMA did not significantly interact with the template in the pre-polymerisation step and, consequently, did not interfere with the recognition properties of the synthesised materials which are exclusively ascribable to MAA. Moreover, higher GMA amount in the polymerisation feed composition (MIP-4) carried out to materials with no recognition properties.

3.3 Surface modification

For the preparation of water compatible MIPs, matrices with the highest imprinting effect (MIP-3 and corresponding NIP-3) were chosen as starting materials.

As before explained, epoxide ring opening with perchloric acid allows to obtain a hydrophilic layer around the imprinted particles (RAM-MIP-3 and RAM-NIP-3), but this step is asked to do not damage the imprinted cavities in the polymeric structure and the spherical shape of the sample (*Figure 5.4b*).

To evaluate the effect of GMA hydrolysis on the recognition properties, binding experiments were also performed using RAM polymers in organic

[11] Gore, M.A.; Karmalkar, R.N.; KulKarni, M.G. *J. Chromatogr. B* **2004**, *804*, 211.

media. These experiments were performed in the same condition of the previous ones, and the data are shown in *Table 5.2*.

According to our hypothesis, epoxide ring opening did not carry out to a decrease in recognition and selectivity properties which remained quite similar.

3.4 Evaluation of the imprinting effect: binding experiments in water media

After the preliminary characterisation in organic media, binding experiments in water were performed by comparing polymers of MIP-3 and RAM-MIP-3 series.

As shown in *Table 5.3*, relevant imprinting effect was observed in RAM-MIP-3 (α_{AMP} equal to 1.3) while MIP-3 was ineffective.

Table 5.3. *Percentage of bound AMP, AP, PA and DF by the imprinted and non-imprinted polymers after 24 h in water media. Data are shown as means ± SD.*

POLYMER	% bound AMP	α_{AMP}	% bound AP	α_{AP}	% bound PA	α_{PA}
MIP-3	92.3 ± 1.1	1.0	67.5 ± 1.1	1.0	96.4 ± 1.2	1.0
NIP-3	91.6 ±1.8		63.8± 0.9		95.7 ± 1.6	
RAM-MIP-3	88.4 ± 1.4	1.3	37.2 ± 1.2	1.0	77.2 ± 1.8	1.0
RAM-NIP-3	68.2 ± 1.3		34.5 ± 1.0		78.3 ± 1.6	

3.5 Water content of spherical polymers

The swelling characteristics of polymers were determined immersing aliquots of microparticles in swelling media: HCl 0.1 N (pH 1, simulating gastric fluid) and phosphate buffer (pH 6.8, simulating intestinal fluid). The WR (%) for all prepared materials are reported in *Table 5.4*.

Table 5.4. *Hydrophilic properties of polymers: water content (%) at pH 1.0 and 6.8 and percentage of bound BSA and DF. Data are shown as means ± SD.*

POLYMER	Water Content (%)		% Bound BSA	% Bound DF
	pH 1.0	pH 6.8		
MIP-3	5.3 ± 1.1	7.3 ± 1.7	60.1 ± 1.9	80.5 ± 2.1
NIP-3	6.1 ± 1.1	5.1 ± 1.2	61.2 ± 1.1	81.5 ± 1.2
RAM-MIP-3	31 ± 0.9	40.4 ± 1.4	4.3 ± 1.2	45.2 ± 1.8
RAM-NIP-3	31.4 ± 1.4	42.1 ± 1.1	5.1 ± 1.1	44.5 ± 1.1

Experimental data confirmed that polymeric microparticles with the external hydrophilic layer were able to absorb relevant amount of water. The values at the different tested pH were quite similar, confirming that water absorption depends on alkoxy groups formed after epoxide ring opening.

3.6 Protein binding

To prove the usefulness of the hydrophilic materials, non-specific hydrophobic adsorption of protein on the different matrices was estimated by injecting 2.0 mL of a BSA standard solution (1.2 mg/mL) on SPE columns packed with MIP-3, RAM-MIP-3 and the corresponding non-imprinted particles[12].

According to the results in *Table 5.4*, RAM-MIP-3 and RAM-NIP-3 showed the lowest protein absorption. This agrees with the expected order of decreasing hydrophobicity where polymers of RAM series possessed the most hydrophilic character and could be less susceptible to fouling by proteins.

The increased hydrophilic properties of RAM materials were also confirmed by performing binding experiment in water using DF. This molecule is structurally different from the template, thus the interactions with matrices

[12] Dirion, B.; Cobb, Z.; Schillinger, E.; Andersson, L.I.; Sellergren, B*J. Am. Chem. Soc.* **2003**, *125*, 1501.

depend just on the hydrophilic/hydrophobic properties of polymers. The binding percentage remarkably decreases moving from MIP-3 to RAM-MIP-3 as shown in *Table 5.4*.

3.7 In vitro release studies

As explained, with RAM-MIPs, because of their restricted access, large molecules, such as proteins, are not destructively accumulated on polymeric surface, while small molecules, such as drugs, can reach the imprinted sites. For this reason, RAM polymers were tested as controlled release device in gastrointestinal simulating fluids.

In vitro release studies were performed immersing aliquots of microparticles loaded with AMP at pH 1.0 (simulating gastric fluid) for 2 h and then at pH 6.8 (simulating intestinal fluid) using the pH change method[13].

Different strategies are applied to determine the possibility to successfully employ a MIP to obtain a sustained/controlled release of the selected drug[14].

In a first approach[15], 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 MIP and NIP according to the imprinting effect. In these conditions, because the releasing profile strongly depends on the loaded drug, MIP particles are able to release a higher amount of drug than NIP ones.

In another approach, more useful to emphasise the differences in the releasing profiles from MIP and NIP, both the polymers are loaded with the same amount of drug by mixing them with a drug standard solution. After the incubation time, samples are dried and release experiments performed. The

[13] Puoci, F.; Iemma, F.; Cirillo, G.; Picci, N.; Matricardi, P.; Alhaique, F. *Molecules* **2007**, *12*, 805.
[14] Puoci, F.; Iemma, F.; Picci, N. *Curr. Drug Del.* **2008**, *5*, 85.
[15] Singh, B.; Chauhan, N. *J. Macromol. Sci. A Pure Appl. Chem.* **2008**, *45*, 776.

percentage of released AMP is calculated considering 100% the AMP content in dried samples[16].

In our work, the second approach was employed and the presence of imprinted cavities in the imprinted polymers makes the release of template more extended over time in comparison with non-imprinted materials.

Our hypothesis was that RAM-MIP-3 has a better ability in controlling drug (AMP) release comparing to RAM-NIP-3. This effect is ascribable to the presence of specific binding sites in the polymeric network which slowly release the drug. The experimental data (*Figure 5.5*) confirm this theory; the drug release from RAM-NIP-3 was remarkably faster than that observed from RAM-MIP-3.

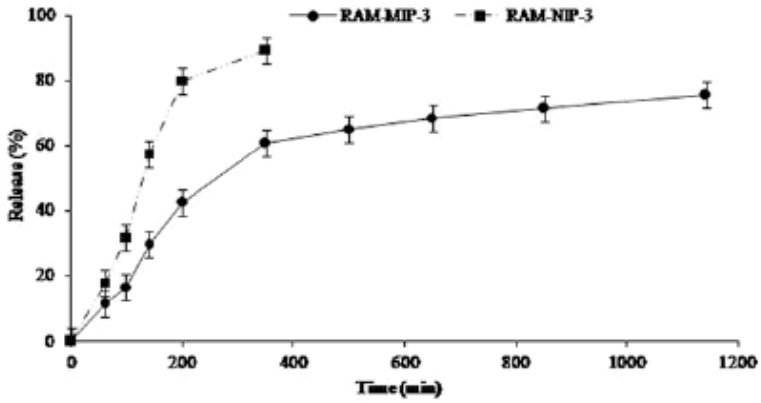


Figure 5.5. *Release profile of p-acetaminophenol from RAM-MIP-3 (●) and RAM-NIP-3 (■) in gastrointestinal simulating fluids. Data are shown as means ± SD.*

In particular, while in the first case the drug was completely released within 400 minutes, at the same time only 60% of AMP was released from RAM-MIP-3 samples and only after 1500 minutes the release was completed.

The differences in the release profile from the two kinds of polymers are ascribable to the different recognition properties of the polymeric matrices. In

[16] Cirillo, G.; Iemma, F.; Puoci, F.; Parisi, O.I.; Curcio, M.; Spizzirri, U.G.; Picci, N. *J. Drug Target.* **2009**, *17*, 72.

the imprinted cavities of RAM-MIP-3, formed during the polymerisation procedure, AMP is strongly bound by non-covalent interactions and the release is retarded. The retention mechanism assumes that acid groups of selective sites have stronger interaction with drug than the non-selective sites. At low pH values (1.0) the carboxylic groups are not ionised and, thus, there is a good interaction with the template. These results might help to understand also the behaviour of these matrices when the pH increases. In these conditions, that simulate the intestinal fluid, in the non-imprinted polymers drug is bound by non-covalent interactions on the surface of the matrices. At pH 6.8 the diffusion rate of the buffer, on the polymer surface, is fast; the carboxylic groups are ionised, and the drug is rapidly released. Instead, the diffusion rate of the buffer into specific cavities of imprinted polymers is slower and the functional groups are ionised more slowly, obtaining a good control of release.

4. Conclusions

Restricted access materials-molecularly imprinted polymers for the selective recognition and the controlled/sustained release of AMP were successfully synthesised by employing a two-step procedure.

In the first step, a typical precipitation polymerisation process was performed using p-acetaminophenol as template, MAA as functional monomer, DVB as crosslinker and GMA as co-monomer, respectively.

The second step was the GMA epoxide ring opening with the formation of a hydrophilic external layer on the polymeric surface. With this reaction, it is possible to modify hydrophobic matrices in more water compatible ones.

These materials are more suitable to be employed in biological media because of the reduction of non-specific hydrophobic interactions.

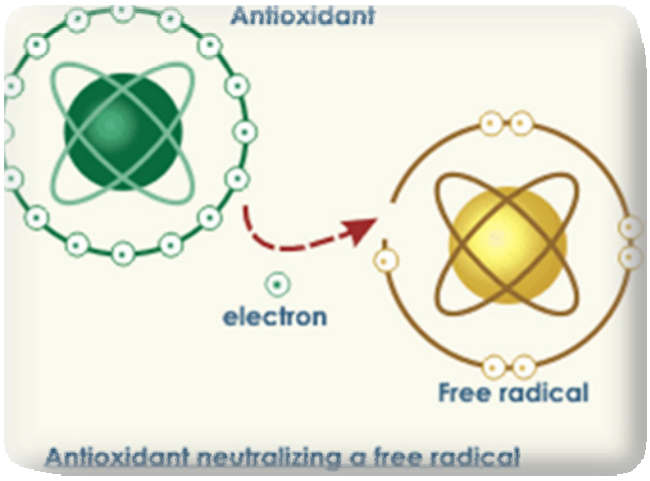
The increased water compatibility of RAM polymers was confirmed by water absorption measurement and protein binding experiments.

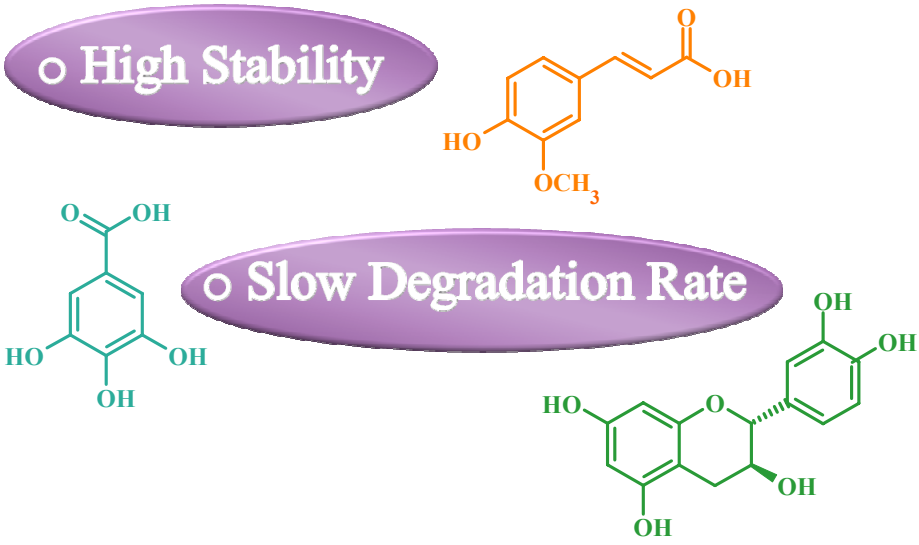
The applicability of this kind of materials as Drug Delivery System was then evaluated. After drug loading, in vitro release experiments were performed, and the results showed the ability of RAM polymers to control AMP release in

gastrointestinal simulating fluids, supporting a release mechanism in which the diffusion rate of the drug from the matrices depends on the selective interaction between drug and imprinted cavities. For this reason, the rate of the release was considerably different, and RAM-MIPs represent a very promising polymeric device for the selective and controlled release of drugs in gastrointestinal simulating fluids.

SECTION II

ANTIOXIDANT POLYMERS





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 ($^{-}O_2^{\bullet}$), 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 focused on the use of antioxidants to inhibit lipid peroxidation, or to protect against the damage of free radicals[5].

Polymeric antioxidants are a particular class of systems characterised by higher stability and slower degradation rate than compounds with low molecular weight[6,7].

Antioxidants with low molecular weight, indeed, are less effective in protecting against free radical damages owing to their poor thermal- and photo-stability. To overcome this limitation, a useful technique is the covalent linkage of these molecules on a polymeric matrix.

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[1] Moskovitz, J.; Yim, M.B.; Chock, P.B. *Arch. Biochem. Biophys.* **2002**, *397*, 354.
[2] Nordberg, J.; Arner, E.S.J. *Free Radical Biol. Med.* **2001**, *31*, 1287.
[3] Auroma, O.I. *Food Chem. Toxicol.* **1994**, *32*, 671.
[4] Halliwell, B.; Gutteridge, J.M.C.; Auroma, O.I. *Anal. Biochem.* **1987**, *165*, 215.
[5] Vendemiale, G.; Grattagliano, I.; Altomare, E. *Int. J. Clin. Lab. Res.* **1999**, *29*, 49.
[6] Kurisawa, M.; Chung, J.E.; Uyama, H.; Kobayashi, S. *Biomacromolecules* **2003**, *4*, 1394.
[7] Pan, J.Q.; Liu, N.C.; Lau, W.W.Y. *Polym. Degrad. Stab.* **1998**, *62*, 165.

Comparing to the antioxidant non-covalent absorption on a polymer system, this is a more advantageous approach because the effects of the antioxidant migration and blooming from the host polymer by mechanical rubbing-off, volatilization or leaching are avoided[8].

Antioxidant polymers could be applied in those fields in which the employment of a single molecule with antioxidant activity is prohibitive[9]; for example, they can be used in haemodialysis application, in particular by their introduction in dialysis membranes. Haemodialysis patients, indeed, are exposed to oxidative stress which contributes to cardiovascular disease and accelerated atherosclerosis, the major causes of mortality in these patients[10]. Another field of application of this kind of materials could be cosmetic formulations, in order to avoid the oxidation of their components, but can also be used as preservative agents in food packaging.

[8] Tseng, T.W.; Tsai, Y.; Lee, J.S.; *Polym. Degrad. Stabil.* **1997**, *58*, 241.
[9] Iemma, F.; Cirillo, G.; Puoci, F.; Trombino, S.; Castiglione, M.; Picci, N. *J. Pharm. Pharmacol.* **2007**, *59*, 597.
[10] Calo, L.A.; Naso, A.; Pagnin, E.; Davis, P.A.; Castoro, M.; Corradin, R.; Riegler, R.; Cascone, C.; Huber, W.; Piccoli, A. *Clin. Nephrol.* **2004**, *62*, 355.

CHAPTER VI

Synthesis of a Novel Polymeric Materials with Biological Properties

1. Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (*Figure 6.1*) is one of the most abundant phenolic acids in plants[1,2]. This compound, that is rarely found in the free form, is usually found as ester cross-linked with polysaccharides in the cell wall, such as arabinoxylans in grasses, pectin in spinach and sugar beet and xyloglucans in bamboo[3]. This molecule can also cross-link proteins[4]. 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[5], pharmaceutical[6], food[7] and cosmetic applications[8].

In recent years, there have been an increasing number of reports on the physiological functions of ferulic acid and its derivatives in human, including antioxidant, antifungal, anti-inflammatory, anti-thrombosis, and anti-cancer activities[9,10,11,12].

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Free ferulic acid is a good antioxidant because of its ability to donate H-atoms of phenolic hydroxyl groups in reaction with peroxyl radicals. In this way stabilised phenoxyl radicals are produced, thus terminating lipid peroxidation chain reactions[13].

This compound, as well as phenolic acid derivatives, is also known to play an important role for the antifungal activity[14]. It could potentially serve as effective alternative to conventional antifungal agents which are frequently perceived to present hazard to human health and environment[15].

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[16].

Moreover, ferulic acid also constitutes the active ingredient in many skin lotions and sunscreens designed for photoprotection[17].

Ultraviolet (UV) radiation, indeed, is well known to exert a variety of deleterious effects on human skin. Chronic and possibly acute exposure to UV radiation increases the risk of skin cancer including basal cell and squamous cell carcinoma as well as malignant melanoma[18].

In recent years, highly advanced synthetic sunscreens have been developed which contains physical and chemical UV filters[19]. These sunscreens have proven to be quite effective, in particular when used for the prevention of the erythema reaction to human skin. Several synthetic sunscreen agents, such as octabenzone, provatene, avobenzone, have been used since years, but studies

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show the possibility of adversely effects on human skin via self inducing reactive oxygen species[20]. Thus, to overcome these serious effects, in recent years the attention is focused on natural biomaterials such as antioxidants from natural sources.

For all these reasons, a polymeric material containing ferulic acid could be very interesting from an industrial point of view.

This work reports on a novel and simple method, involving one-step reaction, to obtain polymers through the direct reaction of an antioxidant molecule and a suitable monomer in presence of water soluble redox initiators. In particular, methacrylic acid (*Figure 6.1*) and ferulic acid were employed as functional monomer and antioxidant molecule, respectively, to obtain a useful material with both antioxidant[21] and antifungal[22] properties.

Furthermore, the possibility to employ the synthesised polymer as photo-protective agent in sunscreen formulations was proposed[23].

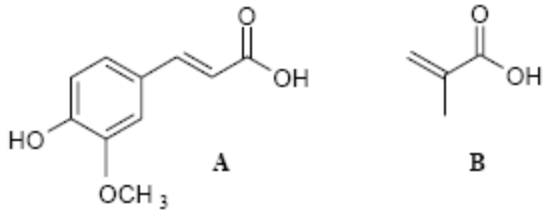

Figure 6.1. *Chemical structures of ferulic acid (A) and methacrylic acid (B).*

2. Materials and methods

2.1 Materials

Ferulic acid (FA), methacrylic acid (MAA), N,N-dimethylformamide (DMF), hydrogen peroxide (H_2O_2), ascorbic acid (AA), linoleic acid, disodium

[20] Palm, M.D.; O'Donoghue, M.N. *Dermatol. Therapy* **2007**, *20*, 360.
[21] Puoci, F.; Iemma, F.; Curcio, M.; Parisi, O.I.; Cirillo, G.; Spizzirri, U.G.; Picci, N. *J. Agric. Food Chem.* **2008**, *56*, 10646.
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hydrogen phosphate, sodium hydrogen phosphate, Tween 20, 2,2′-azobis(2-methyl)propionamidine dihydrochloride (AAPH), ammonium thiocyanate, ferrous chloride, hydrochloric acid (37% w/w), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), sulphuric acid (96% w/w), trisodium phosphate, ammonium molybdate, Folin-Ciocalteu reagent, sodium carbonate, ethyl acetate, acetic acid, poly(methacrylic acid) sodium salt standards, deoxyribose, ferric chloride, ethylenediaminetetraacetic acid disodium salt (EDTA), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich.

All solvents were reagent grade or HPLC-grade.

MAA was purified before use by distillation under reduced pressure.

2.2 Synthesis of the polymer containing ferulic acid (PMAA-FA)

The reaction 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, 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 mantained at 25°C for 3 h under atmospheric air.

