UNIVERSITÀ DEGLI STUDI DI MILANO

Corso di Dottorato di Ricerca in CHIMICA INDUSTRIALE
(XXI Ciclo)

Poly(amidoamine)s of Biotechnological Interest

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Ph.D. Thesis

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Anno Accademico 2007 – 2008

UNIVERSITÀ DEGLI STUDI DI MILANO

Facoltà di Scienze Matematiche, Fisiche e Naturali

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Chapter 1: General Introduction

1.1 What are Biomaterials?

"A biomaterial is a nonviable material used in a medical device intended to interact with biological systems" (D.F. Williams, 1987).

This broad definition include nowadays different substances like metals, ceramics, polymers, and composites that are produced synthetically or biologically for use in the treatment or management of a disease, condition, or injury. Although biomaterials are primarily used for medical applications, they are also used to grow cells in culture, to assay for blood proteins in the clinical laboratory, in processing biomolecules in biotechnology, for fertility regulation implants in cattle, in diagnostic gene arrays, in the aquaculture of oysters and for investigational cell-silicon "biochips." The commonality of these applications is the interaction between biological systems and synthetic or modified natural materials. Moreover, biomaterials are rarely used on their own but are more commonly integrated into devices or implants. Considering this large range of applications is not surprising that the estimated worldwide market for biomaterials that was about $5 billion in 1985, grew to about $23 billion in 2005. [1,2]

Biomaterials require generally several features, that can vary depending on the final application of the device. First, they need to be *non-toxic*, unless it is specifically engineered for such requirements (for example, drug delivery system that targets cancer cells to destroys them). They should be *biocompatible*, that is with a low level of immune response and with a good probability of clinical success of the whole device in which a specific biomaterial is part or its whole. They need the appropriate *mechanical requirements*, like mechanical performance, mechanical durability and physical properties. Moreover, we must consider the pain of the patient related to possible inflammatory reactions due to the introduction of a foreign material or device in the body. Aiming to relieve discomfort and pain, a biomaterial should be *compliant*.

Finally, economical aspects have to be considered, since the research, clinical testing, regulatory clearance, manufacture, quality control, and distribution of medical devices

require large investment from the companies, which need to make a profit to fund all these activities.

The complex balance between the desire to alleviate suffering and death, the excitement of new scientific ideas, the corporate imperative to turn a profit, the risk/benefit relationship and the mandate of the regulatory agencies to protect the public impose the researchers a wide range of ethical considerations in developing new biomaterials.

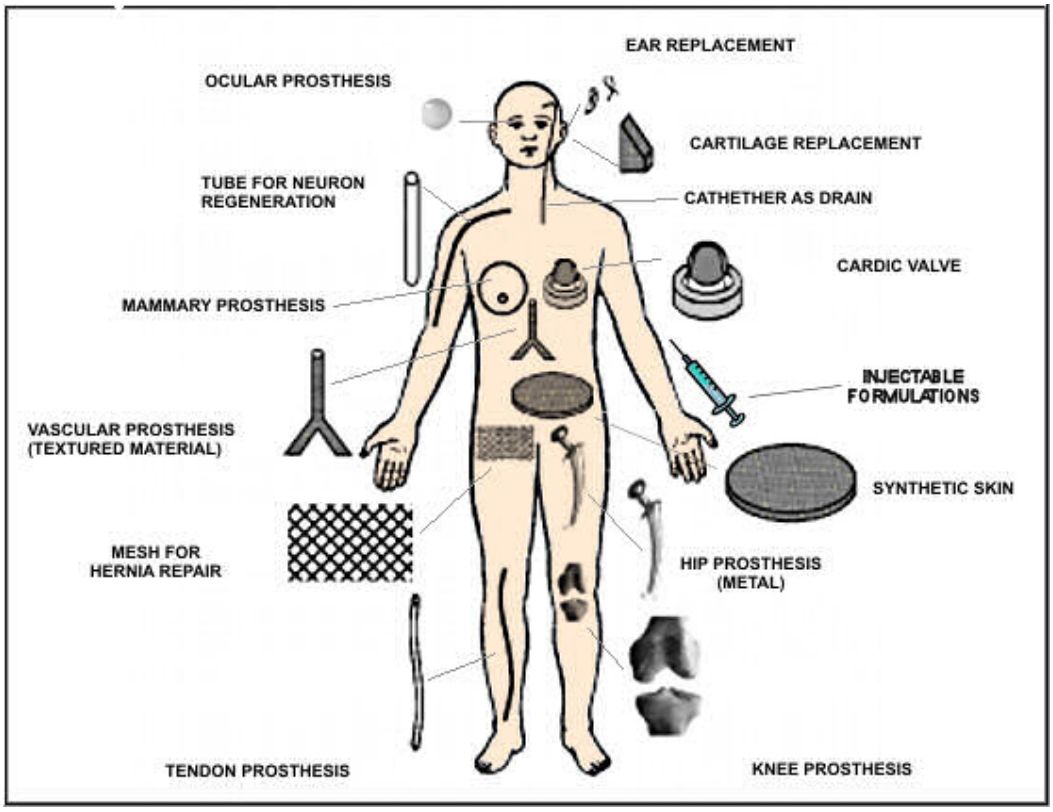


Figure 1.1. Examples of several biomaterial applications. [51]

1.2 Drug Delivery

Drug delivery is the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. [3] To obtain a determined behavior in drug administration, it is necessary to design systems with a specific drug release profile, absorption, distribution and elimination, taking into account safety and patient compliance. Most common methods of delivery include the preferred non-invasive peroral (through the mouth), topical (skin), transmucosal (nasal, buccal/sublingual, vaginal, ocular and rectal) and inhalation routes. Many pharmacological agents such as peptide and protein, antibody, vaccine and gene based drugs have to be delivered by injection, since using other routes they might be susceptible to enzymatic degradation or cannot be absorbed into the systemic circulation efficiently due to molecular size and charge issues. For instance, many bioactive molecules are rapidly degraded by enzymes in the stomach and in the intestine.

Current efforts in the area of drug delivery include the development of targeted delivery in which the drug is only active in the target area of the body (for example, in cancerous tissues) and sustained release formulations in which the drug is released over a period of time in a controlled manner from a formulation.

An important parameter in drug delivery is the release rate of the drug in the organism. The release of the active agent may be constant over a long period, it may be cyclic over a long period, or it may be triggered by the environment or other external events. In any case, the purpose behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under- and overdosing. Other advantages of using controlled-delivery systems can include the maintenance of drug levels within a desired range, the need for fewer administrations, optimal use of the drug in question, and increased patient compliance. While these advantages can be significant, the potential disadvantages cannot be ignored: the possible toxicity or non-biocompatibility of the materials used, undesirable by-products of degradation, any surgery required to implant or remove the system, the chance of patient discomfort from the delivery device, and the higher cost of controlled-release systems compared with traditional pharmaceutical formulations. The ideal drug delivery system should be inert, biocompatible, mechanically strong, comfortable for the patient, capable of achieving high drug loading, safe from

accidental release, simple to administer and remove, and easy to fabricate and sterilize. In general, a controlled-release systems must assure a high blood level of the drug over a long period of time. The main problem of the traditional drug forms is that frequent administration are required to keep the blood level of the agent between a maximum value, which may represent a toxic level, and a minimum value, below which the drug is no longer effective. In controlled drug delivery systems designed for long-term administration, the drug level in the blood remains constant, between the desired maximum and minimum, for an extended period of time. Depending on the formulation and the application, this time may be anywhere from 24 hours (Procardia XL®, for vasospastic angina) to 1 month (Lupron Depot®, for prostate cancer) to 5 years (Norplant®, contraceptive system). In recent years, controlled drug delivery formulations and the polymers used in these systems have become much more sophisticated, with the ability to do more than simply extend the effective release period for a particular drug. For example, current controlled-release systems can respond to changes in the biological environment and deliver (or cease to deliver) drugs with a determined rate, based on the external stimuli. Moreover, new materials are available nowadays that permit the targeting to specific tissues, or cells , or definite sites where the active agent is to be delivered.

1.3 Biomaterials for drug delivery

In the last 50 years, many biomaterials have been discovered, increasingly appropriated to be utilized in drug delivery systems. At the earliest stages of the research (years ’50 -’60) in biomaterials a few compounds displayed features suitable for medical applications. For example:

- Poly(ethylene) for toughness and lack of swelling.
- Poly(methylmethacrylate) for physical strength and transparency.
- Poly(siloxanes) or silicones for insulating ability.
- Poly(urethanes) for elasticity.
- Poly(vinyl alcohol) for hydrophilicity and strength.

- Poly(vinylpyrrolidone) for suspension capabilities.

Afterwards, further materials have been discovered thank to the technological advances. The research focused on chemical inertia, lack of toxic impurities, minimal aging and ease of processing. Some of these are the following:

- Poly(2-hydroxy ethyl methacrylate).
- Poly(N-vinylpyrrolidone).
- Poly(vinyl alcohol).
- Poly(acrylic acid).
- Polyacrylamide.
- Poly(ethylene-co-vinyl acetate).
- Poly(ethylene glycol).
- Poly(methacrylic acid).

Nowadays, new classes of polymers that are able to degrade within the body are available. Moreover, a rational approach to the synthesis of new materials permits to tailor specific features depending on the final application. Promising results were often obtained in clinical trials. Some of these biodegradable polymers are listed below.

- Polyanhydrides (Shieh et al., 1994)
- Polyamidoamines (PAA) (P. Ferruti, A. M. Marchisio, R. Duncan, 2002)
- Polyglycolides (PGA) (Okada et al., 1987).
- Poly(lactide-co-glycolides) (PLGA).
- Polylactides (PLA) (Okada et al., 1987).
- Polyorthoesters (Heller et al., 2000)
- Polyphosphoesters (Richards et al., 1991)
- Polyphosphazenes (Allcock, 1994)

A great advantage of biodegradable polymers is that they are broken down into biologically tolerable molecules that are metabolized and removed from the body via normal metabolic

pathways. However, biodegradable materials do produce degradation by-products that must be tolerated with little or no adverse reactions within the biological environment.
Chemical, physical and processing properties can affect the biodegradation of the polymer in the biologic environment. Some of these factors are the following:

- Site of implantation.
- Morphology (amorphous/semicrystalline, microstructures, residual stresses).
- Storage history.
- Sterilization process.
- Annealing.
- Processing conditions.
- Physical factors (shape and size changes, variations of diffusion coefficients, mechanical stresses, stress- and solvent-induced cracking, etc.).
- Physicochemical factors (ion exchange, ionic strength, pH).
- Chemical structure.
- Chemical composition.
- Distribution of repeat units in multimers.
- Configuration structure.
- Presents of ionic groups.
- Presence of unexpected units or chain defects.
- Molecular weight.
- Molecular-weight distribution.
- Adsorbed and absorbed compounds (water, lipids, ions, etc.).
- Presence of low-molecular-weight compounds.
- Mechanism of hydrolysis (enzymes versus water).

1.4 Controlled Release Systems

A main aspect in drug delivery is represented by the release rate of the active molecule to the target. In most cases a stable blood level of the agent is required; in particular, this value must be between the toxic concentration and the minimum effective concentration. Controlled release systems permit not only a stable blood level, but also improve many aspects compared to the traditional drug forms. By comparison, the controlled release assure (1) a constant rate of drug release, offering a means to circumventing the problems of overdosing and underdosing inherent to conventional formulation. It requires (2) considerably less agent to produce a given duration or effect than does a conventional system. A third important benefit is that (3) this technology makes possible both delivery of the agent locally and its containment at the site of action. In medicine, local delivery and containment reduce the dosage required and the possibility of side effects. A final advantage of controlled release is that (4) fewer application of the agent are required. It is widely recognized that a once-daily dosage regimen results in a significantly better patient compliance than multi-daily dosing, with a consequent improvement in the efficacy of the treatment.

There are three primary mechanisms by which active agents can be released from a delivery system: diffusion, degradation, and swelling followed by diffusion (figure 2). Any or all of these mechanisms may occur in a given release system.

Diffusion occurs when a drug or other active agent passes through the polymer that forms the controlled-release device. The rate controlling step can be the passage of the drug through the polymer matrix or through a selective film or a membrane. In the first case, the rate normally decreases with the time, whereas in the second case the drug delivery rate can remain fairly constant.

The degradable matrices are designed to degrade within the body as a result of natural biological processes. This capability eliminates the need to remove a drug delivery system after release of the active agent has been completed. The polymer degradation occurs usually by hydrolysis of covalent bonds of the macromolecular chain, producing smaller and biologically acceptable compounds. Degradation may take place through bulk hydrolysis, in which the polymer degrades in a fairly uniform manner throughout the

matrix. In other cases, as for example the polyanhydrides and polyorthoesters, the degradation occurs only at the surface of the polymer, resulting in a release rate that is proportional to the surface area of the drug delivery system.

Finally, swelling-controlled release systems are usually designed to be incapable of releasing its agent or agents they are placed in an appropriate biological environment. The swelling increases the aqueous solvent content within the formulation as well as the polymer mesh size, enabling the drug to diffuse through the swollen network into the external environment. One of the most useful feature of a polymer's swelling ability is that the swelling can be triggered by a change (pH, temperature, or ionic strength) in the environment surrounding and for most of these polymers the alteration can be reversible .

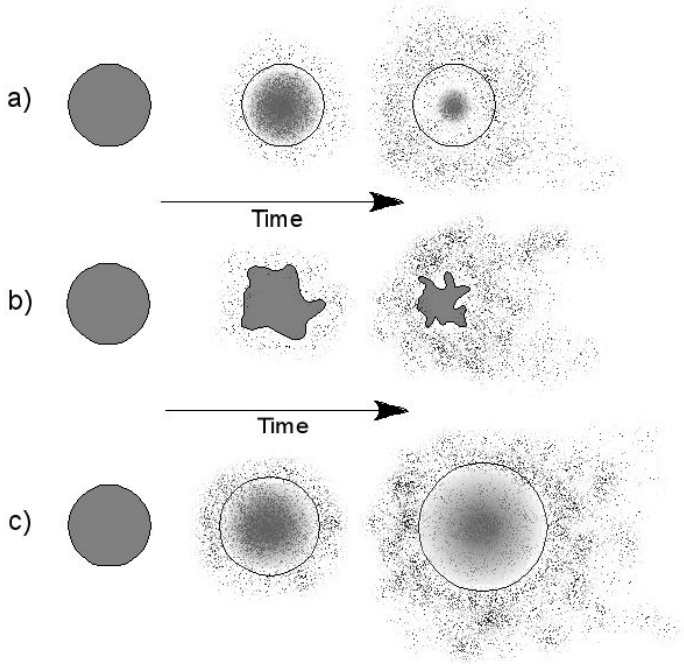


Figure 1.2. Scheme of different drug release strategies: a) diffusion, b) degradation and c) swelling followed by diffusion.

One of the greatest opportunities in controlled drug delivery lie in the sphere of responsive delivery systems, with which it will be possible to deliver drugs through

implantable devices in response to a measured blood level or to deliver a drug precisely to a targeted site. Much of the development of novel materials in controlled drug delivery is focusing on the preparation and use of these responsive polymers with specifically designed macroscopic and microscopic structural and chemical features.

1.5 Characterization of Polymers

As general rule, two parameters are fundamental to characterize a polymer: the number average molecular weight ($\overline{M}_n$) and the weight average molecular weight ($\overline{M}_w$). [24]

$\overline{M}_n$ is defined as the total weight of all molecules in a polymer sample divided by the total number of moles present. Thus the number-average molecular weight is:

$$\overline{M}_n = \frac{\sum_{j=1}^{n} N_j \times M_j}{\sum_{j=1}^{n} N_j} = \sum_{j=1}^{n} n_j \times M_j = \frac{\sum_{j=1}^{n} W_j}{\sum_{j=1}^{n} W_j / M_j}$$

where N_j is the number of moles whose weight is M_j and n_j is the mole fraction (or the number fraction) of molecules of size M_j.

$\overline{M}_w$ is defined as:

$$\overline{M}_w = \frac{\sum_{j=1}^{n} W_j \times M_j}{\sum_{j=1}^{n} W_j} = \sum_{j=1}^{n} w_j \times M_j = \frac{\sum_{j=1}^{n} N_j \times M_j^2}{\sum_{j=1}^{n} N_j \times M_j}$$

where w_j is the weight fraction of molecules whose weight is M_j.

The polydispersity (PD) of a polymer is defined as a ratio of weight average molecular weight divided by number average molecular weight. It is important to note that dealing

with a polymer sample we must always consider a distribution of molecular weights which is described by a ratio (PD). If all molecules of a polymer sample have the same molecular mass this ratio is 1 and the polymer is described as a monodisperse. This ratio is always ≥ 1 and the higher this ratio, the broader is the distribution of molecular weights. Polymer conjugates must be rigorously characterized with respect to their molecular weight and PD because biodistribution and pharmacological activity are known to be molecular weight dependent. The most common method to investigate the average molecular weight of a polymer is the Size Exclusion Chromatography (SEC).

Others fundamental characterizations in polymer chemistry are Nuclear Magnetic Resonance spectroscopy (NMR), MALDI-TOF spectroscopy, viscosimetry and High Performance Liquid Chromatography (HPLC). All the polymers must be fully characterized to be used in clinical evaluation. Moreover, the extensive characterization of the physico-chemical properties of a polymer, is a good starting point for the rationale design of a new compound related to that polymer.

1.6 Bioconjugation of polymers

In the past decade, conjugation of a polymer with a bioactive molecule has become a central issue in pharmaceutical sciences. For many years the starting point for developing a polymeric adduct was the Ringsdorf's Model (Figure 1.3). [25]

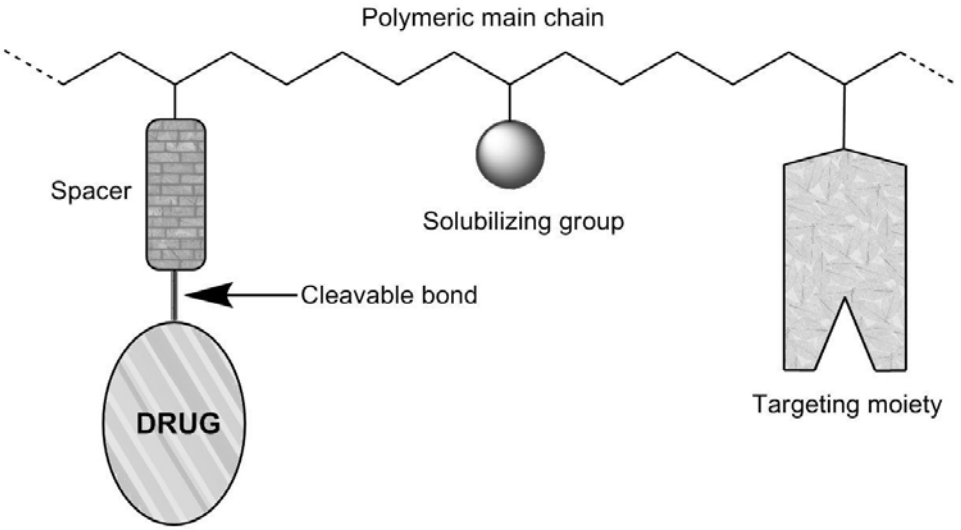


Figure 1.3. Ringsdorf's model of polymer-drug conjugate.

This model included a bioresponsive polymer-drug spacer that is stable during conjugate transport and able to release drug at an optimum rate on arrival at the target site, adequate drug carrying capacity in relation to the potency of the drug that must be carried and the ability to target the diseased cell or tissue by an active or passive mechanism (see EPR effect, Chapter 1.8). In almost all recent structures developed, the "solubilizing group" has been replaced by the use of water soluble polymers. The targeting moiety is used to achieve an active targeting or it can be a tracking group, used for instance as fluorescent- or radio-marker, to follow the behavior of the polymeric adduct *in vitro and in vivo*.

The conjugation of a polymer with a drug or a bioactive molecule (bioconjugation) permits to obtain compounds with many useful features in drug delivery. Several examples of these benefits are the following:

- ✓ stabilization of labile active molecules from chemical and biological degradation.
- ✓ protection from proteolytic degradation.
- ✓ reduction of immunogeni city.
- ✓ decreased antibody recognition.
- ✓ increased plasma half life.
- ✓ modification of organ disposition.
- ✓ drug penetration into selected cell compartments through endocytosis.
- ✓ enhanced possibilities of drug targeting.

The chemistry of bioconjugation is extensive because it deals with a large variety of molecules. Different approaches may be used depending on the properties of the bioactive molecule to be modified and of the ones of the ligand to be coupled.
Despite the large variability that characterizes this field of chemistry, a common feature shared by the reactions used in bioconjugation is the fact that all of them must be carried out in mild conditions, so to avoid disruption of the properties of the conjugated active molecules. Generally, both the drug molecule and the polymer are not reactive by themselves and a preliminary step of activation is needed before coupling. In some cases, it may be necessary to introduce a new functional group in either drug or polymer molecule. This may be achieved either through a specific chemical reaction that transforms a functional group into the desired one, through the use of a difunctional reagent or, as in the case of proteins, through genetic recombinant techniques that allow the introduction of one amino acid with the desired reactivity (e.g.: the thiol function of cysteine or the amino group of lysine). There are many polymers used in the preparation of bioconjugates for pharmaceutical application. Because of their application in the biomedical field, they all share the common properties of being highly hydrated, non toxic, non immunogenic and of having a molecular weight sufficiently low to allow, when they are not biodegradable,

filtration through the kidney. If they have the property to be biodegradable, their degradation products must be non toxic and non immunogenic themselves.

Although the polymers used in bioconjugation present different structures in their polymeric backbone, they have only a limited number of functional residues that are normally exploited in the coupling reaction. The most common anchoring groups are -COOH, -OH and -NH_2. These groups must be activated in order to react with the desired drug molecule and many mild activation methods are presently available. It might be necessary to activate the drug molecule instead of the polymer. The strategy adopted is determined by various factors including the best conditions for preserving drug activity and easiness of conducting the chemical steps of the reaction. Special attention must be paid to the 3-dimensional structure of the polymers, their hydrophobic/hydrophilic balance, their flexibility and biodegradability. All of these factors are important in dictating the fate of the drug in vivo, the rate of elimination from blood and the protection from degradation, both in vivo and in the pharmaceutical formulations.

1.7 Polymer Therapeutics

In recent years, polymer sciences have reached a high technological level in the synthesis of polymers, which can be specifically tailored depending on the target or the final application. The instrumental analysis techniques permit nowadays a reliable characterization of the polymers even at nanometric level. The biotechnology is making possible the production of a large quantity of pharmaceutically active proteins, that was unconceivable in the pre-genomic Era. All these factors contributed to the development of new classes of compounds, which cannot be contained in the definition of "drug delivery systems". To describe these new systems a new term was coined: "Polymer Therapeutics".

Polymer therapeutics (Figure 1.4) have been authoritatively reviewed by Duncan et al. [4] These polymers can act as (1) polymeric drugs, [5] (2) polymer-drug conjugates, [6] (3) polymer-protein conjugates, [7,8] (4) polymeric micelles in which the drug is covalently bound to the polymer, [9] and (5) polymers used as non-viral vectors for intracellular delivery of genes and proteins. [10]

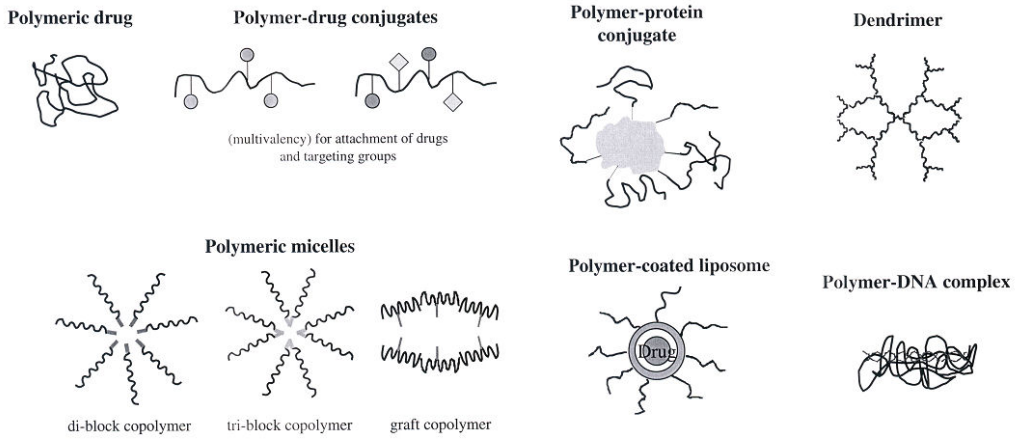


Figure 1.4. Scheme of several polymer therapeutics systems.

To prepare polymer therapeutics different type of polymers can be used. A first division is between natural (e.g. polysaccharides and polyamino acids) [11,12] or synthetic (e.g. HPMA and PEG)[13,14,15]. Depending on the application, they might be inert or essentially biologically active[16]. The use of polymers as targetable drug carriers was first proposed by Ringsdorf. [17] The model is based on a hydrophylic polymer backbone, to which a targeting moiety is attached to promote cell-specific uptake. A polymer-drug linker that can be degraded at specific sites is also incorporated, liberating the drug in the required tissues at an appropriate rate.. The ideal polymeric carrier should possess the following qualities: (i) non-toxic, (ii) non-immunogenic, (iii) biodegradable, (iv) contain functional moieties which allow conjugation to therapeutic compound, (v) high drug loading capacity, (vi) resistant to premature degradation in bloodstream, (vii) simple to manufacture, (viii) able to control the molecular weight distribution, and (ix) stable as pharmaceutical formulation.

Polymer–protein conjugates:

Compound	Name	Status	Comment
SMANCS	Zinostatin Stimalmer	Market	Hepatocellular carcinoma
PEG–L-asparaginase	Oncaspar	Market	Acute lymphoblastic leukemia
PEG–GCSF	Neulasta	Market	Prevention of neutropenia associated with cancer and AIDS chemotherapy
PEG–interferon a 2a	PEG-Introne	Market Phase I/II	Hepatitis B and C Melanoma, chronic myelogenous leukemia and renal cell carcinoma
PEG–interferon a 2b	PEG-Asys	Market Phase I/II	Hepatitis C Phase I/II Melanoma, multiple myeloma and renal cell Carcinoma
PEG–arginine deiminase	ADI-PEG20	Phase I	Hepatocellular carcinoma
PEG–glutaminase combined with a glutamine antimetabolite 6-diazo-5-oxo-L-norleucine	PEG-PGA and DON	Phase I/II	Various

Polymer–drug conjugates:

Compound	Name	Status	Comment
Polyglutamate–paclitaxel	CT-2103; XYOTAX	Phase II/III	Various, particularly non small cell lung cancer; ovarian cancer
Polyglutamate–camptothecin	CT-2106	Phase I	Various
HPMA copolymer–doxorubicin	PK1; FCE28068	Phase II	Various, particularly lung and breast cancer
HPMA copolymer–doxorubicin-galactosamine	PK2; FCE28069	Phase I/II	Particularly hepatocellular carcinoma
HPMA copolymer–carboplatin platinate	AP5280	Phase I/II	Various
HPMA copolymer–DACH–platinate	AP5346	Phase I/II	Various
PEG–camptothecin	PROTHECAN	Phase II	Various

Table 1.1. Examples of polymer–protein and polymer–drug conjugates in clinical use in oncology. [18]

The ideal polymer-drug conjugate (Table 1.1) must facilitate targeting in the body without initiating an adverse toxicological and immunological response. Biodegradable polymers enable repeated administration without concern over accumulation and potential cumulative toxicity. A polymer which has a high drug carrying capacity also helps to increase targeting efficiency. Moreover, for pharmaceutical development an important requisite is the possibility of the up-scaling of the process.

Anyway, several polymers do not have all these characteristics, but they may still be useful as targetable polymeric drug carriers. For example, although HPMA copolymers are non-biodegradable, but limiting molecular weight to less than 40.000 ensures effective renal elimination. [19]

Compared to natural polymers, synthetic polymers have some advantages, particularly the versatility or design with variations in structure for cell-specific interaction. One of the most successful non-biodegradable polymers is PEG, [20] which is used nowadays in many pharmaceutical formulations. However, as a polymeric drug carrier, it has a low drug carrying capacity, therefore limiting its conjugation to potent drugs. Instead, in cancer therapy, biodegradable polymers are preferable because usually the accumulation in the target tissue is a fundamental strategy. The choice of polymers as macromolecular carriers therefore depends on the desired clinical application.

1.8 Polymers for anticancer therapy and EPR effect

Polymer-drug conjugates have many advantages compared to conventional cytotoxic chemotherapy. Conventional chemotherapeutic molecules have low selectivity to tumour tissues due to their random distribution and passive diffusion through cellular membranes in the body. However, a major advantage of polymer-anticancer conjugates is their ability to target tumour tissues passively owing to the hyperpermeability and decreased tissue drainage of the tumour vasculature (EPR effect) (Fig. 1.4). The EPR phenomenon is dependent on both the characteristics of the conjugate, including the molecular weight [25,

26] overall charge [21, 22] and size, and the type of tumour vasculature involved[20]. Many polymer-anticancer compounds rely on the EPR effect for passive targeting to the tumour tissues [27, 28] (Figure 1.5) By targeting to the tumours, polymer-drug conjugates reduce drug toxicity *in vivo* and clinically. [29] In addition, polymer-drug conjugates may also overcome multidrug resistance (MDR) due to the different mechanism of cell uptake (endocytosis) as compared with small therapeutic molecule [30, 31, 32] therefore bypassing resistance at the membrane level. Many polymer-anticancer conjugates have shown effective antitumour activity *in vivo*, including the polyamino acids- [33, 34] PEG- [35] and HPMA [36, 37] copolymer-based systems.

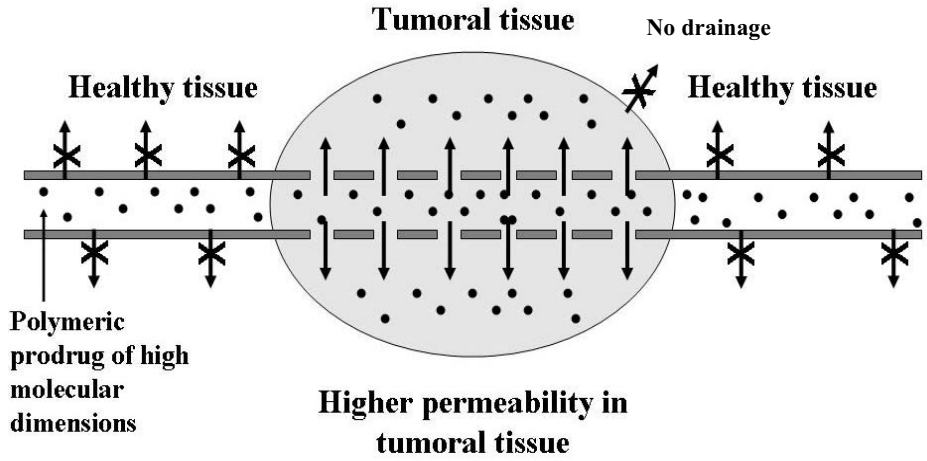


Figure 1.5. "Enhanced Permeability and Retention effect" (EPR). The macromolecules tend to concentrate in tumor tissue much more than in normal tissues, enabling the accumulation of the drug in the tumor.

In order to improve the passive targeting strategy employed by the EPR effect and to minimise toxicity against normal tissues, a targeting ligand attached to the polymer should ghelp to promote better antitumour activity. These targeting motifs can be small peptides [38,39] and proteins. [39,40] As example, the galactosamine-containing HPMA copolymer-doxorubicin (DOX) conjugate (known as PK2, see table 1.1) which targets the hepatocyte

asialoglycoprotein receptor (ASGPR) is currently in phase I/II clinical trial. [36] It is the only targeted polymer-drug conjugate in clinical development to date. This targeting strategy using a polymer-anticancer prodrug has been demonstrated by *in vivo* liver-targeting in both animal [41] and human studies. [39]

The success of delivering polymer-anticancer conjugates such as HPMA copolymer-DOX is based on the concept of lysosomotropic drug delivery. This relies on the endocytic uptake of the polymer-drug conjugate and transfer to the lysosome. Lysosomal cleavage of the GFLG peptidyl linker can release the DOX. Then diffusion of DOX into the cytosol and nuclear interaction results in DOX-mediated cytotoxicity. Enzymatic degradation of peptidyl linker is not the sole mechanism of the drug release. Due to the low pH of the intracellular organelles, drugs can be released from the polymer conjugates using a pH-responsive polymer or an endsomolytic polymer such as PAAs that facilitate cytosolic access of the drug.

1.9 Endosomolytic polymers

Exploiting the unique pH gradient of intracellular organelles, [42] endosomolytic polymers have been designed to be pH-responsive, and many have been used as non-viral gene delivery vectors. These polymers include the polycations e.g. poly(ethylenimine) (PEI), [43] poly(L-lysine) (PLL), chitosan, [44] poly(2-dimethylamino)ethylmethacrylate (PDMAEMA), [44] PAAs, [45] and poly(amidoamine) (PAMAM) dendrimers, [46] polyanions such as poly(propylacrylic acid) (PPAA) [47] and poly(ethylacrylic acid) (PEAA). [48] All rely on the ability to undergo pH-triggered conformational change to initiate membrane disruptive activity.

In general, protonation of the cationic groups in pH-responsive cationic polymers results in polymer expansion and subsequent membrane destabilisation which allows the delivery of therapeutic agents to the cytosol. Such a mechanism is proposed for the membrane destabilisation behaviour of PAAs and other polycations. The “proton sponge” mechanism has been hypothesised to explain PEI membrane disruption. It is suggested that protonation of PEI causes an influx of anions (e.g. Cl- ions) into the endosome, followed by the influx

of water. Subsequent osmotic swelling destabilises the membrane, thereby permits the release of therapeutic agents to the cytosol .

The cationic polymers, such as PEI and PLL are generally one to two orders of magnitude more toxic than PAAs. [49] They also have high haemolytic activity (>50%) at physiological pH (~ pH 7.4), which raises concern in respect of *in vivo* application. In contrast, the lower toxicity of PAAs, their pH-dependent membrane-lytic properties and ability to deliver genes and toxins [49] *in vitro* underline their potential as intracellular delivery. Therefore, the potential of using PAAs for targeted delivery of a toxin, gelonin, was assessed in this investigation.

Internalisation by cells can be both a passive and energy-dependent process, which is crucial for the uptake of nutrients, removal of cell debris, homeostasis maintenance and cell signalling. The discovery of lysosomotropic delivery of macromolecules by de Duve in the 1970s , and the presence of specialised enzymes in the lysosomal compartment, have provided a platform for the development of polymeric therapeutics that exploit the intracellular enzymes and pH gradient (e.g. PAAs) for successful delivery of therapeutics. Therefore understanding the basic principles in the uptake of polymer conjugates into cells and their intracellular transport is essential in order to determine their mechanism of actions and to design better polymer therapeutics in the future.

Since macromolecules are unable to diffuse across the plasma membrane, their cellular uptake is limited by the process of endocytosis. Endocytosis can be broadly defined as the entry of molecules into the cells. It can be generally divided into two groups: phagocytosis (cell eating; uptake of particles) and pinocytosis (cell drinking; uptake of fluid).

Phagocytosis does not occur in all mammalian cell types. It is employed by cells such as macrophages, neutrophils and monocytes for the uptake of large particles (>0.75 μm), including cell debris, apoptotic cells, foreign micro-organisms and parasites etc.

On the other hand, pinocytosis (fluid-phase uptake) occurs virtually in all cells, and involves the constitutive uptake of the extracellular fluid and solutes by specific and non-specific mechanism. Pinocytosis itself can also be subdivided into three main groups: fluid-phase pinocytosis, non-specific adsorptive pinocytosis and receptor-mediated pinocytosis . While fluid-phase pinocytosis is characterised by the constitutive uptake of non-diffusible molecules in the extracellular environment, non-specific adsorptive pinocytosis is mediated

by non-specific binding of solutes to the cell membrane via ionic and hydrophilic/hydrophobic interactions with the cell membrane. Such mechanisms are though to mediate uptake of many polymer-drug conjugates. However, the efficiency of endocytosis can be further improved by receptor-mediated pinocytosis, which involves the capture of ligands via highly specific receptors located on the plasma membrane. Many proteins (e.g. low-density lipoprotein (LDL), transferrin), hormones (e.g. insulin, growth hormone), growth factors (e.g. epidermal GF), viruses (e.g. adenovirus and human immunodeficiency virus) and toxins (e.g. diphteria toxin) also exploit this route of entry into the cells.

The complexity of these broadly classified uptake mechanisms is demonstrated by the fact that these processes are also subdivided into different endocytic pathways, including phagocytosis, pinocytosis, and receptor-mediated endocytosis, which are illustrated in Figure 1.6.

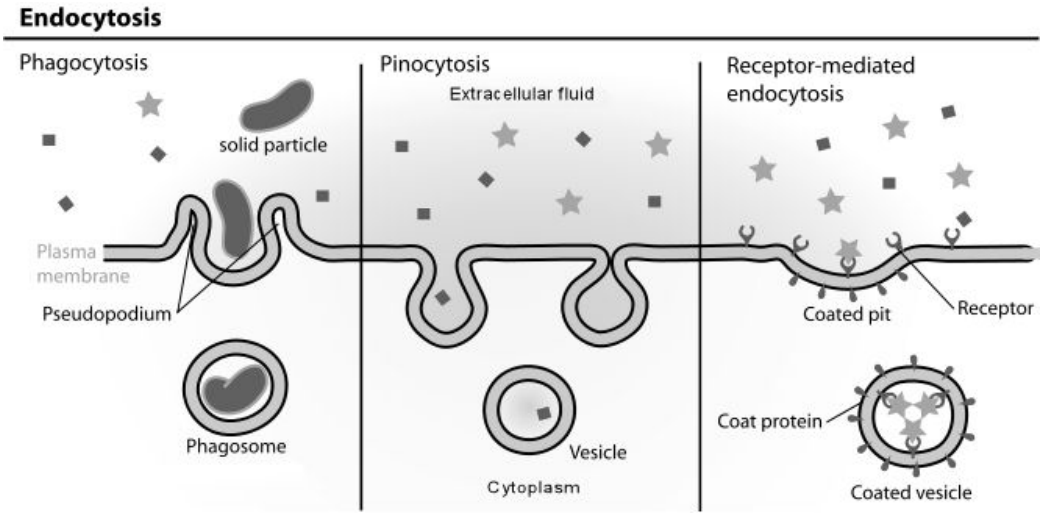


Figure 1.6. Schematic illustration of different endocytic pathways.

1.10 Endocytic pathway of polymer-drug conjugates

The intracellular pathways are complex and dynamic, there is no absolute separation of the different intracellular compartments. The usage of terminology and some vesicle trafficking events studied in literature are still debatable. However, it is generally accepted that endocytosed molecules are processed through different intracellular compartments, principally sorting from early endosome (EE) to late endosome (LE) and subsequent fusion leading to the lysosomal compartment. Other intracellular organelles such as the Golgi apparatus, trans-Golgi network (TGN), cytoskeleton may also be involved. So, after the intracellular uptake of polymers into the cells, the polymers may undergo a series of intracellular sorting, vesicle fusion and degradation, depending on the pinocytosis pathway it undertakes and the nature of the polymer (e.g. endosomotropic or lysosomotropic).

The progressively acidic environment of the intracellular compartments has wide implications for endosomotropic and lysosomotropic delivery systems, particularly when these systems are pH-sensitive or dependent on intracellular enzymes for activity. Upon endocytosis, molecules are first received by the EE, which are characterised by their tubulo-vesicular morphology, mildly acidic pH (ranging between pH 6-6.8) due to the presence of H^+-ATPase pump [50] and specific membrane markers. Endocytosed materials are then sorted for recycling back to the cell surface via the tubules or transport to later compartments via the vesicular part of the EE.

Captured materials are then brought to the late endosome as the final step in the sorting process before delivery to the lysosome. Late endosome contains the lipid lysobisphosphatidic acid and many membrane lamellae (membrane vesicles), which have a lower pH than early endosome, typically between pH 5-6. They also contain lysosomal proteins and degradative enzymes.

The dense lysosomes (~0.5-2 μm) are considered to be the terminal station of the endocytic pathway and are the main site for degradative activity. They contain a high concentration of lysosomal enzymes, whose activities are optimal in the lysosomal membrane or are transported by specific carriers or channels.

References:

[1] B. D. Ratner, A. S. Hoffman, Frederick J. Schoen, Jack E. Lemons, *Biomaterials Science: An Introduction to Materials in Medicine,* Academic Press, p. 137, **2004**.

[2] M.J. Lysaght, , J. O'Laughlin, *The demographic scope and economic magnitude of contemporary organ replacement therapies,* ASAIO, (J46), 515-521, **2000**.

[3] L. Brannon-Peppas, http//www.devicelink.com/mpb/archive/97/11/003.html

[4] R. Duncan, *Nature reviews: drug discovery*, (2), 347-370, **2003**.

[5] L. G. Donaruma, Synthetic biologically active polymers*, Progress in Polymer Science,* (4), 1-25, **1975**.

[6] R. Duncan, in *Handbook of Anticancer Drug Development*, (eds Budman, D., Calvert, H & Rowinsky, E.)(Lippincott Williams & Wilkins, Baltimore, in the press).

[7] J. M. Harris, R. B. Chess, *Nature Rev. Drug Discov*., (2), 214-221, **2003**.

[8] F.M. Veronese, J. M. Harris, (eds), *Adv. Drug Deliv. Systems,* (54), 453-609, **2002**.

[9] M. Yokoyama, et al. *J. Control. Release,* (11), 269-278, **1990**.

[10] A. V. Kabanof, , P.L. Felgner, L. W. Seymour, *Self-assembling complexes for gene delivery. From laboratory to clinical trial* (Wiley, Chichester, **1998**).

[11] A. Al-Shamkhani, R. Duncan, *International journal of Pharmaceutics*, (122), 107-119, **1995**.

[12] C. Li, J.E. Price, L. Milas, N.R. Hunter, S. Ke, D.-F. Yu, C. Charnsangavej, S. Wallace, *Clinical Cancer Research*, (5), 891-897, **1999**.

[13] L.W. Seymour, D.R. Ferry, D. Anderson, S. Hesslewood, P.J. Julyan, R. Poyner, J. Doran, A.M. Young, S. Burtles, D.J. Kerr, *Journal of clinical oncology*, (20), 1668-1676, **2002**.

[14] P.A. Vasey, S.B. Kaye, R. Morrison, C. Twelves, *Clinical cancer research*, (5), 83-94, **1999**.

[15] R. Bukowsi, M.S. Ernstoff, M.E. Gore, J.J. Neumaitis, R. Amato, S. Gupta, C. Tendler, *Journal of clinical Oncology,* (20)**,** 3841-3949 **, 2002.**

[16] L.W. Seymour, *Journal of bioactive and compatible polymers*, (6), 178-216, **1991**.

[17] H. Ringsdorf, *Journal of polymer science, polymer symposium*, (51), 135-153,**1975.**

[18] M.J. Vicent, R. Duncan, *TRENDS in Biotechnology* Vol.24 No.1 January **2006.**

[19] L.W. Seymour, R. Duncan, J. Strohalm, J. Kopecek , *Journal of Biomedical materials Research*, (21), 1341-1358, **1987**.

[20] R. B. Greenwald, Y.H. Choe, J. McGuire, C.D. Conover, *Advanced drug delivery reviews*, (55), 217-250 , **2003.**

[21] H-C. Chiu, C. Konak, P. Kopeckovà, J. Kopecek, *Journal of bioactive and compatible polymers*, (9), 388-341, **1994**.

[22] Danhauser-Riedl, S., Hausmann, E., Schick, H-D., Bender, R., Dietzfelbinger, H., Rastetter, J., Hanauske, A.-R., *Investigational new drugs,* (11), 187-195, **1993**.

[23] G. Odian, *Principles of polymerization*, 4th Ed. Wiley.

[24] H. Ringsdorf, *J. Polym. Sci.,Symp*., (51), 135-153, **1975**.

[25] Seymour, L.W., Miyamoto, Y., Maeda, H., Brereton, M., Strohalm, J., Ulbrich, K., *European journal of cancer,* 31A(5), 766-70, **1995**.

[26] Yamaoka, T., Kuroda, M., Tabata, Y., Ikada, Y., *International journal of pharmaceutics*, (113), 149-157, **1995**.

[27] Vasey, P.A., Kaye, S.B., Morrison, R., Twelves, C., Wilson, P., Duncan, R., Thomson, A.H., Murray, L.S., Hilditch, T.E., Murray, T., Burtless, S., Fraier, D., Frigerio, E., Cassidy, J., *Clinical cancer research*, (5), 83-94, **1999**.

[28] Maeda, H., Sawa, T., Konno, T., *Journal of controlled release*, (74), 47-61 , **2001**

[29] Duncan, R., , Budman, D., Calvert, H., Rowinski, E., *In handbook of anticancer drug development* (Lippincott, Williams & Wilkins: Boston, USA), 239-260, **2003**

[30] Kunath, K., Kopeckovà. P., Minko, T., Kopecek, J., *European Journal of pharmaceutics and biopharmaceutics*, (49), 11-15, **2000**.

[31] Minko, T., Kopeckovà, P., Kopecek, J., *Journal of controlled release*, (54), 223-233, **1998**.

[32] Minko, T., Kopeckovà, P., Kopececk, J., *Pharmaceutical research*, (16), 986-999, **1999**.

