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Novel polymers for biomedical and pharmaceutical applications: Synthesis and characterization

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To my two heroes,

Ivana e Peppino.

<<...chi se lo aspettava, conoscendo Vincenzo

da bambino, che potesse studiare e

addirittura arrivare a fare il dottorato...>>

cit.

I would like to thank every acquaintance, friend, colleague and relative who helped, encouraged and/or hindered me through my own journey up to now.

Index

Thesis outlines.

The aim of this PhD thesis work has been the synthesis of novel antimicrobial amphiphilic polymers to prevent microbial biofilm related-infections and counteract microbial resistance onset. In fact, microbial biofilms are difficultly eradicable due to their high antibiotic resistance. To reach this purpose different strategies along this work were exploited.

In the first Chapter, a briefly dissertation of polymers in medical field and their choice as materials of election for manufacturing a wide range of products, such as implantable medical devices, was reported. Such materials have responded to the need of replacing or repairing damaged human tissues or organs not more active under functional or metabolic point of view. Despite the enormous progress in the optimization of operative procedures for medical device implantation, clinical complications are still associated with the use of medical devices. In fact, when a biomaterial is implanted in the body, a biological response of the organism occurs causing the covering of the medical device surface with a film rich in proteins, polysaccharides and cells. This process, known as biofouling, is usually the first stage of a cascade of biological events which can lead to the development of infective complications up to device failure. Microbial cells can adhere to the material surface and proliferate building up a peculiar tridimensional structure known as biofilm. Microbial biofilms consist of microrganisms embedded in a self-secreted exopolysaccharide matrix (EPS) enabling the diffusion of nutrient and signaling molecules.

Approximately ten thousand deaths per year are related to medical device related infections. This problem is multifold. In fact, on one hand these infections are associated with prolonged hospitalization and growing medical costs and, on the other

hand, the massive use of antibiotics to treat these infections is promoting the development of bacterial resistance at an alarming rate. A worrying feature of biofilm-based infections is represented by the higher resistance of bacterial and fungal cells growing as biofilm with respect to the planktonic ones. The resistance to normal antibiotics that bacteria in biofilms exhibit respect to the planktonic counterpart can get up to 1000 times. For these reasons, a number of different strategies for the prevention of biofilm formation on medical devices have been investigated.

According to the mechanism of biofilm formation, the main approach pursued at this aim is the prevention of microbial adhesion and colonization of the material surface.

Particularly, the development of antifouling or antimicrobial polymers has emerged as the most promising strategy to prevent microbial adhesion and biofilm formation. Such materials either repel microbes (antifouling) or kill bacteria (antimicrobial) present on the surrounding of general surface.

Mainly two strategies can be pursued to develop antimicrobial polymers: i) polymer impregnation with antibiotics or other biocides; ii) polymer functionalization with groups exerting antimicrobial activity. In particular, development of intrinsically antimicrobial cationic polymers were highlighted with emphasis.

In Chapter II, the synthesis and characterization of a novel intrinsically antimicrobial basic polyacrylamide (pAcDED) were reported. In addition, since pAcDED resulted to be water soluble, we have investigated the possible use of pAcDED as carrier for insoluble acidic drugs, such as usnic acid, chosen as a model drug.

The polymer was able to establish multipoint acid-base interactions with the drug phenolic groups leading to a high water solubility of polymer/UA complexes. The shielding of polymer positive charges by usnic acid allowed the obtainment of polymer/UA complexes with size in water smaller than those of polymer alone. Due to

their reduced size, complexes possessed increased activity against *Stahylococcus epidermidis* thanks to an efficient cellular uptake. This work has been recently published as Francolini I., Taresco V., Crisante F., Martinelli A., D'Ilario L., Piozzi A., *Int J Mol Sci.*, **2013**; 14(4), 7356-7369.

In Chapter III, the same polymer, pAcDED, was used as coating for manganese ferrite nanoparticles (MNPs) to obtain hydrophilic core/shell magnetic systems with a good antimicrobial activity. In this regard, an interesting strategy for fighting biofilm formation on device surfaces and surrounding tissues could be represented by the use of MNPs able to target antimicrobial agents in the body sites interested by the infection. In fact, applying an appropriate magnetic field, MNPs can be easily guided and concentrated in a narrow body area, thus reducing both drug administration dose and toxicity. Therefore, in Chapter III the experimental work concerning MNPs coating with either a hydrophilic (pAcDED) or a hydrophobic (a star branched polycaprolactone) polymer was reported. The developed systems were investigated to elucidate their possible role in controlling drug adsorption and release of usnic acid. The activity of usnic acid-loaded polymer-coated MNPs was evaluated against *S. epidermidis*.

In Chapter II and III, the combination of two antimicrobial agents having different mechanisms of action was exploited in order to minimize bacterial resistance onset. Indeed, the polymer positively charge side chain can adsorb on the negative charged bacterial membrane provoking cell disruption while usnic acid can inhibit bacterial metabolic functions.

Recently, it was observed that survival bacteria mechanism is due to genetic mutations provoked by oxidative stress. Indeed, in response to antibiotic administration, bacteria produce reactive oxygen species (ROS). In this regard, to prevent microbial adhesion

and biofilm development a potentially winning strategy could be to combine antibiotics with ROS inhibitors.

Therefore, considering the good antimicrobial and antioxidant properties of phenolic and catecholic moieties, in Chapter IV the synthesis and characterization of a novel hydroxytyrosol-based polyacrylate (pAcHTy) was reported. A water insoluble polymer was obtained possessing nanometric size, good antimicrobial and antioxidant activity. In addition, pAcHTy also showed a good chelating activity with respect to Cu^{2+} ions. This last property can find important application in the medical field, especially in chelation therapy. The use of polymers containing substances bearing cathecolic moieties could be a promising therapeutic approach for developing new agents for neurodegeneration or cancer treatment.

Amphiphilic cationic random copolymers are known to be an alternative strategy to counteract the emergent risk of microbial resistance. To achieve high antimicrobial activity, these polymers have been designed with a suitable balance of hydrophobic content and cationic charges. Generally, this has been obtained by using a monomer containing in side chain primary or tertiary amines as cationic functionality, and a hydrophobic monomer without any biological activity.

In Chapter V, for the first time, novel antimicrobial amphiphilic cationic polymers have been synthesized by copolymerization of two monomers one bearing a positive charge and the other one a hydrophobic compound possessing antimicrobial and antioxidant activity. Also in this case, the use of polymer possessing both antimicrobial and antioxidant properties should reduce the emergence of drug resistant bacteria and better combat biofilm infections. Particularly, the starter monomers introduced in Chapter II and IV in different molar ratio were copolymerized to obtain a suitable amphiphilic

balance. Resulting copolymers showed a discrete water solubility and an enhanced antimicrobial and antioxidant activity compared to homopolymers alone.

Finally, in an appendix of this thesis, a preliminary investigation to obtain a soluble catecholic-moiety-containing homopolymer, possessing antimicrobial and antioxidant properties, was performed through the synthesis of a dopamine-based acrylic monomer. A selective protection of the catecholic group of dopamine was carried out before polymer synthesis by Atom Transfer Radical Polymerization (ATRP).

An interesting development of this work could be the synthesis of soluble block copolymers from this dopamine–based monomer and AcDED with controlled block length in order to tailor physical properties of the materials and to improve their biological activity.

CHAPTHER I

INTRODUCTION

1.1 Polymers in medicine

The numerous advances achieved in the area of synthetic materials has allowed making significant improvements in medical field [1], where polymers have become the materials of choice for manufacturing a wide range of products among which implantable medical devices are the prevailing ones.

Such materials have responded to the need of replacing or repairing damaged human tissues or organs not more active under functional or metabolic point of view. The development and application of new, high-performance polymer materials together with therapeutic innovations have enabled the healing of damaged parts of the body and the survival of a large number of patients.

Both natural, such as collagen and polysaccharides, and synthetic polymers can be used in biomedical applications. Synthetic polymers offer several advantages over the natural ones. In fact, the versatility of the macromolecular chemistry allows tailoring polymer physico-chemical properties (in terms of molecular weight, composition, functionality, mechanical features, degradation rate) to fulfill different applications. In addition, polymers can be easily functionalized with bioactive molecules and processed in different shape and size.

[1] a. Hench L.L., *Biomaterials Science*, **1980**; 208, 826-31.
b. Hanker J.S., Giammara B.L., *Science*, **1988**; 242, 885-92.
c. Sawan S. P., Manivannan G. *Antimicrobial/Anti-infective Materials:principles, applications and devices*, Lavoisier S.A.S., **2013**.
d. Donelli G., Francolini I., Di Carlo V., Di Rosa R., Rapporti ISISTAN 02/34, **2002**.

Therefore, numerous types of medical devices, including intravascular vascular grafts, catheters, heart valves, dental implants and contact lenses, are currently made with polymers and employed in medicine (***Table 1***).

Polymer	Acronym	Mainly applications
Polyglicolic acid	PGA	Biodegradable sutures, plates and intramedullary nails, degradable fracture plates
Polylactic acid	PLA	Plates and intramedullary nails, artificial ligaments, degradable fracture plates, controlled drug release
Styrene-butadiene copolymers	BS	Disposable products, packaging
Acrylonitrile-Styrene copolymers	SAN	Hemodialyzers components
Polyacrylonitrile	PAN	Hemodialysis membrane
Polyamide	PA	Sutures
Polycarbonate	PC	Hemodialysis membrane and oxigenators
Polyhydroxyethylmetha acrylate	PHEMA	Contact lenses, artificial plates
Polyethylene, Low Density Polyethylene	PE; LDPE	Films, packaging, catheters, tubing, connectors, drug controlled administration
Ultra High Molecular Weight Polyethylene	UHMWPE	Articular surfaces, fibers for composites, orthopedic plates.ì
Polyethylenterephtalate	PET	Vascular prostheses, sutures, transdermal steps, components of prosthetic heart valves, cardiac care components
Polymethylmethacrylate	PMMA	Bone cement, contact lenses, intraocular lenses, membranes for hemodialysis, dental materials
Polypropylene	PP	Sterile packs, strings, connectors, oxygenator membranes, sutures, support loops, intraocular lenses
Polysulfone	PSO	Oxigenator and hemodialysis membranes
Polytetrafluoroethylene	PTFE	Vascular prostheses, components of prosthetic valves, artificial ligaments, coatings
Polyurethane	PU	Catheters, cannulae, prosthesis valves, hemocompatible coatings, cardiac assist devices, controlled drug delivery, vascular prostheses
Polyvinyl chloride	PVC	Blood bags, endotracheal tubes, disposable gloves, catheters, disposable accessories
Silicon		Catheters, drains, membranes, artificial leather, plants for plastic surgery

Table 1. *Examples of polymers employed in medicine with relative applications.*

Possible release of substances (such as monomers, catalysts and additives) into the body, the ease adsorption of water and biomolecules from the surrounding environment are some drawbacks associated with use of polymers.

Despite the enormous progress in the optimization of operative procedures for medical device implantation, clinical complications are still associated with the use of medical devices, the most common being the onset of local and systemic infections.

In fact, when a biomaterial is implanted in the body, a biological response of the organism occurs causing the covering of the medical device surface with a film rich in proteins, polysaccharides and cells. This process, known as biofouling, is usually the first stage of a cascade of biological events which can lead to the development of complications, such as thrombosis and infections, and device failure.

1.1.1 Biofouling

Any synthetic or natural material, when exposed to a moist environment, is soon covered by an organic film constituted by biomacromolecules and cells including microorganisms.

Depending on the implantation site, the conditioning film will be composed by a different type of adsorbed proteins. In the case of serum, the adsorbed proteins, consist mainly of albumin, immunoglobulin, fibrinogen and fibronectin.

The presence of such a surface film plays an important role in the early stages of microbial biofilm formation, as it alters the surface properties of the biomaterial thereby influencing the magnitude and speed of microbial adhesion. Moreover, the adsorbed proteins can function as surface receptors facilitating such adhesion [2].

[2] Fitzpatrick F., Humphreys H., O'Gara J. P., *Clin. Microbiol. Infect.*, **2005**, 11, 967–973.

Microbial cells adhered to the material surface proliferate building up a peculiar tridimensional structure known as biofilm. Microbial biofilms consist of microrganisms embedded in a self-secreted exopolysaccharide matrix (EPS) enabling the diffusion of nutrient and signaling molecules.

The biofilm-like growth mode permits the survival of microbial cells under high antibiotic concentration and extreme conditions of temperature, pH, ionic strength and shear stress.

The biofouling process occurs spontaneously and, in many cases, represents an adverse event. Particularly, biofouling is a critical issue in water membrane and wastewater treatment as it greatly compromises the efficiency of the treatment processes. In the food industry, bacterial adhesion to food products or product contact surfaces leads to important hygienic problems and economic losses due to food spoilage [3].

Biofilm formation on the surface of the pipe walls of drinking water distribution systems can be responsible for the decrease of water quality in terms of increasing bacterial levels, reduction of dissolved oxygen, taste and odor change [4].

In the biomedical field, the development of microbial biofilms is a critically important global healthcare issue. In fact, they play a pivotal role in healthcare-associated infections (HAIs), especially those associated to implantable medical devices such as intravascular catheters, urinary catheters and orthopedic implants [5].

Statistic data concerning the incidence of infectious disease in European or USA hospitals clearly show the impact of this issue on public health.

The European Center for Disease Prevention and Control reported in 2008 that approximately 4 million patients per year are estimated to acquire an HAI in European

[3] Kumar C., Anand S., *Int. J. Food Microbiol.*, **1998**, 42, 9-27.
[4] Block J., Haudidier K., Pasquin J., Miazga J., Levi Y., *Biofouling*, **1993**, 6, 333-343.
[5] Francolini I., Donelli G., *FEMS Immunol. Med. Microbiol.*, **2010**, 59, 227-238.

hospitals. The number of deaths occurring as a consequence of these infections is estimated to be at least 37, 000.

In US hospitals, about 2 million people acquire bacterial infections each year with a 5% of mortality. Approximately 50,000 deaths per year are related to catheter infections. This problem is multifold. On one hand, these infections are associated with prolonged hospitalization and growing medical costs. On the other hand, the massive use of antibiotics to treat these infections is promoting the development of bacterial resistance at an alarming rate.

For these reasons, a number of different strategies for the prevention of biofilm formation on medical devices have been proposed in the last three decades. Particularly, since the materials commonly used for manufacturing medical devices are in majority polymers, research interest has been mainly focused on the development of novel selective antifouling or antimicrobial polymers that have the potential to be employed for a wide range of biomedical products including sterile clothing, air filters and medical devices.

1.1.2 Medical device-related infections

In modern medicine, medical devices are used for a wide range of applications. Some of these devices, including heart valves, artificial joints and pace makers, are permanently implanted in the patients to repair or replace damaged parts of the body. Other devices, such as intravascular and urinary catheters, are temporary implanted and allow clinicians to deliver drugs or nutrition fluids into the body, to expel fluids out of the body and to monitor the status of critically ill patients.

In all of these applications, the control of phenomena occurring at the biological tissue/material interface is of paramount importance for the success of the implanted medical device. In fact, as stated above, soon after the exposure of the device to physiologic fluids and tissues, the device surfaces are coated by a film of biological material mainly consisting of proteins, polysaccharides and cells that makes the device more suitable to biofilm formation.

The type of implanted device, the material used for its manufacturing, the body site interested by the implant as well as patient features all contribute to the overall risk of device-related infection.

As for material features, surface hydrophobicity and roughness seem to affect microbial adhesion more than other physico-chemical properties. Particularly, the presence of surface microcavities or microfractures allows bacteria to anchor and provide them with a temporary protection from the action of the host fluids. This permits the irreversible bacterial adhesion to the surface through the expression of specific adhesins. As for material hydrophobicity, given the hydrophobic nature of microbial membrane, usually microorganisms prefer hydrophobic surfaces. However, this is strongly dependent on the bacterial species and generalization at the species or even strain level is often difficult.

The infectious risk as well as the severity of the infections, in terms of morbidity and mortality, are strongly dependent on the type of implanted device. Among employed medical devices, those associated with a high risk of development of infection are intravascular catheters, urinary catheters and orthopedic implants.

Intravascular catheters are extensively used. There are two types: (i) intravascular catheters (ICs) used for short-time vascular access, such as peripheral venous catheters, or the non-tunneled central venous catheters (CVCs); (ii) ICs used for long-term

(indwelling) vascular access, such as the tunneled CVCs. Central venous catheters (CVCs) are responsible for the highest proportion of bloodstream infections. Especially, non tunneled-CVCs account for an estimated 90% of all catheter-related bloodstream infections. Coagulase-negative staphylococci, especially *Staphylococcoccus epiderimidis* and *S. aureus,* and yeasts, especially *Candida albicans*, are the most commonly isolated pathogens in CVC-related infections [6].

The microbial contamination of non-tunneled CVCs is usually extra-luminal, with microrganisms originating from the skin, and, less commonly, intra-luminal from hematogenous seeding of the catheter tip. The intra-luminal colonization of the hub and lumen of the CVC is instead the most common route of infection for tunneled-CVCs.

Urinary catheters are associated with a high rate of development of urinary tract infections (UTIs). In fact, about half of hospitalized patients bearing an urinary catheter for longer than a week will get an urinary tract infection [7].

Catheter-associated urinary tract infections affects 449,334 patients per year in US hospitals [8, 9].

The most common pathogen causing these infections is *Escherichia coli* but other bacterial species can be involved such as *Pseudomonas aeruginosa, Enterococcus spp, Klebsiella spp, or Enterobacter spp* [10, 11].

Orthopedic devices are of great relevance for public health due to the increasing number of aged and disabled people. Thanks to the improvement in the surgical procedures as well as to the availability of novel biocompatible materials, the risk for orthopedic device-related infections is nowadays low and less than 1-2%. However, these

[6] Mermel L.A., Farr B.M., Sherertz R.J., Raad I.I., O'Grady N., Harris J.S., Craven D.E., *Clin. Infect. Dis.*, **2001**, 32, 1249-1272.
[7] Schumm K., Lam T.B., *Neurourol. Urodyn.*, **2008**, 27, 738-746.
[8] Klevens R.M., Edwards J.R., Richards C.L., Horan T.C., Gaynes R.P., Pollock D.A., Cardo D.M., *Public Health Rep,*, **2007**, 122, 160-166.
[9] Lodbell K.W., Stamou S., Sanchez J.A., *Surg. Clin. North Am.*, **2012**, 92, 65-77.
[10] Lohr J.A., Donowitz L.G., Sadler J.E., *Pediatrics*, **1989**, 83, 193-199.
[11] Kalsi J., Arya M., Wilson P., Mundy A., *Int. J. Clin. Pract.*, **2003**, 57, 388-391.

infections are relevant for the healthcare system since a high number of patients is annually subjected to surgery for orthopedic device implantation. In fact, more than 200,000 and more than 400,000 hip replacements and primary total knee arthroplasties are performed each year in the United States, respectively [12].

The early prosthetic joint infection, occurring within the first 3 months after surgery, is usually caused by *S. aureus* or *S. epidermidis*. Delayed infection (within 2 years from surgery) is instead caused by microorganisms of low virulence.

When an implant-related infection occurs, simple chemotherapy with antimicrobial agents is not always effective to eradicate the infection. Therefore, a new surgery for prosthesis removal and replacement is usually needed leading to an increase of the hospitalization time and significant associated health costs [13].

To understand how these complications can be prevented, in the following section a brief overview of phenomena occurring during microbial biofilm formation will be presented since biofilm is considered the causative agent of the device-related infections.

Then, on the basis on the mechanism of biofilm formation, the main strategies to prevent biofilm development will be discussed paying particular attention on those based on antimicrobial polymers that are of interest for this thesis.

1.2 Mechanism of biofilm formation

Understanding the mechanism of biofilm formation has been of fundamental importance in the design of biomaterials able to prevent biofilm growth on their surfaces.

[12] Widmer A.F., *Clin. Infect. Dis.*, **2001**, 33, S94-S106.
[13] Campoccia D., Montanaro L., Arciola C.R., *Biomaterials*, **2006**, 27, 2331-2339.

The use of confocal laser scanning microscopy has clarified the complex morphology that surface-adherent bacteria assume during growth [14]. Biofilms are defined as microbial sessile communities irreversibly attached to a surface in which bacteria are immersed in an exopolysaccharide matrix produced by bacteria themselves [15]. They are characterized by a heterogeneous structure in which individual microcolonies are located in mushroom-like structures. In this particular structure, open channels are present with the function of supplying nutrients and eliminating waste products of cell metabolism. (***Figure 1***).

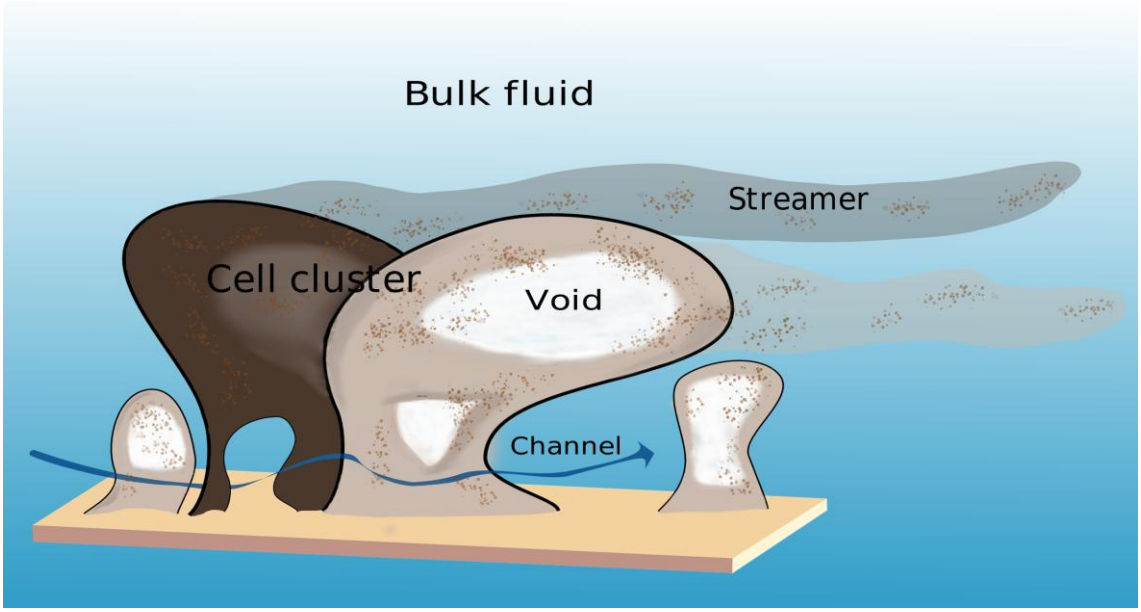

Figure 1. *Schematic representation of the biofilm architecture is reported.*

The formation of biofilm is constituted by a sequence of dynamic events that can be divided into 3 stages (***Figure 2***):

Stage 1. Initial and irreversible adhesion

Stage 2. Maturation of biofilm architecture

Stage 3. Detachment of single cells or cellular aggregates from the mature biofilm.

[14] Stoodley P., Sauer K., Davies D.G., Costerton J.W., *Annu. Rev. Microbiol.*, **2002,** 56,187-209.
[15] Costerton J.W., Lewandowski Z., Caldwell D.E., Korber D. R., Lappin-Scott H.M., *Annu. Rev. Mic.*, **1995**, 49, 711-745.

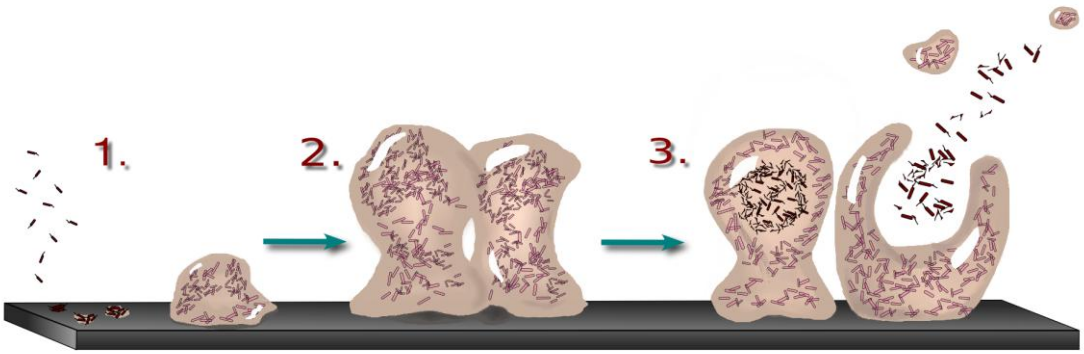

Figure 2: *Schematic representation of the three stages involving biofilm formation.*

1.2.1 Initial microbial adhesion

When floating planktonic cells come into the proximity of a biomaterial, the process of adhesion begins. This process is first reversible and then irreversible [16] (***Figure 3***).

[16] Busscher H.J., Weerkamp A.H., *FEMS Microbiol. Rev.*, **1987**, 46, 165-173.

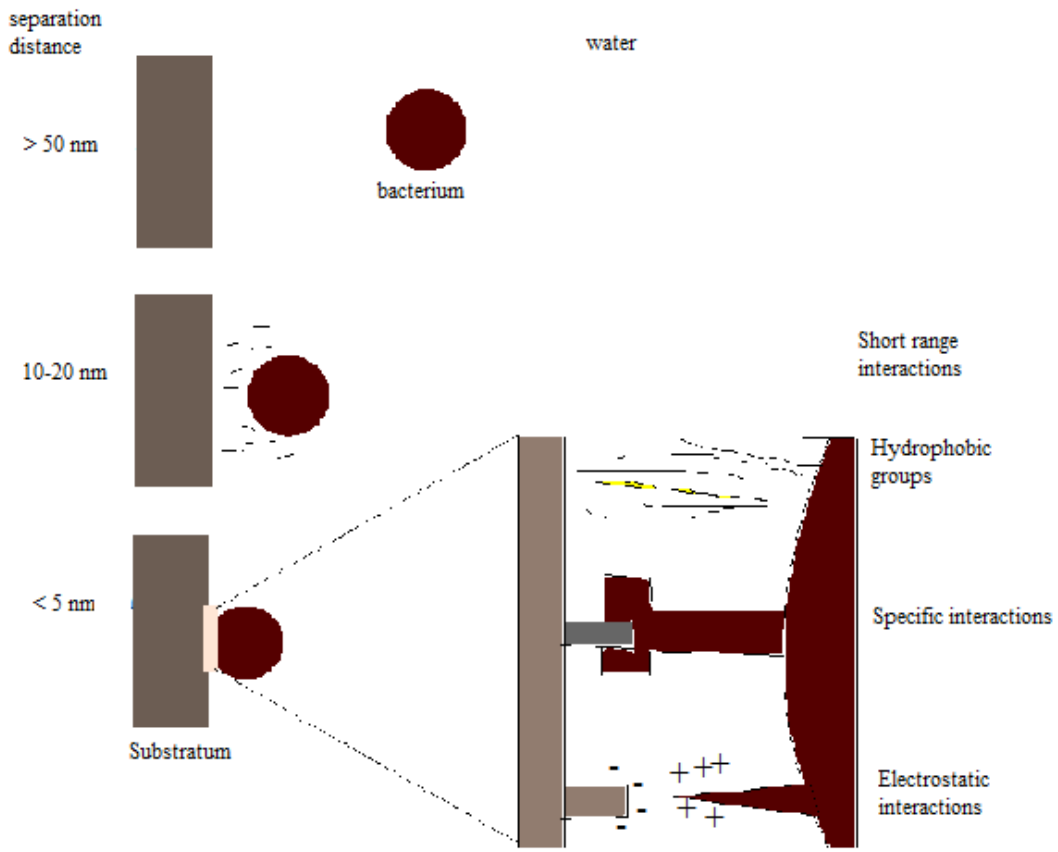

Figure 3. *Interactions that regulate the bacterial adhesion a substrate.*

Due to their small size, low density, variable degree of cell surface hydrophobicity and net negative charge, bacteria can be considered as living colloids. The first interactions occurring between bacterial cell and biomaterial surface are long-range and non-specific such as van der Waals forces or repulsion electrostatic forces and acid-base interactions. The resultant of these different non-specific interaction forces will cause bacterial attraction or repellence by the surface. If bacteria are attracted, short range non-specific interactions occur. These forces are represented by hydrophobic interactions between the biomaterial surface and cell membrane. In fact, many studies have shown that

microorganisms adhere faster on non-polar and hydrophobic surfaces such as Teflon compared to more hydrophilic materials such as glass or metals [17, 18].

Among the short range interactions, there are also specific interactions between the chemical groups present on the surface of the bacterial membrane (carboxyl, hydroxyl, phosphate and amino groups) and biomacromoleucles adsorbed onto the biomaterial surface forming a so-called conditioning film. This film plays an important role in bacterial adhesion since can provide the biomaterial with specific bacterial receptors.

Depending on the type of protein adsorbed on the biomaterial surface, the condition film can either promote or hamper bacterial adhesion. In fact, albumin is known to be a strong adhesion inhibitor while fibrinogen and fibronectin have shown to promote the adhesion of *S. aureus* and some strains of *S. epidermidis* [19, 20].

Finally, material roughness also affects microbial adhesion. Particularly, an increase in surface roughness results in an increase in microbial colonization [21], presumably due a shielding effect from the shear forces present at the surface liquid interface to which bacteria are subject.

1.2.2 Irreversible microbial adhesion and maturation of biofilm architecture

[17] Fletcher M., Loeb G.I., *Appl. Environ. Microbiol.*, **1979**, 37, 67–72.
[18] Pringle J.H., Fletcher M., *Appl. Environ. Microbiol.* **1983**, 45,811–817.
[19] Christensen G.D., Baddour L.M., Hasty D.L., Lowrance J.H., Simpson W.A., *Microbial and foreign body factors in the pathogenesis of medical device infections*. In: Bisno A.L., Waldvogel F.A., *Infections associated with indwelling medical devices,* American Society of Microbiology, Washington DC, **1989**, 27-59.
[20] Y.H., Friedman R.J., *J. Biomed. Mater. Res.*, **1998**, 43, 338-48.
[21] Characklis W.G., McFeters G.A., Marshall K.C., *Physiological ecology in biofilm systems*. In: Characklis W.G., Marshall K.C., *Biofilms*, John Wiley & Sons; **1990**, 341–94.

Profound physiological changes accompany the transition from reversible to permanent attachment onto a surface. In fact, the irreversible adhesion phase involves the secretion of specific bacterial adhesins and their selective binding with substratum receptors. As an example, *S. epidermidis* cells adherent to surfaces were demonstrated to be able to produce a polysaccharide intercellular adhesin (PIA) that binds the cells together and facilitates the formation of microcolonies [22]. During the phase of irreversible adhesion, bacteria can not move perpendicularly to the surface but still retain the ability to move horizontally if endowed with extracellular organelles.

Not only the irreversible adhesion but also the formation of biofilm complex architecture has been shown to be under genetic control. For example, *P. aeruginosa* changes its protein profile when growing in a biofilm than in planktonic state (Brozel et al., 1995) [23]. The regulation of gene transcription during biofilm growth is possible by secretion and accumulation of signaling molecules that allow bacteria to communicate with each other. This process, called quorum sensing (QS), is activated when the concentration of signaling molecules exceeds a certain threshold value and enables bacteria to modulate the expression of genes able to coordinate the group behavior. QS permits bacteria to adapt to environmental changes, such as the variation in nutrient supply, altered levels of oxygen, pH or ionic strength. Also the formation of the extracellular polymeric matrix (EPS, Extracellular Polymeric Substances) in which bacteria are embedded is regulated by quorum sensing mechanism.

Understanding the role of QS systems in clinically relevant strains could represent a new and interesting strategy to prevent biofilm formation. In fact, the ability of *P. aeruginosa* to form the characteristic mushroom-like biofilm structure has been shown

[22]Gerke C., Kraft A., Sussumuth R., Schweitzer O., Gotz F., *J. Biol. Chem.*, **1998**, 273, 18586-18593.
[23] Brozel V.S., Strydom G.M., Cloete T.G., *Biofouling: the Journal of Bioadhesion and Biofilm Research*, **1995**, 3, 195-201.

to be affected by quorum-sensing phenomena [24]. Therefore, the use of quorum sensing inhibitors, such as halogenated and synthetic furanones, caused an attenuation of *P. aeruginosa* virulence and an increase in its susceptibility to tobramycin [25].

1.2.3 Detachment of single cells or cellular aggregates from the mature biofilm

Recently, the detachment of bacterial cells or cellular aggregates from mature biofilm has been demonstrated to be a physiologically regulated event. Time-lapse microscopy has shown that biofilms can release either single cells or visible clumps containing thousands of cells [26].

Therefore, detachment represents a distinct process in the biofilm developmental life cycle. In fact, in case of lack of nutrients or high cell-density, the dispersal of cells from the outer biofilm layers is considered as a survival mechanism for the sessile community. In this way, the network of nutrient exchange channels that runs through the biofilm is preserved.

Also a passive cell detachment can occur by shear forces at the liquid-surface according to different mechanisms such as erosion, abrasion and removal [27].

While detachment has benefits for the biofilm life cycle, it is an adverse event in the biofilm field since the detached bacterial clumps can colonize new body sites and disseminate the infection.

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[24] Davies D.G., Parsek M.R., Pearson J.P., Iglewski B.H., Costerton J.W., Greenberg E. P., *Science*, **1998**, 280, 295-298.

[25] Hentzer M., Wu H., Andersen J.B., Riedel K., Rasmussen T.B., Bagge N., Kumar N., Schembri M.A., Song Z., Kristoffersen P., Manefield M., Costerton J.W., Molin S., Eberl L., Steinberg P., Kjelleberg S., Hoiby N., Givskov M., *EMBO J.*, **2003**, 22, 3803-3815.

[26] Stoodley P., Hall-Stoodley L., Lappin-Scott H. M., *Methods Enzymol.*, **2001**, 337, 306-319.

[27] Brading M.G., Jass J., Lappin-Scott H.M., *Dynamics of bacterial biofilm formation*. In: Lappin-Scott H.M., Costerton J.W., *Microbial biofilms*. Cambridge: Cambridge University Press; **1995**, 46–63.

1.3 Antibiotic resistance of biofilm

A worrying feature of biofilm-based infections is represented by the higher resistance of bacterial and fungal cells growing as biofilm with respect to the planktonic ones [28, 29].

The resistance to normal antibiotics that bacteria in biofilms exhibit respect to the planktonic counterpart can get up to 1000 times.

This finding has been attributed to several factors. First of all, biofilms represent an ideal environment for plasmid exchange among cells. In fact, conjugation frequency appears to be higher in biofilm than in planktonic bacteria. This amplifies both naturally occurring and induced antibiotic-resistance phenomena.

Other factors involved in the biofilm increased drug resistance [30] are:

(1) Slow and scarce penetration of antimicrobial agents through the biofilm matrix;

(2) Physiological response of microorganisms to the heterogeneous chemical environment existing in biofilms [31].

In fact, the growth conditions in biofilms are quite different at lower layers, where nutrients and oxygen are limited, and microbial waste products can be toxic; for example, in the deep layers anaerobic niches are presumably present [32]. These factors promote the development of different bacterial phenotypes, including drug-resistant ones. In addition, also nutrient depletion at lower layers by slowing bacterial growth rate can reduce the number of targets for antimicrobial molecules.

[28] Aslam S., *Am. J. Infect. Control*, **2008**, 36, S175, e 9-11.
[29] Ramage G., Mowat E., Jones B., Williams C., Lopez-Ribot J., *Crit. Rev. Microbiol.*, **2009**, 35, 340-355.
[30] Lewis K., *Curr. Top. Microbiol. Immunol.*, **2008**, 322, 107-131.
[31] Stewart P.S., Franklin M.J., *Nat. Rev. Microbiol.*, **2008**, 6, 199–210.
[32] Borriello G., Werner E., Roe F., Kim A.M., Ehrlich G.D., Stewart P.S., *Antimicrob. Agents Ch.*, **2004**, 48, 2659–2664.

(3) Onset of subpopulations of persistent or dormant bacterial cells in a spore-like, non-dividing state [31, 33].

Therefore, once the biofilm is established, antibiotic treatments for its eradication usually fail. In case of medical device-related infections, the surgical removal and replacement of the infected device is needed. This involves serious inconvenience for the patient and increase in hospital costs.

1.3.1 Strategies to prevent medical device-related infections

In order to prevent infections associated with medical devices, many strategies have been adopted in the last decades.

According to the mechanism of biofilm formation, the main strategies persuade at this aim are:

1) prevention of microbial adhesion and colonization of the material surface;

2) interference with signaling molecules to inhibit biofilm development;

3) biofilm matrix disaggregation.

Since the topic of this PhD thesis concerns the first strategy, a specific section will be focused on the detailed description of the different experimental approaches developed for prevention of microbial adhesion and colonization of medical device surfaces. The first strategy is also, doubtless, the most pursued and successful one.

As for the second strategy, QS plays a crucial role in modulating not only the expression of genes associated with the production of enzymes, virulence factors and metabolites but also the development of biofilm. Therefore, the use of molecules

[33] Lewis K., *Nat. Rev. Microbiol.*, **2007**, 5, 48–56.

interfering with QS has been shown to be a promising strategy to prevent microbial adaptation to the host environment and the establishment of infectious processes.

According to Rasmussen & Givskov [34], in gram-negative bacteria there are basically three possible targets: (1) signal generator, (2) signal molecule and (3) signal receptor. Inhibition to the receptor of binding of the signal molecule by the use of analogues of the signal molecules [35, 36] has been so far the most investigated way to interfere with QS.

Potent inhibitors of gram-negative QS have been shown to be the halogenated furanones purified from *Delisea pulchra* [37] and a series of related synthetic derivatives. These QS inhibitors caused an increase of *P. aeruginosa* susceptibility to tobramycin. Our group showed that, usnic acid, a naturally occurring dibenzofuran derivative, is able to affect the morphology (thickness and roughness) of *P. aeruginosa* biofilm. This phenomenon is presumably associated to usnic acid (UA) ability to interfere with bacterial signaling pathways [38].

In gram-positive bacteria, the QS inhibitor RNAIII- inhibiting peptide (RIP) resulted to be efficacious in preventing and treating staphylococcal medical device-related infections. Particularly, in a rat model, RIP-coated CVCs significantly reduced bacterial adhesion and increased the killing action of antibiotics versus *S. aureus* strains. In fact, when treated with RIP, biofilm growing S. aureus became as susceptible to antibiotics as planktonic cells [39].

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RIP-loaded polymethylmethacrylate beads for orthopedic devices implanted in rats were able to prevent methicillin-resistant *S. aureus* (MRSA) biofilm formation either alone or combined with vancomycin [40].

As far as the third strategy is concerned, the use of substances able to damage the biofilm matrix acting as a protective barrier for bacteria could cause a better exposition of sessile microbial cells to antibiotics as well as to the host immune defenses [41].

In staphylococcal species, Dispersin B, a soluble b-N-acetylglucosaminidase purified from *A. actinomycetemcomitans* by Kaplan et al. [42], was shown to be able to disaggregate the exopolysaccharide matrix produced by biofilm-producing strains since this matrix is rich in a linear poly-N-acetyl- 1,6-b-glucosamine. In addition, our group showed that Dispersin B and the antibiotic cefamandole nafate synergistically prevented staphylococcal biofilm formation on polyurethanes [43].

Depending on the nature of the biofilm constituents, the use of different enzymes can be necessary. In fact, Chaignon et al. [44] demonstrated that two successive treatments, consisting of Dispersin B, followed by a protease (proteinase K or trypsin), were needed for degradation of biofilms produced by clinical staphylococcal strains associated with orthopedic infections.

Engineered bacteriophages able to express Dispersin B were also successfully tested against *E. coli* biofilms [45].

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1.4 Prevention of microbial adhesion and colonization of material surfaces

The development of antifouling or antimicrobial polymers has emerged as the most promising strategy to prevent microbial adhesion and biofilm formation (***Figure 4***). Such materials either repel microbes (antifouling) or kill bacteria (antimicrobial) present in the surface proximity.

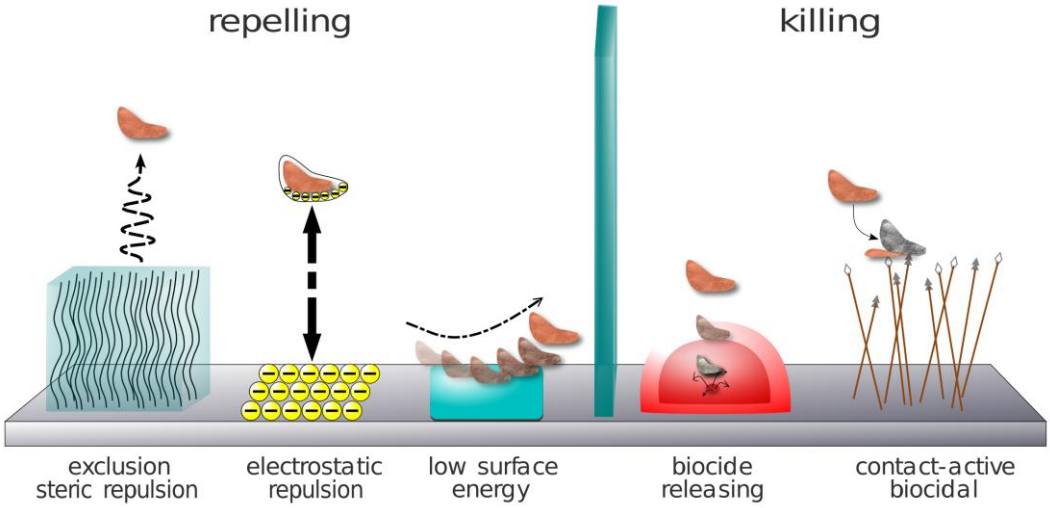

Figure 4. *The different approaches to produce antifouling or antimicrobial polymers.*

1.5 Antifouling polymers

Microbial adhesion is an essential step in the pathogenesis of medical device-related infections. Given the hydrophobic nature of microbial surfaces [46], prevention of bacterial adhesion onto the device surfaces can be achieved by i) coating with hydrophilic polymers, ii) coating with negatively charged polymers.

The development of antifouling polymers is particularly interesting since it avoids the use of drugs (either by systemic administration or by local release from medicated devices) that, when administered over long periods of time, may be associated with undesired side effects.

However, the main limitation of this strategy is that its efficacy is strongly dependent on the bacterial species since the physico-chemical properties of microorganisms can largely vary among species.

1.5.1 Hydrophilic polymer coatings

Several polymer classes have been explored [47] as coating to obtain antifouling polymers, including polyacrylates [48], polyzwitterions [49] and poly(ethylene glycol) (PEG) derivates [50, 51].

Among these, PEG has been the most investigated polymer due to its resistance to protein adsorption, non immunogenicity and antithrombogenicity. PEG antifouling ability is believed to be related to both hydration and steric effects [52]. Also, the lack of

[46] van der Mei H.C., Leonard A.J., Weerkamp A.H., Rouxhet P.G., Busscher H.J., *J. Bacteriol.*, **1988**, 170, 2462-2466.
[47] Chen S., Li L., Zhao C., Zheng J., *Polymer*, **2010**, 51, 5283-5293.
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[52] Chen S., Yu F., Yu Q., He Y., Jiang S., *Langmuir*, **2006**, 22, 8186-8191.

binding sites further hampers the adsorption of proteins and bacterial cells. However, the ability of PEG in reducing bacterial adhesion has had variable degrees of success, depending on the PEG's molecular weight, degree of branching and surface packing density. Also, the bacterial species and the culture media used in the experiments seem to affect PEG antifouling activity.

PEG has been grafted onto the surface of a series of materials, including glass[53], gold [54], poly(ethylene terephthalate) [55] and polyurethanes [56, 57].

Inhibition of *Candida albicans* biofilm formation on polyurethane surfaces was achieved by introducing a 6% polyethylene oxide [58].

Also the adhesion of *S. epidermidis* was prevented by modification of hydrophobic polyurethanes with PEG [59, 60].

Corneillie and colleagues [61] synthesized segmented polyurethanes at different PEG content employing in the polymer synthesis, PEG, polypropylenoxide (PPO) and polytetramethylenoxide (PTMO) in different ratios. Samples containing PEG/PPO or PEG/PTMO in 1/1 molar ratio were able to significantly reduce the adhesion of *S. aureus* and *Enterococcus faecalis* with respect to glass. However, these polymers allowed the adhesion of *P. aeruginosa* unless samples were incubated in urine instead of phosphate buffer, indicating an influence of the culture media and then of the conditioning film.

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[54] KL Prime, GM Whitesides, *Science*, 1991, **252**, 1164.
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The influence of PEG end-groups on bacterial adhesion was investigated by Park K.D. and colleagues by using PEG possessing either -OH, $-NH_2$ or $-SO_3$ as chain-end groups. Results showed that due to a different protein adsorption on the polymer surface, the sulphonated PEG1000-containing polyurethane was the most repellent one. This finding suggests that the sulphonate groups are responsible for the antifouling features of heparin that will be discussed in the following section.

1.5.2 Negative charged polymer coatings

Heparin, a highly sulfated glycosaminoglycan, has always attracted particular attention in the field of hemocompatible biomaterials due to its interesting biological functions, such as anticoagulant activity, cell growth stimulation and antivirus ability. In fact, the physical adsorption or chemical grafting of heparin to artificial surfaces has been shown to improve device hemocompatibility [62, 63, 64]. More recently, the ability of heparin to counteract microbial adhesion and colonization has been demonstrated *in vitro* and *in vivo* [65]. Particularly, the heparinization of both CVCs and dialysis catheters resulted in a significant reduction of catheter-related infections [66, 67].

An alternative strategy to the heparin coating is represented by the introduction of negatively charged chemical groups such as groups sulfates, sulfamates and carboxylates to develop heparin-like polymers[68, 69, 70].

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[62] Mao C., Qiu Y., Sang H., Mei H., Zhu A., Shen J., Lin S., *Adv. Colloid Interface Sci.*, **2004**, 110, 5-17.
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[70] Paulsson M., Gouda I., Larm O., Ljungh A., *J. biomed. mater. res.*, **1994**, 28: 311-317.

The first heparin-like polyurethanes were sulphonated PTMO-based polymers obtained by S.L. Cooper's group [71]. Very recently, also our group has developed segmented polyurethanes containing sulphate or sulphamate groups in the *hard* segment The obtained $–SO_3H$-containing polymers showed good hemocompatibility and inhibited *S. epidermidis* adhesion, these features being related to polymer phase segregation and density of $–SO_3H$ groups [72].

A limitation of this approach is that some bacterial strains have specific receptors for heparin and therefore are able to colonize heparin coated surfaces.

Best results were obtained by adsorption of albumin [73], that similarly to heparin is negatively charged.

1.6 Antimicrobial polymers

Low molecular weight antimicrobial agents have several drawbacks including toxicity, antimicrobial activity limited in time and potential to select resistant bacteria [74]. To overcome these limitations, various efforts have been focused on the development of antimicrobial materials that are effective and selective towards microorganisms. The combined use of drugs and substances at high molecular weight, such as polymers, offers the advantage to better control drug release, and then to extend the residence time of the drug within the body, as well as to limit residual toxicity of the low molecular weight antimicrobial agents. Moreover, polymers are chemically stable, not volatile, and do not to overcome the skin barrier.