The obtained polymer was precipitated by adding the polymeric solution to an excess volume of diethyl ether (5:1), under agitation at room temperature. The suspension was filtered by sintered glass filter funnel (Pyrex, Ø30 mm; porosity 3), washed with diethyl ether and the recovered polymer was dried in a vacuum oven at 40°C. The synthesised material was further purified by dissolution in water and precipitation in diethyl ether (5:1) for three times. The polymer was checked to be free of unreacted FA and any other compounds by HPLC analysis after each purification step. In *Figure 6.2* the chromatogram of a FA standard solution (0.1 mM) in ethanol is reported. Retention time: 3.34 min.

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

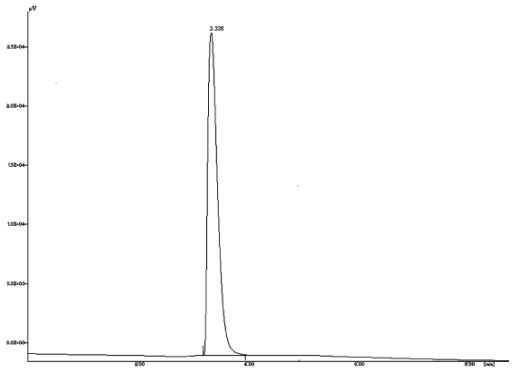

Figure 6.2. *HPLC chromatogram of the FA standard.*

2.3 Instrumentation

The liquid chromatography consisted of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 240 nm. A 250 mm× 4 mm C-18 Hibar® Column, particle size 5µm (Merck, Darmstadt, Germany) was employed. As reported in literature[24], the mobile phase was methanol at a flow rate of 0.5 mL/min and at room temperature.

FT-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.

Molecular weight distributions of synthesised polymers were analysed by a GPC system composed of: µBondagel E-125 and E-500 GPC columns (Millipore, Water Associates) connected in series; a Jasco PU-2080 Plus liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 µl loop); an Agilent ELSD 1200 Light Scattering Detector and a Jasco-Borwin integrator. The mobile phase employed was phosphate buffer saline (pH 7.4) at a flow rate of 0.8 mL/min calibrated with six individual poly(methacrylic acid) sodium salt standards with peak molecular weights ranging from 1670 to 236 000 Da and PDI from 1.02 to 1.11.

[24] Chakravartula, S.V.S.; Guttarla, N. *Nat. Prod. Res.* **2007**, *12*, 1073.

2.4 Evaluation of the antioxidant activity

2.4.1 Determination of scavenging effect on DPPH radicals

Synthesised 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 this material[25].

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 an ethanol solution of DPPH (200 μM) were added obtaining a DPPH solution 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 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 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 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.

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2.4.2 Linoleic acid emulsion system-thiocyanate assay

The antioxidant properties of synthesised PMAA-FA were evaluated through measurement of percent inhibition of peroxidation in linoleic acid system by using the thiocyanate method[154] 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 of 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 PMAA-FA and AAPH (final concentration 25 mM) was added to start the peroxidation of linoleic acid[26]. 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 a 10 mL volumetric flask, 0.2 mL of sample solution, 0.2 mL of an aqueous solution of ammonium thiocyanate (30% w/w), 0.2 mL of a ferrous chloride 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 the 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).

[26] Dufour, C.; Loonis, M.; Dangles, O. *Free Radical Biol. Med.* **2007**, *43*, 241.

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[27].

PMAA-FA (100 mg) was dissolved in distilled water (6 mL) in a volumetric flask. Folin-Ciocalteu reagent (1 mL) was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2% w/v) 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[28].

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 4.0 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

[27] Pan, Y.; Zhu, J.; Wang, H.; Zhang, X.; Zhang, Y.; He, C.; Ji, X.; Li, H. *Food Chem.* **2007**, *103*, 913.
[28] Prieto, P.; Pineda, M.; Aguilar, M. *Anal. Biochem.* **1999**, *269*, 337.

mixture was measured at 695 nm against a control prepared using blank polymer 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 analysed 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.

Each measurement was performed in quintuplicate and the results expressed as means (± SD).

2.4.5 Evaluation of antioxidant activity on hydroxyl radical

PMAA-FA (75 mg) was 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[156]. 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).

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

All samples were assayed in triplicate and data expressed as means (± SD).

2.5 Evaluation of the antifungal activity

The synthesised polymeric material was screened in vitro for their antifungal activity against *Aspergilllus niger*.

Antifungal assays were performed as follows[29]: PMAA-FA was dissolved in distilled water at a concentration of 100 mg/mL. Then, the solution was added to sterilised potato dextrose agar (PDA) to give a final concentration of 10 mg/mL. 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 the following equation (2):

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

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 the polymeric material.

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

3. Results and discussion

3.1 Synthesis of the polymer containing ferulic acid (PMAA-FA)

In this study, PMAA-FA was synthesised by direct reaction of ferulic acid, without any other derivatisation reactions, and ascorbic acid/H_2O_2 as redox initiators at room temperature.

This procedure allows to overcome the limitations of common synthetic strategies for the preparation of functionalised polymers, which involve several different steps. Generally, in order to obtain this kind of materials, two main strategies are used. The first one is based on the functionalisation of a molecule with antioxidant properties by the insertion of a polymerisable group and its subsequent polymerisation or co-polymerisation[30]. The second strategy involves the derivatisation of a pre-formed polymeric structure with an antioxidant[31]. All

[29] Zhong, Z.; Chen, R.; Xing, R.; Chen, X.; Liu, S.; Guo, Z.; Ji, X.; Wang, L.; Li, P. *Carbohydr. Polym.* **2007**, *342*, 2390.
[30] Ortiz, C.; Vazquez, B.; Roman, J.S. *J. Biomed. Mater. Res.* **1999**, *45*, 184.
[31] Atkinson ,D.; Lehrle, R. *Eur. Polym. J.* **1992**, *28*, 1569.

these strategies show some limitations, for example they are time expensive because they require the purification of intermediate products and a difficult optimisation of the reaction conditions. On the contrary, our single-step procedure allows the preparation of highly performing materials in a shorter time.

In this work, methacrylic acid was chosen as functional monomer because of the broad application field of methacrylate polymers in biomedicine and biotechnology. Acrylate and methacrylate polymers, indeed, have been applied in drug delivery systems, contact lenses, food technology, quality control systems and synthetic membranes for biosensors[32].

As before explained, ascorbic acid/hydrogen peroxide redox pair, a water soluble and biocompatible system, was employed as initiator. Conventional initiators, including azo compounds, peroxides and thermal iniferters, require relatively high polymerisation temperature to ensure the rapid decomposition of initiator. By employing a redox initiation system is possible to perform polymerisation processes at lower temperatures, with all the polymer chains initiated almost instantaneously because of the reduction of the induction time[33]. Furthermore, the lower polymerisation temperature reduces the risks of FA degradation and the generation of any kind of toxic reaction products is avoided.

In *Figure 6.3* was reported the mechanism of redox reagents interaction: the AA is oxidised by H_2O_2 forming hydroxyl radical and ascorbate radical intermediates that initiate the reaction[34].

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

[32] Herváz Pérez, J.P.; López-Cabarcos, E.; López-Ruiz, B. *Biomol. Eng.* **2006**, *23*, 233.
[33] Simms, R.W.; Cunningham, M.F. *Macromolecules* **2007**, *40*, 860.
[34] Kitagawa, M.; Tokiwa, Y. *Carbohydr. Polym.* **2006**, *64*, 218.

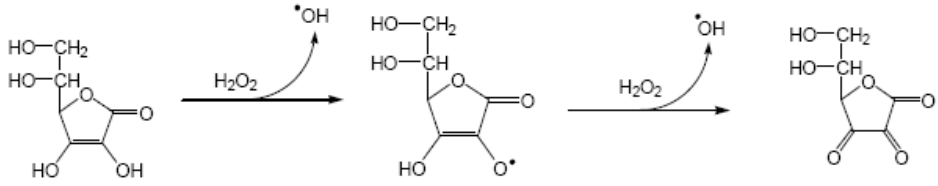

Figure 6.3. *Interaction between ascorbic acid and hydrogen peroxide.*

The obtained polyacrylic systems are characterised by high antioxidant activity, higher stability and slower degradation than natural antioxidants with low molecular weight.

On the basis of chemical structure of ferulic acid, characterised by a carbon-carbon double bond in styrenic position and a phenolic group, a reaction mechanism could be hypothesised. It is known, indeed, that styrenic group of cinnamic acid can undergo free radical polymerisation in a wide range of conditions[35]. Phenolic group compatibility with this kind of reaction was also proved in different research work[36]: monomers with active functional groups (phenolic groups) as side substituents, indeed, were used for the preparation of chelating[37] or grafted polymeric systems using free radical initiators. On the other side, phenolic group could be directly involved in polymerisation process, it is indeed reported that phenolic radical undergoes in dimerisation processes by reaction between hydroxyl radical and aromatic ring[38].

Based on these considerations, our hypothesised reaction mechanism involves the reaction of both styrenic carbon-carbon double bond and phenolic oxygen of ferulic acid.

[35] Esen, H.; Küsefoğlu, S.H. *J. Appl. Polym. Sci.* **2002**, *89*, 3882.
[36] Kim, T.H.; Oh, D.R. *Polym. Degrad. Stab.* **2004**, *84*, 499.
[37] Nanjundan, S.; Selvamalar, C.S.J.; Jayakumar, R. *Eur. Polym. J.* **2004**, *40*, 2313.
[38] Larsen, E.; Andreasen, M.F.; Christensen, L.P. *J. Agric. Food Chem.* **2001**, *49*, 3471.

3.2 Characterisation of PMAA-FA

PMAA-FA and the respective control polymer were characterised by FT-IR and UV-Vis analyses, and molecular weight distributions were analysed by a GPC.

The incorporation of FA in the polymer is proved by the appearance, in the FT-IR spectrum of PMAA-FA (*Figure 6.4*, trace B), of a peak at 1544 cm^{-1} awardable to carbon to carbon stretching within the aromatic ring of ferulic acid.

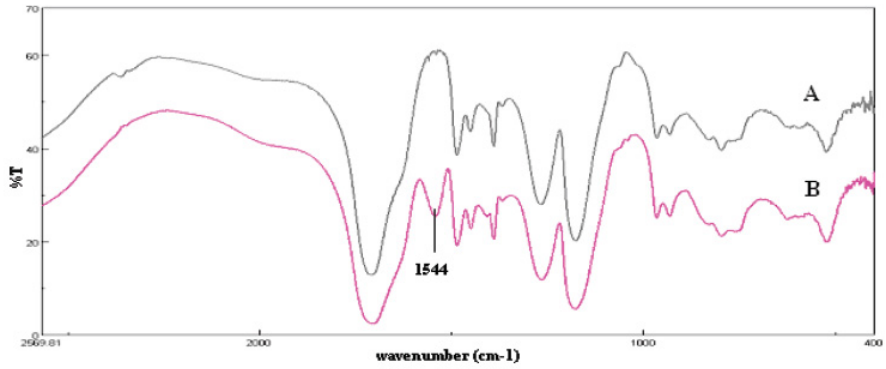

Figure 6.4. *FT-IR spectra of PMAA (**A**) and PMAA-FA (**B**).*

A further confirmation was obtained by comparing UV spectra of FA (25 µM) and PMAA-FA in ethanol (4 mg/mL) as shown in *Figure 6.5*.

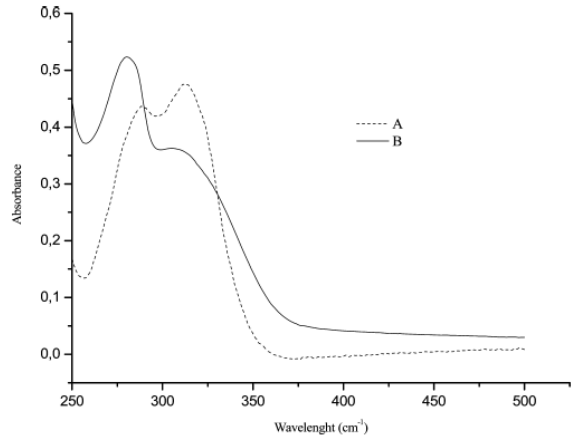

Figure 6.5. *UV spectra of FA (**A**) and PMAA-FA (**B**).*

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 formation of a covalent linkage involving FA was proved by the fact that the wavelength of the aromatic peaks is shorter in PMAA-FA (280 and 305 nm) than in FA (290 and 315 nm) and by the inversion in the magnitude of the two peaks.

Finally, molecular weight distributions of synthesised polymers were analysed by GPC, and the results show an average molecular weight of 70,000 Da and 40,000 Da for PMAA and PMAA-FA, respectively.

3.3 Evaluation of the 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 was 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 6.1*).

Table 6.1. *Inhibition percentages of DPPH radical and linoleic acid peroxidation by PMAA-FA and PMAA[a]. Results are means ± SD of three parallel measurements.*

POLYMER	Inhibition (%)	
	DPPH	Linoleic Acid Peroxidation
PMAA-FA	80.3 ± 1.4	50.2 ± 0.9
PMAA	30.2 ± 1.0	6.9 ± 1.3

3.3.2 Linoleic acid emulsion system-thiocyanate assay

The antioxidant activity of synthesised 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 oxidise 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 6.1*, the effects of polymeric particles (100 mg) on linoleic acid peroxidation compared to ferulic acid after 72 h are shown.

As it is possible to note, ferulic acid-containing polymer is very effective in peroxidation process inhibition.

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

3.3.3 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's phenol reagent is used to obtain a crude estimate of the amount of disposable phenolic groups present in a polymer chain.

Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu's 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 per g of dry polymer.

Control experiments were also performed with blank polymer, and no activity was recovered.

3.3.4 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, the synthesised polymeric material had significant antioxidant activities, and ferulic acid equivalent concentration was found to be 0.59 mM per 1 g of PMAA-FA.

Control experiments were also performed with blank polymer, and no activity was recovered.

3.3.5 Evaluation of antioxidant activity on hydroxyl radical

Hydroxyl radical exhibits very high reactivity and tends to react with a wide range of molecules found in living cells. It can interact with the purine and pyrimidine bases of DNA. It 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[39].

Due to the high reactivity, 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 pro-oxidant in the catalysed decomposition of superoxide and hydrogen peroxide. These radicals are intermediary products of cellular respiration, phagocytic outburst and purine metabolism. Hydroxyl radicals 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 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 radicals was evaluated by the Fenton-mediated deoxyribose assay[40].

Good antioxidant properties were found, with inhibition values of 95 ± 2.1 % for PMAA-FA and 19 ± 1.7 % for PMAA, respectively.

3.4 Evaluation of the 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

[39] Halliwell, B.; Gutteridge, J.M.C.; Aruoma, O.I. *Anal. Biochem.* **1987**, *165*, 215.
[40] Auroma, O.I. *Food Chem. Toxicol.* **1994**, *32*, 671.

infections) which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympani membrane[41].

PMAA-FA showed antifungal activity, as it inhibited the mycelial growth of *A. niger* on PDA. The growth declined with the increase of polymer concentration 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.

3.5 UV protecting properties

In order to evaluate the applicability of the synthesised polymer containing antioxidant moieties as photo-protective agent in sunscreen, UV absorption analyses were performed on PMAA-FA, FA and PMAA (*Figure 6.5*).

The UV absorption of free ferulic acid (trace A) was recorded in the region between 350 and 250 nm, with absorption maximum at 315 and 290 nm. As before mentioned, the incorporation of FA in the polymeric structure determines a hypsochromic shift of the spectral band. The wavelength of the aromatic peaks in PMAA-FA (trace B), indeed, is shorter than in FA (305 and 280 nm, respectively). This effect is ascribable to the reduction of the conjugation between C-C double bond and phenolic ring of ferulic acid due to the formation of a covalent bond between FA and the growth polymeric chain.

The presence of a broad absorption band in the spectral region between 375 and 250 nm, makes the synthesised polymer suitable for application in sunscreen formulations. It is well known that a good photo-protecting agent is asked to absorb the UVA and UVB radiations. In the UV spectrum of PMAA, no absorption band was recorded in the UV region, confirming that the UV protecting properties are ascribable to the presence of ferulic acid.

Thus, PMAA-FA is very effective in this direction, and comparing with free ferulic acid, it is more efficient in protecting against the most dangerous UVB as confirmed by the shift of the absorption peaks to shorter wavelength.

[41] Bisogno, F.; Mascoti, L.; Sanchez, C.; Garibotto, F.; Giannini, F.; Kurina-Sanz, M.; Enriz, R. *J. Agric. Food Chem.* **2007**, *55*, 10635.

The ability of the obtained polymeric material to absorb the UVA radiation is also important because it is reported that the skin damage is ascribable to both the kinds of UV radiations.

4. Conclusions

In this work, PMAA-FA was successfully synthesised by employing redox water soluble initiators. FT-IR and UV spectra performed on the obtained material testified the success of the procedure.

Antioxidant and antifungal properties were evaluated by performing different assays. In particular, linoleic acid emulsion system-thiocyanate assay, determination of scavenging activity on DPPH radicals, disposable phenolic groups in polymeric matrices, total antioxidant capacity, scavenging activity towards hydroxyl radical and determination of antifungal properties against *A. niger* were performed.

Good antioxidant and antifungal properties were recorded in all the tested conditions.

Thus, the adopted synthetic strategy is a very simple approach for preparing macromolecular systems characterised by high antioxidant and antifungal activities. Furthermore, the obtained results clearly show the utility of the proposed polymeric material as preservative in food and in cosmetic fields, as photo-protecting agent and in hospital, medical, pharmaceutical, bioprotective, and related hygienic applications.

CHAPTER VII

Antioxidant and Spectroscopic Studies of Crosslinked Polymers containingFerulic Acid

1. Introduction

In this study, a novel, simple synthetic approach involving one-pot reaction was developed[1]. The synthetic strategy is based on the direct reaction of an antioxidant molecule with suitable functional monomer and crosslinking agent, to build a network bearing antioxidant moieties.

The chosen antioxidant molecule was (E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid (*Figure 7.1*), also known as ferulic acid (FA), one of the most ubiquitous phenolic compounds in nature, especially rich as an ester form in rice bran pitch. This compound has been largely used as a food preservative because of its ability to inhibit the autoxidation of oils. It also constitutes the active ingredient in many skin lotions and sunscreens designed for photoprotection[2].

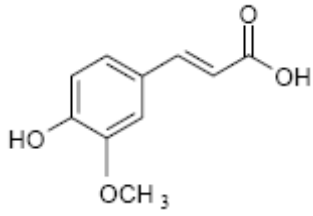

Figure 7.1*. Chemical structure of ferulic acid (FA).*

Two kind of polymeric matrices, with both irregular and spherical shape were synthesised and FA insertion onto the matrices was confirmed by Nuclear Magnetic Resonance-Magic Angle Spinning (NMR-MAS) studies. The

[1] Parisi, O.I.; Puoci, F.; Iemma, F.; De Luca, G.; Curcio, M.; Cirillo, G.; Spizzirri, U.G.; Picci, N. *Polym. Adv. Technol.* **2010**, *21*, 774.
[2] Ou, S.; Kwok, K.C. *J. Sci. Food Agric.* **2004**, *84*, 1261.

polymers were characterised by morphological and dimensional analyses and by water content measurement. Finally, antioxidant properties were evaluated by linoleic acid emulsion system-thiocyanate assay, determination of scavenging activity on 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) radical, determination of available phenolic groups in polymeric matrices and determination of total antioxidant capacity.

The high antioxidant power of the materials, together with the high simplicity of the proposed synthetic strategy, makes these networks very promising for application in several different fields.

2. Materials and methods

2.1 Materials

Ferulic acid (FA), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), 2,2'-azoisobutyronitrile (AIBN), acetic acid, linoleic acid, Tween 20, 2,2′-azobis(2-methyl)propionamidine dihydrochloride (AAPH), ammonium thiocyanate, ferrous chloride, hydrochloridic acid (37% w/w), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), sulphuric acid (96% w/w), ammonium molybdate, Folin-Ciocalteu reagent, sodium carbonate were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA).

Diethyl ether, acetone, ethanol, methanol, acetonitrile were HPLC-grade and provided by Carlo Erba reagents (Milan, Italy).

The monomers were all purified before use.