[33] Li, C., Price, J.E., Milas, L., Hunter, N.R., Ke, S., Yu, D.-F., Charnsangavej, C., Wallace, S., *Clinical Cancer Research*, (5), 891-897 , **1999**.

[34] Sabbatini, P., Aghajanian, C., Hensley, M., Pezzulli, S., Oflaherty, C., Soignet, S., Lovegren, M., Esch, J., Funt, S., Odujinrin, O., Warner, M., Bolton, M.G ., Spriggs, D., In

5th international symposium on polymer therapeutics : from laboratory to clinical practice, (20), **2002**.

[35] Bukowsi, R., Ernstoff, M.S., Gore, M.E., Neumaitis, J.J., Amato, R., Gupta, S., Tendler, C.,*Journal of clinical Oncology,* (20)**,** 3841-3949 **,2002**

[36] Seymour, L.W., Ferry, D.R., Anderson, D., Hesslewood, S., Julyan, P.J., Poyner, R., Doran, J., Young, A.M., Burtles, S., Kerr, D.J., *Journal of clinical oncology*, (20), 1668-1676, **2002**.

[37] Vasey, P.A., Kaye, S.B., Morrison, R., Twelves, C., Wilson, P., Duncan, R., Thomson, A.H., Murray, L.S., Hilditch, T.E., Murray, T., Burtless, S., Fraier, D., Frigerio, E., Cassidy, J., *Clinical cancer research*, (5), 83-94, **1999**.

[38] Mu Y., Kamada H., Kaneda Y., et.al., *Biochem. And biophysical research communications*, (255), 75-79, **1999**.

[39] Duncan R., Seymour L., Wedge S., et. al., *Journal of controlled release*, (19),331-346, **1992**.

[40] Duncan R., Coastworth J., *Human and experimental toxicology*, (17), 93-104, **1998**.

[41] Seymour, L.W., *Journal of bioactive and compatible polymers*, (6), 178-216, **1991**.

[42] Clague M.J. *The Biochem. Journal*, (336), 271-282, **1998.**

[43] O'Hare, K.B., Duncan, R., Strohalm, J., Ulbrich, K., Kopeckovà, P., *Journal of drug targeting*, (1), 217-229, **1993**.

[44] Rihova, B., Jelinkova, M., Strohalm, J., Subr, V., Plocova, D., Hovorka, O., Novak, M., Plundrova, D., Germano, Y., Ulbrich, K., *Journal of controlled release*, (64), 241-261,**1989.**

[45] Richardson, S.C. W., Pattrick, N. G., Mann, Y.K.S., Ferruti, P., Duncan, R., *Biomacromolecules*, (2), 1023-1028, **2001**.

[46] Eichman J.Bielinka A., Baker J., et. al., *Pharmaceutical science and technology today*, (3), 232-245, **2000**.

[47] Murthy N., Robichaud J., Tirrel D., *Journal of controlled release*, (6), 137-143, **1999**.

[48] Lackey C., Murthy N., *Bioconiugate chemistry*, (10), 401-405, **1999**.

[49] Richardson S., Ferruti P., Duncan R., *Journal of drug targeting*, (6), 391-404, **1999**

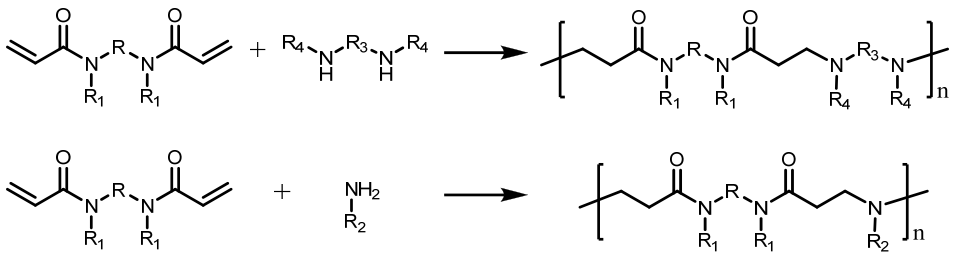
[50] Mellman I., Fuchs R., *Annual review of biochemistry,* (55), 663-700, **1986**.

[51] http://www.mitr.p.lodz.pl/biomat/raport/1_radiation_hydrogels.html

Chapter 2: Poly(amidoamine)s

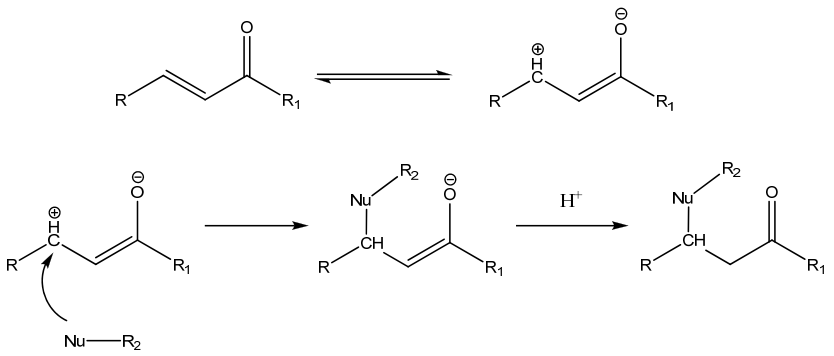
2.1 Introduction

Biomedical polymers and polymer therapeutics rapresent a major impetus for innovation in many therapeutic areas such as cancer, inflammatory, and infective diseases. The search for new drug-delivery concepts and new applications are the major driving force in polymer therapeutics. Functional polymers have found many applications, for instance as ion-exchange resins and agents for surface modification of heparin filters and non thrombogenic surfaces. [1-3] These systems are just entering early clinical evaluation. [4-6] The poly(amido-amine)s (PAAs) are one unique family of synthetic functional polymers that have been widely developed for use both as biomedical materials and polymer therapeutics. PAAs are synthetic ter-amino polymers obtained by stepwise polyaddition of primary or secondary aliphatic amines to bis-acrylamides (Scheme 2.1).



Scheme 2.1 synthesis of PAAs.

The PAAs synthesis is performed in solvents carrying mobile protons, such as water or alcohols, at temperatures over about 10-15° C and without added catalysts. [7-9] High monomer concentrations and relatively low reaction temperatures give the best results. Aprotic solvents, even if highly polar, are unsuitable as reaction media as they yield only low molecular weight products. The amino groups react only if present as free base. The synthetic mechanism is a Michael type addition (Scheme 2.2).



Scheme 2.2. Michael type addition mechanism

Both primary mono-amines and secondary bis-amines lead to linear polymers. Under non-selective conditions bis-primary amines usually give crosslinked products on reaction with bis-acrylamides, that is, they react as tetrafunctional monomers. However, it has been reported that under special conditions including low reactant concentrations, low initial temperatures, and an excess, on a molar basis, of bis-amines, soluble PAAs carrying secondary instead of tertiary amino groups in their main chain can be obtained. These PAAs have been found to provide suitable soluble carriers for drug attachment. [10] The structures of some PAAs are reported in Table 2.1.

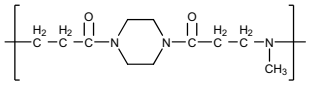
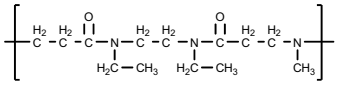
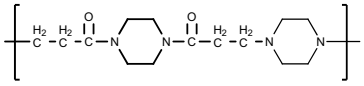
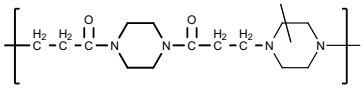
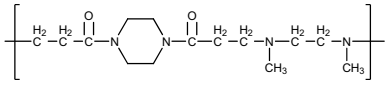
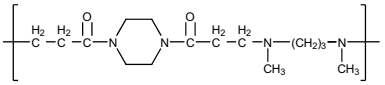
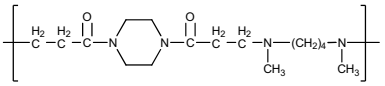
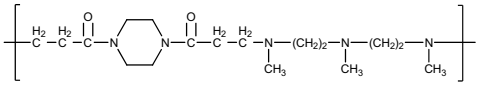
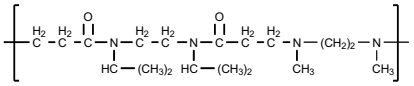
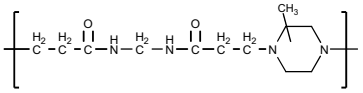
No.	Structure of the repeating unit
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

Table 2.1.Some examples of PAAs. [8]

2.2 Functionalisation of PAAs

PAAs are inherently highly functional polymers. However, further functionalization of PAAs may be useful for special purposes. In many cases, the introduction of additional functions in PAAs as side substituents can be simply achieved starting from the corresponding functionalized monomers. The presence of hydroxy-, *tert*-amino-, allyl-, amido- and ether groups, in the monomers do not interfere with the polymerisation process. The structures of some functionalised PAAs are reported in Table 2.2.

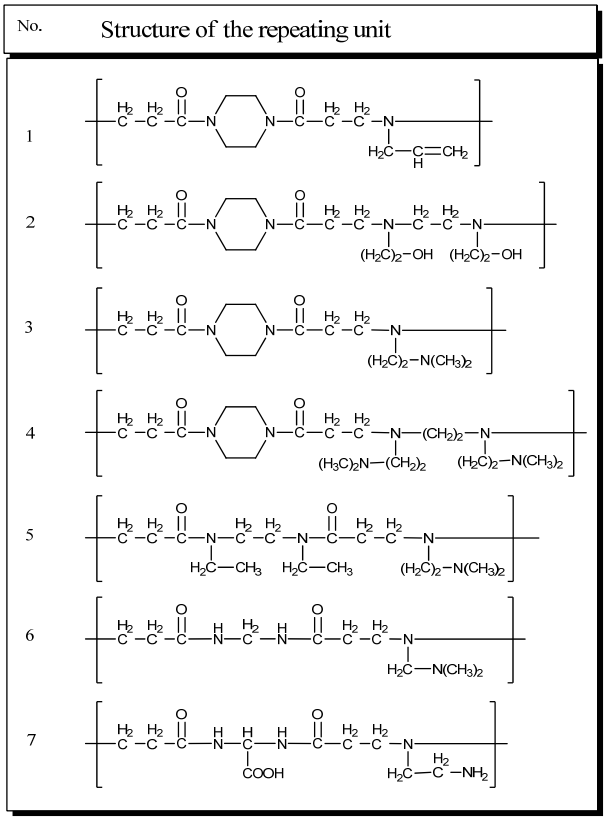


Table 2.2.Functionalised PAAs. [9]

However, to obtain PAAs functionalized with chemical groups as side sostituents such as SH, NH2 NHR and PH2, an indirect pathway is required. In particular, starting from the results of a kinetic study on the addition reaction of N,N-dimethylacrylamide to the side α-

amino groups of partially protonated poly-L-lysine, [11] it has been shown in a previuos work that PAAs carrying primary amino groups as side substituents (exemple 7, Table 2.2), can be prepared by using as monomer 1,2-bis-aminoethane in its mono-protonated form. [12] Owing to the sharp difference among the two basicity constants of 1,2-bis-aminoethane, little or no di-protonated molecules are present in solution and only one amino group per molecule is initially available for the addition reaction. This reaction lowers the pKa of the amino group involved, making it improbable that the same group will subsequently assume a proton by exchange reaction with a protonated one. Only the amino group already substituted remains in its unprotonated form and can react further, and the behaviour of mono-protonated 1,2-bis-aminoethane in PAA synthesis approaches that of a primary monoamine. We have found, in fact, that by adjusting the reaction conditions essentially linear polymer with primary amino groups as side substituents can be obtained from 1,2-diaminoethane and probably also from other bis-primary amines, if a sufficient difference exists between their ionisation constants.

2.*3* Amphoteric PAAs

Amphoteric PAAs are polyamidoamines carrying acidic functions as side substituents, in most cases carboxyl groups.

They are obtained by using either carboxylated bis-acrylamides or aminoacids as monomers. In both cases, a stoichiometric amount of strong base must be added to the monomer mixture. As regards aminoacids, natural α-aminoacids other than glycine yield only oligomeric products, but the polyaddition reaction proceeds reasonably well with all aminoacids bearing no substituents on the carbon atom in α-position, as well as with peptides in which the first aminoacid residue fulfils the same condition.

Amphoteric PAAs present a unique interest as bioactive polymers. Besides being generally less toxic than purely cationic PAAs of similar structure, in solution they change their net average charge as a function of pH. By a proper choice of the starting monomers, the acid and basic strength of the amino and the carboxyl groups can be controlled in such a way that the polymer passes from a prevailingly anionic to a prevailingly cationic state during a

relatively modest pH change. For instance this would occur when amphoteric PAAs are internalised by cells via the endocytic pathway. During this time they are transferred from extracellular fluids of pH 7.4 to intracellular compartments where the pH is lowered to 6.5 (endosomes) and then ~ 5.0 (lysosomes). Moreover, PAAs that become prevailingly cationic at lower pH can also be designed to become membrane-active and thus they display endosomolytic properties.

The structures of some amphoteric PAAs deriving from aminoacids are reported in Table 2.3 and amphoteric PAAs deriving from 2,2-(bis-acrylamido)acetic acid are reported in Table 2.4.

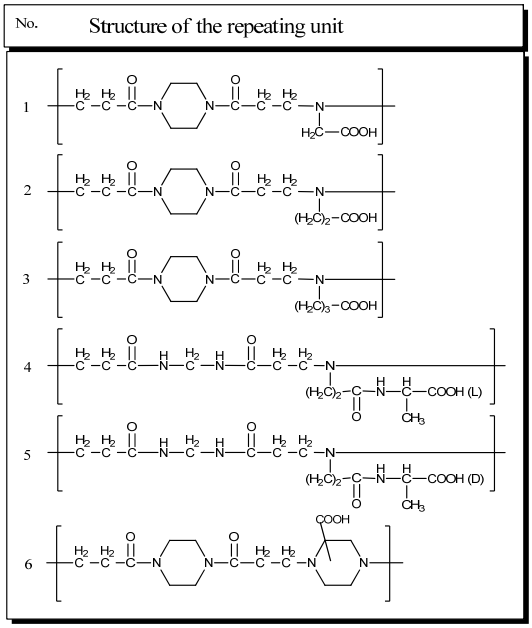


Table 2.3.Amphoteric PAAs deriving from amino acids. [7, 51]

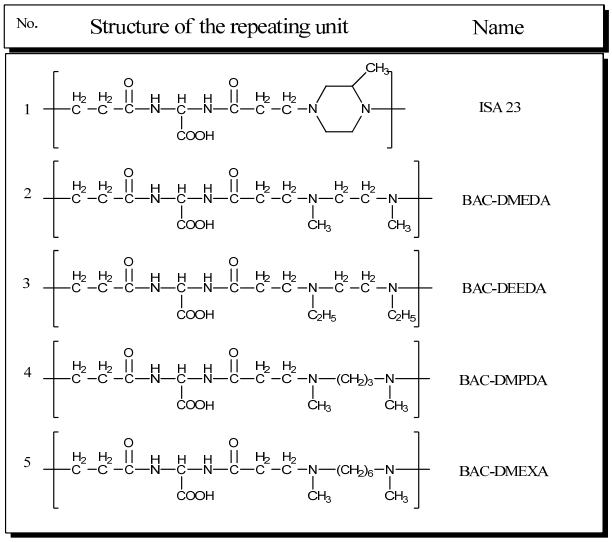


Table 2.4. Amphoteric PAAs deriving from 2,2-*bis*(acrylamido) acetic acid. [13]

PAAs in which the amido and ter-amino groups are not regularly distributed along the macromolecular chain have been synthesised by poly(acylation-addition) of piperazine and other bis-secondary amines, with various activated derivatives of acrylic acid. [14,15] "Alternating" PAAs, in which one amino-group is strictly followed by one amido-group all along the macromolecular chain have also been synthesised. [15]

Polymers structurally related with PAAs were obtained by substituting either bis-acrylic esters, [16] or divinylsulphone [17] for bis-acrylamides or hydrazine [18] or phosphines [19] for amines.Other polymers, of poly(amino-ketone) structure, were obtained by polycondensation of ketonic bis-Mannich bases with bis-amines, [20-22] as reported in Table 2.5.

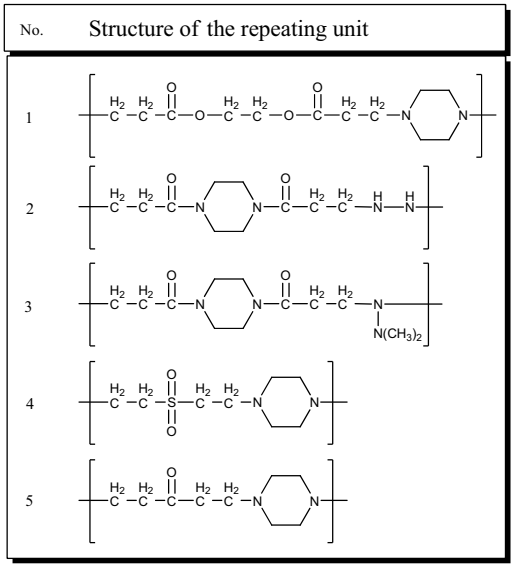


Table 2.5. *tert*-Amino polymers structurally related with PAAs. [20-22]

The ring-opening addition reaction of bis-secondary amines, or primary mono-amines, to ethylene sulphide gave a new family of monomers, namely 2,2'-alkylidenediiminodiethanethiols. [23] These monomers, besides polymerising by polyoxidative coupling, [23] could be employed in polyaddition reactions with bis-acrylamides, bis-acrylic esters, or divinylsulphone, in a way formally similar to that used with bis-secondary amines. [24-26]

The polyaddition proceeded also with bis-methacrylic esters [27] and bis-methacrylamides[28], that are extremely sluggish in reacting with bis-amines under the usual conditions of PAA synthesis. The structures of some selected sulfur-containing polymers structurally related to PAAs are reported in Table 2.6.

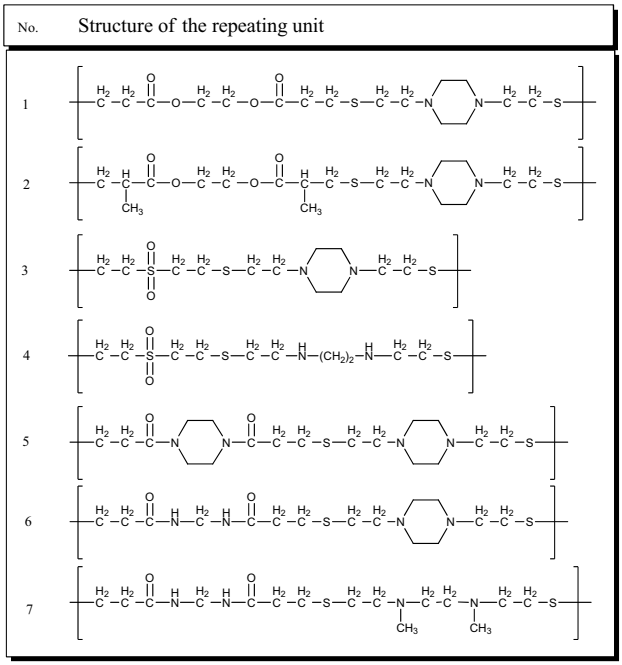


Table 2.6. Sulfur-containing tert-amino polymers structurally related with PAAs. [24-26]

By considering the synthetic process leading to PAAs it's evident that if the hydrolytic cleavage of the main backbone during polymerisation is minimised the end-groups of the products are either sec-amino-, or acrylamido groups. Therefore, by performing the polymerisation reaction with an excess of one of the two monomers, PAAs prevailingly or totally terminated with either of the two groups (A-PAAs and V-PAAs, respectively) can be obtained. End-functionalised PAAs can be employed for preparing block and graft copolymers, as well as crosslinked resins, and therefore can be regarded as macromonomers. A careful end group analysis performed by NMR on samples of a model PAA obtained with different monomer ratios [29] showed that the well known relation between the limit number-average polymerisation degree $\overline{X}_n$ and the initial stoichiometric ratio *r* of the monomers [30]:

$$\overline{X_n} = \frac{1+r}{1-r}$$ Equation 2.1

was true only with excess amine. The average molecular weight (see section 1.5) of the products obtained with initial excess bis-acrylamide was invariably higher than expected. This might be explained by the occurrence, during polymerisation, of some hydrolytic cleavage of the amido groups, preferentially involving the terminal acrylamido groups while sparing internal amido groups. This process would partly substitute amino groups for acrylamido ones, thus reducing the excess of the latter and re-adjusting to some extent the stoichiometric balance. The acrylic acid eventually produced should not compete with the acrylamido groups for the addition of the amino groups. The double bond of acrylic acid, in fact, is comparatively unreactive towards amines under basic conditions.

2.4 Chemical properties of PAAs

The number-average and weight-average molecular weight (see section 1.5) of the PAAs described thus far were in the range 5,000÷30,000 and 10,000÷50,000 respectively, with a dispersity index (depending from the isolation method) usually in the range 1.5 - 2. [34, 35] For example, when the polymerization products of series of differently disubstituted diamines with the same bisacrylamide, 1,4-bis-acryloyl-piperazine or the sodium salt of 2,2-bis-acrylamido-acetic acid, were compared it was shown that their molecular weight diminished with increasing steric hindrance on the nitrogen atoms. The effect of nucleophilicity of the nitrogen atoms apparently plays a minor role in determining overall molecular weight reached. [7] This is not surprising. The polymerisation reaction is the Michael addition, that is, an equilibrium reaction mostly affected by the reactant concentration and steric hindrance.

As mentioned above, when water is used as the polymerisation solvent for the polyaddition reaction, it proceeds fastest and gives the highest molecular weight products. For reactions carried out in aqueous media, the molecular weight increases with the reaction time until it reaches a maximum, after which it steadily decreases. This is not surprising, as in water the polyaddition reaction competes with the hydrolytic cleavage of the amidic bonds. The maximum attainable molecular weight in water depends on both monomers concentration and reaction temperature, being higher for higher concentrations and lower temperatures. At higher temperatures the maximum is attained quicker, but is lower. [7]

PAAs are usually soluble in water as well as chloroform, lower alcohols, dimethylsulfoxide and other polar solvents. However, amphoteric PAAs dissolve only in water. The intrinsic viscosities of PAAs in organic solvents or aqueous media usually range from about 0.15 to 1 dl/g. As a rule, PAAs exhibit relatively large hydrodynamic volumes in solution if compared with polyvinyl polymers of similar molecular weight, indicating a tendency to assume an extended chain conformation in solution.

2.5 PAAs as polyelectrolytes

PAAs can be regarded as polyelectrolytes since they posses ter-amino groups in the main chain. Normally, the values of the protonation constants (logK) of polyelectrolytes depend on the degree of protonation of the whole macromolecule; that is, they follow the modified Henderson-Hasselbach equation:

$$\log K_i = \log K_{i^\circ} + (n-1)\log\left[\frac{(1-\alpha)}{\alpha}\right] \qquad \text{Equation 2.1}$$

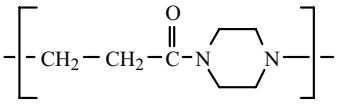
where $\log K_i^\circ$ is the protonation constant of a group present in a completely unionised polymer. The protonation constants of polyelectrolytes are usually referred to as "apparent" constants, as opposed to the "real" constants of non-macromolecular acids and bases. [33] However, in most PAAs the results of the potentiometric titrations are consistent with n values very close to 1. This means that in PAAs the tendency of the aminic nitrogens of each repeating unit to assume a proton in practice does not depend on the degree of protonation of the whole macromolecule. Therefore these groups behave as if belonging to a small molecule. [37-49] Consequently, "real" basicity constants can be determined. The number of the basicity constants of PAAs is equal to the number of the aminic nitrogens present in their repeating unit, and their values are similar to those found for the non-macromolecular models of PAAs, prepared by hydrogen-transfer addition to 4-acryloylmorpholine of the same amines used in the preparation of the corresponding PAA. The basicity constants of some representative PAAs are reported in Table 2.7.

Structure	Polymer $\mathbf{pK_{a1}}$	Polymer $\mathbf{pK_{a2}}$	Model $\mathbf{pK_{a1}}$	Model $\mathbf{pK_{a2}}$
Entry 5 of Table 2.1	8.09	4.54	8.25	4.80
Entry 3 of Table 2.2	8.80	4.11	9.05	4.35
Entry 3 of Table 2.1	7.01	2.98	7.12	2.39
Entry 1 of Table 2.1	7.79	-	8.07	-
Entry 4 of Table 2.1	9.02	7.91[a)]	9.05	8.34[b)]

a) $pK_{a3} = 4.46$, $pK_{a4} = 2.12$
b) $pK_{a3} = 4.42$, $pK_{a4} = 2.43$

Table 2.7. Basicity constants of some PAAs and their non-macromolecular models at 25°C in 0.1 M NaCl solution. [8]

The unusual behaviour of PAAs in the polyelectrolytes domain is probably due to the relatively long distance between the amino groups belonging to different units combined with the high charge-sheltering efficiency of the two amido groups interposed. It is noteworthy that a single amido group bound to a piperazine ring, as in the polymer shown in the scheme 2.3, is not sufficient to minimise interactions between neighbouring units. In fact it exhibits a typical polyelectrolyte behaviour. [50]



Scheme 2.3. Poly(1,4-piperazinediyl-1-oxo-trimethylene)

Differently from "normal" PAAs, amphoteric PAAs deriving from aminoacids and therefore carrying both carboxyl- and amino-groups attached to the same monomer tend to exhibit a typical polyelectrolyte behaviour. [51] This tendency is less pronounced for amphoteric PAAs in which the carboxyl groups and the amino groups are attached to different monomers. [13]

2.6 Heavy metal ions complexing behaviour

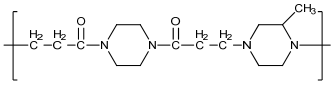
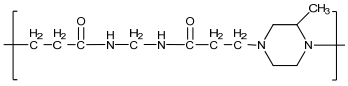
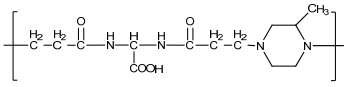
Many PAAs are able to form coordination complexes with heavy metal ions, such as Cu^{2+}, Ni^{2+}, Co^{2+}. "Real" stability constants could be determined for PAAs also in the case of complex formation. [37, 38, 43, 44, 48, 49, 52]

Only those PAAs (with the exception of PAAs derived from aminoacids) containing at least two aminic nitrogens per repeating unit that neither belong to a cyclic monomer nor are separated by more than three methylene groups are capable of complex-formation. The electronic and e.p.r. spectra of both polymeric and non-polymeric complexes obtained from PAA models were similar, and consistent with an octahedral tetragonally distorted structure, in agreement with the substantial independence of the repeating units of the

polymers in the complex formation process. Crosslinked PAA resins retain ion-complexing ability similar to those of the corresponding linear polymers. [53-55]

2.7 Degradation behaviour of PAAs in aqueous media

All PAAs, containing amidic bonds in their main chain are degradable in aqueous solution. The degradation of several PAAs (structures shown in Tables 2.1 and 2.4) has been studied by means of viscometric and chromatographic techniques. [56, 57] The structure of both the aminic and amidic moieties has an influence on the degradation rate. For instance, in PAAs containing 2-methylpiperazine moieties, the rate of decrease in viscosity observed was in the order **4** (Table 2.1) > **10** (Table 2.2) > **1** (Table 2.4) (Figure 2.2).

Name	Structure of the repeating unit	Monomers	$\frac{\eta_{sp}/C}{dl/g}$
PAA-1		BP + 2MeP	0.46
PAA-2		MBA + 2MeP	0.31
PAA-3		BAC + 2MeP	0.27

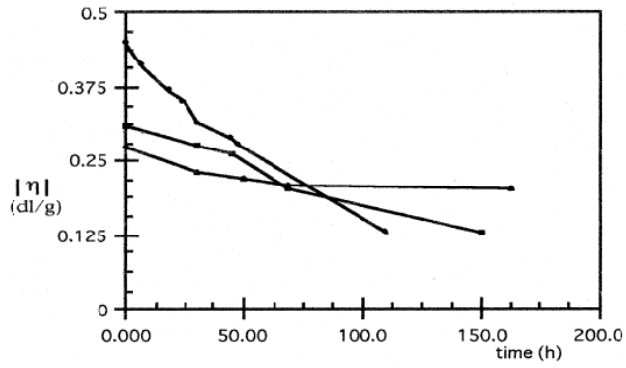


Figure 2.2. Degradation behavior of some representative PAAs at 37°C in 0.2 M phosphate buffer, investigated by viscometric measurements. [83] Initial weight-average molecular weights were approximately: 28000 (PAA-1, squares), 19000 (PAA-2, circles), 16500 (PAA-3, triangles), as measured by means of SEC with PAA-1 calibration.[29]

As the aminic portion of each PAA was the same, it can be concluded that the PAA derived from BAC was the most stable. This trend was confirmed by further studies on several other PAAs having different aminic monomers coupled with the same three amidic monomers. [57, 58] We have observed in many instances that additional *ter*-aminogroups or carboxylate groups, if present as side substituents, increase the degradation rate of PAAs. However, we do not have at present any clear evidence that the degradation rate of PAAs is affected by the basicity of the amino groups present in the macromolecular backbone.

The mechanism of PAA degradation seems to be purely hydrolytic as no vinyl groups, such as those which would have derived from a β-elimination reaction, could be determined by NMR analysis. [29] Moreover, the degradation rate was not affected by the presence of a 20-fold excess 2-mercaptoethanol (on a molar basis). This would have affected degradation if mercaptoethanol was able to act as scavenger of any activated double bonds resulting from a β-elimination-mediated degradation. In addition, it has been demonstrated that degradation of PAAs in aqueous media is strongly influenced by pH but does not seem to be affected by the presence of isolated lysosomal enzymes at pH 5.5. [84]

Bioreducible PAAs have been also recently developed [83, 85] that are able to undergo reductive degradation in presence of a reductive agent such as mercaptoethanol, 1,2-dithiotreitol and glutathione. These PAAs were obtained by using disulfide linkages containing monomers as cystine, cystamine or N,N'-bisacryloylcystamine. Therefore, these polymers are able to be degraded, giving low molecular weight species, in the intracellular environment.

2.8 Biomedical applications of PAAs

PAAs were first developed in the biomedical field in relation with their ability to form stable complexes with heparin. More recently water soluble PAAs used as polymeric drugs, polymer drug carriers (particluarly anticancer conjugates and as pH-responsive polymers which display endosomolytic characteristics and therefore have potential for use as non-viral vectors for intracytoplasmic delivery of proteins and genes.

2.8.1 PAAs as polymer therapeutics

Water soluble PAAs have been examined as anticancer agents in their own right [59] polymer-drug conjugates (particularly as anticancer conjugates) and as endosomolytic vectors for intracellular delivery of genes and toxins. [60, 83, 84] Before water soluble polymers can be seriously studied for parenteral administration it is essential to establish their biocompatibility in respect of route and frequency of administration and the end application.

Although many polycations such as poly-L-lysine, polyethyleneimine and amine terminating PAMAM dendrimers have been explored as drug and oligonucleotide delivery systems, [61-63] they are generally very toxic to cells in culture. For example poly-L-lysine displays IC50 values in the range 1-60 μg/ml depending on the cell type, and incubation time [64] and poly L-lysine, polyethyleneimine and PAMAM dendrimers showed signficant haemolytic activity which is molecular weight- (generation-) dependant. [63-64]

Early studies examined the cytotoxicity of PAAs derived from piperazine or *N,N′*-bis (2-hydroxyethyl)ethylenediamine. Although these polymers were less cytotoxic than poly-L-lysine, [100] the more recently described amphoteric PAAs shown in Table 2.4 are even less toxic (Table 2.8). ISA23, BAC -DMEDA and BAC -DEEDA were >100 times less cytotoxic than the non-amphoteric aminic polymers used as a reference. [13, 66]

Polymer	$\overline{M_w}$	IC_{50} (mg/ml ± SD)
Dextran	72000	>5
ISA 23	42710	>5
BAC-DMEDA	28960	>5
BAC-DEEDA	30310	>5
BAC-DMEPDA	11420	3.55 ± 0.31
BAC-DMEXA	30860	0.23 ± 0.06
Poly(L-lysine)	56500	0.05 ± 0.061
Poly(ethyleneimine)	70000	0.01 ± 0.01

Table 2.8. Cytotoxicity of amphoteric PAAs against B16F10 melanoma cells. [13]

At pH 7.4 ISA23 has ~ 50% of its units in L- and ~50% in LH± form, so the polymer would have a relatively large negative average charge. Similarly at pH 7.4 BAC-DMEDA and BAC-DEEDA would be prevailingly negatively charged with more than 90% of units in the L± form13. This charge effect would explain their lack of toxicity; supported by the observation that BAC-DMEPDA and BAC-DMEXA, which are positively charged at pH 7.4, are more cytotoxic than the other PAAs. Comparing the IC50 values of BAC-DMEDA and BAC-DEEDA it would appear that a modest increase in hydrophobicity does not significantly induce toxicity. Cytoxicity is most closely related to the net positive charge present at physiological pH i.e. the fraction of repeating units present in LH2±+ form. As the log˚K2 (relating to the protonation of the second amino group) increases and approaches physiological pH, so does polymer-mediated toxicity (Table 2.8). This general conclusion is supported by the observation that hydrogen-transfer polyaddition of *N,N*-dimethylacrylamide to the side amino groups of poly L-lysine also resulted in a sharp decrease of toxicity. [11]

As PAAs are degradable in the main chain [31, 32] and are relatively non-toxic this would suggest potential for parenteral administration and use as polymer-based therapeutics. However, before further studies were warranted it was necessary to demonstrate that PAAs could display an appropriate pharmacokinetic profile, i.e. a plasma residence time and tissue- or disease-specific targeting appropriate to proposed application. To study biodistribution analogues of the PAAs ISA 1 and ISA 23 [66] were synthesised to contain approximately 1 mol% 2-*p*-hydroxyphenylethylamine. These polymers were named ISA 4 and ISA 22, respectively. [66] After intravenous (i.v.) injection to rats ^{125}I-labelled ISA 4 was immediately taken up by the liver (> 80% recovered dose at 1h) whereas ^{125}I-labelled ISA 22 was not (liver uptake was <10% recovered dose at 5h) (Table 2.9). The longer circulation time of ISA 22 relative to other cationic polymers provides opportunity for use in tissue targeting either by the incorporation of receptor-targeting ligands e.g. galactose moiety [67] or by passive means such as the enhanced permeability and retention effect (EPR) . [68-70]

Body district	Percent localization			
	ISA 22[a)]		ISA 4[b)]	
	After 1 h	After 5 h	After 1 h	After 5 h
Blood	≈ 70 %	≈ 20 %	≈ 1.5 %	≈ 0 %
Kidney	≈ 2 %	≈ 1 %	≈ 7 %	≈ 4 %
Liver	≈ 10 %	≈ 8 %	≈ 83 %	≈ 85%
Lungs	≈ 2 %	≈ 4 %	≈ 4 %	≈ 3 %
urine	≈ 13 %	≈ 65 %	≈ 0 %	≈ 5 %

a) The structure of ISA 22 is the same as that of Isa 23 (see Table 2.4), in which 3 mol-% of 2-methylpiperazine units have been replaced by tyramine units to allow iodination.
b) The structure of ISA 4 is the same as that of Isa 1 (entry 4 of Table 2.1) in which 3 mol-% of 2-methylpiperazine units have been replaced by tyramine units to allow iodination.

Table 2.9. Body distribution of ^{125}I-labelled PAAs after intravenous injection in rats. [66]

Tumour targeting by the EPR effect occurs due to the fact that circulating macromolecules (proteins or synthetic polymers) are unable to cross the walls of normal capillary vessels but are able to extravasate into tumour tissue due to their leaky angiogenic vasculature. This phenomenon has already been shown to cause significant tumour targeting of N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates containing doxorubicin [69] and platinates [71]. Biodistribution studies in mice bearing subcutaneous B16F10 melanoma showed that ^{125}I-labelled ISA 22 was still accumulating in tumour tissue after 5h (2.5% dose/g). The so-called "stealth" properties of ISA 23 are probably due to its zwitterionic nature with prevailingly negative charge at pH 7.4.

2.8.2 PAAs-anticancer conjugates

Early in the 1970's several PAAs (structures shown in Table 2.10) were shown to display inherent antitumour activity. [59]

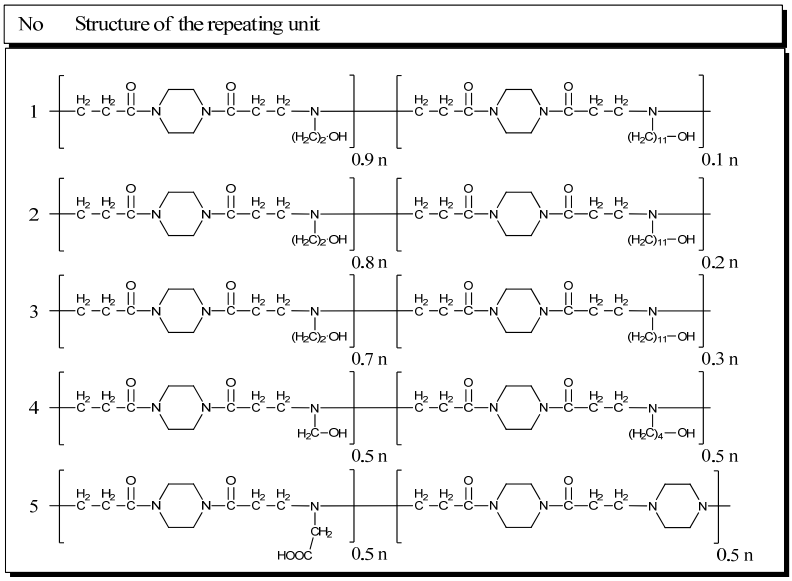


Table 2.10.Structures of PAAs tested for antimetastatic activity. [59]

After i.v. administration PAAs **1-4** of Table 2.10 were toxic. Nevertheless, they could be administered to mice at a dose of 20 mg/Kg, and showed activity in reducing the number and average weight of Lewis lung (but not Sarcoma 180) tumor metastases. However, PAA 5 was not toxic, and moreover at a dose of 200mg/Kg was able to reduce the number and average weight of both Sarcoma 180 and Lewis lung tumor metastases. Although activity was observed in the metastatic models no activity was observed against the primary tumour.

Following the rationale adopted for design of other polymer-drug conjugates [72] more recently PAAs have systematically been developed as water soluble carriers for known anticancer agents including mitomycin C (MMC) [73] and platinates. [74]

2.8.3 Non-viral vectors for intracytoplasmic delivery

Intracytoplasmic delivery of macromolecular drugs, particularly proteins and genes remain a challenge that must be overcome if we are to capitalise on advances in the understanding of the molecular basis of diseases. [75] Although viral vectors mediate effective transfection they have disadvantages including random insertion into host genome, potential immunogenicity [76] and low gene carrying capacity, many non-viral delivery systems including cationic lipids and polymers are relatively toxic and also rapidly localise to lung or liver after i.v. administration thus abrogating their ability to target other tissues. Additionally, in most of the cases they display very poor transfection efficiency. This has in part been attributed to their inability to efficiently permeabilise the endosomal membrane. [77] Efficient cellular targeting and also localisation to the appropriate subcellular compartment is vital if these new approaches are to be converted into useful medicines. Addition of fusogenic peptides to the culture medium can increase transfection efficiency [78, 79] and it has been suggested that polymeric transfection agents such as polyethyleneimine can also facilitate transfection by swelling within the endosome causing membrane rupture, the so-called "proton sponge effect. [79]

Unlike many other polyamines (e.g. polyethyleneimine and poly L-lysine), protonation and de-protonation of the repeating units along the PAA backbone are independent events thus providing the ability to tailor molecules with very sharp basicity constants. This peculiarity means that PAAs bearing two aminic nitrogens in their repeating unit show a marked conformational change during movement from a neutral to acidic pH. This effect is most pronounced when the aminic nitrogens are separated by only two methylene groups. [47]

To investigate whether PAAs are able to permeabilise the endosomal membrane and thus aid cytoplasmic entry, the ability of PAAs to mediate DNA or toxin delivery was studied. [80, 81, 83] Surprisingly, due to their amphoteric nature at pH 7.4, the PAAs ISA 1 and ISA 23 formed torroid shaped interpolyelectrolyte complexes (IPECs) of diameter 80 -150 nm in diameter (10:1 polymer excess) which were visible using TEM [80] (Figure 2.3).

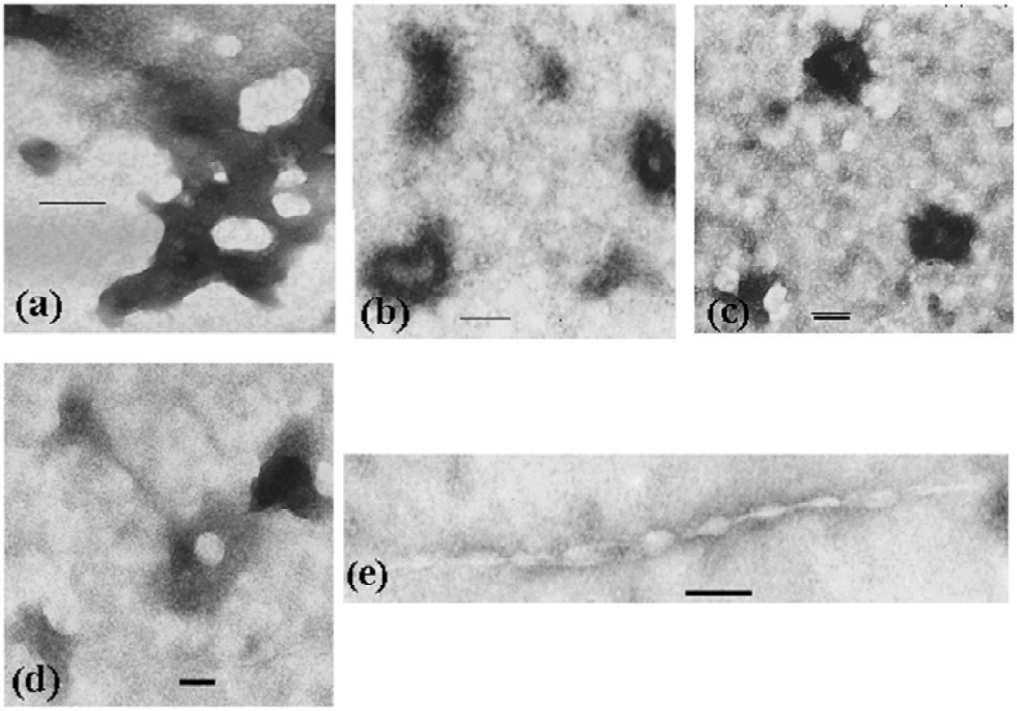


Figure 2.3: TEM images of PAA/DNA complexes. (a) Lipofect ACE (scale bar 67 nm), (b) poly(l-lysine) (scale bar 67 nm), (c) ISA 22 (scale bar 45.5 nm), (d) ISA 4 (copolymeric PAA obtained by polyaddition of a mixture of N,N9-bis(2-hydroxyethyl) ethylenediamine and 2-methylpiperazine (50 : 50) to 1,4- diacryloylpiperazine; scale bar 140 nm), and (e) plasmid (scale bar 67 nm). [80]

The complexes displayed retarded electrophoretic mobility and also the ability to protect DNA from DNAse II degradation. At a polymer:DNA ratio of 10:1 the extent of inhibition of degradation was in the range 92.2 ± 11.2% (ISA 4) - 58.0 ± 10.0% (ISA 23) (Figure 2.11).

In transfection experiments, the PAAs demonstrated the ability to mediate pSV β-galactosidase transfection of HepG2 and COS-7cells. An ISA23 complex of ISA23: DNA mass ratio of 5:1 showed equivalent transfection ability compared with polyethyleneimine and LipofectIN, and was more effective than LipofectACE. [80] Moreover, recently developed cationic PAAs containing disulfide linkages in the main chain showed a significantly higher transfection efficiency compared with polyethyleneimine.[83]

Recent studies are focusing on precise definition of the intracellular trafficking of PAAs with a view to quantitation of their intracellular fate. It is also necessary to find definitive proof that PAAs do break the endosomal membrane. These studies are difficult as it is hard

to find a good marker of the endosomal membrane. Lysosomes are more easily identified due to the presence of lysosomal enzymes in their interior. Isolation of rat liver lysosomes following administration of ISA23 and ISA4 have shown that internalisation of PAA into the lysosomal compartment destabilises the lysosomal membrane resulting in increased fragility and enzyme escape. [82] Interestingly incubation of PAAs on the outside of isolated lysosomes does not have this effect suggesting that differential membrane structure, inside versus outside, must play a role in membrane destabilisation. However, the precise mechanism of PAA membrane perturbation warrants more detailed mechanistic study if PAA structure is to be optimised further.

2.9 Conclusions

In conclusion we can say that PAAs are an extremely versatile family of step-addition polymers. Their facile synthesis and functionalisation allows production of many diverse linear, crosslinked and blocky or grafted polymers. Thus, PAA chemistry can easily be tailored to suit specific applications. PAAs are being developed for many biomedical applications.