[71] Grasel T.G., Cooper S.L., *J. Biomed. Mater. Res.*, **1989**, 23, 311-338.
[72] Francolini I., Crisante F., Martinelli A., D'Ilario L., Piozzi A., *Acta Biomater.*, **2012**, 8, 549-558.
[73] An Y.H., Stuart G.W., McDowell S.J., Mcdaniel S.E., Kang Q., Friedman R.J., *J. orthopaed. Res.*, **1996**, 14, 846-849.
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Mainly two strategies can be pursued to develop antimicrobial polymers: i) polymer impregnation with antibiotics or other biocides; ii) polymer functionalization with groups exerting antimicrobial activity.

In the first approach, the polymer acts as a carrier for one or more antimicrobial agent that once released from the polymer exerts its action (antimicrobial agent-releasing polymers). In the second approach, bactericidal functionalities, such as quaternary amine compounds or phosphonium salts, are introduced in the polymer to obtain intrinsically antimicrobial polymers (biocidal polymers). Biocidal polymers do not release antimicrobial substances but exert their killing action when microorganisms contact the surface.

1.6.1 Antimicrobial agent-releasing polymers

To obtain antimicrobial agent-releasing polymers, antibiotics, antiseptics, surfactants or heavy metals can be applied to the polymer by: a) physical adsorption; b) impregnation, c) complexation or d) conjugation via hydrolytically labile linkages. Particularly, drug adsorption or impregnation are the most pursued methods since they are easy to perform. In addition the impregnation can permit the loading of significant drug amounts.

Although such surfaces work quite efficiently, the main limitation of this strategy is the lack of durability of the antimicrobial activity due to the uncontrolled release of the absorbed antimicrobial agent in the first hours following device implantation.

Generally, the medical device coating with antimicrobial-agent releasing polymers has had successful variable degrees depending on: i) implantation site of the device; ii) type

of antimicrobial agents used for the coatings; iii) concentration of antimicrobial agents on the surface of the coated device and iv) kinetics of drug release.

A large number of antimicrobial agents have been adsorbed onto medical devices and tested in their ability to prevent device-related infections [75, 76, 77]. However, systems based on the use of drug combinations were found to be the most interesting ones since they reduce the onset of antibiotic-resistant microorganisms.

Here following, the antimicrobial-agent releasing systems that have gained more attention in the clinical field are described.

1.6.3 Metal adsorbed/insert on/in polymers

Silver is, doubtless, the most used inorganic antimicrobial agent used to provide polymers with antimicrobial properties.

The antimicrobial activity of silver ions is due to its binding to microbial DNA, thus inhibiting bacterial replication, and to sulphydryl groups, thus provoking the deactivation of metabolic enzymes [78].

Silver in solution has been used for almost a century as a topical antimicrobial agent for the regeneration of damaged tissues [79].

Today, silver is used in the prevention and treatment of a wide number of diseases and particularly for infections. It is also able, acting local, to decrease the concentration of zinc present on the lesion surface, acting locally, due to its metalloproteinase activity [80].

[75] Raad I., Hanna H., Jiang Y., Dvorak T., Reitzel R., Chaiban G., Sherertz R., Hachem R., *Antimicrob. Agents*, **2007**, 51, 1656–1660.
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[80] Lansdown A.B., Silver I., *J. Wound. Care*, **2002**, 11, 125-130.

Ions and free radicals of silver represent the active components in the bactericidal function and, unlike the current antibiotics, do not create problems related to the phenomenon of bacterial resistance [81]. Such active species probably destroy microorganisms instantly, blocking the respiratory enzyme system and causing structural changes in the bacterial cell wall, both in the intracellular environment and onto nuclear membranes. Indeed, the binding of the silver ion to bacterial DNA can inhibit a high number of important transport processes and can modify the processes of cellular oxidation.

So, despite being one of the most powerful metal inhibitors of enzymes and microorganisms, it has a low toxicity to higher organism cells due to its slow adsorption and easy interaction with thiol groups of proteins [82, 83].

Silver antibacterial activity is mainly due to high affinity of Ag^+ ions for thiol groups present on the bacterial cell membrane. This activity increases with the concentration of the ions, although metallic silver has a slight activity against microorganisms thanks to the formation of traces of Ag^+ in the biological environment [84].

In order to counteract the formation of microbial biofilms, silver has been then used for the coating of medical devices (such as intravascular or urinary catheters).

As for CVCs and intravenous catheters, various silver treatments method have been employed [85, 86].

To ensure a low but constant metal dose and then to decrease the possibility of damage to cells and tissues, several products possessing a low silver release are available today.

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[81] Sommonetti N., *Am. Soc. Microbiol.*, **1992**, 1, 834.
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[85] Bach A., Eberhardt H., Frick A., Schmidt H., Bottinger B. W., Martin E., *Crit. Care Med.*, **1999**, 27, 515-520.
[86] Woodyard L.L., Bowersock T.L., Turek J.J., McCabe G.P., DeFord J., *J. Control Release,* **1996**, 40, 23-30.

Silver has a pronounced bactericidal effect on a wide spectrum of organisms, including bacteria and fungi. Indeed, it is effective against bacteria aerobic or anaerobic, Gram-positive (*S. aureus* and *S. epidermidis*) and Gram-negative bacteria (*P. aeruginosa*), fungi (*C. species*) and viruses.

Clinical studies [87] have shown that silver coatings were effective in the reduction of catheter-related infections only for urinary catheters. This finding was confirmed by a recent systematic review and meta analysis by Casey *et al* [88]. In this study, summarizing data on the efficacy of marketed medicated catheters in reducing microbial colonization and preventing CR-BSIs (catheter-related-blood stream infections), it has been demonstrated that silver-alloy-coated, silver-impregnated and silver-iontophoretic CVCs were not able to reduce catheter-related bloodstream infections. These findings are presumably due both to the poor silver antimicrobial activity against gram-positive bacteria, the most frequently involved species in CVC-related infections, and to the albumin presents in the blood that can bind silver ions, thus reducing antimicrobial activity.

Different approaches have been investigated to incorporate silver in polymers. One of these is based on polymer impregnation with silver nanoparticles (AgNps). AgNps-containing systems have been employed to promote wound healing [89] or prevent biofilm formation on dental materials [90, 91].

Another inorganic compound employed to prepare polymer-based antimicrobial composites is titania.

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[87] Lai K.K., Fontecchio S.A., *Am..J..Infect..Control.*, **2002**, 30, 221-225.
[88] Casey A.L., Mermel L.A., Nightingale P., Elliott T,S, *Lancet Infect. Dis.*, **2008**, 8, 763–776.
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Recently, antimicrobial properties of polymer composite systems containing TiO_2-anatase (Titanium oxide) nanoparticles as a biocidal agent have been tested [92, 93]. Particularly, Fernández-García research group incorporated TiO_2 nanoparticles into ethylene-vinyl alcohol copolymer (EVOH) or isotactic polypropylene via a melt process. These nanocomposites showed remarkable antibacterial properties against different bacteria strains not due to biocidal agent release in the media.

Kubacka et *al.* [94] have developed novel, highly efficient antimicrobial nanocomposite films consisted of a commercially EVOH copolymer and embedded with Ag-TiO_2 nanoparticles. In these nanocomposites, different from previously studied TiO_2-EVOH systems, through plasmonic effect, the presence of the noble metal significantly enhanced antimicrobial activity of the system upon UV light. Finally, novel nanocomposites provided with an effective germicide activity toward *E. coli, P. putida, S. aureus* bacteria/cocci and *Pichia jadini* yeasts, by surface doping process of anatase with photodepositated Ag or by addition of Cu and Zn nitrate precursors into EVOH-based films, were also obtained by Kubacka et *al.* [95].

1.6.4 Catheter coated with Chlorhexidine and Silver Sulfadiazine

Chlorhexidine (CH) and silver sulfadiazine (SS) are antiseptic agents commonly used together in topical applications. Indeed, chlorhexidine induces alterations in the bacterial membrane allowing silver ions to penetrate inside the bacterial cell.

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[92] Cerrada M.L., Serrano C, Sánchez-Chaves M., Fernández-García M., Fernández-Martín F., de Andres A., Rioboo R.J.J., Kubacka A., Ferrer M., Fernández-García M., *Adv. Funct. Mater.*, **2008**;18,1949–1960.
[93] Kubacka A., Cerrada M.L., Serrano C., Fernández-García M., Ferrer M., Fernández-García M., *J. Nanosci. Nanotechnol.*, **2008**, 8, 241–246.
[94] Kubacka A., Cerrada M.L., Serrano C., Fernández-García M., Ferrer M., Fernández-García M., *J. Phys. Chem. C.*, **2009**, 113, 9182–9190.
[95] Kubacka A., Ferrer M., Fernández-García M., Serrano C., Cerrada M.L., Fernández-García M., *Appl. Catal. B. Environ.*, **2011**, 104, 346–352.

Catheters having the external surface coated with CH/SS have been produced (Arrowguard Blue® , Arrow International Inc, Reading, Pa., USA) and extensively studied both in vitro, ex vivo and clinical trials [96, 97, 98, 99]. Results of these clinical studies showed that CH/SS coated catheters were effective in reducing catheter-related infections only when catheters were implanted for a short-term (less than 7 days). The main reasons included the low release rate of chlorhexidine after 48 hours of elution and the absence of an intraluminal antimicrobial coating.

1.6.5 Antibiotic-coated devices

In the late 80s', Sherertz *et al.* [100] investigated adsorption of different antibiotics onto catheters as a strategy to reduce device-related infections. Experiments performed, *in vitro* and in animal models, employing dicloxacillin, clindamycin, ciprofloxacin, cefuroxime and cefotaxime, showed a correlation between *in vitro* and *in vivo* findings. Particularly, antibiotic-coated catheters having in *vitro* zones of inhibition ≥ 10-15 mm were efficacious in preventing *S. aureus* infection when implanted in rabbits. To improve the loading of anionic drugs and to increase the long term protection from bacterial infections, coating of device surfaces with cationic surfactants (e.g. tri-iododecylmethyl ammonium chloride, TDMAC), before antibiotic adsorption, was successful performed.

[96] Maki D.G., Stolz S.M., Wheeler S., Mermel L.A., *Ann. Intern. Med.*, **1997**, 127, 257-266.
[97] Tennenberg S., Lieser M., McCurdy B., Boomer G., Howington E., Newman C., Wolf I., *Arch. Surg.*, **1997**, 132, 1348-1351.
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[99] Schierholz J.M., Beuth J., Pulverer G., *J. Hosp. Infect.*, **1999**, 42, 163-165.
[100] Sherertz R.J., Forman D.M., Solomon D.D., *Antimicrob. Agents. Chemother.*, **1989**, 33, 1174-1178.

Raad *et al.* [101] utilized TDMAC for the development of minocycline and rifampin-coated (M/R) catheters. Such devices, tested in clinical trials, were able to reduce catheter colonization and catheter-related infections over a period of 7 days of implantation. Moreover, minocycline and rifampin-coated catheters were less colonized than those impregnated with CH/SS when implanted either for seven days or less (6% vs. 21.4%) or for more than seven days (10.8% vs. 24.4%).

Polymers able to absorb high amount of antibiotics on their surfaces have been also obtained by Piozzi *et al.* by introducing to the side chain of urethane polymers specific functional groups able to interact with the drugs [102]. This approach allowed to obtain high drug-polymer affinity avoiding the use and possible release of surfactants. In addition the loaded and release antibiotic amount could be controlled by ionic interactions established between drug and polymer groups. The increase of drug resistant microorganisms and the occurrence of multispecies biofilm formation require a continuous research of innovative strategies of microbial killing. So far, the clinical treatment of device related infection is based on the combined use of drugs with different antimicrobial spectrum and modes of action. In this regard, Raad and colleagues demonstrated that linezolid and vancomycin were less effective in decreasing the viability of *S. aureus* with respect to daptomycin, minocycline and tigecycline. However, by adding rifampin to either linezolid or vancomycin, a biofilm killing activity increase was observed [103].

[101] Darouiche R.O., Smith J.A., Hanna H., Dhabuwala C.B., Steiner M.S., Babaian R.J., Boone T.B., Scardino P.T., Thornby J.I., Raad I. I., *Urology*, **1999**, 54, 976-981.
[102] Piozzi A., Francolini I., Occhiaperti L., Di Rosa R.R., Ruggeri V., Donelli G., *J. Chemother.*, **2004**, 16, 66-72.
[103] Raad I., Hanna H., Jiang Y., Dvorak T., Reitzel R., Chaiban G., Sherertz R. Hachem R., *Antimicrob. Agents Chemother.*, **2007**, 51, 1656-1660.

Kim and co-workers [104] have showed that combined and sequential treatments with tobramycin and silver enhanced antimicrobial efficacy of more than 200% in *P. aeruginosa*.

Only a few investigations concerning adsorption on polymers of two antimicrobial agents possessing different action mechanism were performed.

Ruggeri *et al.*, [105, 106] incorporated cefamandole and rifampin in polyurethanes together with pore forming agents (polyethylene glycol at different molecular weight and bovine albumin) to increase and control drug release from polymer matrices. Indeed, it has been demonstrated that polyurethanes containing PEG 10000 + cefamandole + rifampicin were active against a rifampicin-resistant *S. aureus* strain up to 23 days.

Antimicrobial-releasing polymer systems allow a more prolonged use of medical device but provide no solution about the onset of the phenomenon of formation of drug resistant strains, unless they release two antimicrobial agents having different action mechanism. From here arises the need for replacement the implant with a new one, treated with a drug able to inhibit the growth of the resistant strain. This problem has, therefore, prompted the search toward the synthesis of intrinsically antibacterial polymers, capable of inhibiting the bacterial proliferation.

1.7 Biocidal Polymers

Cationic and anionic polymers, widely investigated for various therapeutic applications, possess remarkable potential in drug delivery field. In comparison to anionic polymers, cationic polymers can form electrostatic complexes with anionic biomolecules, nucleic

[104] Kim J., Pitts B., Stewart P.S., Camper A., Yoon J., *Antimicrob. Agents Chemother.*, **2008**, 52, 1446-1453.
[105] Ruggeri V., Francolini I., Donelli G., Piozzi A., *J. Biomed. Mater. Res. A*, **2007**, 81, 287-298.
[106] Francolini I., Donelli G., *FEMS*, **2010**, 59, 227-238.

acids and proteins resulting the most promising ones. In addition, since many low molecular weight antimicrobials are positively charged, cationic polymers appear to be also promising as biocidal agents, successfully employing in various areas.

Indeed, microbial contamination is of great concern in medical devices, health care products, textiles, water purification systems, food packaging and storage.

Cationic polymers (biocidal polymers, BCs) are able to kill microorganisms, acting as a source of sterilizing ions or molecules.

To develop biocidal polymers, two strategies have been mainly employed:

i. formation of a covalent bond between an antimicrobial molecule and a polymer matrix [107, 108].

ii. preparation of a monomer having intrinsic antimicrobial activity to be polymerized or copolymerized with a second biological active or no active monomer [109, 110, 111, 112].

As for the first strategy, the introduction of antimicrobial agents into polymers has been performed by different approaches. Following only some examples of this strategy will be reported.

Worley and Sun covalently bonded hydantoins, oxazolidines and imidazolidines in nylon 6,6 and polyester fabrics[113. 114, 115]. The results concerning antimicrobial activity of these materials showed that they were effective without a decrease of their tensile

[107] Woo G.L.Y., Mittelman M.W., Santerre J.P., *Biomaterials*, **2000**, 21, 1235-1246.
[108] Albertsson A.C., Donaruma L.G., Vogel O., *Ann. N.Y. Acad. Sci.*, **1985**, 446, 105-115.
[109] Kanazawa, A., Ikeda T., Endo T., *J. Polym. Sci., Part A: Polym. Chem.*, **1993**, 31, 1467-1472.
[110] Bankova M., Petrova T., Manolova N., Rashkov I., *Eur. Polym. J.*, **1996**, 32, 569-578.
[111] Oh S.T., Han S.H., Ha C.S., Cho W.J., *J. Appl. Polym. Sci.*, **1996**, 59, 1871-1878.
[112] Kanazawa A., Ikeda T., Endo T., *J. Polym. Sci., Part A: Polym. Chem.*, **1994**, 32, 1997-2001.
[113] Lin J., Winkelman C., Worley S.D., Broughton R.M., Williams J.F., *J. Appl. Polym. Sci.*, **2001**, 81, 943–947.
[114] Lin J., Winkelmann C., Worley S.D., Kim J., Wei C.I., Cho U., Broughton R.M., Santiago J.I., Williams J.F., *J. Appl. Polym. Sci.*, **2002**, 85, 177–182.
[115] Ren X., Kocer H.B., Kou L., Worley S.D., Broughton R.M., Tzou Y.M., Huang T.S., *J. Appl. Polym. Sci.*, **2008**, 109, 2756–2761.

strength properties. Park et al. [116] attacked three different antimicrobial acids such as 4-aminobenzoic acid, salicylic acid, 4-hydroxy bezoic acid to ethylene-co-vinyl acetate copolymer. It was observed that copolymer containing salicylic acid was the most active.

Also antibiotics were covalently bonded to polymers. Levofloxacin, penicillin, ampicillin were attacked to polydimethylsiloxane[117] and polytetrafluoroethylene [118, 119]. High antimicrobial activity of the polymers was guaranteed by the antibiotic release.

The second strategy, preparation of a monomer having intrinsic antimicrobial activity to be polymerized or copolymerized with a second biological active or no active monomer, is certainly the most promising and then the most investigated. Most of used synthetic monomers and polymers are acrylic derivatives of pharmacologically active compounds. Among polymers, polyamides, polyurethanes, polyesters and polyacrylamides are more sought after for their versatile structure. As for antimicrobial agents, those containing reactive functional groups such as carboxyl or amino, which may be covalently linked to a variety of polymerizable derivatives, are mainly employed. Besides linear polymers, also dendrimers and hyperbranched polymers containing biguanide, quaternary ammonium salts, and quaternary pyridinium or phosphonium salts exhibit a good antimicrobial activity whose effectiveness is related to charge of the counterion [120]. An intrinsically antimicrobial polymer should be manufactured with the following characteristics [121] :

• facile and inexpensive synthesis;

[116] Park E.S., Kim H.K., Shim J.H., Kim M.N., Yoon J.S., *J. Appl. Polym. Sci.*, **2004**, 93,765–770.
[117] Kugel A., Chisholm B., Ebert S., Jepperson M., Jarabek L., Stafslien S., *Polym. Chem*., **2010**, 1, 442–452.
[118] Aumsuwan N., Heinhorst S., Urban M.W., *Biomacromolecules*, **2007**, 8, 3525–3530.
[119] Aumsuwan N., Danyus R.C., Heinhorst S., Urban M.W., *Biomacromolecules*, **2008**, 9, 1712–1718.
[120] Ikeda T., Hirayama H., Suzuki K., Yamaguchi H., Tazuke S., *Makromol. Chem.*, **1986**, 187, 333-340.
[121] Kenawy E.R., Workley D., Broughton R., *Biomacromolecules*, **2007**, 8, 1359-1383.

• good chemical stability, particularly in the temperature range required for the use;

• water insolubility, in some applications such as water sterilization and medical device manufacturing;

• ability to not decompose giving toxic products, in the biomedical and food packaging fields.

An important feature deeply affecting the physical properties of polymers, including the antibacterial ones, is the molecular weight. In fact, an increase in antimicrobial activity has been shown with increasing polymer molecular weight, for cationic polymers having molecular weights less than 50,000 g mol^{-1}. Otherwise, cationic polymer molecular weights above 120,000 g mol^{-1} negatively influence the antimicrobial activity [122, 123]. This dependence of antibacterial properties on molecular weight is due to poor polymer permeability through cell barrier of target organism. Since the antimicrobial adhesion occurs according to a sequence of events such as absorption on bacterial cell wall, diffusion through the membrane, absorption onto cytoplasmic membrane and its disintegration, polymer molecular weight affects both interactions between polymer and bacterial cell and its penetration and diffusion in cytoplasmic membrane [124].

As for cationic quaternized polymers, another factor can affect their antimicrobial properties. It was found, indeed, a dependence of polymer antimicrobial activity on alkylation type, specially on the length of alkyl chain. In particular, it was observed that a quaternization reaction performed with compounds having long alkyl chains significantly enhances the biocidal properties of final polymer. However, different results have been obtained in literature.

[122] Ikeda T., Hirayama H., Yamaguchi H., Tazuke S., Watanabe M., *Antimicrob. Agents Chemother.*, **1986**, 30, 132-136.
[123] Kanazawa A., Ikeda T., Endo T.J., *Polym. Sci., Part A: Polym. Chem.*, **1993**, 31, 1441-1447.
[124] Ikeda T., Yamaguchi H., Tazuke S., *Antimicrob. Agents Chemother.*, **1984**, 26, 139-144.

Panarin et *al*. [125] evidenced that while the antimicrobial activity of cationic copolymers based on vinylamine, aminoalkyl methacrylate, and N-vinyl pyrrolidone was independent on the alkyl substituent length, the one of monomers considerably improved with chain length increasing.

As for poly(4-vinyl-pyridium)-based polymers (PVPs), Lewis and Klibanov [126] covalently attached to glass slides PVPs differently quaternized with side alkyl chains with length ranging from 0 and 12. They observed that a C_6 chain length was needed to not remarkably decrease the antimicrobial activity of derivatized surfaces.

Therefore, if on one hand, an hydrophobicity polymer increase, obtained by quaternization reaction with long alkyl chains, promotes interactions with cell wall, on the other the variation of hydrophilic/hydrophobic ratio in the polymer can lead to aggregation phenomena of alkyl chains decreasing its antimicrobial action.

As for binding polymers to surfaces, different techniques including chemical grafting techniques [127, 128], layer-by-layer deposition[129, 130] and plasma polymerization [131, 132] have been used.

[125] Panarin E.F., Solovskii M.V., Zaikina N.A., Afinogenov G.E., *Macromol. Chem. Suppl.*, **1985**, 9, 25-33.
[126] Lewis K., Klibanov A.M., *Trends Biotechnol*., **2005**, 23, 343-348.
[127] Advincula R., *Adv. Polym. Sci.*, **2006**, 197, 107-136.
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[129] Hammond P.T., *AIChE J.*, **2011**, 57, 2928-2940.
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[131] Friedrich J., *Processes Polym.*, **2011**, 8, 783-802.
[132] Siedenbiedel F.,Tiller J.C., *Polymers*, **2012**, 4, 46-71.

1.8 The cationic polymers

Cationic antimicrobials, including benzalkonium chloride, chlorhexidine and polymeric biguanides, have been widely employed in many commercial products and in infection control.

The antibacterial action of cationic antimicrobials (CAs) is due to their ability to interact with bacterial cell wall. Indeed, the outer bacterial cell wall is negatively charged and often stabilized by divalent cations such as Mg^{2+} and Ca^{2+}. This negative charge is due to the presence of teichoic acid and polysaccharides in Gram positive bacteria cell wall or lipopolysaccharides and phospholipids in Gram negative bacteria outer and cytoplasmic membrane. In addition, the cytoplasmic membrane, possessing selective permeability and regulating the transfer of solutes and metabolites, is formed by a phospolipid bilayer. Given the different membrane composition, leading to a different protection of cytoplasmic membrane, CAs efficacy could be different versus Gram positive and Gram negative bacteria. However, a high binding affinity of cationic antimicrobial agents, both at low and high molecular weight, for both classes of bacterial cells has been demonstrated. Such affinity is presumably due to strongly electrostatic interactions established between CAs and bacterial cell wall and/or membrane.

Thanks to wide range of obtainable properties, the use of intrinsically antimicrobial polymers (IAPs) is increasingly growing not only in the medical field but also in industrial applications.

Although, action mechanism of IAPs is still under debate, it is generally believed that they can exert their action by cell wall and/or membrane disruption, causing breakdown

of the transmembrane potential, leakage of cytoplasmic material and finally cell death [133, 134].

To better interact with the wall and cytoplasmic membrane, IAPs must possess a hydrophobic region and a strong positive charge as low molecular weight cationic antimicrobial agents. In addition, cationic polymers should be more selective towards bacteria than human cells, since bacterial membrane possesses a high negatively charge concentration due to lipid components. Then, the higher the charge density of the polymer, the greater its antimicrobial efficacy. Although this is true, it has been demonstrated that positive electrical charges are cytotoxic and hemolytic [135].

For these issues, novel cationic and/or amphiphilic polymers were designed and synthesized by employing hydrophobic monomers or neutral macromonomers to be copolymerized with the cationic monomers.

Different parameters affect cationic polymer properties including polymer chain flexibility, hydrogen-bond, hydrophobic and electrostatic interactions, presence of amino groups and pKa.

A wide range of natural or synthetic cationic polymers have been investigated. Among natural polymers, generally non-toxic, biocompatible, biodegradable and with low immunogenicity, Chitosan [136] is the most extensively studied as an antimicrobial agent.

However, the good control over properties and opportunity of easy chemical modifications make synthetic polymers as the best candidates to substitute the currently low molecular weight antimicrobial agents.

[133] Muñoz-Bonilla A., Fernández-García M., *Prog. in Polym. Sci.*, **2012**, 2, 281-339.
[134] Kenawy E.R., Abdel-Hay F.I., El-Shanshoury A.E.R.R, El-Newehy M.H., *J. Polym. Sci. Part A, Polym. Chem.*, **2002**, 40, 2384–2393.
[135] Timofeeva L., Kleshcheva N., *Appl. Microbiol. Biotechnol.*, **2011**, 89, 475–492.
[136] Tan H., Ma R., Lin C., Liu Z., Tang T., *Int. J. Mol. Sci*., **2013**, 1, 1854-1869.

As for synthetic polymers, statistic and amphiphilic block copolymers showed a good antibacterial efficacy and low toxicity for human cells with respect to homopolymers. The poor toxicity of these copolymers is probably due to an increase of their selectivity versus bacterial cells.

In addition, cationic copolymers are very interesting to fight the emergent risk of microbial resistance. Specifically, by using two different monomers one bearing antimicrobial hydrophobic compound and the other one a positive charge, it is possible to obtain copolymers possessing two different action mechanisms, thus hampering the development of resistant strains. Obviously, balance of hydrophobic content and a cationic charges is critical to achieve high activities.

All of polymers having at least one salified or quaternized amino group in their repetitive unit belong to the class of cationic polymers. Generally, these polymers are water-soluble due to the high polarity. In polymers obtained by salification of primary, secondary or tertiary amines, cationic functionality is strictly dependent on pH. Indeed, at pH greater than their pKa occurs deprotonation of nitrogen, which returns to be neutral. Instead, polymers containing a quaternary ammonium salts into repetitive unit, prepared by reaction of tertiary amino group with an alkyl halide, maintain the positive charge at any pH value. The number of the protonable amino groups can vary for each cationic polymer.

Cationic polymers can carry positive charges in the main chain or the side chain. In addition, block copolymers with polyethylene glycol as well as those having a polycationic backbone and grafted hydrophilic side chains have been developed and discussed below in the text.

Although many cationic polymers have been synthesized and investigated for their antimicrobial activity, only some classes of them are reported following.

1.8.1 Acrylic-based polymers

Examples of this polymer class are acrylic or methacrylic derivates including the most investigated PDMAEMA (poly[2-(N,N-dimethylamino)ethyl methacrylate]) [137, 138, 139]. All of these materials possessed excellent antimicrobial properties. In addition, PDMAEMA has been used as a carrier for DNA delivery.

The chemistry and structure of organic groups bonded to nitrogen, the number of nitrogen atoms as well as the counterion remarkably affect the antimicrobial activity of quaternary ammonium compounds [140, 141]. Generally, organic substituents are alkyls, aryls, or heterocyclics. However, to better interact with the lipid bilayer of the cell wall at least one of the substituents should be a long alkyl chain [142].

Lu et al. [143] synthesized novel DMAEMA-based monomers containing quaternary ammonium salts with different length of the alkyl group. After quaternization reaction of tertiary amino group with benzyl chloride (BC), butyl bromide (BB), dodecyl bromide (DB) or hexadecyl bromide (HB), (***Figure 5***). Polymers with longer alkyl chain showed better antimicrobial activity due to their higher affinity for the bacterial membrane.

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[137] Rungsardthong U., Deshpande M., Bailey L., M. Vamvakaki , Armes S.P., Garnett M.C., Stolnika S., *J. Control Release*, **2001**, 2-3, 359-380.

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[139] van de Wetering P., Schuurmans-Nieuwenbroek N.M.E., van Steenbergen M.J., Crommelin D.J.A., Hennink W.E., *J. Control. Release*, **2000**, 1-3, 193-203.

[140] Li G., Shen J., Zhu Y., *J. Appl. Polym. Sci*., **2000**, 78, 668–675.

[141] Gottenbos B., Grijpma D.W., van der Mei H.C., Feijen J., Busscher H.J., *J. Antimicrob. Chemother.*, **2001**, 1, 7-13..

[142] Sauvet G., Dupond S., Kazmierski K., Chojnowski J., *J. Appl. Polym. Sci.*, **2000**, 75, 1005–1012.

[143] Lu G., Wu D., Fu R., *React. Funct. Polym.*, **2007**, 67, 355–366.

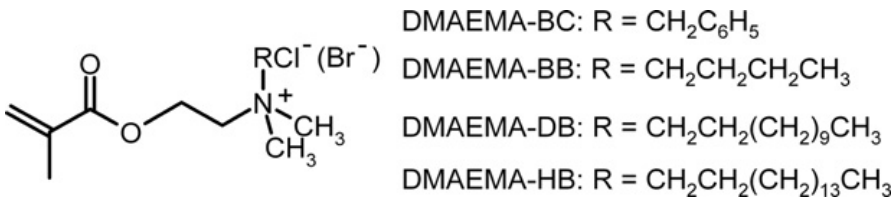

Figure 5. *Chemical structures of DMAEMA-based monomers containing quaternary ammonium salts with different length of the alkyl group.*

The same behavior was found for polymers synthesized from methacrylate monomers based on 1,4-diazabicyclo-[2.2.2.]-octane (DABCO) [144]. The prepared monomers contained two different pendant groups, butyl and hexyl group. It was observed that antimicrobial activity of polymers enhanced with pendant alkyl chain length increasing.

1.8.2 Polymers from monomers bearing aromatic or heterocyclic structures

These polymers are characterized by the presence of aromatic or heterocyclic structures and by a neutral backbone. The quaternary ammonium salt is present in the side chain.
The most studied cationic polymers derive from poly(vinylpyridine), particularly poly(4-vinylpyridine) (P4VP), and polystyrene (PS) and imidazole derivates [145]. As for quaternized P4VP, Tiller et al. [146, 147] showed that polymer surfaces derivatized with poly(vinyl-N-hexyl-pyridium), bonded to glass slides, were more able to kill bacteria when the alkyl chain was longer. The intrinsically antimicrobial property of quaternized P4VP has been also evidenced in block and random copolymers synthesized from P4VP and PS [148].

[144] Dizman B., Elasri M.O., Mathias L.J., *J. Appl. Polym. Sci.*, **2004**, 94, 635–642.
[145] Anderson E.B., Long T.E., *Polymer*, **2010**, 51, 2447–2454.
[146] Tiller J.C., Liao C.-J., Lewis K., Klibanov A.M., *Proc. Natl. Acad. Sci. USA*, **2001**, 98, 5981–5985.
[147] Tiller J.C., Lee S.B., Lewis K., Klibanov A.M., *Biotechnol. Bioeng.*, **2002**, 79, 465–471.
[148] Park E.S., Kim H.S., Kim M.N., Yoon J.S., *Eur. Polym. J.*, **2004**, *40*, 2819-2822.

The antimicrobial activity of N-hexylated P4VP as well as its biocompatibility have been improved by copolymerization with hydrophilic monomers as poly(ethylene glycol) methyl ether methacrylate (PEGMA) [149].

Indeed, with increasing surface wettability, the antibacterial activity of copolymers was 20 times higher than the quaternized homopolymer. The introduction of PEGMA also prevented the hemolysis of polymers [150].

To obtain a suitable balance between antimicrobial activity and toxicity, Sambhy et al. [151] synthesized several amphiphilic pyridium-methacrylate copolymers (***Figure 6***) by varying the ratio between positive charge and alkyl chain length on the polymer. It was observed that for polymers having the same charge/alkyl chain ratio, the spatial separation enhanced both biocidal action and reduced toxicity vs cells.

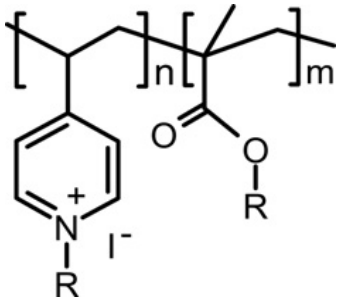

Figure 6. *Chemical structure pyridium-methacrylate copolymers.*

Another antimicrobial polymer showing temperature-responsive behavior was obtained from a methacrylamide monomer containing a pyridine moiety (MAMP) [152]. Besides homopolymer of MAMP, its copolymers with N-isopropylacrylamide (NIPAAm) with two different compositions were synthesized. Polymers with pendant pyridinium groups were obtained by reaction with various bromoalkanes containing 12, 14, and 16 carbon alkyl chains. It was observed that the neutral and quaternized copolymers with low

[149] Sellenet P.H., Allison B., Applegate B.M., Youngblood J.P., *Biomacromolecules*, **2007**, 8, 19–23.
[150] Allison B.C., Applegate B.M., Youngblood J.P., *Biomacromolecules*, **2007**, 8, 2995–2999.
[151] Sambhy V., Peterson B.R., Sen A., *Angew. Chem. Int. Ed.*, **2008**, 47, 1250–1254.
[152] Dizman B., Elasri M.O., Mathias L.J., *Macromolecules*, **2006**, 39, 5738–5746.

MAMP content were water-soluble and showed temperature-responsive behavior in aqueous solutions. In addition, the quaternized water soluble copolymers showed excellent antibacterial activities against *S. aureus* and *E. coli*, whereas the neutral polymers and quaternized water-insoluble homopolymers and copolymers were not active.

1.8.3 Polymers with quaternary ammonium groups in the backbone

Polyelectrolytes belong to this class. In these polymers the quaternary ammonium groups are present in the polymer backbone. Polyelectrolytes can be synthesized by various reaction methods including polycondensation of epichlorohydrin with bis-tertiary or secondary ammine or benzyl amine [153]. As an example, Wynee et al. [154] synthesized a new class of discrete amphiphilic quaternary ammonium compounds as highly effective antimicrobial additives for polyurethane films. The effectiveness of these additives was due to the ability to self-concentrate at the air-polymer interface thanks to the additive hydrophobicity obtained by varying the lengths of the n-alkyl and oxyethylene groups.

1.8.4 Hyperbranched and dendritic polymers

[153] Cakmak I., Ulukanli Z., Tuzcu M., Karabuga S., Genctav K., *Eur. Polym. J.*, **2004**, 40, 2373-2379.
[154] Harney M.B., Pant R.R., Fulmer P.A., Wynne J.H., *ACS Appl. Mater. Interfaces*, **2009**, 1, 39-41.

Branched polyethyleneimines (PEI) represents cationic polymers having the highest positive charge density (***Figure 7***). These hyperbranched polymers generally display a biocidal action like other cationic polymers. However, Helander et al. [155] found that PEI, when applied alone, possessed a permeabilizing effect but no bactericidal effect on gram-negative bacteria. Polyethylenimine is an effective permeabilizer of gram-negative bacteria, while a synergistic antibacterial effect of such a polymer combined with different antibiotics have been shown by Khalil et al. [156].

To improve PEI antibacterial activity, several modifications regarding cationic and hydrophobic groups present in the main chain have been performed [157, 158, 159]. Differently from hyperbranched PEIs, dendrimers possess unique physical and chemical properties including more compact structure and monodisperse molecular weights.

Various polyethyleneglycol diacrylate (PEGDA)-based dendrimers were synthesized by Abid et al. [160] by reaction of PEGDA with ethylene diamine and diethyl amine. Synthesized dendrimers were copolymerized with ethyleneglycol dimethylacrylate (EDGMA) and then quaternized with hydrochloric acid. Quaternary dendritic copolymers showed broad spectrum antimicrobial properties. In addition, their bactericidal properties depended on the concentration of quaternary ammonium groups and surface porosity.

Monodispersity of dendrimer molecular weight allowed Cooper et al. [161] of studying the structure-activity relationship of quaternary ammonium functionalized polypropyleneimine (PPI) dendrimers synthesized by themselves (***Figure 7***). These

[155] Helander I.M., Alakomi H., Latva-Kala K., Koski P., *Microbiology* **1997**, 143, 3193–3199.
[156] Khalil H., Chen T., Riffon R., Wang R., Wang Z., *Antimicrob. Agents Chemother.*, **2008**, 52, 1635–1641.
[157] Gao B.J., Zhang X., Zhu Y., *J. Biomater. Sci. Polym. Ed*., **2007**, 18, 531–544.
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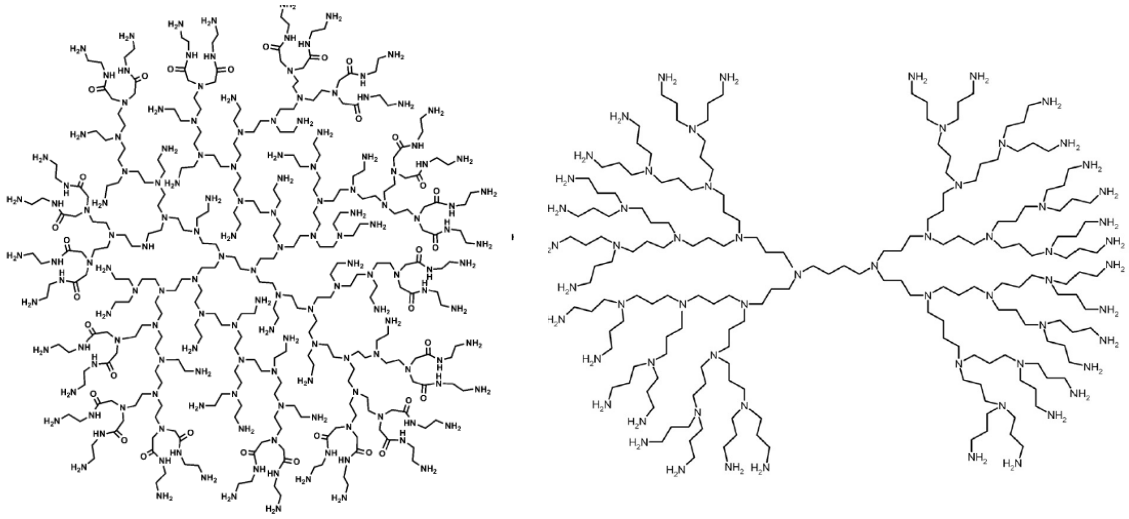
[159] Pasquier N., Keul H., Heine E., Moeller M., *Biomacromolecules*, **2007**, 8, 2874–2882.
[160] Abid C., Chattopadhyay S., Mazumdar N., Singh H., *J. Appl. Polym. Sci.*, **2010**, 116, 1640–1649.
[161] Chen C.Z., Beck-Tan N.C., Dhurjati P., van Dyk T.K., LaRossa R.A., Cooper S.L., *Biomacromolecules*, **2000**, 1, 473–480.

quaternary ammonium dendrimers were very potent biocides. It was demonstrated that the antibacterial properties depended on the size of the dendrimer, the length of hydrophobic chains in the quaternary ammonium groups, and the counteranion [162].



a) **b)**

Figure 7. *Chemical structures of a generic a) PEI and b) PPI dendrimers.*

1.8.5 Polymers with quaternary nitrogen end group

Belong to this class polyoxazolines [163], pseudopeptides prepared by living cationic ring opening polymerization. The positive charge is borne by the initiator used for opening the monomer. Polyoxazolines showed a good antimicrobial activity joined to a low toxicity. In addition, these polymers possess a wide chemical versatility of end functionalities thanks to the control of initiation and termination reactions of polymerization as well as the side chain of the monomer.

[162] Bourne N., Stanberry L.R., Kern E.R., Holan G., Matthews B., Bernstein D.I., *Antimicrob. Agents Chemother.*, **2000**, 9, 2471-2474.
[163] Hoogenboom R., *Angew. Chem. Int. Ed.*, **2009**, 43, 7978-7994.

1.8.6 Polyzwitterions

An interesting class of materials possessing positive and negative charges within the same monomer are polybetaines. Their peculiar ionic structure makes them technologically advanced polymers. Biocompatibility and antifouling features of such polyelectrolytes have been extensively investigated.

Cheng et al. [164, 165] evidenced that zwitterionic poly(sulfobetaine methacrylate) and poly(2-carboxy-N,N-dimethyl-N-(2'-methycryloyoxy)ethyl)-ethanaminium efficiently reduced the colonization of Pseudomonas aeruginosa and Staphylococcus epidermidis.

A new class of materials, cationic polycarboxybetaine esters, having unique properties when interacting with protein, DNAs and bacteria, were prepared by Zhang et co-workers [166]. The synthesized cationic polymers could be converted to nontoxic and nonfouling zwitterionic polymers upon their hydrolysis.

In particular, three positively charged polyacrylamides bearing carboxybetaine ester groups as pendant groups (polyCBAA ester) were synthesized. These three polymers possessing different spacer groups between the quaternary ammonium and the ester groups were then grafted onto a gold-coated surface by atom transfer radical polymerization (ATRP).

The polymer-grafted surfaces showed high protein adsorption. After hydrolysis, the biological surface properties changed dramatically, becoming the surface nonfouling.

[164] Cheng G., Zhang Z., Chen S.F., Bryes J.D., Jiang S.Y., *Biomaterials*, **2007**, 28, 4192-4199.
[165] Cheng G., Li G., Xue H., Chen S., Bryes J.D., Jiang S.Y., *Biomaterials*, **2009**, 30, 5234-5240.
[166] Zhang Z., Cheng G., Carr L.R., Vaisocherovà H., Chen S., Jiang S., *Biomaterials*, **2008**, 29, 4719-4725.

1.8.7 Polymers mimicking natural peptides

Many different organisms produce antimicrobial peptides as part of their first line of defense (host defense antimicrobial peptides). Generally, antimicrobial peptides (AMPs) possess a small molecular size (from 10 to 50 amino acids), amphiphilic features, with cationic and hydrophobic side chains, and are positively charged. Some of these peptides, such as defensins and magainins, are potent antimicrobials because of poor or no susceptibility to bacterial resistant mechanism. The action mechanism of AMPs is different with respect to conventional antibiotics. Indeed, antibiotics generally exert their action inhibiting enzymes and DNA replication, while AMPs attack bacterial cell membranes, making them more permeable with subsequently leakage cellular components and cell death. In addition, cationic AMPs are selective to bacteria over human cells due to their high binding affinity with bacterial surfaces which possess a great negative charge density.

Although the considerable antimicrobial properties exhibit by AMPs, their application as antibiotic substitutes has been hampered by high cost of manufacturing, poorly understood pharmacokinetics and susceptibility to enzyme action. For these reasons, in recent years, an intense research activity has been addressed to development of small molecules and polymers known as synthetic mimics of antimicrobial peptides (SMAPs).

1.8.8 Synthetic peptides

Many synthetic peptides composed of L-α-amino acids (especially L-lysine) or including structures such as D-α-amino acids, β-peptides or peptoids have been obtained. Chain length of 10-30 amino acid residues by using solution-coupling or solid

phase synthesis can be obtained [167]. In both cases it is possible to control precisely amino acid sequence.

To better resemble AMPs, the first synthesized peptides were designed in such a way that a segregation of the hydrophobic and hydrophilic groups of amino acid sequence would occur, thus inducing helix formation, particularly α-helix.

At this aim, analogues of idolidicin or gratisin as well as peptides composed of leucyl and lysyl residues with different composition and sequences were synthesized [168, 169, 170]. All of these peptide-based oligomers showed selectivity and a good antimicrobial activities.

Then, other β-amino acid-based SMAPs were obtained [171, 172, 173]. Although these β-peptides formed a different type of helix, they resulted active and selective.

Successively, many investigation have demonstrated that the helicity of the molecule is not critical for having antimicrobial activity. Instead, it is of paramount importance that the molecule possess a local amphiphilicity and is able to self-organize into hydrophobic and hydrophilic domains on the cell interface.

1.8.9 SMAPs based on synthetic polymers

The main advantage in use of polymer systems over synthetic peptides for developing of SMAPs is that to get materials on large-scale production by few synthetic steps. This makes polymeric SMAPs promising candidates for therapeutical applications.

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[167] Halevy R., Rozek A., Kolusheva S., Hancock R.E.W., Jelinek R., *Peptides*, 2003, 11, 1753-1761.
[168] Halevy R., Rozek A., Kolusheva S., Hancock R.E.W., Jelinek R., *Peptides*, **2003**, 24, 1753–1761.
[169] Tamaki M., Kokuno M., Sasaki I., Suzuki Y., Iwama M., Saegusa K., Kikuchi Y., Shindo M., Kimura M., Uchida Y., *Bioorg. Med. Chem. Lett.*, **2009**, 19, 2856–2859.
[170] Beven L., Castano S., Dufourcq J., Wieslander A., Wroblewski H., *Eur. J. Biochem.*, **2003**, 270, 2207–2217.
[171] Liu D., DeGrado W.F., *J. Am. Chem. Soc.*, **2001**, 123, 7553–7559.
[172] Epand R.F., Raguse T.L., Gellman S.H., Epand R.M., *Biochemistry (Mosc,)*, **2004**, 43, 9527–9535.
[173] Schmitt M.A., Weisblum B., Gellman S.H., *J. Am. Chem. Soc.*, **2004**;126, 6848–6849.

Understanding the influence of some parameters, such as spatial relationship between hydrophobic side chains and cationic groups, charge density, structure of cationic groups, on the biological properties of synthetic polymers, have triggered an intense research activity addressed to the design and development of new synthetic amphiphilic cationic polymers mimicking natural host-defense peptides.

Different amphiphilic copolymers, based on a polymethacrylate or polymethacrylamide backbone having hydrophobic and cationic side chains have been synthesized [174, 175, 176, 177, 178] (***Figure 8***). The hydrophobic groups and the length of the two parts have been changed to obtain non-hemolytic antimicrobials polymers [175]. Primary, tertiary amino groups, or quaternary ammonium salts have been used as sources of cationic moieties. In these studies has been evidenced as the antimicrobial and hemolytic activities of synthesized polymers depend on the properties of amino side chains as much as the hydrophobic nature of the counterpart. In addition, it has also shown that reversible protonation of the amino groups is more appropriate to obtain non-toxic antimicrobial polymers.

[174] Palermo E.F., Kuroda K., *Appl. Microbiol. Biotechnol.*, **2010**, 87, 1605–1615.
[175] Kuroda K., DeGrado W.F., *J. Am. Chem. Soc.*, **2005**, 127, 4128–4129.
[176] Palermo E.F., Kuroda K., *Biomacromolecules*, **2009**, 10, 1416–1428.
[177] Kuroda K., Caputo G.A., DeGrado W.F., *Chem. Eur. J.*, **2009**,15, 1123–1133.
[178] Palermo E.F., Sovadinova I., Kuroda K., *Biomacromolecules*, **2009**, 10, 3098–3107.