2.2 Instrumentation

The liquid chromatograph consisted of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 240 nm. A 250 mm × 4 mm C-18 Hibar® Column, particle size 5μm (Merck, Darmstadt, Germany) was employed. As reported in literature, the mobile phase was methanol at a flow rate of 0.5 mL/min and at room temperature[3].

[3] Chakravartula, S.V.S.; Guttarla, N. *Nat. Prod. Res.* **2007**, *12*, 1073.

The FT-IR spectra were recorded as films or KBr pellets on a Jasco FT-IR 4200.

The UV-Vis absorption spectra were obtained with a Jasco V-530 UV/Vis spectrometer.

The 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. The particles size distribution was carried out using an image processing and analysis system, a Leica DMRB equipped with a LEICA Wild 3D stereomicroscope.

Solid ^{13}C NMR-MAS experiments were conducted on MIP bulk and microsphere polymers in order to characterise the materials and to verify the effective presence of FA into the polymeric networks. The CP-MAS ^{13}C NMR-MAS spectra were recorded at room temperature on a Bruker Avance 500 MHz spectrometer working at 11.74 T equipped with 4 mm probehead. Experiments were conducted on 35-40 mg of samples packed into a ZrO_2 rotor. The rotor was spun at the spinning rate of 9-10 kHz and a contact time of 700 μs was applied to obtain polarization transfer. The 90 degree pulse was 5.0 μs for all the experiments and a recycle delay of 5 s was used.

2.3 Synthesis of bulk polymers

The bulk antioxidant polymers (BAP) were synthesised as follows. FA (0.8 mmol), MAA (3.5 mmol), EGDMA (17.5 mmol) and AIBN (1.2 mmol) were dissolved in acetonitrile (5.5 mL) in a thick-walled glass tube. The obtained solution was purged with nitrogen and sonicated for 10 min. The mixture was, then, incubated under a nitrogen atmosphere at 60°C for 24 h. The resultant bulk rigid polymer systems were crushed, grounded into powder and sieved through a 63 μm stainless steel sieve. The fine particles were removed by repeated sedimentation from acetone (5 x 30 min).

The polymeric particles were Soxhlet extracted with methanol (200 mL) for 48 h. The extracted materials were dried overnight in an oven at 50°C.

The washed particles were checked to be free of FA and any other compound by HPLC analysis.

Bulk control polymers (BCP) were prepared under the same reaction conditions without using FA.

2.4 Synthesis of spherical polymers

The spherical antioxidant polymers (SAP) were prepared by precipitation polymerisation[4]. Briefly, FA (0.8 mmol), MAA (3.5 mmol), EGDMA (17.5 mmol) and AIBN (1.2 mmol) were dissolved in acetonitrile (55.0 mL) in a round bottom flask (100 mL). The polymerisation 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 ethanol (100 mL), acetone (100 mL) and then with diethyl ether (100 mL).

The polymeric particles were Soxhlet extracted as reported for BAP.

Spherical control polymers (SCP) were prepared under the same reaction conditions without using FA.

2.5 Water content measurement

The swelling characteristics of polymers were determined to check hydrophilic affinity of bulk polymers compared to spherical polymers.

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), weighed and left to swell by immersing the filter plus support in a beaker containing the swelling media (PBS pH 7.0, 0.01 M). After 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

[4] Iemma, F.; Cirillo, G.; Spizziri, U.G.; Puoci, F.; Parisi, O.I.; Picci, N. *Eur. Polym. J.* **2008**, *44*, 1183.

bored silicone stopper and then centrifuged at 3500 rpm for 15 min and weighted. The filter tare was determined after centrifugation with only water. The recorded weights were averaged and used to give the water content percent (WR, %) by the following equation (1):

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

where W_s and W_d are weights of swollen and dried polymeric particles, respectively.

Each experiment was carried out in triplicate and the results were in agreement within ± 4% standard error.

2.6 Evaluation of the antioxidant properties

2.6.1 Linoleic acid emulsion system-thiocyanate assay

The antioxidant activity of synthesised polymers was determined in terms of measurement of percent inhibition of peroxidation in linoleic acid system following a reported method with some modifications[5].

Polymers (100 mg) were added to linoleic acid emulsion (2.5 mL, 0.2 M) in phosphate buffer (pH 7.0, 20 mM). The linoleic acid emulsion was prepared by mixing of linoleic acid (0.2804 g), Tween 20 (0.2804 g), as emulsifier and phosphate buffer (50 mL), and then the mixture was homogenised. Peroxidation of linoleic acid was started by the addition of AAPH (final concentration 25 mM) and the solution was incubated at 40°C to accelerate the oxidation process[6]. The degree of oxidation was measured following thiocyanate method. After 3 days incubation, the samples were centrifuged and, in a volumetric flask (10 mL), sample solution (0.2 mL), aqueous solution of ammonium thiocyanate (0.2 mL, 30% w/w), $FeCl_2$ solution (0.2 mL, 20 mM in HCl 3,5 % v/v) and ethanol (75% v/v) to 10 mL were added sequentially. After 3 min stirring, the absorption values of mixtures measured at

[5] Ardestani, A.; Yazdanparast, R. *Food Chem.* **2007**, *104*, 21.
[6] Dufour, C.; Loonis, M.; Dangles, O. *Free Radic. Biol. Med.* **2007**, *43*, 241.

500 nm were taken as peroxide contents. A control was performed with linoleic acid but without polymers.

In order to evaluate the interference of polymeric matrices on linoleic assay, the same experiments were performed using blank polymers.

Percent inhibition of linoleic acid peroxidation was calculated according to the equation (2):

$$Inhibition\,\% = \frac{Abs_0 - Abs_1}{Abs_0} \times 100 \quad (2)$$

where Abs_0 is the absorbance of the control, and A_1 is the absorbance of polymeric or standard sample.

Measurements were carried out in triplicate and data expressed as means.

2.6.2 Determination of scavenging activity on DPPH radicals

To evaluate the free radical scavenging activity, synthesised polymers were allowed to react with a stable free radical, 2,2'-diphenyl-1- picrylhydrazyl radical (DPPH)[175].

In a volumetric flask (25 mL), different amounts of each dry polymer (50, 100, 150, 200 mg) were mixed with an ethanol solution of DPPH (12.5 mL, 200 μM) and ethanol (12.5 mL) to obtain a DPPH solution with a final concentration of 100 μM. The samples were shaken vigorously on a vortex mixer and then incubated for 30 min in a water bath at 25°C. After centrifugation, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm.

The same reaction conditions were applied for blank polymers to evaluate the absorption of DPPH radical on the polymeric surface.

According to equation (2), the scavenging activity of the polymers was measured as the decrease in absorbance of the DPPH.

Measurements were carried out in triplicate and data expressed as means.

2.6.3 Determination of total antioxidant capacity

The total antioxidant capacity of polymeric materials was tested according to a reported method[7].

Polymer particles (100 mg) were combined with 1.2 mL of reagent solution (sulphuric acid 0.6 M, sodium phosphate 28.0 mM and ammonium molybdate 4.0 mM) and methanol (0.3 mL). The reaction mixture was incubated at 95°C for 150 min. After cooling to room temperature and centrifugation, the absorbance of the mixture was measured at 695 nm against a control prepared using blank polymers in the same reaction conditions and the antioxidant activity of polymeric matrices was determined as FA equivalent concentration.

Measurements were carried out in triplicate and data expressed as means.

By using FA standard solutions, a calibration curve was plotted. Each solution (0.3 mL) was mixed with reagent solution (1.2 mL) 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 analysed by UV-Vis spectrophotometer and the correlation coefficient (R^2) of the regression equation, by least square method, was calculated.

2.6.4 Determination of available phenolic groups in polymeric matrices

Amount of available phenolic groups was determined using Folin-Ciocalteu reagent procedure as described[8].

In a volumetric flask (10 mL), dry polymers (100 mg) were dispersed in distilled water (6.0 mL). Then, Folin-Ciocalteu reagent (1.0 mL) and sodium carbonate (3.0 mL, 2 % w/v), were added and the mixture was allowed to stand for 2 h with intermittent shaking. After centrifugation, the absorbance was measured at 760 nm against a control prepared using blank polymers in the same reaction conditions.

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[7] Prieto, P.; Pineda, M.; Aguilar, M. *Anal. Biochem.* **1999**, *269*,337.
[8] Pan, Y.; Zhu, J.; Wang, H.; Zhang, X.; Zhang, Y.; He, C.; Ji, X.; Li, H. *Food Chem.* **2007**, *103*, 913.

Measurements were carried out in triplicate and data expressed as means.

The concentration of available phenolic groups in polymeric matrices was determined as FA equivalent concentration by using an equation that was obtained from a FA calibration curve. This one was recorded by using FA standard solutions. Each solution (0.5 mL) was 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 solutions were analysed by UV-Vis spectrophotometer to obtain a calibration curve and the correlation coefficient (R^2) of the regression equation, by least square method, was calculated.

3. Results and discussion

3.1 Synthesis of antioxidant polymers

Our synthetic strategy involved the direct reaction of the selected antioxidant molecule (FA) with a suitable functional monomer and a crosslinking agent in presence of AIBN as free radical initiator.

To synthesise microparticles with irregular and spherical shape, two different polymerisation processes were performed: the bulk and precipitation polymerisation, respectively. It can be supposed that, in both the reactions, the grown chains consist of MAA units randomly interrupted by EGDMA crosslinker, to build a polymeric network where antioxidant moieties are distributed evenly in the matrix.

In recent years, FA has received much attention in the study of medicine and an increasing number of reports on the physiological functions of FA and its derivatives in human have been related. Free FA carries on its antioxidant function by the formation of a resonance-stabilised phenoxy radical showing high scavenging activity for hydrogen peroxide, superoxide, hydroxyl, and nitrogen dioxide free radicals. In addition, many applications of FA in the food and cosmetic industries have also been discovered. It is usually found as ester

cross-linked with polysaccharides in the cell wall and also with proteins[9]. The cross-linking property of FA with both polysaccharides and proteins suggests that it can be used in the preparation of complex gels in food applications.

On the basis of chemical structure of FA, characterised by a carbon-carbon double bond in styrenic position and a phenolic group, a reaction mechanism could be hypothesised. It is known, indeed, that styrenic group of cinnamic acid can undergo free radical polymerisation in a wide range of conditions and phenolic group compatibility with this kind of polymerisation was also proved in different research work[10,11]. Furthermore monomers with phenolic groups as side substituents were used for the preparation of chelating or grafted polymeric systems using free radical initiators[12]. In addition, the phenolic groups are directly involved in the polymerisation process, as suggested by literature data reporting on dimerisation processes by reaction between phenoxy radical and aromatic ring[13].

In this work, the hypothesised reaction mechanism involves the reaction of both styrenic carbon-carbon double bond and phenolic oxygen of FA.

The choice of methacrylic acid as functional monomer is related to the broad application field of methacrylate polymers in biomedicine and biotechnology.

To obtain the spherical polymers, precipitation polymerisation technique was adopted. The proposed mechanism is characterised by two steps: nucleation and growth of microspheres. The reaction begins as a usual solution polymerisation. The monomers and initiator were dissolved in the organic solvent and during the polymerisation oligomers are formed. After a certain period of time, the concentration of oligomers becomes sufficiently high to allow radical polymerisation of oligomers to form a microgel (Nucleation). Each

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[9] Figueroa-Espinoza, M.C.; Morel, M.H.; Surget, A.; Asther, M.; Moukha, S.; Sigoillot, J.C.; Rouau, X. *Food Hydrocolloid* **1999**, *13*, 65.
[10] Esen, H.; Küsefoğlu, S.H. *J. Appl. Polym. Sci.* **2003**, *89*, 3882.
[11] Puoci, F.; Iemma,F.; Curcio, M.; Parisi, O.I.; Cirillo, G.; Spizzirri, U.G.; Picci, N. *J. Agric. Food Chem.* **2008**, *56*, 10646.
[12] Nanjundan, S.; Selvamalar, C.S.J.; Jayakumar, R. *Eur. Polym. J.* **2004**, *40*, 2313.
[13] Larsen ,E.; Andreasen, M.F.; Christensen, L.P. *J. Agric. Food Chem.* **2001**, *49*, 3471.

seed (microgel) then grows by continuous capture of oligomers. This last effect prevents the occurrence of any further nucleation and hence uniformly-sized particles are produced. During polymerisation the growing polymer chains are separated from the continuous medium by enthalpic precipitation in cases of non favourable polymer-solvent interactions or entropic precipitation in cases where crosslinking prevents the polymer and the solvent from freely mixing.

The employed ratio between FA and MAA represents the optimised value to obtain the polymer with the highest antioxidant efficiency. A higher amount of the FA, indeed, negatively interferes with the polymers formation. In particular, no spherical shape was observed when precipitation polymerisation was performed; and the same ratio was employed in the synthesis of bulk polymer to make a direct comparison between the two kinds of material.

3.2 Polymers characterisation

3.2.1 FT-IR and NMR-MAS analyses

BAP, SAP and the respective control polymers were characterised by Fourier Transform IR spectrophotometry and NMR analyses.

In the FT-IR spectra of BAP and SAP a band at 1513 cm^{-1} is clearly evident. This peak is absent in the spectrum of blank polymers and can be assigned to carbon-carbon stretching within the aromatic ring of FA, confirming the insertion of the antioxidant moieties in the networks.

In *Figure 7.2* is reported only the observed CP-MAS ^{13}C NMR spectrum of bulk polymer because both samples (bulk and spherical polymers) show identical spectra. The aliphatic region is characterised by the presence of several peaks in the range 15-30 ppm not well resolved that can be considered as overlapping signals due to the terminal carbons and to the $–CH_2–$ groups present in the polymeric backbone. The resonance centered at 45 ppm can instead be assigned to the –CH– groups always present in the polymeric matrix, while the peaks at 55 ppm and at 62 ppm are due respectively to the –CH– groups bonded to aromatic moiety of grafted FA and to the phenolic $-O-CH_3$ group. The important region between 110 and 140 ppm, dominated by weak signals, is

specific of aromatic or olefinic carbons and was proper ascribed, along with the signal of C_7, to the inclusion of FA group into the polymeric matrices. Therefore, the peaks at 125 and 136 ppm were assigned to the aromatic carbons C_6, C_1, C_3 and C_4 while the very weak peaks between 115–118 ppm were attributed to the carbons C_2 and C_5. Finally, the well resolved peak observed at 177 ppm and the weak peak at 167 ppm were assigned to carboxylic functions present in the structure.

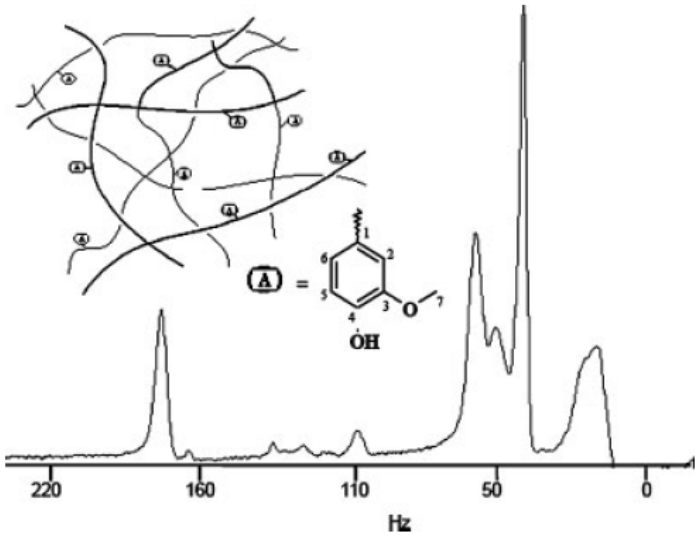

Figure 7.2. *NMR-MAS of BAP.*

3.2.2 Physical characterisation

Polymers were also characterised by morphologic and dimensional analyses and water content measurements. Using scanning electron microscopy (SEM), information about the surface properties of the microparticles were obtained, and spherical shape of materials prepared by precipitation polymerisation was verified (*Figures 7.3*).

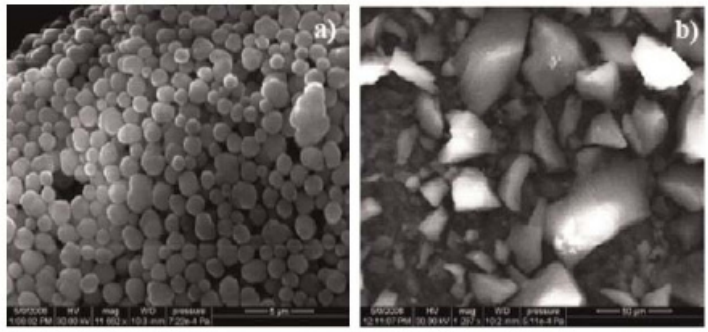

Figure 7.3. *Scanning electron micrograph of SAP (a) and BAP (b).*

Dimensional analysis proved the very narrow distribution of particle size with mean particle size of 1.07 μm ± 0.4 and 1.13 μm ± 0.3 for antioxidant and blank polymers, respectively (*Figure 7.4*).

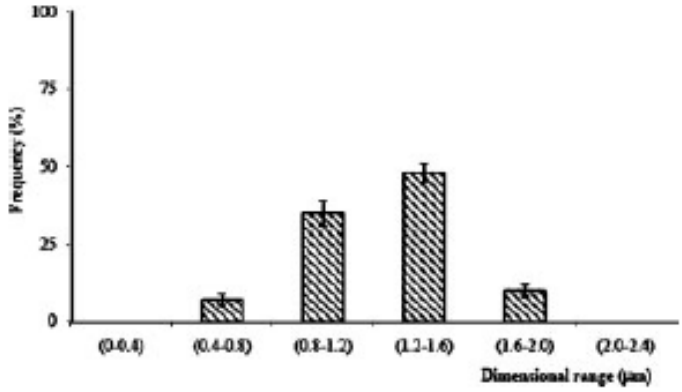

Figure 7.4. *Dimensional analysis of SAP.*

The swelling behaviour of the materials was also studied and the value of contained water percentage was determined in aqueous media (PBS solution pH = 7.0; 0.01 M) after 24 h. Spherical microparticles were able to absorb more water than irregular ones (50.3 ± 1.9 % and 5.2 ± 0.7 %, respectively) because of the higher amount of solvent, acting as porogen in the polymerisation feed, that allows at the water molecules to easily diffuse in the polymeric network. Both polymers show low affinity to aqueous media in consequence of high crosslinking degree of materials.

3.3 Evaluation of the antioxidant properties

3.3.1 Linoleic acid emulsion system-thiocyanate assay

The antioxidant activity of materials 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 oxidise Fe^{2+} to Fe^{3+}. The later Fe^{3+} ions form complex with SCN^{-}, which had maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation, while lower absorbance suggests a higher level of antioxidant activity.

In *Table 7.1*, the effects of polymeric particles (100 mg) on linoleic acid peroxidation after 72 h are shown. Ferulic acid-containing polymers are very effective in peroxidation process inhibition and the values recovered from microspheres are higher than that obtained from irregular microparticles (*Table 7.1*).The lowest inhibition value obtained from the experiments performed with blank polymers, clearly shown that absorbance reduction is ascribable to FA moieties in the polymeric structure.

Table 7.1. *Antioxidant activity of synthesised polymers.*

POLYMERS	Disposable Phenolic Groups (mol L^{-1}/g polymer)	Total Antioxidant Activity (mol L^{-1}/g polymer)	Inhibition (%)	
			DPPH Radicals	Linoleic Acid Peroxidation
BAP	$3.2 \times 10^{-3} \pm 0.1$	$7.8 \times 10^{-3} \pm 0.2$	31 ± 1.4	52.0 ± 1.4
BCP	----	----	1.0 ± 0.2	1.0 ± 0.3
SAP	$5.0 \times 10^{-3} \pm 0.3$	$1.0 \times 10^{-2} \pm 0.1$	49 ± 1.2	63.0 ± 1.3
SCP	----	----	1.0 ± 0.3	2.0 ± 0.4

3.3.2 Determination of scavenging activity 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., FA, hydroquinone, pyrogallol, gallic acid), reduce and decolorise 1,1-diphenyl-2- picrylhydrazine by their hydrogen donating capabilities.

Polymers scavenger ability was evaluated in term of DPPH reduction using FA as reference compound and data are expressed as inhibition (%).