Many PAAs have features rendering them particularly suitable for use as soluble polymer-drug conjugates. Their hydrophilicity allows solubilisation of hydrophobic drugs and functionality allows attachment of pendent drug moieties, targeting groups or diagnostic moieties. The high hydrodynamic volume of PAAs and their ability to prepare 'stealth" polymers provide the opportunity for significant tumour targeting by the EPR effect. As PAAs are bio degradable this allows parenteral injection of high molecular weight polymers without risk of long-term accumulation in the body and moreover the amphoteric polymers display minimum general cytotoxicity or haemolytic activity at pH 7.4. The toxicity of the ultimate degradation products of PAAs was not investigated; however, they are unlikely to be produced in significant amounts within the body since the polymers will be eliminated first upon reduction of their molecular weight.

PAAs are particularly suitable for a use as intracytoplasmic/endosomolytic delivery vectors. They have "real" basicity constants which can be tailored depending on the aminic monomers selected, resulting in a predictable charge situation as a function of pH. This is

particularly important in a sharp change when passing from pH 7.4 (blood) to pH 5.5 or less within the intracellular endosomal and lysosomal compartments. At pH 7.4, amphoteric PAAs behave as zwitterions with a zero- or a slightly negative net average charge. They undergo conformational change as a function of degree of protonation leading to "opening" of PAA coil after cellular internalisation. This unique characteristic can be used to release of a drug payload and assist delivery of macromolecular drugs into the cytosol.

References

[1] P. Ferruti, "Functionalization of Polymers", in: "*Reactions of Polymers*", J.A. Moore, Ed., Reidel Publishing Corp., Dordrecht (Holland), p.73-101, **1973**.

[2] P. Ferruti, E. Ranucci, M.A. Marchisio "Ion chelating polymers for biomedical use",in "*Frontiers of Macromolecular Science*", T. Saegusa, T. Higashimura, A. Abe, Eds, Blackwell Scientific Publications, , p.567-572, **1989**.

[3] P. Ferruti, G. Scapini, M.C. Tanzi, L. Rusconi, "Synthetic or Semisynthetic Polymers of Medical Significance", in: *Macromol. Symposia* (28), 373, **1982**.

[4] R. Duncan, S. Dimitrijevic, E. G. Evagorou, *S.T.P. Pharma Sci.* (6), 237, **1996**.

[5] R. Duncan, “Polymer Therapeutics into the 21st Century”, in: *Drug Delivery in the 21st Century*, K. Park, R. Mrsny, Ed., ACS Books, p.350-363, **2000**.

[6] P. Ferruti et al. *Macromol. Rapid Commun*., (23), 332-355, **2002**.

[7] F. Danusso, P. Ferruti, *Polymer,* (11), 88, **1970**.

[8] P. Ferruti, M.A. Marchisio, R. Barbucci, *Polymer* (26), 1336, **1985**.

[9] P. Ferruti, "Ion-chelating Polymers (Medical Applications)", in: *Polymeric Materials Encyclopedia*, vol. 5, J. C. Salamone, Ed., CRC Press Inc, Boca Raton, Florida, p 3334-3359, **1996**.

[10] G. Caldwell, E. Neuse, A. Stephanou, *J. Appl. Polym. Sci.,* (50), 393, **1993**.

[11] P. Ferruti, S. Knobloch, E. Ranucci, R. Duncan, E. Gianasi, *Macromol. Chem. Phys.* (199), 2565, **1998**.

[12] B. Malgesini, I. Verpilio, R. Duncan, P. Ferruti, *Macromol. Biosci.*, (3), 59–66, **2003**.

[13] P. Ferruti, S. Manzoni, S.C.W. Richardson, R. Duncan, N.G Pattrick, R. Mendichi, M. Casolaro, *Macromolecules* (33), 7793, **2000**.

[14] L. Rusconi, M. Tanzi, P. Ferruti, L. Angiolini, R. Barbucci, M. Casolaro, *Polymer* (23), 1233, **1982**.

[15] M.C. Tanzi, L. Rusconi, C. Barozzi, P. Ferruti, L. Angiolini, M. Nocentini, V. Barone, R. Barbucci, *Polymer* (25), 863, **1984**.

[16] Rusconi, M.C. Tanzi, G. Garlaschelli, P. Ferruti, L. Angiolini, *Makromol.Chem., Rapid Commun.* (3), 909, **1982**.

[17] F. Danusso, P. Ferruti, G. Ferroni, *Chimica e Industria (Milan)* (49), 826, **1967**.

[18] P. Ferruti, Z. Brzozowski, *Chimica e Industria (Milan)* (50), 441, **1968**.

[19] P.Ferruti, R. Alimardanov, *Chimica e Industria (Milan)* (49), 831, **1967**.

[20] F. Andreani, A.S. Angeloni, L. Angiolini, P. Costa Bizzarri, C. Della Casa, A. Fini, N. Ghedini, M. Tramontini, P. Ferruti, *J. Polymer Sci. Polym. Letters* (19), 443, **1981**.

[21] A.S. Angeloni, P. Ferruti, M. Tramontini, M. Casolaro, *Polymer* (23), 1693, **1982**.

[22] A.S. Angeloni, P. Ferruti, M. Laus, M. Tramontini, E. Chellini, G. Galli *Polymer Communications* (24), 87, **1983**.

[23] P. Ferruti, E. Ranucci, *Makromol. Chem., Rapid Commun.* (8), 549, **1987**.

[24] P. Ferruti, E. Ranucci, *J. Polym. Sci.: Part C: Polym. Lett. Ed.* (26), 357, **1988**.

[25] P. Ferruti, E. Ranucci, L. Depero, *Makromol. Chem., Rapid Commun.* (9), 807, **1988**.

[26] P. Ferruti, E. Ranucci, L. Depero, *Polym. Commun.* (30), 157, **1989**.

[27] E. Ranucci, P. Ferruti, *Polymer* (32), 2876, **1991**.

[28] E. Ranucci, F. Bignotti, E. Dancelli, P. Ferruti, *Polymer J.* (25), 625, **1993**.

[29] F. Bignotti, P. Sozzani, E. Ranucci, P. Ferruti, *Macromolecules* (27), 7171, **1994**.

[30] P. J. Flory, "Principles of polymer chemistry", Cornell University Press, London **1953**.

[31] P. Ferruti, L. Provenzale, *Transplantation Proceedings* VIII, (1), 103, **1976**.

[32] E. Martuscelli, M. Palma, F. Riva, P. Ferruti, L. Provenzale*, Chimica e Industria (Milan)* (58), 542, **1976**.

[33] E. Martuscelli, L. Nicolais, F. Riva, P. Ferruti, L. Provenzale, *Polymer* (19), 1329, **1978**.

[34] M. C. Tanzi, B. Barzaghi, R. Anouchinsky, S. Bilenkis, A. Penhasi, D. Cohn, *Biomaterials* (13), 425, **1992**.

[35] M. C. Tanzi, G. Tieghi, P. Botto, C. Barozzi, P. Cardillo, *Biomaterials* (5), 357, **1984**.

[36] M. Mandel, in: "Encyclopaedia of Polymer Science and Technology", vol. 11, H. F. Mark, N. G. Gaylord, N. M. Bikales, Ed., Interscience, New York, p. 739-829, **1992**.

[37] P. Ferruti, "Biomedical and Pharmacological Application of Tertiary Amino Polymers" in: "*Polymeric amines and ammonium salts*", E. J. Goethals, Ed., Pergamon Press, Oxford and New York, p. 305, **1980**.

[38] P. Ferruti, N. Danzo, L. Oliva, R. Barbucci, V. Barone, *J.Chem.Soc.Dalton* 539, **1981**.

[39] P. Ferruti, R. Barbucci, *Advanc. Polym. Sci*. (58), 57, **1984**.

[40] R. Barbucci, P. Ferruti, C. Improta, M. Delfini, A.L. Segre, F. Conti, *Polymer* (19), 1329 **1978**.

[41] R. Barbucci, V. Barone, P. Ferruti, *Atti Acc.Naz.Lincei VIII* (64), 481, **1978**.

[42] R. Barbucci, P. Ferruti, *Polymer* (20), 1061, **1979**.

[43] R. Barbucci, P. Ferruti, C. Improta, M. La Torraca, L. Oliva, M.C. Tanzi, *Polymer* (20), 1298, **1979**.

[44] R. Barbucci, V. Barone, L. Oliva, P. Ferruti, T. Soldi, M. Pesavento, C. Bertoglio Riolo, "Macro-inorganics: Coordination Compounds with Poly(amido-amines)", in: "*Polymeric amines and ammonium salts*", E. J. Goethals, Ed., Pergamon Press, Oxford and New York **1980**, p.263.

[45] R. Barbucci, P. Ferruti, M. Micheloni, M. Delfini, A.L. Segre, F. Conti, *Polymer* (21), 81, **1980**.

[46] R. Barbucci, V. Barone, P. Ferruti, M. Delfini, *J. Chem. Soc. Dalton Transact*. 253, **1980**.

[47] R. Barbucci, M. Casolaro, P. Ferruti, V. Barbone, F. Lelj, L. Oliva, *Macromolecules* (14), 1203, **1981**.

[48] R. Barbucci, V. Barone, P. Ferruti, L. Oliva, *J. Polymer Sci., Polymer Symp.* (69), 49, **1981**.

[49] R. Barbucci, M. Casolaro, N. Danzo, M.C. Beni, V. Barone, P. Ferruti, *Gazz. Chim. It.* (112), 105, **1982**.

[50] R. Barbucci, M. Casolaro, P. Ferruti, M.C. Tanzi, L. Grassi, C. Barozzi, *Makromol.Chem*. (185), 1525, **1984**.

[51] R. Barbucci, M. Casolaro, M. Nocentini, S. Corezzi, P. Ferruti, V. Barone, *Macromolecules* (19), 37, **1986**.

[52] M. Casolaro, P. Ferruti, V. Barone, *Polymer* (23), 148, **1982**.

[53] C. Bertoglio Riolo, T. Soldi, M. Pesavento, *J. Appl. Polymer Sci*. (27), 2239, **1982**.

[54] T. Soldi, P. Ferruti, R. Barbucci, M. Benvenuti, *J.Appl.Polymer Sci.* (28), 3361, **1983**.

[55] M. Benvenuti, M. Pesavento, P. Ferruti, *Polymer Communications* (24), 26, **1983**.

[56] E. Ranucci, L. Sartore, F. Bignotti, M. A. Marchisio, P. Bianciardi, F. M. Veronese, *Biomaterials* (15), 1235, **1994**.

[57] E. Ranucci, F. Bignotti, L. Sartore, P. Bianciardi, M. A. Marchisio, *J. Biomat. Sci. Polym. Ed.* (6), 833, **1994**.

[58] E. Ranucci, G. Spagnoli, P. Ferruti, D. Sgouras, R. Duncan, *J. Biomat. Sci. Polym. Ed.* (2), 303, **1991**.

[59] P. Ferruti, F. Danusso, G. Franchi, N. Polentarutti, S. Garattini, *J. Medicinal Chem*. (16), 497, **1973**.

[60] P. Ferruti, S. Richardson, R. Duncan, "Poly(amidoamine)s as Tailor-made Soluble Polymer Carriers", in: *Targeting of Drugs: Stealth Therapeutic Systems,* G. Gregoriadis, B. McCormack, Eds, Plemun Press, New York **1998**, p. 207–224.

[61] C. F. Chu, S. B. Howell, *J. Pharmacol. Exp. Ther.* (219), 389, **1981**.

[62] G. Citro, C. Szczylik, C. Ginobbi, P. Zupi, B. Calabretta, *Br. J. Cancer* (69), 463, **1994**.

[63] N. Malik, R. Wiwattanapatapee, R. Klopsch, K. Lorenz, H. Frey, J.W. Weener, E.W. Meijer, W. Paulus, R. Duncan, *J. Controlled Rel.* (65), 133, **2000**.

[64] D. Sgouras, R. Duncan, *J. Mater. Sci.: Mater. Med*. (1), 61, **1990**.

[64] E. Ranucci, G. Spagnoli, P. Ferruti, D. Sgouras,, R. Duncan, *J. Biomat. Science, Polymer Edition,* (2), 303, **1991**.

[66] S. Richardson, P. Ferruti, R. Duncan, *J. Drug Targeting* (6), 391, **1999**.

[67] M. Casali, S. Riva, P. Ferruti, *J. Bioactive Compatible* 16(6), 479-491, **2001**.

[68] H. Maeda, "Polymer conjugated macromolecular drugs for tumour-specific targeting" in: *"Polymeric Site Specific Pharmacotherapy",* A. J. Doumb, Ed., John Wiley and Sons Ltd., New York, **1994**.

[69] L.W. Seymour, K. Ulbrich, P.S. Styger, M. Brereton, V. Subr, J. Strohalm, R. Duncan, *Br. J. Cancer* (70), 636, **1994**.

[70] R. Duncan, "Polymer conjugates for tumour targeting and intracytoplasmic delivery. The EPR effect as a common gateway?" in: *Pharmaceutical Science and Technology Today* (2), 441, **1999**.

[71] E. Gianasi, M. Wasil, E.G. Evagorou, A. Keddle, G. Wilson, R. Duncan, *Eur. J. Cancer* (35), 994, **1999**.

[72] R. Duncan, *Anti-Cancer Drugs* (3), 175, **1992**.

[73] E. Schacht, P. Ferruti, R. Duncan, PCT Internat. Pat. Appln. No. PCT/IB94/00259 to the Commission of the European Communities, Octob. 14th **1994**.

[74] P. Ferruti, E. Ranucci, F. Trotta, E. Gianasi, E. G. Evagorou, M. Wasil, G. Wilson, R. Duncan *Macromol. Chem. Phys*. 200, 1644, **1999**.

[75] E. M. Hersh, A. T. Stopeck, "Cancer Gene Therapy Using Non-Viral Vectors: Preclinical and Clinical Observations", in: *Self-assembling complexes for gene delivery from laboratory to clinical trial,* A. V. Kabanov, P. L. Felgner, L. W. Seymour., Ed., Wiley, New York **1998**, p 421–436.

[76] D. C. Gorecki, *Emerg. Drugs* (4), 247, **1999**.

[77] A Kichler, K. Mechtler, J-P. Behr, E. Wagner, *Bioconj. Chem*. (8), 213, **1997**.

[78] J. N. Lozier, A.R. Thompson, P. C. Hu, M. Read, K. M. Brinkhous, K. M, High, D. T. Kuriel, *Human Gene Therap.* (5), 313, **1994**.

[79] O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D.Scherman, B Demeneix,. J-P Behr, *Proc. Natl Acad. Sci. USA* (92), 7297, **1995**.

[80] N.G. Pattrick, S.C.W. Richardson, M. Casolaro, P. Ferruti, R. Duncan, *J Controlled Rel.* (77), 225, **2001**.

[81] S. C. W. Richardson, N. G. Pattrick, Y. K. S. Man, P. Ferruti, R. Duncan, *Biomacromolecules* (2), 1023, **2001**.

[82] P. Ferruti and N.Pattrick, . *Proc. Intl. Symp. Cont. Rel. Bioact. Mat.* 28, **2001**.

[83] C. Lin et al., Novel Bioreducible Poly(amido amine)s for Highly Efficient Gene Delivery, *Bioconjugate Chem*., (18), 138-145, **2007**.

[84] C. Lin, Z. Zhong, M. C. Lok, X. Jiang, W. E. Hennink, J. Feijen, J. F.J. Engbersen, *Journal of Controlled Release*, (116), 130–137, **2006**.

[85] E. Emilitri, P. Ferruti, R. Annunziata, E. Ranucci, *Macromolecules*, (40), 4785-4793, **2007**.

Chapter 3: Polymer-coated ferromagnetic nanoparticles for cancer therapy

3.1 Abstract

A group of novel amphoteric PAAs was synthesized by Michael addition reaction of three amines, 2-methylpiperazine (MP), N,N'-ethylenediamine-diacetic acid (EDDA), 2-aminoethyl-phosphonic acid (AP) with two different bisacrylamides, N,N'-bisacrylamidoacetic acid (BAC) and N,N'-bisacryloylpiperazine (BP). These polymers were obtained with different values of average molecular weight ($\overline{M}_n$: 5000, 15000, 30000, 50000) by varying the stoichiometric ratio of the starting monomers. Each polymer was then evaluated as coating agent of Magnetite and Cobalt-Ferrite nanoparticles. The coating tests showed that only the BAC containing polymers stabilized the colloidal suspension in water and in physiological solution. The BAC-EDDA - coated Magnetite nanoparticles showed the highest stability also after 24 h, yielding particles with an average size around 30 nm (by Dynamic Light Scattering) and good values of hyperthermic efficiency (by a S600 SQUID magnetometer). These results encourage further studies on PAA-nanoparticles systems to investigate the biological properties (i.e. biocompatibility) and magnetic performances (hyperthermia) by in vitro and in vivo tests.

3.2 Introduction

Over the past few decades, nanoparticles, which exhibit significantly distinct physical, chemical and biological properties from their bulk counterparts, have elicited much interest. For cancer diagnosis and therapy there are currently a number of techniques based on different types of nanoparticles [1-6]. Nanotechnological advances in cancer research, diagnostics and therapy, improved direct visualization of malignant cells, targeting at molecular level and safely delivering large amounts of chemotherapeutic agents to the desired cells. These techniques should be capable of rapid and sensitive detection of

malignant cells at early stages. The common feature of all nanoparticle-based cancer therapies is the need of specific NPs for achieving the desired therapeutic effect. However, each diagnostic/therapeutic technique requires a different chemical or physical property of the particles involved, which depends on the specific function played by the NPs in that therapy (e.g., vector, porous receptacle, heating agent, magnetic moment carrier, etc.). Sometimes the particle function is activated using an external agent (magnetic field, light, radiation, etc.) that interacts with the NPs.

Magnetic nanoparticles (MNPs) are one sub-class of this broad cancer-therapy designed NPs. Since the MNPs have a size comparable to the DNA or subcellular structures, this field opened the door for cell separation strategies using magnets as external driving force. Similarly, recent advancements on binding chemistry of biological units onto MNPs surface and the engineering of particle's surface/shape have opened new exciting possibilities for drug delivery with high selective vectors. At present, most applications of MNPs are based on the following physical principles:

- The utilization of the magnetic moment of the MNPs as a disturbance of the proton nuclear resonance (e.g., contrast media for Magnetic Resonance Imaging, MRI)..
- The positioning of MNPs in targeted organs or tissues (e.g. tumor) by application of controlled magnetic field gradients.
- The magnetic losses of nanometric particles in colloids for heating purposes (Magnetic Fluid Hyperthermia, MFH).

MRI is very useful in diagnosis and in following the course of therapies. It can produce images of both hard and soft organs. MRI contrast agents are often used and there are a number of potential compounds for infusion or injection. Basically there are three ways in which a contrast reagent may act. (i)It may not enter or attach to cells and simply act as an indicator of fluid-filled space e.g. the knee capsule. (ii) It may attach to the outside of cells (cell membrane) or (iii) may be taken into the cells. Closely related to these biological differences are the routes by which the agent reaches the target area. The high magnetic moment and the versatility of MNPs make them an attractive platform as contrast agents for MRI[16].

The localizability of the particles by applying external magnetic fields is noteworthy in many types of therapy, for instance drug delivery or hyperthermia. The MNPs can be actively directed to a specific tissue after intravenous (i.v.) injection. The final localisation of the particles could be intracellular in many cases. Though the fate of nanoparticles in the intracytoplasmic environment is not completely clear, it was demonstrated by in vitro and in vivo experiments that cells can survive the presence of such particles in moderate loadings for moderate periods of time [17].

Moreover, MNPs can be heated up by a time varying magnetic field, making them useful for Thermotherapy, and in particular for Magnetic Fluid Hyperthermia.

Magnetic Fluid Hyperthermia (MFH) is a technique in which MNPs are used to induce apoptosis and death in cancer cells exploiting their enhanced heat sensitivity compared to healthy cells, due to the poor development of blood vessels within the cancerous tissues[18]. The heat released by the particles upon application of an alternate magnetic field (usually in the frequency range 50–500kHz) is sufficient to increase the temperature in the tumor mass to values between 42 - 46°C for a few minutes, inducing cell apoptosis [19]. To this purpose, the best performing nanoparticles are those containing metal oxides. Among these oxides, it was found that Magnetite (Fe_3O_4) and Cobalt-Ferrite ($CoFe_2O_4$) can provide nanoparticles with a higher hyperthermic efficiency. Anyway, *in vivo* applications of MNPs involve subtle problems related to the response of a living organism to alien objects as, for instance, the immunological reaction, aggregation in arteries (embolism) and release of toxic metal ions as Nickel or Cobalt.

Polymers Coated Nanoparticles can overcome all these drawbacks. In fact, using amphoteric PAAs (see Section 2.3) it would be possible to obtain nanoparticles with the fundamental features they require to be used in cancer therapy. The properties contribution of PAAs coating to magnetic NPs are the following:

- making NPs colloidal suspension stable.
- making NPs biocompatible.
- making NPs stealth at antibodies.
- enabling NPs to drug delivery.

Amphoteric PAAs can be synthesised containing one or two relatively weak tertiary amino groups and one or more strong carboxyl group per repeating unit. These PAAs are predominantly anionic in extracellular fluids, owing to the incomplete ionisation of the amino groups and complete ionisation of the carboxyl groups at pH 7.4. [7] When injected in experimental animals, they avoid rapid clearance by the reticuloendothelial system; that is, these PAAs are endowed with ‘‘stealth’’ properties, and concentrate by passive targeting at the level of tumors. [8] However, after internalization in cells, the same PAAs undergo conformational changes caused by variation of the pH value, which was around 7.4 in extracellular fluids, and around 5.5 within the cells. [9] It has been also demonstrated that PAAs can act as promoters of the intracellular trafficking of toxins such as Ricin A chain and gelonin.[10] It may be added that, unlike many lipidic vectors [8,11,12] and polycationic polymers,[13] PAAs can be purposely prepared to be nontoxic in vitro [8,14] and in vivo. [15] Furthermore, PAAs do not show the immunogenicity disadvantage of naked nanoparticles. Therefore, amphoteric PAAs can be of fundamental importance in the development of new nanoparticle-based systems for cancer diagnosis and therapy.

In this Chapter the synthesis and the characterization of a series of novel amphoteric PAAs and the method for coating magnetic nanoparticles with these polymers are presented. This study was done in collaboration with Colorobbia Italia S.p.A., (Italy).

3.3 Results and Discussion

A great number of methods is available to produce nanoparticles of magnetite or cobalt ferrite: sol–gel method [21], microemulsion with oil in water micelles [22] or reverse micelles [23], aqueous precipitation and calcinations [24], combustion [25], and forced hydrolysis in a polyol medium [26].

In this widespread scenario, we started a project aimed to design new biocompatible nanomaterials based on PAA-coated ferromagnetic nanoparticles to be used in the magnetic hyperthermic treatment of tumor cells and eventually as contrast agents for MRI. The first step of this project involves the characterisation of the structural and physical properties of the magnetic core before the assembling of coating polymers. Colorobbia optimised the synthesis of ferromagnetic nanoparticles, based on the polyol technique with the seed method proposed by Sun [27], to obtain nanoparticles with controlled size in the range 5-200 nm and high hyperthermic efficiency. [20, 36, 37]

In particular, Magnetite (MAG-NP) and Cobalt-Ferrite (CoFe-NP) nanoparticles were selected for coating tests with PAAs. These nanoparticles were synthesised by Colorobbia and furnished in diethyleneglycol (DEG) solution at 3 wt% of magnetic material.

3.3.1 PAAs synthesis and characterisation

To provide stability in solution and biocompatibility to the ferromagnetic nanoparticles presented above, different kinds of amphoteric PAAs were studied as coating agents. In particular, 5 homopolymers were synthesised by Michael addition reaction of three amines, 2-methylpiperazine (MP), N,N'-ethylenediamine-diacetic acid (EDDA), 2-aminoethyl-phosphonic acid (AP) with two different bisacrylamides, N,N'-bisacrylamidoacetic acid (BAC) and N,N'-bisacryloylpiperazine (BP) (Figure 3.1). Moreover, the effect of the molecular weight of the polymer on coating ability was also investigated. To this purpose, each polymer was obtained with the number average molecular weight ($\bar{M}_n$) of about 5000, 15000, 30000 and 50000 (only for ISA23).

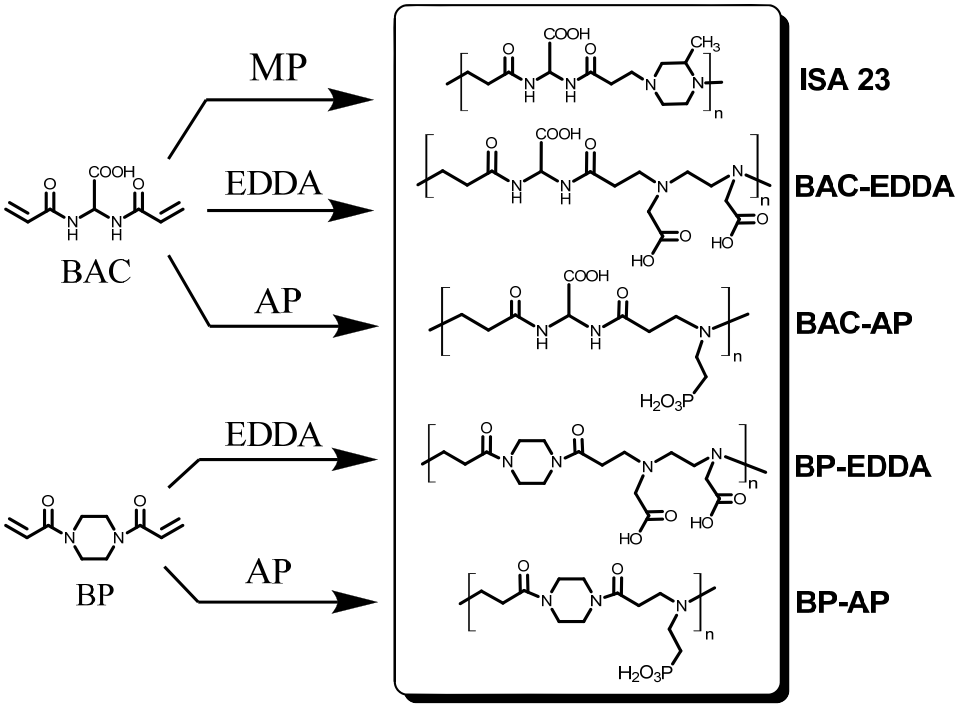


Figure 3.1. Synthetic scheme of PAAs. Abbreviations: **BAC**, 2,2-bisacrilamidoacetic acid; **BP**, N,N'-bisacryloylpiperazine; **MP**, 2-methylpiperazine; **EDDA** N,N'-ethylenediamine-diacetic acid; **AP**, 2-aminoethyl-phosphonic acid. **(a)**, H_2O, LiOH, pH 9.5, N_2, 3 days, r.t.; **(b)**, H_2O, NaOH, pH 9.0, N_2, 7 days, r.t.; **(c)**, H_2O, NaOH, pH 8.5, N_2, 5 days, r.t.

The polymerisations were carried out in water, with a mild base and under inert atmosphere for several days. During the polymerisation the viscosity gradually increased and in all cases gelation was not observed. In order to consume any unreacted acrylamide groups after polymerisation 20 mol% excess morpholine was added into the reaction mixture, lasting the reaction overnight. The polymers were isolated by exhaustive ultrafiltration, followed by freeze-drying. The resulting PAAs have an excellent solubility in water. All the polymers obtained were characterised by NMR spectroscopy and Gel permeation chromatography (GPC) measurements . The ^{1}H NMR spectra showed that the compositions of these polymers are in full accordance with the expected structures (Figure 3.1). No signals between 5 and 7 ppm, corresponding to the acryl group, were observed, indicating that the PAAs are all end-capped with amino groups. The average molecular weights ranged from about 5,000 to 50,000 (calculated by GPC with Pullulan standard).

These monomers were chosen since they displayed a good ion complexation ability (unpublished data). Moreover, is known from previous work that only those PAAs (with the exception of aminoacids derived PAAs) having aminic nitrogens that neither belong to a cyclic monomer nor are separated by more than three methylene groups are capable of complex-formation. The electronic and EPR (Electron Paramagnetic Resonance) spectra of both polymeric and non-polymeric complexes obtained from PAA models were similar, and consistent with an octahedral tetragonally distorted structure, in accordance with the substantial independence of the repeating units of the polymers in the complex formation process. [28-30]

3.3.2 Control of the PAAs average molecular weight

In order to obtain polymers with controlled molecular weight and low polydispersity, three methods were tested: (1) fractionation by solvent/non-solvent chemical separation, (2) fractionation by ultrafiltration, and (3) controlled molecular weight syntheses.

The method (1) was tested on BAC-EDDA and ISA23 to obtain fractions of polymer with controlled molecular weights and weight distributions. To avoid aggregation of the polymeric chains, the polymer was dissolved in a MeOH-H_2O 9:1 saturated solution of LiCl. As it was known that the low solubility of the polymeric chains in acetone was dependant on the molecular weight (i.e. macromolecules with higher molecular weight were less soluble then those of lower molecular weights) the addition of slowly increasing amounts of acetone to a polymer solution gave the fractionation of the polymer sample. Afterwards, the polymer fractions were ultrafiltered by a 1000 cutoff membrane and freeze-dried to eliminate the organic solvents and the LiCl. The GPC traces of the different fractions of ISA23 and BAC-EDDA are shown in Figure 3.2 (a) and (b), respectively.

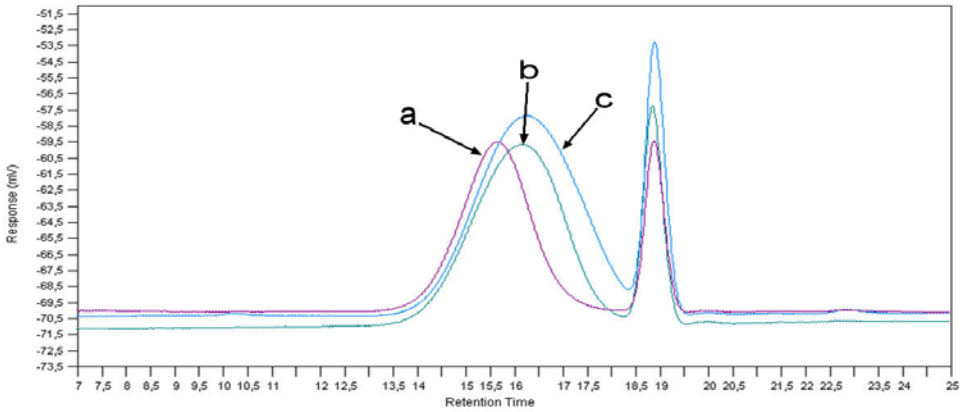


Figure 3.2 (a). GPC chromatograms of three different fractions of ISA 23 obtained by solvent non solvent fractionation. (a), $\overline{M_n}$: 10300; (b), $\overline{M_n}$: 7800; (c), $\overline{M_n}$: 5100. The peaks around 19 minutes are due to air dissolved in the sample's solution.

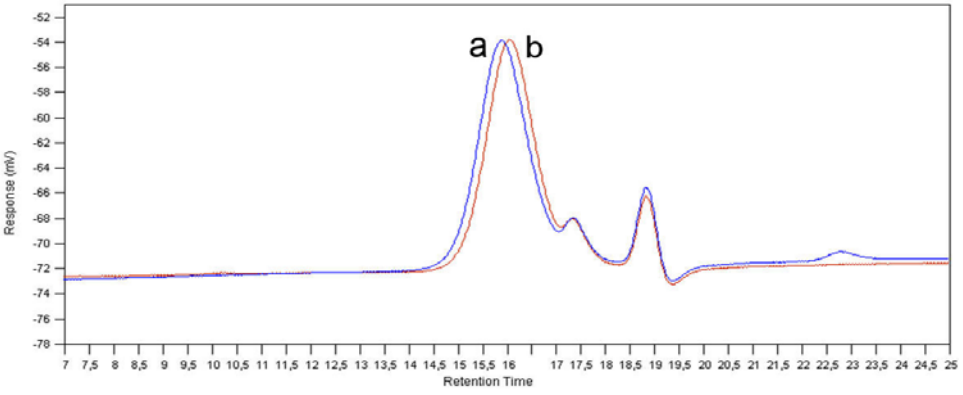


Figure 3.2 (b). GPC chromatograms of two different fractions of BAC-EDDA obtained by solvent/non-solvent fractionation. (a), $\overline{M_n}$: 6200; (b), $\overline{M_n}$: 5600.

The solvent/non-solvent chemical separation yielded a good fractionation of the ISA 23 sample. Nevertheless, with the same method it was not possible to obtain a good reproducibility and fraction separation of BAC-EDDA (Table 3.2). This is probably due to the different solubility in the utilised solvents of the two polymers.

In the method (2), the polymer was ultrafiltered using membranes with a decreasing cutoff value (Amicon ultrafiltration system, Millipore). In particular, the polymer was ultrafiltered firstly with 100,000 cutoff membrane, subsequently the obtained eluate was ultrafiltered again with a 30,000 cutoff membrane and so on, repeating the procedure with 10,000 and

5,000 cutoff membranes. The obtained fractions were freeze-dried and analysed by GPC. As example, the GPC traces of the different fractions of BAC-EDDA are shown in Figure 3.3.

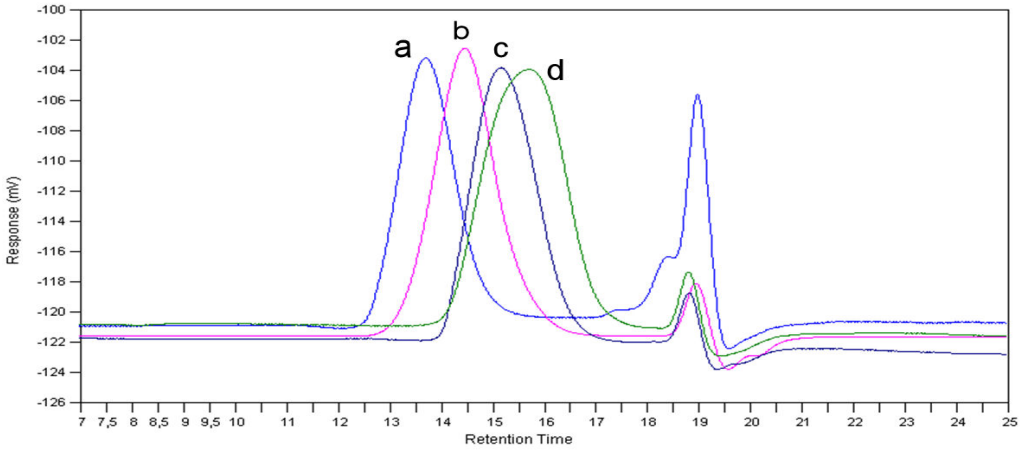


Figure 3.3. GPC chromatograms of four different fractions of BAC-EDDA obtained by ultrafiltration. (a), $\overline{M_n}$: 41000; (b), $\overline{M_n}$: 22600; (c), $\overline{M_n}$: 12300; (d), $\overline{M_n}$: 6500.

A good separation of the fractions of BAC-EDDA was obtained by ultrafiltration. The yield of the fractions compared to the starting polymer were: a) 3%, b) 65%, c) 22%, d) 9%. The average molecular weight of the fractions was lower compared to the nominal cutoff of the related membrane (Table 3.1). This is due to the lower hydrodynamic volume of the proteins used for the membranes' calibration.

In the method (3) the molecular weight was controlled by varying the stoichiometric ratio of the starting monomers in the polymerisation reaction. The average molecular weight of the polymers was controlled by adjusting the stoichiometric ratio "r" of the starting monomers (amines, bisacrylamides) to obtain different degrees of polymerisation "$\bar{X}_n$" (Equation 1). [31]

$$\overline{X_n} = \frac{1+r}{1-r} \qquad \text{Equation 1}$$

Where:

- $r = \frac{N^o_A}{N^o_B}$
- $N°_A$: number of function of monomer in defect
- $N°_B$: number of function of monomer in excess

Each polymer solution was then acidified at pH 4, ultrafiltered with a 5,000 cutoff membrane and freeze-dried. As example, the GPC traces of different batches of BAC-EDDA are shown in Figure 3.4. The polymers thus produced had a number average molecular weight ($\overline{M}_n$) of about 5000, 15000, 30000 and 50000 (only for ISA23) respectively, and good values of polydispersity (PD) were obtained (from 1.5 to 2.0).

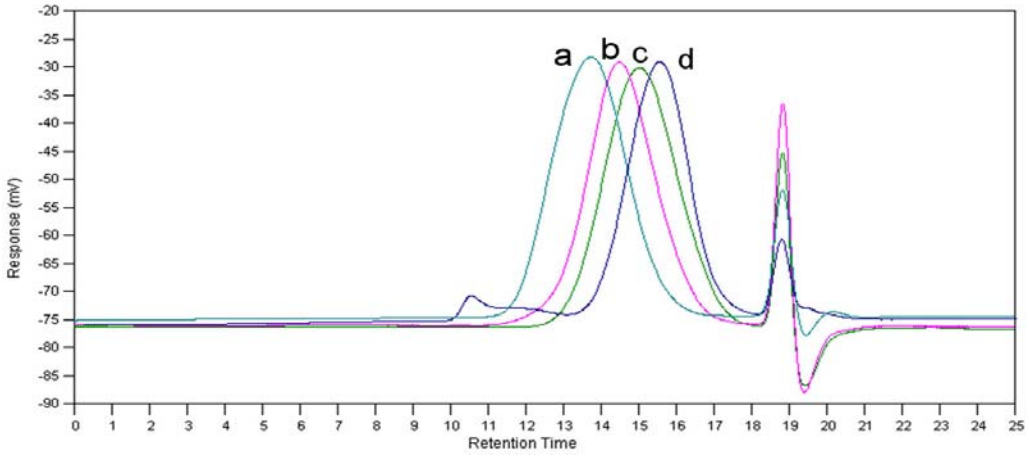


Figure 3.4. GPC chromatograms of four different batches (a, b, c, d) of BAC-EDDA obtained by controlled molecular weight polymerisation. (a), $\overline{M_n}$: 45200; (b), $\overline{M_n}$: 20000; (c), $\overline{M_n}$: 14000; (d), $\overline{M_n}$: 6700.

In table 3.1 the GPC data related to the different fractionation methods are summarised. The method (1) yielded a good fractionation with ISA 23, nevertheless these results were not reproducible with other tested PAAs. A good separation and low PD values were obtained using the methods (2) and (3) with all the polymers synthesised. However, the method (3) resulted the best in order to obtain a specific average molecular weight since this technique allowed a more precise control of the final MW of the polymer. Moreover, this procedure was quicker and gave higher yields compared to method (2).

Method	Sample	Fraction	$\overline{M_n}$	$\overline{M_w}$	*PD*
1	ISA 23	a	10300	13800	1,34
		b	7800	10900	1,40
		c	5100	8500	1,67
1	BAC-EDDA	a	6200	8400	1,35
		b	5600	7500	1,34
2	BAC-EDDA	a	41000	68000	1,66
		b	22600	29800	1,32
		c	12300	15600	1,27
		d	6500	7400	1,14
3	BAC-EDDA	a	45200	77100	1,71
		b	20000	33200	1,66
		c	14000	19700	1,41
		d	6700	9900	1,48

Table 3.1. GPC data of the fractions obtained with the tested methods. **method 1**, fractionation by solvent/non-solvent chemical separation; **method 2**, fractionation by ultrafiltration; **method 3** controlled molecular weight syntheses. The GPC traces of the fractions are showed in the figures 3.5, 3.6 and 3.7.

3.3.3 Functionalisation of PAAs

In order to obtain PAAs able to undergo further functionalisations, we modified the syntheses by adding a small amount of amines carrying functional groups. In particular, ISA 23 and BAC-EDDA were obtained as copolymer with the 5 - 10 mol% of tyramine, ethylenediamine or β-CD respectively. The general synthetic scheme is shown in Figure 3.5.

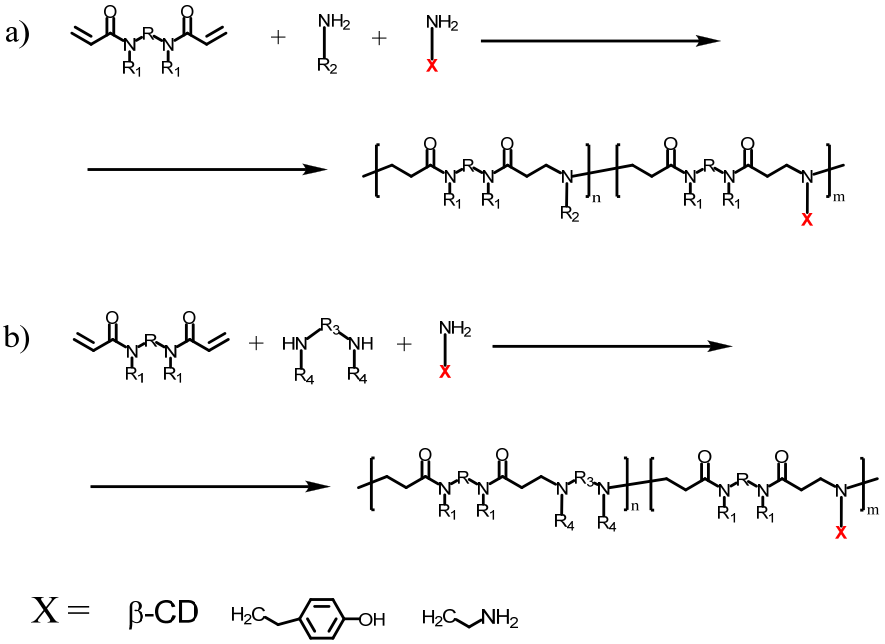


Figure 3.5. General synthetic scheme of the studied copolymers.

Tyramine has a primary amino group, thus it can be used as monomer without further functionalisations or protecting groups.The introduction of tyramine in the PAAs allows the radiolabelling of the polymer, which is a technique of fundamental importance in cancer therapy, e.g. to investigate the polymer distribution after i.v. injection *in vivo* [32].

Ethylenediamine posses two primary amino groups, therefore, to obtain linear polymers it was necessary to protect one of them with a protecting group which must be stable under the conditions of PAA synthesis, that is, under alkaline conditions, but easily removable under conditions not affecting PAA stability. We selected triphenymethyl group as a

protecting group. Thus, the starting monomer was 1-triphenylmethylamino-2-aminoethane (TPHMAE), prepared as previously described [33]. By this procedure, the PAAs become suitable as polymer carriers for carboxylated drugs as well as amenable to the labeling techniques by fluorescent probes commonly employed for proteins.

β-Cyclodextrin (β-CD) has no amino groups itself. Therefore a preliminary functionalisation to obtain the mono-6-deoxy-6-amino-β-cyclodextrin (β-CD-NH_2) was necessary. The synthetic pathway leading to β-CD-NH_2 and the related ^{1}HNMR spectrum are shown in Figure 3.6 and 3.7, respectively.

β-CD has found a number of applications in drug release. In fact, β-CD is able to form host-guest complexes with hydrophobic molecules because of the unique nature imparted by their structure. Thus, a PAA with β-CD as side substituent would be able to complex water insoluble drugs and permit the solubilisation in water, enabling an easier delivery to the target tissue [34].

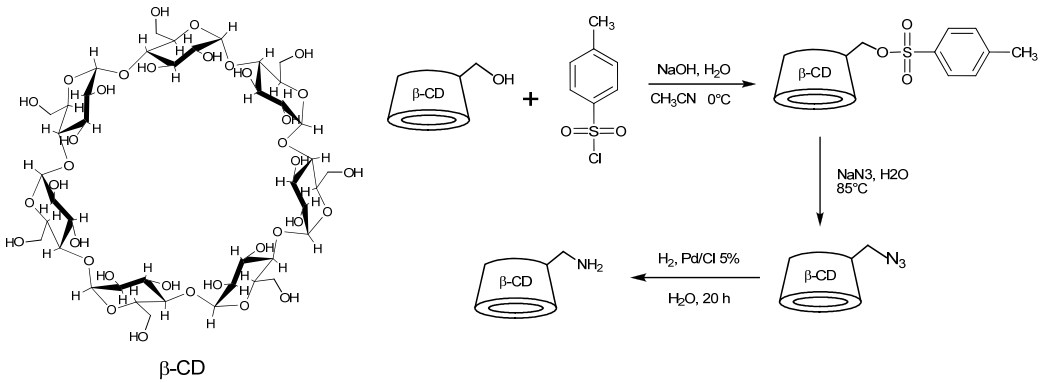


Figure 3.6. Synthesis of β-CD-NH_2

For copolymerisations including tyramine, usual conditions adopted in PAA preparation were used. To the reaction mixture 5 mol% of tyramine was added, together with the required amount of the secondary diamine (MP or EDDA) in order to maintain the stoichiometric ratio between the amine functions and the double bonds of the bisacrylamide.

The copolymerisation reaction including TPHMAE required a different solvent because of its poor solubility in water due to the presence of the triphenylmethyl group. Thus, TPHMAE and the bisacrylamide in a 1/20 ratio were dissolved in methanol. After a 1 day reaction, water-soluble oligomers were obtained. Water was then added to methanol (see the Experimental Part) up to a water/MeOH ratio of 2/1and the secondary diamine (MP or EDDA) was added allowing the polymerisation to proceed in the usual way for PAAs. In all cases, after polymerisation (3 days), the triphenylmethyl protecting group was cleaved by treating with aqueous hydrochloric acid. Pure ISA23-NH_2 and BAC-EDDA-NH_2 were finally obtained as hydrochlorides removing the by-product triphenylmethylcarbinol through solvent extraction, ultrafiltration, and freeze-drying. It can be noticed that the related copolymers (Table 3.2) obtained from the same bisacrylamides and secondary diamines have a considerably higher molecular weight compared to ISA23-NH_2 and BAC-EDDA-NH_2. This indicates a relatively low reactivity of TPHMAE towards the polyadditions reaction. In fact, the TPHMAE/bisacrylamide ratio of 1/20 was used to force the addition reaction. It was considered that after 1 day reaction most of the TPHMAE was transformed into an ABA trimer, in which A and B stay as bisacrylamide with a terminal double bond and a TPHMAE units, respectively.