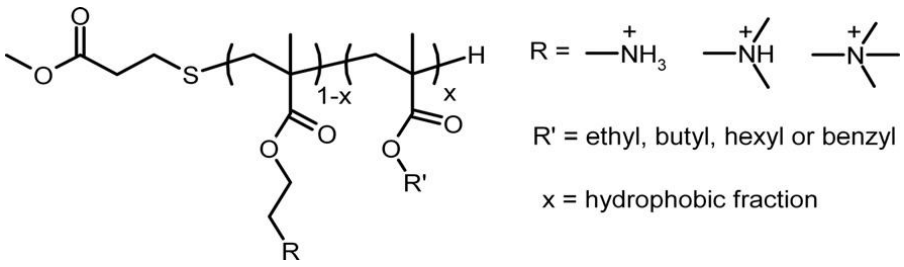


Figure 8. *Chemical structure of amphiphilic copolymers with different amine and hydrophobic side chains.*

1.8.10 Copolymers with neutral sections

After extensively studying the possible applications of cationic homopolymers, searches are heading more and more towards the preparation and characterization of statistical, block or grafted copolymers having neutral hydrophilic or hydrophobic chain sections. Widely used for this purpose is PEG, a very hydrophilic neutral polyether (e.g. segmented copolymers PEG-polylysine, or triblock PEG-PBA-PDMAEMA). Numerous studies have shown PEG effectiveness in facilitating the action of carriers for gene delivery. In fact, it ensures the protection of polycation-DNA complex before its arrival to the cell nucleus, thus preventing the formation of aggregates with serum proteins. Many of the possible applications of cationic amphiphilic copolymers are related to the property of forming micelles. This feature is especially useful in the formation of complexes with DNAs. Indeed, the ability to fold in relation to the affinity with the nucleic acid and the surrounding environment leads to the formation of particular

structures to which the effectiveness of the transport and release of the genetic material of interest is owed [179].

A stimulating approach [180] to give an higher biocompatibility of the quaternary ammonium compounds is the preparation of pegylated-copolymers. These materials are obtained from the copolymerization of DMAEMA and PEGMA. Then, the tertiary amino side chain moieties have been quaternized with different types of functional halides at different degrees of functionalization (***Figure 9***).

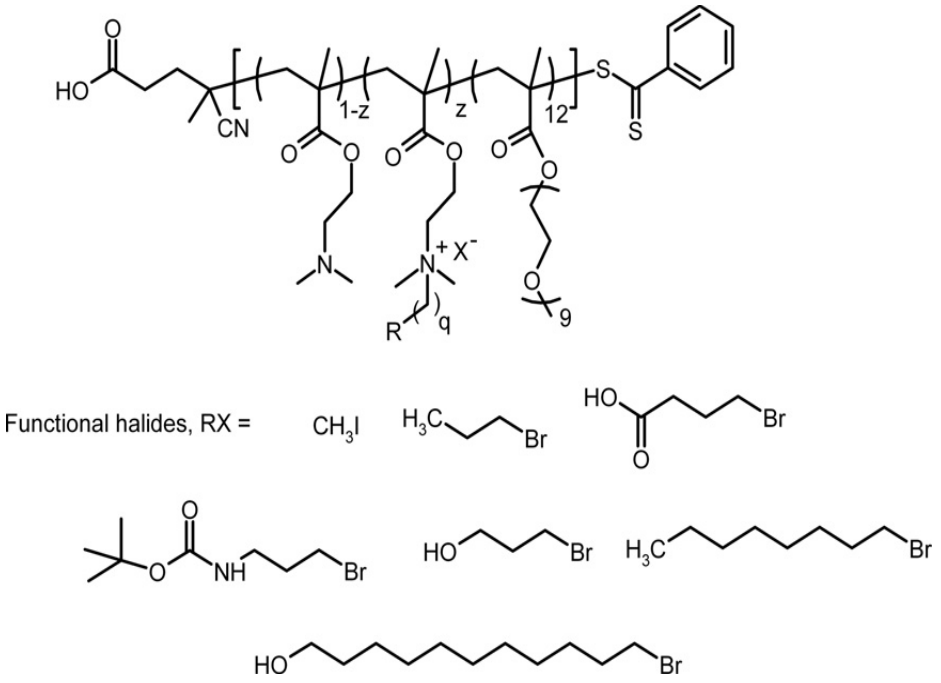


Figure 9. *Chemical structures of pegylated amphiphilic copolymers with different quaternized amine side chains.*

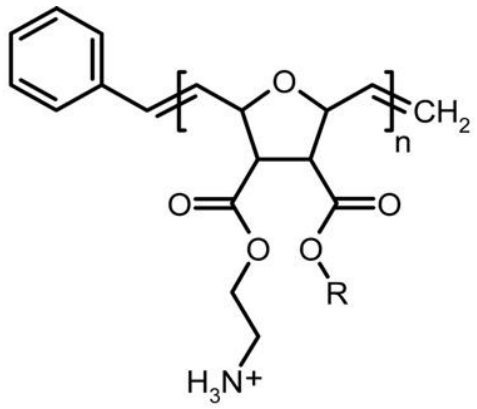
The resulting polymers with shorter chain have shown to possess lower MIC. In addition, the hemolysis resulted to be influenced by chain hydrophobicity.

[179] Sharma R., Lee J.-S., Bettencourt R,C., Xiao C., Konieczny S.F., Won Y.-Y., *Biomacromolecules*, **2008**, 11, 3294-3307.
[180] Venkataraman S., Zhang Y., Liu L.H., Yang Y.Y., *Biomaterials*, **2010**, 31, 1751–1756.

1.8.11 Polynorborene derivatives

The most of synthesized random copolymers have been obtained by employing of a hydrophobic and a hydrophilic comonomer. In these latter years new interesting polynorbornene-based SMAPs have been developed by Tew and coworkers[181, 182]. These polymers contain a facially amphiphilic monomer bearing both the hydrophobic and hydrophilic group. The spatial arrangement of these polymers, that allows the charged and hydrophobic groups to be positioned on opposite sides of the polymer backbone, promotes the interaction with the membrane phospholipids.

Different polynorbornene derivatives have been prepared by using ring opening metathesis polymerization. This synthetic strategy allowed obtaining polymers and copolymers with narrow polydispersity and different molecular weights (***Figure 10***).



R: methly, ethyl, propyl, butyl, isopentyl or hexyl

Figure 10. *Chemical structure of a generic polynorbornene.*

By tuning the hydrophobic/hydrophilic balance, by increasing the number of amine groups on the most hydrophobic polymer, and molecular weight of the synthesized

[181] Ilker M.F., Nüsslein K., Tew G.N., Coughlin E.B., *J. Am. Chem. Soc.*, **2004**, 126, 15870–15875.
[182] Tew G.N., *Mater.s Sci. and Eng.*, **2007**, 57, 28–64.

polymers, it was possible to prepare highly selective, antibacterial and nonhemolytic materials [183, 184].

1.9 Magnetic nanoparticles and drug targeting

In the last thirty years, research in the pharmaceutical industry has been focused on the study of formulations capable of releasing drugs in a controlled manner [185].

Currently, several pharmaceutical formulations are commercially available in which drug release can be either delayed or programmed in terms of rate and duration. Such formulations are defined controlled release therapeutic systems.

The developed systems can be classified in i) delayed release systems; and ii) sustained, site-specific and targeted delivery systems.

Drug targeting systems have the ability to concentrate drugs in organs or tissues in a selective and quantitative manner, regardless from the site and method of administration.

Such systems are characterized by three components: a drug, a targeting unit (capable of selective recognition), and a carrier used to increase the number of drug molecules per singular targeting unit. Pharmaceutical carriers can be soluble polymers, microparticles, microcapsules, cells, lipoproteins, liposomes, micelles and nanoparticles[186, 187].

Currently, nanoparticles are strongly investigated as pharmaceutical carriers since they can be used for intravenous administration, being able to by-pass blood-brain barrier, lung airways and epithelial skin junctions that normally prevent the drugs release.

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[183] Al-Badri Z.M., Som A., Lyon S., Nelson C.F., Nusslein K., Tew G.N., *Biomacromolecules*, **2008**, 9, 2805-2810.
[184] Som A., Choi Y., Tew G.N., *Macromol. Symp.*, **2009**, 283-284, 319-325.
[185] Timko B.P., Whitehead K., Gao W., Kohane D.S., Farokhzad O., Anderson D., Langer R., *Annu. Rev. Mater. Res.*, 2011, 41, 1–20.
[186] Vogelson C.T., *Mod. Drug. Discovery*, **2001**, 4, 49-52.
[187] Linharat R. J. *Controlled release of drugs: polymers and aggregate systems*. Morton Rosoff, VCH, **1989,** 57-62.

Four different targeting mechanisms [188,189] are possible for injectable formulations: i) Passive targeting, ii) active targeting, iii) alternative targeting, and iv) physical targeting.

The passive targeting is based on pharmaceutical formulations able to avoid the removal of the active principle by defence mechanisms of the body such as metabolism, excretion and opsonization. Releasing of such systems into specific body sites occurs via normal physiological processes. The fate of colloidal carriers in the vascular system depends on two fundamental factors that are particle size and surface features. This targeting, however, is not always very efficient since often the injected formulation is incorporated by the mononuclear phagocyte system (MPS) in particular by Kupffer cells of liver.

Generally, particles in the order of nanometers are more able to avoid the entrapping into pulmonary capillaries, but they are blocked by cells belonging to organs such liver or bone marrow. Particle surface features can favor or not opsonization while particle distribution into the body depends on the type of coating, usually based on polymers or surfactants [190].

The active targeting consists in the use of molecules having high affinity for the target zone. To this aim, the surface features of nanocarriers are properly modified. Particularly, hydrophilic particle are less prone to be subjected to MPS attack (many substances naturally present in the body are hydrophilic). The active targeting can be also obtained by binding specific molecules on the particle surface to target the system to the damaged cells. These systems are cellular markers of substances such as sugars, antibodies or lecithins.

[188] Torchilin V.P., *Handb. Exp. Pharmacol.*, **2010**, 197, 3-53.
[189] Torchilin V.P., *Eur. J. Pharma. Sci.*, **2000**, S2, S81-S91.
[190] Owens D.E. , Peppas N.A., *Int. J. Pharma.*, **2006**, 1, 93-102.

In the alternative targeting, to limit particle capture of by MPS system, macrophages are suppressed before particle administration by using a placebo or a suppressing agent such as dextran sulfate, methyl palmitate or salts of gadolino. The clinical potential of this system is however on debate because MPS system inactivation can have dangerous consequences especially in people with cancer.

The physical targeting permits the distribution of the drug-carrier system through variation of parameters, such as temperature and pH, or by applying a magnetic field.

Regions affected by presence of inflammation or tumor often show acidosis and hyperthermia. This makes possible the use of carriers able to release the drug at lower pH values or at temperatures higher than those of healthy tissues.

The targeted delivery of a drug through the application of an external physical force, such as a magnetic field, can be obtained, by employing nanocarriers with ferromagnetic properties [191].

In the context of the physical targeting, magnetic nanoparticles based on metal oxides, mainly magnetite (Fe_3O_4), in single domains of about 5-20 nm, are the carriers most studied for several reasons.

Firstly, they can be obtained with controllable size, from a few nanometers to tens nanometers, and comparable with biological entities. Furthermore, the magnetic characteristics permit to direct the carrier through the application of a magnetic field. This possibility of a "remote control", combined with the intrinsic penetrability of the magnetic field in human tissue, opens many applications involving the transport and/or immobilization of magnetic nanoparticles and the targeting of biological substances.

The main applications of magnetic nanoparticles are in diagnostics, as contrast agents in magnetic resonance imaging (they are able to influence the relaxation time of protons),

[191] Pankhurst Q.A., Connolly J., Jones S.K., Dobson J., *Journal of Physics D: Applied Physics*, **2003**, 36, R167-R181.

in cell biology, for separation and purification of cell populations, and in therapeutics, for cancer treatment by hyperthermia or drug targeting.

In this latter case, nanoparticle systems based on iron oxides, in superparamagnetic conditions [192], coated with biocompatible polymers and functionalized with interest drug, in stabilized aqueous suspension (called ferrofluid) [193], are injected intravenously into the patient and guided up to the target with an external magnetic field , applied in correspondence of the interested body site.

The effectiveness of therapy depends mainly on the strength of the applied magnetic field and the nanoparticle properties, namely:

• homogeneous particle sizes and below a critical diameter;

• high magnetic susceptibility;

• biocompatibility;

• surface properties specific to the application;

• strength of drug-nanoparticle interactions and drug desorption kinetics *in vivo*.

Preliminary studies, employing magnetic nanoparticles loaded with anticancer drugs, have been carried out in animals and men. The results obtained have been very encouraging. In fact, even administering low doses of anticancer agents, a decrease in tumor volume was observed thanks to the targeted drug release [194, 195].

In the literature, several types of ceramic magnets (ferrites with the general formula $MnFe_2O_3$) [196, 197] based on Fe, Ni, Co and Cu have been studied.

Recently, the possibility of using for this type of applications manganese ferrite ($MnFe_2O_4$) [198, 199] has been studied. This ferrite is extremely interesting for its high

[192] Wormuth K., *J. Colloid. Interface Sci.*, **2001**, 241, 366-377.
[193] RajCorresponding K., Moskowitz B., Casciari R., *J. Magn. Magn. Mater.*, **1995**, 1-2, 174-180.
[194] Winter P.M., Schmieder A.H., Caruthers S.D., Keene J.L., Zhang H., Wickline S.A., Lanza G.M., *FASEB J. Reas.Comm.*, **2008**, 22, 2758-2767.
[195] Mc Bain S.C., Yu H.H.P., Dobson J., *Int. J. Nanomedicine*, **2008**, 3, 169-180.
[196] Hans M.L., Lowman A.M., *Curr. Opin. Solid State Mater. Sci*., **2002**, 6**,** 319–327.
[197] Landfester K., Ramírez L.P., *J. Phys. Condens. Matter*, **2003**, 15, S1345-S1361.

magnetization saturation and its critical superparamagnetic size greater than that of iron oxides normally studied. These features allow both synthesizing particles of size greater than 5-10 nm and avoiding a significant reduction of magnetic properties after polymer coating.

Therefore, due to considerable interest covered by such systems, several studies must be performed to optimize the chemical and physical properties of nanoparticles to achieve stabilization of the ferrofluid, biocompatibility of surfaces and to understand the ferrofluid action mechanism.

Magnetic systems could be also used for biofilm fighting from device surfaces and surrounding tissues. By this novel promising strategy, still poorly investigated, nanoparticles capable of targeting antimicrobial agents, alone or possibly in combination with QS-interfering agents or enzymes, could be properly targeted to a localized area in which the drug release is planned to occur. Recently, indeed, it has been demonstrated that magnetic nanoparticles are able to lower *in vitro* the optical density of *S. epidermidis* broth cultures as well as to promote bacterial death up to 48 hrs [200].

[198] Francolini I., Palombo M., Casini G., D'Ilario L., Martinelli A., Rinaldelli V., Piozzi A., *J. Control. Release*, **2012**, 148, e57- e59.
[199] Bellusci M., La Barbera A., Seralessandri L., Padella F., Piozzi A., Varsano F., *Polym. Int.*, **2009**, 58, 1142–1147.
[200] Taylor E.N., Webster T.J., *Int J Nanomedicine*, **2009**, 4, 145–152.

1.10 Antioxidants

1.10.1 Role of antioxidants in fighting bacterial resistance

The emergence of bacteria resistant to antibiotics used in the treatment of device-related infections is a critical issue in the medical field. Generally, bacterial resistance is mediated by the ability of microorganisms to form biofilm on device surface. Employment of alternative therapies using new antimicrobials depends on understanding of complex biological mechanisms underlying drug-mediated cell killing action.

Bacterial adhesion to device and biofilm formation are remarkably affected by various conditions including nutrient availability, low oxygen content, and subinhibitory antibiotic concentration. Particularly, administration of low antibiotic doses can produce mutant strains susceptible to the applied antibiotic but showing cross-resistance to other antibiotics. Recently, it was observed that survival bacteria mechanism is due to genetic mutations provoked by oxidative stress. Indeed, in response to antibiotic administration bacteria produce reactive oxygen species (ROS). At high doses, ROS kill bacteria while at low doses they can lead to mutations.

Although the ROS role in planktonic bacteria has been widely studied and their relation with resistant bacteria has been demonstrated, their influence on cellular stress in biofilm is still debated [201]. However, to prevent microbial adhesion and biofilm development a potentially winning strategy could be to combine antibiotics with inhibitors that prevent the ROS formation.

[201] Høibya N.,Bjarnsholt T., G.Michael, M.Søren, CiofuO., *International Journal of Antimicrobial Agents*, **2010**, 35, 322–332

1.10.2 Reactive Oxigen Species and phenolic compounds

Antioxidants are substances able to counteract oxidative phenomena. According to Halliwell [202], the most widely used definition of antioxidant is: "any substance that when present at low concentration compared with that of an oxydizable substrate, significantly delays or inhibits oxidation of that substrate".

Reactive oxygen species (ROS) include highly reactive molecules such as superoxide anion, hydroxyl radical, peroxy radical and nitric oxide as well as non-radical species, such as hydrogen peroxide, singlet oxygen, hypochlorous acid and ozone. Hydroxyl radical is the most reactive and harmful ROS in biological systems. Such a radical is generated by the reaction of the ferrous ion (Fe^{2+}) with hydrogen peroxide. In this process, known as Fenton reaction, the amount of produced radical is directly proportional to concentration of iron or copper. The endogenous production of ROS mainly takes place in mitochondria, where oxidative processes with electron transport (cellular respiration) occur. In these processes, oxygen acts as the final electron acceptor for energy production. Oxygen, when performing its oxidizing action, is subjected to a series of reduction reactions in which it subtracts electrons to other molecules, giving rise to a series of radical intermediates.

ROS are also bore from arachidonic acid, by the metabolism of polyunsaturated fatty acids, during the production of eicosanoids (prostaglandins, thromboxanes and leukotrienes), molecules playing important functions at level of the vascular apparatus. Another case in which the production of free radicals is considered physiological and useful to the body is when it occurs in macrophages, in which the superoxide radical is used as a "killer" against bacteria and pathogenic viruses.

[202] Halliwell B., *Biochem. Pharmacol*., **1995** ,49, 1341-1348.

In addition to the endogenous mechanisms, factors causing the production of free radicals are: stress, unbalanced diet, alcohol, smoking, strenuous physical exercise, pollution and sunlight. An excess of cellular ROS contributes to the processes of aging [203] and is implicated in development of cancer and chronic diseases, neurodegenerative and cardiovascular diseases, such as ischemia, multiple sclerosis, atherosclerosis, cataracts, diabetes, hepatitis, Parkinson's disease, Alzheimer's disease, dermatitis and muscular dystrophy.

Current research has highlighted the strong contribution of the polyphenols in the prevention of cardiovascular diseases, cancer, osteoporosis, diabetes mellitus and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [204]. Phenolic compounds have remarkable importance for the role they play in foods, especially at nutritional level. They contribute to: i) aroma and flavor of many foods and drinks; ii) stabilization of natural dyes directly present in food or used as additives by food industry; iii) preservation of foods thanks to their antimicrobial and antioxidant features [205]. On the other hand, phenolic compounds if present at low concentrations protect food from oxidative and microbial deterioration, while at high concentrations can interact with proteins, carbohydrates, minerals increasing their bitter taste and astringent feature [206].

The biological activity of polyphenols is determined by several factors, the most important are:

[203] Van Der Loo B., Bachschmid M., Spitzer V., Brey L., Ullrich V., Luscher T. F., *Biochemical and Biophysical Research Communications*, **2003**, 303, 483-487.
[204] Scalbert,A.; Johnson I.T.; Saltmarsh M., *Am. J. Clin. Nutr.*, **2005**, 81, 215S-217S.
[205] Haslam E. J., *Nat. Prod. Reviews*, **1996**, 59, 205-215.
[206] Shinohara T., Kubodera S., Yanagida F.J., *Biosci. Bioeng.*, **2000**, 90, 90-107.

- direct interaction with receptors and enzymes involved in signal transduction that can modify the redox state of cells [207],

- interaction with estrogen receptor with consequent effects on endocrine functions.

A main example is represented by the interaction of soy isoflavones with estrogen receptors, this explaining their role in preventing main manifestations of menopause [208],

- well-known antioxidant activity and free radical scavenger by which they protect cells from oxidative damage;

- pro-oxidant activity which can induce apoptosis of cancer cells and prevent the development of cancers [209].

1.10.3 Antioxidant activity

In every cell of human organism, biochemical processes consuming oxygen for energy production take place. However, these oxidation processes, which are vital for the cell, produce potentially harmful waste products, particularly free radicals, such as molecules or molecular fragments containing one or more unpaired electrons in the more external atomic or molecular orbital [210].

The energy corresponding to this configuration provides these species with instability and high reactivity. As a result, autocatalytic chain reactions trigger. Free radicals are continually produced in human body and are controlled by endogenous enzymes (superoxide dismutase, glutathione peroxidase, catalase, etc.). When a high production of these species occurs, as a result of exposure to oxidizing substances from external

[207] Myhrastad M.C.W., Carlsen H., Nordström O., Blomhoff R., Moskaug, Ø., *Free Radical Bio. Med.*, **2002**, 32, 386-393.
[208] Morabito N., Crisafulli A., Vergara C., Gaudio A., Lasco A., Frisina N., D'Anna R., Corrado F., Pizzoleo M.A., Cincotta M., Altavilla D., Ientile R., Squadrito F.J., *Bone Miner. Res.*, **2002**, 17, 1904-1912.
[209] Sang S., Hou Z., Lambert J.D., Yang C.S., *Antioxid. Redox Sign.*, **2005**, 7, 1704-1714.
[210] Harman D., *J Gerontol*, **1956**, 11(3), 298-300.

sources or alteration of defense mechanisms, valuable biomolecules (DNA, lipids and proteins) can be seriously damaged [211].

Antioxidant agents with a negative reduction potential can supply electrons to free radicals in order to restore the chemical balance of system in which they work. To act as antioxidant, a substance, once oxidized, must originate a not reactive or less reactive species towards other molecules.

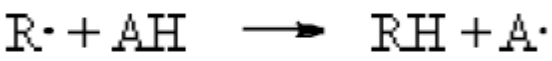
Antioxidants exert their action at rather low concentrations. When their concentration increases, some of them can become pro-oxidants and promote the formation of radicals [212].

In relation to mechanism of action, antioxidants can be distinguished in the following types:

Type I: "chain breaker antioxidants". They act as inactivators of free radicals through two basic processes: transfer of a hydrogen atom (Hydrogen Atom Transfer, HAT) or a single electron (Single Electron Transfer, SET) to radical species. The final result is the same, but kinetics and potential of reactions are different [213].

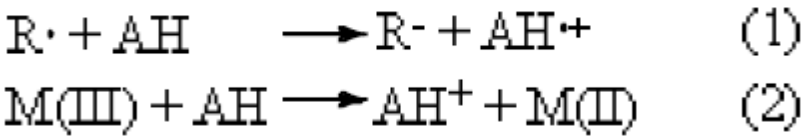
The effectiveness of these antioxidant types depends on stability of radicals in which are transformed [214]. Therefore, the more efficient the relocation of unpaired electrons produced in the reaction with free radicals, the greater their antioxidant power. Really, these mechanisms may also take place simultaneously. HAT reactions are independent on the solvent and pH of medium and, generally, take place quickly enough, finishing in a few minutes (***Scheme 1***).

[211] Boskou D., *Trends Food Sci. Tech.*, **2006**, 17, 505-512.
[212] Shahidi F., Naczk M., *Food phenolic, Technomic Publishing Co.* **1995.**
[213] Huang D., Ou B., Prior R.L.J., *Agric. Food Chem.*, **2005**, 53, 1841-1856.
[214] Saraf S., Ashawat M. S., Saraf S., *Pharmacogn. Rev.*, **2007**, 1(1), 30-40.



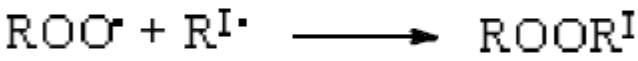
Scheme 1. *Antioxidant of type I for HAT reactions.*

Conversely, SET reactions occur more slowly. In this process, the potential antioxidant transfers an electron reducing compounds such as radicals, metals and carbonyls groups (***Scheme 2***) [215].



Scheme 2. *Antioxidant of type I for SET reactions.*

Type II: "scavenger antioxidants". This type of molecules prevent formation of free radicals, especially for coupling with another radical (***Scheme 3***). This reaction having a very low activation energy occurs with a rate close to diffusion [216]. The scavenger action also includes chelation of metals. Metal traces present in food reduce the activation energy of the reaction in initiation phase of lipid oxidation



Scheme 3. *Antioxidant of type II.*

In nature, the limit between these two classes of antioxidants is not so clear. This because some of mechanisms by which certain antioxidants exert their action have been

[215] Prior R.L., Wu X., Schaich K. J., *Agric. Food Chem.*, **2005**, 53, 4290-4302.
[216] Ingold K.U., *Acc. Chem. Res.*, **1969**, 2, 1-9.

not well defined and in addition substances as phenolic compounds can act simultaneously as first and second type antioxidants.

1.11 Catecholic function

Phenol and benzoic acid derivative polymers are biocidal agents acting into the bacteria membrane. Phenolic and antioxidant compounds are known to cause disruption of cell peptidoglycans or damage the cell membrane, or both [217]. In addition, they can cause intracellular coagulation of cytoplasmic components inhibiting the cell growth or leading to the cell death.

Park et *al*. [218] synthesized and tested in terms of antimicrobial properties different vinyl monomers bearing phenol and benzoic acid as pendant groups. They were effective against bacteria and fungi. However, polymerization of the monomers significantly decreased their antimicrobial activity. This has been explained with the extremely slow diffusion of glass polymer in the medium due to their molecular weight higher than corresponding monomers.

Antimicrobial polymer systems were also prepared from poly(vinyl phenol) (PVP) by Kenawy and Abdel-Fattah [219]. The antimicrobial activity of polymer fibers was examined against different test microorganisms. Generally, it was found that polymer morphology and molecular weight affected the activities against tested microorganisms.

[217] Tranter H.S., Tassou C.C., Nychas G.J., *J. Appl. Bacteriol.*, **1993**, 74, 253-259.
[218] Park E.S., Moon W.S., Song M.J., Kim M.N., Chung K.H., Yoon J.S., *Int. Biodeterior. Biodegrad.*, **2001**, 47, 209–214.
[219] Kenawy E.R., Abdel-Fattah Y.R., *Macromol. Biosci.*, **2002**, 2, 261–266.

Particularly interesting materials, possessing antioxidant and antifungal properties, have been obtained by Iemma et al. [220] by a one-pot free-radical copolymerization of ferulic acid (FA) and methacrylic acid (MAA) (***Figure 11***). After determination of disposable phenolic groups, the macromolecular system was tested in terms of scavenging activity toward hydroxyl radical and antifungal properties against *A. niger*. Good antioxidant and antifungal activities of the polymer were found.

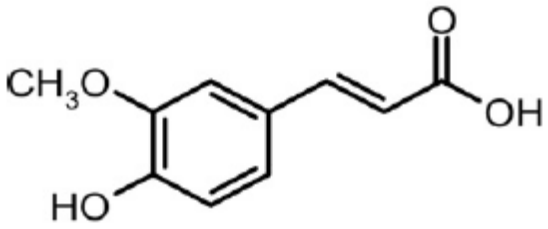


Figure 11. *Ferulic acid molecular structure.*

Also polyphenols from natural sources such as green tea extracts have been found having antimicrobial properties [221]. Mimicking this, Kenawy et *al.* [222] synthesized a copolymer of hydroxystyrene and methylmethacrylate then modified with ethylenediamine (EDA). The amine modified polymer was reacted with two classes of active compounds, particularly aldehydes and phenolic esters. The antimicrobial activities of the polymers and modified polymers were explored with bacteria, fungi and pathogenic molds. *In vitro* studies indicated that the start polymer did not affect the growth of the tested microorganisms, in contrary to its derivatives.

[220] Iemma F., Puoci F., Curcio M., Parisi O.I., Cirillo G., Spizzirri U.G., Picci N., *J. Appl. Polym. Sci.*, **2010**, 115, 784–789.
[221] Ferrazzano G.F., Roberto L., Amato I., Cantile T., Sangianantoni G., Ingenito A., *J. Med. Food*, **2011**, 14, 907-911.
[222] Kenawy E.R., El-Shanshoury A.E.R.R., Omar Shaker N., El-Sadek B.M., Khattab A.H.B., Badr B.I., *J. Appl. Polym. Sci.*, **2011**, 120, 2734-2742.

The main natural antioxidants are characterized by the presence, in their chemical structure, of phenolic groups, particularly, catechol, hydroquinone or gallic moieties (***Figure 12***). Such compounds exhibit high toxicity and thanks to this characteristic they are effective in protecting plants against insects [223].

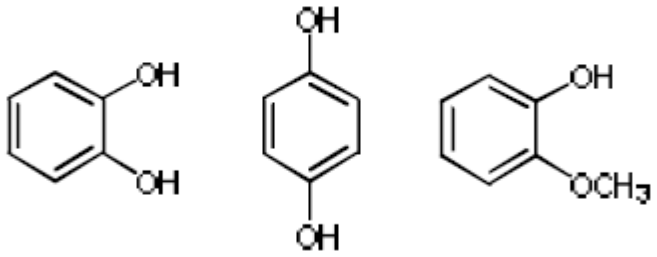
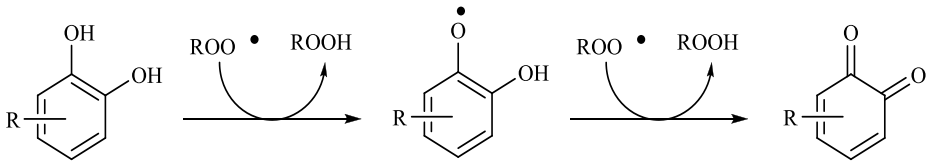


Figure 12*. Derivatives phenolic antioxidant general structures.*

The peculiar antioxidant activity of these phenols, in particular of systems containing catechol structure, is due to the semiquinone formed in the first stage of the reaction which inactivates a second radical turning into a quinone (***Scheme 4***) [224].



Scheme 4*. Antioxidant action mechanism of catecholic systems.*

Examination performed on more than 8000 molecules collected in a database (called Comprehensive Medicinal Chemistry "CMC") has identified 78 pharmacologically active compounds containing one or more catecholic functions. Among them, 12 compounds are bronchodilators, 9 are adrenergic, 7 are antiparkinsonian and other 7 are antihypertensives.

[223] Pokorny J., *J. Eur. J. Lipid Sci. Technol.*, **2007**, 109, 629-642.
[224] Amatori R., Ferrani F., Lucarini M., Pedulli G.F., Valgimigli L.J., *Org. Chem.*, **2002**, 67, 9295-9303.

Since 1% of drugs has one or more catecholic moieties, this suggests that toxicity of this group is, therefore, controllable. A rigorous research, performed in the data base of the U.S. Food and Drug Administration (FDA), showed that there are 17 approved drugs still not in prescription.

Physical-chemical calculations have recently shown that the toxicity of catechol compounds is related to their ability to form the quinine. In particular, the higher the enthalpy of dissociation of the second OH group, the less their toxicity. Obviously, the reduction of polyphenol toxicity often implies a reduction of the activity of radical-scavenger. Therefore, it is often necessary to seek a good compromise.

During the last 50 years, considerable attention has been devoted to the study of new antioxidants to prevent and treat various diseases. They are mostly of natural origin, since natural compounds are preferred by consumers because they are considered healthier.

However, antioxidants can be also produced by many synthetic strategies. Contrary to natural antioxidants, synthetic ones are developed and produced for the exclusive benefit of mankind. An interesting class of antioxidants includes those chemically synthesized having chemical structures mimicking the natural ones. Natural mimicking antioxidants can be obtained at high purity degree, by reproducible and economic procedures.

1.11.1 Reactions of hydroxylation of aromatic compounds: synthesis of catechols

In development of new synthesis strategies to produce synthetic or like natural polyphenols, the main reaction that can be considered is oxidation of aromatic compounds. Given the importance of obtainable products, the reaction of phenol hydroxylation to get catecholic structure molecules is particularly interesting.

Direct and selective hydroxylation of aromatic compounds, especially phenols, is one of the main transformations in organic chemistry and has attracted particular attention in recent years because, as mentioned previously, the hydroxylated aromatic compounds are important precursors of molecules of pharmaceutical interest [225].

Taking natural systems as a model, some oxidative methodologies employing isolated enzymes or microbial cells have been developed [226]. Differently, chemical oxidation has been late to spread as an alternative to enzymatic methods. Oxidant agents such as Fremy's radical, methyltrioxorhenium ($MeReO_3$)/H_2O_2 catalytic system [227] or dimethyldioxirane [228] do not discriminate different sites in the same molecule or even promote the oxidation of the para position if it is free from substituents.

However, few examples of one-pot chemical synthesis of substituted catechols from phenols based on the use of oxidants belonging to the family of hypervalent iodine (V) have been reported [229]. These reagents represent good substitutes to the most common used oxidizing agents because they considerably simplify the oxidative processes, being

[225] Comba P., Knoppe S., Martin B., Rajaraman G., Rolli C., Shapiro B., Stork T., *Chem. Eur. J.*, **2008**, 14, 344-357.
[226] Ullrich R., Hofrichter M., *Cell. Mol. Life Sci.* **2007**, 64, 271-293.
[227] Saladino R., Neri V., Mincione E., Marini S., Coletta M., Fiorucci C., Filippone P., *J. Chem. Soc. Perkin Trans.*, **2000**, 4, 581-586.
[228] Bernini R., Mincione E., Sanetti A., Mezzetti M., Bovicelli P., *Tetrahedron Lett.*, **2000**, 41, 1087-1090.
[229] Hansen T.V., Skattebøl L., *Tetrahedron Lett.*, **2005**, 46, 3357-3358.

extremely active and selective at the same time, versatile, economic and easy to prepare in laboratory [230].

1.12 Hydroxytyrosol

Hydroxytyrosol (HTy), 4-(2-hydroxyethyl) benzene-1,2-diol, is a natural o-diphenolic compound found in olive oil [231], and in the olive processing waste, especially in olive oil vegetation waters (***Figure 13***) [232]. It derives from hydrolysis of oleuropein glycoside by β-glucosidase of cell or by acid catalysis during storage or by pressing of olives [233]. Recently, HTy has been found in some italian red and white wines [234].

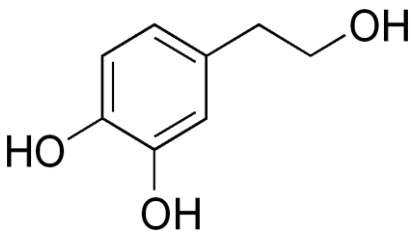


Figure 13*. Hydroxytyrosol chemical structure.*

HTy has a discrete antioxidant capacity and scavenger action versus free radicals, due to o-diphenolic functionality. *In vivo* studies, performed on rats, have shown that low doses of this compound (0.5 mg / Kg) are able to reduce the formation of lipid peroxidation products (isoprostanes) from an oxidative stress [235]. Hydroxytyrosol possesses numerous other biological activities: it prevents some cardiovascular diseases

[230] Zhdankin V.V., *Curr. Org. Synth.*, **2005**, 2, 121-145.
[231] Ragazzi E., Veronese G., *Riv. Ital. Sostanze Grasse*, **1973**, 50, 443−452.
[232] Capasso R., Cristinzio G., Evidente A., Scognamiglio F., *Phytochemistry*, **1972**, 12, 4125-4128.
[233] Briante R., La Cara F., Febbriaio F., Patumi M., Nucci R., *J.Biotechnol*., **2002**, 93, 109-119.
[234] Di Tomasso R., Calabrese R., Rotilio D., *J. High Resol. Chrom.*, **1998**, 21, 549-553.
[235] Owen R.W., Haubner R., Wurtele G., Hull E., Spiegelhalder B., Bartsch H., *Eur. J. Cancer Prev.*, **2004**, 13, 319-326.

[236], platelet aggregation [237], diabetes, cerebral ischemia and several neurodegenerative disorders [238].

Recently, HTy was also used in agrochemical field for controlling the growth of fungi and pathogenic bacteria [239]. *In vitro* studies, performed on cells where a tumor process was induced, have evidenced that this molecule acts as a detoxifying agent against mutagens, increasing cell ability to repair their genetic inheritance. In addition, it has been shown that HTy is also involved in growth phase of tumor avoiding cell proliferation and mitosis, or promoting apoptosis before the neoplasm onset [240]. For all these important properties, HTy is a product of large interest in pharmaceutical field.

Different applications of hydroxytyrosol are known in cosmetic industry, as a component of protective creams for the skin and lipsticks [241], in food industry, as a food supplement or to stabilize and improve the quality of foods and drinks, particularly vegetable oils, in food packaging as a stabilizer of plastics used [242]. Until a few years ago hydroxytyrosol was not commercially available. Today it is produced by some chemical companies with not inexpensive prices.

Given the presence of HTy in vegetable matrices, several protocols of extraction from olives, olive leaves [243], olive oil [244] and the oil mill vegetation water have been developed [245]. These procedures are often laborious and require the use of significant

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[236] Lopez-Huertas L.E., Geerlings A., Morales Sanchez J.C., Boza Puerta J., *PCT WO* 03/082798, **2003**.
[237] Petroni A., Balsevich M., Salami M., Papini N., Montedoro G. F., Galli C., *Thromb. Res.*, **1995,** 78, 151-160.
[238] Assmann G., DeBasker G., Bagnara S., *Eur J. Cancer Prev.*, **1997,** 6, 418–421.
[239] Aziz N.H., Farag S.E., Mousa L.A., Abo-Zaid M.A., *Microbios.*, **1998**, 93, 43-54.
[240] Fabiani R., De Bartolomeo A., Rosignoli P., Servili M., Montedoro G.F., Morozzi G., *Eur. J. Cancer Prev.*, **2002**, 11, 351-358.
[241] Yoshihiro C., Hiroshi A., Akira Y., Seika K., Kazuhiro Y., Kazue D., *JP2004028636*, **2004**.
[242] Peltzer M., Navarro R., Lopez J., Jimenez A., *Polym. Degr. Stabil.*, **2010**, 95, 1636-1641.
[243] Capasso R., Evidente A., Visca C., Gianfreda L., Maremonti M., Greco G. Jr., *Appl. Biochem. Biotech.*, **1996**, 60, 365–377.
[244] Montedoro G.F., M. Servili, M. Baldioli, E. Miniati, *J. Agric. Food Chem.*, **1992**, 40, 1571–1576.
[245] Capasso R., Cristinzio G., Evidente A., Scognamiglio F., *Phytochemistry*, **1992**, 12, 4125-4128.

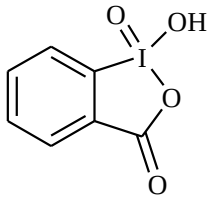
amounts of harmful and flammable organic solvents as well as appropriate extraction and purification equipment. Moreover, in some cases, hydroxytyrosol is not recovered pure but in mixture with other phenolic components, in particular with tyrosol.

From a synthetic point of view, the procedures reported in the literature include the use, as starting materials, the 3,4-dimethoxyphenyl ethanol [246]; 3,4-dimethoxyphenyl acetic acid[247] and 3,4 dimethoxy-benzaldehyde [248]. However, these syntheses require many steps and the final yields are not high in hydroxytyrosol.

1.13 Reaction of hydroxyl insertion in phenolic compounds with IBX acid (2-iodoxybenzoic)

In the last decade, the chemistry of hypervalent iodine (V) compounds has shown an intense development. Indeed, some synthetic applications based on the use of such compounds are reported in literature.

One of the main reactive compound belonging to this family is the 2-iodoxybenzoic acid (IBX). (***Figure 14***)



IBX

Figure 14*. 2-iodoxybenzoic acid chemical structure.*

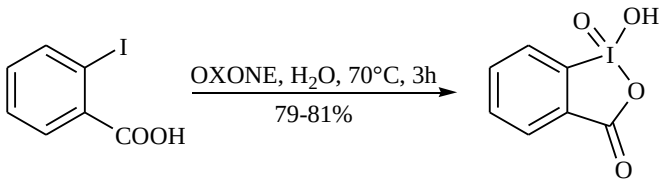
[246] Schopf C., Gottmann G., Meisel E. M., Neuroth L., *Liebigs Ann. Chem.,* **1949**, 563-586.
[247] Baraldi P.G., Simoni D., Manfredini S., Menziani E, *Liebigs Ann Chem*, **1983**, 684-686**.**
[248] Verhe R., Papadopoulos G., Boskou D., *Bull. Liaison – Groupe Polyphenols*, **1992**, 16, 237-244.

IBX has the structure of cyclic benzoiodoxole oxides, as determined by X-ray analysis [249]. It was prepared for the first time by Hartmann and Mayer in 1893 but for a long time its applicability has been limited by poor solubility in most organic solvents. The synthesis was based on the oxidation of 2-iodobenzoic acid with potassium bromate ($KBrO_3$) in aqueous solution of sulfuric acid [250].

This procedure resulted very dangerous because, according to the International Classification of toxic substances, $KBrO_3$ is considered a carcinogenic agent (R45) and the reaction produced bromine vapor with the risk of personal and environmental contamination. Moreover, the presence of bromate and other impurities coming from the synthesis, provided IBX with explosive characteristics at high temperatures (> 200 °C) or for impact.

Therefore, subsequently other oxidants such as peracetic acid [251] and sodium hypochlorite in aqueous solution have been used [252]. More recently, it has been developed an oxidation with oxone, using water as a solvent. This methodology shows several advantages such as employment of harmless reagents, development of sulfates as only reaction byproducts, high yield and a high degree of purity (NMR = 99%) [253]. (***Scheme 5***) Currently, a stabilized formulation of IBX, called SIBX, constituted by a mixture of benzoic acid (22%), isophthalic acid (29%) and 2-iodoxybenzoic (49%) is also commercially available [254].

[249] Gougoutas V.V., *J. Cryst. Struct. Comm.*, **1981**, 10, 489-494.
[250] Boeckman R.K., Shao P., Mullins J., *J. Org. Synth.*, **2000**, 77, 141-152.
[251] Katritzky A. R., Savage J. P., Gallos J. K., Durst H. D., *Org. Prep. Proc. Int.*, **1989**, 21, 157..
[252] Zhdankin V.V., *Curr. Org. Synth.*, **2005**, 2, 121-145.
[253] Frigerio M., Santagostino M., Sputore S., *J. Org. Chem.*, **1999**, 64, 4537-4538.
[254] Pezzella A., Lista L., Napolitano A., D'Ischia M., *Tetrahedron Lett.*, **2005**, 46, 3541-3544.

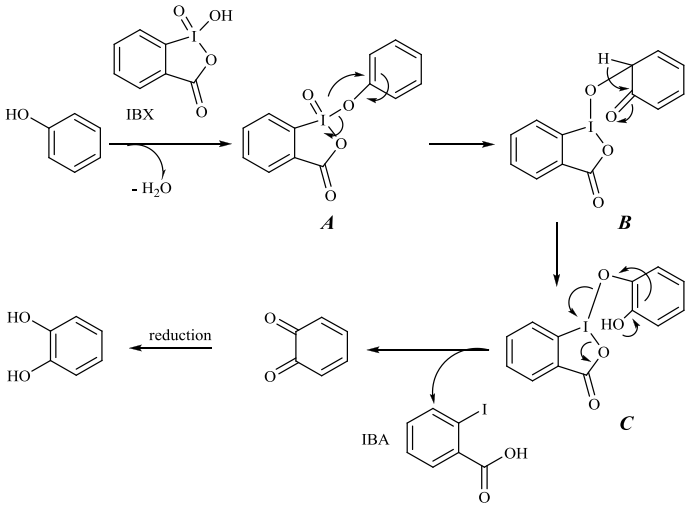


acido 2-iodobenzoico ***IBX***

Scheme 5*. IBX synthesis.*

1.13.1 Iodobenzoic acid reactivity

The first applications of IBX involved the oxidation of alcohols to aldehydes and ketones. With the progress of research on this reagent, other oxidative potentialities of the IBX have been evidenced. An interesting example is hydroxylation of phenols in ortho position. The reaction mechanism, described in the literature, is following reported (***Scheme 6***):



Scheme 6*.Reaction mechanism of ortho phenol oxidation.*

Initially, a coordination between the substrate and IBX occurs. Formation of the intermediate A with pentavalent iodine promotes the shift of oxygen to more

nucleophilic and less hindered intramolecular ortho position of the initial phenol (intermediate B). During this step, iodine is reduced from V to III with subsequent tautomerization (intermediate C). Then, the oxidation of catechol leads to ortho-quinone with reduction of iodine to valence I. Finally, by one-pot mild reduction, catechol is obtained.

1.14 Chelating properties of cathecol antioxidants

Another important application in the medical field of antioxidant substances bearing cathecol moieties is in chelation therapy. In this case, their ability to form complexes with metals is exploited. An example is the study of L-DOPA complexes with some metals [255].

Catechols are well known for their chelating properties towards some metals such as iron (III), cadmium (II), copper (II) etc. with high complexation constants [256].

Different chelating ability of catechol group found for Fe (III), Cu (II) and Ni (II) has been explained with an adsorption of these metals including two aspects [257]:

• Existence of 3d orbital not occupied, interacting with a lone pair of the catechol oxygen forming a coordination bond with the compound;

• catechol OH groups can form with metal ions hydrogen bonds stronger than those formed between water and these ions.

[255] Hamada Y., Rogers C., *J. Coord. Chem.*, **2007**, 60, 2149-2163.
[256] Sillén L.G., Martell A.E., *Stability Constants of Metal-Ion Complexes*, The Chemical Society, Burlington House London, **1964**.
[257] Li L., Li Y., Luo X., Deng J., Yang W., *React. Funct. Polym.*, **2010**, 70, 938-943.

Taking this as a cue, a line of research in the environmental field concerning adsorption of metals by catechol has been developed for a long time [258, 259]. Numerous polymer materials have been provided with a side chain containing the catechol group [260].

Chelation therapy could be a promising therapeutic approach since metals are considered to be a pharmacological target for development of new therapeutic agents for neurodegeneration or cancer treatment.

Indeed, while many neurodegenerative diseases as well as multiple sclerosis are associated with iron elevated levels, cancer patients show significantly high copper levels. Iron under oxidative stress passes from the ferrous state to the ferric one, while copper during the process of cellular copper uptake, being a potent oxidant, is reduced from Cu (II) to Cu (I).

Oxygen is absolutely essential for life. Under certain circumstances, however, the oxygen can also act as a toxic substance. In fact, oxidant events cause the formation of highly reactive species, ROS.

Oxygen circulates in our body in two forms, unstable and stable oxygen molecules. ROS are unstable oxygen molecules, highly reactive produced in the body by several environmental factors and hygienic habits only apparently healthy. ROS react rapidly and indiscriminately with surrounding molecules to capture their missing electrons. Transition metals promote the production of ROS. In biological systems, iron and copper catalysts are particularly important in the production of ROS.

ROS damage proteins, oxidize the bases of the genetic material and cause lipid peroxidation. However, ROS are also useful to fight infection, to kill bacteria and to control the smooth muscle tone, which regulates the functioning of the internal organs

[258] Andjelkovic M., Van Camp J., De Meulenaer B., Depaemelaere G., Socaciu C., Verloo M., Verhe R., *Food Chem.*, **2006**, 98, 23-31.
[259] Brown J.E., Khodr H., Hider R.C., *Biochem J.*, **1998**, 330, 1173–1178.
[260]Bernard J., Branger C., Beurroises I., Denoyel R., Margaillan A., *React. Funct. Polym.*, **2012**, 72, 98-106

and blood vessels. Oxidative stress can be defined as an imbalance between ROS and endogenous antioxidants produced by cells as defense mechanism. When this imbalance occurs, oxidatively modified molecules accumulate in the cells causing dysfunction.