Good results were obtained with both the antioxidant polymers (*Table 7.1*) and also in this case blank polymers did not significantly interfere with the scavenger process. Experimental data clearly suggest that spherical materials, characterised by a higher surface/volume ratio than irregular particles, were more efficient in scavenging free radicals.

3.3.3 Determination of total antioxidant capacity

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

According to the results (*Table 7.1*), materials had significant antioxidant activities: FA equivalent concentration was found to be 7.8×10^{-3} and 1.0×10^{-2} M per gram of bulk and spherical polymers, respectively. The high value recorded for spherical materials could be ascribed to the more homogeneous disposition of FA moieties on the polymeric surface and to the higher water affinity of these materials.

Control experiments were also performed with blank polymers and no activity was recovered.

3.3.4 Determination of available phenolic groups in polymeric matrices

Since the antioxidant activity of polymers is derived from phenolic groups, it is useful express the antioxidant potential in terms of phenolic content. The Folin-Ciocalteu's phenol reagent is used to obtain a crude estimate of the amount of available phenolic groups present in polymers. Phenolic compounds, indeed, undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent.

Available phenolic groups in the samples were expressed as FA equivalent concentration and this value was 3.2×10^{-3} and 5.0×10^{-3} M per gram of bulk and spherical polymers, respectively. Also in this case, the higher homogeneity and water affinity of spherical matrices make microspheres more effective in this assay.

Control experiments were also performed with blank polymers and no activity was recovered.

4. Conclusions

The aim of this work was the development of a novel and simple synthetic approach, involving one-pot reaction, to obtain crosslinked polymeric materials with high antioxidant properties.

The synthetic strategy is based on the direct reaction of the antioxidant molecule with suitable functional monomer and crosslinking agent, to build a network bearing antioxidant moieties.

In particular, FA was chosen as antioxidant agent and two kinds of polymeric matrices, with irregular and spherical shape were prepared.

Ferulic acid insertion onto the matrices was confirmed by FT-IR and NMR-MAS studies. After morphological, dimensional analyses and water absorption measurement, antioxidant properties of materials were evaluated by different assays. In particular, linoleic acid emulsion system-thiocyanate assay,

determination of scavenging activity on DPPH radicals, determination of available phenolic groups in polymeric matrices and determination of total antioxidant capacity were performed.

Good antioxidant properties were recorded, with spherical polymers showing higher efficiency. This effect is ascribable to a more homogeneous disposition of FA moieties on the polymeric surface and to the higher water affinity of these materials.

The planned synthetic strategy is a very simple approach to prepare macromolecular systems with high antioxidant power which could be successfully applied in all that fields in which a consistent reduction of oxidative stress is required.

CHAPTER VIII

Covalent Insertion of Antioxidant Molecules on Chitosan and Gelatin by Free Radical Grafting Procedure

1. Introduction

Grafting polymerisation is a well known method to develop materials with a particular chemical and physical structure; this synthetic strategy allows to improve the properties of natural and synthetic polymers, giving them new characteristics for specific applications[1,2,3]. The aim of graft copolymerisation is to impart some of the desired properties of the molecule used for grafting to a selected macromolecule. The grafting of molecules upon a natural polymers, such as starch, cellulose, chitosan and gelatin, is of great importance to develop new materials combining the properties of both grafted molecule and natural polymers[4,5,6].

Natural macromolecules, such as chitosan and gelatin, are widely employed in industry due to their biocompatibility, biodegradation, non-toxicity and non-immunogenicity.

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[7] and it has been known as a bioactive molecule.

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[2] Dergunov, S.A.; Nam, I.K.; Maimakov, T.P.; Nurkeeva, Z.S.; Shaikhutdinov, E.M.; Mun, G.A. *J. Appl. Polym. Sci.* **2008**, *110*, 558.
[3] Joung, Y.K.; Choi, J.H.; Bae, J.W.; Park, K.D. *Acta Biomater.* **2008**, *4*, 960.
[4] Liu, S.; Sun, G. *Carbohydr. Polym.* **2008**, *71*, 14.
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[7] Chae, S.Y.; Jang, M.; Nah, J. *J. Controlled Release* **2004**, *102*, 383.

Several bioactivities such as antitumor activity[8] immunoenhancing effects[9], wound healing effects[10], antifungal and antimicrobial properties[11], and antioxidant activity[12] of chitosan have been reported.

All these characteristics offer chitosan good potentials for biomedical applications and in food industry as edible coatings for fruit and vegetables[13] packaging films[14] and waste water purification[15].

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 chitosan and gelatin, it is useful to investigate the modification of these natural polymers to develop new materials with improved properties.

It is well known that for some specific polymeric products, especially medical equipment and food packaging, sterilisation via radiation is needed with a potential risk of degradation, i.e., chain scission and/or crosslinking, resulting in discolouration, cracking of the surface, stiffening, and loss of mechanical properties[16].

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,

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[8] Pae, H.O.; Seo, W.G.; Kim, N.Y.; Oh, G.S.; Kim, G.E.; Kim, Y.H.; Kwak, H.J.; Yun, Y.G.; Jun, C.D.; Chung, H.T. *Leukemia Res.* **2001**, *25*, 339.
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[16] Jahan, M.S.; McKinny, K.S. *Nucl. Instrum. Methods Phys. Res., Sect. B* **1999**, *151*, 207.

chemical modifications are generally not preferred for food applications because of the formation of potential detrimental products[17].

Furthermore, 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 photo-oxidation[18,19,20].

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 copolymerisation of gelatin with various monomers is an effective method to improve the properties of this macromolecule. Among the monomers grafted upon gelatin for its modification, the most widely used are methyl methacrylate, acrylonitrile, and ethyl acrylate, polyvinylpirrolidone[21,22].

In literature, several research works report on the applicability of antioxidants as additives for polymers, as they stabilise the polymer from resin extrusion to the moulded pieces production. During processing, the antioxidant retards thermal and/or oxidative degradation[23]. On the other hand, antioxidants with low molecular weight are less effective owing to their poor thermal stability. To overcome this limitation, a 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

[17] Kanatt, R.S.; Chander, R.; Sharma, A. *Food Chem.* **2008**, *106*, 521.
[18] Morgan, P.E.; Pattison, D.I.; Hawkins, C.L.; Davies, M.J. *Free Radical Biol. Med.* **2008**, *45*, 1279.
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[23] Pasanphan, W.; Buettner, G.R.; Chirachanchai, S. *J. Appl. Polym. Sci.* **2008**, *109*, 38.

easily removed from the host polymer by mechanical rubbing-off, volatilization or leaching[24].

Generally, to obtain antioxidant polymers, three main synthetic strategies, involving several different steps, are used. The first one is based on the functionalisation of a molecule with antioxidant properties by the insertion of polymerisable groups and its subsequent polymerisation or copolymerisation[25]. The second strategy involves the derivatisation of a preformed polymeric structure with an antioxidant[26]. Finally, the third approach is the grafting of a synthesised monomeric antioxidant onto a polymeric chain via melt processing with free radical initiators[27]. These strategies show some limitations. The synthesis of a monomeric antioxidant requires the purification of reaction products, whereas in the derivatisation process, especially if the macromolecular system consists of a crosslinked polymer, a difficult optimisation of the reaction conditions is often needed.

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[28,29].

The possibility to graft antioxidant moieties in a polysaccharidic or proteic 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.

In the present work, our challenge was to synthesise novel polymeric antioxidants by a simple method, involving a one-step reaction. The copolymers were obtained through the conjugation of chitosan[30] and gelatin[31] with

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[25] Ortiz, C.; Vazquez, B.; Roman, J.S. *J. Biomed. Mater. Res.* **1999**, *45*, 184.
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antioxidant molecules in presence of water-soluble redox initiators able to generate free radical species at room temperature.

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

Gallic acid (*Figure 8.1*) is a natural phenolic antioxidant extractable from plants, especially green tea[32]. It is widely used in food, drugs, and cosmetics to prevent rancidity induced by lipid peroxidation and spoilage.

Catechins are one of the main classes of flavonoids and 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[33].

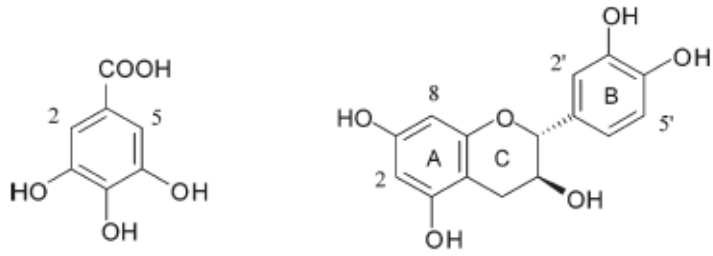

Figure 8.1. *Chemical structures of gallic acid and (+)-catechin.*

The conjugates were characterised by DSC, UV-Vis, fluorescence and FT-IR analyses to confirm the chemical bond between the antioxidant molecule and polymer, then their antioxidant properties were tested by performing different antioxidant assays.

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[31] Spizzirri, U.G.; Iemma, F.; Puoci, F.; Cirillo, G.; Curcio, M.; Parisi, O.I.; Picci, N. *Biomacromolecules* **2009**, *10*, 1923.
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2. Materials and methods

2.1 Materials

Gallic acid (GA), (+)-catechin (CT, *Figure 8.1*), chitosan from crab shells (MW = 95 kDa, 85% deacetylation), gelatin (Ph Eur, Bloom 160), hydrogen peroxide (H_2O_2), ascorbic acid, 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, ferric chloride, ethylenediaminetetraacetic acid disodium salt (EDTA), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid, trichloroacetic acid, hydrochloric acid (37% w/w) were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA).

Methanol, ethanol and chloroform were HPLC-grade and provided by Carlo Erba reagents (Milan, Italy).

2.2 Instrumentation

The liquid chromatography consisted of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 230 nm. A 250 mm × 4 mm C-18 Hibar® Column, particle size 5μm, pore size 120 Å (Merck, Darmstadt, Germany), was employed. As reported in literature[34], the mobile phase adopted for the detection of catechin and gallic acid was a methanol/water/orthophosphoric acid (20/79.9/0.1 v/v) mixture and the flow rate was 1.0 mL/min. The column was operated at 30°C. The sample injection volume was 20 μL.

FT-IR spectra were recorded as films or KBr pellets on a Jasco FT-IR 4200.

Freeze drier Micro Modulyo, Edwards was employed.

Spectrofluorimetric grade solvents were 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

[34] Wang, H.; Helliwell, K.; You, X. *Food Chem.* **2000**, *68*, 115.

photomultiplier tube.

Calorimetric 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 and heating rate 5°C/min.

Molecular weight distributions of gelatin were analysed 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 chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 μl loop); a Jasco UV-2075 HPLC detector and a 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 chitosan conjugates

The synthesis of both catechin-*grafted*-chitosan (CT-CHIT) and gallic acid-*grafted*-chitosan (GA-CHIT) was performed as follows: in a 25 mL glass tube, chitosan (0.5 g) was dissolved in 10 mL of acetic acid water solution (2% v/v). Then, 1 mL of H_2O_2 (1.0 M) containing 0.054 g of ascorbic acid was 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 obtained polymer was checked to be free of unreacted antioxidants and any other compounds by HPLC analysis after the purification step.

The resulting solution was frozen and dried with “freezing-drying apparatus to afford a vaporous solid.

Blank chitosan, that acts as a control, was prepared in the same conditions but in the absence of antioxidant agents.

2.4 Synthesis of gelatin conjugates

The synthesis of both catechin-*grafted*-gelatin (CT-GEL) and gallic acid-*grafted*-gelatin (GA-GEL) was performed as follows: 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 2 h, 0.35 mmol of antioxidant were added to solution. The solution of gelatin and antioxidant, after 24 h, 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 resulting solution was frozen and dried with a freeze drier to afford a vaporous solid.

Purified CT-GEL and GA-GEL were checked to be free of unreacted antioxidant and any other compounds by HPLC analysis after the purification step.

Blank gelatin, that acts as a control, was prepared when grafting process was carried out in the absence of antioxidant agents.

2.5 Determination of scavenging effect on DPPH radicals

In order to evaluate the free radical scavenging properties of synthesised antioxidant conjugates, their reactivity towards a stable free radical, 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), was evaluated[35].

2.5.1 Scavenging properties of chitosan conjugates

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 5 mL of an ethanol solution of DPPH (200 μM) were added, obtaining a DPPH solution with a final concentration of 100 μM. The samples were incubated in a water bath at 25°C and, after 30 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm.

[35] Ardestani, A.; Yazdanparast, R. *Food Chem.* **2007**, *104*, 21.

The same reaction conditions were applied on the blank chitosan in order to evaluate the interference of the 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 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 polymeric samples.

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

2.5.2 Scavenging properties of gelatin conjugates

50.0 mg of functionalised 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 DPPH solution 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).

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

2.6 β-carotene-linoleic acid assay

The antioxidant properties of synthesised functional polymers were evaluated through measurement of percent inhibition of peroxidation in linoleic acid system by using the the β-carotene bleaching test[36].

Briefly, 1 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 mL) was transferred to different test tubes containing 50 mg of each functionalised polymer dispersed in 0.2 mL of 70% ethanol, and 0.2 mL of 70% ethanol in 5 mL 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 blank chitosan and blank gelatin.

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

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

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 (± SD).

[36] Amin, I.; Zamaliah, M.M.; Chin, V.F. *Food Chem.* **2004**, *87*, 581.

2.7 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[37].

20 mg of each conjugate were dissolved in distilled water (6 mL) in a volumetric flask. Folin-Ciocalteu reagent (1 mL) was added and the contents of flask were mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2%, w/w) 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 polymers under the same reaction conditions.

The amount of total phenolic groups in each polymeric material was expressed as gallic acid and catechin equivalent concentrations, respectively, by using the equations obtained from the calibration curves of each antioxidant. These ones were recorded by employing five different gallic acid and catechin standard solutions. 0.5 mL of each solution were added to the Folin-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.

2.8 Determination of total antioxidant activity

The total antioxidant activity of polymeric materials was evaluated according to the method reported in literature[38].

2.8.1 Total antioxidant activity of chitosan conjugates

Briefly, 100 mg of chitosan-antioxidant conjugates were mixed with 2.4 mL of reagent solution (0.6 M sulphuric acid, 28 M sodium phosphate and 4 M

[37] Pan, Y.; Zhu, J.; Wang, H.; Zhang, X.; Zhang, Y.; He, C.; Ji, X.; Li,H. *Food Chem.* **2007**, *103*, 913.
[38] Prieto, P.; Pineda, M.; Aguilar, M. *Anal. Biochem.* **1999**, *269*, 337.

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 assay conditions.

The total antioxidant activity of each polymeric material was expressed as equivalent concentration of the respective antioxidant molecule.

By using five different gallic acid and catechin 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 analysed 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.

2.8.2 Total antioxidant activity of gelatin conjugates

Briefly, 10 mg of grafted polymers 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 M Na_3PO_4, and 4 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 (± SD).

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 analysed by using a UV-Vis spectrophotometer, and the correlation coefficient (R^2), slope, and

intercept of the regression equation obtained by the method of least squares were calculated.

2.9 Determination of scavenginge effect on hydroxyl radical (OH·)

The scavenging effect on hydroxyl radical was evaluated according to the literature[39].

Briefly, 20 mg of each conjugate 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 amount of filtrate, 1 mL of thiobarbituric acid (1% w/v) and 1 mL of trichloroacetic acid (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).

All samples were assayed in triplicate and data expressed as means (± SD).

3. Results and discussion

3.1 Synthesis of antioxidants conjugates

Chitosan and gelatin were chosen as polymeric backbones to synthesise different antioxidant conjugates containing the antioxidative groups of catechin and gallic acid.

The conjugation of the antioxidant moieties on the polymeric 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 components of redox pair involves the oxidation of ascorbic acid by H_2O_2 at room

[39] Halliwell, B.; Gutteridge, J.MC.; Aruoma, O.I. *Anal. Biochem.* **1987**, *165*, 215.

temperature with the formation of ascorbate and hydroxyl radicals that initiate the reaction (*Figure 8.2*)[40].

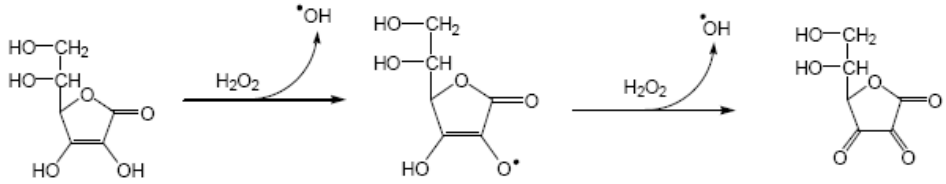


Figure 8.2. *Interaction between ascorbic acid and hydrogen peroxide.*

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 does 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 macromolecule activation toward radical reactions, and a second step for the insertion of the antioxidant molecules on the preformed macroradical.

In *Figure 8.3* and *8.4* a possible mechanism of antioxidant insertion onto chitosan and gelatin, respectively, is proposed.

In the chitosan case, 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 (**STEP 1**)[41].

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[42]. Even in free radical-initiated copolymerisation, NH_2 groups of

[40] Kitagawa, M.; Tokiwa, Y. *Carbohydr. Polym.* **2006**, *64*, 218.
[41] Mun, G.A.; Nurkeeva, Z.S.; Dergunov, S.A.; Nam, I.K.; Maimakov, T.P.; Shaikhutdinov, E.M.; Lee, S.C.; Park, K. *React. Funct. Polym.* **2008**, *68*, 389.
[42] Chen, T.; Kumar, G.; Harris, M.T.; Smith, P.J.; Payne, G.F. *Biotechnol. Bioerg.* **2000**, *70*, 564.

chitosan involve in macroradical formation. At those sites, the insertion of the antioxidant molecules can occur (**STEP2**).

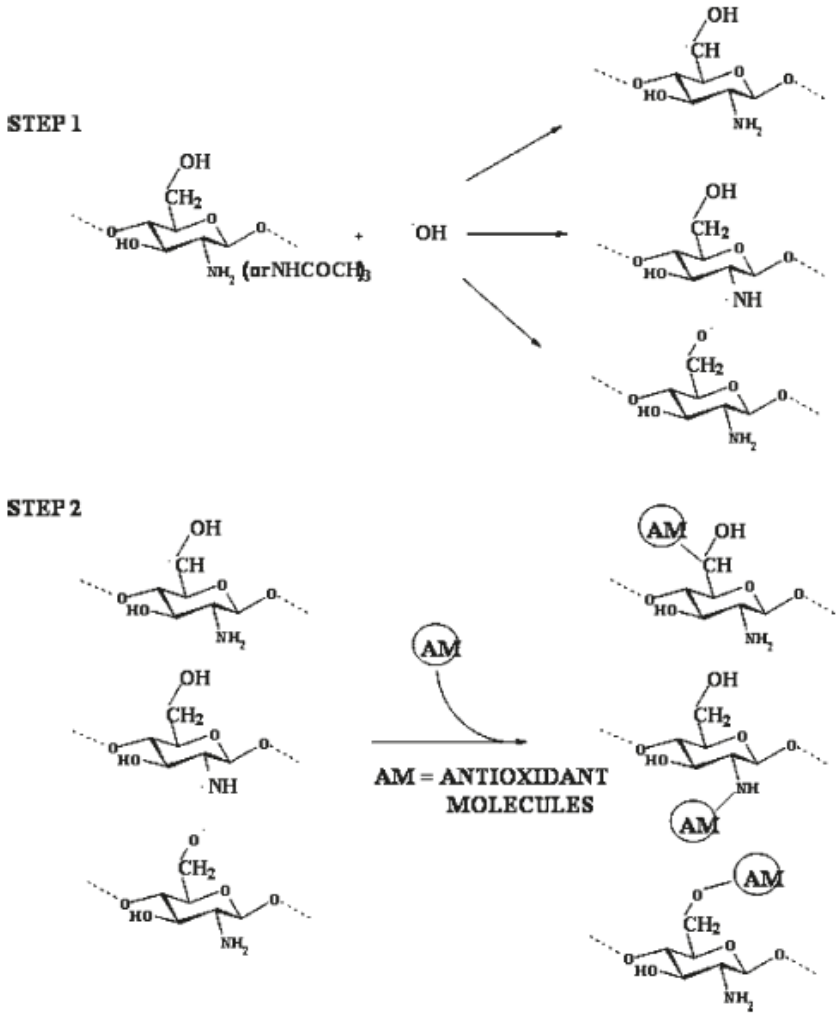


Figure 8.3. *Insertion of antioxidant molecules in chitosan backbone.*

In the gelatin case, hydroxyl radicals, generated by the initiators pair, attack the sensible residues in the side chains of the protein, producing radical species on the aminoacidic structure. These ones react with the antioxidant molecules inducing an antioxidant-gelatin covalent bond.