The copolymerisation of β-CD was carried out under normal condition used for the synthesis of the related homopolymers. We encountered some reproducibility problems due to hygroscopicity of β-CD, since a stoichiometric ratio between amines and bisacrylamides is necessary to obtain polymers with high molecular weight. β-CD-NH_2 was then dried under N_2 flow at 40°C for 24 h and the purity was checked by titration of the amino groups with HCl 0.1 M (see appendix) just before the addition to the reaction mixture. Copolymers of ISA 23 and BAC-EDDA were synthesised with 5 or 10 mol% of β-CD-NH_2 (compared to the total amount of amine). The structures and the average molecular weights of the synthesised polymers are shown in Table 3.2. The polymers were characterised by NMR spectroscopy (^{1}H, ^{13}C) and GPC analysis.

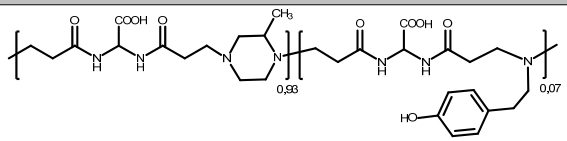
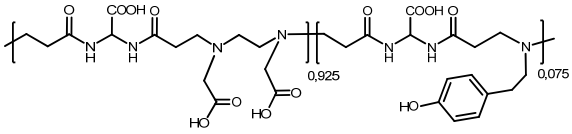
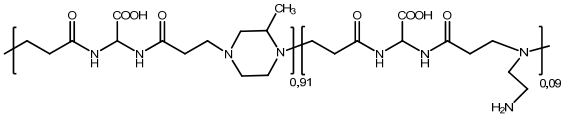
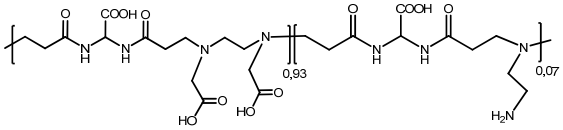
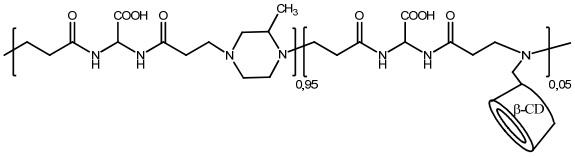
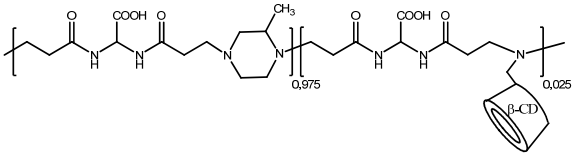
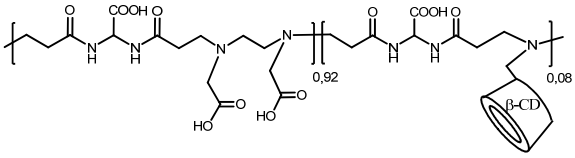
Name	Structure	$\overline{M_n}$	$\overline{M_w}$	PD
ISA23-tyr		11300	14700	1,30
BAC-EDDA-tyr		15300	19000	1,24
ISA23-NH2		3500	4900	1,40
BAC-EDDA-NH2		3200	4100	1,28
ISA23- β-CD-5		25100	47300	1,88
ISA23- β-CD-3		24100	39600	1,64
BAC-EDDA-β-CD		21800	29700	1,36

Table 3.2. Structures and average molecular weights of the obtained copolymers.

As example, the ^{1}HNMR spectra (Figure 3.8) and the GPC traces (Figure 3.9) of copolymers ISA 23-β-CD-5 and ISA 23-β-CD-3 (obtained from BAC, MP and 5 or 10 mol% of β-CD-NH_2, respectively), are shown.

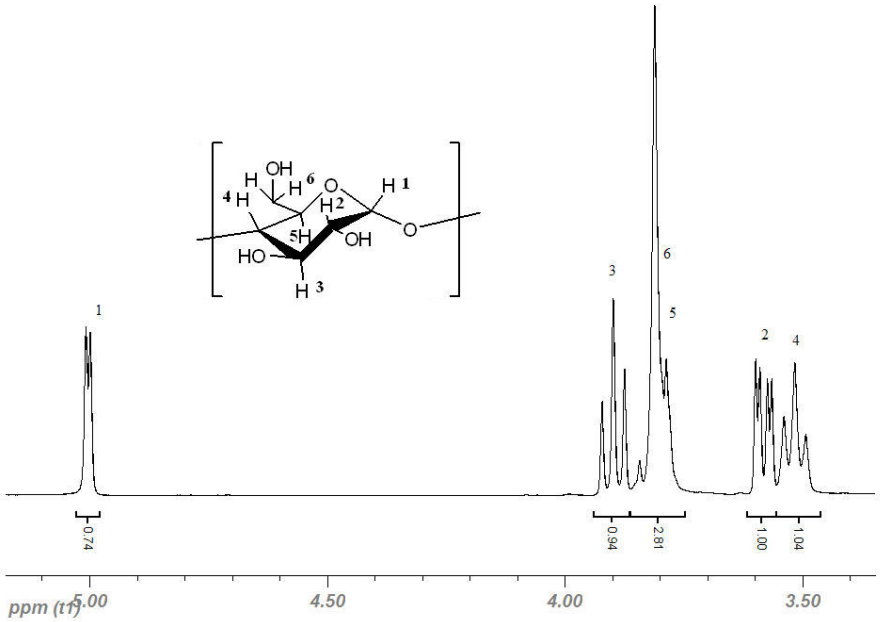


Figure 3.7. HNMR spectra of β-CD-NH_2.

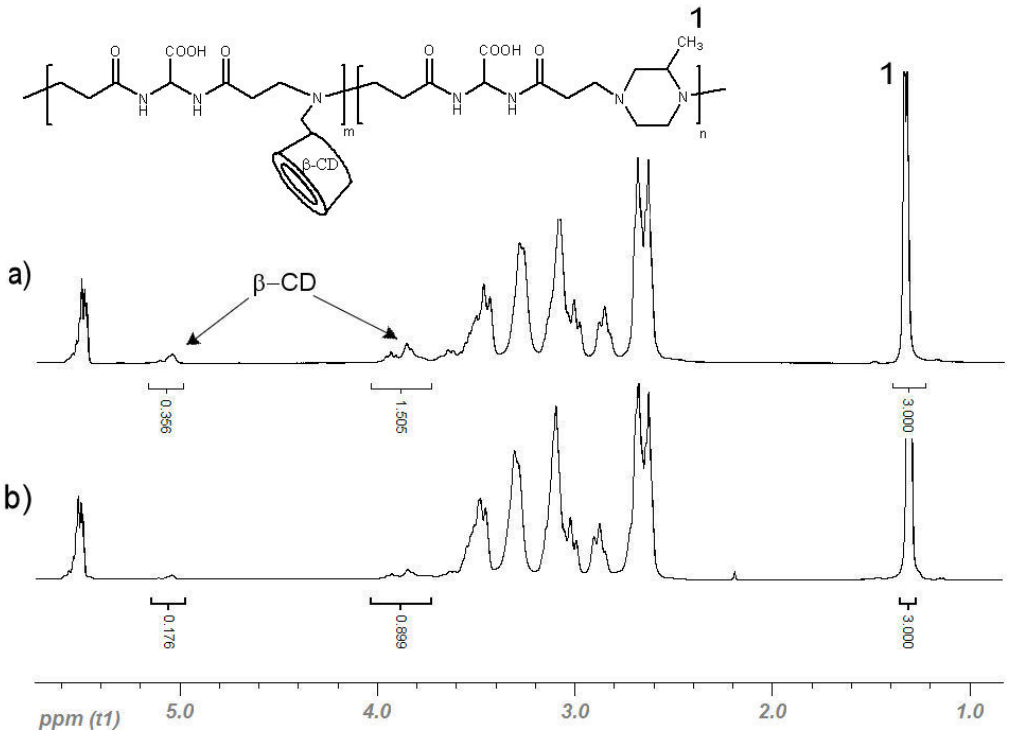


Figure 3.8. HNMR spectra in D_2O of a) ISA 23-β-CD-5 and b) ISA 23-β-CD-3

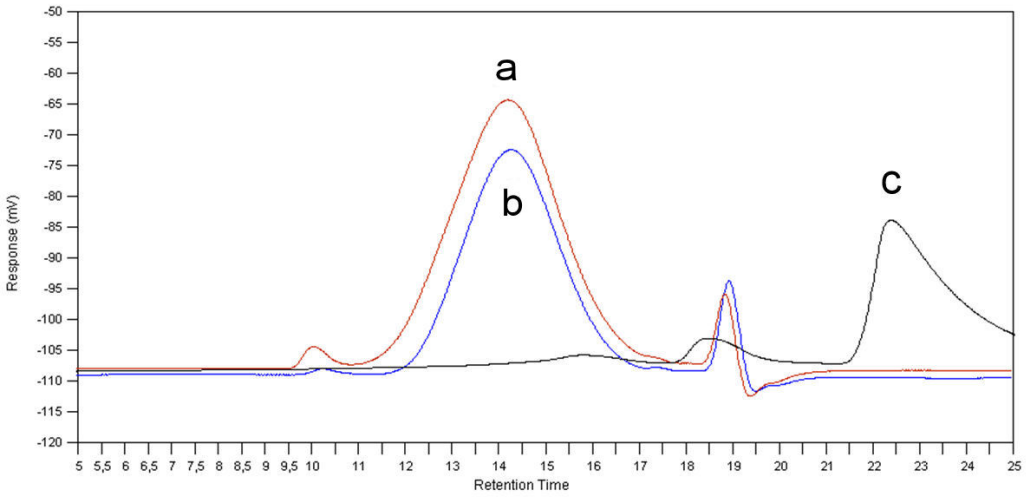


Figure 3.9. GPC traces of a) ISA 23-β-CD-5, b) ISA 23-β-CD-3 and c) β-CD-NH_2.

The GPC chromatograms show that there is no free β-CD in the polymer samples. Moreover, from the NMR spectra it was possible to calculate an amount of 2.5 mol% of β-CD in the ISA 23-β-CD-3 and of 5.0 mol% in the ISA 23-5-β-CD. Thus, only the 50 % of the starting monomer (5 mol% and 10 mol%, respectively) reacted with the bisacrylamide. In the case of BAC-EDDA-β-CD the final amount of β-CD in the polymer was 8 mol%, starting from 10 mol%. This is probably due to the different reactivity of the β-CD-NH_2 monomer compared with 2-methylpiperazine or EDDA, respectively. The related ^{1}HNMR spectrum is shown in Figure 3.10.

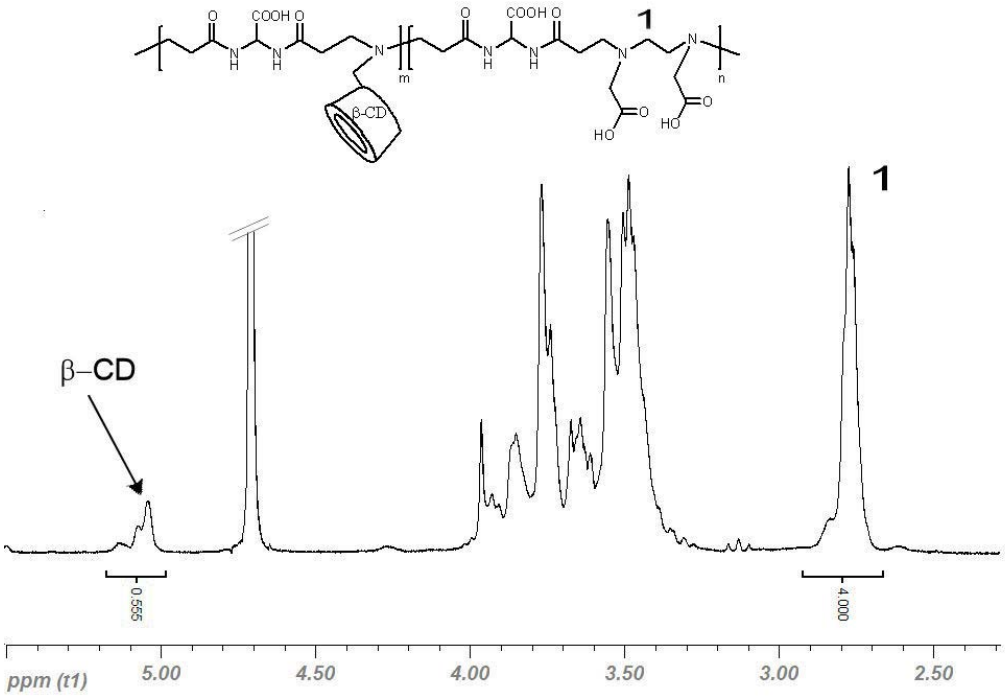


Figure 3.10. HNMR spectrum in D_2O of BAC-EDDA-β-CD.

3.3.4 Nanoparticles Coating tests

The coating tests were performed in collaboration with Colorobbia. The particle size (by Dynamic Light Scattering, DLS) and hyperthermic efficiency measurements were carried out by Colorobbia.

The magnetic nanoparticles selected for coating tests were Magnetite (Mag-NP) and Cobalt-Ferrite (CoFe-NP) nanoparticles. The NPs were obtained from the synthesis [20] as stable colloidal solution in diethyleneglycol (DEG). Preliminary tests showed that the NPs were not able to produce stable suspension in water, therefore complexation tests were run as follows: nanoparticles in diethyleneglycol (DEG) (0.2 g in 40 ml of solvent) were added dropwise to an aqueous solution of the polymer (1.2 g in 200 ml) under stirring. Ultrafiltration tests with Amicon (Millipore) were not appropriate since caused fast aggregation and then precipitation of the NPs. This is due to the magnetic field necessary to

the rotation of the stirring bar. Good results were obtained instead with dialysis tubes (30.000 cutoff) that allowed the elimination of low molecular weight species and the organic solvent (final concentration of DEG in water, ≤ 0.1%)..

All the polymers shown in Figure 3.1 were used in coating tests and for each polymer, batches with different average molecular weights were also tried (Table 3.3).

Polymer	$\overline{M_n}$	$\overline{M_w}$	**PD**	Stabilisation of the NPs solution
ISA 23	4500	7900	1.76	Stable solution
	16800	21300	1.27	
	33900	55000	1.62	
	49000	97000	1.98	
BAC-EDDA	6700	9900	1.48	Stable solution
	11000	15500	1.41	
	20000	33200	1.66	
BAC-AP	3700	5000	1.35	Stable solution
	12400	17900	1.44	
	22500	33500	1.49	
BP-EDDA	5800	9300	1.60	Non stable solution
	14300	22500	1.57	
	25200	41900	1.66	
BP-AP	5100	7200	1.41	Non stable solution
	17300	23000	1.33	
	20600	25800	1.25	

Table 3.3. PAAs used in nanoparticles coating tests.

Stable colloidal solutions of coated nanoparticles were obtained with BAC containing polymers (ISA23, BAC-EDDA, BAC-AP). In particular, BAC-EDDA showed the higher stability. Instead, BP containing polymers caused aggregation of the particles and then precipitation.

It was also found that generally the polymers with a value of $\overline{M_n}$ around 15000 yielded the narrowest particle size distribution. However, all the polymers tested were not able to minimise the Cobalt release in Cobalt-Ferrite nanoparticles under non toxic values. Therefore, further investigations were carried out only on coated magnetite nanoparticles (average size: 10 nm by DLS). The particle size measurements (by DLS) of Magnetite NPs with BAC-EDDA and BAC-AP in water and in physiological solution are shown in Figure 3.11 a-d.

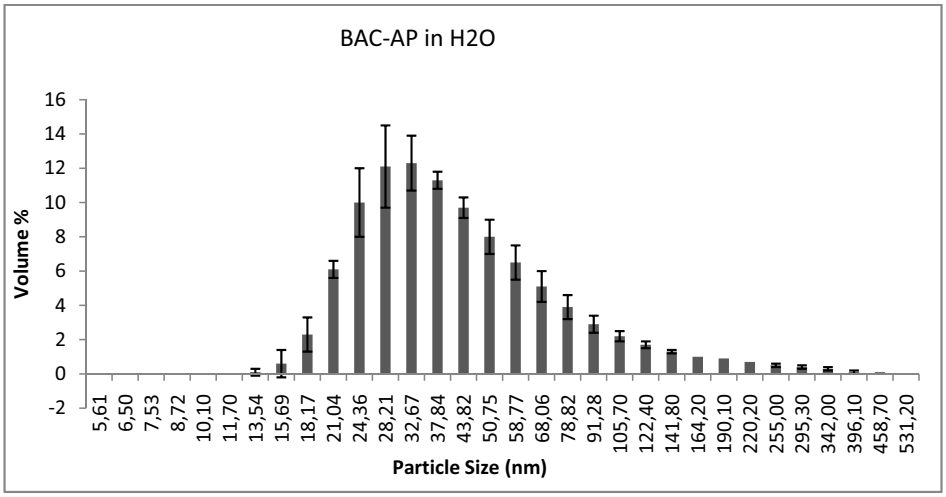


Figure 3.11 (a). DLS measurement of Mag-BAC-AP ($\overline{M_n}$: 12400) in water

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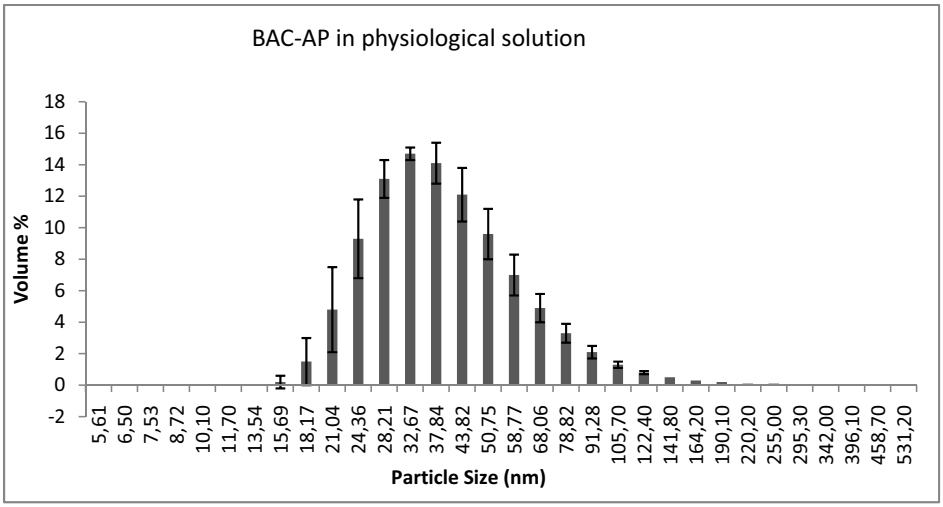


Figure 3.11 (b). DLS measurement of Mag-BAC-AP ($\overline{M_n}$: 12400) in physiological solution.

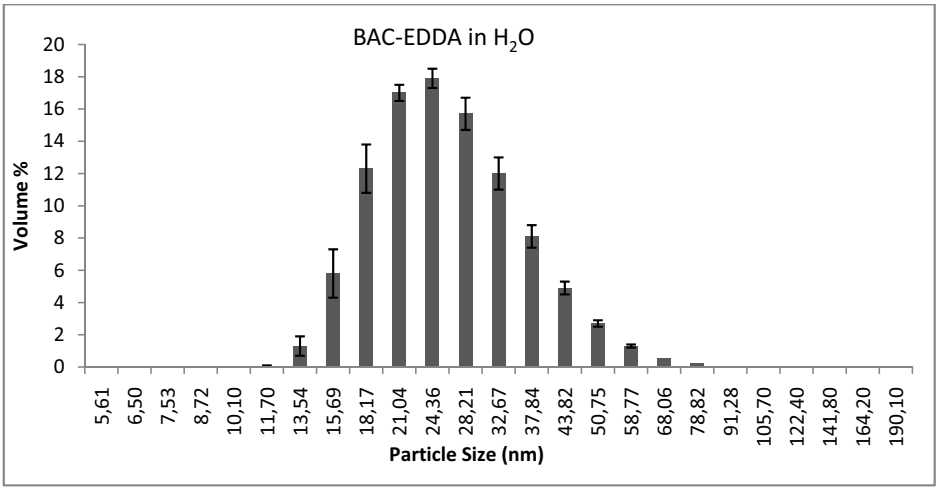


Figure 3.11 (c). DLS measurement of Mag-BAC-EDDA ($\overline{M_n}$: 11000) in water.

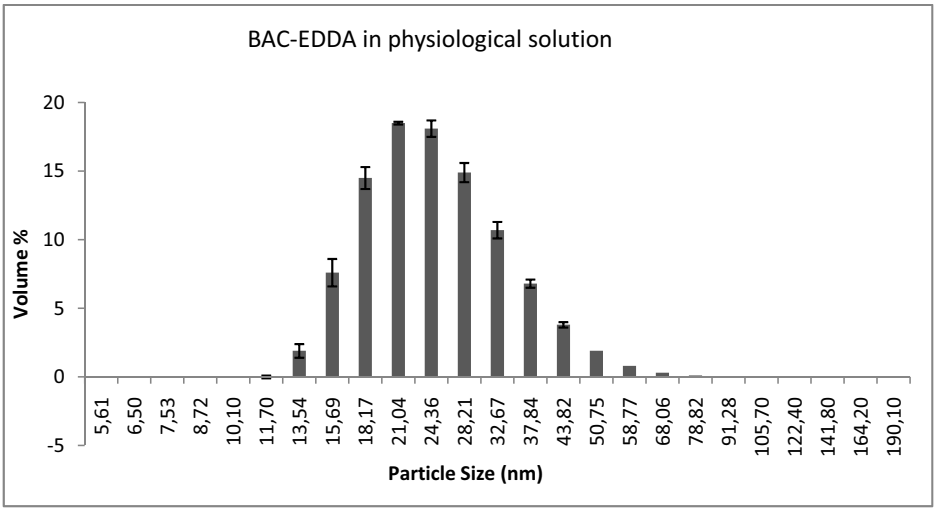


Figure 3.11 (d). DLS measurement of Mag-BAC-EDDA ($\overline{M_n}$: 11000) in physiological solution.

BAC-EDDA showed a slightly narrower particle size distribution both in water and in physiological solution compared to BAC-AP, whereas the effect of the solvent on the size distribution was negligible.

The Magnetite nanoparticles coated with BAC-EDDA (Mag-NP-BAC-EDDA) were used for hyperthermic efficiency measurement. This test was performed also on Mag-NP-BAC-EDDA in presence of cis-diammineplatinum (II) dichloride (Cisplatin), an anticancer agent

(Table 3.4). The Cisplatin/nanoparticles weight ratio in the coating reaction mixture was 1/2.

Sample	Solvent	Hyperthermic efficiency of Non-coated NPs (°C)	Hyperthermic efficiency of Coated NPs (°C)
Mag-NP-BAC-EDDA	Physiological solution	5.05	4.51
Mag-NP-BAC-EDDA + Cisplatin	Physiological solution	5.05	3.89

Table 3.4. Hyperthermic efficiency of BAC-EDDA coated Mag-NP with and without Cisplatin. Hyperthermic efficiency measurements have been carried out with irradiation at 170 KHz and with a magnetic field of 21 KA/m^2 for 30 seconds.

The conjugates showed an average size compatible with other kind of nanoparticles used in clinical trials for cancer therapy. The hyperthermic efficiency was decreased by the polymer coating (10%), but is still comparable with the values relevant to other systems commonly studied for magnetic hyperthermia. [35] At the moment *in vitro* tests are in progress. In particular, the stability of the coated nanoparticles in DMEM medium, and subsequently, the hyperthermic efficiency on different cell lines will be investigated.

3.4 Conclusions

A series of new amphoteric PAAs was synthesised and characterised. Different methods to vary the average molecular weight of the polymers and to obtain a low polydispersity were investigated. By using the method of the molecular weight control by varying the stoichiometric ratio of the starting monomers it is possible to finely tune the MW of the polymer and obtain high yields and good PD values.

These PAAs were used as coating agents in nanoparticles coating tests. The BAC containing polymers (ISA23, BAC-EDDA and BAC-AP) yielded stable colloidal solutions. Among the

others, BAC-EDDA, and in particular the one with a $\overline{M_n}$ of 11000, conferred the highest stability to nanoparticles.

Magnetite nanoparticles coated using BAC-EDDA yielded very stable solutions and good values of hyperthermic efficiency. The decrease of the NP's hyperthermic efficiency due to the coating polymer was 10 %, thus the final value is still comparable with other systems studied in Magnetic Hyperthermia.

We obtained PAAs also as copolymers with 5-10 mol% of β-Cyclodextrin, tyramine and ethylenediamine. These copolymers, used as coating agents, enable the nanoparticles to drug delivery, radiolabelling and further functionalisations. Moreover, the carboxylic acid and amine functional groups of the polymer can facilitate attachment of binding partners (e.g., antibodies) to the polymer, which can allow the polymer-coated nanoparticle to be used in a variety of applications including protein detection and cell labeling. Further developments of functionalised magnetite NPs are under investigation for hyperthermic applications in locoregional cancer therapy and as carriers of pharmaceutical compounds for magnetic drug targeting. Part of this work has been published in an international patent [36].

References

[1] G. Barrat, *Cell. Mol. Life Sci.*, (60), 21, **2003**.

[2] Q. Pankhurst, , I. Connolly, S.K. Jones, I. Dobson, *J . Phys. D: App/. Phys.* , (36), R167, **2003**.

[3] U. Hafeli, W. Schutt, l. Teller, M. Zborowski, (Eds), *"Scientific and Clinical Applications of Magnetic Carriers",* Plenum Press , New York, **1997**.

[4] M. Klostranec, , W.C.W. Chan, *Adv. Mater.,* (18), 1953, **2006**.

[5] G.F. Goya, V. Grazù and M.R. Ibarra *Current Nanoscience,* , (4), 1-16, **2008**.

[6] P. Tartaj, M.D.Morales, S. Veintemillas-Verdaguer, T. Gonzalez-Carreno, C.J. Serna, *J. Phys. D-App/. Pbys.,* (36), R182, **2003**.

[7] P. Ferruti, S. Manzoni, S. Richardson, R. Duncan, N. G. Pattrick, R. Mendichi, M. Casolaro, *Macromolecules*, (33), 7793, **2000**.

[8] S. Richardson, P. Ferruti, R. Duncan, *J. Drug Targeting*, (6), 391, **1999**.

[9] S. C.W. Richardson, N. G. Pattrick, Y. K. S. Man, P. Ferruti, R. Duncan, *Biomacromolecules*, (2), 1023, **2001**.

[10] N. G. Pattrick, S. C.W. Richardson, M. Casolaro, P. Ferruti, R. Duncan, *J. Controlled Release*, (77), 225, **2001**.

[11] M. B. Bally, P. Harvie, F. M. P.Wong, S. Kong, E. K.Wasan, D. L. Reimer, Adv. Drug *Delivery Rev*., (38), 291, **1999**.

[12] J. Smith, Y. Zhang, R. Niven, Adv. *Drug Delivery Rev*., (26), 135 **1997**.

[13] W. T. Godbey, K. K.Wu, A. G. Mikos, *J. Controlled Release*, (60), 149 **1999**.

[14] E. Ranucci, G. Spagnoli, P. Ferruti, D. Sgouras, R. Duncan*, J. Biomater. Sci. Polym*., (2), 303, **1991**.

[15] [15a] P. Ferruti, S. Knobloch, E. Ranucci, R. Duncan, E. Gianasi*, Macromol. Chem. Phys*., (199), 2565; **1998**; [15b] D. C. Gorecky, Emerg. *Drugs*, (4), 247, **1999**.

[16] C. Sun, J. S.H. Lee, M. Zhang, *Advanced Drug Delivery Reviews,* (60) 1252–1265, **2008**.

[17] C. Berry, and A. Curtis *Europhysics News*, Vol. 34, No. 6, **2003.**

[18] I. Hilger, R. Hergt, and W. A. Kaiser, IEE Proc.: *Nanobiotechnol*., (152), 33, **2005**.

[19] A. H. Habib, C. L. Ondeck, P. Chaudhary, *JOURNAL OF APPLIED PHYSICS* (103), 07, A307, **2008**.

[20] G. Baldi, D. Bonacchi, C. Innocenti, G. Lorenzi, C. Sangregorio, *Journal of Magnetism and Magnetic Materials,* (311), 10–16, **2007.**

[21] F.X. Cheng, et al., *Solid State Commun*. (107), 471, **1996**.

[22] N. Moumen, P. Veillet, M.P. Pileni, *J. Magn. Magn. Mater*. (149), 67, **1995**.

[23] A.T. Ngo, P. Bonville, M.P. Pileni, *Eur. Phys. J.* B, (9), 583, **1999**.

[24] K.J. Davis, et al., *J. Magn. Magn. Mater*., (149), 14, **1995**.

[25] C.H. Yan, Z.G. Xu, F.X. Cheng, et al., *Solid State Commun*., (111), 287, **1999**.

[26] S. Ammar, A. Helfen, N. Jouini, et al., *J. Mater. Chem*., (11), 186, **2001**.

[27] S. Sun, et al., *J. Am. Chem. Soc*., (126), 273, **2004**.

[28] C. Bertoglio Riolo, T. Soldi, M. Pesavento, *J. Appl. Polymer Sci*. (27), 2239, **1982**.

[29] T. Soldi, P. Ferruti, R. Barbucci, M. Benvenuti, *J.Appl.Polymer Sci.* (28), 3361, **1983**.

[30] M. Benvenuti, M. Pesavento, P. Ferruti, *Polymer Communications,* (24), 26, **1983**.

[31] P. J. Flory, *Principles of Polymer Chemistry*, Published by Cornell University Press, **1953** (688 pp.).

[32] N.G. Pattrick, S.C.W. Richardson, M. Casolaro, P. Ferruti, R. Duncan , *Journal of Controlled Release,* (77), 225–232, **2001**.

[33] B. Malgesini, I. Verpilio, R. Duncan, P. Ferruti, *Macromol. Biosci*., (3), 59–66, **2003.**

[34] P. Ferruti, E. Ranucci, F. Trotta, E. Gianasi, E. G. Evagorou, M. Wasil, G. Wilsona , R. Duncan, *Macromol. Chem. Phys*., (199), 1644–165, **2000.**

[35] J. R. McCarthy, R. Weissleder, *Advanced Drug Delivery Reviews,* (60), 1241–1251, **2008.**

[36] International Patent **WO 2008/074804 A2** , *G. Baldi,D. Bonacchi,F. Innocenti, G. Lorenzi, M. Bitossi, P. Ferruti, E. Ranucci, A. Ricci, M. Comes Franchini. (Colorobbia Italia S.p.A., Italy).* Magnetic nanoparticles of magnetic metal oxides in polymer particle constructs for the application in hyperthermia, preparation thereof and use in constructs having a pharmacological application.

[37] G. Baldi, D. Bonacchi, M. Comes Franchini, D. Gentili, G. Lorenzi, A. Ricci, C. Ravagli, *Langmuir,* (23), 4026-4028, **2007**.

Chapter 4: Poly(amidoamine)s containing peptides inserted in the main chain.

4.1 Abstract

A general method for preparing linear high polymers bearing in their main chain peptides is reported in this section. Peptides can be transformed into α,ω-difunctional monomers in Michael-type stepwise polyadditions with bis-acrylamides by adding one or two cysteine as terminal residues. This provides a general method for preparing linear high polymers of peptides where the peptide moieties, connected by short hydrophilic bis-acrylamide-derived linkers, constitute the main portion of the polymer chain. To demonstrate the feasibility of this synthetic procedure the following model compounds for cysteine-modified peptides were used: reduced and oxidised glutathione, RGDC, CRGDC, ISLHAC and CISLHAC peptide sequences. Novel series of polymers structurally related to PAAs in which the peptide represents the main constituent, were obtained. Moreover, the kinetic aspects of these reactions were considered.

In preliminary biological tests these polymers showed a negative zeta potential at physiological pH, negligible values of cytotoxicity and haemolytic activity. Further In vitro tests to investigate the biological properties of these polymers in comparison with the parent peptides are in progress.

4.2 Introduction

Malignant cellular transformation is largely viewed as the consequence of autonomous cell genetic alterations, leading to the activation of oncogenes and the inactivation of tumor suppression genes and increasing genomic instability, which gives rise to a cell capable of limitless proliferation and survival, invasion, and metastasis. [1] In addition, the process of formation of a tumor-associated vasculature (angiogenesis) has been recognised as an essential event promoting tumor progression. In the absence of tumor angiogenesis, tumors

enter a state of dormancy, characterised by a balance between cell proliferation and apoptosis. [2] During the last decade, significant advances have been made in the understanding of the tissue, cellular, and molecular events that regulate and mediate tumor angiogenesis. Many extracellular, cell surface, and intracellular molecules modulating angiogenesis have been identified and characterised, including growth factors and growth factor receptors, and especially vascular integrin inhibition showed very promising characteristics in antiangiogenic therapy.

Integrins are heterodimer transmembrane receptors for the extracellular matrix composed of an alpha and beta subunit. Antagonists of integrin $\alpha_v\beta_3$ have been shown to inhibit tumor angiogenesis and disrupt metastasis [4]. In addition, $\alpha_v\beta_3$ integrin antagonist can be radiolabeled and selectively localised in the tumors for diagnostic imaging. These encouraging preclinical results stimulated researchers and industry to develop pharmacologic inhibitors of integrin function for clinical testing.

The binding site between the integrins and the related ligands consists in a short aminoacid stretches on exposed loops, particularly the arginine-glycine-aspartic acid (RGD) sequence (Figure 4.1).

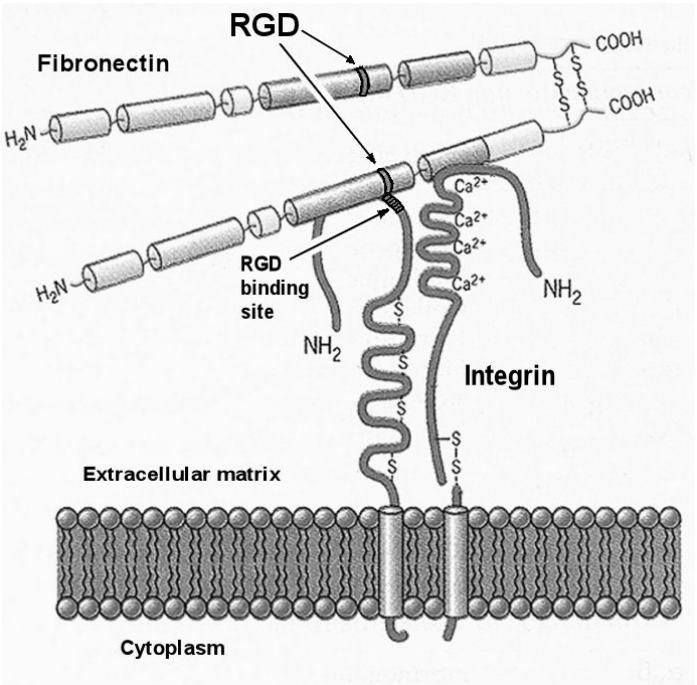


Figure 4.1. Binding of fibronectin (integrin ligand) by integrin (transmembrane receptor).

The RGD sequence was identified in 1984 by Pierschbacher and Rouslahti as a minimal essential cell adhesion peptide sequence in fibronectin. [5] Since its discovery a great number of RGD containing drugs has been developed for different applications in cancer therapy. [3] The numerous advantages of RGD towards the use of entire proteins were suddenly recognised. The use of proteins, in fact, bears some disadvantages in the view of medical applications, e.g. the required purification, immune response, proteolytic degradation. [6-18] By using small peptides as cell recognition motifs most of the problems listed above can be overcome. [19-26]

In recent years, RGD-targeted drugs and imaging agents have been developed by covalent conjugation of the peptide to drug or to a carrier device that has been equipped with drug molecules or in gene delivery by viral and non-viral vectors. Among this broad class of RGD-targeted drugs, the polymeric vectors showed the most promising features in cancer therapy because of their versatility and ease of synthesis. The most used functional groups to provide a stable linkage between RGD and the polymer are amino, hydroxy and carboxy group. [27-31] The main obstacle to the coupling reactions is that there are further reactive functional groups in the RGD peptide. This problem can be overcome by using protecting groups or activating agents.

In a more recent approach named chemoselective ligation, selected pairs of functional groups are used to form stable bonds without the need of an activating agent and without interfering with other functional groups. [32–34] As example, thiol (cysteine) bearing RGD peptides can be linked in a Michael addition reaction to acrylic esters or acryl amides with good yields. [7]

In our group, poly(amidoamines)s (PAAs) containing RGD mimicking moieties were previously synthesised. [35] These polymers were obtained by polymerisation of agmatine, the de-carboxylation product of arginine, with N,N'-bisacryloyl-acetic acid (BAC) and by copolymerisation of agmatine and methylpiperazine (MP) with BAC. In biological tests these PAAs showed no cytoxicity and low or no haemolytic activity. Thus, we supposed that RGD containing PAAs could enhance the efficiency of RGD peptide in biological applications, without affecting its biochemical properties.

4.3 Rationale of the study

In a general sense, active peptides or peptido-mimetic substances constitute at present one of the frontier sectors of medicinal chemistry. Synthetic methods leading to polymers in which the peptide motif, α,ω-linked by short connecting segments, is the major constituent could expand the range of applications in this sector. In this project, a novel synthetic procedure to obtain linear polymers, structurally related to PAAs, with short peptides inserted in the main chain is investigated.

Poly(amidoamine)s (PAAs) are synthetic polymers characterised by the presence of *tert*-amine and amide groups regularly arranged along the polymer chain (see Section 2). Linear PAAs are obtained by Michael-type polyaddition of primary monoamines or secondary bis-amines to bis-acrylamides. [36,40] Primary bis-amines yield cross-linked polymers. [37,39] PAA-like polymers can be also prepared from bis-acrylamides and bis-thiols. [41] In early studies we observed that PAAs cannot be prepared using α-aminoacids as monomers, with the exception of glycine, because their primary amino group does not undergo a double addition step to bis-acrylamides. [36,37] Moreover, peptides initiating with substituted α-aminoacid residues do not polymerise with bis-acrylamides. Glycine-initiated peptides give comb-like polymers with bisacrylamides with peptide residues as side substituents. [36-39, 42]

L-Cystine is a natural bis-α-aminoacid derived from the oxidative coupling of two cysteine molecules. Its terminal primary amino groups are both substituted in the α position by the short chain interconnecting the two L-cysteine moieties. In our recent study, we have reported that L-cystine gives linear PAAs with typical bis-acrylamides, acting as a difunctional monomer in spite of having four mobile hydrogens. A kinetic study confirmed that each amine group of L-cystine undergoes a single addition step. [43] Moreover we have recently found that L-cysteine can be used as difunctional monomer with bisacrylamides to yield linear PAAs. [52]

Summing up the above data, we concluded that any peptide carrying either two amino groups belonging to α-substituted aminoacid residues, or one of such groups plus a cysteine residue, or two cysteine residues will act as a difunctional monomer in stepwise Michael-type polyadditions to bis-acrylamides giving linear polymers in which the peptide motifs

constitute the larger portion of the polymer chain (Figure 4.2). In the latter case, it is possible to obtain linear polymers also in the presence of amine groups in the peptide by carrying out the polymerisation at slightly acidic pH (see section 4.4). It follows that any natural or synthetic peptide can be converted into an α,ω-difunctional monomer by adding one or two suitably selected aminoacid residues.

In this section, a novel method for preparing linear high polymers of peptides is presented. The feasibility of this synthetic method was demonstrated by obtaining linear polymers using different molecules of high biological interest. The biological properties of the polymer synthesised are also discussed.

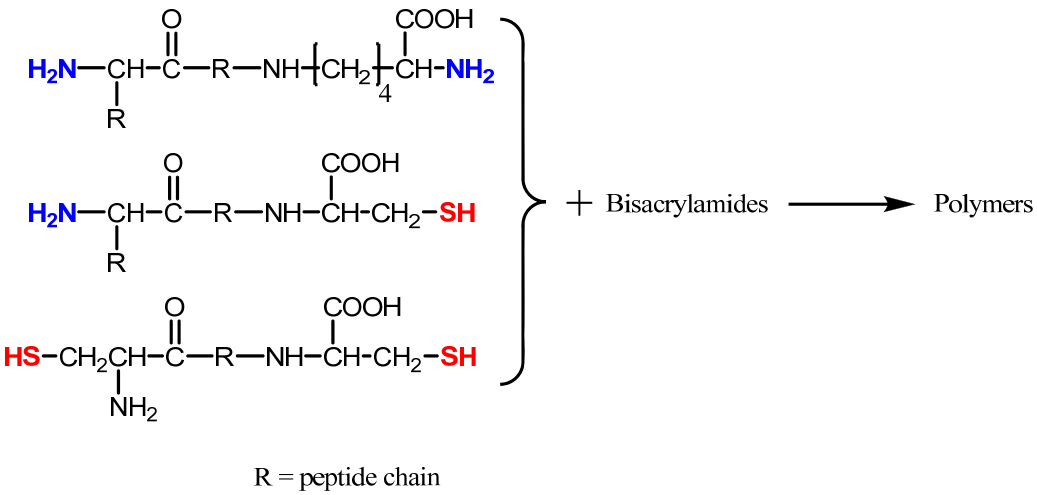


Figure 4.2: general synthesis of PAAs containing peptides in the main chain.

4.4 Results and discussion

To investigate the proposed synthetic method, the following peptide sequences were considered: reduced and oxidised glutathione, RGD, and ISLHA. In particular, 3 different series of polymers structurally related to PAAs were obtained by Michael polyaddition between N,N'-bisacryloylpiperazine (BP) or N,N'-bisacrylammidoacetic acid (BAC) with the following cysteine containing peptides, respectively: (i) reduced and oxidised glutathione, (ii) RGDC, CRGDC, (iii) ISLHAC and CISLHAC peptides (Figure 4.3). Moreover, the synthesis of polymers containing peptides by polyoxidation of the thiol groups in α,ω position of the peptide was investigated. Kinetic studies of these reactions and preliminary biological characterisation were also performed.

Reduced L-glutathione (**α**-L-glutamyl-L-cysteinyl-glycine) (GSH) is a natural peptide initiating by **α**-carboxy-substituted aminoacid residue and containing a L-cysteine residue. Oxidised (dimerised) glutathione (GSSG) is a natural peptide carrying two **α**-carboxy-substituted aminoacid residues at the opposite termini. RGDC/CRGDC and ISLHAC/CISLHAC are derivatives of the well known biologically active RGD (L-arginyl-glycyl-L-aspartic acid) and ISLHA (L-isoleucyl-L-seryl-L-leucyl-L-histidyl-L-alanine) peptides, [44, 45] converted into difunctional monomers by introducing one and two L-cysteine units, respectively.

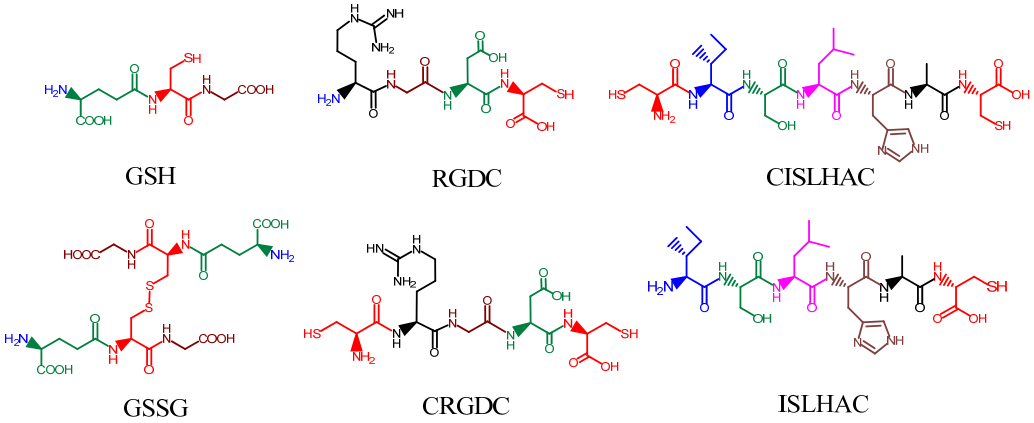
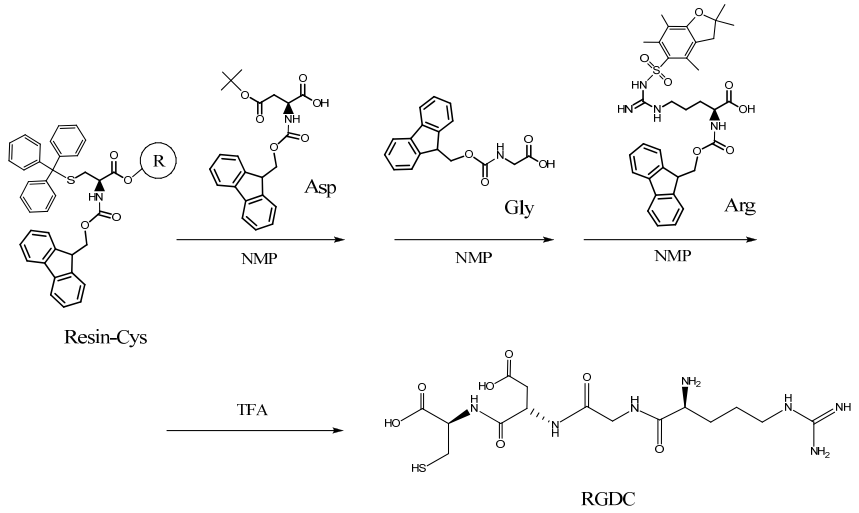


Figure 4.3: general synthesis of PAAs containing peptides in the main chain.