CHAPTER II

WATER SOLUBLE USNIC ACID-POLYACRYLAMIDE COMPLEXES WITH ENHANCED ANTIMICROBIAL ACTIVITY AGAINST STAPHYLOCOCCUS EPIDERMIDIS

2.1 Introduction

Drug oral bioavailability can be reduced by several physiological factors such as metabolic degradation prior to absorption, poor absorption from gastrointestinal tract or first-pass metabolism [1]. Low solubility and subsequent unsatisfactory dissolution rate often compromise the development of new active molecules hindering their usage in clinics.

In an attempt to improve drug oral bioavailability, various pharmaceutical formulation technologies have been developed, including particle size reduction, use of surfactants or complexes formation.

Usnic acid, one of the most extensively studied lichen derivates, is known to possess several interesting biological properties [2]. It has a good antimicrobial activity against planktonic Gram-positive bacteria [3, 4], such as *Staphylococcus epidermidis, S. aureus*, *Enterococcus faecalis, Mycobacterium tuberculosis*, and some pathogenic fungi. [5] Usnic acid was also recently shown to be able to prevent biofilm formation by

[1] Veber D.F., Johnson S.R., Cheng H.Y., Smith B.R., Ward K.V., Kopple K.D., *J Med. Chem* **2002**, 45, 2615-2623.
[2] Cocchietto M., Skert N., Nimis P., Sava G., *Naturwissenschaften* **2002**, 89, 137-146.
[3] Lauterwein M., Oethinger M., Belsner K., Peters T., Marre R, *Antimicrob. Agents Chemother.* **1995**, 39, 2541-2543.
[4] Ramos D.F. and Almeida da Silva P.E., *Pharm. Biol.* **2010**, 48, 260-263.
[5] Pires R.H., Lucarini R., Mendes-Giannini M.J., *Antimicrob. Agents Chemother.* **2012**, 56, 595-597.

Staphylococcus spp. when incorporated in polyurethanes [6, 7] or in bone cements [8]. Therefore, this drug is under investigation for the prevention of medical device-related infections as antimicrobial agent to be adsorbed on the surface of medical devices in alternative to conventional antibiotics [9].

Besides the antimicrobial activity, usnic acid also possesses anticancer properties. Its antiproliferative activity was first reported against lung carcinoma [10] and then demonstrated against a wide variety of human cancer cell lines [11, 12]. A recent study showed that usnic acid works in a p53-independent manner [13], making it a potential candidate for novel therapies.

Despite these recognised features, usnic acid therapeutic application has not yet been introduced due to its low water solubility [10] and high hepatotoxicity[14, 15]. Thus, its use in a safe and efficient manner requires the development of suitable dosage forms able to improve its therapeutic index.

Natural or synthetic water-soluble polymers have been extensively investigated as carriers either to solubilise drugs [16] or to control and target their release to specific cells [17, 18] . The potential advantage of the use of synthetic polymers over the natural ones

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[6] Francolini I., Norris P., Piozzi A., Donelli G., Stoodley P., *Antimicrob. Agents Chemother.* **2004**, 48, 4360-4365.

[7] Labib M.E., Brumlik C.J., Stoodley P., Dukhin S.S., Davidson T., Tabani Y., *Colloids Surf. A Physicochem. Eng. Asp,.* **2010**, 254, 331-337.

[8] Kim S., Greenleaf R., Miller M.C., Satish L., Kathju S., Ehrlich G., Post J.C., Sotereanos N.G., Stoodley P., *J. Mater. Sci. Mater. Med.*, **2011**, 22, 2773-2780.

[9] Piozzi A., Francolini I., Occhiaperti L., Di R.R., Ruggeri,V., Donelli G., *J Chemother.*, **2004**, 16, 446-452.

[10] Takai M., Uehara Y., Beisler J.A., *J Med. Chem.*,**1979**, 22, 1380-1384.

[11] Song Y., Dai F., Zhai D., Dong Y., Zhang J., Lu B., Luo,J., Liu M., Yi Z., *Angiogenesis.*, **2012**,

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[15] Guo L., Shi Q., Fang J.L., Mei N., Ali A.A., Lewis S.M., Leakey J.E., Frankos V.H., *J Environ. Sci. Health C. Environ. Carcinog. Ecotoxicol. Rev.* **2008**, 26, 317-338.

[16] Craig D.Q.M., *Int. J. Pharm.* **2002**, 231, 131-144.

[17] Mahmud A., Xiong X.B., Aliabadi H.M., Lavasanifar A. *J. Drug Target* **2007**, 15, 553-584.

[18] Pillai O., Panchagnula R., *Curr. Opin. Chem Biol.* **2001**, 5, 447-451.

relies on the versatility of the macromolecular chemistry which allows to tailor the polymer molecular weight, to control its composition and to introduce specific functional groups or bioresponsive elements.

A number of polymer/anticancer drug conjugates are being tested clinically, most of which has N-(2-hydroxypropyl)methacrylamide copolymers as carriers [19]. So far, only a few studies concern the development of usnic acid/polymer systems. Particularly, PEG 400 or polypropylene glycol were used as co-solvents to increase usnic acid solubility [20]. However, only a poor or no effect on drug effectiveness was achieved. Better results were instead obtained by drug complexation with 2-hydroxypropyl-β-cyclodextrin in terms of both solubility and anti-proliferative activity [20]. The entrapping of a usnic acid/β-cyclodextrin complex in liposomes allowed the development of a long-term controlled-release system [21]. However, this system did not show better antimicrobial activity with respect to the free usnic acid when tested against Gram-positive bacteria.

The injection of usnic acid-loaded poly(D,L-lactic acid-co-glycolic acid) nanoparticles in Swiss mice produced a 26% increase in tumour inhibition and a reduction in drug hepatotoxicity [22], as a consequence of drug targeting.

In a more recent study [23], usnic acid was derivatized with various amine moieties, including the three natural low molecular weight polyamines putrescine, spermidine and spermine, in order to improve drug cellular uptake by targeting the polyamine transport system (PTS). Although results showed an increase in drug cytotoxicity against human

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[19] Duncan R., *Nature Rev.*, **2003**, 2, 347-360.

[20] Kristmundsdottir T., Aradottir H.A., Ingolfsdottir K., Ogmundsdottir M., *J. Pharm. Pharmaco.,l* **2002**, 54, 1447-1452.

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[22] da Silva Santos N.P., Nascimento S.C., Wanderley M.S., Pontes-Filho N.T., da Silva J.F., de Castro C.M., Pereira E.C., da Silva N.H., Honda N.K., Santos-Magalhães N.S. *Eur. J. Pharm. Biopharm.*, **2006**, 64, 154-160.

[23] Bazin M., Le Lamer A., Delcros J., Rouaud I., Uriac P., Boustie J., Corbel J., Tomasi S. *Bioorgan. Med. Chem.*, **2008**, 16, 6860-6866.

cancer cell lines, drug targeting to the PTS was unsuccessful. The authors explained the failure by an unfavourable architecture of the conjugates.

Therefore, since the encapsulation or conjugation of the usnic acid with polymers can enhance its bioavalaibility reducing its toxicity, we wanted to develop water soluble cationic polymers as drug carriers possessing a high affinity with the drug thanks to acidic-base interactions.

It is known that cationic polymers have good antimicrobial features thanks to the presence of pendent charged groups such as phosphonium, biguanide or nitrogen groups. Different factors affect their activity such as polymer molecular weight, the type of counterion and the alkyl chain length attached to charged group [24, 25]. Although synthetic cationic polymers can be an alternative to the use of low molecular weight antimicrobials, they suffer from their cytotoxicity if considered for therapeutic applications [26].

In fact, in spite of the positive charges facilitate the polymer cellular internalization, the non-specific electrostatic interactions with blood components lead undesired side effects. It was seen that the complexation of cationic polymers with biomolecules such as DNA, leading to the partial neutralization of charges, reduces the toxicity of such polymers [27].

A widely investigated polyacrylamide in the literature is the poly-N-[2-(N,N-dimethylamino)ethyl]acrylamide containing charged tertiary amine groups bearing methyl residues [24, 28].

[24] Kenawy E., Worley S.D., Broughton R., *Biomacromolecules* **2007**, 8, 1359-1384.
[25] Palermo E.F., Kuroda K., *Appl. Microbiol. Biotechnol.* **2010**, 87, 1605-1615.
[26] Fischer D., Lib Y., Ahlemeyerc B., Krieglsteinc J., Kissela T. *Biomaterials* **2003**, 24, 1121-1131.
[27] Cherng J., van de Wetering P., Talsma H., Crommelin D. J. A., Hennink W. E., *Pharm Res.* **1996**, 13, 1038-1042.
[28] Salloum D. S., Olenych S. G., Keller T. C. S., Schlenoff J. B., *Biomacromolecules* **2005**, 6, 161-167.

In this work, an ethylacrylic amide containing charged tertiary amine groups bearing ethyl residues was first synthesized. We chose to introduce longer alkyl residues to enhance the polymer hydrophobicity and then improve its interaction with bacterial membrane. Pursuing this strategy, propyl and butylacryl amides always bearing ethyl residues on the charged amine groups were also synthesized.

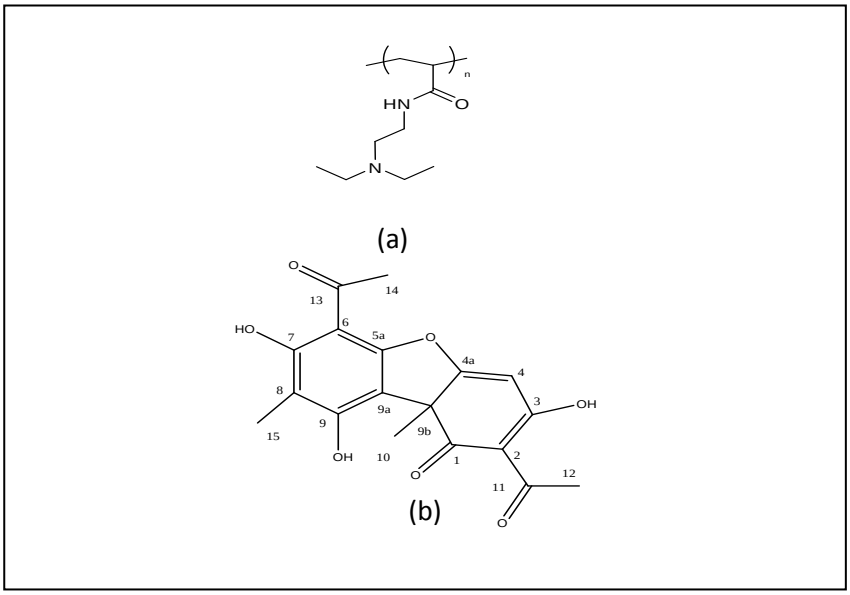
The water-soluble monomers were used for radical polymerization in order to obtain polymers to be employed as carriers for usnic acid. Differently from the other systems developed in literature, such cationic polymers can establish multipoint acid-base interactions with the drug phenolic groups. These interactions could improve not only the drug bioavalaibility but also polymer antimicrobial activity as well as lower polymer toxicity by decreasing charge density.

Finally, since one of the main issue related to the use of antimicrobial agents is the development of drug resistant bacteria, the employment of an intrinsically antimicrobial carrier rather than an inert polymer could address this problem by exerting a synergistic effect in bacterial killing. Indeed, the most pursued strategy to reduce the drug resistance is the combined use of antimicrobial agents having different mechanisms of action. In our case, the cationic polymers adsorb on the negative charged bacterial membrane provoking cell disruption while the usnic acid inhibits bacterial metabolic functions. The developed polymer/usnic acid systems were characterized by thermal and spectroscopic analysis and tested against *Staphylococcus epidermidis*.

2.2 Experimental methods

2.2.1 Monomer and polymer synthesis

Three acrylic monomers were synthesized by reaction between acryloyl chloride (Ac, Fluka) and N,N-diethylethylendiamine (DED, Sigma Aldrich) or N,N dipropylethylendiamine (PED, Santa Cruz Biotechnology, Inc.) or N,N dibutylethylendiamine (BED, Fluka). The obtained monomers were called AcDED, AcPED and AcBED respectively. The synthesis procedure was: 0.029 moles of each diamine was added to a solution of Ac (0.038 moles) in dimethylcarbonate (DMC, 75 ml, Sigma Aldrich) containing K_2HPO_4 (0.08 moles, Carlo Erba), used as base. The reaction was carried out for 4 h at room temperature. In all reactions no side product was found. After reaction, the solution was filtered to remove the inorganic and organic salt and the monomer recovered by solvent evaporation (yield ranging from 85 to 90%).



Scheme 1. *Structure formula of pAcDED (a) and usnic acid (b).*

Differently from AcDED monomer, AcPED and AcBED resulted no water soluble. Since the object of our work was to develop water soluble antimicrobial polymers to be employed as carriers for hydrophobic drugs, only AcDED monomer was used for radical polymerization.

Polymer synthesis was carried out at 25 °C for 24 h by using a 1 M water solution of AcDED (5 ml) and $K_2S_2O_8$ ($2.8x10^{-4}$ mmoles, Carlo Erba) plus $FeSO_4$ ($2.4x10^{-4}$ mmoles, Carlo Erba) as radical initiators. The resulting polyacrylamide (***Scheme 1a***) was called pAcDED (pK_b=8.61).

To evaluate the effect of polymer molecular weight on usnic acid dissolution in water, polymers at different chain length were obtained by adding in the synthesis sodium metabisulfite ($Na_2S_2O_5$, Carlo Erba) as chain transfer agent (T). The following chain transfer agent/monomer molar ratios were employed: [T]/[AcDED] = 0, 0.05, 0.1, 0.3 and 0.5. Resulting polymers were called $pAcDED_{T=0}$, $pAcDED_{T=0.05}$, $pAcDED_{T=0.1}$, $pAcDED_{T=0.3}$ and $pAcDED_{T=0.5}$.

2.2.2 Polymer characterization

^{1}H-NMR spectra were performed employing a Varian XL 300 instrument and Chloroform ($CDCl_3$) or water (D_2O) as solvents. The obtainment of AcDED monomer and the respective polymer (Figure 1a) was confirmed by ^{1}H-NMR (300MHz, $CDCl_3$ and D_2O): AcDED δ= 0.95 (t, J =7.2 Hz, 6H, $N(CH_2CH_3)_2$), 2.56-2.47 (m, 6H, $CH_2N(CH_2CH_3)_2$), 3.34-3.28 (m, $CONHCH_2$), 5.52 (dd, J_1=9.9 Hz, J_2=2.1 Hz, 1H, CHH=CH), 6.07 (dd, J_1 = 9.9 Hz, J_3 = 16.8 Hz, 1H, CHH=CH), 5.52 (dd, J_3=16.8 Hz,

J_2=2.1 Hz, 1H, CH*H*=CH), 6.70 (s, 1H, CON*H*); pAcDED$_{T=0}$ δ= 0.76 (s, 6H, N(CH_2CH_3)$_2$), 1.64-1.20 (m, 3H, CH_2CH), 2.47 (s, 6H, $CH_2N(CH_2CH_3)_2$), 2.99 (s,2H, CONHCH_2).

Molecular weight measurements were carried out at 25°C using a gel permeation chromatography (GPC) system equipped with a Shimadzu RID-10A differential refractive index detector and Tosoh TSK polyacrylamide gel columns. The calibration curve was obtained by using polyethylene oxide standards. Aqueous sample solutions containing 0,1 M NaCl were injected by an autosampler Hitachi AS-2000 at a flow rate of 0.8 mL min^{-1}.

pAcDED$_{T=0}$ possessed a M_n of 120x10^3 g/mol and 1.35 polydispersity index. By increasing the [T]/[AcDED] molar ratio the polymer molecular weight decreased, reaching a M_n value of 69x10^3 g/mol for a 0.5 molar ratio (***Table 1***).

The size of polymers was evaluated by dynamic light scattering analysis (Coulter LS-13320) employing water solutions at 0.1 mg/ml concentration.

Differential scanning calorimetry (DSC) measurements were carried out by a METTLER TA 3000 calorimeter provided with a TC 10 A processor, by keeping the cell (DSC30) under N_2 flow. The explored temperature range was -100-250° C and the employed heating rate was 10 °C/min. For each sample two scans were performed.

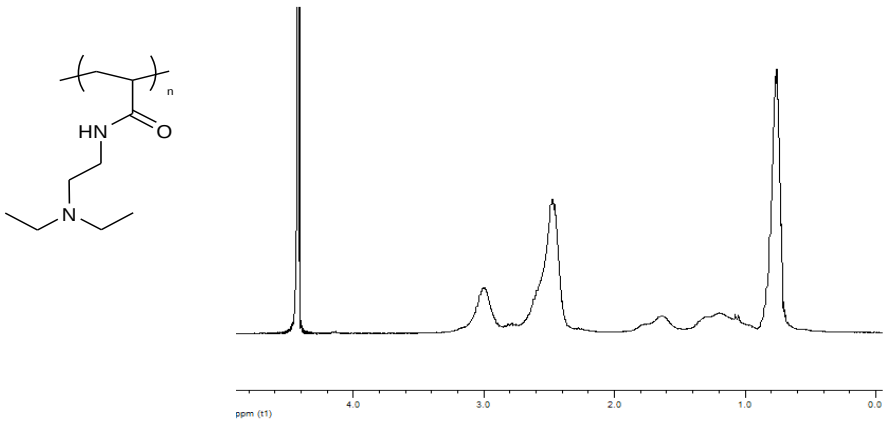
2.2.3 Preparation of pAcDED/UA complexes and their characterization

Polyacrylamide/Usnic acid (UA, Sigma Aldrich, M=344 g/mol, pKa_3=4.4, pKa_9=8.8, pKa_7=10.7, (***Scheme 1b***) complexes were prepared by dissolving in water the pAcDEDs having different molecular weights with the drug at the following polymer/UA weight

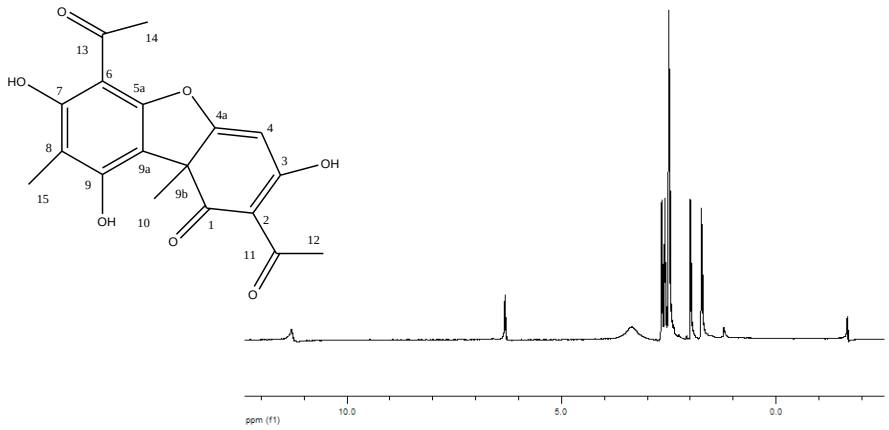
ratios, 70/30, 60/40, 50/50 and 30/70. In particular, 50 mg of UA were added to 5 ml polymer solutions containing different amounts of polymer ranging from 116 to 22 mg. Considering that pAcDEDs have one basic group per repetitive unit while the drug has three acidic groups, the employed weight ratios correspond to equiv$_{(basic\ group)}$/equiv$_{(acidic\ group)}$ ratio of 1/0.5, 1/1, 1/2 and 1/3. After water evaporation under vacuum, powders were recovered and stored at 4 °C.

^{1}H-NMR measurements were carried out dissolving the complexes in dimethyl sulfoxide (DMSO-d_6).

Thermal and DLS analyses were performed on the polymer/UA complexes by employing the same conditions used for the polymers.



(a)



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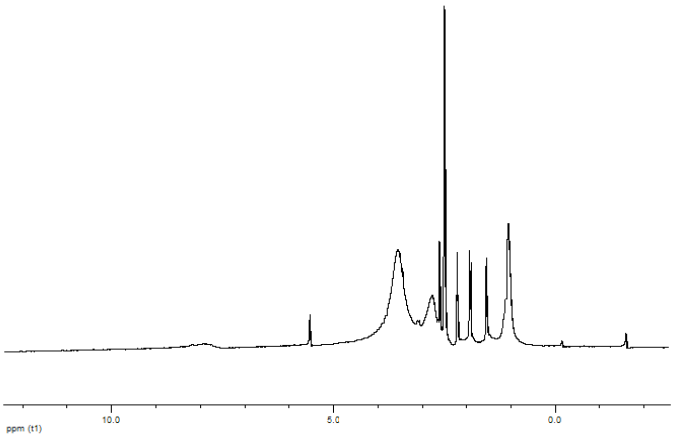


(c)

Figure 1: *^{1}H-NMR spectra of pAcDED$_{T=0}$ (a), usnic acid (b) and pAcDED$_{T=0}$/UA 70/30 complex (c).*

To evaluate UA dissolution in water, tablets containing 50 mg of drug and variable amount of pAcDEDs were prepared and kept in water up to complete dissolution. At specified time frame, aliquots of the solutions were collected and tested by UV-spectroscopy (290 nm).

The antibacterial activity of samples was assessed against *Staphylococcus epidermidis* (ATCC 35984) by the disk diffusion test and broth microdilution assay. The former test was carried out on cellulose disks embedded with 20 µL of a 1 mg/mL polymer alone or

polymer/UA solution and placed in Petri plates previously seeded with 10^8 CFU/mL bacterial concentration. Following incubation at 37°C for 24 h, diameters of inhibition zones of bacterial growth were measured.

The broth microdilution assay allowed instead to determine the minimum inhibitory concentration (MIC) of UA, pAcDEDs and polymer/UA complexes. As for UA, because of its limited water solubility, dimethylsulfoxide (2% wt) was used as solvent mediator for the antimicrobial agent, after ruling out any intrinsic activity of dimethylsulfoxide by plating viability [6]. As for polymer/UA complexes, tablets containing a fixed amount of drug (500 μg) and variable amounts of polymer to obtain 70/30, 60/40, 50/50 or 30/70 pAcDED/UA weight ratios were prepared and dissolved in water (1 mL). Then, a series of dilutions was prepared and inoculated with a 10^6 CFU/mL bacterial concentration. After 24 h-incubation at 37 °C, bacterial growth was determined by measuring the adsorbance at 600 nm. All the experiments were done in triplicate. Analysis of variance was performed using MiniTab. Differences were considered significant for $P < 0.05$.

2.3 Results and Discussion

Infectious disease is a critically important healthcare issue. The extensive use of antibiotics to eradicate the infections has contributed to the emergence of multidrug-resistant pathogens [29, 30]. In the last years, the need of suitable alternatives to replace or support systemic therapies has promoted both the use of antibiotic combinations and the

[29] Davies J., Davies D., *Microbiol Mol Biol Rev* **2010**, 74, 417-433.
[30] Francolini, I., Donelli, G. *FEMS Immunol. Med Microbiol* **2010**, 59, 227-238.

development of new classes of antimicrobial compounds [24, 25, 31]. In this regard, antimicrobial polymers offer the promise for enhancing the efficacy of existing antimicrobial agents by increasing their efficiency and selectivity [24].

Cationic polymers based on ammonium salts are undoubtedly the most investigated macromolecular antimicrobial compounds. Similarly to host defense peptides [25], cationic polymers accumulate at the level of the polyanionic bacterial cell surface. They have a broad-spectrum of activity since both Gram-negative and Gram-positive bacteria display on their surfaces anionic charges.

In this study, a hydrophilic polyacrylamide (pAcDED) was obtained by polymerization of a novel acrylic monomer that resulted water soluble thanks to the short alkyl chain (ethyl chain) bearing the cationic amine group. Therefore, this polymer was employed as carrier for usnic acid.

The use of a chain transfer agent in the synthesis controlled polymer molecular weight as demonstrated by the data reported in ***Table 1***.

In the same table, DLS data show that $pAcDED_{T=0}$ and $pAcDED_{T=0.5}$ in water possessed a remarkable size of 920 and 500 nm respectively. These values suggest that the polymer chains interact each other leading to large aggregates. In GPC analysis, these aggregation phenomena were avoided by adding NaCl in the eluent.

[31] Francolini, I., Ruggeri, V., Martinelli, A., D'Ilario, L., Piozzi, A., *Macromol. Rapid Comm.* **2006**, 27, 233-237.

POLYMER	M_n (10^3 g/mol)	$I=M_w/M_n$	Size (nm)*
pAcDED $_{T=0}$	120	1.35	920±40
pAcDED $_{T=0.05}$	100	1.30	880±80
pAcDED $_{T=0.1}$	94	1.32	780±50
pAcDED $_{T=0.3}$	78	1.29	680±70
pAcDED $_{T=0.5}$	69	1.31	500±70

* The data, representative of at least six measurements, are reported as arithmetic mean ± standard deviation.

Table 1. *Molecular weight, polydispersity index and DLS size of synthesized polymers*. *The data, representative of at least six measurements, are reported as arithmetic mean ± standard deviation.*

In ***Figure 2***, the size of inhibition zone of bacterial growth for the polymers synthesized with different amount of chain transfer agent is reported. As can be observed, all of polymers showed small inhibition zones indicating a poor diffusion ability probably due to their large molecular size and possible interaction with ionic components of the agar medium. The lowest molecular weight polymer ($pAcDED_{T=0.5}$) displayed the largest inhibition zone (4 mm).

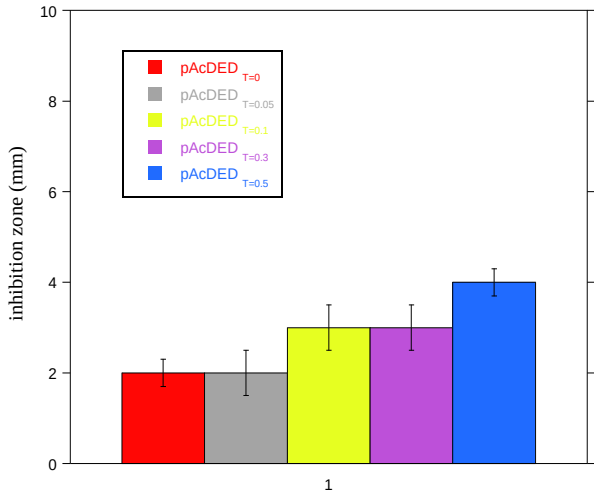
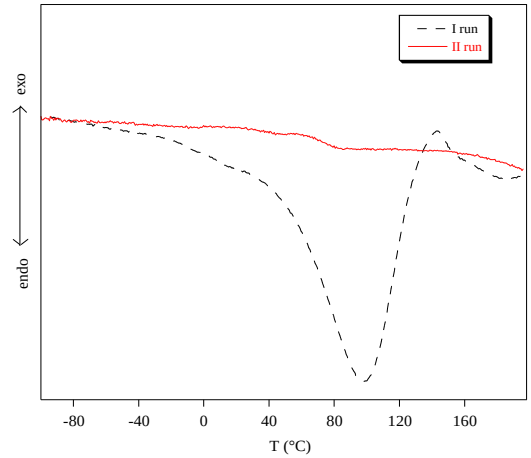


Figure 2: *Inhibition zones of bacterial growth of polymers synthesized with different amount of chain transfer agent (T).*

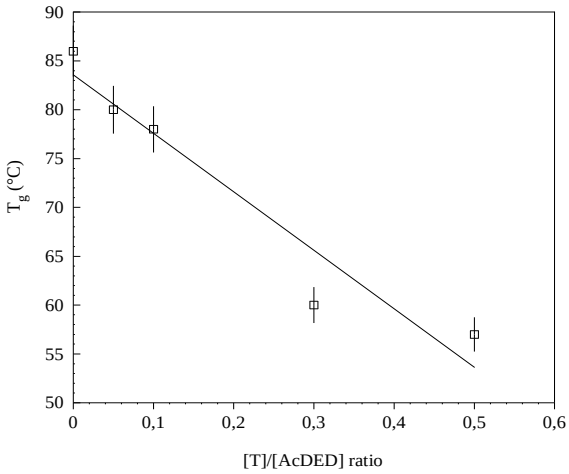
For all pAcDED polymers a MIC value of 100 µg/mL was found. This minimum inhibitory concentration is comparable to those reported in literature for polymers based on ammonium salts [32].

Differential scanning calorimetry evidenced that pAcDEDs were amorphous polymers. As an example, the thermogram of $pAcDED_{T=0}$ is reported in ***Figure 3a***. In the first scan, a wide endothermic peak related to the adsorbed water can be observed. This peak in the second scan disappeared. To evidence if any transition was present, the first derivative of the curves was done. A glass transition temperature at 86 °C was found.

[32] Palermo, E.F., Kuroda, K., *Biomacromolecules* **2009**, 10, 1416-1428.



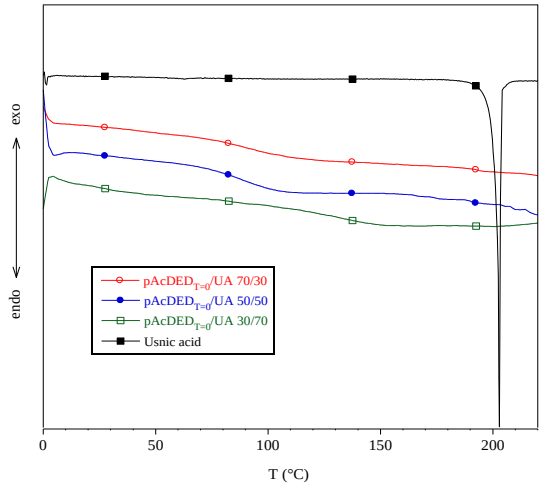
(a)



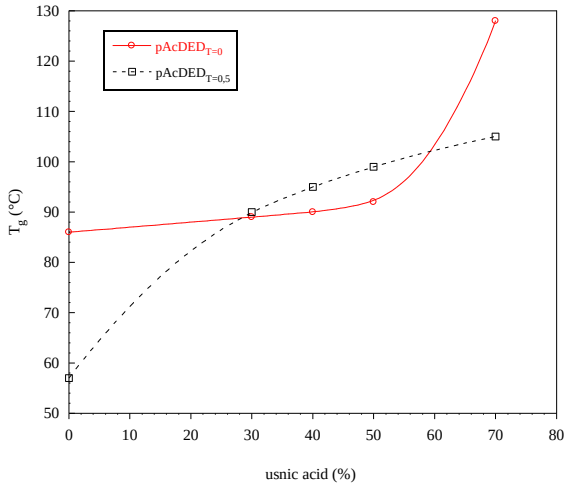
(b)

***Figure 3**: pAcDED$_{T=0}$ thermograms related to the first and second heating cycle (a); dependence of polymer glass transition temperature (T_g) on the chain transfer agent/monomer [T]/[AcDED] ratio (b). The T_g values were determined by the first derivative of the curve obtained in the second heating cycle.*

This transition remained unchanged in third scan. With increasing of [T]/[AcDED] molar ratio employed in the synthesis a decrease in glass transition temperature (***Figure 3b***) was observed indicating a higher chain flexibility of the lower molecular weight pAcDEDs. This was presumably due to the higher concentration of the chain-ends.



(a)



(b)

Figure 4: *DSC curves of usnic acid and pAcDED$_{T=0}$/UA complexes (a); dependence of polymer glass transition temperature on usnic acid content for pAcDED$_{T=0}$ and pAcDED$_{T=0.5}$ (b). The T_g values were determined by the first derivative of the curve obtained in the first heating cycle.*

DSC also allowed to assess the establishment of polymer/drug interactions. In fact, the disappearance or shifting of exothermic or endothermic peaks as well as variation of

enthalpy values can give information on the compatibility/incompatibility of the components in the formulation. In ***Figure 4a***, DSC curves of UA, $pAcDED_{T=0}$/UA 30/70, 50/50 and 70/30 are reported.

As can be observed, UA is a crystalline drug with a melting temperature of 203 °C (melting enthalpy = 98 J/g). When UA was dispersed in pAcDED, the absence of the melting peak in all the tested formulations indicates that UA was in an amorphous state. This is probably due to the establishment of polymer/drug acidic-base interactions hindering UA crystallization. These interactions also caused a stiffening of polymer chains as demonstrated by the increase in polymer T_g (***Figure 4b***). This stiffening occurs at a 30% UA content for the lower molecular weight polymer ($pAcDED_{T=0.5}$) and only at high UA content (70%) for $pAcDED_{T=0}$. Thus, due to its basic and amorphous features, pAcDED seems a good carrier for UA since a molecular dispersion of the drug and a complete drug/polymer miscibility in the amorphous phase was achieved.

A further confirm of the polymer/drug interactions was given by ^{1}H-NMR measurements. ^{1}H-NMR spectra of usnic acid and the $pAcDED_{T=0}$ /UA 70/30 complex ($equiv_{(basic\ group)}/equiv_{(acidic\ group)}$ ratio =1/0.5) are reported in ***Figure 4b*** and ***4c*** respectively.

The UA spectrum showed a resonance at 11.3 ppm related to the protons of the hydroxyl groups in 3, 7 and 9 position (***Figure 1b***). The signal at 6.2 ppm was attributed to the proton in position 4 while the resonances at 2.65, 2.57, 1.98 and 1.71 ppm to the methyl groups in 12, 14, 15 and 10 position, respectively. In the spectrum of the $pAcDED_{T=0}$ /UA 70/30 complex, the disappearance of the signal at 11.3 ppm indicated the establishment of acid-base interactions between UA and pAcDED (***Figure 1c***). These interactions also caused shifts of the signals attributed to the CH in position 4 (from 6.2 to 5.4 ppm) and to the CH_3 in position 12 (from 2.65 to 2.20 ppm). Similar

results were found for the other complexes where the $equiv_{(basic\ group)}/equiv_{(acidic\ group)}$ ratio was ≥1/1 (data not shown).

Also experiments of drug water dissolution showed the strong polymer/drug interactions. Indeed, differently from the free UA, a complete tablet dissolution was achieved after 30 min of immersion in water for all the tested formulations. This indicates that the polymer promotes UA solubilization in water. However, since a significant variation of the UV spectrum was observed after tablet dissolution, it was not possible to determine the increase in UA water solubility. In fact, other than the adsorption band of the free drug (290 nm), a new band, strongly dependent on the polymer/drug ratio, appeared at 240 nm (data not shown). This suggests the formation of a stable polymer/UA complex both at low and high drug concentrations.

As for the size of complexes in solution, DLS analysis showed a decrease in complex sizes with respect to those of polymers alone due to the ability of usnic acid to shield polymer positive charges breaking the inter-chain interactions (***Table 2***). Indeed, the drug can establish multipoint interactions with polymer chains promoting the formation of small structures.

	Size (nm)		
pAcDED/UA	**[T]/[AcDED]=0**	**[T]/[AcDED]=0.1**	**[T]/[AcDED]=0.5**
100/0	920±40	780±50	500±70
70/30	530±40	70±7	60±10
60/40	600±30	90±8	80±10
50/50	580±50	95±15	90±9
30/70	610±70	100±15	100±10

Table 2. *DLS size of polymer/UA complexes.The data, representative of at least six measurements, are reported as arithmetic mean ± standard deviation.*

Such size decrease was higher for the polymers at lower molecular weight ($pAcDED_{T=0.1}$ and $pAcDED_{T=0.5}$) than the polymer obtained without chain transfer agent. This finding can be attributed to the large initial size of $pAcDED_{T=0}$ aggregates that did not allow UA to interact efficiently polymer cationic groups. The greater size of $pAcDED_{T=0}$ with respect to other polymers is due to not only the higher length of the polymer chains but also probably to the lower concentration of chain ends that involving a minor excluded volume allows each chain to interact with nearby chains.

Moreover, the complex size depended also on usnic acid content (***Table 2***). Generally, a slightly increase of the complex size was observed with increasing of drug amount. The size of the $pAcDED_{T=0.1}$/UA and $pAcDED_{T=0.5}$/UA complexes could be suitable for a good cellular uptake [22].

To verify the effect of polymer charge density on the antibacterial properties of complexes, the $pAcDED_{T=0}$/UA and $pAcDED_{T=0.5}$/UA samples at different polymer/UA ratio were submitted to the disk diffusion tests. The $pAcDED_{T=0}$/UA complexes showed a diameter of inhibition zone ranging from 4 mm for polymer/UA 70/30 to 2 mm for polymer/UA 30/70, values comparable to that of UA (2 mm). This finding indicates a poor diffusion ability of $pAcDED_{T=0}$ /UA complexes due to their remarkable size (***Table 2***).

On the contrary, as shown in ***Table 3***, the antibacterial activity of $pAcDED_{T=0.5}$/UA complexes resulted improved with respect to both UA and polymer alone, particularly for the $pAcDED_{T=0.5}$/UA 70/30. This result confirms that the inhibition zone depends mainly on size of the complexes rather than drug amount. Therefore, UA, being strongly linked to the polymer, cannot directly act against bacteria but can potentiate polymer activity by reducing the size of aggregates.

The broth microdilution assay was performed on UA, after its solubilisation with dimethylsulfoxide (2% wt), pAcDEDs and on $pAcDED_{T=0.5}$/UA complexes, these latter being chosen for the better antimicrobial activity showed in the disk diffusion tests. For all pAcDED polymers a MIC value of 100 µg/mL was found. This minimum inhibitory concentration is comparable to those reported in literature for polymers based on ammonium salts [32]. In ***Table 3*** the MIC values of UA, $pAcDED_{T=0.5}$ and $pAcDED_{T=0.5}$/UA complexes against *S. epidermidis* are reported. All $pAcDED_{T=0.5}$/UA complexes possessed a lower MIC with respect to UA ($P<0.01$).

Also in this case, the good activity of the complexes can be attributed to their reduced size. Indeed, the killing effect extent seems to be not significantly dependent on UA content in the samples (***Table 3***).

Sample	Inhibition zone (mm)	MIC (µg/ml)
Usnic acid	2 ± 0.5	16 ± 4
pAcDED $_{T=0.5}$	4 ± 1	100 ± 3
pAcDED $_{T=0.5}$/UA 70/30	15 ± 2	5 ± 1
pAcDED $_{T=0.5}$/UA 60/40	9 ± 1	7 ± 1
pAcDED $_{T=0.5}$/UA 50/50	9 ± 2	8 ± 1
pAcDED $_{T=0.5}$/UA 30/70	9 ± 2	10 ± 2

Table 3. *Inhibition zones of bacterial growth and MICs of usnic acid, pAcDED$_{T=0.5}$ and pAcDED$_{T=0.5}$/UA complexes.*

Then, the UA complexation with a cationic amorphous polymer at low molecular weight allowed not only a successful drug solubilization but also an improvement of the polymer activity thanks to the reduction of the aggregate size. Since the best results were obtained for the complex at a low drug content (pAcDED$_{T=0.5}$/UA 70/30), this strategy is particularly winning given the known UA toxicity. Also the polymer toxicity could be reduced since the interaction with the drug partially neutralizes its positive charges. In addition, the combined use of two antimicrobial agents at different mechanism of action reducing the risk of emergence of drug-resistant microorganisms makes these systems particularly interesting for clinical application.

2.4 Conclusions

To increase the bioavalaibility of water insoluble drugs novel basic polyacrylamides (pAcDEDs) at different molecular weight were synthesized and used as carriers. All obtained polymers were amorphous, water-soluble and able to complex usnic acid, chosen as model drug.

DSC and ^{1}H-NMR measurements showed that the polymers established multipoint acid-base interactions with the drug phenolic groups. The shielding of polymer positive charges by usnic acid allowed the obtainment of polymer/UA complexes smaller in size than the polymers alone particularly for polymers with lower molecular weight. The drug-polymer multipoint interactions were more favored in the polymers with a higher concentration of chain ends. Indeed, a complex size of about 60 nm was obtained with the lowest molecular weight polymer at low usnic acid content ($pAcDED_{T=0.5}$/UA 70/30).

The polymer molecular weight affected also the antimicrobial activity against *S. epidermidis* of both the polymer alone and polymer/UA complexes. Indeed, $pAcDED_{T=0.5}$ displayed the largest inhibition zone of bacterial growth both alone and when combined with UA.

The good activity of the complexes is probably due to their reduced size that allows an efficient cellular uptake of the antimicrobial complexes. The killing effect extent seems to be not significantly dependent on UA content in the samples.

Novel polymers here reported could be used as carrier for different types of molecules (drugs, DNA, vaccines) that similarly to usnic acid have toxicity issues or need a targeted release. Further investigations are needed to determine whether this complexation allows a safer use of the cationic polymer and usnic acid in therapeutics.

CHAPTER III

USNIC ACID LOADED CORE-SHELL MANGANESE FERRITE NANOCOMPOSITE

3.1 Introduction

Magnetic nanoparticles (MNPs) are interesting materials for many applications in different fields such as the environment, medical and electronic one. In particular, in the medical field MNPs can be used for drug delivery[1, 2], magnetic resonance imaging (MRI)[1, 3], cell labeling [1, 3] and hyperthermia [4].

In fact, development of magnetic particles with a mean diameter of few nanometers is a subject of intense research in the biomedical field since they can be alternatively used in magnetically targeted therapy or diagnostics [1, 3] .

Using magnetic nanoparticles in drug targeting requires not only production of particles with a narrow size distribution and superparamagnetic properties but also with engineered surfaces such to ensure the adsorption and release of active molecules at a specific body site. However, because of their large surface area/volume ratio, the nanometer-sized iron oxide particles tend to agglomerate into large clusters and loose the specific properties associated with their initial nanometer dimensions [5] . Therefore, an appropriate surface modification and particle stabilization have become critical challenges for extending the utility of magnetic nanoparticles into biomedical field [5]. In

[1] Laurent S., Forge D., Port M., Roch A., Robic C., Vander Elst L., Muller R. N., *Chem. Rev.*, **2008**, 108, 2064–2110.
[2] Dobson J., *Nanomedicine*, **2006**, 1, 31–37.
[3] Veiseh O., Gunn J. W., Zhang M., *Adv. Drug Delivery Rev.*, **2010**, 62, 284–304.
[4] Barakat N. S., *Nanomedicine*, **2009**, 4, 799–812.
[5] Dong H., Huang J., Koepsel R. R., Ye P., Russell A. J., Matyjaszewski K., *Biomacromolecules*, **2011**, 12, 1305–1311.

fact, despite natural accumulation in target tissues (tropism of host cell), surface engineering of magnetic particles with proteins, target ligands or polymers can improve nanoparticle selectivity towards tissues or organs.

In particular, the ability of hydrophilic polymers of increasing the uptake of micro-and nano-particles into lipid bilayer of cell membrane has already been demonstrated [6] . Indeed, nanoparticle coating with hydrophilic materials such as PEG[7] has been able to increase the uptake of nanoparticles in cancer cells compared to the uncoated system. In recent years, many efforts have been directed towards surface modification of various nanostructured carriers with hydrophilic polymers to increase their biocompatibility, circulation time in bloodstream, resistance to protein absorption (which would allow system recognition by the reticuloendothelial system (RES)) and efficiency in cellular internalization [7] .

A common strategy employed to prepare a ferrofluid is to coat magnetic nanoparticles with either a surfactant, inorganic material or polymer [8]. Surface coating may be also useful to improve nanoparticle biocompatibility as well as to permit a better drug conjugation and delivery to the target area.

Magnetic particles offer a high potential for developing effective drug delivery systems. In general, when drugs are systemically administered only a part of the active principle reaches the desired site owing to poor absorption and loss in metabolism. In addition, the drug dosage is critical in all therapies since the drug concentration must be included between the toxic and ineffective levels. The development of targeted release (TR) systems solves these drawbacks since the drug is release directly at the target site.

[6] Romberg B., Hennink W.E., Storm G., *Pharm. Res.*, **2008**, 25, 55–71.
[7] Francolini I., Palombo M., Casini G., D'Ilario L., Martinelli A., Rinaldelli V., Piozzi A., *J. Control. Release*, **2012**, 148, e57- e59
[8] Raj K., Moskowitz B., Casciari R., *J. Magn. Magn. Mater.*, **1995**, 1-2, 174-180.

TR systems can be extremely useful for the treatment of diseases localized in specific body areas such as tumors [9] . Recently, magnetic nanoparticles have been also proposed for the treatment of microbial infections related to implantable medical devices [10, 11]. These infections are caused by the formation of a sessile bacterial community, known as biofilm [12], on the device surfaces . Microbial biofilms are difficultly eradicable due to their high antibiotic resistance [13]. The resistance to normal antibiotics that bacteria in biofilms exhibit respect to the planktonic counterpart can get up to 1000 times.

This finding has been attributed to several factors. First of all, biofilms represent an ideal environment for plasmid exchange among cells. In fact, conjugation frequency appears to be higher in biofilm than in planktonic bacteria. This amplifies both naturally occurring and induced antibiotic-resistance phenomena.

Other factors involved in the biofilm increased drug resistance [14] are i) slow and scarce penetration of antimicrobial agents through the biofilm matrix; ii) physiological response of microorganisms to the heterogeneous chemical environment existing in biofilms [15]. In fact, the growth conditions in biofilms are quite different at lower layers, where nutrients and oxygen are limited, and microbial waste products can be toxic; for example, in the deep layers anaerobic niches are presumably present [16]. These factors promote the development of different bacterial phenotypes, including drug-resistant ones. In addition, also nutrient depletion at lower layers by slowing bacterial growth rate can reduce the number of targets for antimicrobial molecules; iii) onset of

[9] Alexiou C., Schmid R.J., Jurgons R., Kremer M., Wanner G., Bergemann C., Huenges V, Nawroth T., Arnold W., Parak F.G., *Eur. Biophys J.* , **2006**, 35, 446–450.
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[16] Borriello G., Werner E., Roe F., Kim A.M., Ehrlich G.D., Stewart P.S., *Antimicrob. Agents Chemoter.*, **2004**, 48, 2659–2664.

subpopulations of persistent or dormant bacterial cells in a spore-like, non-dividing state [17].

Therefore, once the biofilm is established, antibiotic treatments for its eradication usually fail. In case of medical device-related infections, the surgical removal and replacement of the infected device is needed. This involves serious inconvenience for the patient and increases in hospital costs.

The localization of magnetic nanoparticles releasing antimicrobial agents (alone or in combination with quorum sesing-interfering agents and biofilm-matrix disaggregating enzymes) at the infected area surrounding the implanted medical device has emerged as a novel promising strategy to potentiate the activity of the employed antimicrobial agents against the biofilm.

In this study, manganese ferrite nanoparticles were obtained by coprecipitation of Fe^{3+} and Mn^{2+} from water-in-toluene micelle system. Magnetic nanoparticles were coated with pAcDED an amorphous and water-soluble polymer, bearing tertiary amino groups [18]. These groups, besides providing the polymer with intrinsic antimicrobial activity, can be employed for the adsorption of drugs with acidic properties by establishing acid-basic interactions. In addition, the pAcDED hydrophilic shell attached to the MNPs can contribute to avoiding MNPs recognition by mononuclear phagocyte system, thus prolonging their circulation time into the bloodstream.