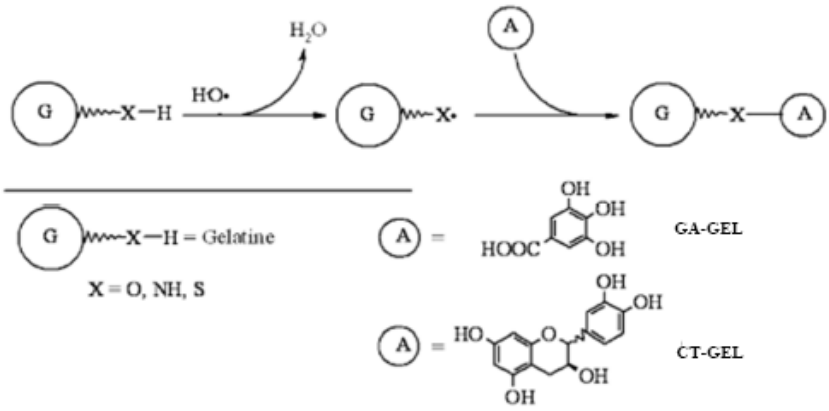


Figure 8.4. *Insertion of antioxidant molecules in gelatin backbone.*

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[43] using free radical initiators. However, phenolic group could be directly involved in polymerisation process; it is reported, indeed, that phenolic radical undergoes in dimerisation processes by reaction between hydroxyl radical and aromatic ring in the orto- or para-position relatively to the hydroxyl group[44].

On the basis of these data, it can be reasonably hypotesised that the insertion of antioxidants on chitosan and gelatin 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 8.1*), respectively.

In the reaction feed the amount of antioxidant was 0.7 mmol/g of polymer for all the conjugate systems; this value represents the optimum to obtain a material with the highest efficiency. A lower amount of antioxidant in the reaction feed, indeed, is not enough to obtain a material with significant antioxidant properties; on the other hand, a higher amount carries out to a material with no improvement in antioxidant properties.

[43] Nanjundan, S.; Selvamalar, C.S.J.; Jayakumar, R. *Eur. Polym. J.* **2004**, *40*, 2313.
[44] Uyama, H.; Maruichi, N.; Tonami, H.; Kobayashi, S. *Biomacromolecules* **2002**, *3*, 187.

3.2 Characterisation of chitosan-antioxidant conjugates

In order to verify the covalent insertion of (+)-catechin and gallic acid into chitosan chains, the functionalised materials and the control polymer were characterised by Fourier Transform IR spectrophotometry, UV and DSC analyses.

FT-IR-spectra of both chitosan-antioxidant conjugates show the appearence of new peaks at 1538 cm^{-1} and at 1558 cm^{-1}, respectively, awardable to carbon to carbon stretching within the aromatic ring of gallic acid and catechin; moreover, in the IR spectrum of gallic acid-*grafted*-chitosan, 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). These last ones were recorded using blank chitosan at the same concentration as baseline, in order to remove the interference of the native polysaccharide. As depicted in *Figure 8.5* and in *Figure 8.6*, both the UV spectra of both conjugates show the presence of absorption peaks in the aromatic region that can be related to the presence of gallic acid and catechin 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.

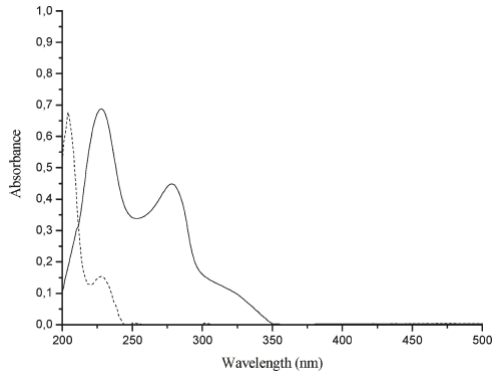


Figure 8.5. *UV spectrum of catechin (- - -) and catechin-grafted-chitosan (−).*

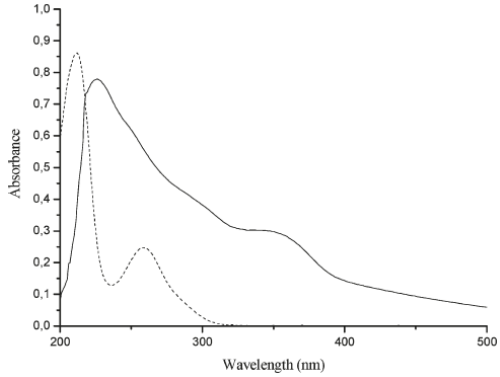


Figure 8.6. *UV spectrum of gallic acid (- - -) and gallic acid-grafted-chitosan (−).*

Finally, DSC analyses of pure antioxidants, blank chitosan and each chitosan conjugate were performed (*Figures 8.7* and *8.8*). The calorimetric analysis of pure gallic acid shows a sharp melting endotherm at 266.5°C, corresponding to the melting point of antioxidant molecule (*Figure 8.7C*), while for pure catechin a melting endotherm at 155.8°C was displayed (*Figure 8.8C*).

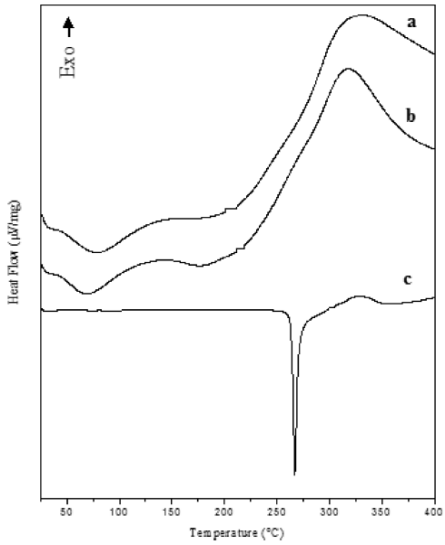


Figure 8.7. *DSC of gallic acid (c), blank chitosan (b), and gallic acid-grafted-chitosan (a).*

As far as DSC of blank chitosan is concerned (*Figures 8.7B* and *8.8B*) a broad endotherm, located around 39-151°C, are clearly visible and have been assigned to the glass transition of polysaccharidic chain; ΔC_p associated to this transition was -195 J/g. DSC thermograms of gallic acid-*grafted*-chitosan (*Figure 8.7A*) displays the disappearance of melting endotherm of gallic acid and a ΔC_p value (-241 J/g), associated to the polysaccharidic gel transition, higher than that observed in blank chitosan.

Similar results were observed in the DSC thermogram of catechin-chitosan conjugate (*Figure 8.8A*). Then different thermal behaviours between blank chitosan and these conjugated systems were observed and can be ascribable to the covalent doping of chitosan with antioxidant compounds.

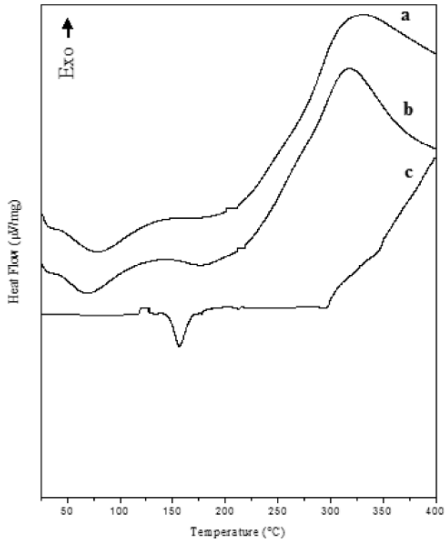


Figure 8.8. *DSC of catechin (c), blank chitosan (b), and catechin-grafted-chitosan (a).*

3.3 Characterisation of gelatin-antioxidant conjugates

The grafting procedure carried out in large amount of solvent (water) and functional molecules (GA and CT), allows to obtain water soluble gelatin conjugates. In these conditions, 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 8.9*).

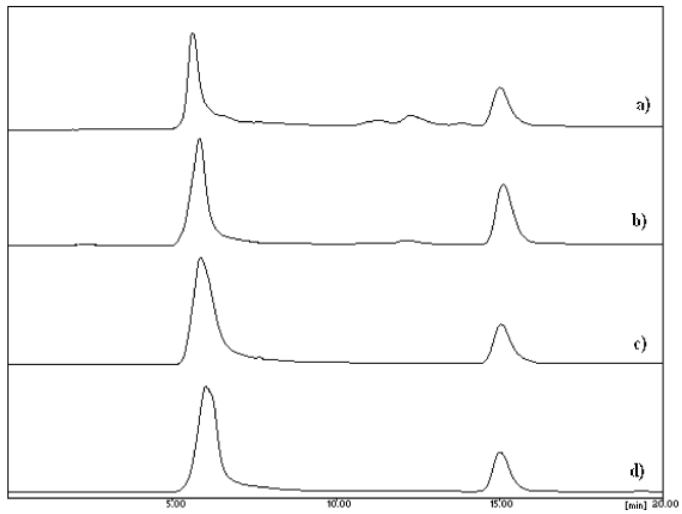


Figure 8.9. *Gel permeation chromatograms of native gelatin (a), blank gelatin (b), GA-GEL (c), and CT-GEL (d).*

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 GA-GEL conjugate, 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 spectrum the wavelength of the aromatic peaks is lower (211 and 258 nm) than in grafted protein, as depicted in *Figure 8.10a*. The same results appear in the UV-Vis spectrum of CT-GEL conjugate, where it is evident the displacement of the adsorption bands to higher wavelength (230 and 275 nm) respect to free CT (204 and 229 nm), as showed in *Figure 8.10b*. 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.

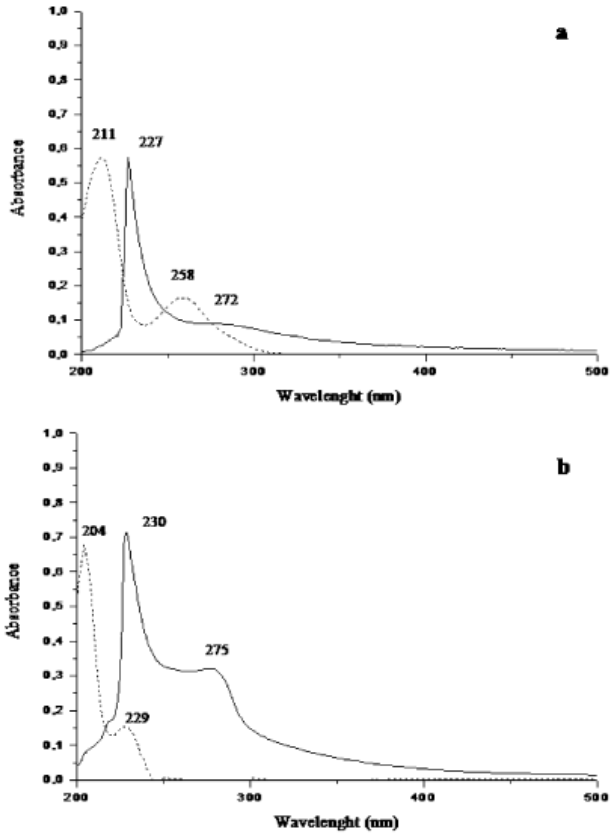


Figure 8.10. *UV-Vis spectra: (a) GA (- - -) and GA-GEL (—); (b) CT (- - -) and CT-GEL (—).*

The emission spectra of antioxidants and conjugate polymers also confirm the covalent functionalisation of the protein. In the spectra of GA-GEL and CT-GEL conjugates, bathochromic shifts of the emission peaks of GA (from 354 to 416 nm) and CT (from 318 to 409 nm), are detected (*Figure 8.11*). These spectral red shifts are due to the covalent conjugation, because no emission peak is detected in the same wavelength range for blank gelatin.

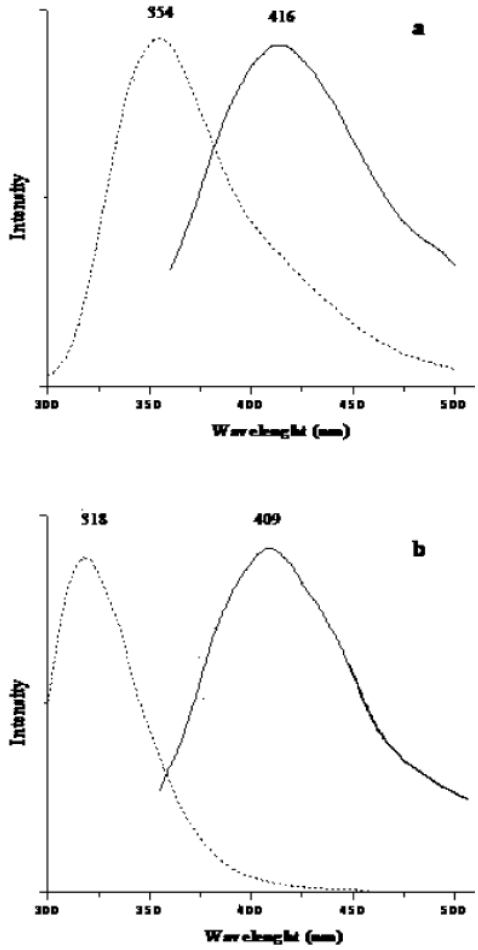


Figure 8.11. *Emission spectra: (a) GA (- - -) and GA-GEL (—); (b) CT (- - -) and CT-GEL (—).*

Thermal characterisation of the synthesised 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 8.12* and *8.13*, for GA-GEL and CT-GEL, respectively.

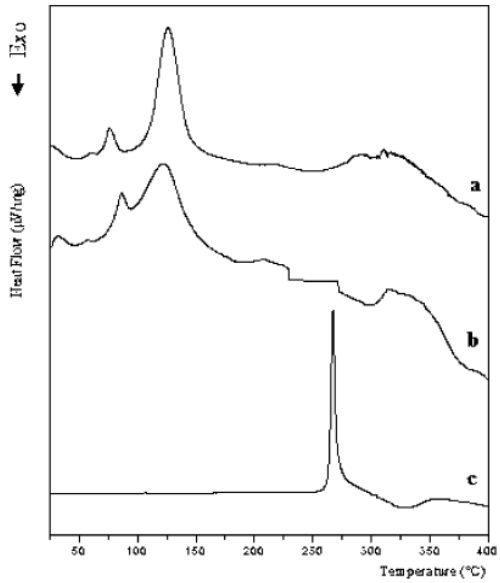


Figure 8.12. *Calorimetric analyses of GA-GEL (a), blank gelatin (b), and GA (c).*

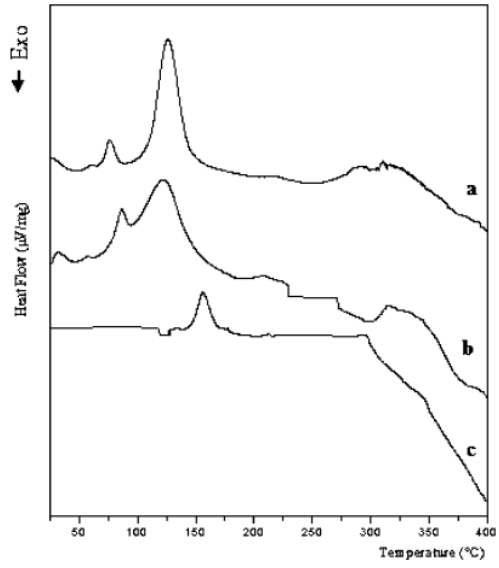


Figure 8.13. *Calorimetric analyses of CT-GEL (a), blank gelatin (b), and CT(c).*

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; ΔC_p associated to this transition was -242.1 J

per grams of grafted protein (*Figures 8.12b* and *8.13b*). The calorimetric analysis of pure GA shows a narrow melting endotherm at 266.5°C, corresponding to the melting point of antioxidant molecule (*Figure 8.12c*), while for pure CT a melting endotherm at 155.8°C was displayed (*Figure 8.13c*). Since the grafting of GA produces structural modification onto the proteic chains, in the DSC thermogram of GA-GEL conjugate (*Figure 8.12a*) marked differences appear. The calorimetric analysis displays the absence of melting endotherm of GA, while, the ΔC_p 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 of GA-GEL conjugate needs 25% more heat respect to unmodified protein. The same results were observed in the DSC thermogram of CT-GEL conjugate (*Figure 8.13a*), where the endotherm due to melting of CT disappears, while the endotherm at 40-180°C shows a ΔC_p equal to -285.5 J per grams of grafted protein; in this case to produce glass transition in the CT-GEL conjugate about 18% more heat is necessary respect to unmodified polymer. Thus, the conjugation of gelatin with GA and CT causes an increasing in the thermal stability of the native protein.

3.4 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.

Polymers scavenger ability were evaluated in terms of DPPH reduction using, for each synthesised polymer, gallic acid and (+)-catechin as reference compounds and data are expressed as inhibition (%).

As reported in *Table 8.1*, in our operating conditions, all the conjugates showed high scavenging activity.

Table 8.1. *Inhibition percentages of linoleic acid peroxidation, DPPH radical and hydroxyl radical.*

SAMPLE	Inhibition (%)		
	Linoleic Acid Peroxidation	**DPPH Radical**	**Hydroxyl Radical**
Blank Chitosan	23± 1.2	14± 1.1	17± 1.4
CT-CHIT	98 ± 0.8	98 ± 1.1	95 ± 0.9
GA-CHIT	85 ± 0.9	92 ± 1.3	60 ± 1.1
Blank Gelatin	43 ± 3	36 ± 2	28 ± 2
CT-GEL	99 ± 2	98 ± 3	76 ± 3
GA-GEL	98 ± 1	66 ± 3	64 ± 3

3.5 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 attack 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, all the conjugates exhibited good antioxidant properties (*Table 8.1*).

3.6 Evaluation of disposable phenolic groups by Folin-Ciocalteu procedure

Since the antioxidant activity of all the conjugates 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 chains. 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 complex to form chromogens in which the metals have lower valence.

For each biopolymer, disposable phenolic groups were expressed as mg equivalent of the respective functionalising antioxidant.

Particularly, for gallic acid- and catechin-chitosan conjugates these values were 7 mg/g and 4 mg/g of dry polymers, respectively, while for gallic acid- and catechin-gelatin conjugates these values are equal to 0.7 mg/g and 0.9 mg/g.

3.7 Determination of total antioxidant activity

The assay is based on the reduction of Mo(VI) to Mo(V) by the antioxidant agent 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 control polymers, which contained no antioxidant component.

The high absorbance values indicated that the synthesised materials had significant antioxidant activities, and gallic acid and catechin mg equivalents in the respective functionalised polymers were found to be 3 mg/g and 5 mg/g for chitosan conjugates, and 0.5 mg/g and 0.4 mg/g for gelatin conjugates.

3.8 Hydroxyl radical (OH·) scavenging activity

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

This radical exhibits very high reactivity and tends to react with a wide range of molecules found in living cells. It can interact with the purine and pyrimidine bases of DNA. It 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. 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 pro-oxidant in the catalysed 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 by donation of hydrogen or electron from the antioxidant to the radical or by direct reaction with it. Consequently, the ability of the syntesised 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 and blank gelatin (*Table 8.1*).

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 and gelatin, two of the most widely used natural biopolymers.

The rapidity of the reaction, together with the absence of toxic reaction products, makes this procedure very useful to exalt the biological properties of the two macromolecules.