4.4.1 Peptide Synthesis

The non commercially available peptides (RCGD, CRGDC, ISLHAC, CISLHAC) were synthesised by solid phase synthesis method (SPS) with a ABI 433 Peptide Synthesiser and Fmoc based chemistry (see Chapter 6). As example, the synthesis of RGDC is reported (Scheme 4.1). The final step of the reaction is the cleavage of the peptide from the resin by stirring 4 h in TFA, with small amounts of water, thioanisol and ethane-1,2-dithiol as scavengers. The use of concentrate TFA permits the cleavage of all the acid-labile protecting groups of the aminoacid residues in the same step. Considering the conditions used for polimerisation reactions (see later), the peptides thus obtained can be used as difunctional monomer with the exception of ISLHAC peptide, since we found that the imidazole ring of the hystidine could react with the double bond of the bisacrylamide at pH 9.5. (see Section 4.4.4).



Scheme 4.1. Synthesis of RGDC by solid phase synthesis (SPS) method.

After cleavage of protecting groups, the peptides were purified firstly by double precipitation with methanol/diethylic ether, to remove the thiols, secondly by HPLC (eluent: H_2O/CH_3CN), to remove the fractions of peptide with wrong sequence. The peptides were thus obtained with 70% - 80% of yield. All the peptides were characterised

by ESI Mass spectroscopy, NMR spectroscopy, Elmann's assay and iodimetric titration (thiol groups concentration).

4.4.2 Glutathione containing PAAs

Reduced glutathione was used as monomer in polimerisation reaction, under standard PAA synthesis conditions, with N,N'-bisacryloylpiperazine (BP) and N,N'-bisacrylammidoacetic acid (BAC) obtaining polymers named BP-GSH and BAC-GSH, respectively. Moreover, each polymer was obtained in two different regioisomeric forms: in the first one, the GSH is randomly oriented along the macromolecular chain ("random" BP-GSH or BAC-GSH), in the second one, the GSH is orderly oriented along the main chain of the polymer ("ordered" BP-GSH or BAC-GSH) with a "head-to-head" sequence (Figure 4.4).

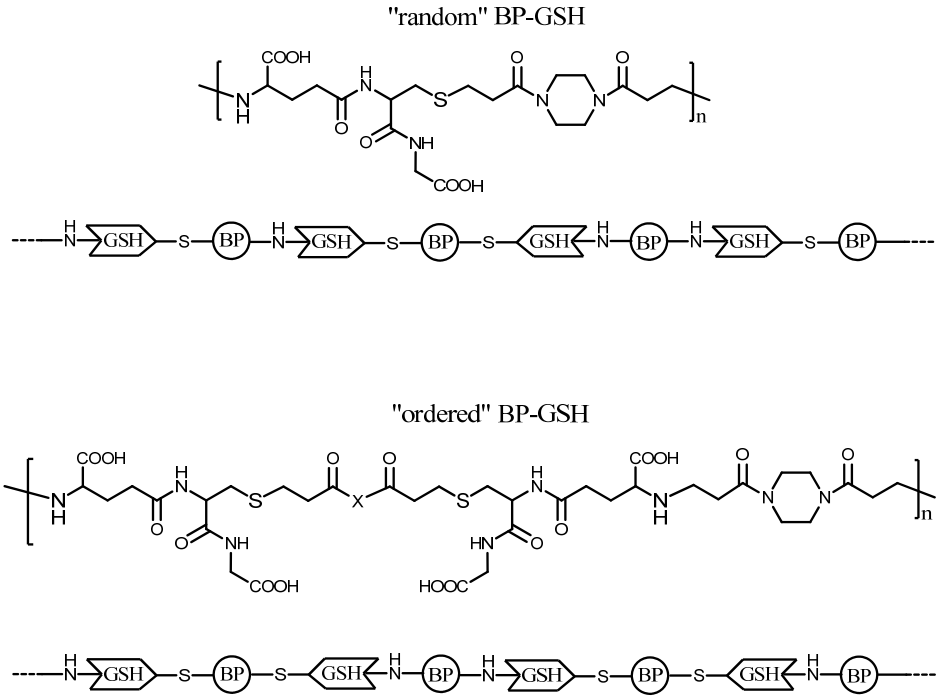
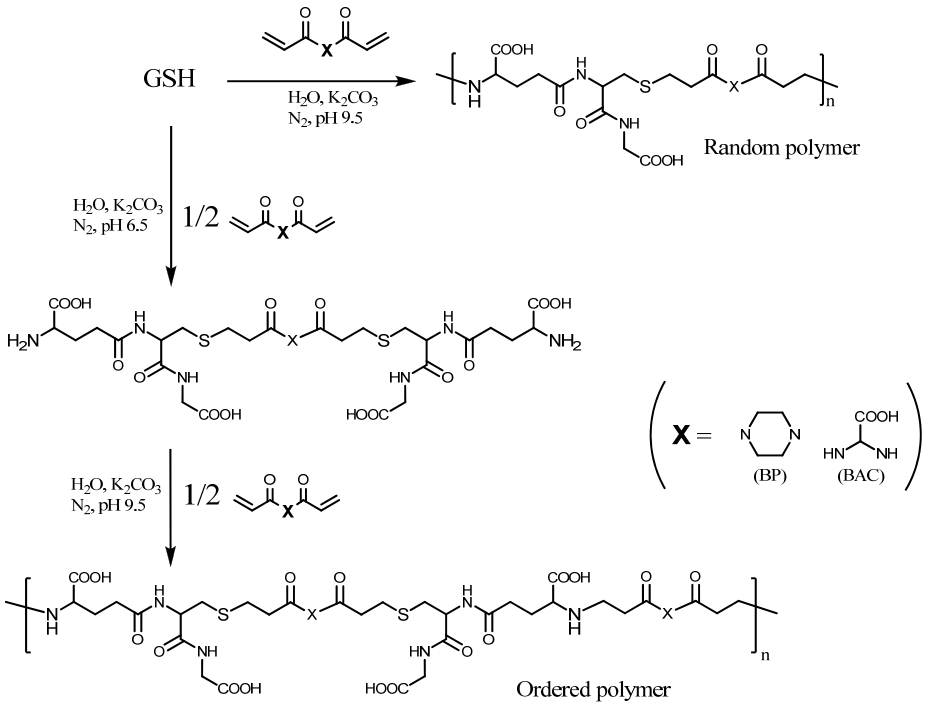


Figure 4.4. Structures of "random" and "ordered" BP-GSH. BP is N,N'-bisacryloylpiperazine and GSH is reduced glutathione.

This was achieved by carrying out the reactions with two different procedures (Scheme 4.2): (1)The random polymer was obtained with a one step reaction, performed for 6 days at pH 9.5 with equimolar amount of GSH and bisacrylamide. At this value of pH, both thiol and amino group can react with the double bond of the bisacrylamide, giving a linear polymer in which the GSH residues are randomly oriented along the polymeric chain. (2)The ordered polymer is obtained in a two steps reaction: the first step is carried out for 24 hours at pH 6.5 in presence of 1 equivalent of GSH and half equivalent of bisacrylamide. At this value of pH the amino group of GSH ($pK_a \sim 9.6$) is completely in its protonated form, then is not able to react with the double bond. On the contrary, the thiol group can react quickly with the double bond at pH 6.5. Therefore, a trimer ABA (where A is a GSH residue and B is a bisacrylamide residue), carrying two amino groups in the α,ω position, is obtained. In the second step, carried out for 6 days, the pH value is increased to 9.5 and another half equivalent of bisacrylamide is added to the reaction mixture. In these conditions, the ABA trimer behaves like a secondary diamine, yielding a linear polymer in which the GSH residues are orderly oriented along the polymer main chain. As example, the ^{1}HNMR spectrum of BP-RGDC is showed in Figure 4.5. Further studies will be performed to investigate possible differences between the behaviour of the "ordered and "random" polymers obtained, as for example in solubility and in degradation rate in physiological environment.



Scheme 4.2. One step and two steps synthesis method of GSH containing PAAs.

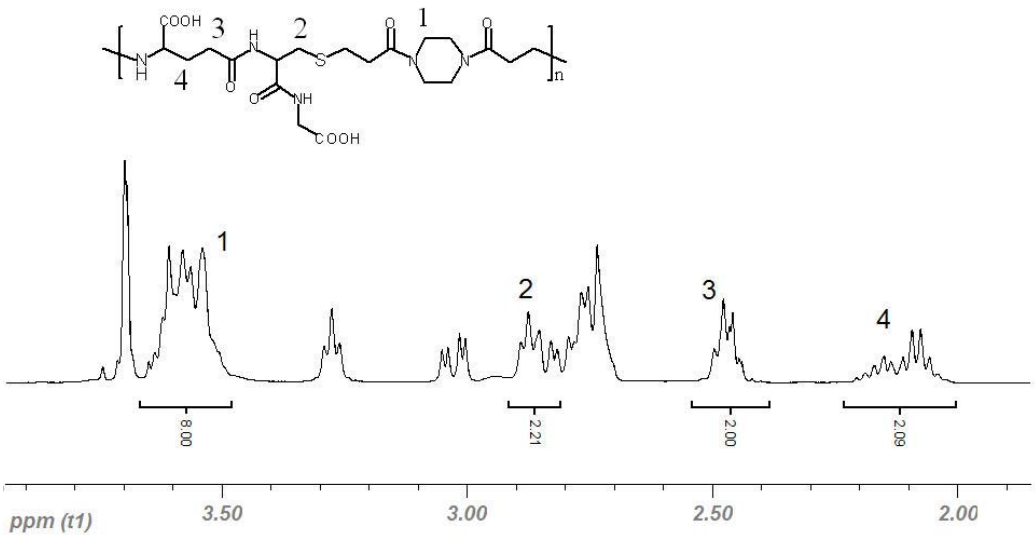
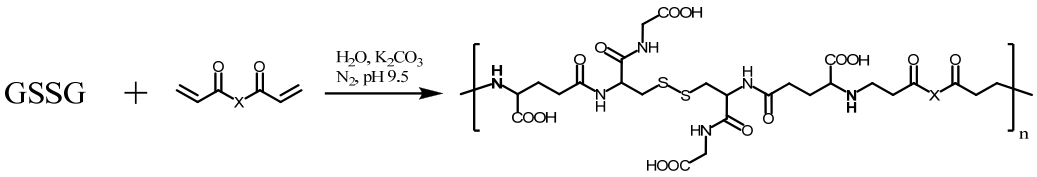


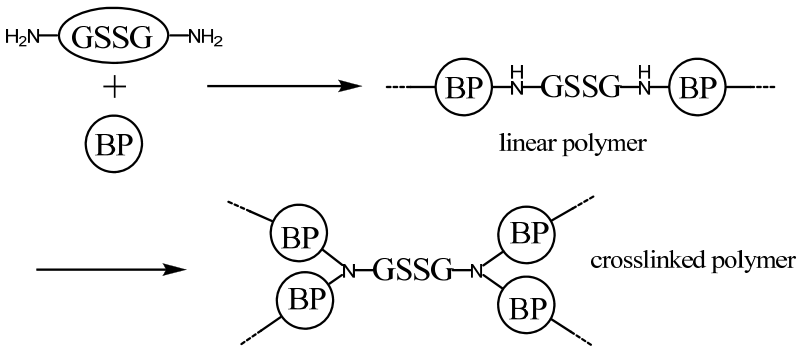
Figure 4.5 HNMR spectra of random BP-GSH in D_2O.

Oxidised glutathione (GSSG) was also used as monomer in polymerisation reactions with BP and BAC (Scheme 4.3), with the same conditions used for GSH containing PAAs ("random" BP-GSH). In this case, due to the structure of GSSG, carrying two amino groups with identical reactivity toward the double bond of the bisacrylamide, only the polymer with a random orientation of the GSSG residues along the polymeric chain was obtainable.



Scheme 4.3. Synthesis of GSSG containing PAAs.

By performing the reactions for 6 days, linear polymers with a number average molecular weight ($\overline{M_n}$) up to 18600 (see Table 4.1) were produced. In order to increase the final average molecular weight of the polymers, we extended the reaction time for more than 6 days. However, after 8 days of reaction, the formation of partially crosslinked polymer was noticed in the reaction mixture. The insoluble product was filtered off, and the soluble fraction showed very high polydispersity (PD) values by GPC analysis. This is due to the fact that the secondary amino groups generated from the addition of the primary amino groups (of GSH or GSSG) to the double bond of the bisacrylamide can react again with the double bond. It follows that GSH and GSSG can behave as tri- and tetrafunctional monomers in polymerization reaction (Scheme 4.4).



Scheme 4.4. Formation of crosslinked BP-GSSG polymer. BP is N,N'-bisacryloylpiperazine and GSSG is oxidised glutathione.

Anyway, the reaction rate of the addition of the secondary amino group is order of magnitude lower than that of primary amino group (see Section 4.4.5). Thus, below a reaction time of 6 days, we can consider that the obtained polymers are prevailingly linear. Therefore, all the polymerisation reactions were carried out for 6 days obtaining polymers with good yields (70% – 80%) and a $\overline{M_n}$ ranging from 7800 to 18600 (Table 4.1). All the synthesised polymers were characterised by Gel Permeation Chromatography (GPC) and ^{1}HNMR spectroscopy.

Polymer	$\overline{M_n}$	$\overline{M_w}$	PD	yield
BP-GSH random	12900	50700	3.9	87 %
BP-GSH ordered	7800	18600	2.4	79 %
BAC-GSH	10400	35000	3.4	75 %
BAC-GSH	8300	23100	2.9	71 %
BP-GSSG	18600	33100	1.8	80 %
BAC-GSSG	10900	20200	1.8	85 %

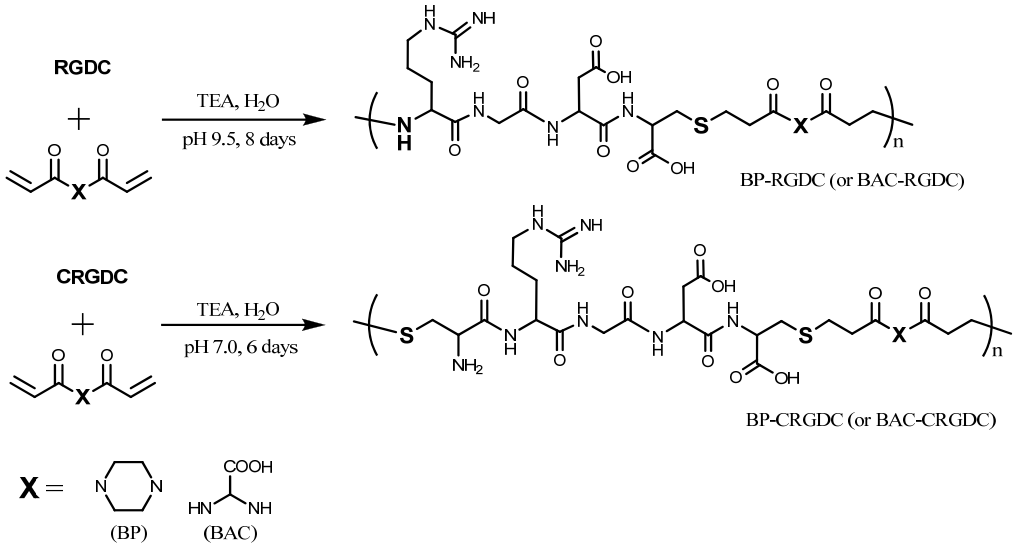
Table 4.1. GPC data of glutathione containing PAAs. Average molecular weights were measured by online Right (90°) and Low (7°) Angle Light Scattering detectors.

4.4.3 RGD containing PAAs

The RGD peptide was converted into difunctional monomers suitable for the Michael polyaddition with bisacrylamides, by adding either one cysteine moiety at the C-terminal, thus obtaining the RGDC peptide or two cysteine moieties at both C- and N-terminal, thus obtaining the CRGDC peptide. The polymerisation reactions were carried out in conditions similar to those used for glutathione containing PAAs (Scheme 4.5). RGDC containing polymers were obtained in both “random” and “ordered” form (see Section 4.4.2), applying the same method used for GSH containing PAAs (Section4.4.2). However, low molecular weight polymers were obtained. This is likely due to the strong intermolecular interactions generated in the reaction solution using K_2CO_3 as base to adjust the pH.

In fact, we have found that using triethylamine (TEA), instead of K_2CO_3, to adjust the pH, the viscosity of the starting reaction solution was decreased, and the average molecular

weight of the polymer increased 4-5 times (see Table 4.2). Moreover, since the oxidation by atmospheric oxygen of the thiol groups of RGDC and CRGDC occurs at a relatively high rate, degassed water was used as reaction solvent and the reaction mixture was maintained under inert atmosphere.



Scheme 4.5. Synthesis of RGD containing PAAs.

In the case of RGDC containing PAAs, the polymerisation reaction was carried out at pH 9.5. At this pH, both thiol and amino groups can take part in the polyaddition reaction with the bisacrylamide. Indeed, about the 50 % of the amino group of RGDC ($pK_a \sim 9.5$) is in its unprotonated form, thus able to react with the double bond. Previous studies have shown that the side guanidine residue of arginine (pKa ~ 12.5) under the pH conditions adopted does not participate in the addition reaction. Similar conditions were adopted for CRGDC containing PAAs, except the pH value, which was around 7, since at this value only the thiol groups are able to react, whereas the aminic nitrogen of the cysteine residue is completely in its protonated form.

Compared to glutathione, the modified RGD monomers displayed a lower reactivity towards the double bond of the bisacrylamide, resulting usually in longer reaction times, lower conversion and lower average molecular weights. The lower reactivity can be ascribed to the probable zwitterionic form of the modified RGD, which could alter the conformation of the molecule in solution. Moreover, these reactions were performed with a higher dilution (0.4 g of bisacrylamide per ml of water) of bisacrylamides compared to normal conditions for PAAs (1.6 g of bisacrylamide per ml of water) in order to limit the viscosity of the starting reaction mixture, that was higher than the one usually observed for GSH polymerisation reactions. In this case, the high viscosity is due intermolecular hydrogen bonds between the peptide molecules in water. Thus, to minimize these interactions we performed the polymerisation of RGDC and CRGDC also with BAC. In fact, the carboxy group of BAC can limit peptide-peptide interactions. However, although the viscosity of the starting reaction mixture was visibly reduced, BAC exhibited lower reactivity compared to BP towards the peptides resulting in a longer reaction time and yielding polymers with lower molecular weight. All these polymers were characterised by GPC and ^{1}HNMR. The ^{1}HNMR spectra of BP-RGDC is showed in Figure 4.6.

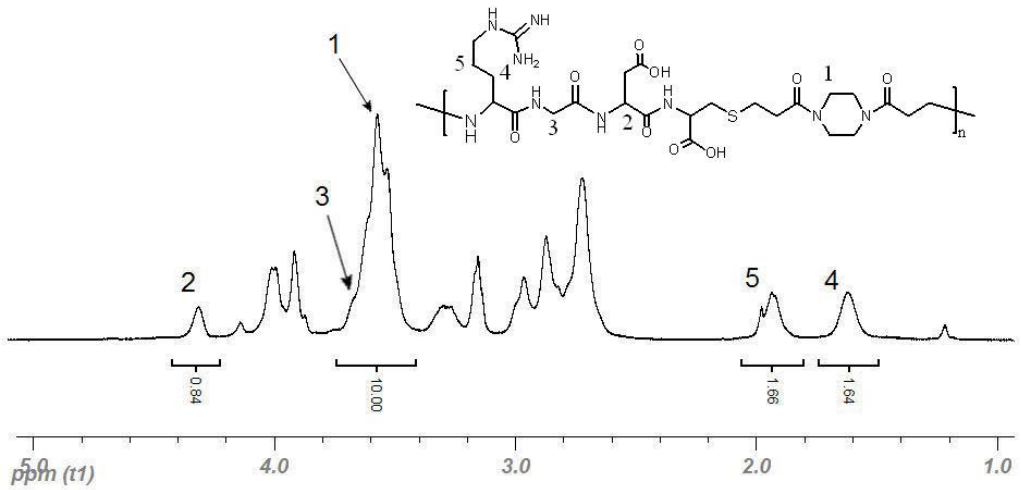
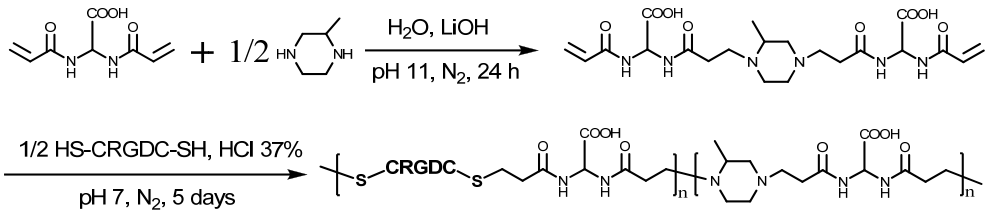


Figure 4.6 HNMR spectra of BP-RGDC in D_2O.

In order to vary the amount of RGD in the polymer, we performed the reaction between BAC and equimolar amounts of CRGDC and 2-methylpiperazine (MP), obtaining a copolymer ISA23-CRGDC (Scheme 4.6). The obtained polymers showed the presence of terminal double bonds. This is mainly due to the rapid oxidation of the thiol groups of the peptides that diminish the real concentration of thiol groups available for the addition reaction. It follows that, during the polymerisation reaction, the real concentration of thiol groups is slightly lower than that of double bonds, resulting in the presence of terminal double bonds in the final polymer. In these polymers terminal double bonds have to be eliminated since can lead to undesirable crosslinking of the polymer chains. Thus, at the end of the reaction, a 10 % of MP was added to terminate residual double bonds.

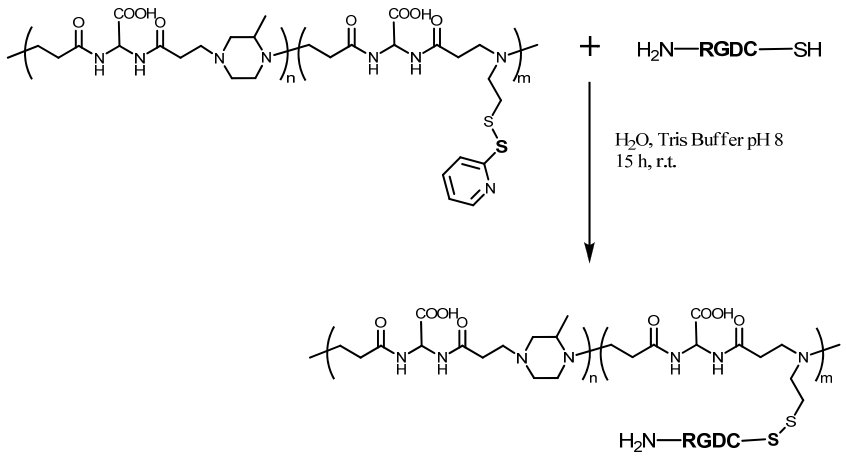
This copolymer was obtained with high yield (75 %) and, thanks to the ISA23 repeating unit, showed a higher solubility in water compared to the others homopolymers containing RGD. By the ^{1}HNMR spectra it was possible to calculate a CRGDC amount of 36 wt% in the polymer.



Scheme 4.6. Synthesis of ISA23-CRGDC copolymer.

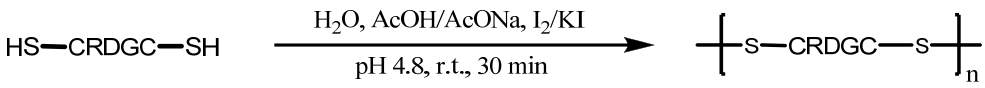
All the polymers shown so far in this study contain amide groups in the main chain, therefore they can undergo hydrolytic degradation (e.g. in biologic environment). In order to obtain an RGD containing PAA able to release the RGD peptide by reductive degradation, we have synthesised an ISA 23 containing RGDC as side substituent (Scheme 4.7). The RGDC monomer was used in a substitution reaction involving the thiol group of the cysteine residue and the pendent disulfide linkages of the polymer ISA23-SS-Py ($\overline{M_n}$, 9500), that was synthesised as previously described. [47] The obtained polymer carries RGDC as side substituent and with a free amino group, suitable for further

functionalisations. Moreover, the disulfide linkage can be cleaved by a reductive agent, releasing the peptide.



Scheme 4.7. Synthesis of ISA23-SS-RGDC.

A different synthetic strategy was used to obtain a polymer in which the CRGDC peptide is the unique constituent. Exploiting the high reactivity of thiol groups toward the oxidation to give disulfide linkages, we performed a polymerisation reaction by oxidating the thiol groups of CRGDC peptide in a concentrated solution (0.5 M). Unexpectedly, no polymerisation was observed by simply stirring the reaction mixture, containing CRGDC (at pH 7), under air atmosphere. Instead, by using I_2/KI as oxidating agent, polymerisation occurred with by far shorter reaction time (30 min) compared with the traditional method used for PAAs (Scheme 4.8).



Scheme 4.8. Synthesis of p(CRGDC).

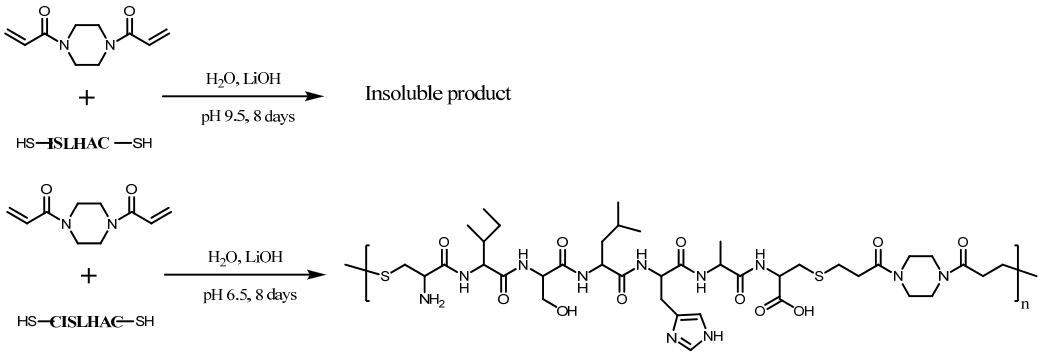
The acetate buffer was used to quench the hydroiodic acid formed as by product of the reaction. The p(CRGDC) displayed, as expected, a slightly lower solubility in water compared with the CRGDC peptide. Despite the high reaction rate observed, the molecular weight was in the range 3000 – 5000. The low average molecular weight can be addressed to the low monomer concentration (0.5 M) and to a probable cyclisation of the oligomers. The average molecular weights of the RGD containing PAAs are summarised in Table 4.2.

Polymer	$\overline{M_n}$	$\overline{M_w}$	**PD**	**yield**	**peptide wt%**
BP-RGDC random	7200	22300	3.1	56 %	69 %
BP-RGDC ordered	3200	6100	1.9	45 %	69 %
BAC-RGDC random	2400	3400	1.6	52 %	69 %
BAC-RGDC ordered	2800	4000	1.4	35 %	69 %
BP-CRGDC	16700	39200	2.3	65 %	74%
BAC-CRGDC	3000	7500	2.5	58 %	74%
ISA23-CRGDC	5500	8000	2.3	75 %	36 %
ISA23-SS-RGDC	12600	21000	1.7	91 %	60 %
p(CRGDC)	3200	5000	1.6	86 %	100 %

Table 4.2. GPC data of RGD containing PAAs. Average molecular weights were measured by GPC with online Right (90°) and Low (7°) Angle Light Scattering detectors. The yields are referred to the weight amount of the polymers ultrafiltered with a 1000 cutoff membrane, compared to the starting monomers.

4.4.4 ISLHA containing PAAs

To obtain an α,ω-difunctional monomer, the ISLHA peptide was modified by adding one cysteine moiety (ISLHAC) or two (CISLHAC) at the C-terminal or both C- and N-terminal of the peptide, respectively. By using the same procedure adopted for cysteine modified RGD, ISLHAC and CISLHAC were used as monomer in polymerisation reaction with BP (Scheme 4.9).



Scheme 4.9. Synthesis of BP-ISLHAC and BP-CISLHAC.

The polymerisation of ISLHAC with BP, performed at pH 9.5, resulted in an insoluble product. This resulted from the cross-linking reaction of hystidine rings with bisacrylamide double bonds. By contrast, the polymerisation reaction involving CISLHAC peptide and BP, carried out at pH 6.5, produced a linear polymer. Therfore, the imidazole ring of hystidine is not able to react with the double bond at pH 6.5. To prove this, the reactivity of imidazole, chosen as a model compound for hystidine, with BP was investigated in D_2O at 9.5 and 6.5 by means of ^{1}HNMR, which allowed monitoring the decrease in concentration of BP double bonds. As comparison, other two reactivity tests were carried out using 2-mercaptoethanol, as model compound for the thiol group of the cysteine residue (Figure 4.7). These results confirm that imidazole can react with BP at pH 9.5. Therefore the imidazole ring of the histidine could react with the double bond of BP at pH 9.5 and,

consequently, we can suppose that the ISLHAC monomer behaves as trifunctional monomer reacting with bisacrylamides at pH 9.5.

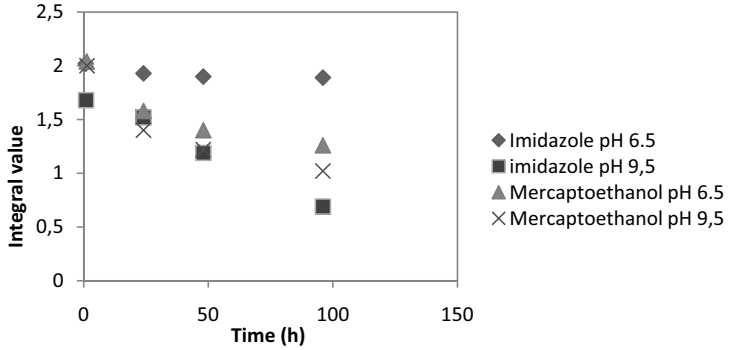


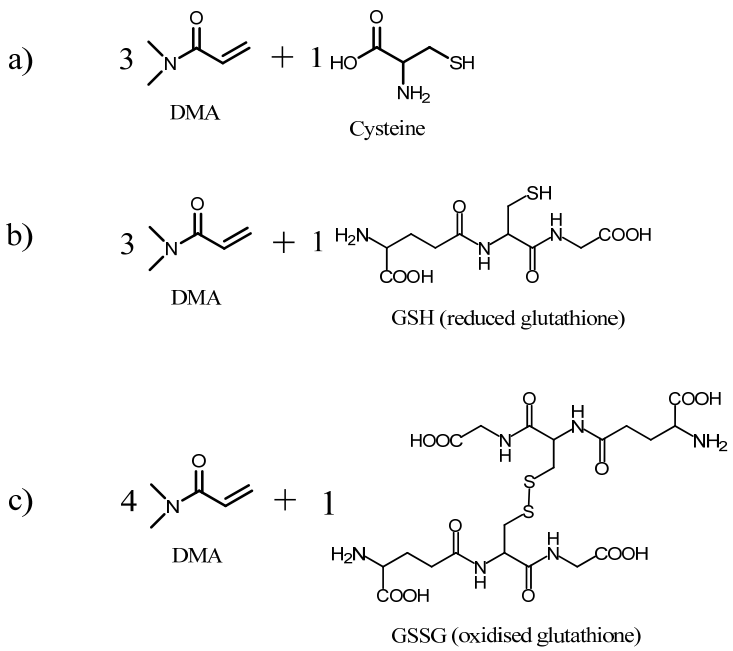
Figure 4.7. Reactivity test between imidazole and BP at pH 6.5 and 9.5, and 2-mercaptoethanol with BP at pH 6 and 9.5. The integral value related to the double bond of BP is compared with the reference integral (CH_3 of *tert*-butanol).

Instead, a linear polymer was obtained by performing the polymerisation at pH 6.5 with BP and CISLHAC. The polymer was obtained with low molecular weight (Mn, 3000; Mw, 4400; PD 1.47) and low yield (35 %). This is probably due to the low solubility of the peptide, caused by the presence of cysteine, leucine and isoleucine moieties, and to high viscosity of the reaction mixture, caused by intermolecular hydrogen bonds of hystidine and serine moieties. Anyway, thanks to the BP moiety, the polymer resulted more soluble in water than the related monomer.

Based on the results presented above, we can conclude that the possibility to insert a peptide motif in PAAs by using it as α,ω–difunctional monomer is strongly dependent on the number and the type of aminoacids constituting the peptide. However, since it is well known that additional aminoacids flanked to the peptide do not affect its biological properties, it is possible to modify the peptide to adjust its solubility and to allow the reactivity in the polymerisation reaction at different pH.

4.4.5 Kinetic studies

Extensive kinetic tests were performed to study the behaviour of the peptide sequences used as α,ω-difunctional monomer in polyadditions reactions with bisacrylamides. In particular, L- cysteine, reduced glutathione (GSH) and oxidised glutathione (GSSG) were used as model compound in Michael addition with dimethylacrylamide (DMA) ((a), (b) and (c) in Scheme 4.10, respectively).



Scheme 4.10. Studied reactions for kinetic tests.

DMA was utilised instead of the traditional bisacrylamides used in PAA synthesis (e.g. BP, BAC) to avoid the increase in viscosity with the reaction progress. The kinetic tests were carried out at pH 9.5, 25°C and with a DMA concentration of 1.6 g per ml of water (the usual conditions for PAA synthesis). In these tests, 3/1 ratio between DMA and cysteine and GSH and 4/1 between DMA and GSSG were used. This ratios were chosen considering DMA as a monofunctional reagent, cysteine and GSH as trifunctionals, and GSSG as tetrafunctional (see Section 4.4.2). The decrease in double bond concentration during reaction was monitored by UV-visible spectrometry.

To understand the behaviour of these monomers in Michael addition we referred to our previous works on hydrogen transfer addition and polyaddition reactions involving acrylamides, bis-acrylamides, and/or bis-acrylic esters [47-50]. These kind of reactions exhibits pseudo-second-order kinetics, with the kinetic constants that include the concentration of the catalytic protonic species, when performed in the presence of hydroxyl solvents. Based on these premise, the decrease in DMA concentration ([DMA]) were plotted in conversion / time and $[DMA]^{-1}$ / time graphs (see later).

Kinetic "A": DMA + cysteine

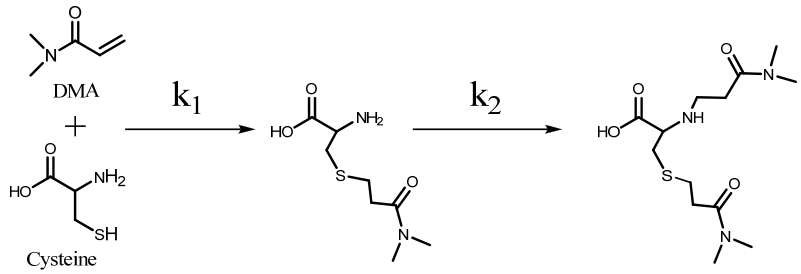
For this test, a 3/1 DMA/cysteine ratio was used. In this case, we expected to observe two different conversion rates, since it is known from previous work that the thiol group can react faster than the amino group at pH 9.5. Effectively, it was found that by plotting the conversion rate of DMA in 1/[DMA] *versus* time graph, the obtained curve was not linear, but diverged at about 50 % conversion (Figures 4.8 and 4.9). This result indicates the presence of two different reaction steps with different rates. In the first one the thiol group react with the double bond of DMA, until one equivalent of DMA is reacted. After that, the addition of the amino group to the double bond becomes prevailingly, consuming another equivalent of DMA. The best fitting conversion curve was calculated by numerically integrating the following differential equation:

$$\frac{d[DMA]}{dt} = -k_1[DMA][SH] - k_2[DMA][NH2] \qquad \text{Equation 4.1}$$

With $k_1 = 1.1 \times 10^{-3}$ and $k_2 = 4 \times 10^{-5}$

where [DMA], [SH] and [NH2] represent the concentration of double bonds (of DMA), thiol groups and primary amino groups (of cysteine), respectively. The kinetic constants k_1 and k_2 of the two reaction steps were obtained by determining the slopes of the very initial and the very final segments of the 1/[BP] versus *t* curves (Figure 4.9), with the assumption that in either regions the reaction of one amino group of MP prevails over the other.

At the end of the reaction (40 days), only two equivalents of DMA were consumed, indicating that cysteine behaves as difunctional monomer (Scheme 4.11) in Michael addition reaction to the double bond. This behaviour is consistent with the one found for reaction between L-cystine (the L-cysteine dimer) and BP in a previous work. [47]



Scheme 4.11. Addition steps in kinetic "A".

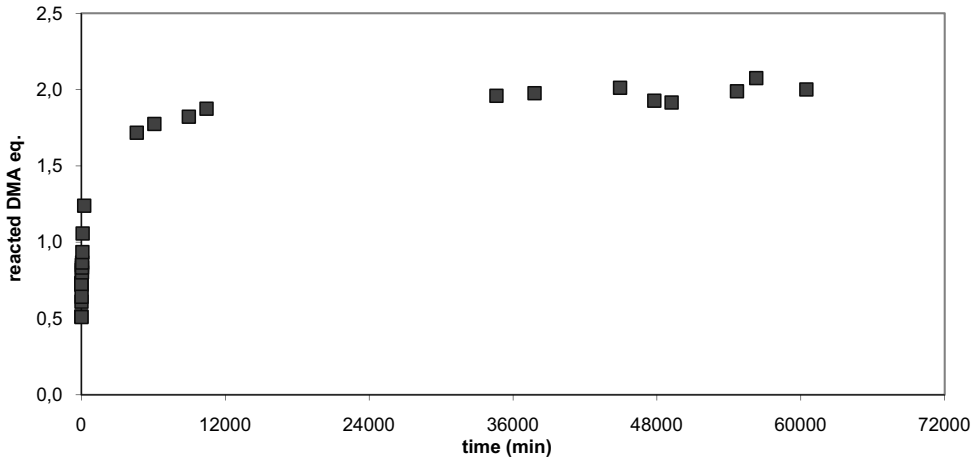


Figure 4.8. Kinetic test "A". After about 40 days (60000 min) only two equivalent of DMA were reacted.

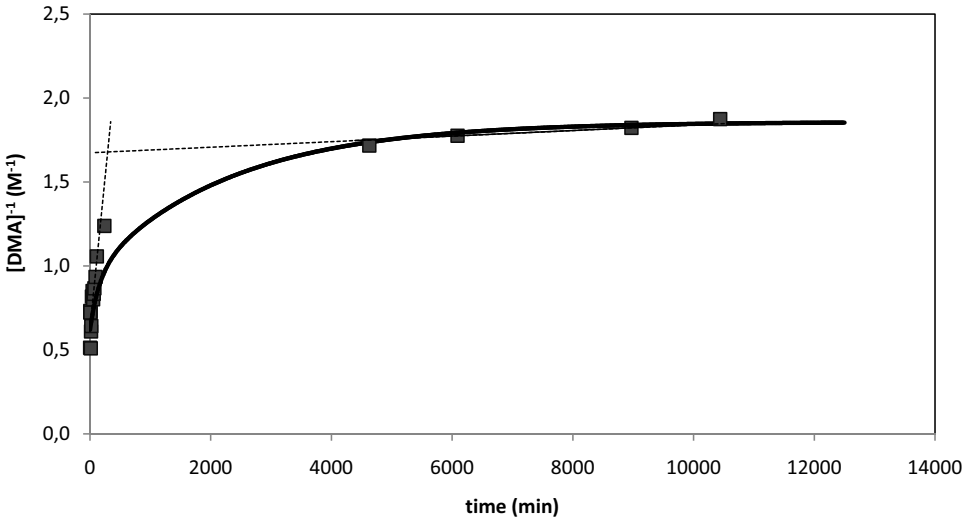


Figure 4.9. Kinetic test "A". Calculated conversion curves using the equation 4.1 (enlarged view).

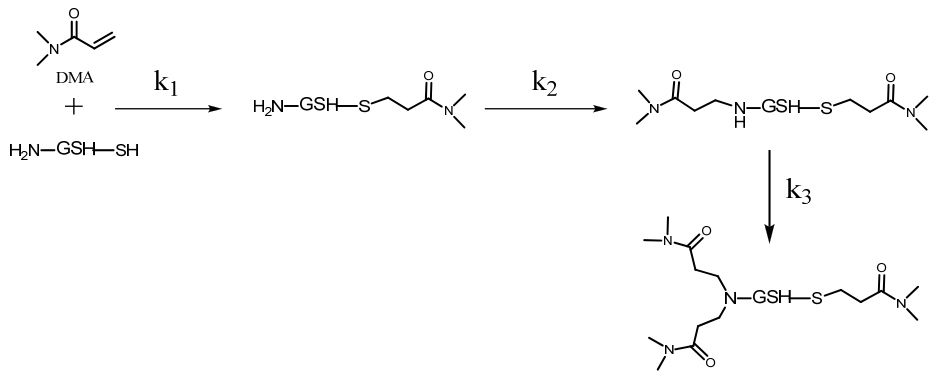
Kinetic "B": DMA + GSH

This kinetic test was performed with a 3/1 DMA/GSH ratio. GSH has one thiol and one primary amino group like L-cysteine. However, it was unexpectedly noticed that after approximately 10 days of reaction, more than 2 equivalents were reacted (Figure 4.10). This is due to multiple addition of the amino group to the double bonds of DMA. These results indicate that GSH could behave as trifunctional monomer in Michael addition with the double bond of DMA. In fact, by plotting the kinetic data in 1/[DMA] *versus* time graph, three different reaction steps were observed (Figure 4.11). Therefore, the conversion curve was calculated by numerically integrating the following equation:

$$\frac{d[DMA]}{dt} = -k_1[DMA][SH] - k_2[DMA][NH2] - k_3[DMA][NH2'] \qquad \text{Equation 4.2}$$

where $\frac{d[NH2']}{dt} = k_2[DMA][NH2]$, $k_1 = 2.6 \times 10^{-3}$, $k_2 = 4 \times 10^{-4}$ and $k_3 = 6 \times 10^{-6}$

where [DMA], [SH], [NH2] and [NH2'] represent the concentration of double bonds, thiol groups, primary amino groups and secondary amino groups (generated from the addition of the primary amino groups to DMA), respectively. The kinetic constants k_1, k_2 and k_3 of the two reaction steps were obtained with the same method and the same assumptions of kinetic "A" (see above). In particular, k_1, k_2 and k_3 are referred to addition to the double bonds by thiol groups, primary and secondary amino groups, respectively (Scheme 4.12).



Scheme 4.12. Addition steps in kinetic "B".

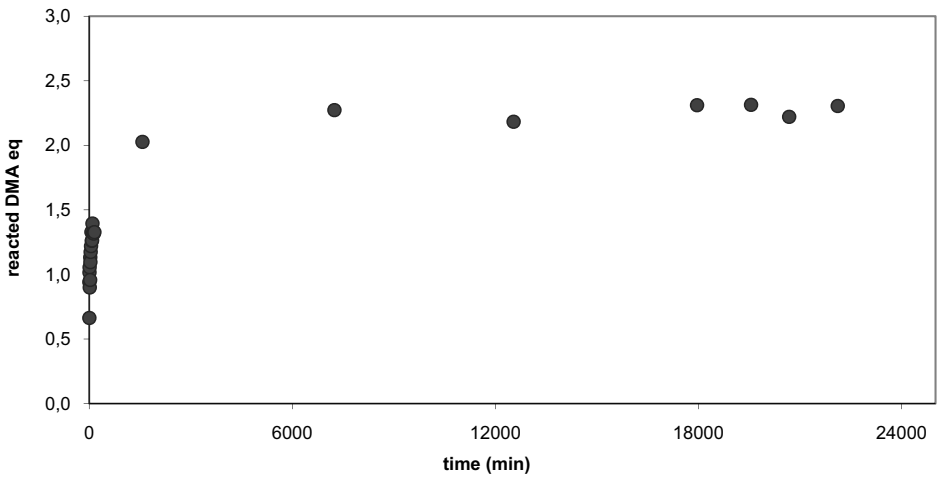


Figure 4.10. Kinetic test "B". After about 5 days (7000 min) more than two equivalent of DMA were reacted.