To evaluate the ability of pAcDED-coated MNPs to adsorb and release drugs, usnic acid was chosen as a model drug due to both its chemical features, enabling it to establish acid-base interactions with pAcDED, and its antimicrobial and antitumoral activity. In fact, usnic acid has a good activity against planktonic Gram-positive

[17] Lewis K., *Nat. Rev. Microbiol.*, **2007**, 5, 48–56.
[18] Francolini I., Taresco V., Crisante F., Martinelli A., D'Ilario L., Piozzi A., *Int J Mol Sci.*, **2013**; 14(4), 7356-7369.

bacteria, [19, 20] such as *Staphylococcus epidermidis*, *S. aureus*, *Enterococcus faecalis*, *Mycobacterium tuberculosis*, and some pathogenic fungi [21] . This drug was also recently shown to be able to prevent biofilm formation by *Staphylococcus spp.* when incorporated in polyurethanes [22, 23] or in bone cements [24]. Therefore, usnic acid is under investigation for the prevention of medical device-related infections as an antimicrobial agent to be adsorbed on the surface of medical devices in alternative to conventional antibiotics [25].

Besides antimicrobial activity, usnic acid also possesses anticancer properties. Its antiproliferative activity was first reported against lung carcinoma [26] and then demonstrated against a wide variety of human cancer cell lines [27, 28].

Despite these recognised features, usnic acid therapeutic application has not yet been introduced due to its low water solubility and high hepatotoxicity[29, 30]. Thus, its use in a safe and efficient manner requires the development of suitable dosage forms able to improve its therapeutic index.

Moreover, since one of the main issue related to the use of antimicrobial agents is the development of drug resistant bacteria, employing pAcDED-coated core-shell MNPs loaded with usnic acid could overcome this problem by exerting a synergistic effect in

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[28] Backorova M., Backor M., Mikes J., Jendzelovsky R., Fedorocko P., *Toxicol. In Vitro* **2011**, 25, 37-44.
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[30] Guo L., Shi Q., Fang J.L., Mei N., Ali A.A., Lewis S.M., Leakey J.E., Frankos V.H., *J. Environ. Sci. Health C. Environ. Carcinog. Ecotoxicol. Rev.* **2008**, 26, 317-338.

bacterial killing. Indeed, the most pursued strategy to reduce the drug resistance is the combined use of antimicrobial agents having different mechanisms of action. In our case, the positively charge polymer can adsorb on the negative charged bacterial membrane provoking cell disruption while usnic acid can inhibit bacterial metabolic functions.

The activity of usnic acid-loaded, pAcDED-coated MNPs was evaluated against *Staphyloccoccus epidermidis*. The results were compared to those obtained by the use of MNPs coated with a star branched hydrophobic polymer based on polycaprolactone [31].

3.2 Materials and Methods

3.2.1 Preparation of magnetic manganese ferrite nanoparticles ($MnFe_2O_4$)

Manganese ferrite nanoparticles were synthesized by coprecipitation of Fe^{3+} and Mn^{2+} from a water-in-toluene micro-emulsion system followed by thermal treatment as previously described [27]. Briefly, 12.5 mL of a 0.4 M sodium dodecyl benzene sulfonate (NaDBS, Aldrich) solution were added to 11.5 mL of a water solution containing 0.4 M $Fe(NO_3)_3$ (Fluka) and 0.2 M $Mn(NO_3)_2$ (Fluka). Later, excess toluene (1:20 water:toluene volume ratio) was added to the mixture and the resulting microemulsion was stabilized by stirring for 24 h. Finally, 20 mL of 1 M NaOH solution (in stoichiometric amount to obtain $MnFe_2O_4$) were added to the microemulsion under nitrogen flow to avoid Mn^{2+} oxidation. After 2 h stirring, the microsuspension was kept at 100 °C for 90 min under nitrogen flow (digestion process). Then, the precipitated powder was collected by centrifugation and repeatedly washed with a 1:1 water/ethanol

[31] Yanga J., Parkb S.B., Yoonc H.G., Huhd Y.M., Haama S., *International Journal of Pharmaceutics*, **2006**, 324, 185-190.

solution. The obtained dark brown powder was first dried at 40 °C and then submitted to a thermal treatment in oven at 600 °C. The employed procedure allowed the obtainment of magnetic nanoparticles ($MnFe_2O_4$) with a mean size of 6 nm and a saturation magnetization of 29 emu/g [32].

3.2.2 Polymer synthesis

To obtain magnetic nanoparticles with different surface properties, $MnFe_2O_4$ were coated with either a hydrophilic basic polyacrylamide (pAcDED) or a hydrophobic star-branched poly-ε-caprolactone (sbPCL).

The polyacrylamide pAcDED was synthesized as previously described [18]. The polymer was obtained by radical polymerization of the monomer AcDED obtained by reaction between acryloyl chloride (Ac, Sigma) and N,N-diethylethylendiamine (DED, Sigma), as described elsewhere. Briefly, for monomer synthesis DED (0.029 moles) was added to a solution of Ac (0.038 moles) in dimethylcarbonate (DMC, 75 ml) containing K_2HPO_4 (0.08 moles). The reaction was carried out for 4 h at room temperature. After reaction, the solution was filtered and AcDED recovered by solvent evaporation.

The polymerization was carried out at 25 °C for 24 h by using a 1 M water solution of AcDED and $K_2S_2O_8$ ($2.8x10^{-4}$ mmoles) plus $FeSO_4$ ($2.4x10^{-4}$ mmoles) as radical initiators. The polymer was purified by dialysis and recovered after lyophilization. The obtained polymer possessed M_w = 120000 g/mol [18].

The star-branched poly-ε-caprolactone was obtained by ring opening polymerization (ROP) of ε-caprolactone (CL, Sigma) by using tetraethylenepentamine (TEPA, Fluka)

[32] Bellusci M., Canepari S., Ennas G., La Barbera A. Padella F., Santini A., Scano A., Seralessandri L., Varsano F., *J. Am. Ceram. Soc.*, **2007**, 90, 3977-3983.

as a polyfunctional initiator (5 reactive functionality) and $Sn(Oct)_2$ as a catalyst (0.1% with respect to monomer). The length of polymer arms was varied by employing different [CL]/[TEPA] molar ratios ranging from 2 to 100. ROP reaction was carried out at 110 °C for 24 h, under stirring and nitrogen flow. Nitrogen flow was needed to have the anhydrous environment and avoid the water to act as an initiator of polymerization. Acronym of the synthesized polymers was $sbPCL_n$, where n the obtained arm length. In this work, only the most hydrophobic polymer ($sbPCL_{50}$) having longer arms with the same length was chosen.

3.2.3 MNPs coated with pAcDED

Nanoparticle coating with pAcDED was carried out by employing two methodologies: i) physical adsorption and ii) *in situ* microemulsion-polymerization and cross-linking.

As for the first method, nanoparticles (50 mg) were put in contact with a pAcDED water solution ($1x10^{-3}$ M) for 24 h under sonication. Then, the coated nanoparticles were recovered by centrifugation, repeatedly washed in water and dried. The nanocomposite was called pAcDED-$MnFe_2O_4$.

As for the second coating procedure, the monomer AcDED was first physically adsorbed onto the nanoparticle surface and then polymerized and cross-linked by N,N methylenebisacrylamide (Bis-A, Sigma). Particularly, 50 mg of nanoparticles were suspended by sonication in 1 mL of AcDED water solution (10 mg/mL) and kept in aqueous environment for 6 hr. This suspension was added to a solution of sorbitan monoleate (SPAN80 2 mg) in 40 ml of ciclohexane. Once reached a stable emulsion under stirring at 15000 rpm, Bis-A (1 mg) and $K_2S_2O_8$ (1 mg) were added. Polymerization and cross-linking was carried out at room temperature under stirring for

15 min, till disappearance of double bond absorbance in ATR-IR (Attenuated total reflection infrared spectroscopy) spectrum.

The coated nanoparticles were recovered by centrifugation and washed with water to eliminate the unreacted monomer and the not-physically adsorbed polymer. This nanocomposite containing pAcDED cross linked was called $pAcDED_{CL}$-$MnFe_2O_4$.

3.2.4 MNPs coated with sbPCL

The adsorption of the hydrophobic polymer $sbPCL_{50}$ on ferrite surface was carried out by suspending 50 mg of nanoparticles in THF and by adding a $1x10^{-3}$ M $sbPCL_{50}$ solution in THF. After 24 hr under sonication, the coated nanoparticles were recovered by centrifugation and submitted to repeated washings in THF and water to eliminate the unbound polymer. Finally, the $sbPCL_{50}$ coated nanoparticles were dried under vacuum at 40 °C. The nanocomposite was called $sbPCL_{50}$-$MnFe_2O_4$.

3.2.5 Characterization of the core-shell nanocomposites

Attenuated total reflection infrared spectroscopy (ATR-IR) was accomplished by using a Thermo Nicolet 6700 instrument equipped with a Golden Gate diamond single reflection device (Specac). Spectra were acquired with a resolution of 2 cm^{-1}, in the range 4000-650 cm^{-1}.

Thermogravimetric analysis was carried out employing a Mettler TG 50 thermobalance, provided with the same TC 10 A processor. The explored temperature range was 25 - 600 °C, and the heating rate 10 K/min, under N_2 flow.

Size distribution of nanoparticles was assessed by a Laser Diffraction Particle Size Analyzer (LS 13320, Beckman Coulter) while their morphology was investigated by scanning electron microscopy (SEM, Assing LEO1450VP).

3.2.6 Usnic acid loading and release

Due to the poor solubility of usnic acid (UA, 344.315 g/mol) in water, a sodium salt of the drug was prepared by adding NaOH (0.1 N) to a UA solution in a 3.5/1 NaOH/UA molar ratio.

For UA adsorption, either uncoated or coated MNPs were kept in contact for 24 h with a 0.015 M drug solution under moderate stirring. Later, UA not adsorbed to the nanoparticles was rinsed out with water, lefting it in contact with the nanoparticles for 2 h. The amount of adsorbed drug was determined as the difference of drug concentration in the solution before and after the adsorption plus washings. Drug amount in solution was determined by UV-Vis spectroscopy (290 nm). The kinetics of drug release was studied by keeping drug-loaded nanoparticles in water for increasing times. At defined times, aliquots of solution were collected and analyzed by UV-Vis spectroscopy.

3.2.7 Evaluation of antimicrobial activity of UA-loaded MNPs

For biological assay, *Staphylococcus epidermidis* ATCC 35984 was grown in Triptic Soy Agar (TSA) and Tryptic Soy Broth (TSB). The antimicrobial activity of nanocomposites was assessed by the Kirby Bauer test. MH agar plates were seeded with 10^8 CFU/ml of *S. epidermidis*, cellulose discs were loaded with different concentrations

of various drug loaded MNPs and then placed in these Petri plates. Following incubation at 37 °C for 24 h, the diameters of inhibition zones of bacterial growth around the discs were measured. All the experiments were done in triplicate. Analysis of variance was performed using MiniTab. Differences were considered significant for $P < 0.05$.

3.3 Results and Discussion

The coating of MNPs with a polymer shell does not only decrease MNPs aggregation and flocculation, but can be also exploited for functionalization with specific components, such as other functional groups, specific binding sites, or various drugs [33]. To limit MNPs interaction with host-immune defences, different surface engineering have been proposed. Particularly, surface modification with hydrophilic molecules such as polyethylene glycol (PEG) [34], hyaluronic acid, polyvinyl alchool[35], polyethyleneimine [36], dextran[37] and chitosan[38] have been shown to reduce MNPs opsonization through steric repulsion, thus prolonging circulation time.

Hydrophobic/hydrophilic interactions have been proved to be useful when adsorbing hydrophobic drugs onto MNPs. For this application, MNPs have been engineered with hydrophobic layers, such as oleic acid[39], to adsorb hydrophobic drugs [39,40]. This strategy

[33] Veiseh O., Gunn J.W., Zhang M., *Adv. Drug Deliv. Rev.*, **2010**, 62, 284-304.
[34] Harris J.M., Chess R.B., *Nature Rev.Drug Dis.*, **2003**, 2, 214–221.
[35] Indira T.K., Lakshmi P.K., *Int. J. Pharma. Nano.*, **2010**, 3, 1035-1042
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[40] Yu M.K., Jeong Y.Y., Park J., Park S., Kim J.W., Min J.J., Kim K., Jon S., *Angew. Chem. Int. Edition*, **2008**, 47, 5362–5365.

has some drawbacks such as MNPs sensitivity to environmental conditions and low control over molecular orientation of bound ligands.

In this study, $MnFe_2O_4$ were coated with either a hydrophilic basic polyacrylamide (pAcDED) or a hydrophobic star-branched poly-ε-caprolactone ($sbPCL_{50}$).

Poly-ε-caprolactone was chosen because it has good characteristics of biocompatibility and high thermal stability and is widely used in biomedical applications [41, 42]. Additionally, the use of a hydrophobic polymer such as the PCL for covering MNPs could ensure a good affinity of UA for the nanostructured system, due to high drug hydrophobicity.

A star branched PCL was obtained by ROP of of ε-caprolactone by using TEPA as polyfunctional initiator. Among the different experimented [CL]/[TEPA] molar ratios, the [CL]/[TEPA]=50 allowed obtaining a polymer with 5 arms and an experimental number-average degree of polymerization ($DP_{n,exp}$) of 50 each. The use of lower molar ratios resulted in polymers with lower degree of branching (data not shown). Instead, when higher molar ratios were employed, a decrease in the $DP_{n,exp}$ was evidenced presumably due to possible degradation phenomena occurring during the synthesis (data not shown).

The [CL]/[TEPA]=50 molar ratio permitted the obtainment of the best fitting between the experimental number-average molecular weight, determined by ^{1}H-NMR ($M_{n\text{-}NMR}$=27.600 Da), and the theoretical one calculated from the used [CL]/[TEPA] molar ratio ($M_{n\text{- theoretical}}$=28.700 Da). The occurred reaction was also confirmed by ATR-IR analysis. In addition, thermal analysis performed by differential scanning calorimetry

[41] Hutmacher D., Hürzeler M.B., Schliephake H., *Int. J. Oral. Maxillofac. Implants*, **1996**, 11, 667-678.
[42] Dash T.K., Konkimalla V.B., *J. Control. Rel.*, **2012**, 158, 15-33.

evidenced the semicrystalline features of $sbPCL_{50}$. In particular, a melting peak with a temperature of 54°C was observed (thermogram not shown).

pAcDED-coating was chosen both for its hydrophilicity and intrinsic antimicrobial activity displayed against *S. epidermidis*. In fact in a previously work [18], a MIC value of 100 µg/ml was found for pAcDED against this strain. Furthermore in the same work, it was highlighted as pAcDED was able to complex usnic acid by establishing polymer-drug multipoint interactions thus promoting drug solubilization in water and increasing antimicrobial activity.

To evaluate the success of MNPs coating, ATR-IR analysis was used. As can be observed in ***Figure 1***, the uncoated magnetic nanoparticles show a strong peak in the range 850 cm^{-1} and 1000 cm^{-1} attributed to the metal-OH bond of the iron and manganese oxide skeleton, a peak between 1340 and 1650 cm^{-1} attributed to the H-O-H bending of the water adsorbed or entrapped in the structure, and a wide band between 2500 and 3650 cm^{-1} attributed to the O-H stretching.

The $sbPCL_{50}$-coated MNPs spectrum showed, besides the ferrite signals, also the signals related to the polymer. Particularly, it can be observed the absorbance related to the stretching of polymer C=O groups (1720 cm^{-1}) and the stretching (2800-3000 cm^{-1}) and bending (1450-1100 cm^{-1}) of the polymer CH_2 groups (***Figure 1A***).

ATR-IR spectrum of the pAcDED-$MnFe_2O_4$ sample displayed the peak relative to the stretching of the amide C=O at 1650 cm^{-1}, the absorption at 1550 cm^{-1} related to the stretching and bending of amide C-N, and the peak at 1100-1450 cm^{-1}, related to the bending of the CH_2 (***Figure 1B***).

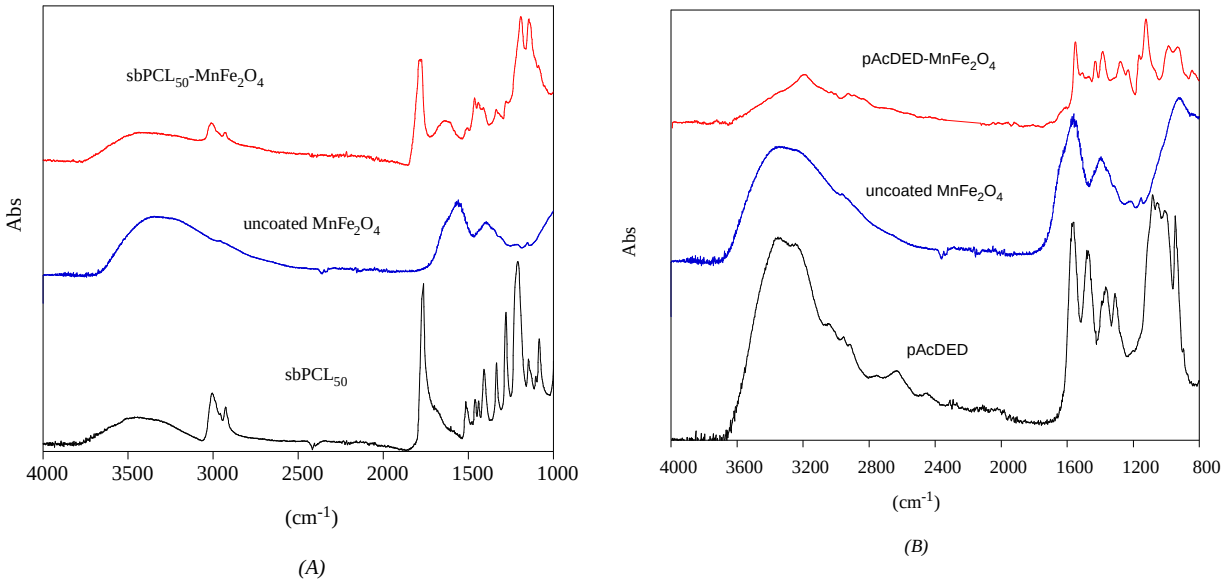

Figure 1. *ATR-IR spectra of uncoated $MnFe_2O_4$ nanoparticles, $sbPCL_{50}$ polymer and $sbPCL_{50}$-$MnFe_2O_4$ nanocomposite (A); uncoated $MnFe_2O_4$ nanoparticles, pAcDED polymer and pAcDED-$MnFe_2O_4$ nanocomposite (B).*

SEM analysis showed that MNPs were spherical, with a narrow size distribution and a average size of about 30 nm, but arranged in aggregates (***Figure 2A***). SEM micrographs of nanocomposites (***Figure 2B*** and ***C*** made at 15k magnification) showed that the polymer coating increased the size of the nanoparticles up to about 50-60 nm. Also, it could be observed that the coated-nanoparticles appeared more separated than the ferrite alone [43]. This latter finding confirms the ability of positively charged polymers to de-aggregate MNPs already reported in the literature for polyetylenimmine (PEI) that, when used for MNPs coating, was able to de-aggregate MNPs without re-aggregation in any pH conditions [43]. The further advantage of using positively charged polymers for MNPs coating is that the net negative charge of MNPs can be neutralized thus permitting the loading of negative molecules, such as DNA and proteins.

[43] Wang X., Zhou L., Ma Y., Li X., Gu H., *Nano Res.*, **2009**, 2, 365-372.

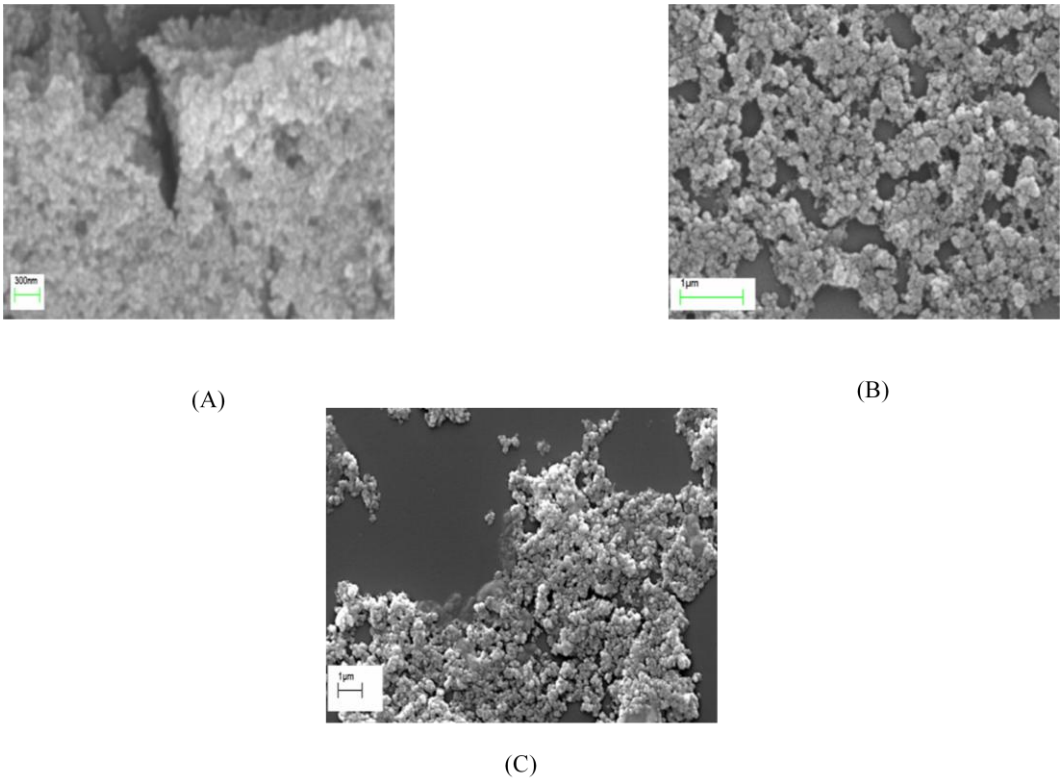

Figure 2. *SEM micrographs of uncoated MNPs (A), sbPCL$_{50}$-MnFe$_2$O$_4$ (B) and pAcDED-MnFe$_2$O$_4$ (C).*

In order to evaluate the coating content, Thermogravimetric Analysis (TGA) and Derivative Thermogravimetric Analysis (DTGA) were used, given that these techniques have been reported to be suitable for assessing presence of polymer coating [44, 45]. In Figure 3 the TGA and DTGA curves of uncoated $MnFe_2O_4$ are reported. As it can be observed, the uncoated magnetic nanoparticles present a slight loss (10%) between 25 ° and 250 ° C corresponding to water either weakly adsorbed or strongly linked to oxide structure. This loss in weight is gradual and continues up to 600 ° C (***Figure 3***).

[44] Osuna Y., Jauregui K.M.G., Gaona-Lozano J.G., de la Garza-Rodríguez I.M., Ilyna A., Díaz Barriga-Castro E., Saade H., López R.G., *Journal of Nanomaterials*, **2012**, 2012, 1-7.
[45] Juríková A., Csach K., Mikuf J., Koneracká M., Záviová V., Kubovíková M., Kopanský P., *ACTA PHYS. POL. A*, **2012**, 121, 1296-1299.

As for the nanocomposite sbPCL$_{50}$-MnFe$_2$O$_4$ (***Figure 4***), the loss in weight in the temperature range of 30-110 ° C (about 13%) was attributed to weakly bound water, while the second gradual loss in the range of 150-300 ° C to polymer degradation. This latter loss in weight was of about 20 %.

Also for the pAcDED-MnFe$_2$O$_4$ sample (***Figure 5***), having the first weight loss related to bound water (about 13%) at about 100 °C, a more complex polymer degradation between 130 ° C to 550 ° C corresponding to about 20% was observed. The nanocomposite with cross linked pAcDED (pAcDED$_{CL}$-MnFe$_2$O$_4$) showed the same percentage losses of the pAcDED-MnFe$_2$O$_4$ (thermogram not shown).

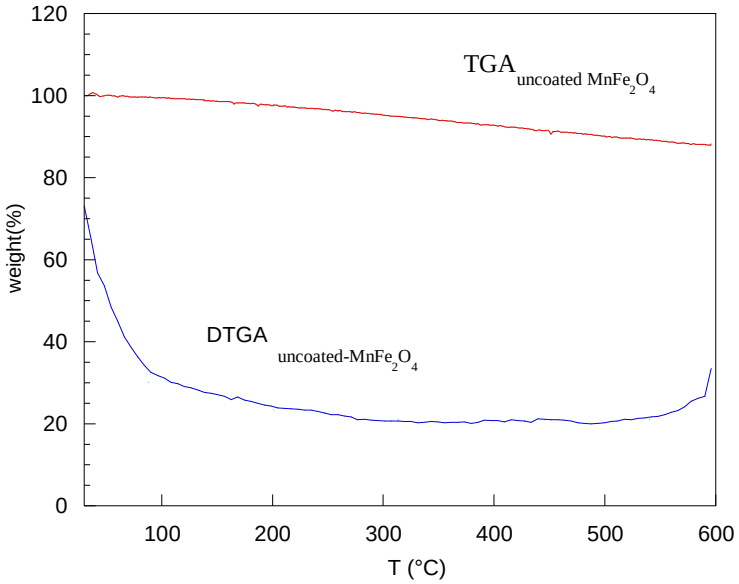

Figure 3*. Uncoated MnFe$_2$O$_4$ TGA and DTGA curves.*

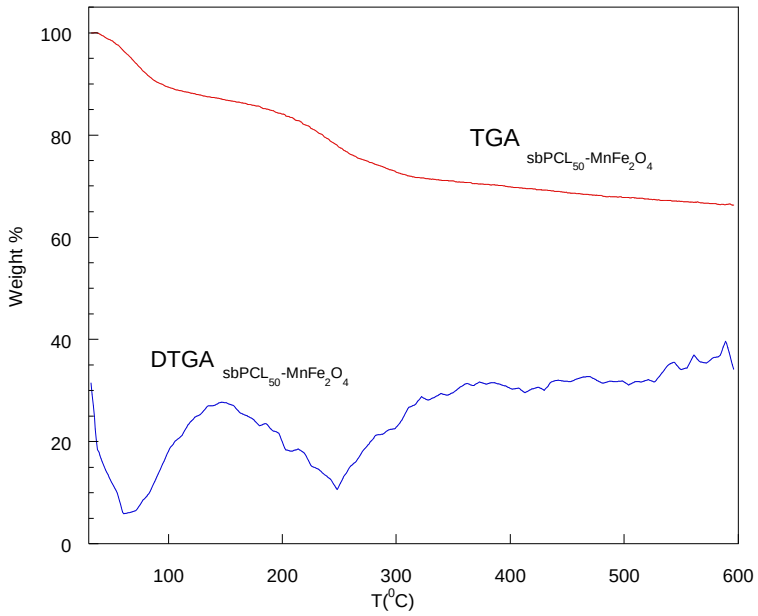

Figure 4. *$sbPCL_{50}$-$MnFe_2O_4$ TGA and DTGA curves.*

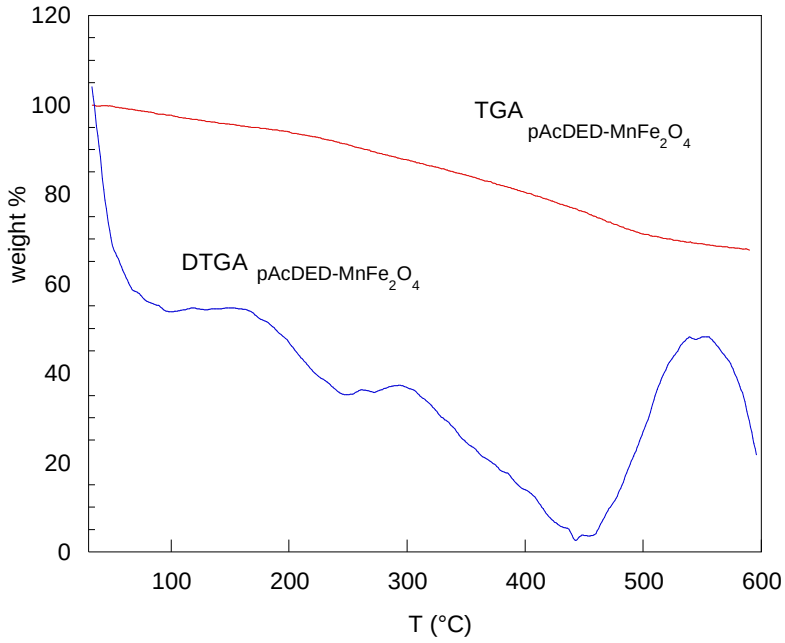

Figure 5. *pAcDED-$MnFe_2O_4$ TGA and DTGA curves.*

After physical characterization, the MNPs were subjected to drug adsorption. Generally, drug stability in vivo as well as adsorption by target tissues can be greatly improved by the use of targeted-release systems. In fact, these delivery systems can permit efficient pharmacokinetics and offer protection for easily degradable molecules, such as

peptides, proteins and nucleic acids that usually have short in vivo half-lives [46]. In addition, an appropriate nanoparticle coating can permit a higher drug loading with respect to uncoated nanoparticles and a suitable drug release. In particular, hydrophilic coatings can enhance drug loading thanks to their good swelling in water and ability to establish hydrogen bonds with drugs [47]. Moreover, by introducing a polymer shell with functional groups, such as amino groups, it is possible to conjugate targeting ligands thus providing a controlled and persistent release [46].

Sample	**(mg_{UA}/mg_{MNPs})**
uncoated-$MnFe_2O_4$	0.22± 0.04
sbPCL_{50}- $MnFe_2O_4$	0.49 ± 0.07
p$AcDED_{CL}$- $MnFe_2O_4$	0.48 ± 0.05
pAcDED- $MnFe_2O_4$	0.62 ± 0.02

Table 1. *Usnic acid loaded amount.*

Usnic acid was loaded on both the uncoated and coated MNPs. Usnic acid loaded amount was expressed as mg_{drug} / mg_{MNPs} ratio. The values are the result of arithmetic averages performed on at least four loading experiments.

As can be observed from data reported in ***Table 1***, uncoated MNPs absorbed a small amount of drug compared to coated MNPs. Particularly, pAcDED-$MnFe_2O_4$ sample

[46] Chronopoulou L., Massimi M., Giardi M.F., Cametti C., Devirgiliis L.C., Dentini M., Palocci C., *Colloids Surf. B*, **2013**, 103, 310-317.
[47] Francolini I., Palombo M., Casini G., D'Ilario L., Martinelli A., Rinaldelli V., Piozzi A., *J. Control. Release*, **2012**, 148, e57- e59.

showed greater loading of drug. In this case, probably, the drug can also penetrate from the surface up to the inner layers due to the significant swelling of the polymer. This hypothesis can be confirmed by comparing the data of pAcDED-$MnFe_2O_4$ which those of $pAcDED_{CL}$-$MnFe_2O_4$. The less amount of drug adsorbed by the latter system is probably due to the formation of a more compact structure that prevented drug diffusion into the underlying polymer layers thus allowing a more superficial drug adsorption (as shown by TGA data the amount of polymer in the two nanocomposites was the same).

As for $sbPCL_{50}$-$MnFe_2O_4$, the discrete absorbed drug amount can be ascribed to the high drug affinity for this hydrophobic material.

In general, drugs to perform their action must be released into biological fluids. In our case such release is ensured by the lack of a covalent bond between the nanocomposite and the sodium usniate.

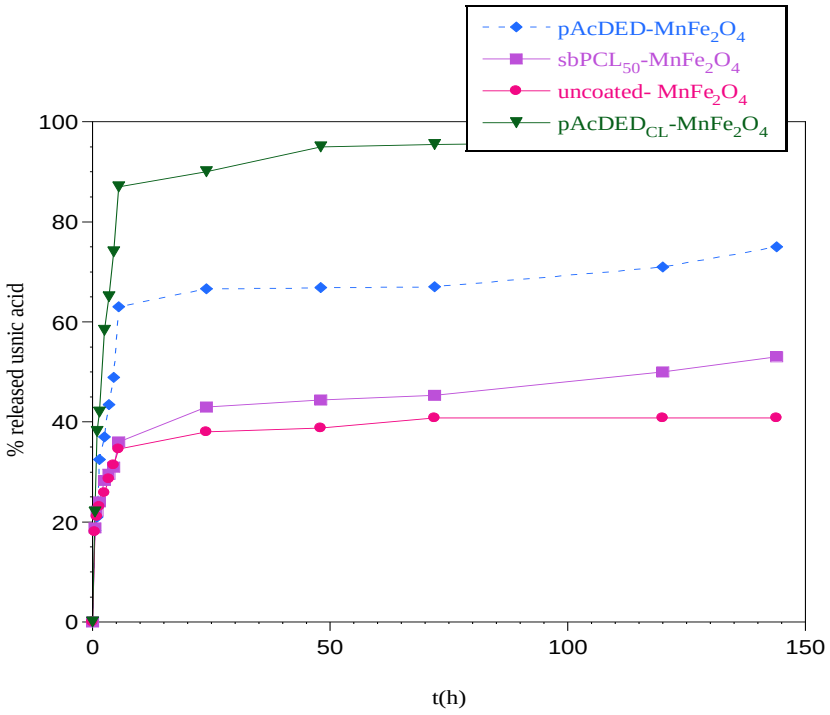

***Figure 6**. Usnic acid release kinetics of each nanostructured sample.*

From the kinetics (***Figure 6***), it can be observed that the nanocomposite pAcDED$_{CL}$-$MnFe_2O_4$ released more than 90% of drug in 24 h as a demonstration of a drug absorption on the surface polymer layers.

In the case of pAcDED-$MnFe_2O_4$, the lower quantity of released drug in 24 h (about 65%) is probably due to a diffusion controlled release exerted by acid-base interactions established between the tertiary amine groups of the polymer and the acidic OH groups of the drug. Among the nanocomposites, sbPCL$_{50}$-$MnFe_2O_4$ showed the minor release probably due to the poor hydrophilicity of the polymer matrix. However, all systems reached the plateau in 24 h.

To confirm that the interaction forces between nanocomposites and usnic acid controlled the drug diffusion and release, a defined amount of nanoparticles were embedded on cellulose disks in order to have an equal content of usnic acid (1 mg) for all systems. Such systems were then subjected to Kirby-Bauer assay. In this test, the size of inhibition zone is due to the diffusion of drug released amount. This amount depends on drug-nanocomposite interactions.

Sample	Inhibition zone (mm)
uncoated- $MnFe_2O_4$	22 ± 3
sbPCL -$MnFe_2O_4$	18 ± 3
pAcDED$_{CL}$-$MnFe_2O_4$	11 ± 4
pAcDED-$MnFe_2O_4$	6 ± 2

Table 2. *Inhibition zones of uncoated and coated MNPs before release kinetics.*

In ***Table 2***, the inhibition zones of nanocomposites and uncoated-$MnFe_2O_4$ are reported. The high zones of inhibition observed for MNPs and sbPCL_{50}-$MnFe_2O_4$ indicated a poor interaction between the matrices and the drug. On the contrary, the lower inhibition zones observed for the two nanocomposites based on pAcDED demonstrated strong ionic interactions between UA acid groups and polymer tertiary amine groups. In addition, the higher inhibition zone measured around p$AcDED_{CL}$-$MnFe_2O_4$ confirmed the hypothesis of drug adsorption only on surface polymer layers, then more superficial with respect to the pAcDED-$MnFe_2O_4$.

To verify the potential use of these systems *in vivo*, it must be considered that when drug-loaded ferrofluids are administered in patients, in order to allow nanoparticles achieving the target site a magnetic field is externally applied for a sufficiently long period of time, generally ranging from 30 to 60 min [48]. Once the drug is released, nanoparticles are degraded and excreted by the body. At this aim, evaluations of the drug released from the systems at 30 and 60 minutes were performed (***Table 3***).

[48] Alexiou C., Schmid R.J., Jurgons R., Kremer M., Wanner G., Bergemann C., Huenges V, Nawroth T., Arnold W., Parak F.G., *Eur. Biophys J.* , **2006**, 35, 446–450.

Sample	mg_{UA}/mg_{MNPs} 30 min	mg_{UA}/mg_{MNPs} 60 min
Uncoated- $MnFe_2O_4$	0.04 ± 0.01	0.05 ± 0.005
sbPCL - $MnFe_2O_4$	0.09 ± 0.01	0.12 ± 0.04
$pAcDED_{CL}$-$MnFe_2O_4$	0.11 ± 0.04	0.21 ± 0.02
pAcDED-$MnFe_2O_4$	0.11 ± 0.03	0.14 ± 0.02

Table 3. *Drug release after 30 and 60 minutes.*

As it can be observed the nanocomposites based on pAcDED released drug amounts higher than uncoated and $sbPCL_{50}$-coated MNPs. In addition, since at 60 min $pAcDED_{CL}$-$MnFe_2O_4$ released the highest drug quantity (0.21 ± 0.07 mg_{UA}/mg_{MNPs}), this system seemed to be the most suitable for drug targeting.

In particular, these drug-loaded MNPs could be used either for the prevention or for the treatment of medical device-related infections. In fact, they could be injected in the patient soon after device implantation, to prevent biofilm formation, or, later, in presence of signs of infection, to kill the biofilm grown on the device surfaces.

The significant drug amount released in 60 minutes by $pAcDED_{CL}$-$MnFe_2O_4$ is encouraging for a successful biofilm eradication. In fact, the treatment protocol adopted in clinical studies for cancer patients [49, 50, 51, 52] consists in the injection of about 50 ml of ferrofluid (0.5-1% total blood volume), at a concentration of 10-20 mg/ml, containing 1-3 mg of drug / kg of patient. If we hypothesize a similar treatment protocol for patients

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suffering from a localized infection caused by staphylococci, an extremely high dose of antimicrobial agent, approximately 1000 times higher than the usnic acid MIC (16 μg/ml), can be concentrated in the infected area surrounding the medical device. This opens interesting perspective for a potential future *in vivo* application of the developed UA-loaded $pAcDED_{CL}$-$MnFe_2O_4$ system.

3.4 Conclusions

In order to increase MNPs biocompatibility and circulation time in blood as well as to provide them with functional groups able to interact with biologically active substances, manganese ferrites were coated with hydrophilic and hydrophobic polymers. The hydrophilic and water-soluble polymer pAcDED, bearing tertiary amino groups, should have allowed nanostructured systems exerting antimicrobial action and binding an antimicrobial drug by ionic interactions (usnic acid). The combination of two antimicrobial agents should contribute to prevent the development of drug resistant bacteria. MNPs coating with a novel star-branched polymer based on poly-ε-caprolactone should have only increased UA loading thanks to its hydrophobic features.
To demonstrate that nanocomposites could be potentially used to target a drug at a specific body site, usnic acid was adsorbed on the surface of uncoated and coated MNPs. The amount of drug adsorbed on nanocomposite was higher than uncoated MNPs. This phenomenon was accentuated in presence of hydrophilic matrices. The hydrophilic features also affected the release kinetics of nanostructured systems. Indeed, MNPs coating with polymers based on pAcDED, either crosslinked or not, besides increasing the amount of released drug, controlled drug release by establishing strong

ionic interactions between UA acid groups and polymer tertiary amine groups, as evidenced by Kirby-Bauer assay. In addition, findings regarding the UA release indicated that pAcDED$_{CL}$-$MnFe_2O_4$ could be the most suitable system for drug targeting thanks to the greater presence of the drug on the polymer surface.

CHAPTER IV

SYNTHESIS OF AN ACRYLIC-BASED POLYESTER BEARING CATECHOLIC MOIETIES WITH ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES.

4.1. Introduction

Implanted biomedical devices are of increasing importance in modern medical care. Different materials, generally inert and non-toxic, including metals, ceramics and polymers are used for manufacturing of these devices. However, since the biological response to material is related to its surface features, to avoid the device failure the chemical, physical, and morphological properties of biomaterial must be carefully considered [1, 2, 3] .

The foreign body reaction includes a cascade of cellular events such as protein adsorption, adhesion and activation of immune cells, fibrosis and infection[4]. In particular, material-mediated inflammatory responses induce the production of reactive oxygen species (ROS) which besides contribute to oxidative degradation of material itself [5].

ROS including hydroperoxide, hypochloride, hydrogen peroxide and hydroxyl anions, are released by activated phagocytes together with other molecules having killing and degradative activities [6].

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The inflammatory reaction is beneficial if its effects are limited to the pathogen agents. The insufficiency of a component of the inflammatory reaction such as the production of ROS leads indeed to bacterial infections. On the other hand, an overproduction of ROS is deleterious because it leads to oxidative stress (imbalance of oxidants and antioxidants). High levels of free radical species can damage lipids, proteins, enzymes, carbohydrates, and DNA in cells and tissues. These damages are involved in many pathological conditions such as cancer, diabetes, cardiovascular and autoimmune diseases, and neurodegenerative disorders such as Alzheimer's disease [7, 8, 9, 10] .

To slow or inhibit the process of oxidative degradation, antioxidant substances such as superoxide dismutase, catalases, ubiquinol and glutathione are synthesized naturally in biological systems [11].

Natural or synthetic polymers are better candidates for the development of medical devices thanks to their physico-chemical properties that allow to fulfill different applications.

It is well known that polymers become more resistant to photo-oxidation reactions if small amounts of antioxidants are added. Taking this as a cue, many studies have shown that oxidative stress induced by body response to polymer device can be slowed down or suppressed by conjugation of antioxidant molecules to polymer matrix [12, 13, 14, 15, 16, 17]

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Among antioxidants, cathecolic compounds have gained increasing research attention due to their ortho-diphenolic moiety that extends their biological activity from free radical scavangers to metal chelators. In this regard, hydroxytyrosol (HTy), the main natural polyphenolic compound occurring in virgin olive oil and in olive oil wastewaters, possesses important pharmacological potential related to its antioxidant, anti-inflammatory and antiplatelet aggregation activity. In addition, hydroxytyrosol and its derivates possess a strong chelating ability of metals and a broad-spectrum antimicrobial activity against a series of microorganisms including *Staphylococcus epidermidis* and *aureus*, *Vibrio cholerae* and *Salmonella* species [18].

The chelating properties of catechols towards some metals such as iron (III), cadmium (II), copper (II) with high complexation constants are well known [19]. A line of research in the environmental field concerning adsorption of metals by catechol has been developed for a long time [20,21]. In addition, also polymer materials have been provided with a side chain containing the catechol group to be used as resins [22].

The ability to form complexes with metals can be exploited in chelation therapy. An example is the study of L-DOPA complexes with some metals [23]. Chelation therapy could be a promising therapeutic approach since metals are considered to be a pharmacological target for development of new therapeutic agents for neurodegeneration or cancer treatment.

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[16] Wattamwar P.P., Biswal D., Cochran D.B., Lyvers A.C., Eitel R.E., Anderson K.W., Hilt J.Z., *Acta Biomater*, **2012**, 8, 2529-2537

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Indeed, while many neurodegenerative diseases as well as multiple sclerosis are associated with iron elevated levels, cancer patients show significantly high copper levels. Copper during the process of metal cellular uptake, being a potent oxidant, is reduced from Cu (II) to Cu (I).

In this work, to develop a multi-function polymer endowed with antioxidant and antimicrobial activity a novel hydroxytyrosol-based polyacrylate (pAcHTy) was synthesized. After physical characterization, the antimicrobial and antioxidant activities of pAcHTy as well as its chelanting ability for copper ions were evaluated.

The scavenging and antibacterial properties tested toward 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *Staphylococcus epidermidis* respectively, have demonstrated that the pAcHTy polymer can be employed in the field of medical devices as biomaterial able not only to suppress the oxidative stress but also to prevent infections frequently associated to the implant of medical devices. In addition, the high chelating capacity for copper ions shown by this polymer suggests a possible use of pAcHTy in chelating therapy.

4.2. Materials and Methods

4.2.1 Materials

Sodium metabisulphite was purchased from Carlo Erba. Tyrosol (2-(4-Hydroxyphenyl)ethanol, Ty) was purchased from Wako Chemicals GmbH. Acrylic acid (AA), hydroquinone, potassium persulfate, sodium metabisulphite, *p*-toluensyulfonic acid (pTSOH), sodium dithionite, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich as were all of other solvents and reagents. All chemicals

were of analytical grade and used as received. 2-iodoxybenzoic acid (IBX) was prepared in the laboratory as described in the literature [24]. 2-(3′,4′-dihydroxyphenyl)ethanol (Hydroxytyrosol, HTy) was prepared in the laboratory as described in the literature [25]. *Staphylococcus epidermidis*, strain ATCC 35984, and Muller Hinton broth (MH, Oxoid) were employed for microbiologic tests. The methanol used for the antioxidant activity and tetrahydrofuran for the GPC analysis were of HPLC grade from Sigma Aldrich.

4.2.2 Synthesis of poly 2-(3,4-hydroxyphenyl)ethyl acrylate

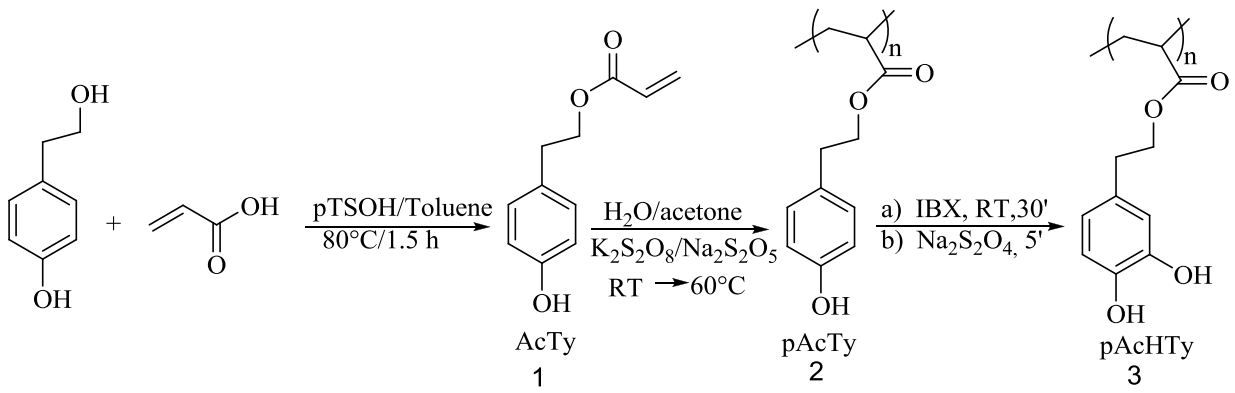
The procedure to obtain the hydroxytyrosol-containing polymer is reported in the scheme 1. The first step consisted in the Fischer esterification of acrylic acid with Tyrosol (Ty) to obtain 2-(4-hydroxyphenyl)ethyl acrylate (AcTy) (product 1 in the ***Scheme 1***).

Particularly, Ty (0.1 mol) was dissolved in 75 ml of toluene in presence of 20 mg of hydroquinone and 750 mg of *p*-toluensulfonic acid (pTSOH). Then, acrylic acid (0.12 mol) was added and the mixture was refluxed for 1.5 h in a flask equipped with Dean-Stark apparatus. At the end of the reaction, the mixture was cooled and treated with sodium bicarbonate (6 g) and water (1.5 ml) until carbon dioxide evolution ended. The mixture was then dried with sodium sulfate anhydrous, filtered and toluene was removed under vacuum.

[24] Frigerio M., Santagostino M., Sputore S., *J. Org. Chem.*, **1999**, 64, 4537–4538.