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

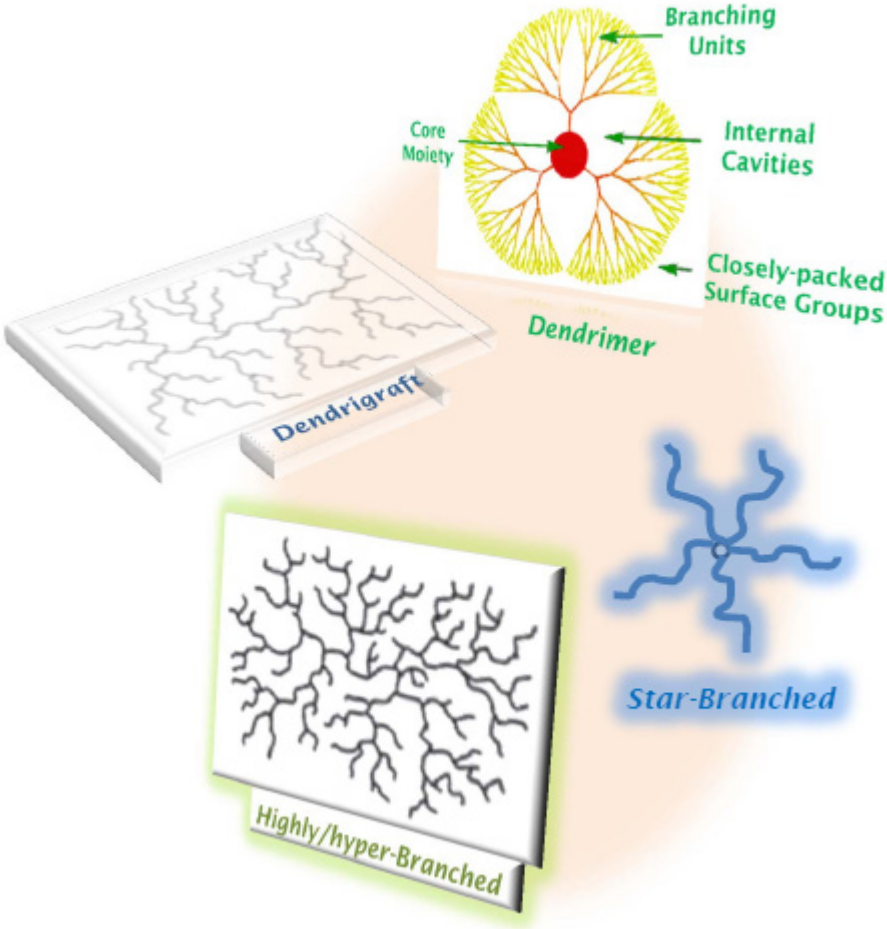
The covalent insertion of gallic acid and catechin in the polymeric chains was confirmed by UV-Vis, fluorescence and FT-IR analyses, while the enhanced thermal stability of the functional materials was demonstrated by DSC thermograms.

Finally, antioxidant properties of all the conjugates were evaluated by performing five different assays. Particularly, determination of scavenging activity on DPPH and hydroxyl radicals, 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 adopted synthetic strategy allows to improve the properties of natural polymers, such as chitosan and gelatin, and to introduce new particular features for specific applications in pharmaceutical, cosmetic and food industry.

SECTION III

BRANCHED POLYMERS



INTRODUCTION

Macromolecular architecture plays an important role to obtain polymeric materials characterised by specific properties: the properties of a polymer are indeed considerably influenced by its end groups[1]. Due to the significantly larger number of end groups per single polymer chain, this effect is more evident for a branched structure compared to a linear polymer. The introduction of large amounts of branching units into linear polymers dramatically changes the physical properties. In fact, it has been demonstrated that the chemical nature of the end-group functionalities of a branched polymer influences not only the solubility in various solvents[2,3], but also melt and thermal properties such as the glass transition temperature[4,5] and crystallinity[6].

Polymers with a branched architecture, which include dendrimers and hyperbranched (HB), show interesting and versatile properties that drive synthetic efforts to their production[7].

Dendrimers are well defined synthetic macromolecules, with regularly placed branched repeat units[8], often considered as perfect branched architectures with at least one branch point per monomer unit. This kind of materials is characterised by a high degree of symmetry and a high number of functional groups on the surface. Dendrimers are synthesised in a step-wise fashion from simple branched monomer units and consist of three different parts: a central core or moiety, internal layers which contain the repeat branching units for each

[1] *Cyclic Polymers*, 2nd ed.; Semlyen, J.A., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000.
[2] Wooley, K.L.; Hawker, C.J.; Lee, R.; Fréchet, J.M.J. *Polym. J.* **1994**, *26*, 187.
[3] Kim, Y.H.; Webster, O.W. *Macromolecules* **1992**, *25*, 5561.
[4] Uhrich, K.E.; Hawker, C.J.; Fréchet, J.M.J.; Turner, S.R. *Macromolecules* **1992**, *25*,4583.
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[7] England, R.M.; Rimmer, S. *Polym. Chem.* **2010**, DOI: 10.1039/c0py00154f.
[8] Roovers, J.; Comanita, B. *Adv. Polym. Sci.* **1999**, *142*, 179.

generation and which form internal voids and channels, and a surface of terminal units which are closely packed[9]. (*Figure 1.*)

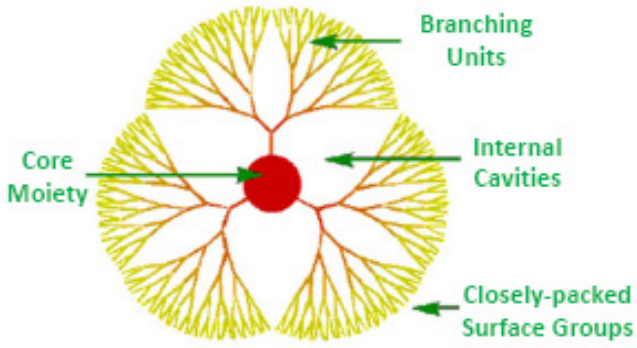

Figure 1. *Schematic representation of a dendrimer.*

The core is the central unit and represents the center of symmetry for the entire molecule. Connected to the core is a first layer of branched repeat units or monomers that is considered to be the first generation of the dendrimer. Each generation, usually but not necessarily, contains the same branched repeat units. The process of growth is extendable to several more generations. Because of the multifunctionality of each repeat unit, the number of segments in each generation grows exponentially. The end-standing groups of the outermost generation are called peripheral or terminal groups. The unique architecture and monodisperse structure of dendrimers result in improved physical and chemical properties, such as excellent solubility and very low solution viscosities, when compared to traditional linear polymers. These characteristics make them particularly good candidates for a wide range of applications, from catalyst supports to drug carriers.

Branched and hyperbranched polymers are three-dimensional macromolecules which are closely related to dendrimers. These systems exhibit defects in the structure, such as varying amounts of linear segments, and this

[9] Tomalia, D.A.; Fréchet, J.M.J. *J. Polym. Sci.: Part A: Polym. Chem.* **2002**, *40*, 2719.

leads to properties that are modified relative to those of dendrimers. The structure of branched and hyperbranched polymers, is less regular than that of dendrimers in a number of ways (*Figure 2.*)[10].

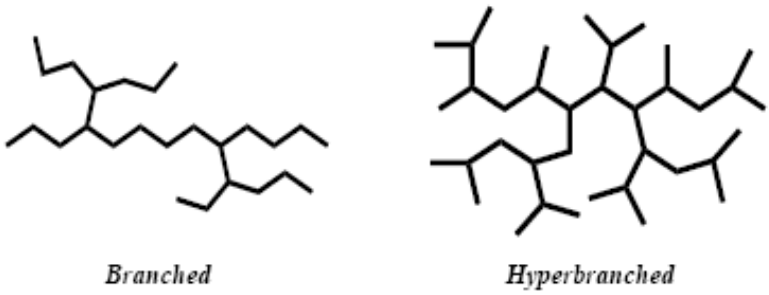

Figure 2. *Schematic representation of a branched polymer and a hyperbranched polymer.*

First of all, they usually show broad molecular weight distributions and irregular branching, but the key point is an uneven distribution of functional groups throughout the molecule that are not all involved in branching[11].

Although hyperbranched polymers lack in the uniformity of monodisperse dendrimers, they still possess many attractive dendritic features such as good solubility, low solution viscosity, globular structure, and multiple end groups[12]. Furthermore, the synthesis of dendrimers is labour (multistep procedures) with time consuming purifications and therefore expensive. Based on these considerations, their production is prohibitive and not suitable to scale-up. On the other hand, the usually inexpensive, one-pot synthesis of hyperbranched macromolecules makes them particularly interesting from an industrial point of view.

[10] Gao, C.; Yan, D. *Prog. Polym. Sci.* **2004**, *29*, 183.
[11] Unal, S.; Yilgor, I.; Yilgor, E.; Sheth, J.P.; Wilkes, G.L.; Long, T.E. *Macromolecules* **2004**, *37*, 7081.
[12] Wooley, K.L.; Fréchet, J.M.J. Hawker, C.J. *Polymer* **1994**, *35*, 4489.

Within the past ten years the development of new synthetic strategies to hyperbranched polymers has surpassed the detailed investigation of these materials.

Crucially, Sherrington and co-workers (Costello et al.[13], O'Brien et al.[14], Isaure et al.[15]) have developed a new facile, generic and cost effective route for the synthesis of branched polymers (*Strathclyde route*) via conventional free radical polymerisation. The synthetic strategy involves the use of commercially available vinyl monomers in the presence of chain transfer agents (CTAs) to inhibit cross-linking and gelation processes. The stoichiometric balance between the branching agent and the CTA represents the key point of this strategy that holds out good promise in terms of scale-up and exploitation.

[13] Costello, P.A.; Martin, I.K.; Slark, A.T.; Sherrington, D.C.; Titterton, A. *Polymer* **2002**, *43*, 245.
[14] O'Brien, N.; McKee, A.; Sherrington, D.C.; Slark, A.T.; Titterton, A. *Polymer* **2000**, *41*, 6027.
[15] Isaure, F.; Cormack, P.A.G.; Sherrington, D.C. *J. Mater. Chem.* **2003**, *13*, 2701.

CAPITOLO IX

Synthesis of Branched Polymers with Antifungal and Antioxidant Properties

1. Introduction

In the recent period, antioxidant and antifungal polymers have aroused an increasing and considerable interest due to their higher stability and slower degradation rate than compounds with low molecular weight[1,2]. This kind of macromolecular systems could be useful for a broad range of applications in which the employment of a single molecule with antioxidant or antifungal properties is prohibitive[3,4]. For example, they could find application as preservative agents in food packaging and in pharmaceutical and cosmetic formulations, in order to avoid the oxidation of their components or the contamination by pathogenic fungi. These microorganisms, indeed, are characterised by a strong ability to survive on different surfaces[5] and contaminated materials are often associated with increased risk of infections. Medically important fungi, such as *Candida, Aspergillus, Fusarium, Mucor,* and *Paecilomyces spores*, survive on hospital fabrics from one day to several weeks, thus, the development of antifungal polymers as infection-resistant materials for hospital, medical, pharmaceutical, bioprotective, and related hygienic applications[6], could be very interesting.

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[1] Kurisawa, M.; Chung, J.E.; Uyama, H.; Kobayashi, S. *Biomacromolecules* **2003**, *4*, 1394.
[2] Pan, J.Q.; Liu, N.C.; Lau, W.W.Y. *Polym. Degrad. Stab.* **1998**, *62*, 165.
[3] Puoci, F.; Iemma, F.; Curcio, M.; Parisi, O.I.; Cirillo, G.; Spizzirri, U.G.; Picci, N. *J. Agric. Food Chem.* **2008**, *56*, 10646.
[4] Abdou, E.S.; Elkholy, S.S.; Elsabee, M.Z.; Mohamed, E. *J. Appl. Polym. Sci.* **2008**, *108*, 2290.
[5] Narasimhan, B.; Belsare, D.; Pharande, D.; Mourya, V.; Dhake, A. *Eur. J. Med. Chem.* **2004**, *39*, 827.
[6] Cao, Z.B.; Sun, Y.Y. *ACS Appl. Mater. Inter.* **2009**, *1*, 494.

The synthesis of antioxidant and antifungal polymers, presenting also a branched structure, could be very attractive to obtain materials with unique biological, chemical and physical properties.

Branched polymers are macromolecules characterised by the presence of numerous branch points and more than two end groups. These materials, representing a class of polymers somewhere between linear polymers and polymeric networks, usually show broad molar mass distributions. In these systems, the functional groups are randomly distributed throughout the macromolecule and not all of them are involved in branching.

Numerous studies have revealed that branched polymers offer significantly different physical properties from linear polymers and polymer networks, such as melt rheology, mechanical behavior, and solution properties[7]. The introduction of large amounts of branching units in these macromolecules is responsable for lower solution and melt viscosities, increased solubility in appropriate solvents and higher functional group density, features that can be very different from those of their linear analougues[8,9,10,11].

The aim of this study is the synthesis of novel branched polymers characterised by antifungal and antioxidant properties, and the evaluation of their biological activities.

In recent years, there has been an increasing number of reports on the physiological functions of ferulic acid and its derivatives, such as 3,4-dimethoxycinnamic acid (DMCA), in humans including antioxidant, antifungal, anti-inflammatory, antithrombosis, and anticancer activities[12,13,14,15]. In this work the adopted strategy was the functionalisation of DMCA by the insertion of a

[7] McKee, M.G.; Unal, S.; Wilkes, G.L.; Long, T.E. *Prog. Polym., Sci.* **2005**, *30*, 507.
[8] Burchard, W. *Adv. Polym. Sci.* **1999**, *143*, 113.
[9] McLeish, T.C.B.; Milner, S.T. *Adv. Polym. Sci.* **1999**, *143*, 195.
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[14] Xu, L.N.; Xu, D.C.; Zhang, Z.J. *acta Acad. Med. Sinicae* **1984**, *6*, 414.
[15] Panizzi, L.; Catalano, S.; Miarelli, C.; Cioni, P.L.; Campeol, E. *Phytother. Res.* **2000**, *14*, 561.

polymerisable group and its subsequent copolymerisation to obtain branched polymers. Methyl methacrylate (MMA) and ethylene glycol dimethacrylate (EGDMA) were employed as functional monomer and branching agent, respectively, in the presence of a chain transfer agent (1-dodecanethiol, DDT) to inhibit cross-linking and gelation processes. All the polymers were synthesised by a conventional solution phase free radical polymerisation procedure using 2,2'-azoisobutyronitrile (AIBN) as initiator.

Control polymers for the evaluation of biological properties were synthesised under the same reaction conditions but using 2-hydroxyethyl methacrylate (HEMA) instead of the antioxidant and antifungal co-monomer.

The obtained polymeric materials were characterised by solubility tests in chloroform, determination of isolated recoveries, NMR spectroscopy and GPC analyses.

2. Materials and methods

2.1 Materials

3,4-dimethoxycinnamic acid (DMCA), thionyl chloride, 2-hydroxyethyl methacrylate (HEMA), triethylamine (TEA), sodium hydroxide, hydrochloric acid, sodium sulfate, methyl methacrylate (MMA), ethylene glycol dimethacrylate (EGDMA), 1-dodecanethiol (DDT), 2,2'-azoisobutyronitrile (AIBN) were obtained from Sigma-Aldrich Chemical Co.

All solvents were reagent or HPLC-grade and provided by Sigma-Aldrich Chemical Co.

Methyl methacrylate and ethylene glycol dimethacrylate were purified by passing down an alumina column, 2-hydroxyethyl methacrylate was purified by distillation and 2,2'-azoisobutyronitrile (AIBN) by recrystallisation.

2.2 Monomer synthesis

The acid chloride of 3,4-dimethoxycinnamic acid was prepared by refluxing 2.1 g (10 mmol) with 10 mL (137 mmol) of thionyl chloride with a

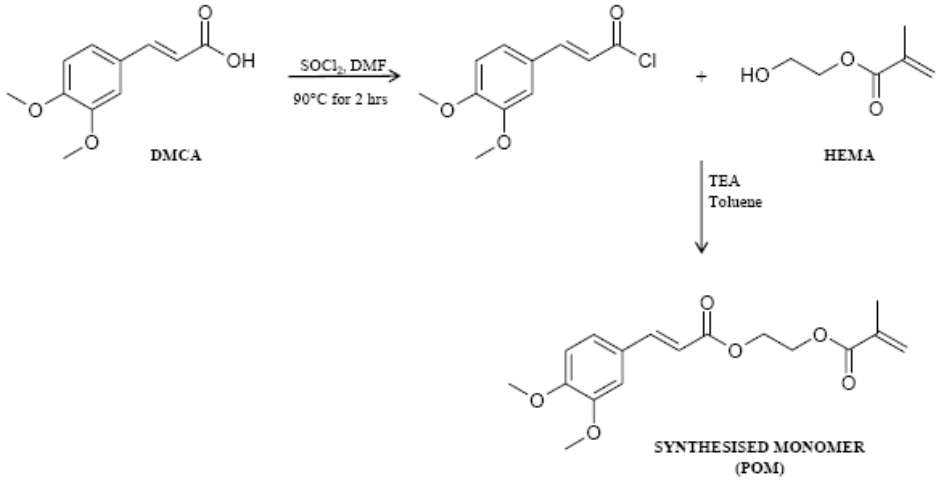
few drops of anhydrous DMF at 90°C for two hours[16]. Then, excess thionyl chloride was removed by evaporation under reduced pressure.

A two-necked round bottom flask (250 mL) was charged with 1.21 mL (10 mmol) of freshly distilled 2-hydroxyethyl methacrylate (HEMA), 1.5 mL (11 mmol) of triethylamine (TEA) and 50 mL of anhydrous toluene. The flask was fitted with a mechanical stirrer and kept in an ice bath. The obtained acid chloride was dissolved in 50 mL of toluene and added dropwise to the two-necked flask. The stirring was continued overnight and the preparation of acid chloride and its esterification were carried out in a nitrogen atmosphere.

The precipitated salt (TEA·HCl) was filtered off and the filtrate was extracted with distilled water, NaOH (5% w/v), HCl (5% w/v) and distilled water several times. The aqueous phase was discarded every time. The organic phases were combined and dried over Na_2SO_4. On evaporating the solvent from the organic phase, the monomer (POM) was obtained.

The monomer was further purified by column chromatography. The column was packed with silica gel and toluene:ethyl acetate (9:1 v/v) mixture. The eluting medium was toluene:ethyl acetate (9:1 v/v) mixture.

The monomer synthesis is shown in *Scheme 9.1*.



Scheme 9.1. *Monomer synthesis.*

2.3 Synthesis of linear and branched polymers

The copolymerisations were carried out using a Carousel 6 Place Reaction Station (Radley Discovery Technology, U.K.).

Linear and branched polymers were synthesised in absence and in presence of different amounts of chain transfer agent (*Table 9.1*). MMA (20 mmol) and all the other reactants were added to 10 mL of anhydrous THF and the mixtures were purged with nitrogen. The reaction vessels, each equipped with a condenser, were maintained at 60°C for 16 h with magnetic stirring. Each polymer was isolated by precipitation into an excess volume of methanol, collected by filtration using a filter funnel (porosity 4) and washed with methanol. Finally, the recovered polymers were dried overnight in a vacuum oven set at 40°C.

Control polymers were synthesised for the evaluation of biological properties under the same reaction conditions but using 2-hydroxyethyl methacrylate (HEMA) instead of the antioxidant and antifungal co-monomer.