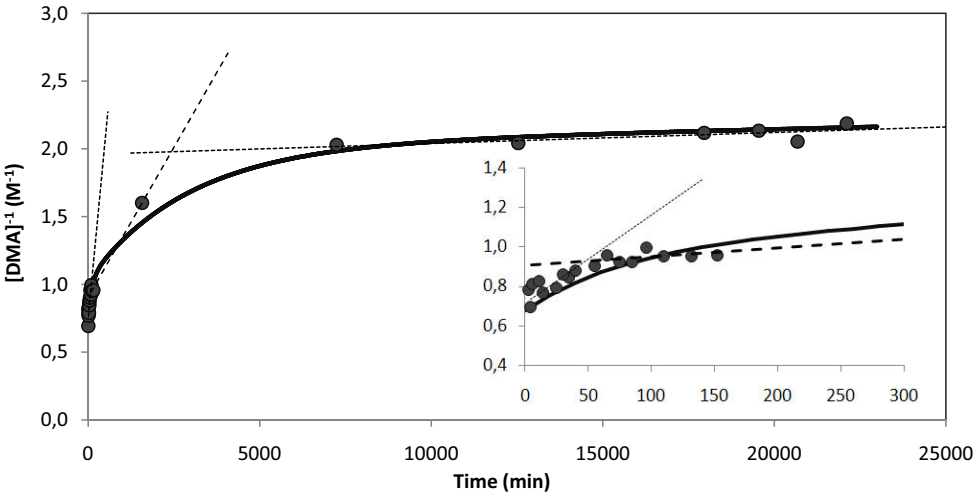


Figure 4.11. Kinetic test "B". Calculated conversion curves using the equation 4.2. In the inset: enlarged view.

Kinetic "C": DMA + GSSG

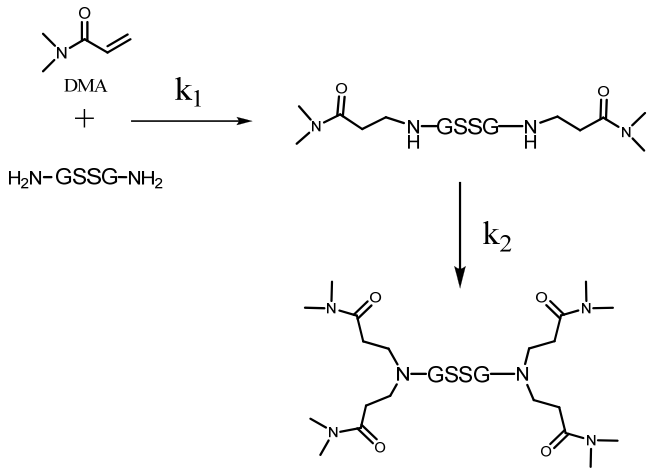
In this case, a 4/1 DMA/GSSG ratio was used. GSSG has only two identical primary amino groups and no thiol groups. As in the case of GSH a multiple addition step was observed for the amino groups and more than 2 equivalents of DMA were consumed after 10 days of reaction.. However, only two different reaction steps were identified (Figure 4.12 and 4.13), since in this case the addition of the thiol group is not present. Thus, the following differential equation was used to calculate the conversion curve by numerical integration:

$$\frac{d[DMA]}{dt} = -k_1[DMA][NH2] - k_2[DMA][NH2'] \qquad \text{Equation 4.3}$$

where $\frac{d[NH2']}{dt} = k_2[DMA][NH2]$, $k_1 = 1 \times 10^{-4}$ and $k_2 = 2 \times 10^{-6}$

where [DMA], [NH2] and [NH2'] represent the concentration of double bonds, primary amino groups and secondary amino groups (generated from the addition of the primary

amino groups to DMA), respectively. The kinetic constants k_1 and k_2 indicate the addition to the double bonds of primary and secondary amino groups, respectively (Scheme 4.13).



Scheme 4.13. Addition steps in kinetic "C".

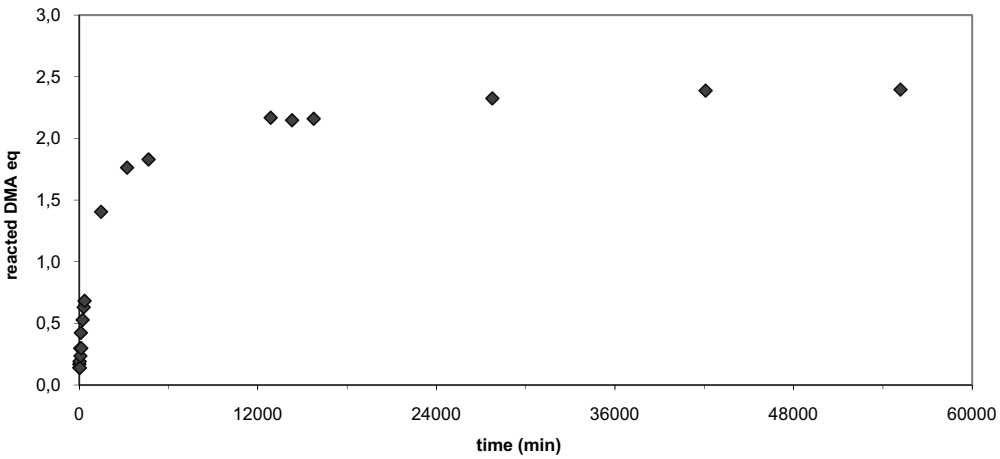


Figure 4.12. Kinetic test "C". After about 8 days (12000 min) more than two equivalent of DMA were reacted.

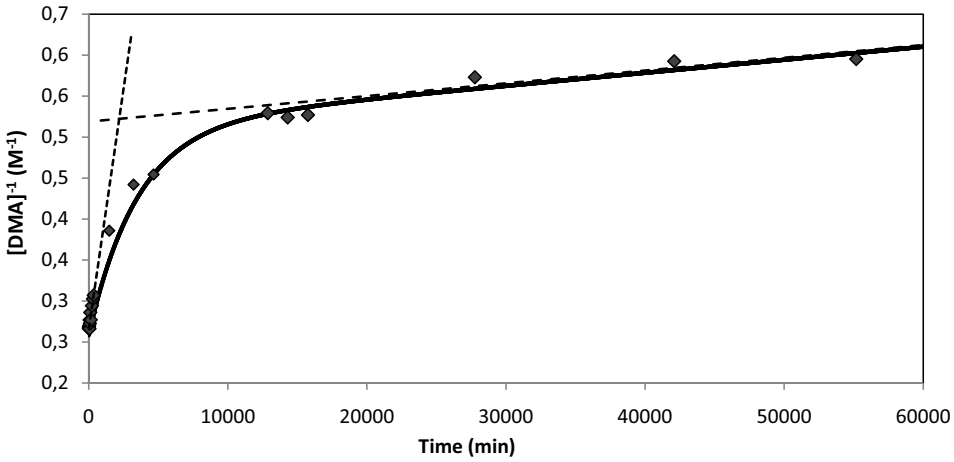


Figure 4.13. Kinetic test "C". Calculated conversion curves using the equation 4.3.

Kinetic test	Tested molecules	k_1	k_2	k_3
A	DMA + cysteine	1.1×10^{-3}	4×10^{-5}	-
B	DMA + GSH	2.6×10^{-3}	4×10^{-4}	6×10^{-6}
C	DMA + GSSG	1×10^{-4}	2×10^{-6}	-

Table 4.3. Calculated kinetic constants for kinetic tests (a), (b) and (c).

The values of the k_1, k_2, and k_3 kinetic constants obtained for tests "A", "B" and "C" are summarised in Table 4.3. It was previously demonstrated that the thiol group reacts with the double bond of a bisacrylamide faster than the amine group. [49] Thus, in cases "A" and "B", the higher kinetic constant (k_1) can be addressed to the thiol group and, consequently, the lower (k_2) refers to the amine group. Moreover, in the case "B" the lowest constant (k_3) refers to the addition of the secondary amine group to the double bond (see above). In the case "C", k_1 and k_2 indicate the addition of the primary and the secondary amino group, respectively.

No kinetic evidence of multiple addition steps to the primary amino group of L-cysteine molecule was found, confirming the previous studies on reaction between L-cystine and BP. [47] In contrast, multiple addition steps to the double bond were observed for amino groups of GSH and GSSG. Thus, in these molecules, the secondary amino group generated from the addition of the primary amino group to the double bond of the DMA can react again with another double bond. This is probably due by a catalytic effect of glutathione. This results are consistent with the experimental evidence of partial crosslinking in polymerisation reaction of GSH and GSSG with BP (see Section 4.4.2). However, the reaction rate of the second addition of the amine group is some order of magnitude lower than that of primary addition, and the effect (crosslinking) is evident only after at least 5 days of reaction. Therefore, by adjusting the reaction time, is still possible to obtain linear polymers using these molecules as difunctional monomers.
Finally, based on the structural resemblance of glutathione with RGD, and on the comparable results obtained in polymerisation reactions (see Section 4.4.2, 4.4.3), we can suppose that the behaviour of glutathione in this kind of polyaddition reaction is analogous to RGD peptide.

4.4.6 Biological properties

Preliminary biological characterisations were carried out on BP-RGDC and BP-CRGDC by the group of prof. R. Cavalli (University of Turin). It was shown that both polymers have no haemolytic activity at concentrations lower than 1.73 mg/ml (BP-RGDC) and 2.32 mg/ml (BP-CRGDC). At the same concentration there was no evidence of cytotoxicity.
The zeta potential measurement for both the polymers are shown in Figure 4.13. The values are negative (-5mV /-34 mV) in the pH range 5.0 – 7.4.

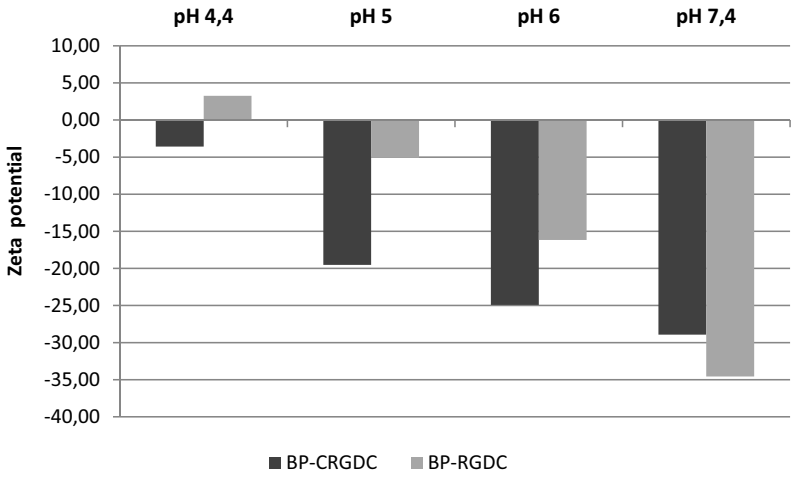


Figure 4.14. Zeta potential of BP-RGDC and BP-CRGDC.

The negative values were expected since the carboxy group of the aspartic acid residue of RGD is deprotonated at physiologic pH. Moreover, negatively charged polymers usually show stealth properties when injected *in vivo*. Further in vitro tests are in progress to investigate the ability of these polymers in promoting cellular adhesion, compared with the parent peptide.

4.5 Conclusions

A general synthetic methods to introduce a short peptide motif as main constituents of a polymer backbone was established. The new polymers were obtained by polyaddition of NH_2- or SH –terminated peptides to bis-acrylamides and were therefore named "poly(peptamidoamine)s" (PPAAs) since they are based on peptides and are structurally related to PAAs.

BP and BAC were used as comonomers in polymerisation reaction with reduced and oxidised glutathione, cysteine modified RGD and cysteine modified ISLHA peptides, respectively, yielding polymers with a relatively high molecular weight.

Our experimental results suggest that this method could be extended, by adding the suitable aminoacid residues, to the preparation of polymeric peptides irrespective of the aminoacid sequence and the molecular weight of the parent peptide. In the resultant polymers, as in proteins, the peptide moieties constitute an integral part of the polymer chain in which they are inserted in α,ω fashion. The polymerisation reaction takes place under remarkably friendly conditions, preserving the basic character of the amine group involved. Polymers obtained with BP as comonomers do not alter the acid-base properties of the parent peptides, as previously demonstrated. [46] By contrast, the presence of BAC can increase the anionic character and confer stealth-like properties to the polymer. [52] In addition, the RGD containing polymers showed no cytotoxicity and no haemolytic activity. The possible applications of PPAAs in biology, medicine and physics cannot be fully estimated at present, but are probably not negligible. Part of the work presented in this chapter has been published in an Italian Patent. [51]

References

[1] D. Hanahan, R.A. Weinberg, *Cell,* (100), 57-70, **2000**.

[2] J. Folkman, *Semin. Oncol.,* (29), 15-18, **2002.**

[3] K. Temming, R. M. Schiffelers, G. Molema, R. J. Kok, *Drug Resistance Updates*, (8), 381–402, **2005**.

[4] P.C. Brooks, M.P. Montgomery, M. Rosenfeld, R.A. Reisfeld, T. Hu, G. Liker, et al. *Cell*, (79), 1157, **1994.**

[5] M. D. Pierschbacher & E. Ruoslahti, *Nature*, (309), 30-33, **1984**.

[6] X. N. Wei, D. Klee, H. Hocker, *Achen. Biomaterialien*; (2), 81–86, **2001.**

[7] D.L. Elbert, J.A. Hubbell, *Biomacromolecules*, (2), 430–441, **2001**.

[8] G.B. Fields, J.L. Lauer, Y. Dori, P. Forns, Y.C. Yu, M. Tirrell, *Biopolymers*, (47), 143–151, **1998**.

[9] A.M. Rouhi, *Chem Eng News*, (77), 51–59, **1999**.

[10] J.B. Lhoest, E. Detrait, P. van den Bosch de Aguilar, P. Bertrand, *J. Biomed. Mater. Res.*, (41), 95–103, **1998.**

[11] V. V. Hlady, J. Buijs. *Curr. Opin. Biotechnol.*, (7), 72–77, **1996**.

[12] G. Altankov, F. Grinnell, T. Groth *J. Biomed. Mater. Res.*, (30), 385–391, **1996.**

[13] D. K. Pettit, A.S. Hoffman, T.A. Horbett, *J. Biomed. Mater. Res.,* (28), 685–691, **1994**.

[14] P. A. Underwood, J.G. Steele, B.A. Dalton. *J. Cell. Sci.*, (104), 793–803, **1993**.

[15] D. J. Iuliano, S. S. Saavedra, G. A. Truskey, *J. Biomed. Mater. Res.,* (27), 1103–1113, **1993.**

[16] K. Lewandowska, E. Pergament, C. N. Sukenik, L. A. Culp. *J. Biomed. Mater. Res.*, (26), 1343–1363, **1992.**

[17] D. L. Elbert, J.A. Hubbell, *Annu. Rev. Mater. Sci.* (26), 365–394, **1996.**

[18] T. A. Horbett, K. R. Lew, *J. Biomater. Sci. Polym. Ed.*, (6), 15–33, **1994.**

[19] K. M. Shakesheff, S. M. Cannizzaro, R. Langer, *J. Biomat. Sci. Polym. Ed.* (9), 507–518, **1998.**

[20] Y. Ito, M. Kajihara, Y. Imanishi, *J. Biomed. Mater. Res.*, (25), 1325–1337, **1991.**

[21] T. Boxus, R. Touillaux, G. Dive, J. Marchand-Brynaert, *Bioorg. Med. Chem.,* (6), 1577–1595, **1998.**

[22] J. A. Neff, K. D. Caldwell, P. A. Tresco. *J. Biomed. Mater. Res.* (40), 511–519, **1998.**

[23] E. Ruoslahti, *Annu. Rev. Cell. Dev. Biol.*;(12), 697–715, **1996.**

[24] Aumailley M, Gurrath M, Muller G, Calvete J, Timpl R, Kessler H. *FEBS Lett*, (291), 50–54, **1991.**

[25] M. Gurrath, G. Muller, H. Kessler, M. Aumailley, R. Timpl, *Eur. J. Biochem.*, (210), 911–921, **1992.**

[26] B. Ivanov, W. Grzesik, F. A. Robey, *Bioconjug. Chem.*, (6), 269–77, **1995.**

[27] D. Anderheiden, O. Brenner, D. Klee, R. Kaufmann, H. A. Richter, C. Mittermayer, H. Hocker, *Angew. Makromol. Chem.*, (185), 109–127, **1991.**

[28] M. J. Moghaddam, T. Matsuda *J. Polym. Sci. Pol. Chem.*, (31), 1589–97, **1993.**

[29] B. K. Brandley, R. L. Schnaar. *Anal. Biochem.* (172), 270–280, **1988.**

[30] R. L. Schnaar, B. G. Langer, B. K. Brandley. *Anal. Biochem.*, (151), 268–281, **1985.**

[31] P. Banerjee, D.J. Irvine, A.M. Mayes, L.G. Griffith. *J. Biomed. Mater. Res.*, (50), 331–339, **2000.**

[32] J.P. Tam, Q. Yu, Z. Miao. *Biopolymers*, (51), 311–332, **1999.**

[33] G. Tuchscherer. *Tetrahedron Lett.*, (34), 8410–8422, **1993.**

[34] F. Wahl, M. Mutter. *Tetrahedron Lett.*, (37), 6861–6864, **1996.**

[35] J. Franchini et al., *Biomacromolecules,* (7), 1215-1222, **2006.**

[36] F. Danusso, P. Ferruti, *Polymer*, (11), 88, **1970.**

[37] P. Ferruti, M. A. Marchisio, R. Barbucci, *Polymer* (26), 1336, **1985.**

[38] P. Ferruti, "Ion-Chelating Polymers (Medical Applications"), in: *"Polymeric Materials Encyclopedia*", J. C. Salamone, Ed., CRC Press Inc., Boca Raton, Florida, (vol 5), 3334, **1996**.

[39] P. Ferruti, M. A. Marchisio, R. Duncan, *Macromol. Rapid Commun*., (23), 332. **2002.**

[40] J. Franchini, P. Ferruti, *J. Bioactive Compat. Pol*. (19), 221, **2004**.

[41] P. Ferruti, E. Ranucci, L. Depero, *Polym. Commun.* (30), 157, **1989.**

[42] N. Lavignac, M. Lazenby, J. Franchini, P. Ferruti, R. Duncan, *Int. J. Pharm*., 300 (1-2), 102, **2005.**

[43] E. Emilitri, P. Ferruti, R. Annunziata,E. Ranucci, M.Rossi, L. Falciola, P. Mussini, F. Chiellini, C. Bartoli, *Macromolecules*, (40), 4785, **2007.**

[44] A. L. Dunehoo, M. Anderson, S. Majumdar, N. Kobayashi, C. Berkland, T. J.Siahaan, *Journal of Pharmaceutical Sciences*, 95 (9), 1856, **2006.**

[45] R. Stupp, C. Ruegg, *Journal of Clinical Oncology*, 25 (13), 1637, **2007.**

[46] P. Ferruti, S. Manzoni, S. C. W. Richardson, R. Duncan, N. G. Pattrick, R. Mendichi, M. Casolaro, *Macromolecules,* (33), 7793, **2000.**

[47] E. Emilitri, P. Ferruti, R. Annunziata, E. Ranucci, *Macromolecules,* **2007,** (40)*,* 4785-4793.

[48] A. Manfredi, E. Ranucci, M. Suardi, P. Ferruti, *Journal of Bioactive and Compatible Polymers,* Vol. 22, March **2007**, 219.

[49] P. Ferruti, S. Knobloch, E. Ranucci, R. Duncan, E. Gianasi, *Macromol. Chem. Phys.*, (199), 2565, **1998**.

[50]. E.Ranucci, and P.Ferruti, *Polymer*, 32(15): 2876–2879. **1991**.

[51].R. Cavalli,; M. Trotta,; P. Ferruti,; E. Ranucci (Università di Torino), Polimeri biocompatibili di bisacrilamidi e aminoacidi Brevetto Italiano TO2007A000415, **2007.**

[52] P. Ferruti, S. Richardson, R. Duncan, *Targeting of Drugs: Stealth Therapeutic Systems*, G. Gregoriadis, B. McCormack, Eds., Plenum Press, New York **1998**, p. 207-224.

Chapter 5: Hyperbranched poly(amidoamine)s for gene delivery

5.1 Abstract

In this work gene delivery properties of new hyperbranched PAAs containing disulfide linkages in the main chain were investigated in comparison with the relevant linear polymers. Eight different bioreducible poly(amidoamine)s (PAAs) were prepared by hydrogen-transfer polyaddition of N,N'-bisacryloylcystamine (CBA) with ethylenediamine (EDA) or N,N'-dimethylethylenediamine (DMEDA) and of N,N'-bisacryloylpiperazine (BP) with cystamine (CYST) or N,N'-dimethylcystamine (DMC). Each polymer was terminated with 4-aminobutanol (ABOL) and 2-ethanolamine (ETA). Remarkable results were obtained in DNA complexation tests, including particle size and zeta-potential measurement. DNA transfection experiments with COS-7 cells showed that polyplexes from hyperbranched CBA containing PAA give transfection higher or comparable with the relevant linear polymers and the branched pEI (reference polymer), and XTT assays displayed low or no toxicity. Further tests are in progress to investigate the influence of terminal groups on the transfection capabilities of these polymers.

5.2 Introduction

Almost all human diseases have a genetic component, from untreatable monogenic disorders to cancer and heart disease. In recent years, gene therapy led to very promising results in many biomedical applications. The main objective in gene therapy is successful in vivo transfer of the genetic materials to the targeted tissues. Unfortunately, a suitable vector to deliver genes into cells which is both effective and safe is not yet available.[1] Vectors for gene delivery can be divided in two classes, viral and non viral. Viral vectors are obtained by modification of viruses to allow the delivery of foreign DNA. They possess a high transfection efficiency but, in the other hand, they have some drawbacks like immunologic reaction and low gene carrying capacity, resistance to repeated infection and

difficulty in production and quality control. Non viral vectors include naked DNA, oligonucleotide, lipoplexes and polymer DNA complexes (polyplexes). They showed interesting features in gene delivery. In fact, although they possess generally a lower efficiency in transfection compared to viral vectors, they have lower host immunogenicity, higher gene carrying capacity and can be obtained with simple large scale synthesis. In particular, synthetic polymers are very promising for applications in gene delivery since they allow a high level of design flexibility for biomaterial construction. Although the mechanism involved in gene delivery is not fully elucidated, it is known there are several extra- and intracellular barriers that a polymeric vector has to overcome in the delivery pathway. The cationic polymer-mediated gene delivery is illustrated Figure 5.1.

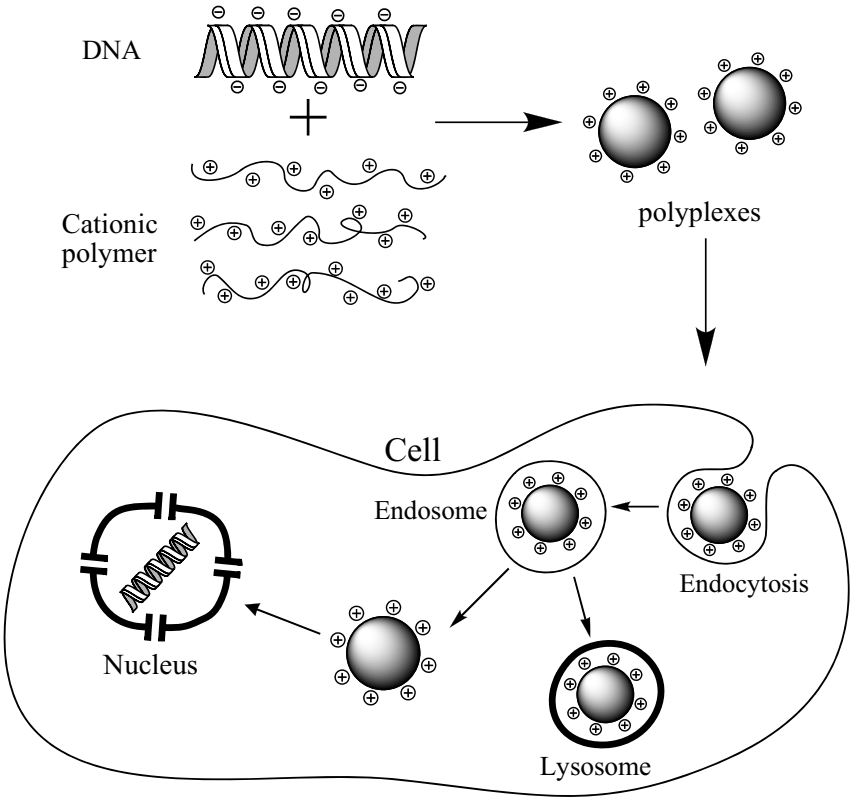


Figure 5.1. Schematic illustration of cationic polymer-mediated gene delivery.

Nano-sized polyplexes are formed by a self-assembling process between negatively charged DNA and cationic macromolecules due to electrostatic interaction. These polyplexes display a cationic surface charge and facilitate DNA cellular uptake by endocytosis. Within the endosome, the pH gradually decrease from 7.4 to about 4.5 (lysosome). To avoid degradation by lysosomal enzymes, the polyplexes must escape from the endosome by some transmembrane mechanism or endosomolytic process like the "proton sponge effect". [2] Once in the cytoplasm, the DNA must unpack from the particle and be carried into the nucleus, although these mechanisms are not fully understood. When DNA is introduced within the nucleus, gene expression and thus production of proteins occur.

It was found that good transfection efficiency is generally correlated with several features of polyplexes. In particular, polyplexes with size smaller than 200 nm and high stability in the extracellular environment display good transfection efficiency compared with bigger (or less stable) polyplexes. [3] Moreover, polyplexes' efficient endosomal escape is achieved by using polymers with high buffer capacity [4] in the range of endosomal pH change (pH 7.4 to 5.1) by "proton sponge effect". Finally, therapeutic applications of these polymers can be seriously hampered by cytotoxicity.

Two of the most widely investigated vectors for gene delivery are the cationic polymers polyethylenimine (PEI) and poly(L-lysine) (PLL) (Figure 5.1). PEI is a gene carrier with high transfection efficiency and high cytotoxicity. [5] Many factors affect the efficiency/cytotoxicity profile of PEI polyplexes such as molecular weight, degree of branching, ionic strength of the solution, zeta potential and particle size. [6,7] It was demonstrated that it is possible to minimize the toxicity of PEI by introducing acid-labile imine linkers in the polymer. [8] The acid-labile PEI may be rapidly degraded into low molecular weight PEI in acidic endosomes. PEG-grafting of PEI also led to a decrease of the toxicity in vitro. [8,9]

By contrast, PLL have a biodegradable nature since it is a linear polypeptide with the amino acid lysine as the repeat unit. However, when entered into the circulatory system, PLL polyplexes were rapidly bound to plasma proteins and cleared from the circulation. [10] This may cause lower transfection efficiency. Dendritic poly(L-lysine) of the 6th generation (KG6) showed high transfection efficiency, without significant toxicity or cell specificity.

As in the case of PEG-grafted PEI, the creation of amphiphilic PLL, by linking both PEG and palmitoyl groups to the polymer, reduced toxicity without compromising the gene delivery efficiency. [11]

More recently, poly(amidoamine)s (PAAs) (Figure 5.1) begun to be studied as vector for gene delivery, showing promising results. As example, several amphoteric PAAs, carrying a carboxylic acid as side group in the bisacrylamide segment, synthesised by our group showed a DNA transfection ability comparable with PEI [12] and endosomolytic ability. [13] This can be explained by the capacity of these polymers to undergo conformational changes in the endosomal pH range. We also developed bioreducible PAAs containing disulfide linkages in the main chain, able to degrade in a reductive environment. [23]

Moreover, Engbersen et al. developed a series of bioreducible PAAs containing disulfide linkages in the main chain with DNA transfection ability higher than PEI. [14] These polymers were more efficient in DNA transfection than the related polymers lacking disulfide linkages. This can be ascribed to the biodegradability of the polymer in the intracellular environment. It was found also that, despite the cationic nature, these PAAs have low or no cytotoxicity. Thus, poly(amidoamine)s can be regarded as promising vectors for the development of high efficient and nontoxic polymeric gene carriers.

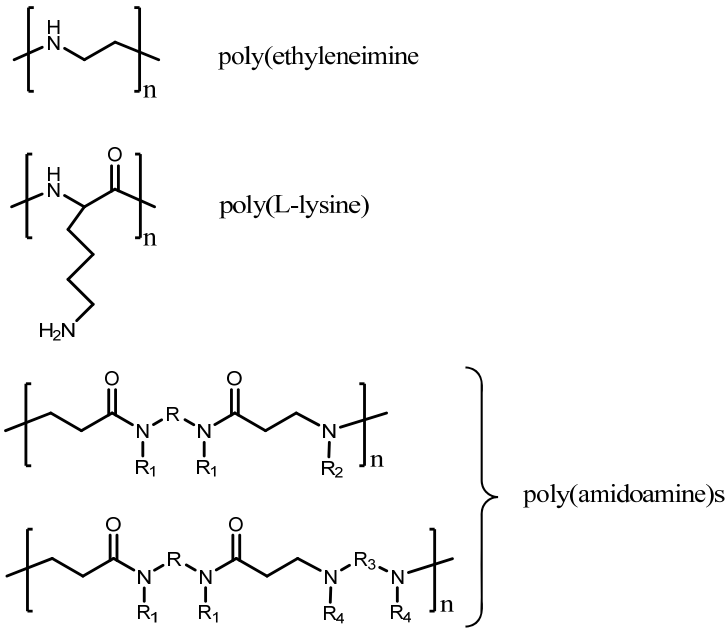


Figure 5.1. Structures of polyethylenimine (PEI), poly(L-lysine) (PLL) and poly(amidoamine)s (PAAs).

5.3 Rationale of the study

Poly(amidoamine)s (PAAs) are polymers that can be easily synthesised by the Michael–type polyaddition between bisacrylamides and primary mono-amines or secondary bis-amines (See Section 2). PAAs displayed in different studies very promising features in gene delivery. [14, 15] Recently developed bioreducible PAAs containing disulfide linkages in the main chain showed better transfection than the related counterparts lacking disulfide linkages. This effect can be addressed to the biodegradability of the polymer in the intracellular environment. In previous works, it has been shown that partially degraded poly(amidoamine)s dendrimers ("activated PAMAM") were able to transfect DNA more efficiently than non degraded dendrimers. [16] This is likely due to the higher structural flexibility in solution of partially degraded dendrimers. In a recent work, it was found that hyperbranched poly(esteramine)s can transfect DNA with improved efficiency if compared with polyethylenimine (PEI) and poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA). Moreover, these hyperbranched poly(esteramine)s showed low or no toxicity in vitro. [17]

Therefore, we supposed that an hyperbranched PAAs containing disulfide linkages in the main chain can have different advantages in DNA transfection compared with traditional linear PAAs. Firstly, in hyperbranched polymers the ratio between the number of terminal functions and the total number of monomeric units is higher than in linear polymers. Thus, by adding a suitable terminal group it is possible to improve the solubility in water of the polymer, and adjust the interaction between the polymer and the DNA. Secondly, the high density of secondary and tertiary amines can increase the buffering capacity over a wide pH range, promoting endosomal escape of polymer/DNA complexes by proton sponge effect. It was found also that the terminal groups of linear polymer can affect the transfection efficiency. [18] Thus, Hyperbranched PAAs could also be used as platform to investigate the effect of terminal groups.

Based on these premises, we supposed that the hyperbranching can affect the transfection efficiency of PAAs. To investigate this hypothesis, we synthesized a new series of biodegradable PAAs (Figure 5.2) and tested their biological properties as well as the DNA transfection efficiency of polymer-DNA polyplexes. I performed this study at the Polymer Chemistry and Biomaterials (PBM) group (University of Twente, The Netherlands), in collaboration with the group of professor J.F.J. Engbersen.

5.4 Results and Discussion

5.4.1 PAAs Synthesis and characterisation

In this study, 8 different biodegradable PAAs were synthesised and characterised (Figure 5.2). In particular, 4 hyperbranched PAAs were obtained by Michael polyaddition between N,N'-bisacryloylpiperazine (BP) and cystamine (CYST) or N,N'-bisacryloylcystamine (CBA) and ethylenediamine (EDA). Each polymer was terminated with two different primary amines, 4-aminobutanol (ABOL) and 2-aminoethanol (ETA). In order to investigate the effect of hyperbranching, 4 linear PAAs, but structurally related to the above hyperbranched PAAs, were synthesised by polyaddition of N,N'-dimethylethylenediamine (DMEDA) to CBA and of N,N'-dimethylcystamine (DMC) to BP. Also in this case each polymer was terminated with ABOL and ETA. All the synthesised polymers were characterised by Gel Permeation Chromatography (GPC) and ^{1}HNMR spectroscopy.

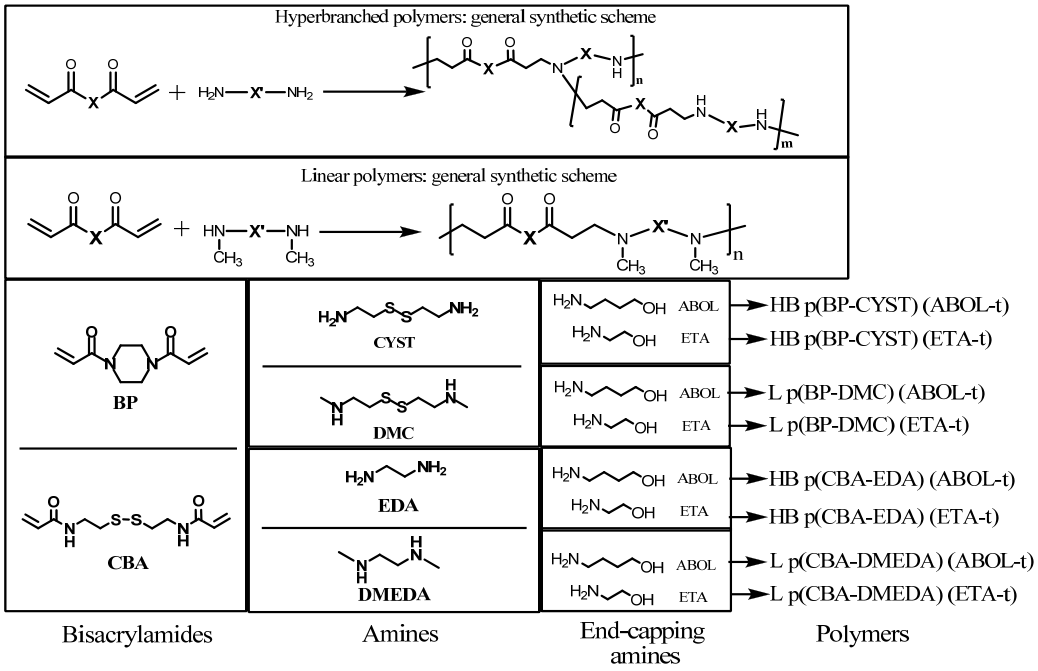


Figure 5.2. General synthetic schemes and monomers utilised to obtain linear and hyperbranched PAAs. Abbreviations: **HB**, hyperbranched; **L,** linear; **CBA,** N,N'-bisacryloylcystamine; **BP**, N,N'-bisacryloylpiperazine; **CYST**, cystamine; **DMC**, N,N'-dimethylcystamine; **EDA,** ethylenediamine; **DMEDA**, N,N'-dimethyl-ethylenediamine; **ETA**, ethanolamine; **ABOL**, aminobuthanol; **-t**, terminated.

Linear PAAs were synthesised with reaction conditions similar to those of traditional PAAs (see Chapter 6). In the case of L-p(CBA-DMEDA) the polymerisation reaction was carried out in a 4/1 methanol/water mixture at 40 °C, since CBA monomer is not soluble in water. As secondary diamines, DMEDA and DMC were chosen because of their structural similarity with the amines used for the hyperbranched polymers syntheses (EDA and cystamine). In order to obtain hyperbranched PAAs, the difunctional amines used in the synthesis of linear PAAs were substituted with primary diamines (EDA and cystamine), that behave as tetrafunctional monomers under the reaction conditions adopted. The structures of the related polymers are shown in Figure 5.3.

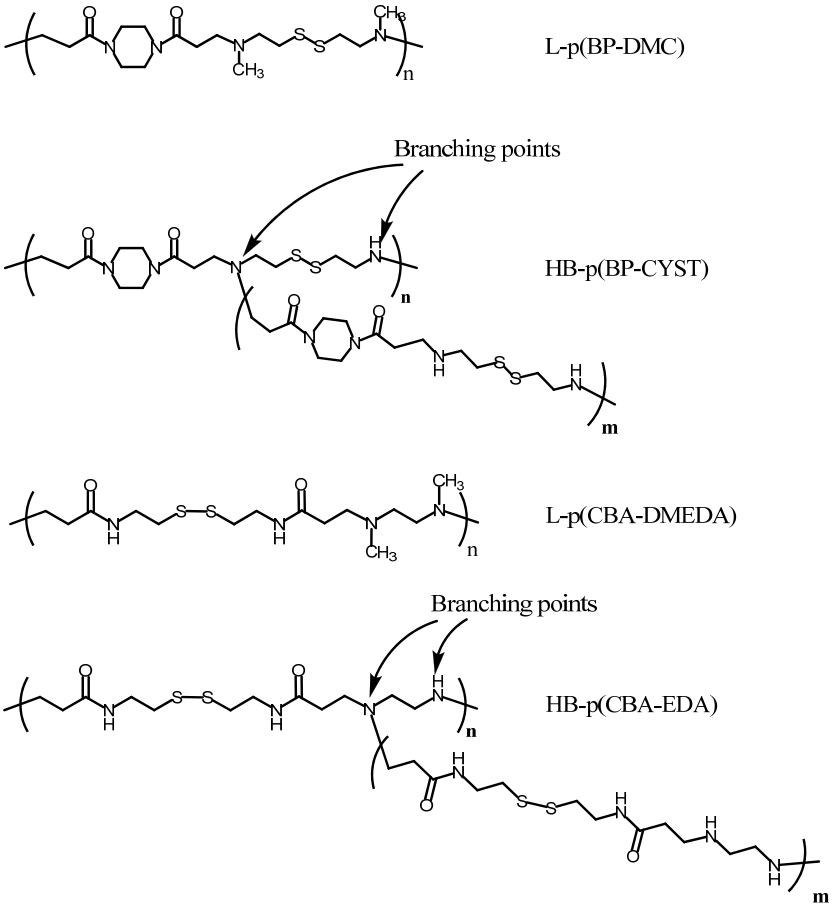


Figure 5.3. Structures of linear and hyperbranched PAAs..

Since the mechanism of polymerization of tetrafunctional monomers is more complicated than that of difunctional monomers, some consideration about this aspect are needed.

If the reactive functions of a tetrafunctional monomer have equal reactivity, nonlinear polymers and infinite networks as manifested by gelation are formed with predictable gel points [19,20]. In our case, however, the reactivity of tetrafunctional groups is unequal and many possible reaction routes exist. In particular, the addition rate of the primary amino group to the double bond is higher than that of the secondary amino group generated from the first addition. The lower reaction rate related to the third and the fourth additions is presumably biased by a combination of steric hindrance and decrease in nucleophylicity due to substituents carrying electron-withdrawing carbonyl groups in β position. Therefore, the polymerisation reaction can be divided in three phases; in the phase one the linear chain growth is prevailing, in the second phase the branching occurs, leading to crosslinking in the third phase. Moreover, second and third phases occur only with high conversion degrees.

In our recent work [21], a new approach was studied to determine the reactivity of different functions of a multifunctional monomers in order to obtain hyperbranched PAAs. In particular, stoichiometrically imbalanced reactant mixtures with excess of double bonds were used to adjust the degree of branching of the polymers. To determine the critical stoichiometric ratio "r_c" , under which the system is unable to gel and yields hyperbranched but still soluble polymers, the following equation was used [20]:

$$r_c = \frac{1}{1 + \rho(f - 2)} \qquad \text{Equation 5.1}$$

Where:

r_c = critical stoichiometric ratio (number of functions in defect / number of functions in excess)

ρ = the fraction of functions belonging to the monomer with functionality >2 with respect to the total amount of the functions of the same type initially present in the system.

f = number of functions of the monomer with functionality >2

Operatively, a series of reactions with progressively larger excess of double bonds were carried out for 6 days, and the reaction with the minimum double bonds excess inhibiting gelation of the polymer led to the r_c value for that couple of monomers. The r_c values found for the reactions involving the couples BAP/cystamine and CBA/EDA were 0.3083 and 0.3312, respectively.

All the synthesised PAAs showed good solubility in water and in buffer solutions. In the case of hyperbranched polymers, it was noticed the presence of insoluble particles at the end of the reaction. This particles were filtered off by ultrafiltration with a 100.000 cutoff membrane. The average molecular weight was measured by GPC with an online Refractive Index (RI) detector, PEG standards calibration, and a 8.5/1.5 sodium acetate buffer/methanol mixture as mobile phase. The average molecular weight of the obtained PAAs ranged from 7800 to 30800. It must be noted that the average molecular weights calculated for hyperbranched polymers could be underestimated, since they usually show a lower hydrodynamic volume compared with the linear counterparts.

^{1}HNMR spectra confirmed the expected structures of linear and hyperbranched polymers. Moreover, there was no evidence of residual double bonds, that usually result in cytotoxicity of the polymer. To determine the degree of branching (DB) following equation was used [15]:

$$DB = \frac{D+T}{D+T+L} \qquad \text{Equation 5.2}$$

Where:

D = number of branching units
T = number of terminal units
L = number of linear units.

A semiquantitative determination of the amount of terminal, linear and branched units was performed by ^{1}HNMR. For all the hyperbranched polymers it was calculated a DB value of about 0.50. As example the ^{1}HNMR spectrum of HB-p(CBA-EDA) (ABOL-t) is showed in Figure 5.4.

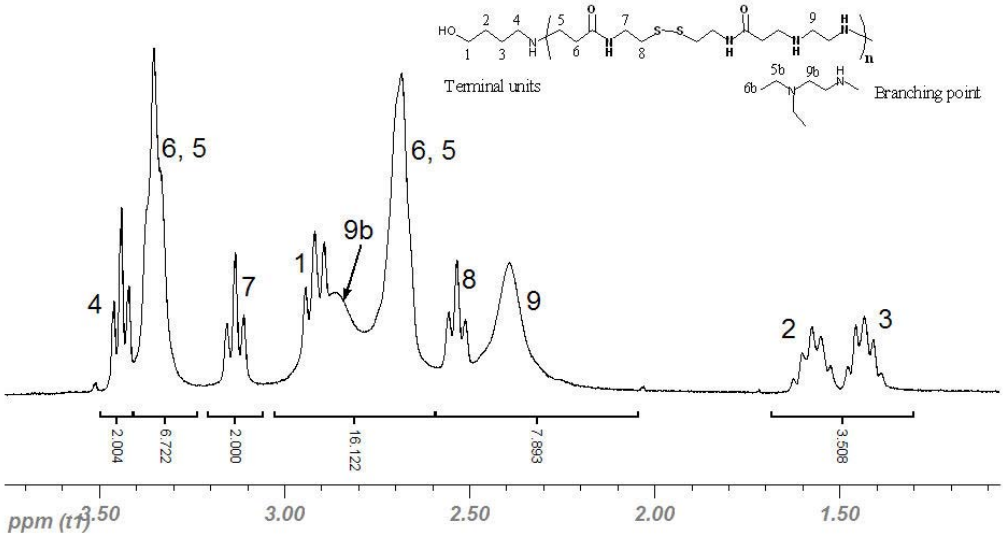


Figure 5.4. [1]HNMR spectrum of HB-p(CBA-EDA) (ABOL-t)

It has been shown that high buffer capacity can promote endosomal escape of polymer/DNA polyplexes by "proton spong effect". Therefore, the buffer capacity of the polymers, defined as the percentage of amine groups becoming protonated in the pH range from pH 7.4 to 5.1, is a relevant parameter in the overall transfection process. The buffer capacity of the synthesised PAAs, together with PEI as a reference, is showed in Figure 5.5. The buffer capacity of the tested PAAs was generally higher compared to that of the reference polymer (commercial branched 25k polyethyleneimine (PEI)) and was slightly higher in the case of ETA terminated polymers compared with the ABOL terminated. Moreover, the BP seems to increase the buffer capacity compared to CBA, and there is no evidence of any influence of the branching.

The degradability of the polymers in a reductive environment was also verified by [1]HNMR specroscopy. In particular, the polymer solutions in D_2O (0.1 M of disulfide linkages) were incubated with 2.4 equivalents of mercaptoethanol respect to the disulfide linkages concentration in the solution. After one hour, both hyperbranched and linear PAAs were completely degraded. Thus, it can be deduced that the hyperbranching does not affect the reductive degradation rate of PAAs.

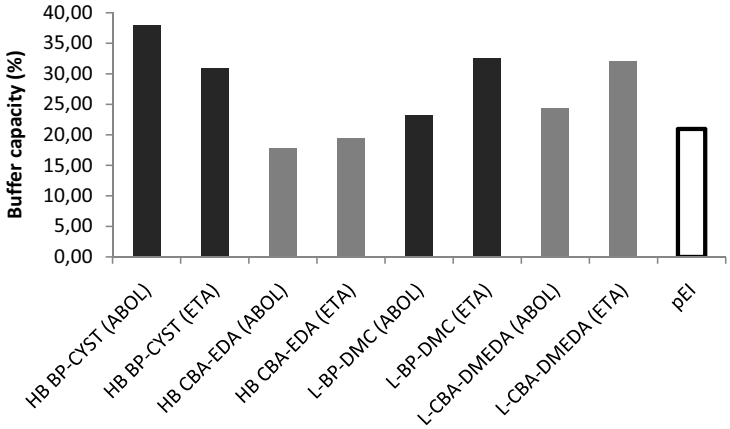


Figure 5.5. Buffer capacity of the synthesised PAAs. Black bars, BP-containing PAAs; grey bars, CBA containing PAAs.