[25] Applicant: Università degli Studi della Tuscia. Inventors/Applicant: Bernini R., Inventors: Mincione E., Barontini M., Crisante F. "Method for preparing hydroxytyrosol and hydroxytyrosol derivatives" Number: PCT/IB2008/000598. International Pubblication Number: WO2008/110908 A1, **2008**. b) *J. Agric. Food Chem.* **2008,** *56,* 8897–8904

In the second step, the monomer AcTy (1 mmol) was dissolved in a mixture water/acetone (1:1 w/w) and submitted to radical polymerization by employing potassium persulfate (0.03 mmol) and sodium disulfite (0.1 mmol). After stirring for 50 min at room temperature, acetone was distilled off by heating at 60 °C. The reaction mixture was kept at 80 °C for 2 hr. At the end of reaction, a stable emulsion of nanoparticles of tyrosol-containing polymer (pAcTy) was obtained (product 2 in the ***Scheme 1***). This emulsion was then one-pot oxidized with 2-iodoxybenzoic acid (IBX, 1.2 mmol) and then reduced by sodium dithionite (2 mmol) in order to obtain nanoparticles of the hydroxytyrosol-containing polymer (pAcHTy) (product 3 in the ***Scheme 1***). After centrifugation at 3500 rpm for 15 min, water was eliminated and the polymer was recovered and washed many times with deionized water to remove sulfur by-products of the reduction reaction. Finally, pAcHTy nanoparticles were dried in vacuum oven at 25 °C for 1 day.



Scheme 1*: pAcHTy synthesis.*

While the pAcTy polymer was soluble in some organic solvent such as tetrahydrophuran, chloroform and toluene, the pAcHTy polymer was totally insoluble in both water and normal organic solvent.

4.2.3 Characterization of pAcTy and pAcHTy polymers

Size distribution of pAcTy and pAcHTy nanoparticles was assessed by a Laser Diffraction Particle Size Analyzer (LS 13320, Beckman Coulter), while their morphology was studied by scanning electron microscopy (SEM, LEO1450VP, Assing). For SEM observations, pAcHTy films were prepared by casting a THF polymer suspension on Teflon plates. Following solvent evaporation under vacuum at 30 °C, round shaped discs were obtained.

4.2.4 Evaluation of pAcHTy oxidation percentage

To determine the pAcHTy oxidation percentage, 200 mg of polymer were placed in contact with 10 ml of 1M NaOH at room temperature for 48 hours. Later, the solution was acidified with 1M HCl. The aqueous solution was extracted three times with ethyl acetate and the organic phase was washed with saturated NaCl solution and subsequently with distilled water. The crude product was recovered and then analyzed by ^{1}H-NMR.

4.2.5 NMR spectroscopy and elemental analysis

^{1}H-NMR spectra were performed employing a Varian XL 300 instrument and chloroform-d as solvent. All chemical shifts are expressed in ppm (δ scale). Elemental

analysis of the synthesized polymers was carried out by Carlo Erba EA 1110 instrument and data were reported as C, H, N, S percentages.

Attenuated Total Reflection spectra (ATR) were acquired by employing a Nicolet 6700 FT-IR spectrometer and a Golden Gate diamond single reflection device (Specac). The spectra were obtained by co-adding 200 interferograms in the range 4000–650 cm^{-1} at a resolution of 4 cm^{-1}.

4.2.6 Thermal analysis and GPC measurement

Differential scanning calometry (DSC) analysis was performed from –100 to 200 °C under N_2 flux using a Mettler TA-3000 DSC apparatus. The scan rate used for the experiments was 10 °C/min and the sample weight about 6-7 mg. Thermogravimetric analysis (TGA) was carried out employing a Mettler TG 50 thermobalance, at the heating rate of 10 °C/min, under N_2 flow and in temperature range 30 - 500 °C.

GPC measurements were carried out at 30 °C using a 150-C Waters GPC apparatus equipped with a differential refractive index detector. The calibration curve was obtained by using 9 standard monodisperse polystyrene samples of known molecular weight ranging from $1.3x10^3$ to $1.5x10^6$ g/mol. Two crosslinked polystyrene (PS) columns (Water Ultrastyragel) which were able to separate in the range $2x10^3$–$1x10^6$ g/mol (linear) and 200–$3x10^4$ g/mol were used. The time required for each analysis was 30 min with a mobile phase flux of 1mL/ min. The transport solvent was HPLC grade chloroform (Fluka).

4.2.7 Evaluation of antimicrobial activity of monomer and polymers

The antimicrobial activity of the acrylic monomer AcTy and pAcTy was assessed by a disk diffusion test by impregnating cellulose disks with compound solutions (1 mg/ml). MH agar plates were seeded with 10^8 CFU/ml of *S. epidermidis* (ATCC 35984) and cellulose disks were placed in these Petri plates. Following incubation at 37 °C for 24 h, the diameters of inhibition zones of bacterial growth around the discs were measured.

Antimicrobial activity of the pAcTy and insoluble polymer pAcHTy was also estimated by turbidimetric analysis. Briefly, various amount of polymer (5, 10 and 15 mg) were placed into test tubes containing 2 ml of *S. epidermidis* suspension having an optical density of 0.1-0.2 at 600 nm. A tube containing the bacterial suspension without polymer was used as control. Control and test tubes were then incubated overnight at 37 °C. Since the amount of adsorbed light increases with an increase in cell population, the antimicrobial activity of the polymers was correlated to control tube. Analysis was repeated until absorbance reached that of the control tube.

The evaluation of pAcHTy ability to control bacterial adhesion was also evaluated by scanning electron microscopy (SEM). Particularly, pAcHTy films, prepared by casting method, were put in contact with a bacterial suspension (optical density 0.1-0.2 at 600 nm) and incubated overnight. Following incubation, films were washed twice with PBS (pH=7.4), fixed with 2.5% glutaraldehyde in 0.1 M PBS buffer (pH 7.4) at room temperature for 30 min, dehydrated by dipping with different concentrations of ethanol (30, 50, 70, 90 %) for 5 min and with hexamethyl disilazano for 30 seconds. For SEM observations, samples were gold coated by sputtering.

4.2.8 Antioxidant activity

The antioxidant activity of hydroxytyrosol and polymer pAcHTy was determined by using DPPH as free anionic radical [26]. For each antioxidant compound, different concentrations were tested (expressed as the number of moles of antioxidant/mole DPPH).

As for hydroxytyrosol, antioxidant solution in methanol (4 mL) was added to 10 mL of a 1.5×10^{-4} mol/L methanol DPPH solution. The decrease in absorbance was determined at room temperature at 520 nm after 30 min, time in which a plateau was reached. The amount of DPPH was evaluated from a calibration curve (absorbance vs. concentration). Then, for each concentration tested, the residual DPPH as a function of antioxidant agent/DPPH molar ratio was plotted. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (Efficient Concentration = EC_{50}, moles of antioxidant/mole DPPH). EC_{50} values were extrapolated from the graph.

As for the pAcHTy antioxidant activity, due to polymer insolubility, measurements were performed by using the DPPH method with some modifications [27].

Different amount of the powdered polymer were transferred into a vial, and the reaction was started by adding 60 mL of methanol and 20 mL of methanol DPPH solution (0.5 M). The vials was stirred for 30 min to facilitate the surface reaction between the insoluble polymer and the DPPH reagent. Following centrifugation at 3500 g for 2 min, the absorbance of the optically clear supernatant was measured at 520 nm.

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4.2.9 Copper adsorption kinetics

The chelanting activity of pAcHty was evaluated versus copper ions. Particularly, copper adsorption kinetics were followed in batch at 20 °C by using 20 ml of a copper solution at a 350 ppm concentration containing 30 mg of pAcHty. To study the influence of pH and temperature on copper adsorption, experiments were performed at different pH and T, specifically in the range 2 and 8 for pH and in the range 20 and 50 °C for temperature.

During test, solutions were kept in continuous magnetic stirring. At regular time intervals, 100 µl of solution were sampled, stored in a refrigerator at 5 °C and subsequently analyzed by Atomic Absorption Spectrophotometry (AAS) by a Shimadzu atomic absorption spectrophotometer AA-6300, with flame dual beam optical and range of wavelengths between 185 and 900 nm.

For analysis of solutions a flame continuous method was used. The combustion air/fuel mixture consisted of air and acetylene. Different standard solutions of copper were used for calibration.

The amount of metal ion adsorbed was determined by the equation:

$$\frac{mg_{(Me)}}{g_{(resina)}} = \frac{(C_0 - C)}{C_0} \frac{Vol\ (ml)}{peso\ (g)}$$

while the efficiency of the treatment was assessed by the percentage removal:

$$\%\ R = \frac{(C_0 - C)}{C_0}\ 100$$

Where C_0 is the initial concentration of the solution and C the concentration of the solution at the different sampling times.

To evidence the affinity of copper towards pAcHTy polymer, after the adsorption cycle a desorption cycle was performed by putting in contact the copper ion-containing polymer with 20 ml of 0.1 HCl for 3 hr under stirring. Later, the supernatant was collected and 100 µl were taken and analyzed by AAS.

Analysis of variance comparisons were performed using MiniTab. Differences were considered significant for *P* values of <0.05. Data were reported as means ± 1 SD.

4.3 Results and Discussion

In the last two decades, there is increasing interest in cathecolic compounds thanks to their various biological activities related to the ortho-diphenolic moiety. Particularly, hydroxytyrosol, the main natural polyphenolic compound occurring in virgin olive oil and in olive oil wastewaters, possesses important pharmacological potential related to its antioxidant [28, 29], anti-inflammatory [29, 30] and antiplatelet aggregation activity [31], which may help the prevention of cardiovascular and neurodegenerative diseases. In addition, hydroxytyrosol and its derivates possess a strong chelating ability of metals. Given elevated copper and oxidative stress levels in a variety of malignances, including

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solid tumor and blood cancer, the use of copper chelating material as anti-angiogenic agent could be useful in cancer treatment [32].

In the field of biomaterials, the oxidative stress resulting from tissue-material interaction plays a central role in the biological toxicity of many materials, including polymers and metals. Recently, it has been demonstrated that also polymers generally considered biocompatible can induce the oxidative stress response [33, 34]. At this regard, the synthesis of antioxidant polymers could represent a new and promising strategy to tune the biological response thus improving material biocompatibility.

In this study, a new antioxidant and antimicrobial polyacrylate containing hydroxytyrosol was synthesized. This dual-function polymer could be employed in the field of medical devices as a biomaterial able to suppress the oxidative stress biological response, to prevent infections frequently associated to the implant of medical devices [35] as well as to reduce copper concentration in cancer patients [36].

The obtainment of a hydroxytyrosol containing polymer was verified by FTIR and ^{1}H-NMR analysis. Particularly, the infrared spectroscopic analysis was useful for a qualitative confirmation of the occurred synthesis of the monomer AcTy and the corresponding polymer pAcHTy.

In the IR spectrum of the monomer AcTy (***Figure 1***), a band at approximately 3400-3300 cm^{-1} relative to OH stretching can be observed. In addition, in the region between 1730-1500 cm^{-1}, the absorbance bands corresponding to the ester stretching C = O, the stretching of the double bond of the acrylic moiety and the stretching of the C=C

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[33] Jiang W. W., Su S. H., Eberhart R. C., Tang L., *J. Biomed. Mater. Res. A,* **2007**, 82, 492-497
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[35] Maeyama R., Kwon Il K., Mizunoe Y., Anderson J. M., Tanaka M., Matsuda T., *J. Biomed. Mater. Res. A,* **2005**, 75, 146-155
[36] Francolini I., D'Ilario L., Guaglianone E., Donelli G., Martinelli A., Piozzi A., *Acta Biomater*., **2010**, 6, 3482-90.

belonging to the phenyl-ring are present. At 800 cm^{-1} a broad peak related to the bending in the plane and out of plane due to the aromatic CH is also observable.

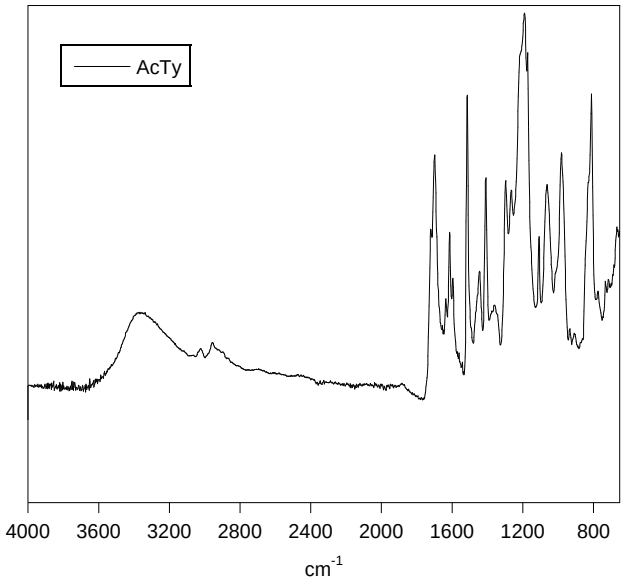

Figure 1. *IR spectrum of the monomer AcTy*

In the IR spectrum of pAcHTy (***Figure 2***), the disappearing of the acrylic double bond peak at around 1410 cm^{-1} confirmed the successful of polymerization.

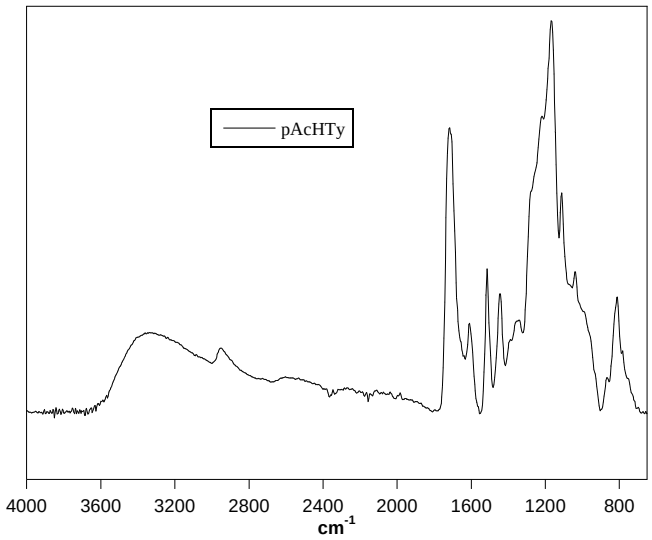

Figure 2. *IR spectrum of pAcHTy*

In ***Figure 3***, the ^{1}H -NMR spectrum of the monomer AcTy is reported. The obtainment of the monomer was confirmed by the presence of the resonance peaks at 4.3 ppm due to the CH_2 adjacent to the ester oxygen and those in the range 5.8-6.4 ppm due to the protons of the acrylic moiety. These last signals were absent in the spectrum of the tyrosol-containing polymer pAcTy (Inset of ***Figure 3***), thus confirming monomer polymerization.

After oxidation and reduction, the polymer pAcHTy was obtained as an insoluble resin presumably caused by cross-linking of the quinones [37].

In literature, it has been highlighted that conjugate systems such as cinnamic acid can undergo free-radical polymerization obtaining thermoset resins [38]. Also compounds containing phenolic groups were used for the preparation of chelating [39] or grafted [40] insoluble polymer systems. Indeed, the phenolic group could be directly involved in the polymerization process forming a phenolic radical that undergoes a dimerization processes by reaction between OH and the aromatic ring [41]. On the basis of these findings, it is possible to hypothesize that the polymerization mechanism may involve the reaction of both acrylic double bonds and OH of hydroxytyrosol leading to the formation of crosslinks.

Therefore, the ^{1}H-NMR spectrum of this sample (Inset of ***Figure 3***) was recorded on the poor water soluble fraction. A variation of the integral intensities relative to the aromatic signals (6.4-6.6 ppm), due to the achievement of a metha-para- arrangement after reaction of the pAcTy (para-structure) with IBX and $Na_2S_2O_5$, was observed.

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[37] Yu M., Hwang J., Deming T. J., *J. Am. Chem. Soc.*, **1999**, 121, 5825-5826.
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[41] Larsen E., Andreasen M.F., Christensen L.P., *J Agric Food Chem*, **2001**, 49, 3471-3475.

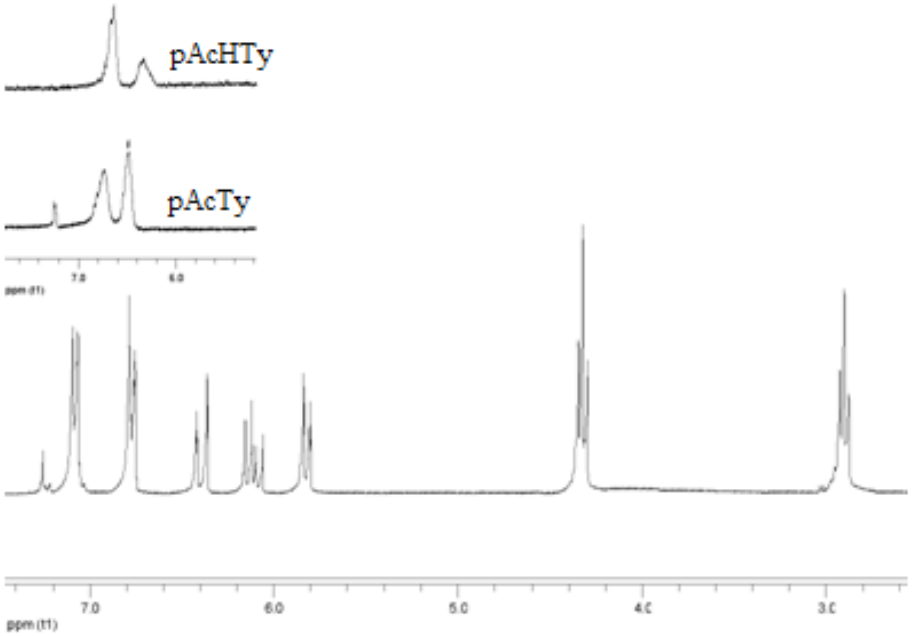

Figure 3. *[1]H-NMR of AcTy. In the inset, spectra of pAcTy and pAcHTy in the range 6-7 ppm are reported.*

To determine the pAcHTy oxidation percentage, a de-esterification reaction was carried out under conditions described in Materials and Methods. Following de-esterification, the sample was submitted to [1]HNMR analysis (spectrum not shown). A yield of oxidation of about 60% was determined. Elemental analysis confirmed the composition of the polymer repetitive units.

DSC analysis evidenced that the synthesized polymers were mainly amorphous showing a glass transition at approximately 60 °C for pAcTy and 135 °C for pAcHTy (***Figure 4***). The higher glass transition temperature of pAcHTy is in agreement with the cross-linking hypothesis [42, 43].

[42] Stutz H., Illers K-H, Mertes J., *J. Polym Sci., Part B: Polym Phys*., **1990**, 28, 1483-1498.
[43] Stejny J., *Polym Bull*, **1996**, 36, 617-621.

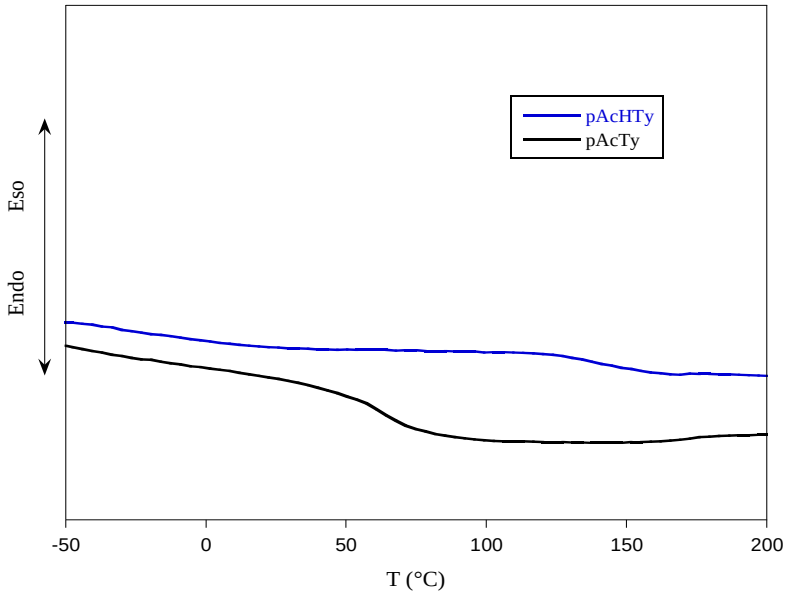

Figure 4. *DSC curves of synthesized polymers*

To verify polymer thermal stability, thermogravimetric analysis (TGA) was carried out. The thermal profile of the two polymers (***Figure 5***) showed less stability of pAcHTy than pAcTy. The presence of two decompositions, one around 230 °C and the other at 330 °C, in the pAcHTy, can be probably attributed to the coexistence of the two type of side residues, tyrosol and catechol moieties, that do not allow a good "packing "of the polymer chains.

GPC analysis, performed only on the soluble polymer pAcTy, evidenced a molecular weight of pAcTy of about 5000 g/mol.

Radical polymerization lead to a stable emulsion of pAcTy nanoparticles having an average size, measured by Laser Diffraction Analyzer, of about 60±5 nm. SEM observations, instead, showed nanoparticles possessing average size of about 400 nm (***Figure 6***), probably due to aggregation phenomena occurring during drying in vacuum oven.

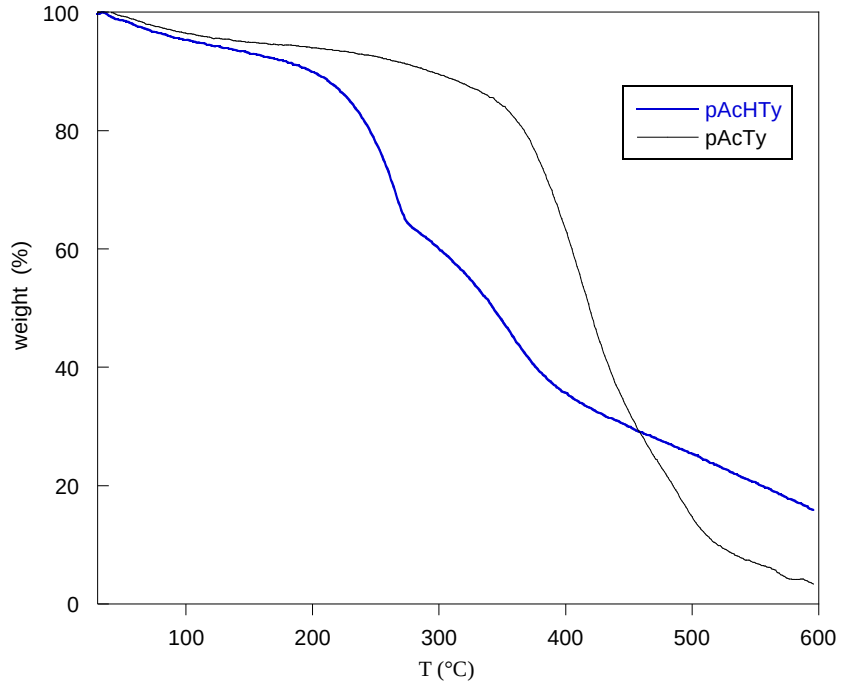

Figure 5. *TGA curves of synthesized polymers*

Indeed, the sample observed by SEM was obtained by preparing pAcHTy films by a solvent casting method. Breakable films were obtained showing differently colored surfaces: the face in contact with the teflon plate showed a white coloration while the face exposed to air a pale brown. This latter effect suggested a partially oxidation phenomenon. As can be observed from the figure 6, the film was made of spherical nanoparticles attached one another, with a rather narrow size distribution.

The subsequent reaction of oxidation/reduction carried out on pAcTy did not significantly changed size and morphology of nanoparticles (data not shown).

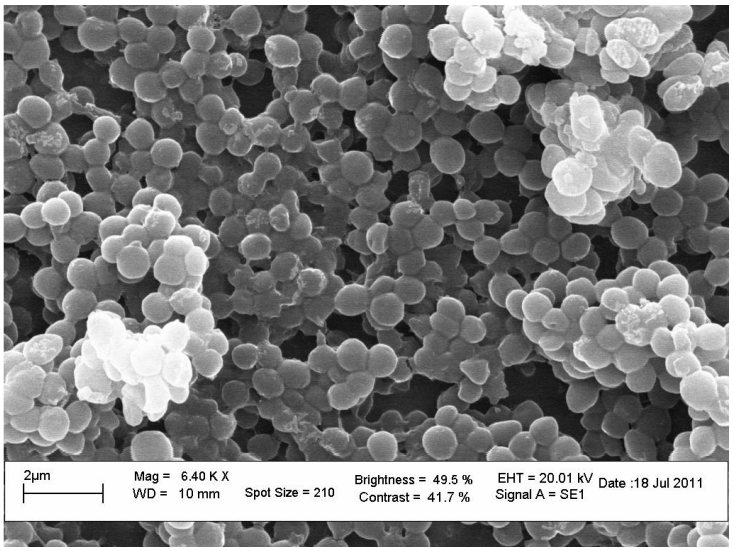

Figure 6. *SEM micrograph of pAcHTy nanoparticles.*

When submitted to disc diffusion test, AcTy monomer, pAcTy and pAcHTy polymers did not show any inhibition zone probably due to their poor diffusion in the agar. To evaluate the efficacy in the prevention of bacterial colonization and biofilm formation, pAcHTy films were observed by SEM after 24 hr of incubation with a bacterial suspension. SEM micrographs of the films evidenced a total absence of microorganisms (data not shown).

The antimicrobial activity of pAcTy and water-insoluble pAcHTy nanoparticles was also assayed by turbidimetric analysis. Despite the totally insolubility in water of these polymers the absorbance of the bacterial solutions in contact with nanoparticles (test tubes) did not reached the value of the control tube in 24 hr of incubation. A MIC value of 2.5 mg/ml and 7.5 mg/ml against *S. epidermidis* for pAcHTy and pAcTy respectively was determined.

Polyphenols possess biocide activity against a wide number of pathogenic bacteria [44]. Oxidized polyphenols, such as hydroxytyrosol, also have inhibitory activity against bacterial growth. The MIC value reported in literature for hydroxytyrosol against the

[44] Cowan M. M., *Clin. Microbiol. Rev*., **1999**, 12, 564-582.

Gram-positive (*S. pyogenes* and *S. aureus*) and the Gram negative bacteria (*E. coli* and *K. pneumoniae*) was of about 0.4 mg/ml [45]. Since the antimicrobial activity of the insoluble pAcHTy was only due to surface functional groups, it is possible to hypothesize a lower MIC value in case of soluble polymers. In addition, taking into account the different molecular weight of the antioxidant polymer and hydroxytyrosol, the good MIC value found for pAcHTy suggests its potential use for prevention or treatment of bacterial infections.

Oxidative stress may be ascribed to the unbalancing in the oxidant-antioxidant equilibrium, favoring the oxidant side [28]. Oxidative stress has been associated to disorders such as cancer [46], diabetes mellitus [47], atherosclerosis [48] , neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [49], autoimmune disorders such as arthritis [57] and has also been indicated to be involved in aging [50].

The antioxidant activity of HTy and pAcHTy was determined by using DPPH as free anionic radical. In the DPPH method, the reaction degree depends on hydrogen-donating ability of antioxidant species.

It has been well documented that cysteine, glutathione, ascorbic acid, tocopherol, and polyhydroxy aromatic compounds (e.g., ferulic acid, hydroquinone, pyrogallol, gallic acid) are able to reduce and decolorize 1,1-diphenyl-2-picrylhydrazine thanks to their hydrogen-donating capabilities [51] .

[45] Tafesh A., Najami N., Jadoun J., Halahlih F., Riepl H., Azaizeh H., *Hindawi Publishing Corporation* **2011**, doi:10.1155/2011/431021.
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[51] Ardestani, A.; Yazdanparast, R., *Food Chem.*, **2007**, 104, 21–29

Also, synthetic and natural polymers modified with antioxidant molecules have been reported to have a good scavenging activity on DPPH radicals [52, 53].

In our case, by plotting the residual DPPH percentage as a function of antioxidant agent/DPPH molar ratio, EC_{50} values of 0.18 ± 0.02 mmol HTy/mmol DPPH and 0.80 ± 0.02 mmol pAcHTy/mmol DPPH were found (***Figure 7***). The less activity of PAcHTy than HTy is presumably due to insolubility of material that partially hampered interactions between DPPH and antioxidant polymer groups. The antioxidant activity found for pAcHTy was rather high compared to that reported in literature for modified natural or synthetic polymers [52, 53].

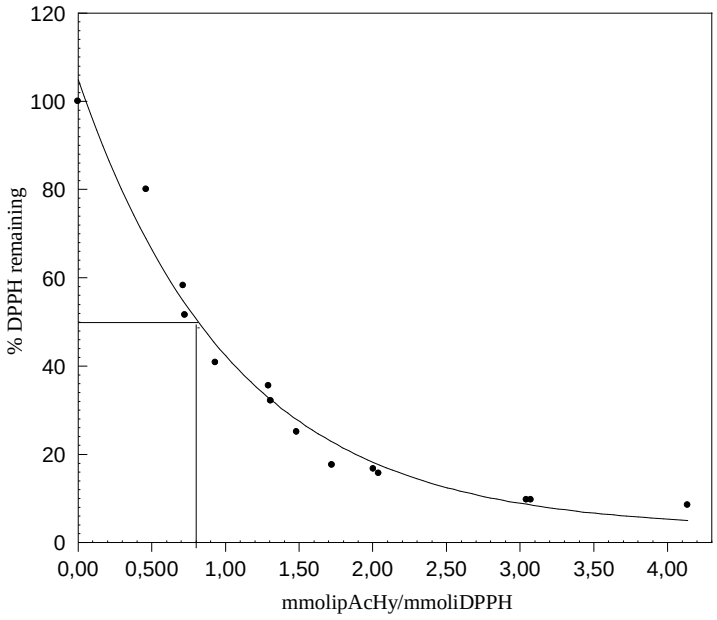

Figure 7. *Residual DPPH percentage as a function of pAcHTy/ DPPH molar ratio.*

[52] Curcio M., Puoci F., Iemma F., Parisi O.I., Cirillo G., Spizzieri U.G., Picci N., *J. Agric. Food Chem.*, **2009**, 57, 5933–5938

[53] Iemma F., Puoci F., Curcio M., Parisi O.I., Cirillo G., Spizzirri U.G., Picci N., *J. Appl. Polym. Sci.*, **2010**, 115, 784-789.

As previously mentioned, several studies have reported that in tumor patients copper levels are over the normal concentration threshold and that such high copper levels can be directly correlated to cancer advancement. In addition, transition metals (copper, iron) promote the production of ROS in biological systems. To play a positive action in the body, a good balance between ROS and endogenous antioxidants able to swallow the excess ROS is needed.

Radicals can give chain reactions such as formation of superoxide anion (radical anion). The hydrogen peroxide is converted by catalase in H_2O, but can also generate highly reactive hydroxyl radicals via the Fenton reaction. This reaction is catalyzed by Fe^{2+} or Cu^{2+}, which can be generated by the same anionic radicals. An example of chain reaction is the lipid peroxidation, which leads to the fragmentation of lipids of cell membranes. In genetic diseases causing extremely low levels of cholesterol and triglyceride, deficiency of selenium and vitamin E, peroxidized radicals are able to reach vital structures such as DNA and mitochondria, thus creating damage sometimes irreversible. These diseases can however be modulate by chelating therapy with antioxidants that bind and remove iron and copper excess. In particular, therapies based on copper reduction by chelation are currently under investigation [54, 55] as alternative ways to develop new anti-cancer agents able to change oxidative-stress conditions.

For these reasons, in this study the ability of the hydroxytyrosol-containing polymer pAcHTy to chelate copper ions was also evaluated.

The amount of adsorbed metal at different pH is shown in ***Figure 7***. The maximum copper removal percentage (Rem %) at different pH was 12 %, 65 % and 100% at pH=3, 6.5 and 8 respectively.

[54] Pan Q., Kleer C.G., van Golen K.L., Irani J., Bottema K.M., Bias C., *Cancer Res*, **2002**, 62, 4854–4859.

[55] Camphausen K., Sproull M., Tantama S., Venditto V., Sankineni S., Scott T., *Bioorg Med Chem*, **2004**, 12, 5133–5140.

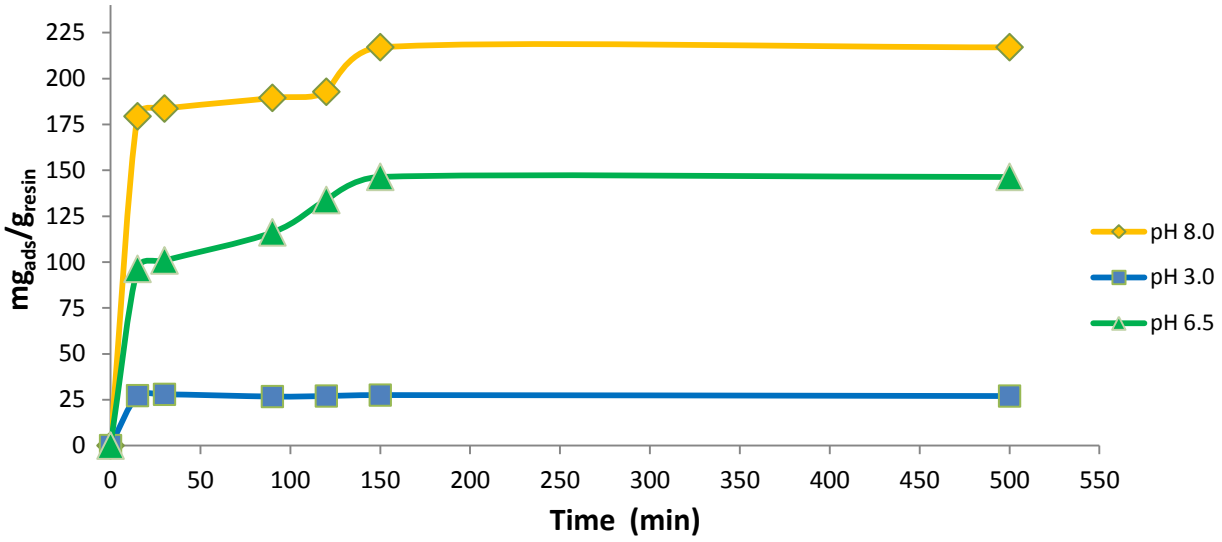

Figure 7. *Kinetics of Cu^{+2} adsorption on pAcHTy at different pH.*

At all of the experimented pH, the equilibrium was reached in about 150 minutes. In fact, even after 24 hours of contact, the concentrations of the supernatant remained constant in all of cases. It should be noted that kinetics at pH 6,5 and 8 have a first plateau in the range of 15-120 minutes, probably due to the organization of surface groups complexing the metal. In particular, given the coexistence of phenolic and catechol groups, it can be assumed that the first metal complexation occurs with catechol groups while the second one with phenol groups. The structural variation of polymers induced by metals has already been highlighted in other studies [56].

In general, pH significantly influences the ability of polymer to chelate copper ions. In our case, high pH values caused an increase in polymer ability to remove copper ions in solution. Probably this is due to deprotonation of catechol and phenol groups occurring at basic pH that make them available for adsorption of metal ions.

[56] Crescenzi V., Airoldi C., Dentini M., Pietrelli L., Rizzo R., *Makromol. Chem.*, **1981**, 182, 219-223

In fact, the hydrogen ion concentration is one of the factors affecting the adsorption capacity of a material. At acidic pH, [H_3O^+] ions can compete with the metal cations in the adsorption mechanism while, at alkaline pH, phenomena of precipitation of metal in the form of hydroxides can occur.

Temperature is another factor affecting the polymer adsorption ability. To verify this effect, tests at different temperatures (20, 30, 40 and 50 °C) were carried out (***Table 1***).

Temperature (K)	***Time (min)***	***Ce (ppm)***	***Rim (%)***	***x/M (mg/g)***	***ln x/M***
293.15	150	0	100	217	5,38
303.15	150	131	77	171	5,14
313.15	150	241	57	126	4,83
323.15	150	267	52	116	4,75

Table 1. *Adsorption of Cu +2 by pAcHTy as a function of time. Operating conditions: 30 mg of pAcHTy, 20 ml of 350 ppm copper, pH = 8.*

Knowing that $\ln \frac{x}{M} = \frac{\Delta S°}{R} - \frac{\Delta H°}{RT}$ and plotting the ln x/M as a function of 1/T, the trend reported in ***Figure 8*** was obtained.

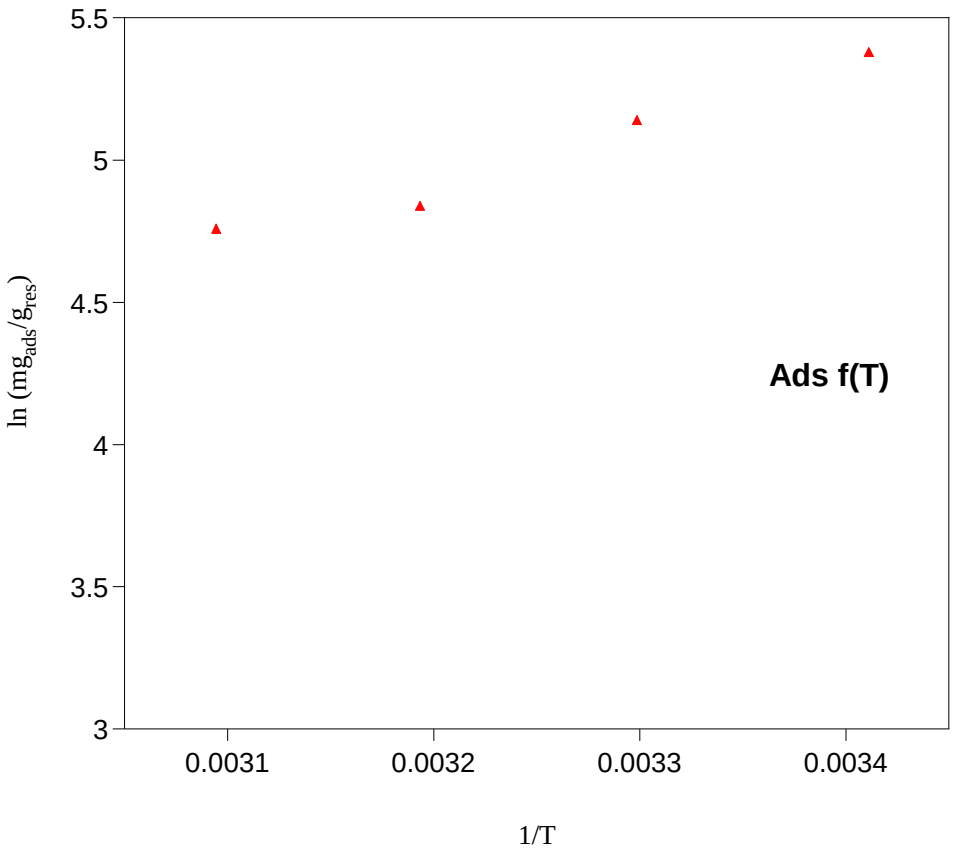

Figure 8. *Copper removed by pAcHTy as a function of temperature.*

From this curve, the values of ΔH ° and ΔS °, respectively, from the slope and the intercept of the straight line, can be obtained:

$$\frac{\Delta H^{\circ}}{R} = -2063.7 \qquad \frac{\Delta S^{\circ}}{R} = -1.676$$

From these values, it can be stated that the process of copper adsorption by pAcHTy is exothermic. Indeed, the concentration of the adsorbed cations decreases with increasing temperature, and is spontaneous at low temperatures. The increase in temperature causes, probably, a structural change in surface making the OH catechol less available for the adsorption process.

In conclusion, the high radical scavenger activity and metal ion chelating ability, comparable to those of other phenolic-based polymers reported in literature, [57, 58] demonstrate the potential application of pAcHTy in the medical field.

4.4 Conclusions

A novel hydroxytyrosol-based polyacrylate (pAcHTy) endowed with antioxidant and antimicrobial activity was synthesized. Since hydroxytyrosol is only produced by some chemical companies with not inexpensive prices, in this work the polymer was obtained by free radical polymerization of a monomer containing its precursor, Tyrosol (Ty). Hydroxylation of Ty phenol group in ortho position by IBX reagent, with a yield of 60%, leaded to an insoluble polymer (pAcHTy) possessing nanometric size (about 60 nm). Besides the strong antioxidant activity in DPPH assay, pAcHTy showed a good chelating activity with respect to Cu^{2+} ions. Such properties suggest that this polymer can be employed in the medical field to suppress the oxidative stress, to prevent infections frequently associated to the implant of medical devices as well as to reduce copper high levels in tumor patients.

[57] Iemma F., Cirillo G., Puoci F., Trombino S., Castiglione M., Picci N., *J. Pharm.Pharm.*, **2007**, 59, 597-601.
[58] Spizzirri U.G., Iemma F., Puoci F., Cirillo G., Curcio M., Parisi O.I., Picci N., *Biomacromolecules*, **2009**, 10, 1923–1930.

CHAPTER V

AMPHIPHILIC RANDOM COPOLYMERS AS ANTIMICROBIAL AND ANTIOXIDANT AGENTS.

5.1 Introduction

Increasing antibiotic resistance and development of medical device-related infections, caused by multiple bacterial species, require a continuous update in the strategies of microbial killing[1].

In addition, the bacterial and fungal cells grown embedded in microbial biofilms have resistance higher than to the planktonic ones[2, 3].

The efficacy of devices coated with various antimicrobial agents such as rifampicin, clindamycin, chlorhexidine and silver has been widely demonstrated[1, 4].

However, the main limitation related to these use of the drug-coated devices is the rapid release of the antimicrobial agent absorbed (in first hours from the implant) and as a consequent short antibacterial action. In addition, the low systemic antibiotic concentration reached in the patient after few days from the device implant can increase the risk for selecting of antimicrobial resistant strains.

[1] Francolini I.,Donelli G., *FEMS*, **2010**, 59, 227-238.
[2] Aslam S. *Am J Infect Contro*, **2008**, 36, 175-211.
[3] Ramage G., Mowat E., Jones B., Williams C., Lopez-Ribot J., *Crit Rev Microbiol*, **2009**, 35, 340-355.
[4] Raad I., Hanna H., Jiang Y., Dvorak T., Reitzel R., Chaiban G., Sherertz R., Hachem R., *Antimicrob Agents Chemother* **2007**, 51, 1656-1660.

A valid approach to prevent the emergence of resistant pathogens should require the use of two antimicrobial agents possessing different antimicrobial spectrum and mechanism of action[1, 5].

During the last decades, numerous efforts has been focused on the synthesis of antimicrobial polymers able to inhibit the bacterial adhesion or kill parasite cells.

Mainly two strategies have been pursued to develop antimicrobial polymers: i) polymer impregnation/adsorption with antibiotics or other biocides; ii) polymer functionalization with groups exerting antimicrobial activity [6].

In the first approach, the polymer acts as a carrier for one or more antimicrobial agent that once released from the polymer exerts its action (antimicrobial releasing polymer).

Polymers able to adsorb high amount of antibiotics have been generally obtained through modifications of their surface with specific functional groups capable to interact directly with the drug [5]. The binding of the active compound to the polymer offers the opportunity to extend the residence time of the drug within the body, limit problems of drug residual toxicity as well as increase the efficacy and selectivity of the drug.

In the second approach, bactericidal functionalities, such as phosphonium salts or quaternary amine compounds, are introduced in the polymer to obtain intrinsically antimicrobial polymers (biocidal polymers).

Since quaternary ammonium compounds are by far the most useful antiseptics and disinfectants developed [7, 8], tertiary amine groups then quaternized have been introduced in the side chain of polymers, thus developing cationic polymers (polymer disinfectants).

[5] Ruggeri V., Francolini I., Donelli G., Piozzi A., **2007**, *J Biomed Mater Res A*, 81, 287-298.
[6] Kenawy E.R., WorkleyD., Broughton R., *Biomacromolecules*, **2007**,8, 1359-1383.
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Polymer disinfectants bearing cationic quaternary ammonium salt (QAS) have been often modified with hydrophobic long alkyl chain capable of breaking the bacterial membrane [9].

In literature, different derivatives of polyvinyl-pyridine, polyacrylates (or polyacrylamides), polyvinyl-alcohol (or polyphenols) and other polymers containing as side chain QAS modified with long hydrophobic chain have been reported [6, 10, 11].

Although the antimicrobial moieties in these synthetic polymers have been designed to confer high selectivity and sensibility towards bacterial membranes respect to the mammalians ones, often they showed toxic effects even for human cells [12,13].

For this issue, the synthesis of novel cationic and/or amphiphilic polymers obtained by employing hydrophobic monomers or neutral macromonomers to be copolymerized with the cationic monomers has been needed.

Since three decades, a class of novel intrinsically antimicrobial polymers has been under investigation: host defense peptides.

The host defense peptides [14], importants component of innate immune system, are released to counteract the invasion of pathogenic and virulent microorganisms by mammalian or plant cells. They are potent antibiotics possessing an unusual broad-spectrum, antimicrobial activity and showing a lower onset of bacterial resistance compared to common drugs [15].

[9] Ikeda T., Tazuke S., Suzuki Y., *Makromol. Chem. Macromol. Chem. Phys.*, **1984**, 185, 869–876.
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[14] Zasloff M., *Nature*, **2002** 415, 389–395
[15]Hancock R. E**.,** Sahl H. G**.,** *Nat.Biotechnol.***, 2006,** 24, 1551-1557.

However, their application in the medical field as well as their marketing has been hampered by high manufacturing costs, partially unknown pharmacokinetics and high enzymatic deactivation [16].

The understanding of the correlation between structural parameters and biological activity of the host defense peptides and synthetic polymer disinfectants allowed the development of synthetic materials called peptido-mimetic antimicrobial polymers or amphiphilic cationic polymers (synthetic mimics of antimicrobial peptides SMAMPs) [17, 18].

SMAMPs have emerged as promising candidates for the developments antimicrobial agents that not only show high efficacy but also are less susceptible to resistant development in the bacteria they target.

Although, action mechanism of SMAMPs is still under debate, it is generally believed that they can exert their action by cell wall and/or membrane disruption, causing breakdown of the transmembrane potential, leakage of cytoplasmic material and then cell death[19,20].

It has been widely demonstrated that the key parameters which regulate the antimicrobial and hemolytic activity, for both the host defense peptides and synthetic polymers, are positive charge at neutral pH [21,21], low molecular weight [22], spatial arrangement [23] and amphiphilicity [24, 25].

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[16]Marr A.K., Gooderham W.J., Hancock R.E.W., *Curr. Opin. Pharmacol.*, **2006**, 6, 468–472.
[17]Palermo E.F., Kuroda K., *Appl. Microbiol. Biotechnol.*, **2010**, 87, 1605–15.
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[25]Palermo E.F., Sovadinova I., Kuroda K., *Biomacromolecules*, **2009**, 10, 3098– 3107.

To obtain antimicrobial amphiphilic cationic polymers from the common QAS's polymers, three different strategies have been employed: i) decreasing of polymer hydrophobicity via coupling with neutral hydrophilic moieties such as PEG [26,27], ii) modification of the nature of the hydrophobic groups [17] and iii) variation of the ratio of cationic and hydrophobic side chains in random copolymers [9, 28].

Many of these amphiphilic random and block copolymers showed a good antibacterial efficacy and low toxicity for human cells with respect to homopolymers [17]. The poor toxicity of these copolymers has been attributed to an increase of their selectivity versus bacterial cells.

The resistance to normal antibiotics which bacteria in biofilms exhibit compared to the planktonic counterpart can raise up to 1000 times [1]. This feature has been attributed to several factors such as poor drug penetration through the biofilm or exchange of plasmids and ROS [29] among the biofilm-associated microorganisms in order to increase the natural mutation phenomena.