Table 9.1. *Polymers feed composition.*

POLYMER	MOLAR RATIO MMA/CO-MONOMER/EGDMA/DDT/AIBN	RECOVERY (%)
PMMA in absence of DDT		
POI 9	100/0/0/0/1	*77*
POI 10	100/0/0/0/1	*78*
PMMA in presence of DDT		
POI 17	100/0/0/0.5/1	*79*
POI 16	100/0/0/0.8/1	*76*
POI 15	100/0/0/1/1	*71*
POI 14	100/0/0/1.2/1	*67*
POI 13	100/0/0/1.5/1	*65*
POI 12	100/0/0/2/1	*55*
MMA/HEMA Linear Copolymer in absence of DDT		
POI 37	H-100/2/0/0/1	*77*

MMA/HEMA Linear Copolymers in presence of DDT		
POI 47	H-100/2/0/0.5/1	*65*
POI 46	H-100/2/0/0.8/1	*66*
POI 38	H-100/2/0/1/1	*64*
POI 39	H-100/2/0/2/1	*56*
POI 40	H-100/2/0/3/1	*44*
MMA/POM Linear Copolymer in absence of DDT		
POI 34	POM-100/2/0/0/1	*69*
MMA/POM Linear Copolymers in presence of DDT		
POI 49	POM-100/2/0/0.5/1	*75*
POI 48	POM-100/2/0/0.8/1	*67*
POI 43	POM-100/2/0/1/1	*74*
POI 44	POM-100/2/0/2/1	*57*
POI 45	POM-100/2/0/3/1	*50*
Control Polymer with EGDMA (2%) in absence of co-monomer		
POI 58	100/0/2/2/1	*60*
MMA/HEMA Branched Polymers (EGDMA 2%)		
POI 54	H-100/2/2/0.5/1	*92*
POI 55	H-100/2/2/0.8/1	*98*
POI 56	H-100/2/2/1/1	*76*
POI 59	H-100/2/2/1.8/1	*64*
POI 57	H-100/2/2/2/1	*69*
POI 60	H-100/2/2/2.2/1	*60*
POI 61	H-100/2/2/2.4/1	*55*
POI 62	H-100/2/2/2.6/1	*54*
POI 63	H-100/2/2/2.8/1	*53*
MMA/POM Branched Polymers (EGDMA 2%)		
POI 67	POM-100/2/2/1.8/1	*70*
POI 64	POM-100/2/2/2/1	*69*
POI 65	POM-100/2/2/2.2/1	*64*
POI 66	POM-100/2/2/2.4/1	*63*
POI 68	POM-100/2/2/2.6/1	*65*
POI 69	POM-100/2/2/2.8/1	*57*

MMA/HEMA Branched Polymers (EGDMA 5%)		
POI 70	H-100/2/5/5/1	*46*
POI 71	H-100/2/5/5.2/1	*40*
POI 72	H-100/2/5/5.4/1	*48*
MMA/POM Branched Polymers (EGDMA 5%)		
POI 73	POM-100/2/5/5/1	*52*
POI 74	POM-100/2/5/5.2/1	*55*
POI 75	POM-100/2/5/5.4/1	*54*
MMA/HEMA Branched Polymers (EGDMA 10%)		
POI 78	H-100/2/10/10/1	*58*
POI 79	H-100/2/10/10.2/1	*53*
POI 80	H-100/2/10/10.4/1	*56*
MMA/POM Branched Polymers (EGDMA 10%)		
POI 83	POM-100/2/10/10/1	*63*
POI 84	POM-100/2/10/10.2/1	*63*
POI 85	POM-100/2/10/10.4/1	*62*

*H: HEMA; **POM: Synthesised Monomer.

2.4 Characterisation

2.4.1 Solubility tests

Solubility tests were performed by weighing ~10 mg of each polymer and attemping to dissolve them in 1 mL of solvent. The samples were left to stand overnight at room temperature and, then, filtered off with an Acrodisc® 0.2 μm PTFE membrane. The solvent tested was chloroform.

2.4.2 Elemental microanalyses

Elemental microanalyses were carried out on a Perkin-Elmer 2400 analyser by the Elemental Microanalytical Service available within the Department of Pure and Applied Chemistry at the University of Strathclyde.

2.4.3 FT-IR spectroscopy

FT-IR spectra were recorded as films or KBr pellets on a Jasco FT-IR 4200.

2.4.4 NMR spectroscopy

^{1}H NMR and ^{13}C NMR spectra were recorded on a 500 MHz Oxford - AV500 spectrometer using $CDCl_3$ as the solvent in 5 mm NMR tubes. In all the spectra the signal of $CDCl_3$ was used as a reference.

2.4.5 Dual detection size exclusion chromatography (DD-SEC)

The instrument package was supplied by Optokem and comprised the following equipment: (i) a Jones Chromatography 760 series Solvent D-Gasser, (ii) a Waters 515 HPLC pump operating at room temperature, (iii) a Jasco AS-950 autosampler with 50 position sample racks, (iv) a column oven, (v) a set of three Styragel HR 2, HR 4 and HR 6 designation 7.8 x 300 mm GPC columns, and (vi) two detectors connected in a serial configuration: a multiangle light scattering detector (mini-Dawn) supplied by Wyatt Technology and an interferometer refractomer detector (Optilab DSP) supplied by Wyatt Technology.

Chloroform was the mobile phase, the column oven temperature was set to 30ºC, and the flow rate was 1 mL/min.

The samples were prepared for injection by dissolving 10 mg of polymer in 1 mL of HPLC-grade chloroform and filtered off with an Acrodisc® 0.2 µm PTFE membrane. 0.2 mL of this mixture was then injected, and data were collector for 40 min. The wavelength used was 690 nm. The d*n*/d*c* value used was 0.090 (Chloroform/PMMA). Astra for Windows was used to collect and process the signals transmitted from the detectors to the computer and to produce the molar mass distribution plots.

3. Results and discussion

3.1 Monomer characterisation

The synthesised monomer (34% yield) showed a melting point of 72-73°C and a R_f value equals to 0.27.

The FT-IR spectrum (*Figure 9.1*) shows several peaks in the region around 3000-3100 cm^{-1} ascribable to the aromatic −C−H stretching, peaks at 1585 and 1601 cm^{-1} awardable to −C−C− stretching within the aromatic ring, a characteristic peak at 1714 cm^{-1} for the conjugated ester carbonyl group and a peak at 1637 cm^{-1} due to −C=C− stretching. The absence of a broad peak at 3450 cm^{-1} for the –OH stretching confirms the formation of the monomer.

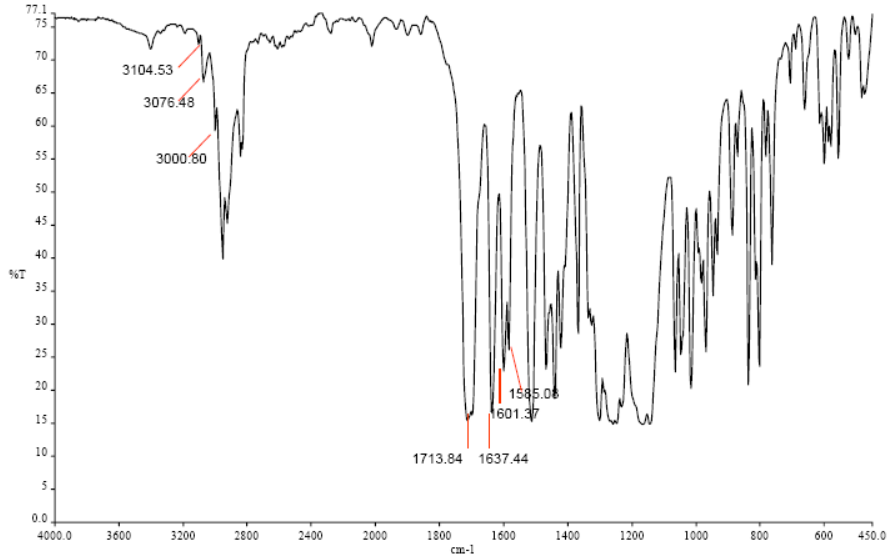


Figure 9.1. *FT-IR spectrum of POM.*

^{1}H NMR and ^{13}C NMR spectra also confirm the structure of the synthesised monomer.

All the peaks were assigned to their corresponding hydrogen atoms as shown in *Figure 9.2.*

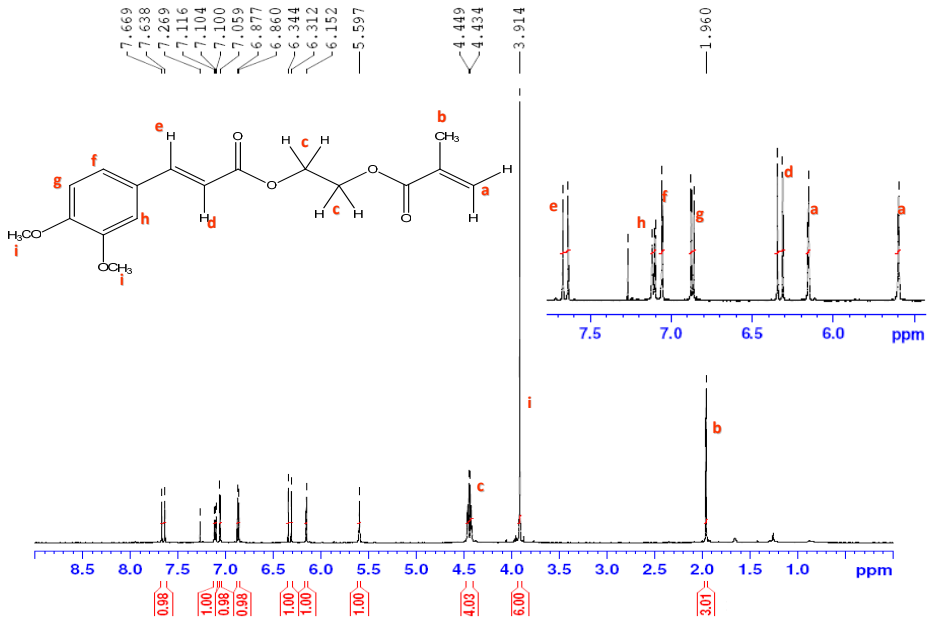


Figure 9.2. *^{1}H NMR spectrum of POM.*

The ^{13}C NMR spectrum (*Figure 9.3.*) shows 17 peaks corresponding to 17 different carbons.

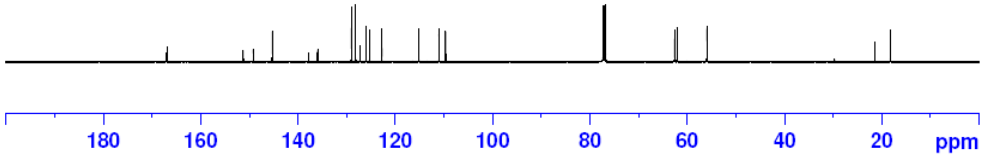


Figure 9.3. *^{13}C NMR spectrum of POM.*

Elemental Analysis: calcd for $C_{17}H_{20}O_6$: C, 63.74; H, 6.29. Found: C, 63.80; H, 6.42.

MALDI-TOF analysis: m/z 320.11.

3.2 Polymers characterisation

3.2.1 Solubility tests

Solubility tests were performed using chloroform as solvent. As shown in *Table 9.2*, all the synthesised polymers were soluble in chloroform except for POI54, POI55 and POI56. In the first two cases, indeed, macroscopic gels were obtained at the end of the reaction, while, in the last case, a very viscous solution was obtained and the polymer POI56 isolated by precipitation into methanol was not soluble in chloroform.

Table 9.2. *Appearance of the polymerisation mixture at the end of the reaction and solubility in chloroform.*

POLYMER	APPEARANCE OF THE REACTION MIXTURE	SOLUBILITY (10 mg/mL)
		$CHCl_3$
POI 58	Solution with some gel particles	Soluble
POI 54	Macroscopic gel	Insoluble
POI 55	Macroscopic gel	Insoluble
POI 56	Very viscous solution with some gel particles	Microgel
POI 59	Solution with a gel portion	Soluble
POI 57	Solution with some gel particles	Soluble
POI 60	Solution with some gel particles	Soluble
POI 61	Solution with some gel particles	Soluble
POI 62	Solution with some gel particles	Soluble
POI 63	Solution with some gel particles	Soluble
POI 67	Solution with a gel portion	Soluble
POI 64	Solution with a gel portion	Soluble
POI 65	Solution with some gel particles	Soluble
POI 66	Solution with some gel particles	Soluble
POI 68	Solution	Soluble
POI 69	Solution with some gel particles	Soluble
POI 70	Solution with a gel portion	Soluble
POI 71	Solution with a gel portion	Soluble
POI 72	Solution	Soluble
POI 73	Solution	Soluble
POI 74	Solution	Soluble

POI 75	Solution with some gel particles	Soluble
POI 78	Solution	Soluble
POI 79	Solution with some gel particles	Soluble
POI 80	Solution	Soluble
POI 83	Solution with some gel particles	Soluble
POI 84	Solution	Soluble
POI 85	Solution	Soluble

3.2.2 NMR spectroscopy

The ^{1}H NMR spectra (*Figure 9.4*) of linear PMMA homopolymers confirm the incorporation of DDT transfer agent with the multiplet at 1.3 ppm corresponding to the methylene H atoms in the -$(CH_2)_9$- group. The intensity of this signal increases with the amount of DDT used in the polymer formulation.

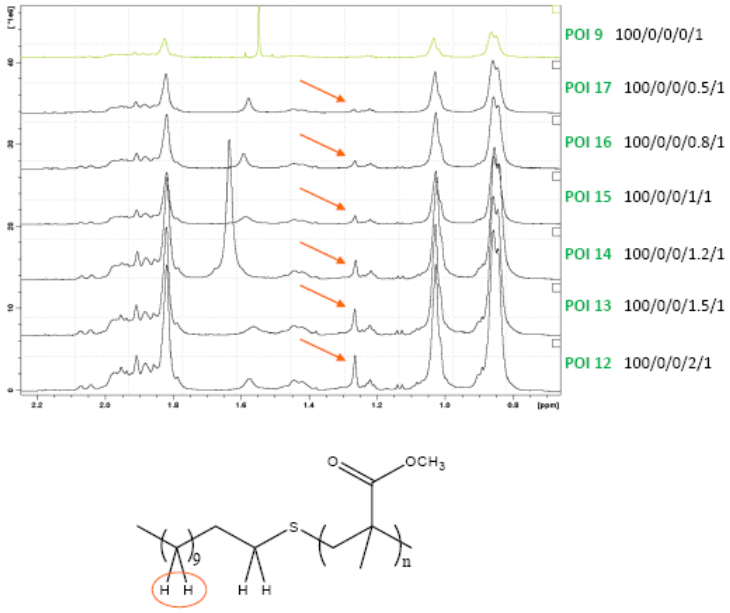


Figure 9.4. *^{1}H NMR spectra of linear PMMA homopolymers.*

In *Figure 9.5* the ^{1}H NMR spectrum of POI58 (branched polymer synthesised in absence of co-monomer) is shown. Signals at 4.1, 4.2 and 4.36

ppm are assigned to the methylene H atoms (H^5) in fully reacted (branching) EGDMA residues and to the methylene H atoms (H^1) and (H^2) in pendent unreacted EGDMA residues, though the 4.2 ppm signal is often overlapped by the 4.1 ppm signal. This spectrum shows evidence for unreacted pendent methacrylate groups with vinylic H atoms resonances at 5.65 (H^3) and 6.17 (H^4) ppm.

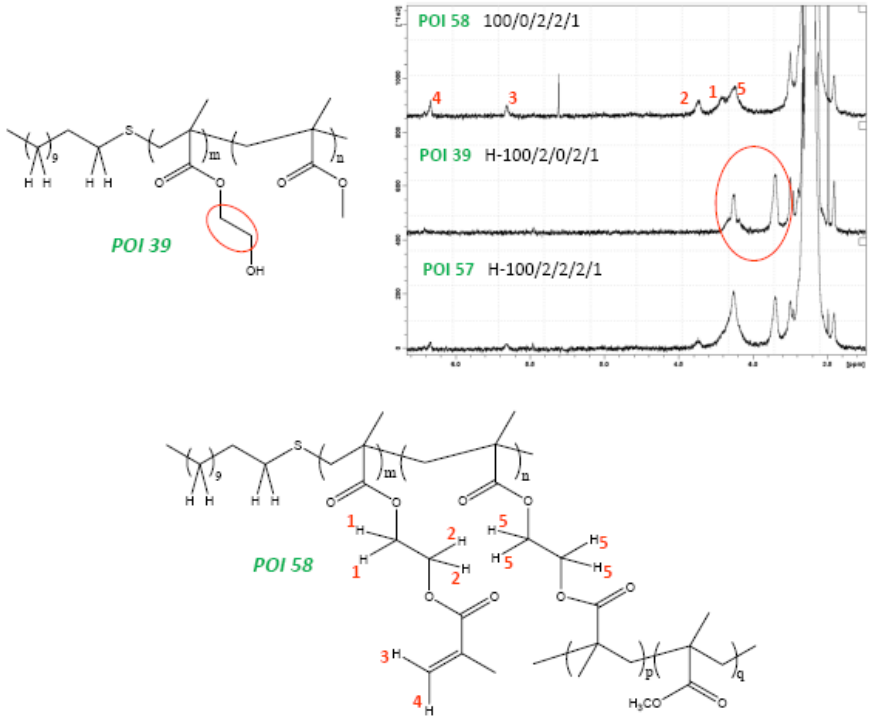


Figure 9.5. *^{1}H NMR spectra of HEMA polymers.*

In POI39 (linear MMA/HEMA copolymer) ^{1}H NMR spectrum, signals at 3.85 and 4.12 ppm are ascribable to methylene H atoms of HEMA; in POI57 ^{1}H NMR spectrum, signals from methylene H atoms of HEMA and EGDMA are overlapped confirming the incorporation of both the co-monomer and the branching agent in the polymer.

POI44 ^{1}H NMR spectrum clearly confirms the incorporation of POM monomer in the polymeric material, in POI64 ^{1}H NMR spectrum the presence of signals from POM and EGDMA is evident (*Figure 9.6*).

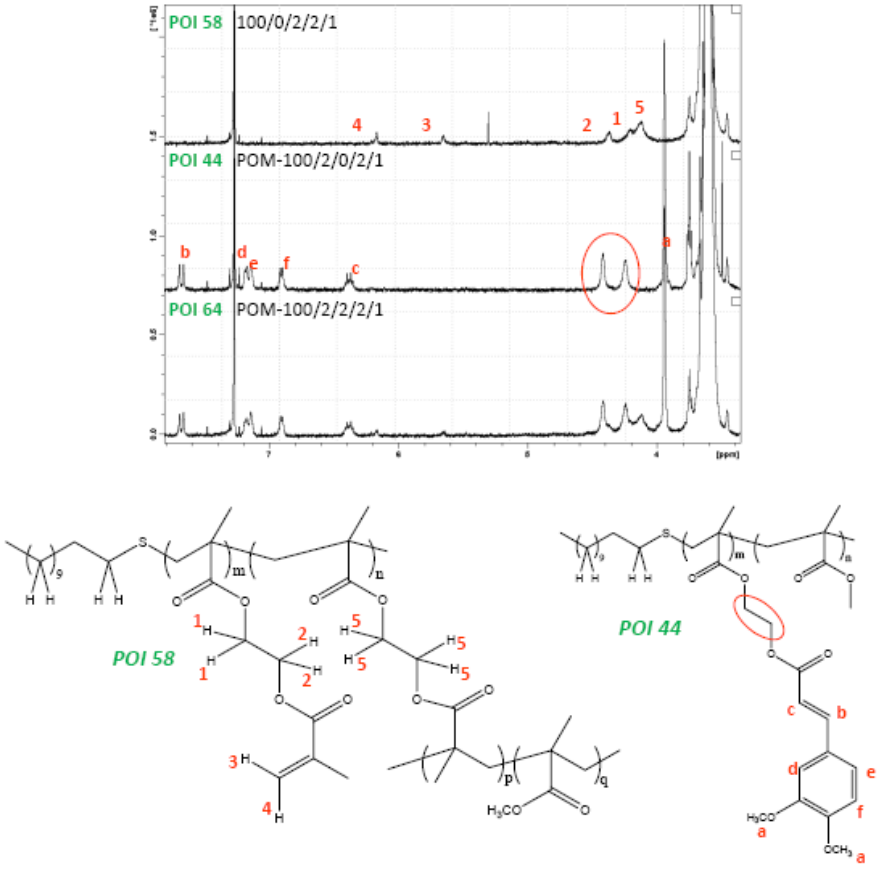


Figure 9.6. *^{1}H NMR spectra of POM polymers.*

3.2.3 Evaluation of DD-SEC data

Different kinds of distribution curves, essential for the characterisation of the synthesised polymers, can be obtained from SEC data (*Table 9.3*).