The GPC data and the buffer capacity values related to the obtained PAAs are summarised in Table 5.1.

	polymer	$\overline{M}_p$	$\overline{M}_n$	$\overline{M}_w$	PD	Buffer capacity (%)
Hyperbranched	HB-p(BAP-CYST) (ABOL-t)	9400	10800	16300	1.51	37.96
	HB-p(BAP-CYST) (ETA-t)	12900	16900	22700	1.34	30.96
	HB-p(CBA-EDA) (ABOL-t)	6100	7800	14100	1.80	17.78
	HB-p(CBA-EDA) (ETA-t)	8900	11300	16400	1.45	19.39
Linear	L-p(BAP-DMC) (ABOL-t)	9300	9600	14800	1.54	23.12
	L-p(BAP-DMC) (ETA-t)	30700	30800	43300	1.41	32.44
	L-p(CBA-DMEDA) (ABOL-t)	12900	12500	18200	1.46	24.41
	L-p(CBA-DMEDA) (ETA-t)	10700	10500	14800	1.41	32.00

Table 5.1. GPC data and buffer capacity of the synthesised PAAs.

5.4.2 DNA complexation and release

A fundamental feature that a polymer needs in gene delivery is the ability of complex DNA into nanosized polyplexes. Generally, to be efficiently endocytosed by cells, polyplexes must have an overall positive surface charge and a average size smaller than about 200 nm. Therefore, zeta-potential and particle size of polymer-DNA polyplexes were measured with polymer/DNA ratios of 48/1, 12/1, 3/1 and 1/1. The zeta-potential and particle size values of the PAAs, measured by Dynamic Light Scattering (DLS), are showed in Figures 5.6 and 5.7.

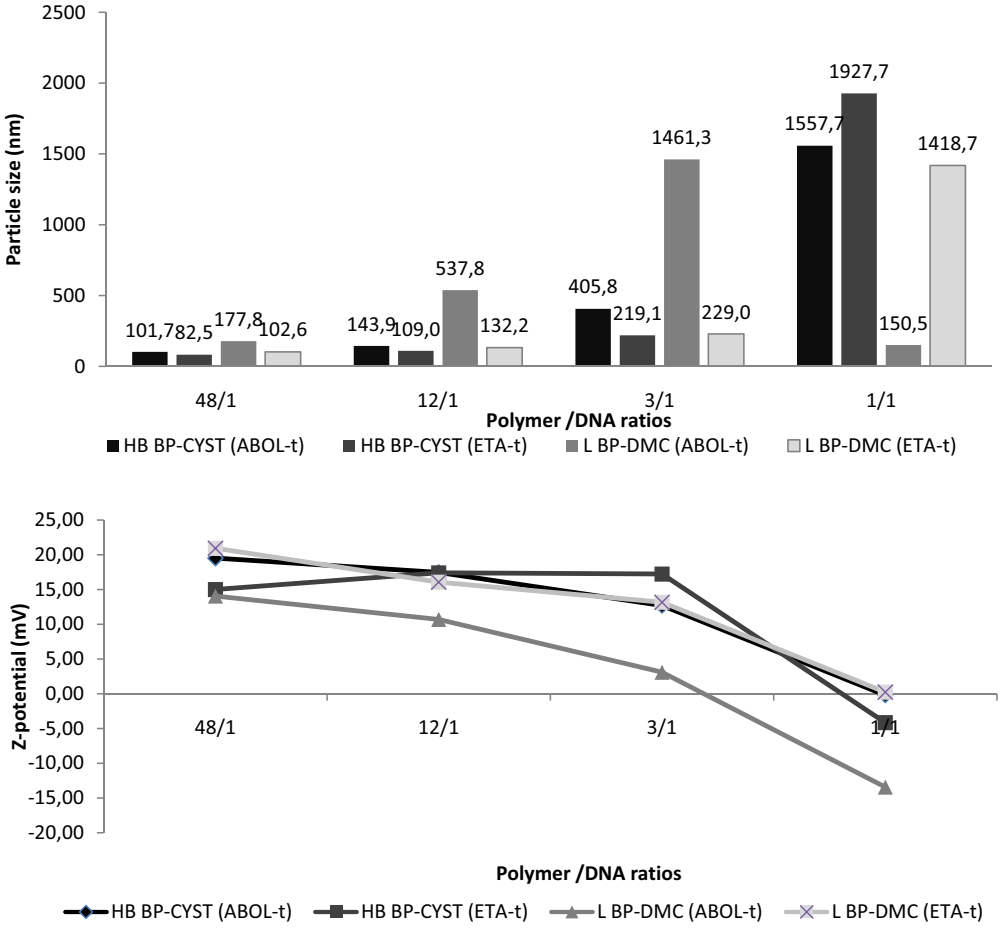


Figure 5.6. Particle size (above) and zeta-potential (below) values of the BP containing PAAs.

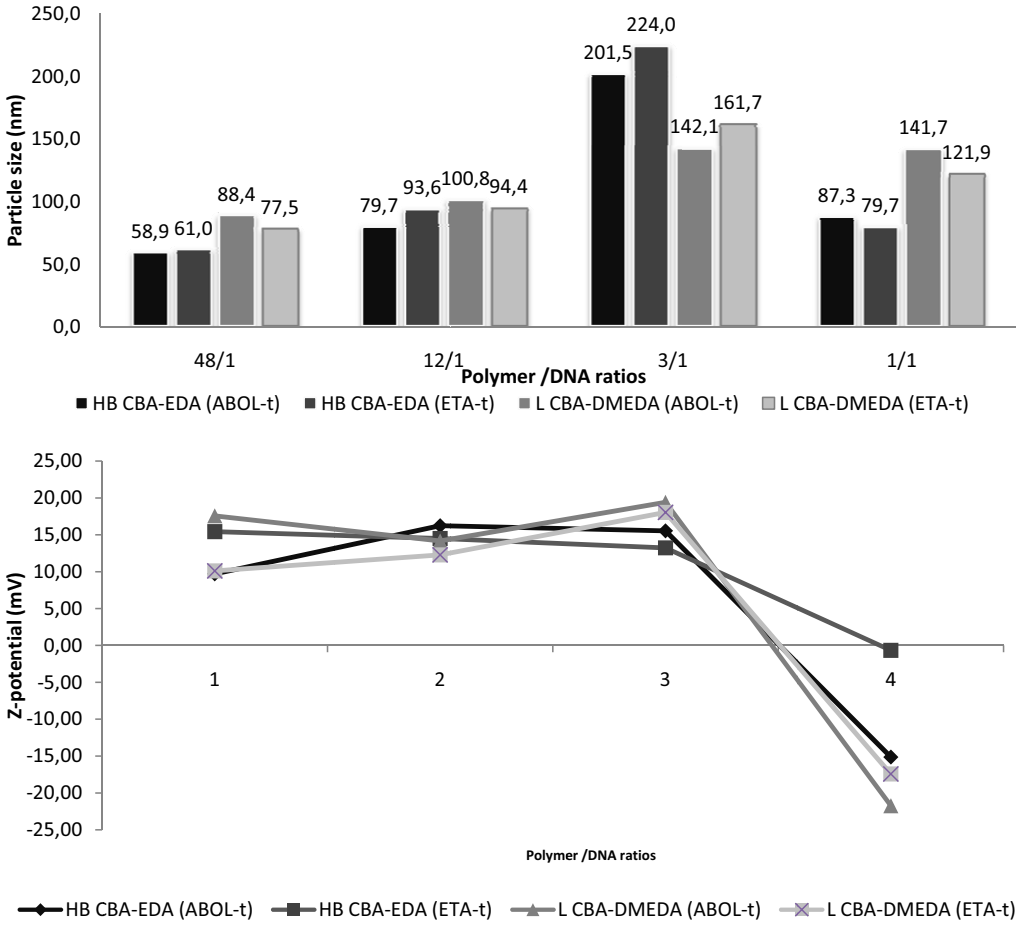


Figure 5.7. Particle size (above) and zeta-potential (below) values of the CBA containing PAAs.

In general, hyperbranched polymers produced smaller particles than linear polymers, and CBA containing polymers showed smaller particle size compared to BP containing polymers. All the polymers showed optimal values of zeta potential (from +15 mV to +35 mV) at any polymer/DNA ratios, except with 1/1 ratio (from 0 mV to -25 mV). Since negative values of zeta potential and particle size higher than 200 nm are normally related to poor transfection efficiency, we performed the following DNA transfection tests on polyplexes with polymer/DNA ratio from 48/1 to 3/1.

5.4.3 In vitro transfection efficiency and cytotoxicity

The transfection of polyplexes based on the synthesised PAAs was studied in vitro by using COS-7 cells and the plasmid pCMV-LacZ as reporter gene. Transfection efficiencies were measured for polyplexes with polymer/DNA ratio of 48/1, 12/1, 6/1 and 3/1 by ONPG assay (Figure 5.8, 5.9). As reference, polyplexes based on commercial 25k branched PEI with a polymer/DNA ratio of 6/1 were used. Transfection tests were performed in presence and without Fetal Bovine Serum (FBS).

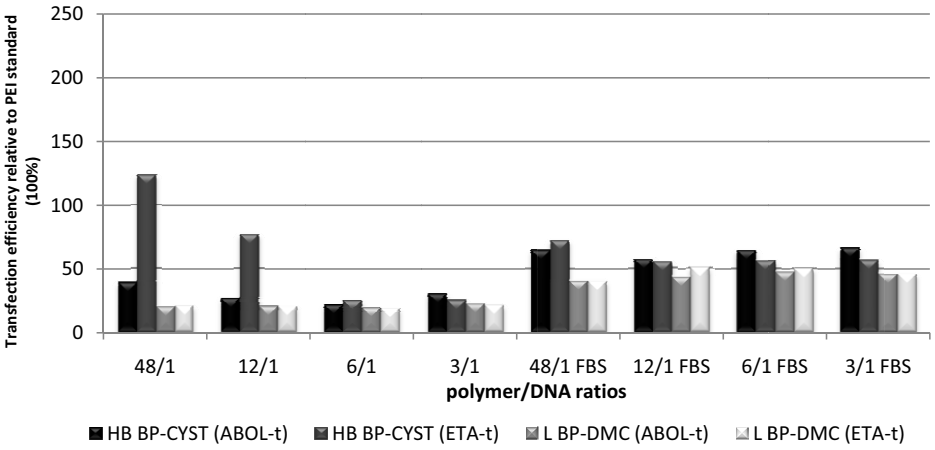


Figure 5.8: DNA transfection efficiency of polyplexes of BP containing polymers at polymer/DNA ratio 48/1, 12/1, 6/1, 3/1.

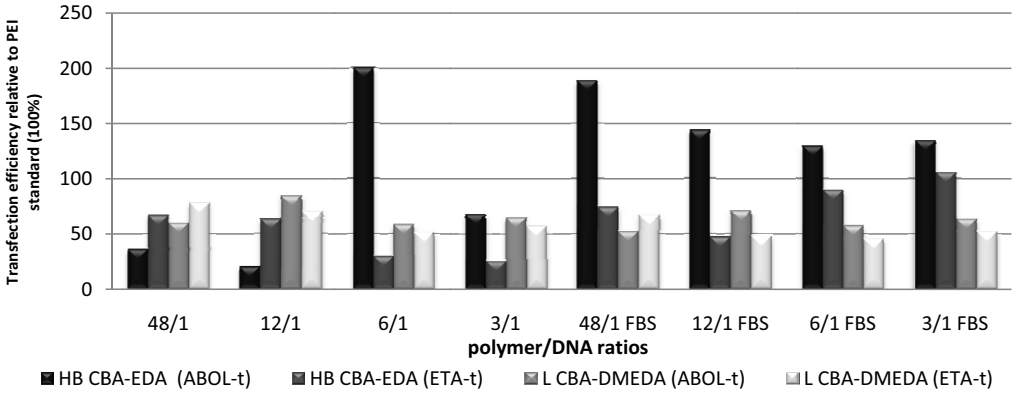


Figure 5.9. DNA transfection efficiency of polyplexes of CBA containing polymers at polymer/DNA ratio 48/1, 12/1, 6/1, 3/1.

As expected from a previous work, [22] CBA containing PAAs were more effective in transfection than BP containing PAAs. This could be attributed to the lower flexibility of the macromolecular chain, due to the piperazine ring. HB-(BP-CYST) (ETA-t) showed a transfection efficiency comparable with PEI only at polymer/DNA ratios of 48/1 and 12/1(only without FBS).

The best transfection efficiency was obtained with the polymer HB-p(CBA-EDA) (ABOL-t) at a polymer/DNA ratio of 6/1, without FBS. Unexpectedly, the transfection efficiency of hyperbranched (HB-) polymers was generally enhanced in presence of FBS than without. This result is encouraging for *in vivo* testing of these polymers.

In general, the effect of hyperbranching on transfection is not constant, although in most of the cases hyperbranched (HB-) polymers showed better transfection than the related linear (L-) polymers. Moreover, the effect of the terminal group (ABOL and ETA) was negligible and only in the case of HB-p(CBA-EDA) (ABOL-t) the transfection efficiency was clearly enhanced by ABOL if compared with ETA.

The cytotoxicity is another fundamental parameters affecting the transfection process.

The polyplexes cytotoxicity for all the polymers, at the same polymer/DNA ratios used in transfection tests, was also measured by XTT assay (Figures 5.10 and 5.11).

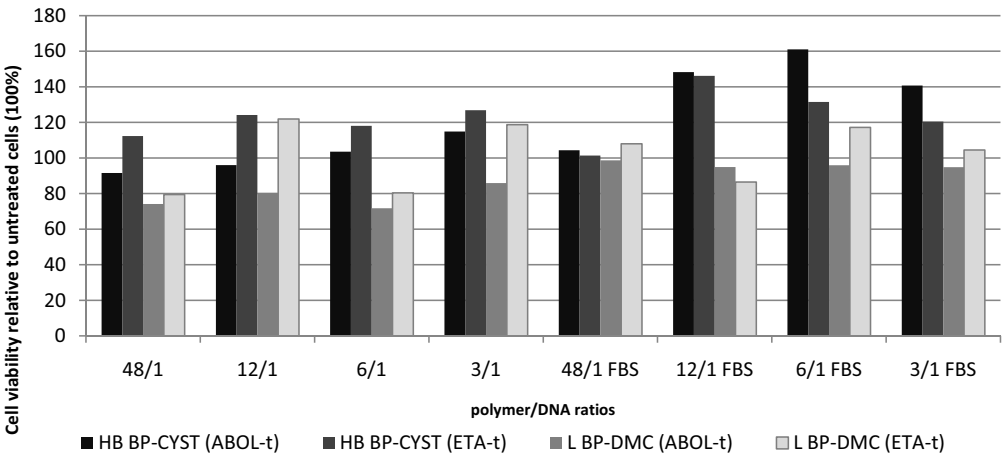


Figure 5.10. Cell viabilities related to BP containing polymers.

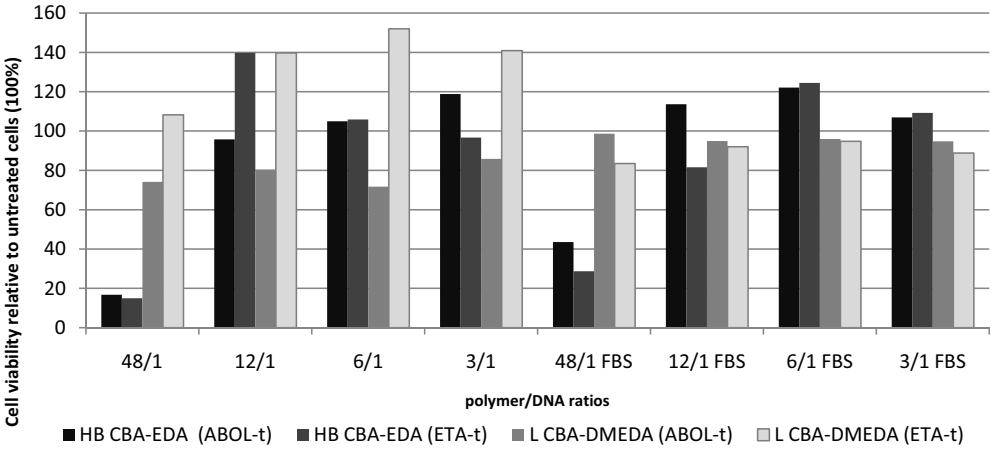


Figure 5.11. Cell viabilities related to CBA containing polymers.

All the PAAs tested displayed low or no cytotoxicity. Moreover, it was unexpectedly found that hyperbranched (HB-) PAAs show generally less cytotoxicity than the related linear (L-) PAAs. The presence of FBS caused for most of the PAAs a slight decrease in toxicity. Remarkable toxicity was found only for HB-(CBA-EDA) at a polymer DNA ratio of 48/1, irrespective of the terminal group. A constant effect on cell viability of the terminal groups (ABOL and ETA) is not evident. Moreover, the difference in toxicity between PAAs ABOL or ETA terminated in the linear PAAs is not enhanced in the related hyperbranched PAAs.

Summarising the results discussed above, the studied PAAs showed low or no cytotoxicity at all the tested polymer/DNA ratios. In transfection tests, the best performing polymer was HB-p(CBA-EDA) (ABOL-t) at a polymer/DNA ratio of 6/1 with transfection efficiency higher or comparable with PEI. In general, the hyperbranched polymer showed a higher transfection efficiency than the linear counterparts, although the enhancement seems to be strongly dependent on the polymer structure and the polymer/DNA ratio.

5.5 Conclusions

Eight novel kind of hyperbranched and linear PAAs containing disulfide linkages in the main chain, and different terminal functions were synthesized and characterized. These PAAs are able to undergo reductive degradation in a reductive environment (i.e. inside cells). The polymers' transfection efficiency was tested and compared. CBA containing polymers showed a DNA transfection efficiency comparable with PEI and performed mostly better than polymers containing BP. Better results were obtained with hyperbranched PAAs in comparison with the linear PAAs and, in particular, the hyperbranched CBA containing PAA ABOL terminated (HB CBA-EDA (ABOL-t)) showed the highest transfection efficiency. Anyway, clear correlations between the hyperbranching, and the transfection efficiency are not readily apparent. Despite of their cationic nature, these hyperbranched PAAs displayed low or no toxicity.

At the moment further tests are in progress with these hyperbranched and linear polymers terminated with ethylenediamine (EDA). In the future, it could be interesting to investigate the effect in transfection of the hyperbranched polymers varying the amount of the disulfide linkages, or varying the terminal functions.

References

[1] R. Langer et al. *Accounts of Chemical Research* ,Vol. 41, No. 6, June **2008**, 749-759.

[2] O. Boussif, F. Lezoualch, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, Proc. Natl. Acad. Sci. U.S.A., (92), 7297-7301, **1995**.

[3] S. Grosse, Y. Aron, G. Thevenot, *J. Gene. Med.*, (10), 1275-1286, **2005**.

[4] P. Midoux, M. Monsigny, *Bioconj. Chem.*, (19), 406-411, **1999**.

[5] Y.H. Kim, J.H. Park, M. Lee, Y.H. Kim, T.G. Park, S.W. Kim, *J. Control. Release,* (103), 209–219, **2005**.

[6] K. Kunath, A. von Harpe, D. Fischer, H. Petersen, U. Bickel, K. Voigt, T. Kissel, J. *Control. Release,* (89), 113–125, **2003**.

[7] R. Kircheis, S. Schuller, S. Brunner,M. Ogris, K.H. Heider,W. Zauner, E. Wagner, *J. Gene Med.,* (1), 111–120, **1999**.

[8] H. Petersen, T. Merdan, K. Kunath, D. Fischer, T. Kissel, *Bioconjug. Chem*., (13), 812–821, **2002**.

[9] H. Petersen, M.F. Fechner, A.L. Martin, K. Kunath, S. Stolnik, C.J. Roberts, D. Fischer, M.C. Davies, T. Kissel, *Bioconjug. Chem*., (13), 845–854, **2002**.

[10] C.M. Ward, M.L. Read, L.W. Seymour, , *Blood,* (97), 2221–2229, **2001**.

[11] M.D. Brown, A. Schatzlein, A. Brownlie, V. Jack,W.Wang, L. Tetley, A. I. Gray, I.F. Uchegbu, *Bioconjug. Chem*., (11), 880–891, **2000**.

[12] S. C. W. Richardson, N. G. Pattrick, Y. K. S. Man, P. Ferruti, and R. Duncan, *Biomacromolecules*, (2), *1023-1028,* **2001**.

[13] S. C. W. Richardson,; Y. K. S. Man,; P. Ferruti,; R. Duncan, *Proc. 26th Int. Symp. Control. Relat. Bioact. Mater.*, (26), 434-435, **1999**.

[14] C. Lin, Z. Zhong, M. C. Lok, X. Jiang, W. E. Hennink, J. Feijen, J. F. J. Engbersen, *Bioconjugate Chem*., (18), 13̃8-145 , **2007**.

[15] S. C. W. Richardson, N. G. Pattrick, Y. K. Stella Man, P. Ferruti, R. Duncan, *Biomacromolecules*, (2), 1023-1028, **2001**.

[16] J. Dennig, *Top. Curr. Chem.,* (228), 227–236, **2003**.

[17] Z. Zhong, *Journal of Controlled Release*, (109), 317–329, **2005**.

[18] G. T. Zugates, N. C. Tedford, A. Zumbuehl,S. Jhunjhunwala, C. S. Kang, L. G. Griffith, D. A. Lauffenburger, R. Langer, D. G. Anderson, *Bioconjugate Chem.*, (18), 1887–1896, **2007**.

[19] Odian G. Principles of polymerization. New York: Wiley, **1991** [chapter 2].

[20] P. J. Flory, Principles of Polymer Chemistry, Published by Cornell University Press, **1953** (688 pp.).

[21] Ferruti, P.; Ranucci, E.; Trotta, F.; Cavalli, R., Polimeri iperramificati a base di ciclodestrine e poli(amidoammine) per il rilascio controllato di farmaci insolubili, Italian Patent MI2007A1173, **2007**.

[22] E. Emilitri, P. Ferruti, R. Annunziata, E. Ranucci, *Macromolecules*, (40), 4785-4793, **2007**.

Chapter 6: Experimental Part

Materials

All solvents were of analytical grade purchased from ALDRICH, FLUKA or LAB-SCAN and used as received. Lithium chloride monohydrate, NaOH pellets, 37% hydrochloric acid (HCl), 1,2-diaminoethane (EDA), sodium bicarbonate, potassium bicarbonate, triphenychloromethane, N,N'-ethylenediamine-diacetic acid (EDDA), 2-aminoethyl-phosphonic acid (AP), N,N'-dimethylethylenediamine (DMEDA), N,N'-bisacryloylcystamine (CBA), cystamine (CYST), 4-aminobutanol (ABOL), 2-ethanolamine (ETA) were purchased from FLUKA at the highest purity and used without further purifications. Fmoc-protected aminoacids (L-cysteine, L-aspartic acid, glycine, L-arginine, L-hystidine, L-leucine, L-isoleucine, L-alanine, L-serine), cysteine loaded Wang resin, N-methylpirrolydone (NMP), HBTU, diisopropylethylamine (DIPEA), tri-fluoroacetic acid, ethylic ether, piperidine, dichloromethane, dimethylformamide, thioanisol, 1,2-ethanedithiol, acetonitrile (HPLC grade) were purchased at peptide grade from Biosolve and used without further purifications.

2-methylpiperazine (MP) (from FLUKA) was re-crystallised from n-heptane and its purity determined titrimetrically just before use.

Methods

Polymers average molecular weight: The molecular weight and polydispersity ($\overline{M_n}/\overline{M_w}$) of the synthesized poly(amido amine)s were determined by GPC with three different systems:

- System 1: WATERS 515 HPLC Pump instrument, with Toso-Haas 486 columns, pullulan standards (Aldrich) calibration, using Tris buffer pH 8.00 ±0.05 as mobile phase. Conditions: sample concentration 10mg/ml; flow rate 1ml/min.; Detectors: Waters 486 Tunable Absorbance Detector (UV), wavelength 230 nm and Waters 2410 Refractive Index Detector (RI); room temperature (~24°C). Data collected with Cirrus GPC Software (MS Windows).

- System 2: Knauer Pump 1000 with a Knauer Autosampler 3800, with Toso-Haas 486 columns, using Tris buffer pH 8.00 ± 0.05 as mobile phase. Conditions: the same used in System 1; Detectors: Right (90°) Angle Light Scattering, Low (7°) Angle Light Scattering detector and Waters 2410 Refractive Index Detector (RI); room temperature (~24°C). Data collected with Omnisec software (MS Windows).
- System 3: PL-GPC 120 system (Polymer Laboratories) and two thermostated (30ºC) PL aquagel-OH 30 columns (8 μm, 300×7.5 mm, Polymer Labs, with a low-molar-mass separation range (200~100,000) and PEG standards (Polymer Labs) calibration. 0.3 M sodium acetate aqueous solution (pH 4.5) was used as eluent at a flow rate of 0.5 mL/min. Data were collected using a Refractive Index detector (RI) with Cirrus GPC Software (MS Windows).

NMR: ^{1}HNMR and ^{13}CNMR spectra were recorded on Bruker spectrometers operating at and 400 MHz (Bruker 400 Ultrashield), respectively. Polymer samples were prepared by dissolving about 10 mg of polymer in salt-free form, in 1 mL of D_2O.

Buffer capacity: the buffer capacity of the PAA polymers was determined by acid-base titration. An amount equal to 5 mmol of amine groups of the PAA polymer was dissolved in 10 mL of 150 mM NaCl aqueous solution. The pH of the polymer solution was set at 2.0 and the solution was titrated with 0.1 M NaOH solution using an automatic titrator (Metrohm 702 SM Titrino). For comparison, branched PEI (Mw=25 kDa) dissolved in 150 mM aqueous solution adjusted to pH 2.0, was also titrated using the same method. The buffering capacity defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.4, was calculated from equation 1:

$$BufferCapacity(\%) = \frac{\Delta V_{NaOH} \times 0.1M}{NMol} \qquad \textbf{Equation 6.1}$$

Wherein ΔV NaOH, is the volume of NaOH solution (0.1 M) required to bring the pH value of the polymer solution from 5.1 to 7.4, and N mol (5 mmol), is the total moles of protonable amine groups in PAA polymer.

Particle size and zeta-potential measurements: the surface charge and the size of polyplexes were measured at 25 °C with a Zetasizer 4000 (Malvern Instruments Ltd., Malvern, UK). PAA/plasmid DNA polyplexes at a polymer/DNA mass ratio of 12/1 were prepared by adding a HEPES buffer solution (20 mM, pH 7.4, 5 wt% glucose) of poly(amidoamine)s (800 µL, 225 µg/mL) to a HEPES buffer solution of plasmid DNA (200 µL, 75 µg/mL), followed by vortexing for 5 s and incubating at room temperature for 30 min.

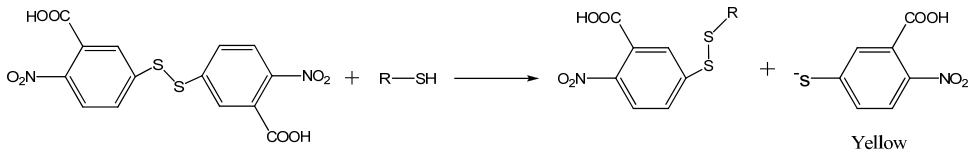
In vitro transfection and cell viability assays: Transfection experiments were performed with COS-7 cells (SV-40 transformed African Green monkey kidney cells) by using the plasmid pCMV-LacZ. [1,2] Two parallel transfection series, one for the determination of reporter gene expression (β-galactosidase) and the other for the evaluation of cell viability by XTT assay, were carried out in separate 96-well plates (ca. 1.0×10^4 cells per well). Different polymer/plasmid DNA weight ratios, ranging from 12/1 to 48/1 (w/w), were used to prepare the polyplexes. In brief, polyplexes were prepared by adding 200 µL of a HEPES buffer solution (20 mM, 130 mM NaCl, pH 7.4) of poly(amidoamine) with varying concentrations (from 37.5 to 150 µg/mL) to 50 µL of a HEPES buffer solution of plasmid DNA (50 µg/mL), followed by gentle shaking and incubating at room temperature for 30 min. All transfection and toxicity assays were carried out in triplo. In a standard transfection experiment, the cells were incubated with the desired amount of polyplexes (100 µL dispersion with 1 µg plasmid DNA per well) for 1 h at 37 °C in a humidified 5% CO_2-containing atmosphere. Next, the polyplexes were removed. 100-µL of fresh culture medium was added and the cells were cultured for 2 days. The transfection efficiency was determined by measuring the activity of β-galactosidase using the ONPG assay. [1] A poly(ethyleneimine)(PEI)/DNA formulation prepared at a polymer/DNA ratio of 3/1 (w/w) was used as a reference. [1] The number of viable cells was measured using an XTT assay [3]. The XTT value for untreated cells (i.e. cells not exposed to the transfection medium) is taken as 100% cell viability.

Reductive degradation studies of PAAs:

The selected PAA (700 mg, 0.4 mmol of repeating unit) was dissolved in a D_2O (4 ml). The homogeneous solution obtained was maintained at 25°C under nitrogen atmosphere while stirring, and then 2-mercaptoethanol (70 mg; 0.88 mmol) added. An aliquot of solution (1 ml) was taken after 1 h and analysed by ^{1}HNMR.

Determination of free –SH groups with Ellman's reagent:

The unknown concentration of thiol groups in peptide samples were determined by comparison with an amino acid standard, L-cysteine. First, various concentrations of L-cysteine (10^{-2} – 10^{-6} mol/L) were prepared in TRIS buffer (pH 8). A solution of Ellman's reagent was freshly prepared in TRIS buffer (4.2 mg in 1 ml). 250 µl of L-cysteine solution Were diluted with 2.5 ml of TRIS and 50 µl of Ellman's reagent solution were added. The sample is incubated at room temperature for 15 min. Subsequently, absorbance is read at 412 nm using TRIS as blank. A concentrated solution of L-cysteine alone in TRIS buffer did not show any absorbance at 412 nm.



Scheme 6.1. Ellman's reagent addition reaction to thiols.

Fractionation procedure with a solvent non solvent method: a solution of MeOH and H_2O 9:1 was saturated with LiCl. The polymer batch was dissolved in this solution in the highest concentration affordable. A solution of acetone saturated with LiCl was added drop wise into the polymer batch until a precipitate is formed. The solid was recovered through centrifugation and the liquid was kept in a flask. The white solid was washed 3 times with a solution of the same composition of the solution where it precipitated and dried under vacuum. The liquid part kept in a flask was used to start again the procedure adding drop wise acetone and waiting for a precipitation.

Nanoparticles coating tests: diethyleneglycol (DEG) solution of nanoparticles in (0.2 g in 40 ml of solvent) was added dropwise to an aqueous solution of the polymer (1.2 g in 200 ml) under stirring. The resulting solution, was stirred for about 10 minutes and then was ultrafiltered with a 30000 cutoff dialysis tube to eliminate low molecular weight species, uncoated nanoparticles and polymer in excess. Ultrafiltration with Amicon (Millipore) system gave rapid precipitation of nanoparticles due to the magnetic field generated by the stirrer.

Synthesis of RGDC peptide:

The peptides were synthesized by the solid-phase method [4] with trityl polymer-bound resin cysteine-functionalized at a substitution level of 0.9 mmol/g, 0.3 g of resin was used for each synthesis. All amino acids were protected with (9-fluorenylmethyl)carbamate (Fmoc). Aminoacids were loaded in this order: first, aspartic acid, second, glycine and third, arginine. Each synthetic cycle consisted of (i) a 20-min Fmoc deprotection of the aminoacid bound to the resin with piperidine, (ii) washing of the resin with N-methylpyrrolidone (NMP), (iii) double coupling with each aminoacid activated with a 0.5 M solution of both O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluoro phosphate (HBTU) and N-Hydroxybenzotriazole (HOBt) in DMF *(3* eq. of the Fmoc-amino acid respect to 1 eq. of resin) for 1 h each in NMP. All couplings were monitored by conductimetric measuring of Fmoc concentration on waste solvent. Protected peptide-resin was first cleaned with CH_2Cl_2, then dried *in vacuo* up to a constant weight (0.3-0.4 g); afterwards the peptide cleavage from both the resin and all the protective groups was performed with tri-fluoroacetic acid (TFA) (20 mL) and H_2O, 2,2-ethandithiol and thioanisole (1 ml of every) for 2 hours. The unblocked resin was filtered, and the peptide was precipitated with diethyl ether and filtered off. Later the peptide was purified by gradient HPLC (solution A: H_2O with 0.1 % v/v of TFA; solution B: CH_3CN/Sol.A 1/1) and finally was obtained with yield of 85% w/w. Purity (Ellman's assay), 98.4 %. The product was stored at -18°C under inert atmosphere. ^{1}H NMR (D_2O) δ=2.85 (dd, HS-**CH_2**-CH); 4.76-4.78 (m, HS-CH_2-**CH**(COOH)-NH); 2.91-2.97 (m, HN-CO-**CH_2**-CH); 4.56 (t, CH_2-**CH**(COOH)-NH); 4.06 (dd, HN-CO-**CH_2**-NH); 3.95 (t, CO-**CH**(NH_2)-CH_2); 1.8-1.71 (m, CH(NH_2)-**CH_2**-CH_2); 1.91-1.93 (m, CH_2-**CH_2**-CH_2-NH); 3.22 (t, CH_2-**CH_2**-NH-C(NH_2)=NH).

^{13}C NMR (D_2O): δ=35 (HS-**CH_2**-CH); 50 (HS-CH_2-**CH**(COOH)-NH); 172 (HS-CH_2-CH(**CO**OH)-NH); 172 (HN-**CO**-CH_2-CH); 25 (HN-CO-**CH_2**-CH); 55 (CH_2-**CH**(COOH)-NH); 174 (CH_2-CH(**CO**OH)-NH); 171 (HN-**CO**-CH_2-NH); 52 (HN-CO-**CH_2**-NH); 170 (NH-**CO**-CH(NH_2)-CH_2); 42 (NH-CO-**CH**(NH_2)-CH_2); 23 (CH(NH_2)-**CH_2**-CH_2); 28 (CH_2-**CH_2**-CH_2-NH); 40 (CH_2-**CH_2**-NH-C(NH_2)=NH).

MS (70 eV): *m/z* = 450 (M+) (calculated, 449.5).

Synthesis of CRGDC peptide:

The synthesis was carried out by using the same procedure of RGDC synthesis, but with this sequence of aminoacids: aspartic acid, glycine, arginine, and cysteine. Yield 82 % w/w. Purity (Ellman's assay), 97.6 %. The ^{1}H NMR spectra of CRGDC is qualitatively the same of RGDC, but in that of CRGDC all cysteine peaks (^{1}H: 2.85, 4.76) have double intensity. The ^{13}C spectra are also similar.

MS (70 eV): *m/z* = 552.3 (M+) (calculated, 552.3).

Synthesis of CISLHAC peptide:

The synthesis was carried out by using the same procedure of RGDC synthesis, but with this sequence of aminoacids: alanine, hystidine, leucine, serine, isoleucine, and cysteine. Yield 71 % w/w. Purity (Ellman's assay), 98.2 %. ^{1}H NMR (D_2O) δ=0.84 (b, **$(CH_3)_2$**-CH, Ile, Leu); 1.34 (d, **CH_3**-CH, ala); 2.92 (m, CH-**CH_2**-SH, cys); 3.75 (d, CH-**CH_2**-OH, ser); 4.30 (m, CH-**CH_2**-CH, hys).

^{13}C NMR (D_2O): δ=39.0 (CH-**CH_2**-CH, hys); 16.3 (**$(CH_3)_2$**-CH, Ile); 10.4 (**CH_3**-CH, ala); 36.2 (CH-**CH_2**-SH, cys); 22.0 (CH-**CH_2**-OH, ser); 25.7 (**$(CH_3)_2$**-CH, Leu);

MS (70 eV): *m/z* = 746 (M+) (calculated, 745.91).

Synthesis of ISLHAC peptide:

The synthesis is carried out by using the same procedure of RGDC synthesis, but with this sequence of aminoacids: alanine, hystidine, leucine, serine, and isoleucine. Yield 75 % w/w. Purity (Ellman's assay), 96.5 % . The ^{1}H NMR spectra of CISLHAC is qualitatively the same of ISLHAC, but in that of CISLHAC all cysteine peaks (^{1}H: 2.85, 4.76) have double intensity. The ^{13}C spectra are also similar.

MS (70 eV): *m/z* = 642 (M+) (calculated, 642.77).

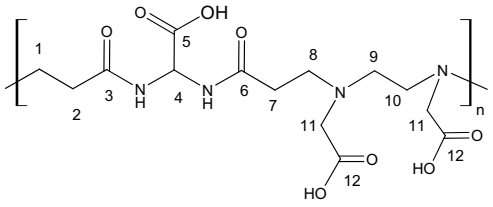
Synthesis of the monomers:

2,2-Bisacrylamido acetic acid (BAC) and 1,4-bisacryloylpiperazine (BP) were synthesised as previously described,(BP, [5] BAC, [6]) and their purity determined titrimetrically (BAC) or by NMR spectroscopy (BP) just before use. N,N'-dimethylcystamine (DMC) was synthesised as described in literature [7]. N-triphenylmethyl-monosubstituted 1,2-diaminoethane was synthesized as previously described [8] .

Syntheses of PAAs:

ISA 23 and the related copolymers were synthesised as previously described: ISA 23 and ISA23-Tyr, [9] ISA23-β-CD [10] and ISA23-NH2. [8]

BAC-EDDA: In a 10 ml round bottom flask, bidistilled water (1.6 ml) was added. Under stirring, $LiOH*H_2O$ (618mg; 14.75 mmol) and EDDA (865 mg; 4.91 mmol) were added in this order. After the reagents were completely dissolved, BAC (purity, 97.4 %; 1.0 g; 4.91 mmol) was added to the solution. Then the reaction was gently stirred for 48 hours under inert atmosphere (N_2) and in the dark. After this time the mixture was diluted to 20 ml with water, acidified to pH 4.5 with 37% HCl and ultrafiltered through 3000 cut-off membrane to eliminate the excess of low molecular weight species (unreacted monomers, LiCl). Finally the product was freeze-dried and stored at -20°C. $\overline{M_n}$: 20000; PD: 1.66 (by GPC System 1). Yield: 87 % w/w. ^{1}HNMR (D_2O): δ (ppm) = 2.75 (H9, H10) (4H, m,); 5.53 (H4) (1H, s,); 3,50 -3,70 (H7, H8, H11)(12H, b).



Scheme 6.2. Structure of BAC-EDDA

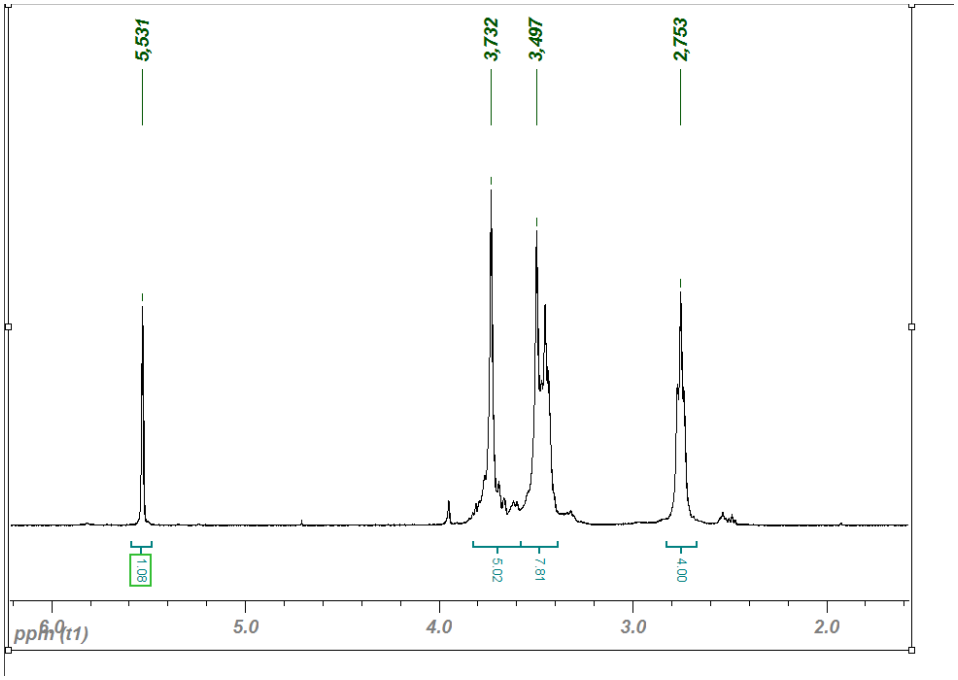
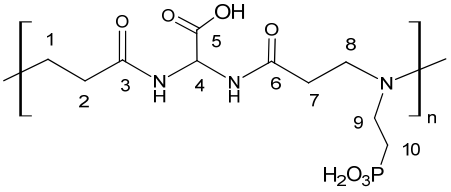


Figure 6.1. [1]HNMR spectrum of BAC-EDDA in D_2O at 25 °C.

BAC-AP: In a 10 ml round bottom flask, 5 M aqueous NaOH solution (2.980 g) and then AP (621.1 mg; 4.96 mmol) were added under stirring. After the reagent was completely dissolved, BAC (purity, 97.4 %; 1.0 g; 4.91 mmol) was added to the solution. Then the reaction was gently stirred for 72 hours under inert atmosphere (N_2) and in the dark. After this time the mixture was diluted to 20 ml with water, acidified to pH 4.5 with 37% HCl and ultrafiltered through 3000 cut-off membrane to eliminate the excess of low molecular weight species (unreacted monomers, NaCl). Finally the product was freeze-dried and stored at -20°C. $\overline{\boldsymbol{M_n}}$: 22500; PD: 1.49 (by GPC System 1). [1]HNMR (D_2O): δ (ppm) = 2.05(H10) (2H, m,); 2.85 (H1, H8) (4H, b); 3.39 (H9)(2H, b); 3.49 (H2, H8) (4H, b); 5.70 (H4) (1H, s)



Scheme 6.3. Structure of BAC-AP

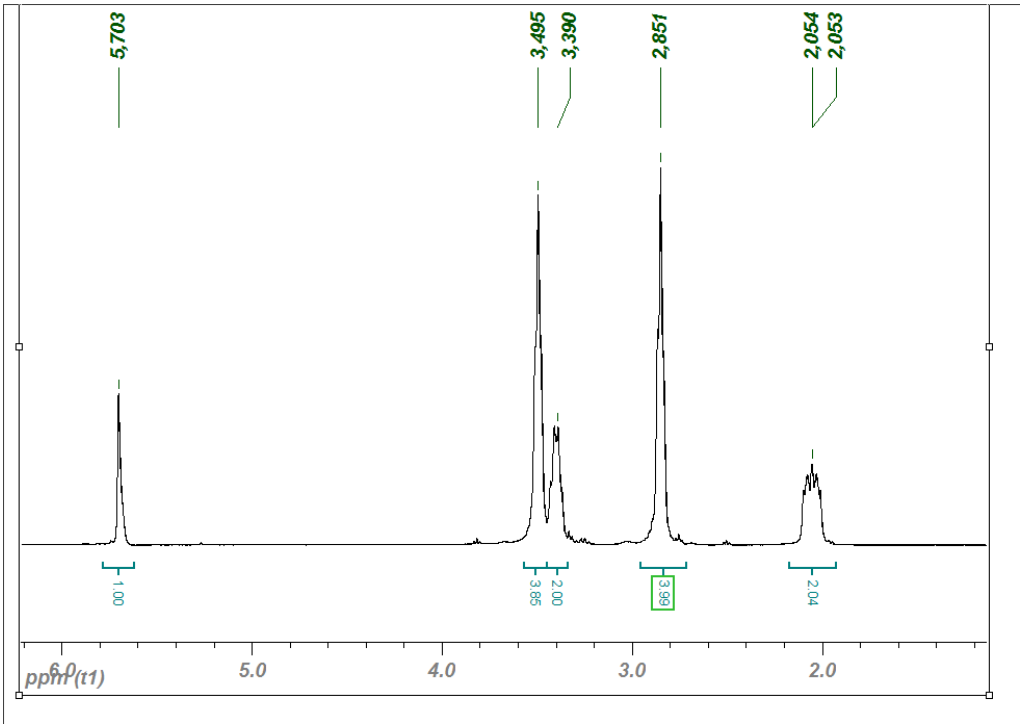
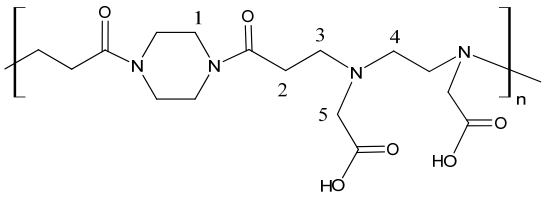


Figure 6.2. [1]HNMR spectrum of BAC-AP in D_2O at 25 °C.