ROS including hydroperoxide, hypochloride, hydrogen peroxide and hydroxyl anions are released by activated phagocytes together with other molecules having killing and degradative activities [30]. Such radicals are generated by reaction of ferrous ion (Fe^{2+}) with hydrogen peroxide. In this process, known as Fenton reaction, the amount of radical produced is directly proportional to the concentration of iron or copper.

An excess of cellular ROS contributes to the processes of aging [31] and is implicated in the development of cancer and chronic diseases, neurodegenerative and cardiovascular

[26] Sellenet P.H., Allison B., Applegate B.M., Youngblood J.P., *Biomacromolecules*, **2007**, 8, 19–23.
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diseases, such as ischemia, multiple sclerosis, atherosclerosis, cataracts, diabetes, hepatitis, Parkinson's disease, Alzheimer's disease, dermatitis and muscular dystrophy [32, 33, 34, 35].

As previously mentioned, random amphiphilic cationic copolymers are very interesting to fight the emergent risk of microbial resistance. Generally, in these polymers, to achieve high antimicrobial activity, a suitable balance of hydrophobic content and cationic charges was obtained by employing a monomer containing in side chain primary or tertiary amines as cationic functionality, and a hydrophobic monomer without any activity.

In this work, novel antimicrobial amphiphilic cationic polymers have been synthesized by copolymerization of two monomers one bearing a positive charge and the other one a hydrophobic compound possessing antimicrobial activity, in particular antioxidant activity. The development of polymer possessing both antimicrobial and antioxidant properties could represent a promising approach to reduce the emergence of drug resistant bacteria and better combat biofilm infections.

Our group has recently synthesized a new acrylamide monomer (AcDED) by reacting acryloyl chloride (Ac) with N,N-diethylethylendiamine (DED)[36]. The radical polymerization [37] of this monomer possessing tertiary amine groups, allowed the synthesis of a water-soluble, intrinsically antimicrobial cationic polymer (pAcDED) [38]. In order to obtain a material with also antioxidant properties, the monomer AcDED

[32] Halliwell B., Gutteridge J. M. C., *Methods Enzymol*, **1990**, *186*, 1-85.
[33] Ames B. N., Shigenaga M. K., *Ann. N.Y. Acad. Sci.*, **1992**, 663, 85-96.
[34] Cestaro B., *Etas Libri-RCS Medicina*, **1994**.
[35] Chen C. H., Fischer A., Reagan J. D., Yan L. J., Ames B. N., *Proc. Nat. Acad. Sci.*, **1995**, 92, 4337-4341.
[36] Zhang L., Wang X., Grinberg N., Krishnamurthy D.K., Senanavake C.H., *Tetrahedron Letters*, **2009**, 50, 2964-2966
[37] A.S. Sarac, *Prog. Polym. Sci*., **1999**, 24, 1149–1204
[38] Francolini I., Taresco V., Crisante F., Martinelli A., D'Ilario L., Piozzi A., *Int J Mol Sci.*, **2013**; 14(4), 7356-7369.

were copolymerized with a second acrylic monomer possessing a catecholic functionality.

Among antioxidants, cathecolic compounds have gained increasing research attention due to their ortho-diphenolic moiety that extends their biological activity from free radical scavangers to metal chelators as well as to antimicrobial agents [39]. In this regard, hydroxytyrosol (HTy), the main natural polyphenolic compound occurring in virgin olive oil and in olive oil wastewaters, possesses important pharmacological potential related to its antioxidant, anti-inflammatory and anti-platelet aggregation activity. In addition, hydroxytyrosol and its derivatives possess a strong chelating ability towards metals and a broad-spectrum antimicrobial activity against a series of microorganisms including *Staphylococcus epidermidis* and *S. aureus*, *Vibrio cholerae* and *Salmonella* species [40].

The only drawbacks related to the use of HTy are its poor availability and high cost.

For these reasons, in this work, Tyrosol (Ty), a low-cost phenol also present in olive oil, was used as a precursor molecule of hydroxytyrosol.

Particularly, a Tyrosol containing monomer was first synthesized. Then, the catecholic function was inserted by a patented chemo- and regio-selective aromatic hydroxylation [41]. To obtain polymers with a various amphiphilic balance, different ratios between the antioxidant-hydrophobic monomer and the hydrophilic AcDED one were used.

After physical characterization, the antimicrobial properties of obtained copolymers in terms of minimal inhibitory concentration (MIC) and antifouling characteristics were tested against *S. epidermidis*. It was observed that the most hydrophobic copolymer

[39] Faure E., Falentin-Daudré C., Jérôme C., Lyskawa J., Fournier D., Woisel P., Detrembleur C., *Progress in Polymer Science*, **2012**, 38, 236-270.

[40] S. Granados-Principal, J. L. Quiles, C. L. Ramirez-Tortosa, P- Sanches-Rovira, M. C. Ramirez-Tortosa., *NutRev*, **2010**, 68, 191-206)

[41] Bernini R, Mincione E, Barontini M, Crisante F. MI2007A000519, 2007; Applicant: Università degli Studi della Tuscia. Inventors/Applicant: Bernini, R.; Mincione, E.; Barontini, M.; Crisante, F. Number: PCT/IB2008/000598. International Pubblication Number: WO2008/110908 A1, **2008**.

containing 30% mol/mol of HTy possessed the lowest MIC. In addition, this material also displayed good antifouling characteristics after coating of a commercial catheter.

5.2 Materials and methods

5.2.1 Materials

Sodium metabisulphite ($Na_2S_2O_5$) and potassium monobasic phosphate (K_2HPO_4) were purchased from Carlo Erba. Achryloyl chloride 96% and N, N-diethylethylendiamine (DED) were supplied from FLUKA. Tyrosol (2-(4-Hydroxyphenyl)ethanol, TY), acrylic acid (AA), hydroquinone, potassium persulfate ($K_2S_2O_8$), *p*-toluensyulfonic acid (pTSOH), sodium dithionite ($Na_2S_2O_4$), Ferrozine, Ferrous sulphate (Fe_2SO_4), Ferrous chloride ($FeCl_2$), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich as well as other solvents and reagents. DMSO-d_6 100% were supplied from CIL (Cambridge Isotope Laboratories, Inc). All of chemicals were of analytical grade and used as received. 2-iodoxybenzoic acid (IBX) was prepared in the laboratory as described in the literature [42]. *Staphylococcus epidermidis*, strain ATCC 35984, and Muller Hinton broth (MH, Oxoid) were employed for microbiologic tests.

[42]Applicant: Università degli Studi della Tuscia. Inventors/Applicant: Bernini R., Inventors: Mincione E., Barontini M., Crisante F. "Method for preparing hydroxytyrosol and hydroxytyrosol derivatives" Number: PCT/IB2008/000598. International Pubblication Number: WO2008/110908 A1, **2008**. b) *J. Agric. Food Chem.* **2008,** *56,* 8897–8904

5.2.2 Monomer and homopolymer synthesis

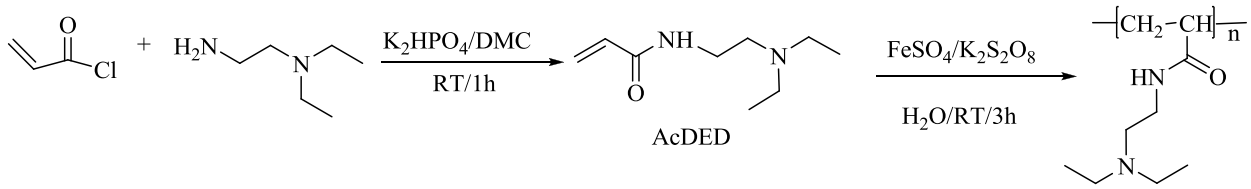
Synthesis of AcDED and pAcDED

Synthesis of acrylate monomers based on N,N-diethylethylendiamine (AcDED) and tyrosol (AcTy), as well as synthesis of respective homopolymers have been already described. Following, shortly, the main synthetic steps were reported.

AcDED was synthesized by reaction between acryloyl chloride (Ac, Fluka) and N,N-diethylethylendiamine (DED, Sigma Aldrich). Briefly 0.029 moles of diamine was added to a solution of Ac (0.038 moles) in dimethylcarbonate (DMC, 75 ml, Sigma Aldrich) containing K_2HPO_4 (0.08 moles, Carlo Erba), used as base [36] . The reaction was carried out for 4 h at room temperature. Then, the solution was filtered to remove the inorganic and organic salt and the monomer recovered by solvent evaporation (yield ranging from 85 to 90%).

Homopolymer synthesis was carried out at 25 °C for 24 h by using a 1 M water solution of AcDED (5 ml) and $K_2S_2O_8$ ($2.8x10^{-4}$ mmoles, Carlo Erba) plus $FeSO_4$ ($2.4x10^{-4}$ mmoles, Carlo Erba) as radical initiators. The resulting polyacrylamide (***Scheme 1***) was called pAcDED (pK_b=8.61).

In previous work [38] , homopolymers at different chain length were obtained by adding in the synthesis sodium metabisulfite ($Na_2S_2O_5$, Carlo Erba) as chain transfer agent (T). In this work, the [T]/[AcDED]=0.1 molar ratio (chain transfer agent/monomer) was employed. The resulting polymer, herein called pAcDED, possessed a molecular weight of $94x10^3$ g/mol and a polydispersity index of 1.32.

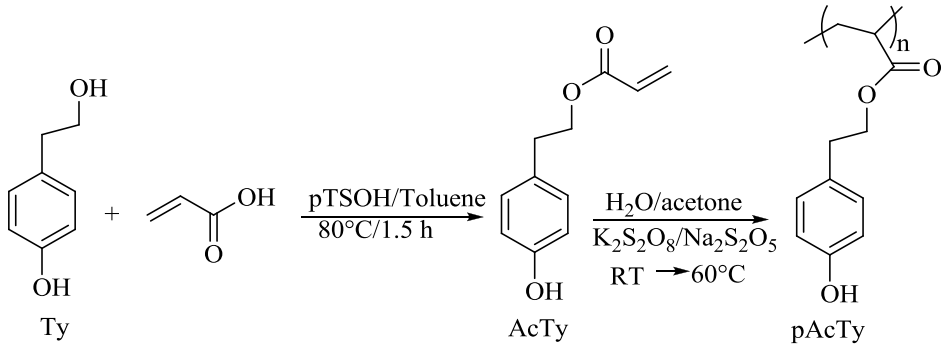


Scheme 1. *Synthesis procedure for pAcDED.*

Synthesis of AcTy and pAcTy

As for tyrosol-based acrylic monomer (AcTy) synthesis, 0.1 mol of Ty was dissolved in 75 ml of toluene in presence of 20 mg of hydroquinone and 750 mg of *p*-toluensulfonic acid (pTSOH). Then, 0.12 mol of acrylic acid was added and the mixture was refluxed for 1,5 hrs in a flask equipped with Dean-Stark apparatus. At the end of the reaction, the mixture was cooled and treated with sodium bicarbonate (6 g) and water (1.5 ml), until carbon dioxide evolution ended. The mixture was then dried with sodium sulfate anhydrous and filtered. The solvent (toluene) was removed under vacuum. In the second step, the monomer AcTy (1 mmol) was dissolved in a mixture water/acetone (1/1 w/w) and subjected to radical polymerization by employing potassium persulfate (0.03 mmol) and sodium disulfite (0.1 mmol). After stirring for 50 min at room temperature, acetone was distilled off by heating at 60 °C. The reaction mixture was kept at 80 °C for 2 h. At the end of reaction, a stable emulsion of nanoparticles of tyrosol-containing polymer (pAcTy) was obtained (***Scheme 2***). The

reaction yield and the presence of the monomer within the repeat unit were assessed by ^{1}H-NMR (data previously reported).



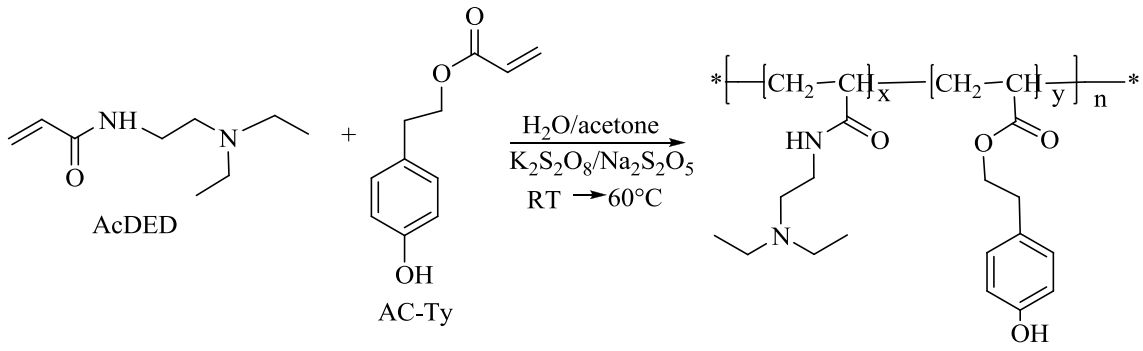
Scheme 2. *Synthesis procedure for pAc-Ty.*

Synthesis of pAcDED-co-pAcTy copolymer

To obtain copolymers containing both the tertiary amine and the phenolic functions a variation of pAc-Ty polymerization procedure was performed.

After dissolution in water (3 ml) of the AcDED monomer (in variable quantity according to the chosen ratio between co-monomers), the aqueous solution was added to an acetone solution (3 ml) containing AcTy monomer. The mixture was kept under continuous stirring until homogeneity. At this point, the initiator (potassium persulfate) and the chain transfer agent (sodium metabisulfite) were added. In order to distillate acetone, the solution was heated up to a temperature of 80 ° C. Finally, the aqueous solution containing the copolymer (named pAcDED-co-AcTy, ***Scheme 3***) was dialyzed in water by using a membrane of regenerated cellulose (Spectr/por membrane

BIOTECH) with a cut off of 3,500 Dalton. The conversion and the ratio of the monomers within the repeat unit of the copolymer were assessed by ^{1}H-NMR.



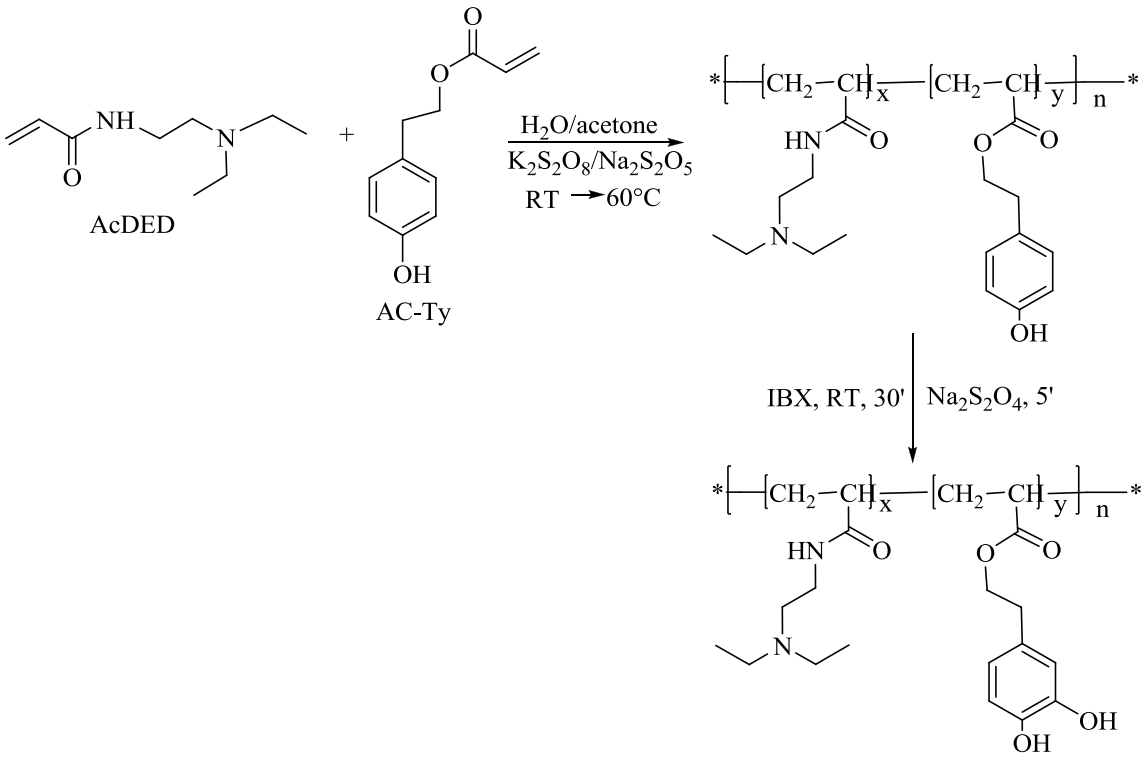
Scheme 3. *Synthesis procedure for pAcDED-co-pAcTy.*

Oxidation/reduction of pAcDED-co-pAcTy copolymer

IBX (in stoichiometric amount to the phenolic fraction) was added to an aqueous solution of pAcDED-co-pAcTy copolymer (200 mg/ml), at 0 °C under magnetic stirring. The immediate appearance of opalescence and orange coloration evidenced the oxidation of the phenol to ortoquinone group. After adding of an aqueous solution of sodium dithionite to the reaction batch (stoichiometric to IBX), the mixture was kept under stirring until to observe a chromatic variation from orange to white. Also in this case, the obtained solution was purified by dialysis in water. Different molar ratios between the monomers AcDED and AcHTy were used. Particularly, AcDED/AcHTy molar ratios of 90/10, 80/20, 70/30, 60/40 and 50/50.

As for copolymers containing AcDED/AcHTy molar ratios of 60/40 and 50/50, after reduction of the ortoquinone function to the catecholic one, polymer preipitation was

observed. Therefore, the purification of these water-insoluble copolymers was performed through several washes in water. All polymers obtained (named pAcDED-co-pAcHTy, ***Scheme 4***) were further dried in vacuum oven at 25 °C for 1 day.



Scheme 4. *Synthesis procedure for pAcDED-co-pAcHTy.*

5.3 Polymer characterization

5.3.1 NMR spectroscopy and elemental analysis

^{1}H-NMR spectra were performed employing a Varian XL 300 instrument and chloroform-d or DMSO-d_6 100% CIL as solvent. Elemental analysis of the synthesized polymers was carried out by Carlo Erba EA 1110 instrument and data were reported as C, H, N, S percentages.

5.3.2 Thermal analysis

Differential scanning calometry (DSC) analysis was performed using a Mettler TA-3000 DSC apparatus in the temperature range from –100 to 200 °C, under N_2 flux. The scan rate used for the experiments was 10 °C/min and the sample weight of about 6-7 mg. Thermogravimetric analysis (TGA) was carried out employing a Mettler TG 50 thermobalance, at the heating rate of 10 °C/min, under N_2 flow and in temperature range 30 - 500 °C.

5.3.3 FT-IR spectroscopy

Attenuated Total Reflection spectra (ATR) were acquired by employing a Nicolet 6700 FT-IR spectrometer and a Golden Gate diamond single reflection device (Specac).The spectra were obtained by co-adding 200 interferograms in the range 4000–650 cm^{-1} at a resolution of 4 cm^{-1}.

5.3.4 Scanning electron microscopy (SEM)

The morphological analysis of polymer nanoparticles and film was performed by LEO1450VP scanning electron microscope (SEM), having a resolution of 3.5 nm. The samples were sputtered with gold using a SEM coating unit (PS3 coater) at 6 mA for 6 min.

5.3.5 Particle size determination

Nanoparticle size determination was assessed by a Laser Diffraction Particle Size Analyzer (LS 13320, Beckman Coulter). Water or organic solvents were used as suspending media. The acquisition time for measurements was 90 s.

5.3.6 UV-Visible spectroscopy

Electronic absorbance spectra in the UV-Vis region and photometric measurements were performed by using a HP U2000 singular beam spectrophotometer. The instrument works in the wavelength range between 190-1100 nm with resolution of 0.004 nm.

5.3.7 Gel permeation chromatography (GPC)

Molecular weight measurements were carried out at 25 °C using a gel permeation chromatography (GPC) system equipped with a Shimadzu RID-10A differential refractive index detector and Tosoh TSK polyacrylamide gel columns. The calibration curve was obtained by using polyethylene oxide standard. Aqueous sample solutions containing 0.1 M NaCl were injected by an autosampler Hitachi AS-2000 at a flow rate of 0.8 mL/min^{-1}.

5.3.8 Antiradical activities of compounds by DPPH

The antioxidant activity of copolymers was determined by using DPPH as free anionic radical [43]. For each antioxidant compound, different concentrations were tested (expressed as the number of moles of antioxidant/mole DPPH).

Antioxidant-containing copolymer solutions in methanol (4 mL) were added to 10 mL of a 1.5×10^{-4} mol/L methanol DPPH solution. The decrease in absorbance was determined at room temperature at 520 nm after 30 min, time in which a plateau was reached. The amount of DPPH was evaluated from a calibration curve (absorbance vs. concentration). Then, for each concentration tested, the residual DPPH as a function of antioxidant agent/DPPH molar ratio was plotted. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (Efficient Concentration = EC_{50}, moles of antioxidant/mole DPPH). EC_{50} values were extrapolated from the graph.

As for antioxidant activity of insoluble copolymers, measurements were performed by using the DPPH method with some modifications [44].

Different amount of the powdered polymer were transferred into a vial, and the reaction was started by adding 60 mL of methanol and 20 mL of methanol DPPH solution (0.5 M). The vials were stirred for 30 min to facilitate the surface reaction between the insoluble polymer and the DPPH reagent. Following centrifugation at 3500 rpm for 2 min, the absorbance of the optically clear supernatant was measured at 520 nm.

5.3.9 Chelating activity versus ferrous ions

The chelanting activity of copolymer solutions versus ferrous ions was estimated by measuring the formation of Fe^{2+}-ferrozine complex exploiting a modification of Dinis

[43] Brand-Williams W., Cuvelier M. E., Berset C., Lebensm.-Wiss.u.,*Technol.*, **1995**, 28, 25-30.
[44] Serpen A., Capuano E., Fogliano V., Gökmen V., *J. Agric. Food Chem*., **2007**, 55, 7676–7681.

method (and Carter method 1971) [45]. Briefly, 50 µl of 2 mM $FeCl_2$ was added to 1 ml of polymer solution at different concentrations. The reaction was initiated by addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the coloured complex, indicating the bond formation between Fe(II) ions and ferrozine, was measured at 562 nm. The inhibition percentage of the ferrozine–Fe^{2+} complex formation was calculated as [(A_0-A_s)/As]x100, where A_0 was the absorbance of the control and A_s the one of the polymer solutions.

5.4 Biological characterization

5.4.1 MIC determinations

The antibacterial activity of samples was assessed against *Staphylococcus epidermidis* (ATCC 35984) by the disk diffusion test and broth microdilution assay [46]. The former test was carried out on cellulose disks embedded with 20 µL of a 1 mg/mL polymer solution and placed in Petri plates previously seeded with 10^8 CFU/mL bacterial concentration. Following incubation at 37°C for 24 h, diameters of inhibition zones of bacterial growth were measured.

Broth microdilution assay allowed to determine the minimum inhibitory concentration (MIC) of all copolymer series. *Staphylococcus epidermidis* ATCC 35984 was grown in Triptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) at 37 °C. Briefly, 1 mL of polymer solution at various concentrations was added to 1 mL of microorganism solution at a concentration which gave an optical density reading of 0.1–0.2 at 600 nm.

[45] Carrasco-Castilla J., Hernández-Álvarez A.J., Jiménez-Martínez C., Jacinto-Hernández C., Alaiz M., Girón-Calle J., Vioque J., Dávila-Ortiz G., *Food Chemistry*, **2012**, 135, 1789-1795.
[46] Venkataraman S., Zhang Y., Liu L.H., Yang Y.Y., *Biomaterials*, **2010**, 31, 1751–1756.

Then, a set of dilutions was prepared and inoculated with a 10^6 CFU/mL bacterial concentration. After 24 h incubation at 37 °C, bacterial growth was determined by measuring the absorbance at 600 nm. Broth containing cells alone was used as control. *S. epidermidis* was chosen for its implications in medical device-related infections. All the experiments were done in triplicate. Differences were considered significant for $P < 0.05$.

5.4.2 Evaluation of antifouling polymer properties

The evaluation of the ability of copolymers to control bacterial adhesion was performed by scanning electron microscopy (SEM). Particularly, a section of commercial catheter was coated with pAcDED-co-pAcHTy 70/30 and immersed for 24 h in 2.5 ml of bacterial suspension (0.5 Mac Farland) grown in TSB broth. After incubation the coated device was washed twice with PBS (pH=7.4), fixed with 2.5% glutaraldehyde in 0.1 M PBS buffer (pH 7.4) at room temperature for 30 min, dehydrated through graded ethanol and gold coated by sputtering.

Statistics

Analysis of variance comparisons were performed using MiniTab. Differences were considered significant for *P* values of <0.05. Data were reported as means ± 1 SD.

5.5 Results and discussion

Intrinsically antimicrobial polymers exert their action according to a sequence of events such as absorption on bacterial cell wall, diffusion through the membrane, absorption onto cytoplasmic membrane and its disintegration [19, 20]. To better interact with the wall and cytoplasmic membrane, intrinsically antimicrobial polymers must possess both a hydrophobic region and a hydrophilic one containing strong positive charges. The balance of hydrophobic content and cationic charges is critical to achieve high activity since it has been demonstrated that positive electrical charges are cytotoxic and hemolytic [13].

In the last years, different statistic and amphiphilic block copolymers, showing a good antibacterial efficacy and low toxicity for human cells with respect to their homopolymers, have been developed [47, 48] .

Among amphiphilic antimicrobial polymers, those containing primary or tertiary amines and an inert hydrophobic side have been widely investigated. Polymers and copolymers possessing tertiary ammonium groups, such as polystyrenes [49] and polydiallyamines [50] showed a wide spectrum antimicrobial activity higher than the same series containing quaternary ammonium groups. However, such materials lysed human red blood cells at relatively low concentrations. The lack of discrimination between prokaryotic and eukaryotic cells was ascribed to high hydrophobicity of polymer backbone.

To obtain a suitable balance between antimicrobial activity and toxicity, Sambhy et *al.* [51] synthesized several amphiphilic pyridium-methacrylate copolymers by varying the

[47] Palermo E.F., Vemparala S., Kuroda K., *Biomacromolecules*, **2012**, 13, 1632–1641.
[48] Kuroda K. and Caputo G.A., *WIREs Nanomed Nanobiotechnol*, **2013**, 5, 49-66.
[49] Gelman M.A., Weisblum B., Lynn D.M., Gellman S.H., *Organic letters* 2004, 6, 557-560.
[50] Timofeeva L.M., Kleshcheva N.A., Moroz A.F., Didenko L.V., *Biomacromolecules*, 2009, 10, 2976-2986.
[51] Sambhy V., Peterson B.R., Sen A., *Angew Chem Int Ed*, **2008**, 47,1250–1254.

ratio between positive charges and the alkyl chain length on the polymer. It was observed that for polymers having the same charge/alkyl chain ratio, the spatial separation enhanced biocidal action and reduced toxicity vs cells. In these copolymers the positive charges were born by secondary and tertiary amines.

Palermo et *al.*[52], by comparing polymethacrylate derivatives containing either protonate amines or quaternary ammonium groups, revealed that primary amines were the most effective at conferring good antimicrobial properties to polymers. In addition, primary amines resulted less hemolytic than tertiary ones. As for copolymers containing quaternary ammonium groups, a greater amount of hydrophobic comonomer was required to express activity and more selectivity for *E. coli* versus human red blood cells [53].

Most of investigated polymers and copolymers are based on acrylic monomers containing charged tertiary amine groups bearing methyl residues [6].

Amphiphilic cationic copolymers can be also very interesting to fight the emergent risk of microbial resistance. Specifically, by using two different monomers one bearing an antimicrobial hydrophobic compound and the other one a positive charge, it could be possible to obtain materials hampering the development of resistant strains because of the different action mechanisms exerted against microorganisms.

The main aim of this work was the synthesis of dual-function polymers endowed with antimicrobial and antioxidant properties obtained by copolymerization of two monomers containing tertiary amine and catecholic groups.

Particularly, an ethylacrylic amide monomer containing tertiary amine groups bearing ethyl residues [38], previously synthesized by our group, was copolymerized with a novel active hydrophobic acrylic monomer. The introduction in the tertiary amine group of

[52] Palermo E.F., Lee D.K., Ramamoorthy A., Kuroda K., *J. Phys. Chem. B*, **2011**, 115, 366-375.
[53] Palermo E.F., Kuroda K., *Biomacromolecules*, 2009, 10, 1416-1428.

alkyl residues longer than those used in literature [6] was intended to enhance the polymer hydrophobicity and then improve its interaction with bacterial membrane [38]. Instead the presence of an active hydrophobic spacer exerting not only biocide action but also antioxidant action should ensure the reduction of the emergence of drug resistant bacteria, by suppression of ROS present in the biofilm, and better combat biofilm infections.

A first qualitative evaluation of the synthesized copolymers was performed by ATR-IR spectroscopy. In ***Figure 1***, the spectra of two homopolymers (pAcDED and pAcTy) and 70:30 copolymer are reported in the 4000-650 cm^{-1}spectral region.

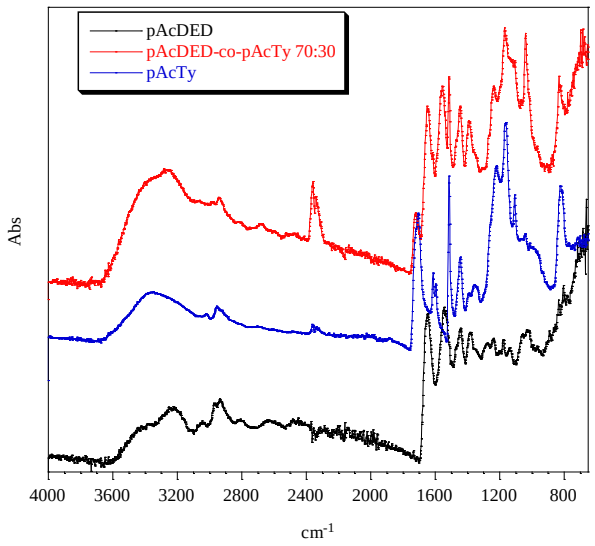


Figure 1: *ATR-IR spectra of pAcDED, pAcTy and pAcDED-co-pAcTy 70:30 copolymer in the spectral range between 4000-650 cm^{-1}.*

In the copolymer spectrum, characteristic absorptions of both homopolymers are observed. In particular, it can be noted at 1720 cm^{-1} the peak related to the ester carbonyl stretching, at 1650 cm^{-1} the stretching of amide carbonyl, at 1554 cm^{-1} the peak relative to the amide NH bending and CN stretching and at around 1510 cm^{-1} the

stretching of C=C bond belonging to tyrosol aromatic ring. In addition, in the range 1150-750 cm^{-1} it was possible to evidence the absorptions related to the C-O-C bending of ester group, to the CN bending of amide group and finally to the CH bending of p-aromatic ring.

Plotting the ratio between the absorbance value at 1720 cm^{-1}, referred to the carbonyl ester of aromatic moiety, and that one at 1554 cm^{-1}, attributed to second amide-band, a linear decrement of such ratio with hydrophobic moiety decreasing in the repeat unit of (pAcDED-co-pAcTy)s was observed (***Figure 2***). In this way, by easy spectroscopic measurements, it was possible to carry out a semi-quantitative evaluation of the molar ratio of the two comonomers in the polymer chain.

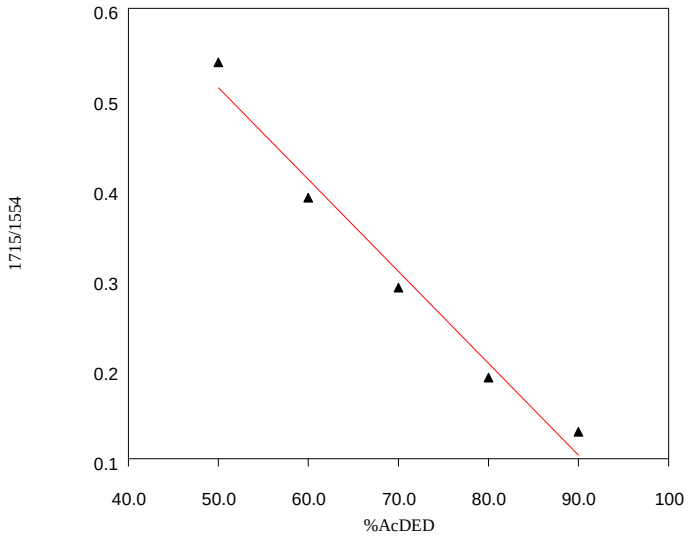


Figure 2: *Abs_{1720}/Abs_{1554} ratio versus content of AcDED comononer*.

The same profile was observed for copolymer bearing hydroxytyrosol moiety, pAcDED-co-pAcHTy (data not shown).

To determine the occurred polymerization reaction and the comonomer molar ratio in the repeat unit, ^{1}H-NMR measurements were carried out on the various synthesized copolymers. First of all, peaks relative to double bond of acrylic group were absent,

confirming that the monomer conversion was quantitative. In addition, for all of copolymers containing phenolic residues, resonances between 6.5-6.6 ppm, relative to aromatic protons, were observed. In the spectral range between 4.2 - 3.1 ppm, the copolymer resonances were covered or modified by the peak of water; while in the 1.0 - 1.8 ppm range, the two broad and very low intensity peaks were attributed to alkylic protons of polymer backbone. Finally, at 0.9 ppm the methyl hydrogen resonance belonging to AcDED moiety was observed (***Figure 3***).

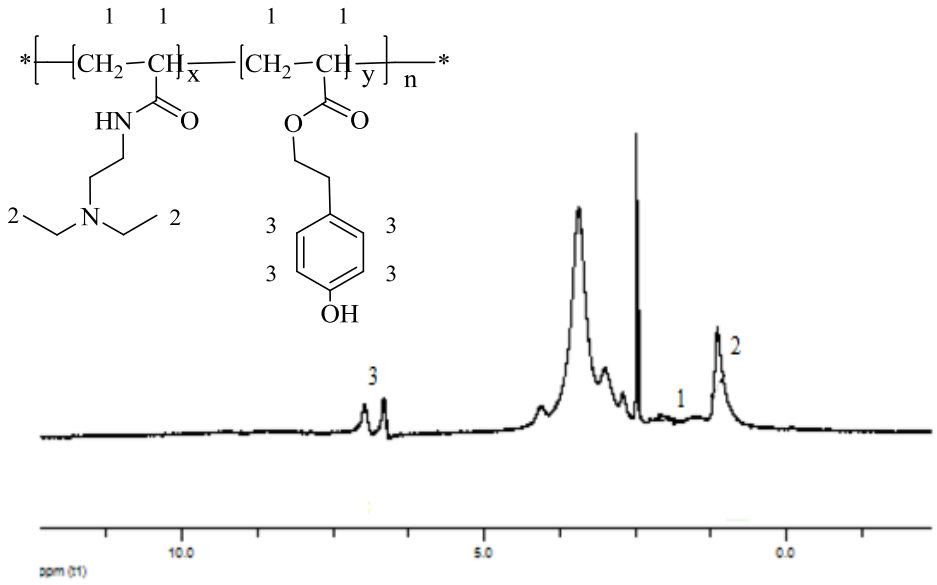
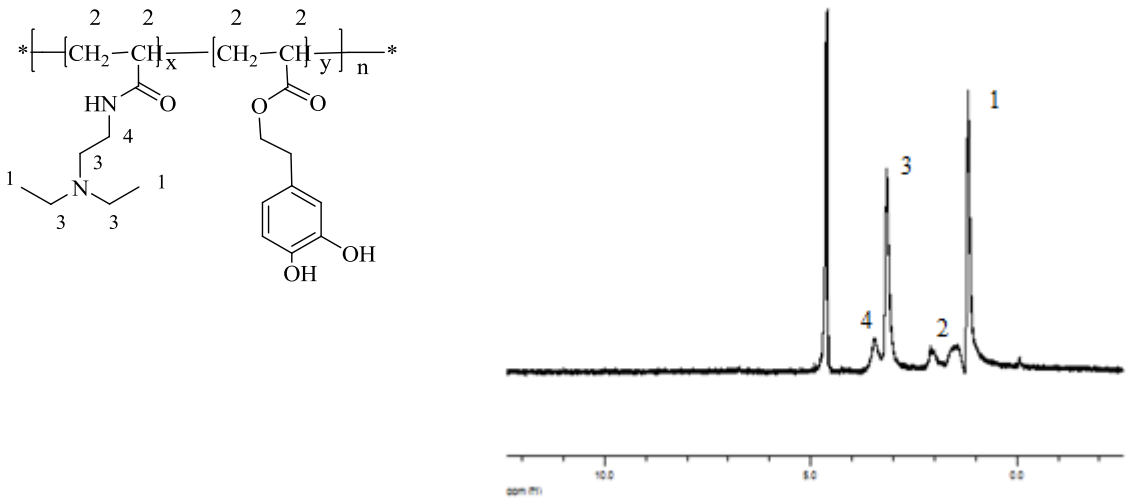


Figure 3*: Repeat unit of the copolymer and* ^{1}*H-NMR spectrum relative to copolymer pAcDED-co-pAcTy 70:30 .*

To evaluate the molar ratio of the comonomers in the repeat unit, the following equation that takes into account the integral values of resonances at 0.9 ppm and 6.5-6-6 ppm was used:

$$AcDED / AcTy = \frac{\frac{\int_{0.9\,ppm}(CH_3 - CH_2 - N)_2}{6}}{\frac{\int_{0.9\,ppm}(CH_3 - CH_2 - N)_2}{6} + \frac{\int_{6.5-6.6\,ppm}(-\mathrm{Ar}\text{-})}{4}}$$

The obtained values were in agreement with the monomer molar ratios used for polymerization. Also elemental analysis confirmed ^{1}H-NMR results (data not reported). ^{1}H-NMR analysis was also exploited to verify the obtainment of catecholic function by regio and chemoselective one-pot oxidation/reduction reaction. The resonances of methylene protons bound to amide nitrogen and to tertiary amine-related nitrogen could be observed between 3-3.5 ppm; while aliphatic protons attributed to polymer main aliphatic chain were between 1.5 and 2 ppm. Finally, at about 0.9 ppm the methyl proton resonance of tertiary amine residue was observed.



(a) ***(b)***

Figure 4*: Repeat unit of the oxidated copolymer* ***(a)****;* ^{1}*H-NMR spectrum of pAcDED-co-pAcHTy 70:30 copolymer after the catecholic function insertion* ***(b)****.*

In all of pAcDED-co-pAcHTy copolymer spectra, the peaks relative to aromatic protons disappeared (***Figure 4***). This is probably due to a supramolecular re-structuring of pseudo- flower-like micellar morphology. Likely, phenyl rings interact each other by stacking interactions leading aromatic protons to be shielded against magnetic field.

Proton shielding effects were already observed by Bütün et *al.* [54] on block copolymers containing two different tertiary amine groups in the side chain of the two employed acrylic monomers. In particular, the authors confirmed the micelle formation from the disappearance of some ^{1}H-NMR signals relative to one of the two blocks containing quaternized tertiary amine groups.

Micellization behaviour of amphiphilic random copolymers have been widely investigated. In this regard, Kawata et *al.* [55] proposed flower micelle model of the minimum loop size. Particularly, they have investigated dependences of hydrophobicity of both hydrophobic and ionic monomer units on the micellar structure. The statistical copolymer micelles had a single hydrophobic core and assumed compact conformations comparable to typical globular proteins. Tominaga et *al.* [56] studied flower micelle arrangement of sodium (2-acrylamido)-2-methylpropanesulfonate and N-dodecylmethacrylamide p(AMPS/C12) random copolymer, in 0.05 M aqueous NaCl by light scattering and atomistic molecular dynamic simulation.

Summarizing, when dissolved in water, amphiphilic random copolymers, bearing hydrophobic and electrolyte units attached to polymer chain, tend to self-assemble to form a hydrophobic core, and to force the main chain to take the loop conformation.

[54] Bütün V., Armes S.P., Billingham N.C., *Macromolecules* **2001**, 34, 1148-1159.
[55] Kawata T., Hashidzume A., Sato T., *Macromolecules* **2007**, 40, 1174−1180.
[56] Tominaga Y., Mizuse M., Hashidzume A., Morishima Y., Sato T., *J. Phys. Chem. B*, **2010**, 114, 11403–11408.

From ^{1}H-NMR data, therefore, a pseudo- flower-like micellar arragment could be also hyphothesized for our pAcDED-co-pAcHTy copolymers.

DLS data (***Table 1***) showed that the copolymer series soluble in water possessed size slightly higher than normal flower micelles reported in literature [57]. These findings could be due to polymer chain swollen-aggregates interacting each other [58]. Indeed, pAcDED-co-pAcAHTy 70:30 bearing the highest aromatic moiety content had size smaller with respect to the other soluble copolymers, suggesting greater hydrophobic interactions. In order to elucidate particle size and the real structure of polymeric aggregates, a more in depth investigation of these systems at different pH by DLS analysis and X-rays measurements is needed. In ***Table 1***, the molecular weight and polydispersity index of water-soluble copolymers, determined by GPC analysis, were also reported. In these measurements, the aggregation phenomena were partially reduced by adding NaCl in the eluent.

POLYMER	$\mathbf{M_n}$ x10^3 (g/mol)	**I=M_w/M_n**	**Size (nm)**
pAcDEDcopAcAHTy7030	8.0	1.85	90±10
pAcDEDcopAcAHTy8020	13.0	1.70	215±15
pAcDEDcopAcAHTy9010	15.0	1.55	300±25

Table 1*. Molecular weight, polydispersity index and DLS size of synthesized water-soluble polymer.*

A particular feature of polymers, especially of copolymers, having hydrophilic and hydrophobic regions, is the microphase separation due to the different chemical nature

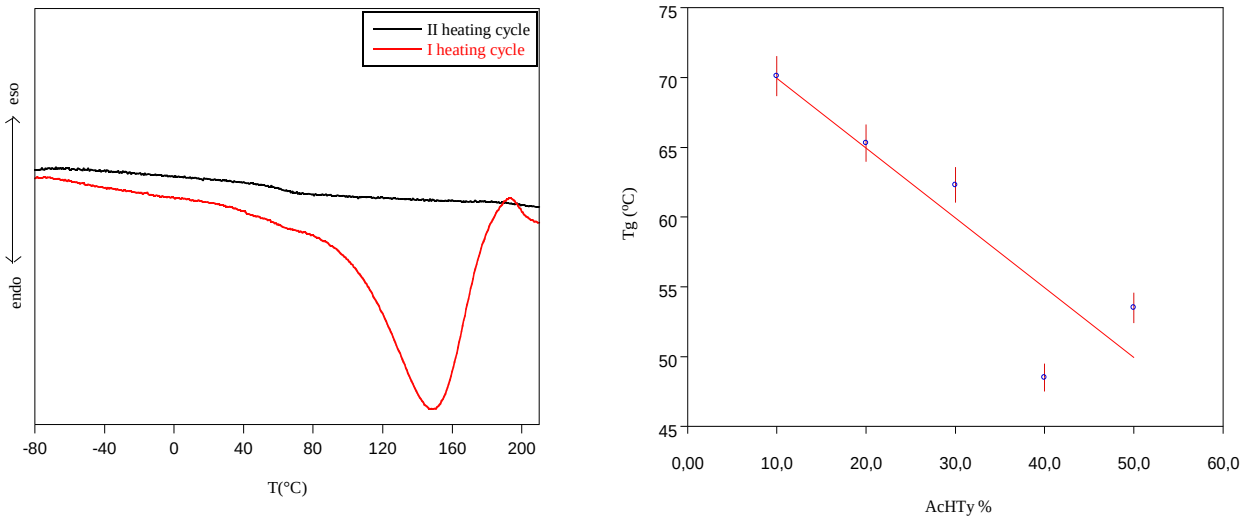
[57] JeongY.II, Nah J.W., Lee H.C., Kim S.H., Cho C.S., *Int. J. Pharm.*, **1999**, 188, 49-58.
[58] Lee E.S., Oh K.T., Kim D., Youn Y.S., Bae Y.H., *J Control Release*, **2007**, 123(1), 19-26.

of the two sides. Thermal transitions, particularly the glass transition temperature (T_g), are affected by possible formation of different intermolecular interactions between hydrophilic/hydrophobic sides [59]. In addition, such interactions can be promoted by the presence of functional groups in the main or side chain. If phase mixing occurs, the mobility of the segments decreases with a consequent increase of T_g value. On the contrary, high interactions between hydrophobic segments or steric hindrance of bulky substituents can lead to an enhancement of phase separation and then of T_g value [60].

The study of hydrophobic/hydrophilic phase separation of the synthesized copolymers was carried out by DSC analysis. Such measurements evidenced substantially amorphous materials with a high hygroscopicity. For this latter issue, two heating cycles in the temperature range from -50 °C to 200 °C were performed (***Figure 5 a***).

In first scan, a broad endothermic peak, around 100 °C, related to weakly adsorbed water can be observed. The disappearance of this peak in the second scan permitted the observation of the glass transition. The profile of glass transition temperature (T_g) versus the content of aromatic monomer (AcHTy) is shown in ***Figure 5 b***.

[59] Quattrociocchi G., Francolini I., Martinelli A., D'Ilario L., Piozzi A., *Polym Int* , **2010**, 59, 1052–1057.
[60] Francolini I., D'Ilario L., Guaglianone E., Donelli G., Martinelli A., Piozzi A., *Acta Biomaterialia* , **2010**, 6, 3482–3490.



(a) ***(b)***

Figure 5: *DSC thermograms of pAcDED-co-pAcHTy 70:30 copolymer (a); T_g profile versus AcHTy content (b).*

As it can be noted, a decrease of T_g value with incrising of hydrophobic monomer amount was observed. This can be due to a plasticizer effect exerted by AcHTy monomer presumably caused by the steric hindrance of the hydroxytyrosol aromatic rings. The stacking interactions among aromatic rings probably improved the chain flexibility of cationic region leading to a greater phase separetion. In fact, the pAcDED-co-pAcHTy 70:30 copolymer possessing the lowest molecular size showed the lowest glass transition and then the highest phase separation.

To evaluate the effect of amphiphilic copolymer structure and composition on their antibacterial activity against *S.epidermidis*, the broth microdiluition test was performed on all of copolymers bearing catecholic moiety. (pAcDED-co-pACHTy)s showed lower MIC against *S.epidermidis* with respect to both previously synthesized homopolymers [38, CHAPTER IV] (***Figure 6***). This finding could be explained by considering several factors. Generally, biological activity increases with hydrophobic side increasing, then with aromatic groups [17] . In fact, in the copolymer pAcDED-co-pAcHTy 70:30 a probable

optimum amphiphilic balance could have been reached[17] . Also the introduction of high amount of a second antimicrobial group (catecholic moiety), such as in pAcDED-co-pAcHTy 70:30, could have improved the biocidal activity by a synergistic action with ammine groups. Moreover, the good killing activity of the copolymers could be attributed to their reduced sizes with respect to homopolymers [38] that permit an efficient cellular uptake of the antimicrobial complexes.