Table 9.3. *DD-SEC Data.*

POLYMER	MOLAR RATIO MMA/CO-MONOMER/ EGDMA/DDT/AIBN	DD-SEC DATA		
		M_n	M_w	M_w/M_n
PMMA in absence of DDT				
POI 9	100/0/0/0/1	24 130	33 550	1.39
POI 10	100/0/0/0/1	24 230	34 700	1.43
PMMA in presence of DDT				
POI 17	100/0/0/0.5/1	14 810	19 660	1.33
POI 16	100/0/0/0.8/1	12 770	16 630	1.30
POI 15	100/0/0/1/1	12 170	15 450	1.27
POI 14	100/0/0/1.2/1	12 170	15 000	1.23
POI 13	100/0/0/1.5/1	9 915	12 100	1.22
POI 12	100/0/0/2/1	9 761	11 520	1.18
MMA/HEMA Linear Copolymer in absence of DDT				
POI 37	H-100/2/0/0/1	22 560	32 750	1.45
MMA/HEMA Linear Copolymers in presence of DDT				
POI 47	H-100/2/0/0.5/1	18 650	24 490	1.31
POI 46	H-100/2/0/0.8/1	14 810	18 490	1.25
POI 38	H-100/2/0/1/1	12 780	15 930	1.25
POI 39	H-100/2/0/2/1	9 048	11 270	1.25
POI 40	H-100/2/0/3/1	7 927	8 793	1.11
MMA/POM Linear Copolymer in absence of DDT				
POI 34	POM-100/2/0/0/1	31 390	45 040	1.44
MMA/POM Linear Copolymers in presence of DDT				
POI 49	POM-100/2/0/0.5/1	17 190	23930	1.39
POI 48	POM-100/2/0/0.8/1	13 910	18 810	1.35
POI 43	POM-100/2/0/1/1	13 780	18 930	1.37
POI 44	POM-100/2/0/2/1	10 430	13 310	1.28
POI 45	POM-100/2/0/3/1	-	-	-
Control Polymer with EGDMA (2%) in absence of co-monomer				
POI 58	100/0/2/2/1	19 240	91 710	4.77

MMA/HEMA Branched Polymers (EGDMA 2%)				
POI 59	H-100/2/2/1.8/1	23 950	126 600	5.29
POI 57	H-100/2/2/2/1	20 900	141 700	6.78
POI 60	H-100/2/2/2.2/1	19 580	71 490	3.65
POI 61	H-100/2/2/2.4/1	18 880	56 310	2.98
POI 62	H-100/2/2/2.6/1	18 630	47 300	2.54
POI 63	H-100/2/2/2.8/1	17 370	41 950	2.42
MMA/POM Branched Polymers (EGDMA 2%)				
POI 67	POM-100/2/2/1.8/1	24 870	189 300	7.61
POI 64	POM-100/2/2/2/1	22 400	115 600	5.16
POI 65	POM-100/2/2/2.2/1	17 450	59 640	3.42
POI 66	POM-100/2/2/2.4/1	16 060	58 120	3.62
POI 68	POM-100/2/2/2.6/1	14 710	43 270	2.94
POI 69	POM-100/2/2/2.8/1	14 350	38 280	2.67
MMA/HEMA Branched Polymers (EGDMA 5%)				
POI 70	H-100/2/5/5/1	23 030	68 860	2.99
POI 71	H-100/2/5/5.2/1	19 060	45 840	2.41
POI 72	H-100/2/5/5.4/1	19 530	47 800	2.45
MMA/POM Branched Polymers (EGDMA 5%)				
POI 73	POM-100/2/5/5/1	20 370	77 860	3.82
POI 74	POM-100/2/5/5.2/1	16 480	50 960	3.09
POI 75	POM-100/2/5/5.4/1	17 410	60 260	3.46
MMA/HEMA Branched Polymers (EGDMA 10%)				
POI 78	H-100/2/10/10/1	28 110	157 500	5.60
POI 79	H-100/2/10/10.2/1	25 000	103 200	4.13
POI 80	H-100/2/10/10.4/1	25 130	104 200	4.15
MMA/POM Branched Polymers (EGDMA 10%)				
POI 83	POM-100/2/10/10/1	21 470	205 200	9.56
POI 84	POM-100/2/10/10.2/1	-	-	-
POI 85	POM-100/2/10/10.4/1	18 990	105 000	5.53

*H: HEMA; **POM: Synthesised Monomer.

The most informative way to present these data is in terms of molar mass distribution curves and molar mass vs elution volume plots. Differential weight fraction *versus* molar mass, which is also known as the molar mass distribution, gives information on the M_w, M_n and PDi; molar mass *versus* elution volume is important to prove if a polymer is effectively branched or not. Comparing it with the same plot of a linear polymer, which is equivalent to the branched one, at a given elution volume, it should produce higher molar masses than the linear polymer. It is also important to appreciate that, for example, a molar mass datum point at a single SEC elution volume potentially still relates to a complex mixture of macromolecules of differing molar mass and branching architecture, all of which coincidentally possess very similar hydrodynamic volume.

In this study, the obtained branched polymers give rise to complex SEC data. The resulting macromolecular products are, indeed, a complex mixture in terms of molar mass and backbone architecture.

The effect of the amount of the chain transfer agent (DDT) on the molar masses of linear PMMA homopolymers is shown in *Figure 9.7*: with an increasing amount of DDT in the polymer formulation, the molar masses decrease.

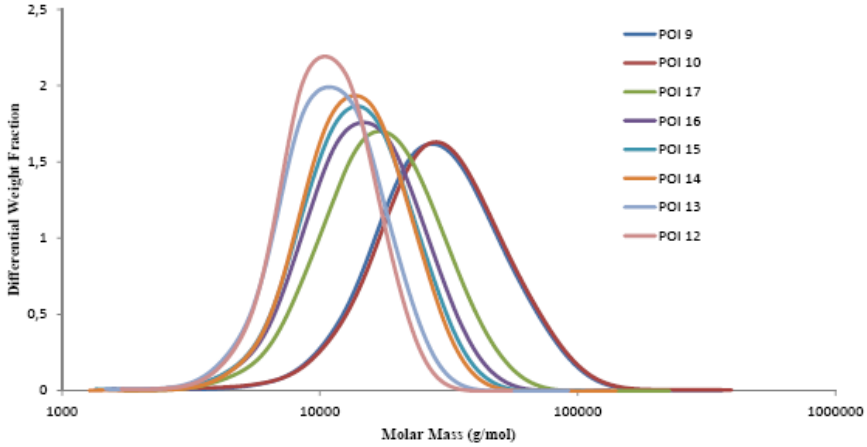


Figure 9.7. *Molar mass distributions showing the effect of increasing amount of DDT on the molar masses of linear PMMA homopolymers.*

The reproducibility of the polymerisation procedure was confirmed by the analyses of POI9 and POI10, synthesised employing the same molar ratio MMA/CO-MONOMER/BRANCHER/DDT/AIBN (100/0/0/0/1). These samples, indeed, present approximately the same molar masses and polydispersities.

Also for linear MMA/HEMA and MMA/POM copolymers (*Figures 9.8* and *9.9*, respectively), the distributions show a shift to higher molar mass as the level of DDT used in reactions reduced.

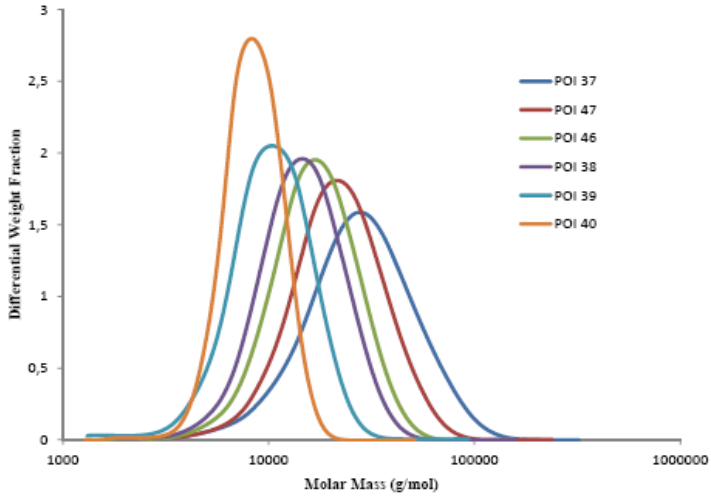


Figure 9.8. *Molar mass distributions for linear MMA/HEMA copolymers.*

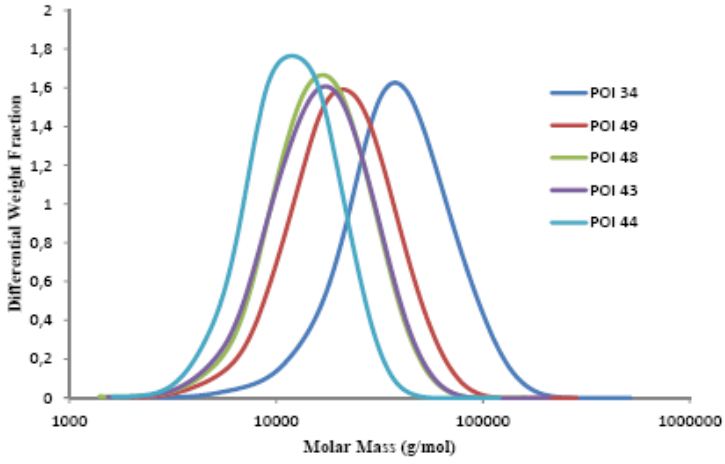


Figure 9.9. *Molar mass distributions for linear MMA/POM copolymers.*

Branched polymers containing HEMA or POM as co-monomer and EGDMA (2%) as brancher have broader molar masses than those of the correspondent linear analougues (POI37 and POI34, respectively), denoting higher PDi values. The distributions of the branched polymers are shifted towards lower molar masses as the level of DDT increased (*Figures 9.10* and *9.11*).

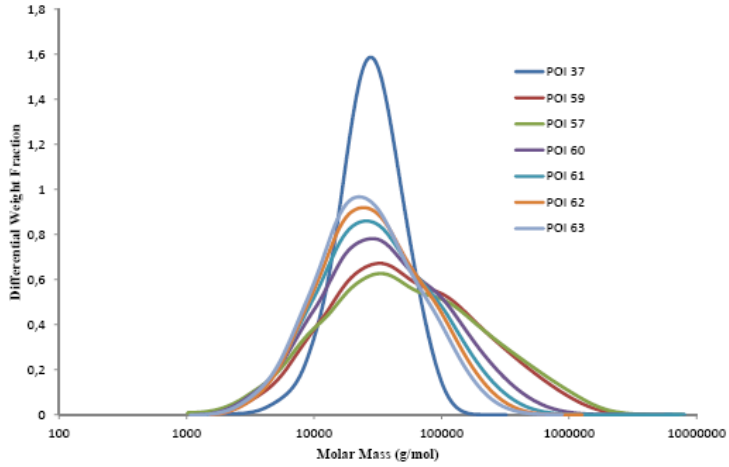


Figure 9.10. *Molar mass distributions for branched polymers containing HEMA as co-monomer and EGDMA (2%) as brancher.*

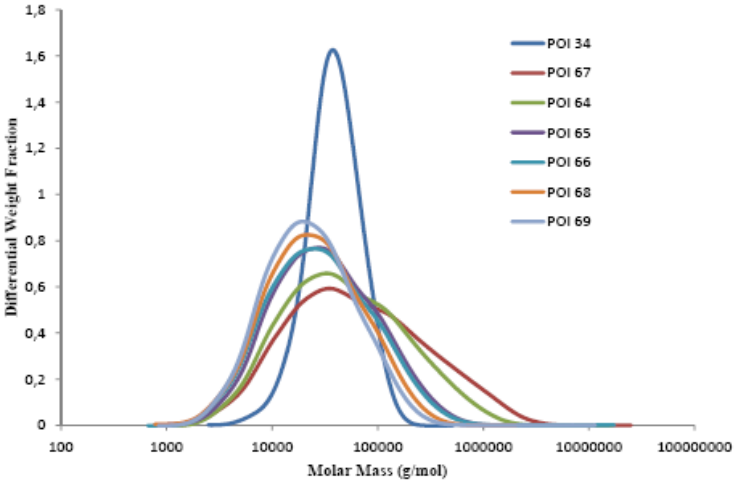


Figure 9.11. *Molar mass distributions for branched polymers containing POM as co-monomer and EGDMA (2%) as brancher.*

For the same polymers, comparing molar masses at different elution volumes, the elution volume plots are obtained (*Figures 9.12* and *9.13*).

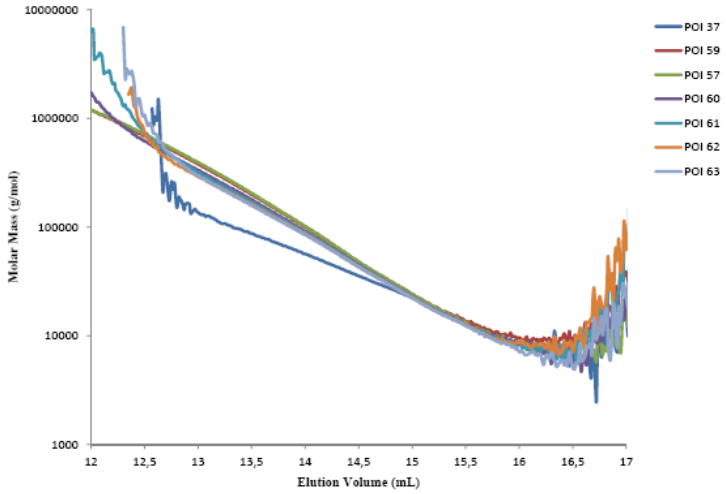


Figure 9.12. *Elution volumes plots for branched polymers containing HEMA as co-monomer and EGDMA (2%) as brancher.*

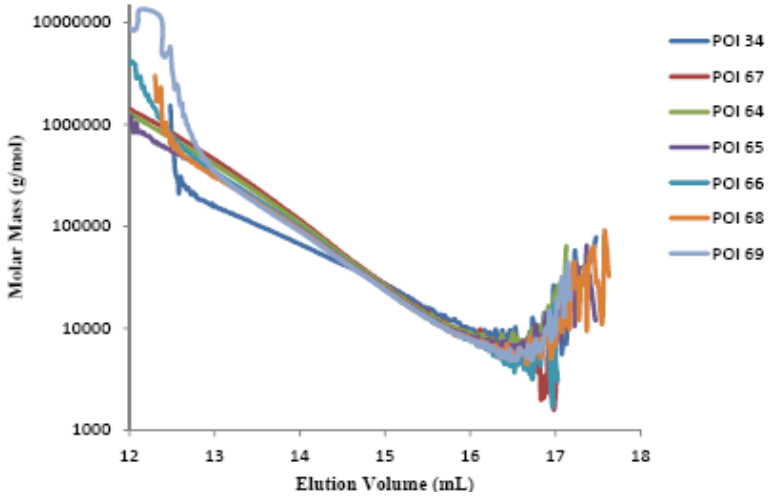


Figure 9.13. *Elution volumes plots for branched polymers containing POM as co-monomer and EGDMA (2%) as brancher.*

Polymers containing EGDMA, at given elution volumes, have higher molar mass than the linear references (POI37 and POI37). This effect is evident at

lower elution volumes, in the higher molar mass fractions. A difference in the polymers architecture, indeed, leads to different hydrodynamic volumes resulting with different elution profiles. It is therefore conclusive that polymers POI59-63 and polymers POI67-69 are branched in relation to linear polymers POI37 and POI34, respectively.

Molar mass *versus* elution volume plots of branched polymers synthesised employing POM as co-monomer and EGDMA (5% and 10%) as brancher are shown in *Figures 9.14* and *9.15*, respectively. Also in this case, all the polymers with EGDMA incorporated show evidence of branching. Comparing molar masses at different elution volumes, it is noticeable that these materials have, indeed, higher molar masses than the correspondent linear references (POI37 and POI34).

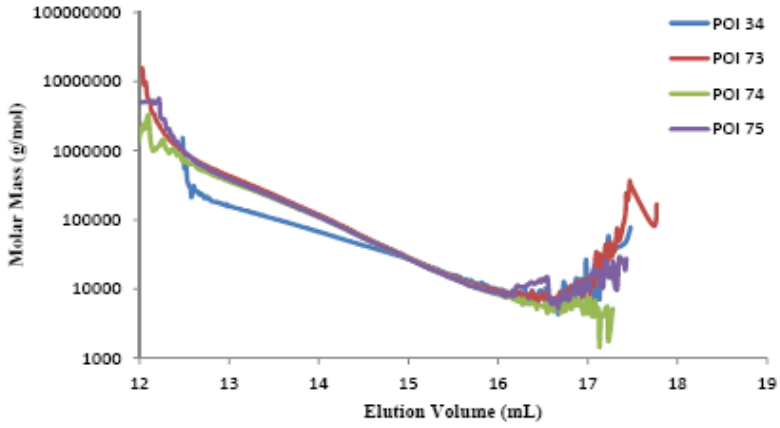


Figure 9.14. *Elution volumes plots for branched polymers containing POM as co-monomer and EGDMA (5%) as brancher.*

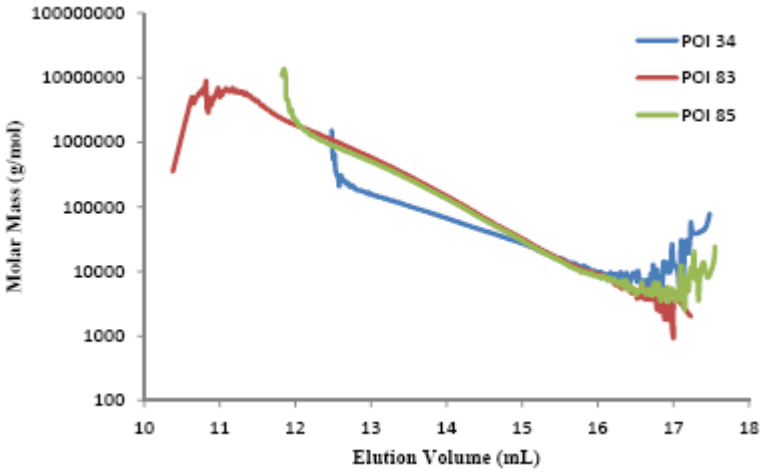


Figure 9.15. *Elution volumes plots for branched polymers containing POM as co-monomer and EGDMA (10%) as brancher.*

4. Conclusions and future work

In this study, novel branched polymers were synthesised employing methyl methacrylate and ethylene glycol dimethacrylate as functional monomer and branching agent, respectively, and in presence of an antioxidant and antifungal co-monomer.

The co-monomer was synthesised by the introduction of a polymerisable functionality into the 3,4-dimethoxycinnamic acid molecule and was characterised in terms of isolated yield, melting point, R_f value, NMR spectroscopy, MALDI-TOF mass spectrometry, FT-IR and elemental analyses.

In order to synthesise branched polymers, a facile, generic and cost effective route was employed (*Strathclyde route*). This synthetic strategy involves the use of vinyl monomers in the presence of chain transfer agents (CTAs) to inhibit cross-linking and gelation processes. The stoichiometric balance between the branching agent and the CTA represents the key point of this strategy that holds out good promise in terms of scale-up and exploitation.

The obtained polymeric materials were characterised by solubility tests in chloroform, determination of isolated recoveries, NMR spectroscopy and

GPC analyses and SEC data confirmed the branched nature of the polymers containing EGDMA.

In order to verify the antioxidant and antifungal properties of the synthesised branched polymers specific assays will be performed.

Suggestions for possible future work could include:

- ✓ the post-modification of the control polymers synthesised employing HEMA as co-monomer;
- ✓ the demethylation and conversion of the polymers, obtained using the synthesised co-monomer, to the corresponding hydroxy derivatives.

In the first case, it could be possible to obtain bioactive polymers avoiding steps such as the synthesis of the antioxidant and antifungal monomer and its subsequent purification. In the second case, the demethylation allows to obtain a new series of materials to test in terms of antioxidant and antifungal properties.

CONCLUSIONS

In the last decades, the development of new, facile and cost effective synthetic strategies to prepare polymeric systems has received great attention due to the broad range of application fields of these materials.

Natural and synthetic polymers play a key role in several aspects of the human life. The main molecules of biological interest, such as DNA, proteins and polysaccharides, are polymers as well as natural fibers (cotton, silk, cellulose) and plastic materials.

On the basis of these considerations, this PhD work wants to be an exploration of different synthetic routes for the preparation of novel functional polymers potentially useful in fields, such as biomedical, pharmaceutical, cosmetic and food.

For this purpose, Molecular Imprinted Polymers (MIPs), polymeric antioxidants and branched polymers were synthesised and their potential applications as Drug Delivery Systems (DDS), stationary phases in Solid Phase Extraction (SPE) and as antioxidant and antifungal agents were investigated confirming the efficiency of the employed synthetic procedures.