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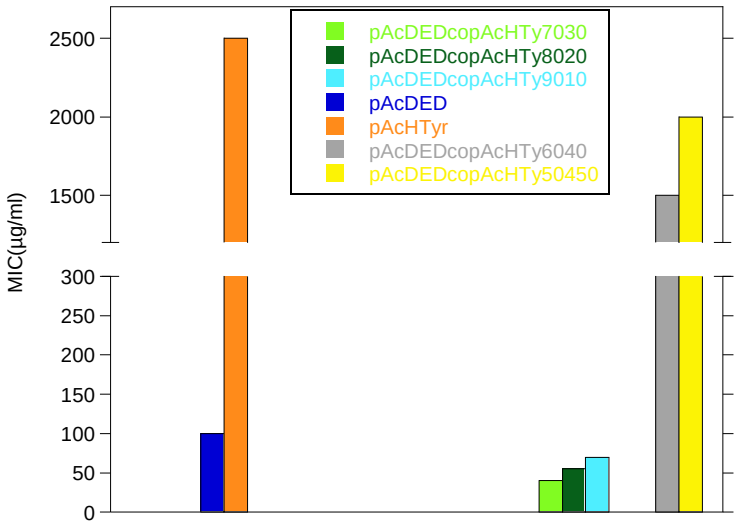

Figure 6: *MIC values of all synthesized copolymers compared to those of pAcDED and pAcHTy homopolymers.*

In general, MIC values found for all copolymers were comparable to those reported in literature for amphiphilic polymers or polymers based on ammonium salts [13, 24]. In addition, since phenolic groups were found to exert biocidal activity against many fungi [61, 62] and different Gram-positive and negative, we can hypothesize that our hydroxytyrosol-based copolymers could have a broad spectrum antimicrobial activity.

[61] Iemma F., Puoci F., Curcio M., Parisi O.I., Cirillo G., Spizzirri U.G., Picci N., *J. Appl. Polym. Sci.*, **2010**, 115, 784-789.
[62] Park E.S., Moon W.S., Song M.J., Kim M.N., Chung K.H., Yoon J.S., *Int. Biodeterior. Biodegrad.*, **2001**, 47, 209–214.

To assess the possible application of synthesized copolymers as materials for coating of medical devices, a commercial intravascular catheter in polyurethane was coated with the most active copolymer pAcDED-co-pAcHTy 70:30. The coating procedure involved the immersion of a catheter segment (approximately 2 cm) in a solution of the oxidized copolymer but not yet reduced by sodium dithionite. To obtain an adequate coating thickness such a procedure was repeated several times. The reduction reaction was then carried out on the polymer film adhered on the catheter surface. The resulting coating was assessed by ATR-IR while coating thickness resulted of about 50 microns was measured with a gauge. Finally, the ability of copolymer pAcDED-co-pAcHTy 70:30 to inhibit bacterial adherence was evaluated through SEM observations.

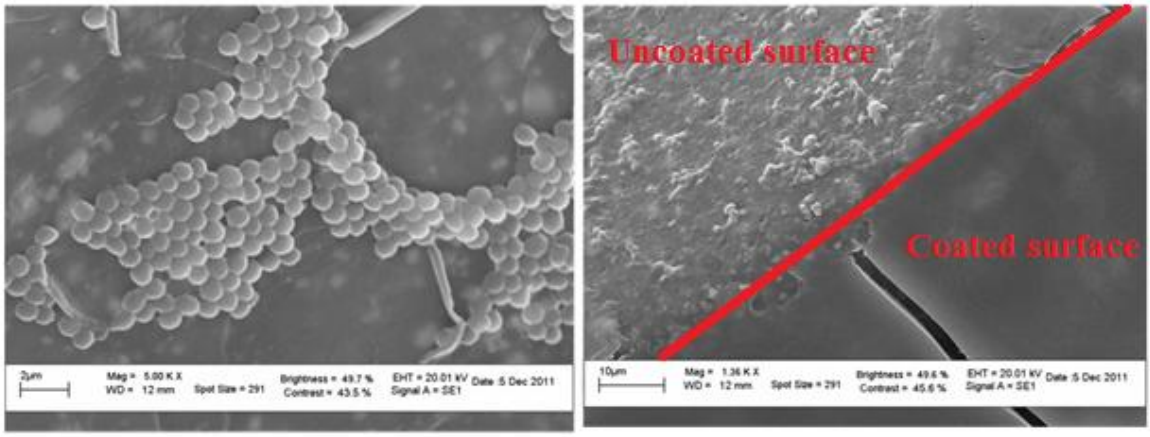

Figure 7: *SEM micrographs of a) uncoated and b) polymer-coated catheter surface after contact with bacteria.*

Figure 7 shows SEM micrographs of uncoated and polymer-coated catheter surface after contact with bacteria. In particular, the image related to the coated catheter (***Figure 7 b***) was taken on a boundary region where the separation between the coated and

uncoated surface was evident. As it can be noticed, uncoated catheter was totally colonized (***Figure 7 a***) contrary to the coated catheter region (***Figure 7 b***). However, in the ***Figure 7 b***, specifically in the uncoated region, bacterial colonization with biofilm formation was observed. These evidences showed that the coating of medical devices with the most active copolymer synthesized by us is promising for counteracting *S. epidermidis* biofilm formation.

The use of these copolymers should also permit to fight microbial resistance not only because they act against bacteria through two action mechanisms but also because they are able to block ROS production inside the biofilm.

To evaluate this latter property, the antioxidant features of synthesized copolymers were evaluated by the DPPH method. DPPH is a stable organic radical that may be converted to a colorless neutral molecule when accepting a proton from any hydrogen donor (***Figure 8***). Among the synthesized copolymers, those water-soluble maintained a high scavenging activity with respect to hydroxytyrosol-based polymer (EC_{50} = 0.80 ± 0.02) . They exerted their action in a dose dependent manner due to the different amount of catecholic moieties (***Table 2***).

Figure 8: *Color variation of DPPH solutions after contact with different amount of copolymers.*

POLYMER	EC_{50}*	% chelate ferrous
pAcDED-co-pAcAHTy 50:50	0.93 ± 0.06	95 ± 5 %
pAcDED-co-pAcAHTy 60:40	0.88 ± 0.05	90 ± 6 %
pAcDED-co-pAcAHTy 70:30	0.48 ± 0.02	85 ± 5 %
pAcDED-co-pAcAHTy 80:20	0.64 ± 0.03	65 ± 3 %
pAcDED-co-pAcAHTy 90:10	0.76 ± 0.03	25 ± 7 %

*EC_{50}= moles of antioxidant/mole DPPH

Table 2. *Antioxidant activity versus DPPH and chelating capacity versus ferrous ions of the synthesized copolymers.*

The main strategy to prevent catalysis of hydroperoxide decomposition and Fenton type reactions is chelation of transition metals. Particularly, the transition metal ion Fe^{2+} permits the formation and propagation of many radical reactions.

To estimate ferrous ion chelating activity of the synthesized copolymers the ferrozine assay was used. Ferrozine normally forms stoichiometric complexes with Fe^{+2} ions. In presence of other chelating agents, the Ferrozine-Fe^{+2}-complex formation is not allowed thus causing a reduction of complex red coloration. Therefore, the chelating activity can be easily estimated by spectroscopic UV-Vis measurements. The obtained results suggested that copolymers had a good ferrous ion chelating ability, thus forming a more stable complex than ferrozine (***Table 2***). In particular, the ferrous ion chelating percentage improved with increasing of catecholic moiety concentrationin the copolymer repeat unit.

5.6 Conclusions

Herein, we have reported for the first time the synthesis of amphiphilic copolymers bearing two different antimicrobial moieties. Particularly, a tertiary amine and a catecholic functional group in the side chain of copolymers were introduced at different molar ratios. All of the obtained copolymers had amorphous features and up to 0.3 molar ratio of cathecolic groups they were also water soluble. ^{1}H-NMR and DSC measurements showed that aromatic pendant groups interact each other probably leading to a supra-macromolecular architecture and an enhanced phase separation. The phase separation could justify the copolymer size decreasing with the aromatic amount increasing. Indeed, the water soluble copolymer with the highest catecholic content (pAcDED-co-pAcHTy 70:30) possessed a mean size value of 90 nm, as shown by DLS measurements. The better biological activity of the water soluble copolymers was probably due to several factors such as their reduced size permitting an efficient cellular uptake of the antimicrobial complexes, an optimum amphiphilic balance as well as a synergistic biocidal action. In addition, all of the copolymers showed both good antioxidant activity and ferrous ion chelating ability. To evaluate the possible use of these novel biocidal copolymers in the medical field, the toxicity of these materials on animal and human cells will be assessed in future investigations.

APPENDIX

SYNTHESIS AND PRELIMINARY CHARACTERIZATION OF SOLUBLE RANDOM COPOLYMERS BASED ON DOPAMINE.

A.1 Introduction

Amphiphilic random copolymers have been widely employed for developing antimicrobial polymer mimics of amphiphilic active structures of antimicrobial helix-forming peptides such as magainin[1]. Such copolymers can arrange in conformations showing separated domains or facially amphiphilic structures obtained by separation of cationic side chains and hydrophobic segments under the influence of the cell membrane interface[2,3].

It has been recently demonstrated [4,5] that amphiphilic block copolymers showing selective activity against bacteria over human cells can be also good candidates to substitute antibiotics[1,4].

Indeed, vinyl ether di-block copolymers prepared by cationic-activated polymerization[4] and di-block copolymers of 2-(N,N-dimethylamino)ethyl methacrylate and butyl methacrylate, obtained by Atom Transfer Radical Polymerization (ATRP) and possessing cationic and hydrophobic segments, displayed both biocidal effects against bacteria and poor hemolysis. Random copolymers with the same ratio of cationic and hydrophobic groups were instead more hemolytic, although they showed similar biocidal activity.

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[1] Kuroda K. and Caputo G.A., *WIREs Nanomed Nanobiotechnol*, **2013**, 5, 49-66.
[2] Palermo E.F., Vemparala S., Kuroda K., *Biomacromolecules*, **2012**, 13, 1632–1641.
[3] Mowery B.P., Lee S.E., Kissounko D.A., Epand R.F., Epand R.M., Weisblum B., Stahl S.S., Gellman S.H., *J Am Chem Soc*, **2007**, 129, 15474–15476.
[4] Oda Y., Kanaoka S., Sato T., Aoshima S., Kuroda K., *Biomacromolecules*, **2011**, 12, 3581–3591.
[5] Wang Y.Q., Xu J.J., Zhang Y.H., Yan H.S., Liu K.L., *Macromol Biosci*, **2011**, 11, 1499–1504.

Even if these block copolymers seem to form vesicles or micelles at high concentration, it was observed that they display antimicrobial activity even below the critical micelle concentration (CMC)[1]. Such a finding suggested that the conformation of single polymer chains can be responsible for the biological activity. [6] Indeed, it was hypothesized that water soluble segments, shielding the hydrophobic segments, lead to the formation of a core-shell structure thus avoiding the interaction of the hydrophobic core with the aqueous media. As a result, an intramolecular or intermolecular micellar structure is formed.

Therefore, these micelles are constituted of nano-aggregates with a core of insoluble blocks surrounded by flexible soluble blocks. These micelles can be spherical, or differently shaped, with narrow size distribution depending on the experimental conditions. These sophisticated materials offer many advantages including high drug loading efficiency without chemical modification, ease preparation, colloidal stability with low critical micelle concentration (CMC), predictable size with narrow size distribution, ability to protect drugs from possible deactivation and improved pharmacokinetics[7, 8, 9].

In order to achieve suitable molecular architectures, it is crucial the synthesis of a copolymer with a well-defined structure. This can be obtained employing synthetic strategies able to give control of stoichiometry and regioselectivity of each chain segment. Therefore, anionic[10, 11] or cationic polymerization[4], ring-opening polymerization (ROP)[12,13], radical living polymerizations, such as Reversible Addition

[6] Chu B., *Langmuir*, **1995**, 11, 414–421.
[7] Mikhail A.S., Allen C., *J Control Release*, **2009**;138, 214–23.
[8] Yoon H.J., Jang W.D., *J Mater Chem*, **2010** , 20, 211–22.
[9] Pokorny J., *Eur. J. Lipid Sci. Technol.*, **2007**, 109, 629-642.
[10] Tessmar J.K., Mikos A.G., Gopferich A., *Biomacromolecules*, **2002**, 3, 194– 200.
[11] Zhang S., Qing J., Xiong C., Peng Y., *J. Polym. Sci., Part A, Polym. Chem.*, **2004**, 42, 3527– 3536.
[12] Kang N., Leroux J.C., *Polymer*, **2004**, 45, 8967–8980
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Fragmentation Transfer (RAFT)[14] or ATRP[15,16,17], as well as combinations of two of these techniques[18] were used for the synthesis of these antimicrobial polymer mimics of natural peptides.

Polymeric micelles thanks to their ability to self-assemble into core-shell nanostructures offer attractive features not only for delivery of a large array of drugs but also as emulsion stabilizers, surface modifiers and carriers in gene therapy.[19,20]

The aim of this work was to obtain a dopamine –based homopolymer possessing antimicrobial and antioxidant properties. Differently from the insoluble hydroxytyrosol-based one, obtained in the work described in (Chapter IV), this new dopamine-based homopolymer is water-soluble. To avoid cross-linking reactions, the catecholic groups of dopamine (DOPA) were protected with t-butyldimethylsilyl (TBDMSiCl) chloride. Instead, to obtain a homopolymer with narrow molecular weight distribution, ATRP polymerization was employed. In addition, in order to improve the biological activity in terms of poor cytotoxicity and hemolysis of final materials, random copolymers with defined size and segment length were synthesized by using polyethylene glycol methacrylate (PEGMA).

In this appendix, the synthesis and characterization of the DOPA-based monomer, homopolymer and three DOPA-PEGMA-based random copolymers was reported. This work was carried out in the laboratory of Prof. Nicola Tirelli in Manchester.

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[14] Hong C.Y., You Y.Z., Pan C.Y., *J. Polym. Sci., Part A, Polym. Chem.*, **2004**, 42, 4873– 4881.
[15] Ranger M., Jones M.C., Yessine M.A., Leroux J.C., *J. Polym. Sci., Part A, Polym. Chem.*, **2001**, 39, 3861– 3874.
[16] Dufresne M.H., Le Garrec D., Sant V., Leroux J.C., Ranger M., *Int. J. Pharm.*, **2004**, 277, 81– 90.
[17] Sant V.P., Smith D., Leroux J.C., *J. Control. Release*, **2004**, 97, 301–312.
[18] Vo C.D., Cadman C.J., Donno R., A.C.M. Goos J. , Tirelli N., *Macromol. Rapid Commun.*, **2013**, 34, 156−162.
[19] Riess G., *Prog. Polym. Sci.*, **2003**, 28, 1107–1170.
[20] Gaucher G., Dufresne M.H., P. Sant V., Kang N., Maysinger D., Leroux J.C., *Journal of Controlled Release*, **2005**, 109, 169–188.

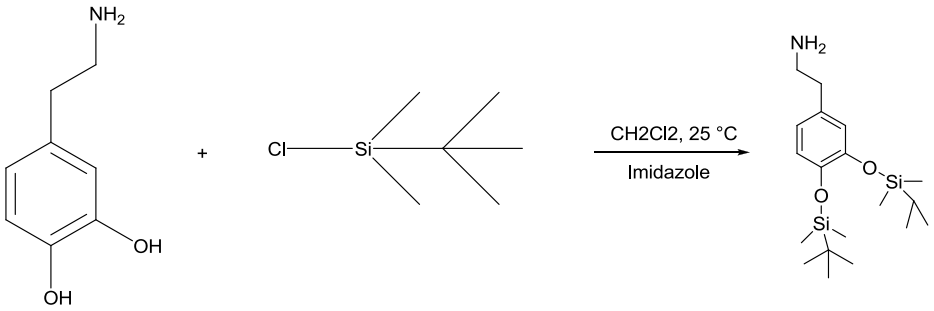
A.2 Experimental methods

A.2.1 Materials

Hydrochloride Dopamine (DOPA), Imidazole, t-butyldimethylsilyl chloride (TBDMSi), acryloyl chloride (Ac), thrietylamine, ethyl 2-bromo-2-methylpropanoate (EBIB), 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA), polyethyleneglycolmethacrylate (M_n = 360 Da) (PEGMA).

A.2.3 Monomer and polymer synthesis

2-(3,4-bis((tert-butyldimethylsilyl)oxy)phenyl)ethanamine (DOPA-TBDMSi)



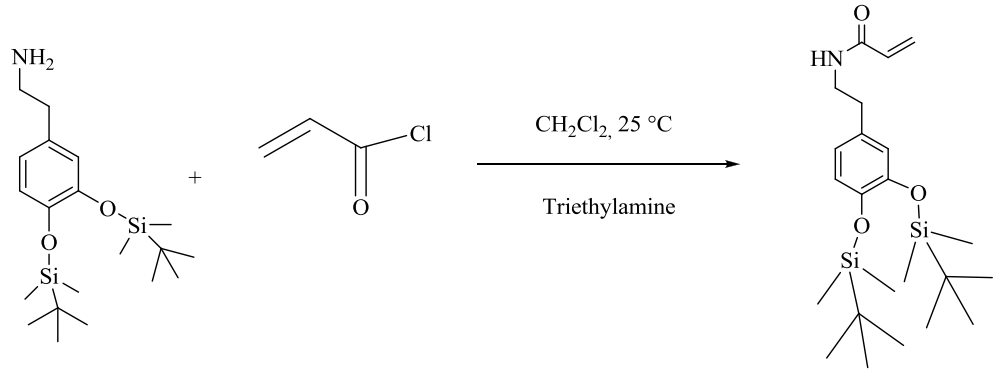
Sheme1. *Synthesis of DOPA-TBDMSi*

A mixture of hydrochloride Dopamine (2.12 mmol) and Imidazole (10.6 mmol) was added to a dry round-bottom flask equipped with a stir bar. After sealing it with a rubber septum, the flask was degassed and filled with argon three times and then left under argon flux. Then, dichloromethane (20 ml) was added via a syringe. A solution of t-butyldimethylsilyl chloride (6.37 mmol in 10 ml of dichloromethane) was added to the mixture and the solution was stirred overnight at RT (***Scheme 1***). After precipitation

and filtration of imidazole salt, the filtrate was extracted twice with $NaHCO_3$ saturated aqueous solution and twice with 1 N HCl aqueous solution. Finally, the evaporation of extracted under vacuum provided a pale yellow oil that was then subjected to flash chromatography on SiO_2 and CH_2Cl_2-CH_3OH 9/1.

^{1}H-NMR spectra were performed employing Chloroform ($CDCl_3$) as a solvent. The obtainment of DOPA-TBDMSi monomer-precursor was confirmed by ^{1}H-NMR (300MHz, $CDCl_3$) : DOPA-TBDMSi δ= 0.17 (s, 12H, -Si(CH_3)$_2$C-), 0.95 (s, 18H, -Si(CH_3)$_2$C(CH_3)$_3$), 2.75 (t, J= 9 Hz, 2 H, $NH_2CH_2CH_2$Ph), 2.93 (t, J= 9Hz, 2H, $NH_2CH_2CH_2$Ph), 6.67-6.79 (m, 3H, aromatic protons).

N-(3,4-bis((tert-butyldimethylsilyl)oxy)phenethyl)acrylamide (DOPA-TBDMSi-Ac)



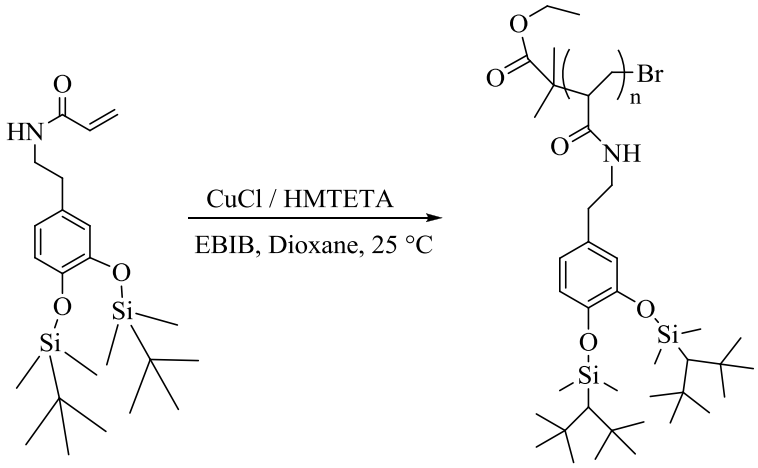
Sheme2. *Synthesis of DOPA-TBDMSi-Ac*

A solution of DOPA-TBDMSi (2.13 mmol in 20 ml of CH_2Cl_2) was added to a dry and previously degassed round-bottom flask equipped with a stir bar and dropping funnel. Triethylamine (4.26 mmol) and, drop to drop, a solution of acryloyl chloride (Ac, 4.26 mmol in 15 ml of CH_2Cl_2) were further added under argon flux. The solution was stirred overnight at RT (***Scheme 2***).

After filtration, the precipitate was subjected to flash chromatography on SiO_2 and CH_2Cl_2:CH_3OH, 98%:2%.

To confirm the coupling reaction occurred between the monomer DOPA-TBDMSi and the Ac, ^{1}HNMR analysis was carried out: DOPA-TBDMSi-Ac δ= 0.01 (s, 12H, -Si(C*H*$_3$)$_2$C-), 0.80 (s, 18H, -Si(CH$_3$)$_2$C(C*H*$_3$)$_3$), 2.54 (t, J= 6 Hz, 2 H, -CONHCH$_2$C*H*$_2$Ph), 3.36 (t, J= 6Hz, 2H, -CONHC*H*$_2$CH$_2$Ph), 5.43(dd, J_1= 1.3 Hz, J_2= 10.3Hz, 1H, C*H*H=CH), 5.82 (dd, J_2= 10.3 Hz, J_3= 17 Hz, 1H, CHH=C*H*), 6.07 (dd, J_1= 1.3 Hz, J_3= 17 Hz, 1H, C*H*H=CH), 6.43-6.59 (m, 3H, aromatic protons).

DOPA-TBDMSi-Ac homopolymer



Scheme 3. *Synthesis of DOPA-TBDMSi-Ac homopolymer.*

ATRP of DOPA-TBDMSi-Ac was carried out by employing 30%wt in Dioxane solutions at 25 °C. Typically, EBIB as initiator (0.04 mmol), DOPA-TBDMSi-Ac (1.2 mmol, target DP = 30), and HMTETA as ligand (0.04 mmol) were introduced into a 25 ml schlenk tube.

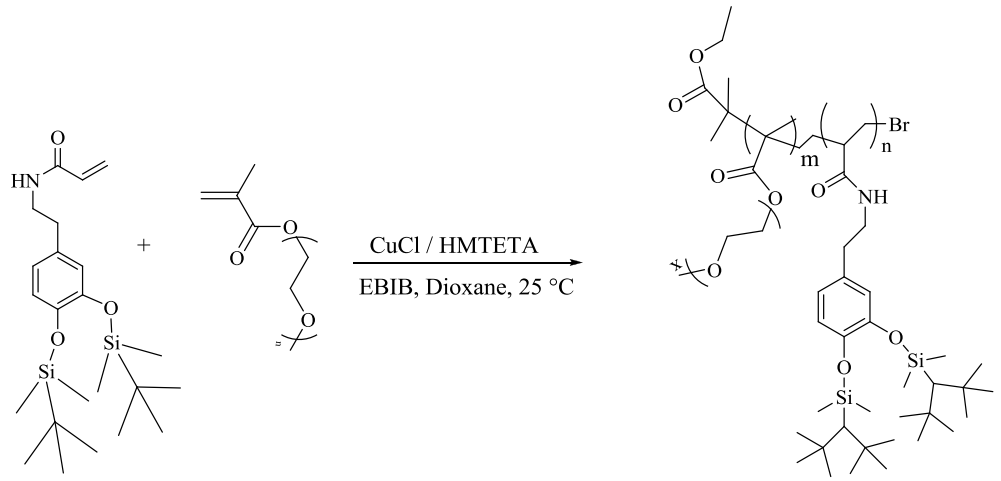
The mixture was bubbled with argon for 30 min prior the addition of degassed 1,4-dioxane. Finally, the copper catalyst was added to mixture under argon purging. The

mixture colour turned dark blue-green, indicating the onset of polymerization. The polymerization was carried out under magnetic stirring at 20 °C for a required time and terminated by dilution with aerated THF. At this point, the solution turned yellow-green, indicating the copper oxidation.

The polymerization kinetics was followed by ^{1}H-NMR analysis. During the polymerization the strong aromatic stacking interactions lead to a split of the aromatic proton signals, and then different chemical shifts for the polymer and free monomer were observed.

DOPA-TBDMSi-Ac-homopolymer δ= 0.06 (m, 12H, -Si(C*H*$_3$)$_2$C-), 0.86 (m, 18H, -Si(CH$_3$)$_2$C(C*H*$_3$)$_3$), 2.64 (t, J= 6 Hz, 2 H, -CONHCH$_2$C*H*$_2$Ph), 5.51(dd, J$_1$= 1.3 Hz, J$_2$= 10.3Hz, 1H, C*H*H=CH), 5.92 (dd, J$_2$= 10.3 Hz, J$_3$= 17 Hz, 1H, CHH=C*H*), 6.19 (dd, J$_1$= 1.3 Hz, J$_3$= 17 Hz, 1H, C*H*H=CH), 6.5-6.7 (m, 3H, aromatic protons), 7.01-7.2 (m, 3H, aromatic protons belonging to the polymer).

DOPA-TBDMSi-Ac PEG methacrylate(PEGMA) copolymers



Scheme4. *Synthesis of DOPA-TBDMSi-Ac PEG methacrylate(PEGMA) copolymers*

PEGylation reaction concerns the covalent attachment of polyethylene glycol (PEG) chains to another molecule. The first example of "PEGylation" was reported in the 70's [21]. PEG covalent attachment to agents such as drugs, proteins or inorganic-nano-systems [22] is used to mask the agent to the host's immune system (reduced immunogenicity and antigenicity), and to increase the hydrodynamic volume (size solution) of the agent. This can enhance the circulation time of active systems in the blood avoiding an immune response.

PEGylation can also provide hydrophobic drugs and proteins with more water solubility and higher mobility in solution, as well as decrease their toxicity and immunogenicity.

ATRP copolymerisation of DOPA-TBDMSi-Ac and PEGMA (Mw=360 Da) was performed in 30%-wt dioxane solutions at 25°C by employing different PEGMA: DOPA-TBDMSi-Ac molar ratio, precisely 0.65, 0.75 and 0.85. As an example, to achieve a PD=30 (polymerization degree), EBIB as an initiator (7.9 mg, 0.04 mmol), DOPA-TBDMSi-Ac (80 mg, 0.18 mmol), PEGMA (370 mg, 1.02 mmol, 0.85) and HMTETA as a ligand (9.3 mg, 0.04 mmol) were introduced into a 25 ml schlenk tube (***Scheme 4***).

From this point, the experimental procedure was the same of that used for the homopolymer synthesis. Also in this case, the polymerization kinetics was followed by ^{1}H-NMR analysis.

DOPA-TBDMSi-Ac-copolymer δ= 0.62 (s, 12H, -Si(C*H*$_3$)$_2$C-), 1.42 (s, 18H, -Si(CH$_3$)$_2$C(C*H*$_3$)$_3$), 1.49-1.58 (m, 3H, C*H*$_3$-CH$_2$-O- of EBIB), 1.63-1.72 (m, 6H, poly-C(C*H*$_3$)$_2$- of EBIB; 2H, polymer backbone), 2.34 (d, J=6.5 Hz, 3H, CC*H*$_3$=CH of PEG), 2.62(d, J=7.2 Hz, 3H, CC*H*$_3$=CH of PEG into the polymer), 2.78 (t, J= 9 Hz, 2 H, -

[21] Siegwarta D.J., Oh J.K., Matyjaszewski K., *Progress in Polymer Science*, **2012**, 37, 18-37.
[22] Kotsokechagia T., Zaki N.M., Syres K., Leonardis P.D., Thomas A., Cellesi F., Tirelli N., *Langmuir*, **2012**, 28, 11490-11501.

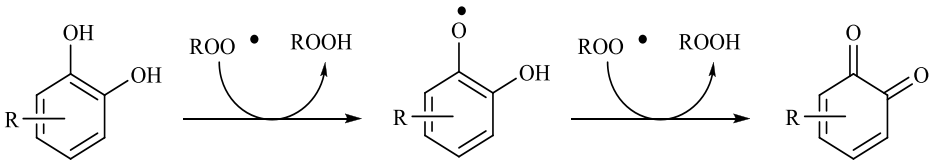
CONHCH$_2$C*H*$_2$Ph into the polymer) 3.11 (t, J= 6.9 Hz, 2 H, -CONHCH$_2$C*H*$_2$Ph), 3.78-4.27 (m, PEG monomer and the same protons in the polymer backbone, dioxane, 4.67 (t, J= 6Hz, 2H, O-C*H*$_2$CH$_2$-), 6.02 (s, 1H, C*H*CH$_3$=CH), 6.5 (s, 1H, CHCH$_3$=C*H*), 7.08-7.20 (m, 3H, aromatic protons).

A.3 Results and discussion

Phenol- or benzoic acid-based polymers are biocidal agents having the bacteria membrane as a target. They can either interact with the surface of the cell, causing the membrane disintegration, or promote intracellular coagulation of cytoplasmic components, leading to the cell growth inhibition or death.

The main natural antioxidants are characterized by the presence, in their chemical structure, of phenolic groups, particularly, catechol, hydroquinone or gallic moieties. Such compounds are very effective in protecting plant against insects [9] but are highly toxic.

The peculiar antioxidant activity of these phenols, in particular of the catechol structure system, is due to semiquinone, formed in the first stage of the reaction, which can inactivate a second radical converting to quinone (***Scheme 5***) [23] .

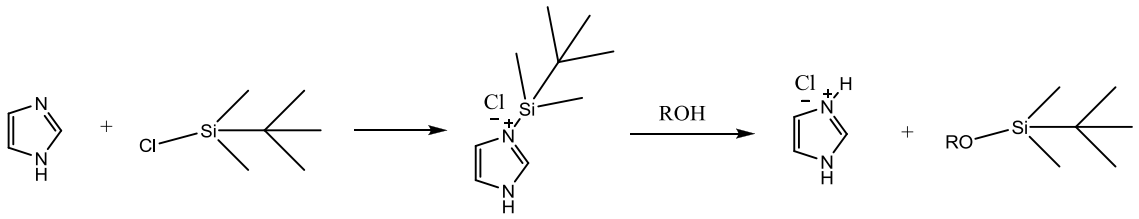


Scheme 5. *Antioxidant activity of catechol moieties.*

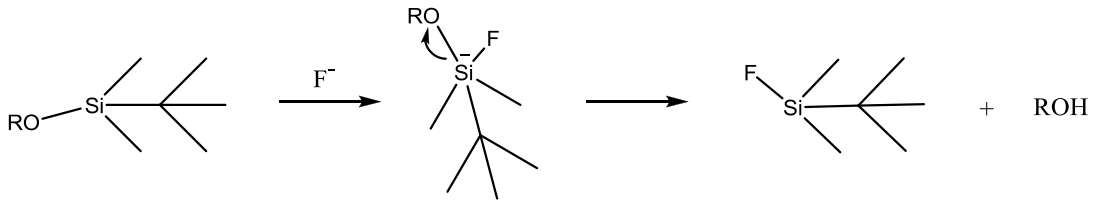
The monomer precursor DOPA-TBDMSi was obtained taking advantage of the Corey's reaction[24] where imidazole behaves both as a base and catalyst with a yield of 75 %.

[23] Amatori R., Ferrani F., Lucarini M., Pedulli G. F., Valgimigli L., *J. Org. Chem.*, **2002**, 67, 9295.

The reaction proceeds via *N*-tert-butyldimethylsilylimidazole, a very reactive silylating agent:



Corey discovered[25] the rapid cleavage of the silyl ethers to alcohols by treatment with 2-3 eq. tetra-*n*-butylammonium fluoride (TBAF) in THF at 25 °C. Nucleophilic attack of fluoride anion leads to a pentavalent silicon centre which is permitted due to hybridization of the vacant d-orbitals of silicon. Moreover, the formation of the strong Si-F bond is the driving force for a fast and quantitative cleavage



Tert-butyldimethysilyl ether protectors are stable in alkaline environment but can be converted back to alcohols under acidic conditions (2:1 acetic acid / water at 25 °C).

The coupling reaction between Ac and DOPA-TBDMSi was carried out in CH_2Cl_2 with 55% yield, after flash chromatography purification. The resulting monomer DOPA-TBDMSi-Ac was a viscous yellow oil.

The occurred reaction was verified by ^{1}H-NMR and ATR-FTIR spectroscopy. In particular, in the spectrum of DOPA-TBDMSi-Ac new peaks around at 1663 cm^{-1} related to the carbonyl stretching (first amide peak) and at 1550 cm^{-1} due to the second amide peak (C-N stretching and NH bending) were observed (Figure 1).

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[24] Corey E. J., *J. Am. Chem. Soc.* **1972**, *94*, 6192
[25] Corey E. J., *J. Am. Chem. Soc.*, **1972**, *94*, 6190-6191

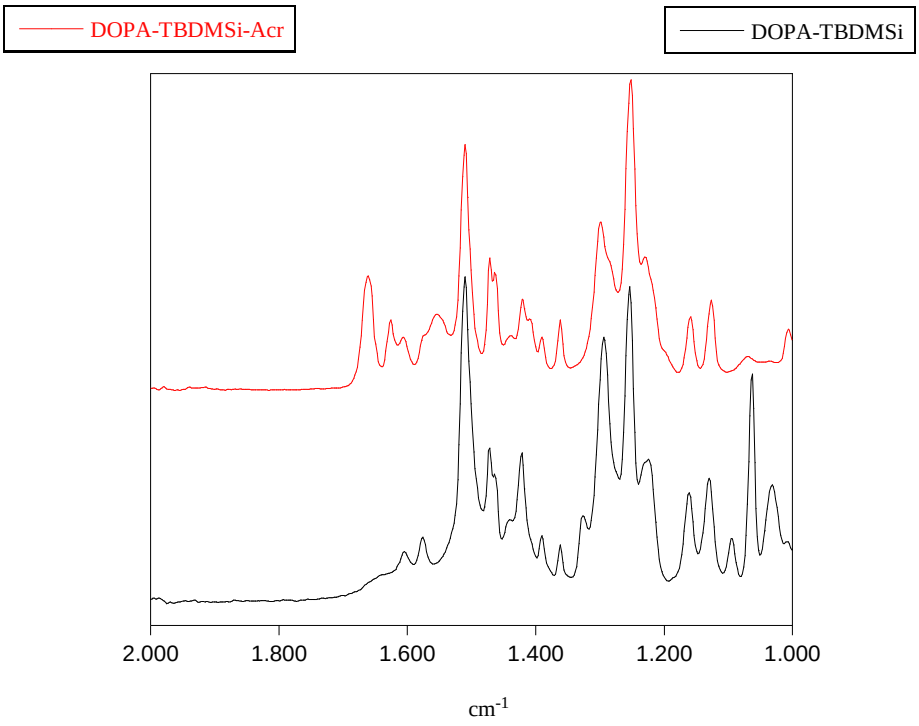
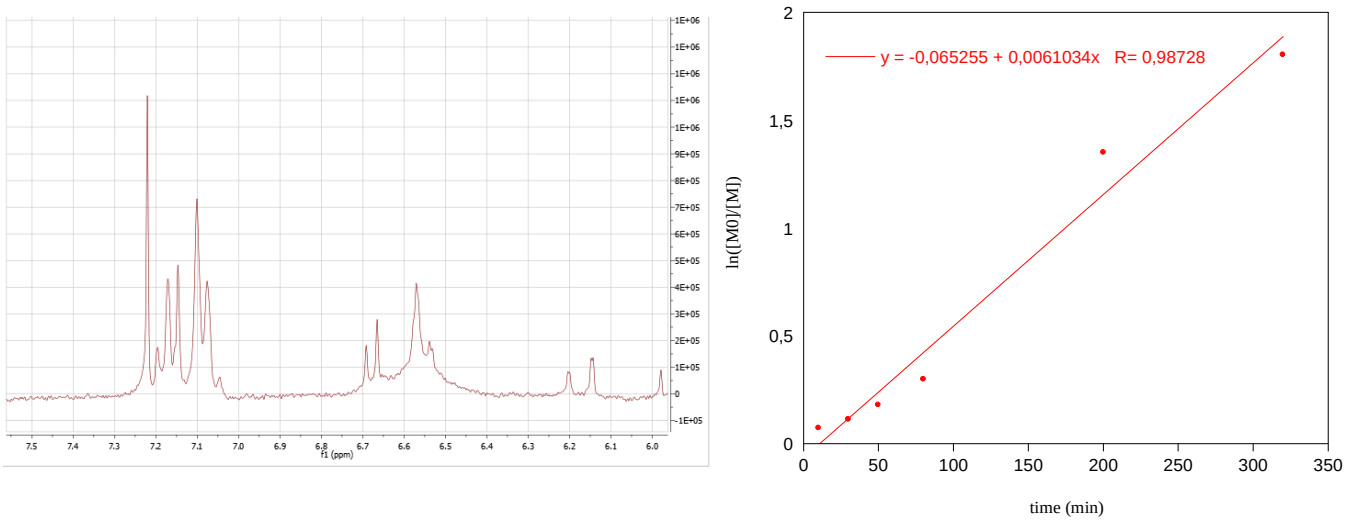


Figure 1: *Spectra of DOPA-TBDMSi and DOPA-TBDMSi-Ac.*

Polymerization kinetics were performed directly in sealed NMR tubes by adding CuBr to the reactant mixture under vigorous argon flux. The ^{1}H-NMR spectra were collected after 20 minutes in order to achieve the complete monomer conversion. As for copolymer series, the reactivity ratio (r_1 and r_2) was also taken out.

For evaluating the monomer conversion, ln([Mo]/[M]) vs time was plotted, where [Mo] was the monomer concentration in the feed and [M] was the monomer residue concentration after each ^{1}H-NMR measurements. As it can be observed in ***Figure 2 b***, a straight line showing a 1st order kinetics was obtained. To determine the [M] value, the ratio between the peak integral at 5.51 ppm, due to one of the vinylic proton, and the integral peak at 0.06 ppm, related to the 12 protons of the silicon group, which remained stable during the polymerization, was exploited (***Figure 2 a***).



(a) (b)

Figure 2: *^{1}H-NMR spectrum of homopolymer and ln([Mo]/[M]) vs time plot.*

Conversion studies showed that methacrylic monomer resulted by far more reactive than the acrylamide one. The same result was obtained by using the Fineman-Ross and Kelen-Tudos methods.

The fastest and oldest model is the Fineman-Ross [26] one. The model is due to the following equation:

$G=r_1H-r_2$ (a)

where $G=\frac{X(Y-1)}{Y}$, $H=\frac{X^2}{Y}$, $X=\frac{f_1}{f_2}$, and $Y=\frac{F_1}{F_2}$ (b)

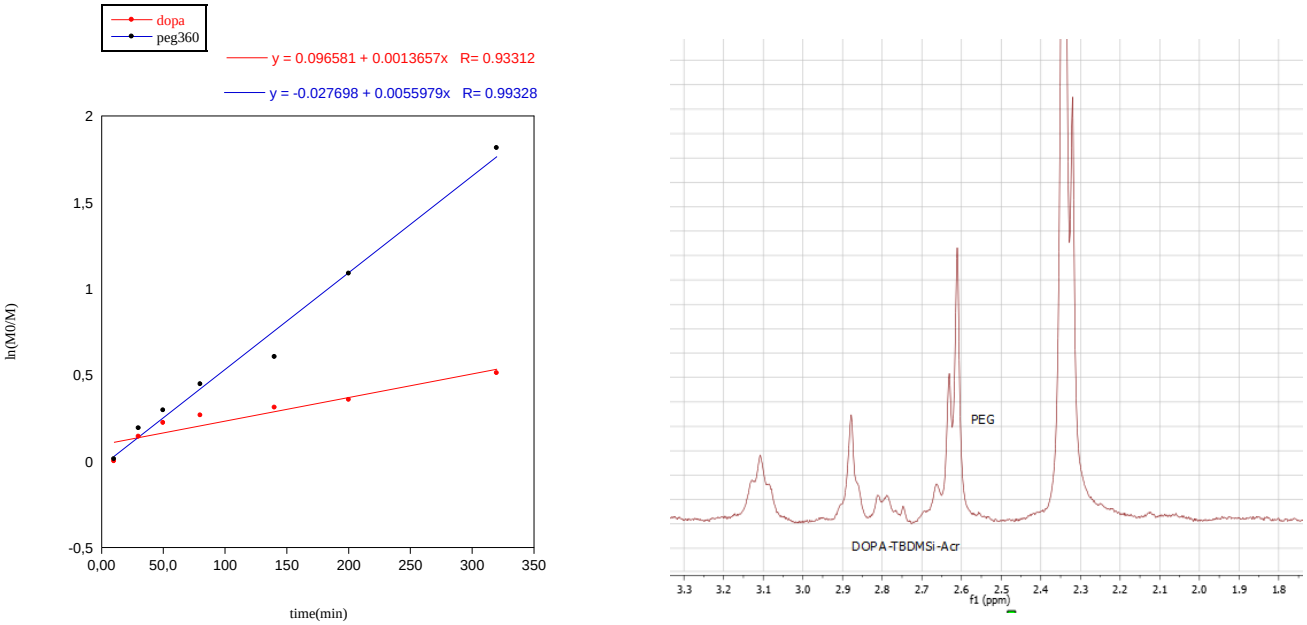
*f*1, *f*2, *F*1 and *F*2 represent PEG-360 and DOPA-TBDMSi-Ac molar fractions in the feed (*f*1, *f*2), determined from the monomers ratio, and in the copolymer (*F*1, *F*2), determined by ^{1}H NMR. In particular, F1 is due to the equation

[26] Finemann M., Ross S., *J. Polym. Sci. A*, **1964**, 2, 1687-1692.

$$F1(^{1}\text{H NMR}) = \frac{\frac{\int_{2.62ppm}(C\boldsymbol{H}_3 - C)}{3}}{\frac{\int_{2.62ppm}(C\boldsymbol{H}_3 - C)}{3} + \frac{\int_{1.5ppm}(-\text{CONHC}\boldsymbol{H}_2)}{2}}$$

while F2 is calculated as 1-F1.

By plotting *G* versus *H* (***Figure 4***) a straight line was obtained, the slope of which provided $r_{PEG\text{-}360}$ while $r_{DOPA\text{-}TBDMSi\text{-}Ac}$ was deduced from the negative intercept value. Values of $r_{PEG\text{-}360}$=**1.35** and $_{DOPA\text{-}TBDMSi\text{-}Ac}$ =**0.41** were found respectively.



a) *b)*

Figure 3: *Kinetics studies were carried out by ^{1}H-NMR. a) ln([Mo]/[M]) vs time for both monomers. B) Copolymer peaks chosen for Fineman-Ross and Kelen-Tudos's models.*

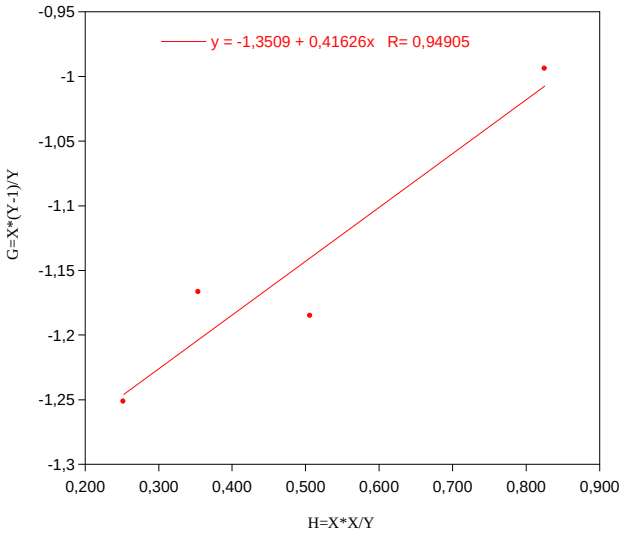


Figure 4: *G versus H for Fineman-Ross plot (where G and H are given by equation (a) and (b))*

The Kelen-Tüdos model [27] can also be applied to the experimental data. For that, the factor α has to be introduced from (c) equation:

$$\alpha = \sqrt{H_{\min} . H_{\max}} \qquad \text{(c)}$$

where $H_{\min}$ and $H_{\max}$ correspond to the lowest and the highest values of H defined in the Fineman-Ross model. The Kelen-Tüdos model is based on the following equation:

$$\eta = \left(r1 + \frac{r2}{\alpha} \right) \xi - \frac{r_2}{\alpha} \qquad \text{(d)}$$

$$\text{where } \eta = \frac{G}{\alpha + H},\ \xi = \frac{H}{\alpha + H} \qquad \text{(e)}$$

and *G* and *H* are the values obtained from the Fineman-Ross model.

The plotting of η versus ξ (***Figure 5***) led to a straight line, the slope of which was $r_{PEG360} + r_{DOPA\text{-}TBDMSi\text{-}Ac} / \alpha$ and the intercept was $-\alpha r_{PEG360}$. Values of $r_{PEG360} = 6.45$ and $r_{DOPA\text{-}TBDMSi\text{-}Acr} = 1.42$ were found.

[27] Kelen T., Tüdos F., *J. Macromol. Sci. Chem.*, **1975**, A9, 1-27.

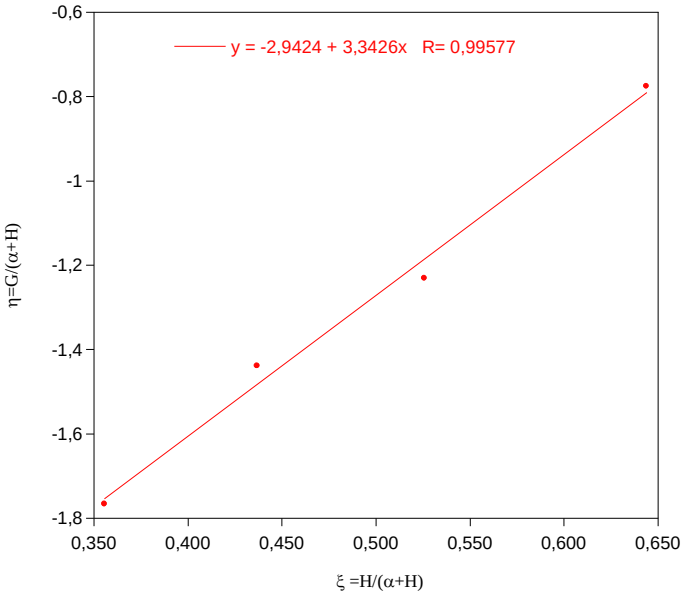


Figure 5: *η versus ξ for the Kelen-Tüdos plot (where η and ξ are given by equation (d))*

It was verified the occurred cleavage of the silyl-moieties using the NH_4F in methanol or the already mentioned acidic condition. In both cases after 20h of reaction, it was possible to notice a black precipitate onset due to the dopamine-group oxidation. After the lysis reaction, every dopamine ^{1}H-NMR signals disappeared leading to a whole cleavage.

The here synthesized pegylated-macromonomer possessing a well defined chain length will be used to prepare water soluble block copolymers. Such copolymers should display both selective biocidal effects against bacteria and poor hemolysis and then they could be excellent substitutes of antibiotics.