BP-EDDA: In a 10 ml round bottom flask, bidistilled water (1.6 ml) was added. Under stirring, LiOH*H_2O (425mg; 10.1 mmol) and EDDA (907.2 mg; 5.15 mmol) were added in this order. After the reagents were completely dissolved, BP (1.0 g; 5.15 mmol) was added to the solution. Then the reaction was gently stirred for 48 hours under inert atmosphere (N_2) and in the dark. After this time the mixture was diluted to 20 ml with water, acidified to pH 4.5 with 37% HCl and ultrafiltered through 3000 cut-off membrane to eliminate the excess of low molecular weight species (unreacted monomers, LiCl). Finally the product was freeze-dried and stored at -20°C. $\overline{M_n}$: 25200; PD: 1.66 (by GPC System 1). [1]HNMR (D_2O): δ (ppm) = 2.93(H4) (4H, m,); 3.54 (H1) (8H, b); 3,58 -3,65 (H2, H3)(8H, b); 3.80 (H5) (4H, b).



Scheme 6.4. Structure of BP-EDDA

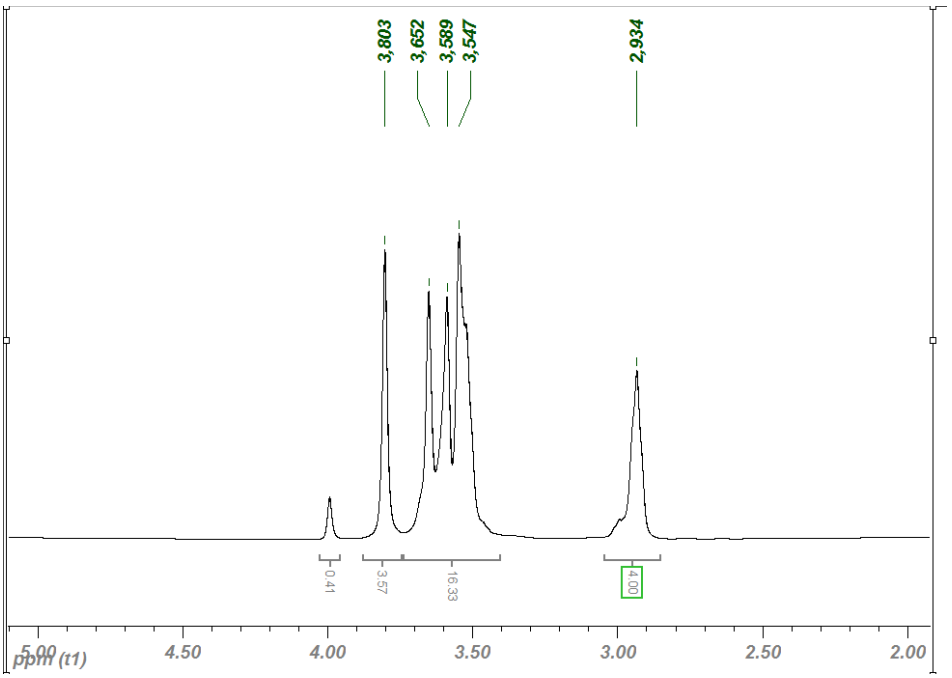
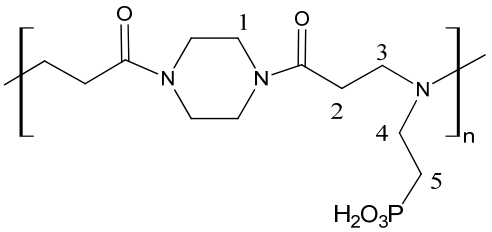


Figure 6.3. ^{1}HNMR spectrum of BP-EDDA in D_2O at 25 °C.

BP-AP: In a 10 ml round bottom flask, 5 M aqueous NaOH solution (2.060 g) and then AP (644.0 mg; 5.15 mmol) were added under stirring. After the reagent was completely dissolved, BP (1.0 g; 5.15 mmol) was added to the solution. Then the reaction was gently stirred for 72 hours under inert atmosphere (N_2) and in the dark. After this time the mixture was diluted to 20 ml with water, acidified to pH 4.5 with 37% HCl and ultrafiltered through

3000 cut-off membrane to eliminate the excess of low molecular weight species (unreacted monomers, NaCl). Finally the product was freeze-dried and stored at -20°C. $\overline{M_n}$: 20600; PD: 1.25 (by GPC System 1). ^{1}HNMR (D_2O): δ (ppm) = 1.99 (H5) (2H, m); 2.98 (H3) (4H, b); 3.36 (H4)(2H, b); 3.43 (H2) (4H, b); 3.54 – 3.59 (H1) (8H, s).



Scheme 6.5. Structure of BP-AP

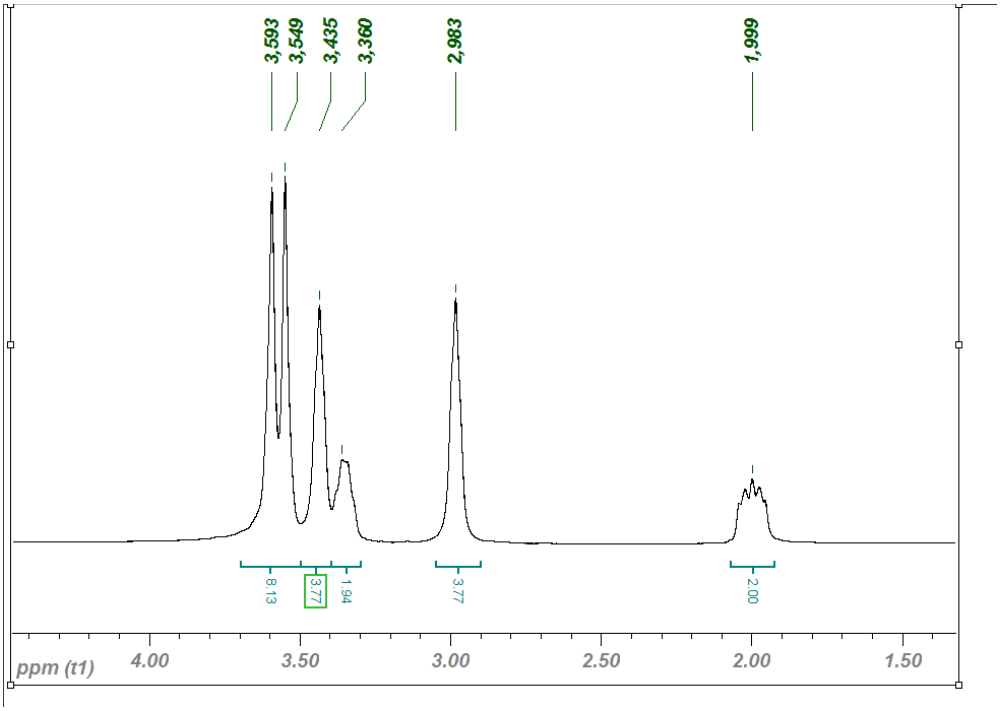


Figure 6.4. ^{1}HNMR spectrum of BP-AP in D_2O at 25 °C.

BAC-EDDA-Tyr: working as described in BAC-EDDA synthesis, the following reagents were added to the reaction flask in this order: H_2O (1.6 ml), LiOH*H_2O (211.4 mg; 5.04

mmol), BAC (purity, 97.4 %; 1.0 g; 4.91 mmol), EDDA (778.5 mg; 4.419 mmol), tyramine (purity: 98 %; 67.4 mg; 0.511 mmol). The polymer thus obtained can be radiolabeled with ^{125}I as previously described [11]. The polymer was purified as described in BAC-EDDA synthesis. $\overline{M_n}$: 15300; PD: 1.24 (by GPC System 1). ^{1}HNMR (D_2O): The spectrum of BAC-EDDA-Tyr is qualitatively the same of BAC-EDDA, but in that of BAC-EDDA-Tyr the peaks of tyramine (δ (ppm) = 7.19 – 6.86) are also evident. Amount of tyramine: 7.5 % of the tyramine containing repeating units over total number of repeating units.

BAC-EDDA-NH2: working as described in BAC-EDDA synthesis, the following reagents were added to the reaction flask in this order: H_2O/MeOH 2/1 (1.6 ml), TEA (purity 99 %; 515 mg; 5.04 mmol), BAC (purity, 97.4 %; 1.0 g; 4.91 mmol), EDDA (778.5 mg; 4.419 mmol), N-triphenylmethyl-1,2-diaminoethane (148.48 mg; 0.491 mmol). After 24 h 0.5 ml of water are added to the solution, stirring for 3 days. The protecting group of the primary amino group was removed by adding to the solution 2ml of water and adjusting to the pH of 2 with HCl 2M and stirring for 2 hours more. The polymer was purified as described in BAC-EDDA synthesis. $\overline{M_n}$: 3200; PD: 1.28 (by GPC System 1). ^{1}HNMR (D_2O): The spectrum of BAC-EDDA-NH2 is qualitatively the same of BAC-EDDA, but in that of BAC-EDDA-NH2 the peaks of EDA (δ (ppm) = 3.80 – 3.96 (-$\mathbf{CH_2}$-$\mathbf{CH_2}$-NH_2) (4H, b)) are also evident. Amount of EDA: 7.0 % of the EDA containing repeating units over total number of repeating units.

BAC-EDDA-β-CD: working as described in BAC-EDDA synthesis, the following reagents were added to the reaction flask in this order: H_2O 1.6 ml, $NaHCO_3$ (412.5 mg; 4.91 mmol), BAC (purity, 97.4 %; 1.0 g; 4.91 mmol), EDDA (778.49 mg; 4.419 mmol), mono-6-deoxy-6-amino-β-cyclodextrin (556.7 mg; 0.491 mmol). The polymer was purified as described in BAC-EDDA synthesis. $\overline{M_n}$: 21800; PD: 1.36 (by GPC System 1). ^{1}HNMR (D_2O): δ (ppm) = 5.01 (anomeric hydrogen of β -CD) (7H, b); see BAC-EDDA spectrum. ^{1}HNMR (D_2O): The spectrum of BAC-EDDA-β-CD is qualitatively the same of BAC-EDDA, but in that of BAC-EDDA-β-CD the peaks of β-CD (δ (ppm) = 5.01 (anomeric

hydrogen of β -CD) (7H, b)) are also evident. Amount of β -CD: 8 % of the β -CD containing repeating units over total number of repeating units.

BP-GSH (random): in a 10 ml round bottom flask, BP (306.5 mg, 1.579 mmol) was dissolved in water (0.54 ml). Reduced L-glutathione (GSH) (500.2 mg, 1.578 mmol) and potassium carbonate (220.3 mg, 1.578 mmol), in this order, were added under nitrogen and the resultant solution (pH ≅ 9.5) was gently stirred 6 days at 20° under nitrogen. The mixture was then diluted to 100 ml with water, acidified to pH ≅ 4.5 with hydrochloric acid, ultrafiltered through a membrane with nominal cut-off 5000 and freeze-dried. $\overline{M_n}$: 12900; PD: 3.9 (by GPC System 2). Yield 86.9 % w/w.

^{1}H NMR (D_2O): δ = 3.62 (CH_2-**CH**(NH_2)-COOH); 3.6 (NH-**CH_2**-COOH); 4.5 (CH_2-**CH**(NHCO)-CONH); 2.75-3 (S-**CH_2**-CH); 2.4 (CONH-**CH_2**-CH_2); 3.3 (NH-**CH_2**-CH_2); 2.95 (CH_2-**CH_2**-CON); 2.10 (CH_2-**CH_2**-CH); 3.75 (piperazine ring); 3.0 (NCO-**CH_2**-CH_2); 2.8 (CH_2-**CH_2**-S).

^{13}C NMR (D_2O): δ = 62.5 (CH_2-**CH_2**-COOH); 45 (NH-**CH_2**-COOH); 53 (CH_2-**CH**(NHCO)-CONH); 33 (S-**CH_2**-CH); 32 (CONH-**CH_2**-CH_2); 43 (NH-**CH_2**-CH_2); 29 (CH_2-**CH_2**-CON); 25 (CH_2-**CH_2**-CH); 42 (piperazine ring); 28 (NCO-**CH_2**-CH_2); 34 (CH_2-**CH_2**-S); 170-174 (quaternary carbons)

Elemental analysis $(C_{20}H_{32.51}N_5O_{8.7}Cl_{0.125}S)_n$ $(501.56)_n$: Calculated, C = 46.31, H = 6.32, O = 26.83, N = 13.50, S = 6.18, Cl = 0.85; Found C = 44.02, H = 5.85, O = 26.34, N =12.72, S = 5.84; Cl = 0.82.

Specific rotation $[\alpha]^{20}_{589}$ = -17.5 deg·dm^{-1}·g^{-1}·cm^3

BP-GSH (ordered): this reaction is carried out in two steps. In the first step, water (0.54 ml), BP (153.3 mg; 0.7895 mmol), GSH (500.2 mg; 1.578 mmol) and potassium carbonate (110.1 mg; 0.7895 mmol), in this order, were added in a 10 ml reaction flask and the resultant solution (pH ≅ 6.5) was stirred for 24 hours at 20° under nitrogen. Afterwards, BP (153.3 mg; 0.7895 mmol) and potassium carbonate (110.1 mg; 0.7895 mmol) were added and the resultant solution (pH ≅ 9.5) was stirred for 6 days at 20° under nitrogen. Purification was done as BP-GSH (random). $\overline{M_n}$: 7800; PD: 2.4 (by GPC System 2). Yield

79.4 % w/w. The ^{1}H NMR and ^{13}C NMR spectra of the BP-GSH (ordered) is qualitatively the same of BP-GSH (random).

BAC-GSH (random): this polymer was prepared as BP-GSH (random) starting from water (0.54 ml), BAC (purity 97.4 %; 321.26 mg; 1.579 mmol), GSH (500.2 mg, 1.578 mmol) and potassium carbonate (440.6 mg, 3.156 mmol).). $\overline{M_n}$: 10400; PD: 3.4 (by GPC System 2). Yield 75 % w/w. The ^{1}H NMR and ^{13}C NMR spectra of the BAC-GSH (random) is qualitatively the same of BP-GSH (random) but in that of BAC-GSH (random) the peaks of BP are substituted by those of BAC (^{1}H NMR (D_2O): δ =3.07 (4H, b); 3.27 (4H, b); 5.47 (1H, s)).

BAC-GSH (ordered): this polymer was obtained as BP-GSH (ordered) starting from water (0.54 ml), BAC (purity: 97.4; 160.63 mg; 0.7895 mmol), GSH (500.2 mg; 1.578 mmol) and potassium carbonate (110.1 mg; 0.7895 mmol) in the first step, and BAC (purity: 97.4; 160.63 mg; 0.7895 mmol) and potassium carbonate (110.1 mg; 0.7895 mmol) in the second step. $\overline{M_n}$: 8300 PD: 2.9 (by GPC System 2). Yield 71.8 % w/w. The ^{1}H NMR and ^{13}C NMR spectra of the BAC-GSH (ordered) is qualitatively the same of BAC-GSH (random).

BP-GSSG: this polymer was prepared as BP-GSH starting from BP (194.0 mg, 0.999 mmol), water (0.75 ml), oxidized L-glutathione (645.2 mg, 1.000 mmol) and potassium carbonate (152.7 mg, 1.093 mmol). $\overline{M_n}$: 18600; PD: 1.8 (by GPC System 2). Yield 80.3 % w/w.

^{1}H NMR (D_2O): δ = 3.65 (CH_2-**CH**(NH_2)-COOH); 3.6 (NH-**CH_2**-COOH); 4.75 (CH_2-**CH**(NHCO)-CONH); 2.8-3.2 (S-**CH_2**-CH); 2.5 (CONH-**CH_2**-CH_2); 3.9 (piperazine ring); 2.9 (NCO-**CH_2**-CH_2); 3.25 (NH-**CH_2**-CH_2).

^{13}C NMR (D_2O): δ = 62 (CH_2-**CH**(NH_2)-COOH); 53 (NH-**CH_2**-COOH); 39 (CH_2-**CH**(NHCO)-CONH); 29 (S-**CH_2**-CH); 43 (CONH-**CH_2**-CH_2); 29 (piperazine ring); 25 (NCO-**CH_2**-CH_2); 44 (NH-**CH_2**-CH_2); 170-175 (quaternary carbons)

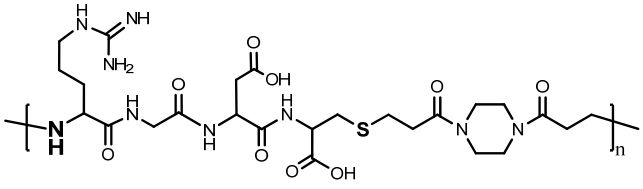
Elemental analysis $(C_{30}H_{49.7}N_8O_{15.8}Cl_{0.3}S_2)_n$ $(806.89)_n$: Calculated, C = 42.33, H = 5.89, O = 29.70, N = 13.16, S = 7.53, Cl = 1.37; Found C = 39.95, H = 5.63, O = 29.36, N =12.57, S = 7.4; Cl = 1.36.

Specific rotation $[\alpha]^{20}_{589}$ = -70.3 deg·dm^{-1}·g^{-1}·cm^3

BAC-GSSG: this polymer was prepared as BP-GSSG starting from BAC (purity 97.4 %; 321.26 mg; 1.579 mmol), GSSG (970.45 mg; 1.579 mmol) and potassium carbonate (220.59 mg, 1.579 mmol). $\overline{M_n}$: 10900; PD: 1.8 (by GPC System 2). Yield 85 % w/w. The ^{1}H NMR and ^{13}C NMR spectra of the BAC-GSSG is qualitatively the same of BP-GSSG but in that of BAC-GSSG the peaks of BP are substituted by those of BAC (^{1}H NMR (D_2O): δ =3.07 (4H, b); 3.27 (4H, b); 5.47 (1H, s).

BP-RGDC: In a 10 ml round bottom flask RGDC (purity: 98.4 %; 91.26 mg; 0.20 mmol) was added under a moderate nitrogen flow, followed by 0.300 ml of degassed and bidistilled water. Afterwards, BP (38.8 mg; 0.2 mmol) was added under stirring to the flask. After the reagents were completely dissolved, triethylamine (TEA) (purity: 99%; 20.44 mg; 0.2 mmol) was added to the solution (pK: 9.0-9.5). Then the reaction was gently stirred for 8 days under inert atmosphere (N_2) and in the dark. After this time the mixture was diluted to 20 ml with water, acidified to pH 4.5 with 37% HCl and ultrafiltered through 1000 cut-off membrane to eliminate the excess of low molecular weight species (unreacted monomers, TEA). Finally the product was freeze-dried and stored at -20°C. $\overline{M_n}$: 7200; PD: 3.1 (by GPC System 2). Yield: 56 % w/w.

^{1}H NMR (D_2O, all broad peaks): δ = 1.62 (2H, CH(NH_2)-**CH_2**-CH_2-NH, Arg); 1.93 (CH(NH_2)-CH_2-**CH_2**-NH, arg); 3.57-3.67 (8H, piperazine ring); 4.29 (S-CH_2-**CH**(COOH)-NH, CYS); 3.99 (CO-**CH_2**-CH(COOH)-NH, asp).



Scheme 6.6. Structure of BP-RGDC.

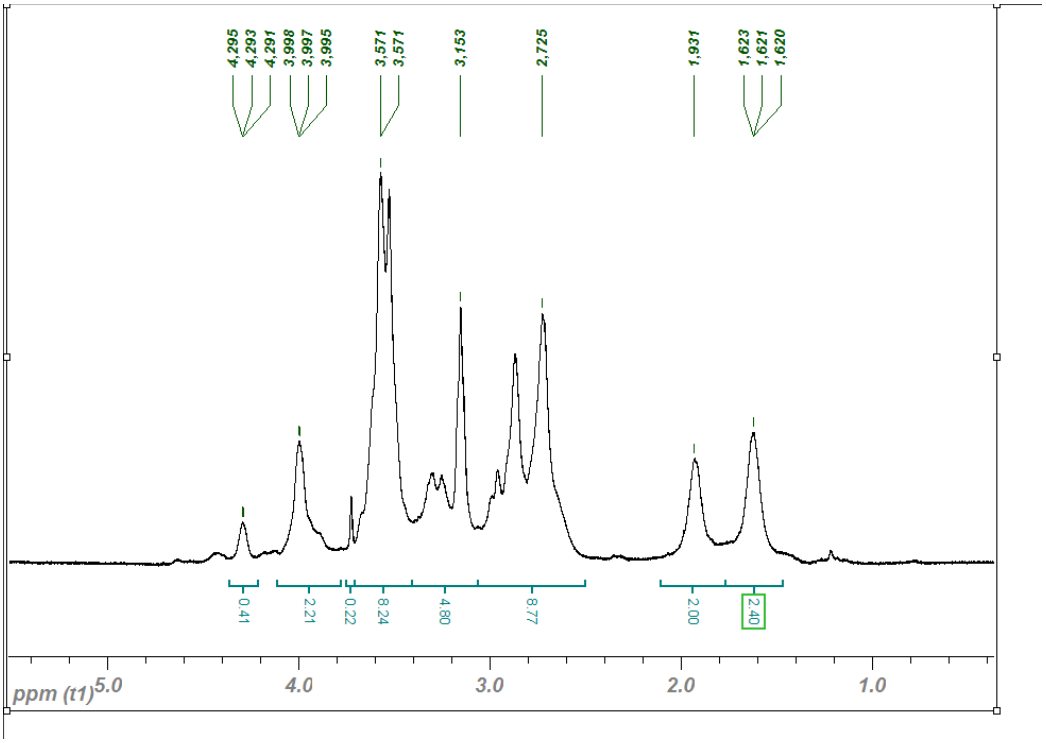
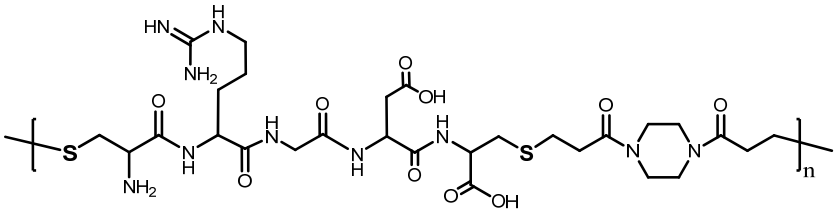


Figure 6.5. ^{1}HNMR spectrum of BP-RGDC in D_2O at 25 °C.

BP-CRGDC: this polymer was prepared as BP-RGDC starting from CRGDC (purity: 97.6 %; 113.24 mg; 0.2 mmol), bidistilled and degassed water (0.300 ml), BP (38.8 mg; 0.2 mmol) and TEA (20.44mg; 0.2 mmol) to obtain a final solution pH of 6.5-7.0. The reaction was terminated after 6 days. $\overline{M_n}$: 16700; PD: 2.3 (by GPC System 2). Yield: 65 % w/w.

^{1}H NMR (D_2O, all broad peaks): δ = 1.60 (2h, $CH(NH_2)$-**CH_2**-CH_2-NH, Arg); 1.90 ($CH(NH_2)$-CH_2-**CH_2**-NH, arg); 3.57-3.67 (8H, piperazine ring); 4.29 (S-CH_2-**CH**(COOH)-NH, cys); 3.99 (CO-**CH_2**-CH(COOH)-NH, asp).

^{13}C NMR (D_2O): δ=35 (HS-**CH_2**-CH, cys); 50 (HS-CH_2-**CH**(COOH)-NH, cys); 173 (HS-CH_2-CH(**CO**OH)-NH, cys); 172 (HN-**CO**-CH_2-CH, gly); 27 (HN-CO-CH(**CH_2**-COOH-NH, asp); 60 (HN-CO-**CH**(CH_2-COOH)-NH, asp); 173 (CH_2-CH(**CO**OH)-NH, cys); 170 (HN-**CO**-CH_2-NH, gly); 52 (HN-CO-**CH_2**-NH, gly); 168 (NH-**CO**-$CH(NH_2)$-CH_2, cys); 42 (NH-CO-**CH**(NH_2)-CH_2, cys); 23 ($CH(NH_2)$-**CH_2**-CH_2, arg); 28 (CH_2-**CH_2**-CH_2-NH, arg); 40 (CH_2-**CH_2**-NH-$C(NH_2)$=NH, arg); 32 (piperazine ring).



Scheme 6.7. Structure of BP-CRGDC.

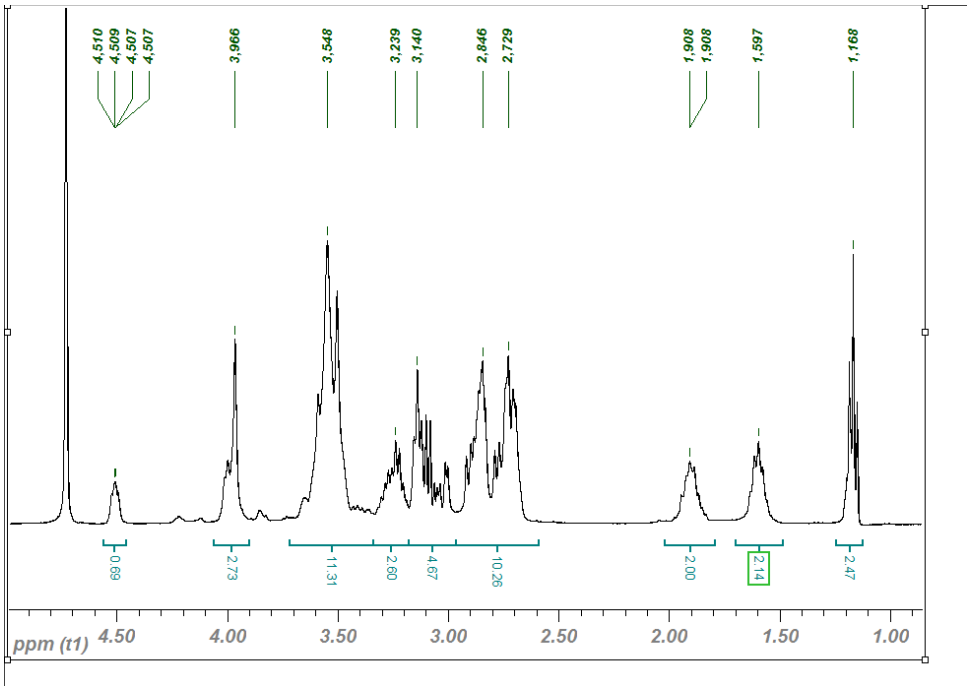


Figure 6.6a. ^{1}HNMR spectrum of BP-CRGDC in D_2O at 25 °C.

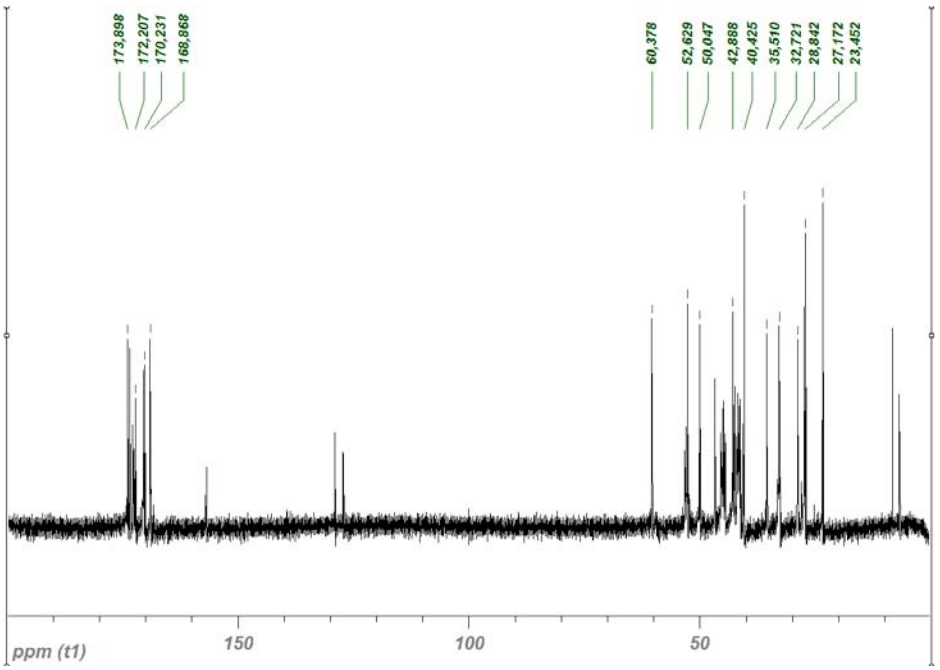


Figure 6.6b. ^{13}CNMR spectrum of BP-CRGDC in D_2O at 25 °C.

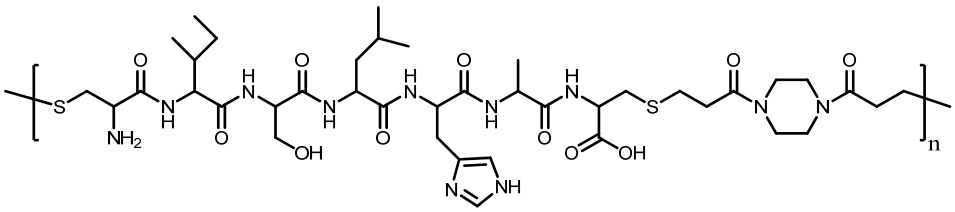
BAC-RGDC : this polymer was prepared as BP-RGDC starting from RGDC (purity: 98.4 %; 91.26 mg; 0.20 mmol), BAC (purity: 97.4%; 40.69 mg; 0.20 mmol) and TEA (purity: 99%; 20.44 mg; 0.2 mmol). $\overline{M_n}$: 2400; PD: 1.6 (by GPC System 2). Yield 52.5 % w/w. The ^{1}H NMR and ^{13}C NMR spectra of the BAC-RGDC is qualitatively the same of BP-RGDC but in that of BAC-RGDC the peaks of BP are substituted by those of BAC (^{1}H NMR (D_2O): δ =3.07 (4H, b); 3.27 (4H, b); 5.47 (1H, s).

p(CRGDC): in a 10 ml round bottom flask, CRGDC (purity: 97.6 %; 46.0 mg; 0.08142 mmol) was dissolved in water (0.300 ml) and TEA (purity: 99%; 10 mg; 0.0978 mmol) was added to the solution. Afterwards, a sodium acetate buffer solution (pH ≅ 6.0) (0.700 ml) containing I_2 (10.5 mg/ml of buffer) and KI (25 mg/ml of buffer) was added to the reaction mixture under stirring. The polymer was then precipitated by adding ethanol, centrifuged, washed again with ethanol and then diethyl ether, and finally dried with N_2 flow.). $\overline{M_n}$: 3200; PD: 1.6 (by GPC System 2). Yield 86 % w/w.

ISA23-SS-RGDC: in a 10 ml round bottom flask, the polymer ISA23-SS-Py [12] (149.5mg; 0.4727 mmol of repeating units) and RGDC (purity: 98.4 %; 50.79 mg; 0.1112 mmol) were dissolved in Tris buffer (pH ≅ 8.0) and stirred overnight at room temperature. . $\overline{\boldsymbol{M_n}}$: 12600; PD: 1.7 (by GPC System 2). Yield 91 % w/w. The ^{1}H NMR and ^{13}C NMR spectra of the ISA23-SS-RGDC are qualitatively the same that ISA23, but in that of ISA23-SS-RGDC characteristic peaks of RGDC are also evident (see above).

BP-CISLHAC: : this polymer was prepared as BP-CRGDC starting from CISLHAC (purity: 98.2 %; 151.91 mg; 0.2 mmol), bidistilled and degassed water (0.400 ml), BP (38.8 mg; 0.2 mmol) and TEA (20.44mg; 0.2 mmol) to obtain a final solution pH of 6.5-7.0. The reaction was terminated after 8 days. $\overline{\boldsymbol{M_n}}$: 3000; PD: 1.47 (by GPC System 2). Yield: 35 % w/w.

^{1}H NMR (D_2O, all broad peaks): δ = 0.84 (b, **$(CH_3)_2$**-CH, Ile); 1.27 (CH_3-**CH**, ala); 4.33 (m, CH-**CH_2**-CH, hys); 3.55 (8H, piperazine ring).



Scheme 6.8. Structure of BP-CISLHAC.

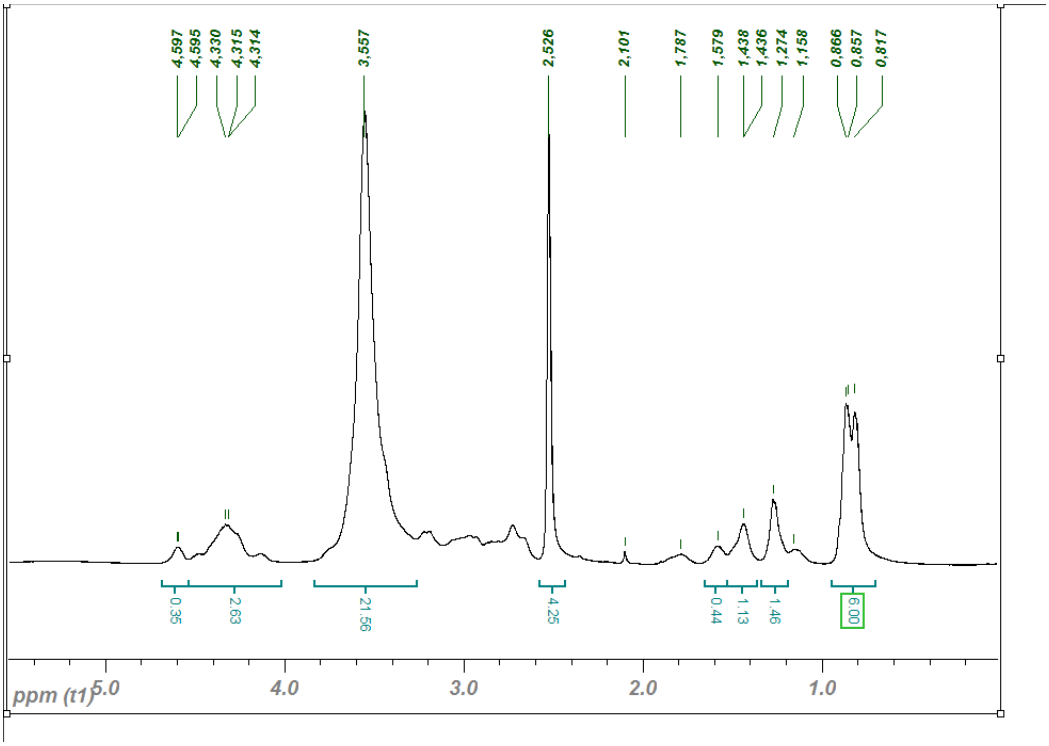
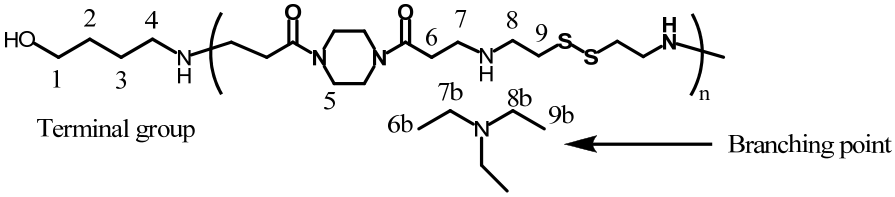


Figure 6.7. ^{1}HNMR spectrum of BP-CISLHAC in D_2O at 25 °C.

HB-p(BP-CYST): cystamine dihydrocloride (purity: 98 %; 309.0 mg; 1.345 mmol) and BP (1694.3 mg; 8.723 mmol) were dissolved in water (5.0 ml) in a 10 ml round-bottom flask. The pH was then adjusted to 9.0-9.5 by adding $CsCO_3$. The reactant solution was stirred at room temperature for 3 days under inert atmosphere and in the dark. The solution was then diluted with 20 ml of water, acidified at pH 4.5 with HCl 37 %, ultrafiltrated (1000 cut-off membrane) and freeze-dried. Afterwards, the polymer (510 mg; 1.47 mmol of repeating units) was dissolved in MeOH (5.20 ml) and then 40 mol% excess of ABOL (or ETA) was added to consume the terminal acrylamide groups and stirring was continued for 24 hours at room temperature. Finally, the solution was diluted with water (20 ml), acidified to pH 4.5 with HCl 37 %, ultrafiltered (1000 cut-off membrane) and freeze-dried. $\overline{M_n}$: 10800; PD: 1.51 (by GPC System 3). Yield 40% w/w. Insoluble aggregates were eliminated by ultrafiltration (100,000 cut-off membrane). ^{1}H NMR (D_2O, all broad peaks): δ = 1.44 (H3) (2H, m); 1.58 (H2) (2H, m); 3.44 (H5, piperazine ring) (8H, b); 2.50 – 3.0 (H7b, H8, H9)

(6H, b); 3.0 – 3.60 (H6b, H8b, H9b, b) (6H, b); 2.93 (H1) (2H, t); 3.44 (H4) (2H, t); 3.15 (H6) (4H, b); 2.75 (H7) (4H, b).



Scheme 6.9. Structure of HB-p(BP-CYST).

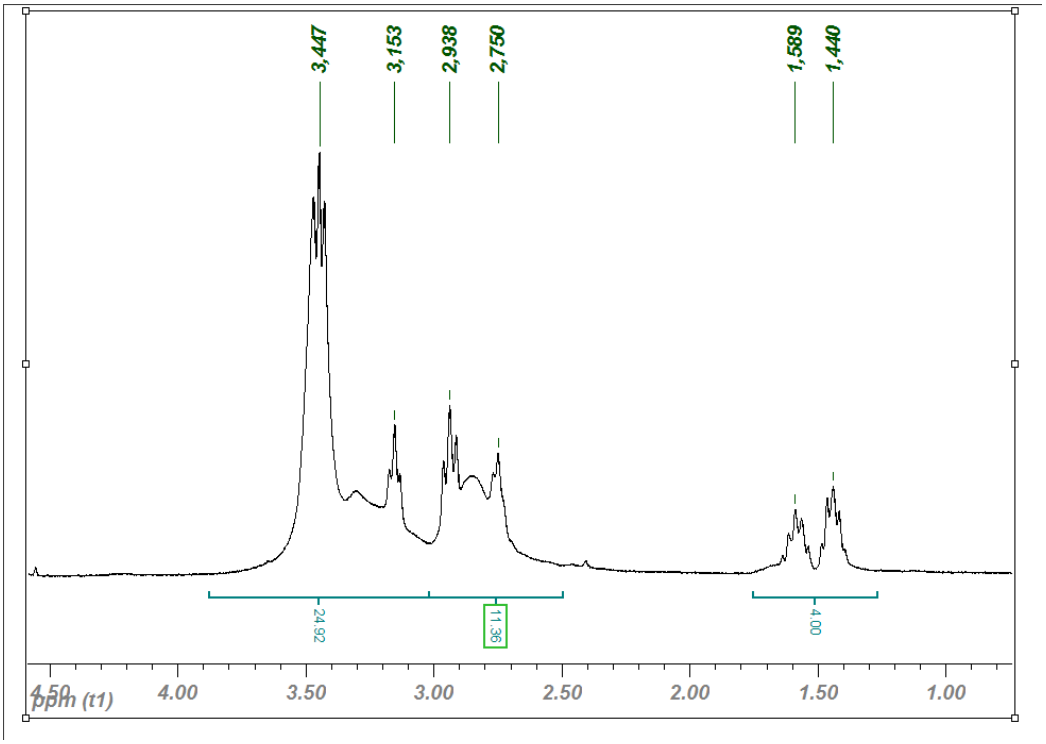
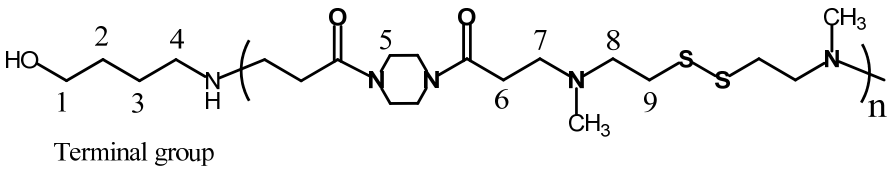


Figure 6.8. ^{1}HNMR spectrum of HB p(BP-CYST) (ABOL-t) in D_2O at 25 °C.

L-p(BP-DMC): in a 10 ml round-bottom flask, bidistilled water (1.80 ml), N,N'-dimethylcystamine dihydrocloride (DMC) (599.6 mg; 2.367 mmol) and N,N'-bisacryloyl piperazine (BP) (460mg; 2.368 mmol) were added in this order. The pH was then adjusted to 9.5-10 by adding TEA (479.3 mg; 4.760 mmol). The reactant solution was stirred at

room temperature for 3 days under inert atmosphere and in the dark. Afterwards, 10 mol% excess ABOL (or ETA) was added to consume any unreacted acrylamide groups and stirring was continued for 24 hours. The solution was then diluted with 20 ml of water, acidified at pH 4.5 with HCl 37%, ultrafiltrated (cutoff membrane 1000) and freeze-dried. $\overline{M_n}$: 9600; PD: 1.54 (by GPC System 3). Yield 87% w/w.^{1}H NMR (D_2O, all broad peaks): δ = 1.43 (H3) (2H, m); 1.66 (H2) (2H, m); 3.44 (piperazine ring) (8H); 3.49 (H4) (2H, b); 3.08 (H1) (2H, b); 3.31 (H6, H9) (8H, b); 2.87 (H7, H8) (8H, b).



Scheme 6.10. Structure of L-p(BP-DMC).

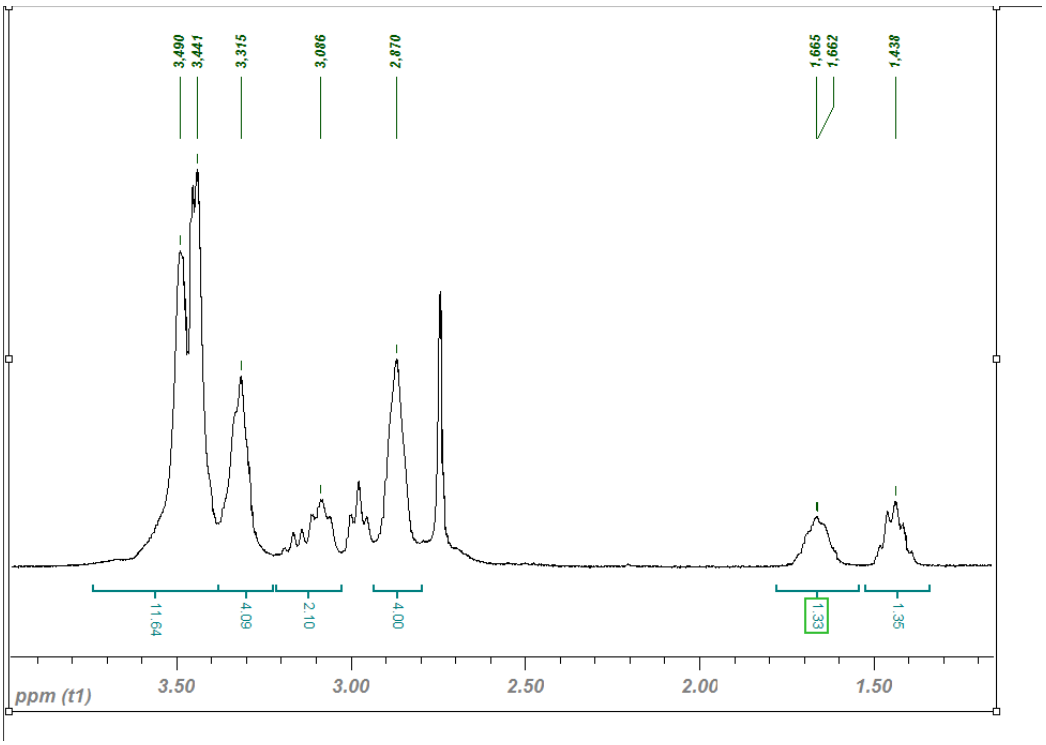
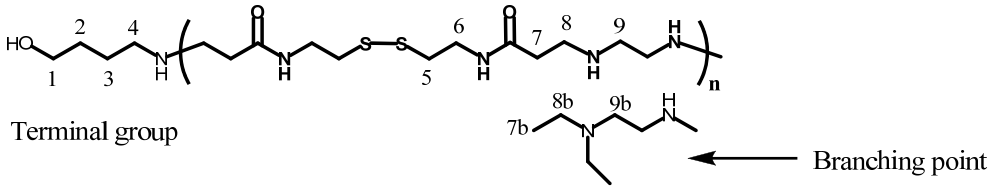


Figure 6.9. ^{1}HNMR spectrum of L-p(BP-DMC) (ABOL-t) in D_2O at 25 °C.

HB-p(CBA-EDA): this polymer was prepared as HB-p(BAP-CYST) starting from a solution MeOH/bidistilled water 5.395/1, EDA (purity 99.5 %; 82 mg; 1.3576 mmol) and CBA (2143 mg; 8.2303 mmol). The reaction was carried out at 40 °C for 5 days. End-capping of the terminal double bonds and purification were performed as HB-p(BAP-CYST). $\overline{M_n}$: 7800; PD: 1.80 (by GPC System 3). Yield 38% w/w. Insoluble aggregates were eliminated by ultrafiltration (cutoff 100,000). ^{1}H NMR (D_2O, all broad peaks): δ = 3.35 (H7, H7b) (4H, b); 2.68 (H8, H8b) (4H, b); 3.13 (H6) (4H, t); 2.53 (H5) (4H, t); 2.7 – 3.0 (H9b) (4H, m); 2.4 – 2.5 (H9) (4H, b); 2.91 (H1) (2H, t); 1.57 (H2) (2H, m); 1.43 (H3) (2H, m); 3.44 (H4) (2H, t).



Scheme 6.11. Structure of HB-p(CBA-EDA).

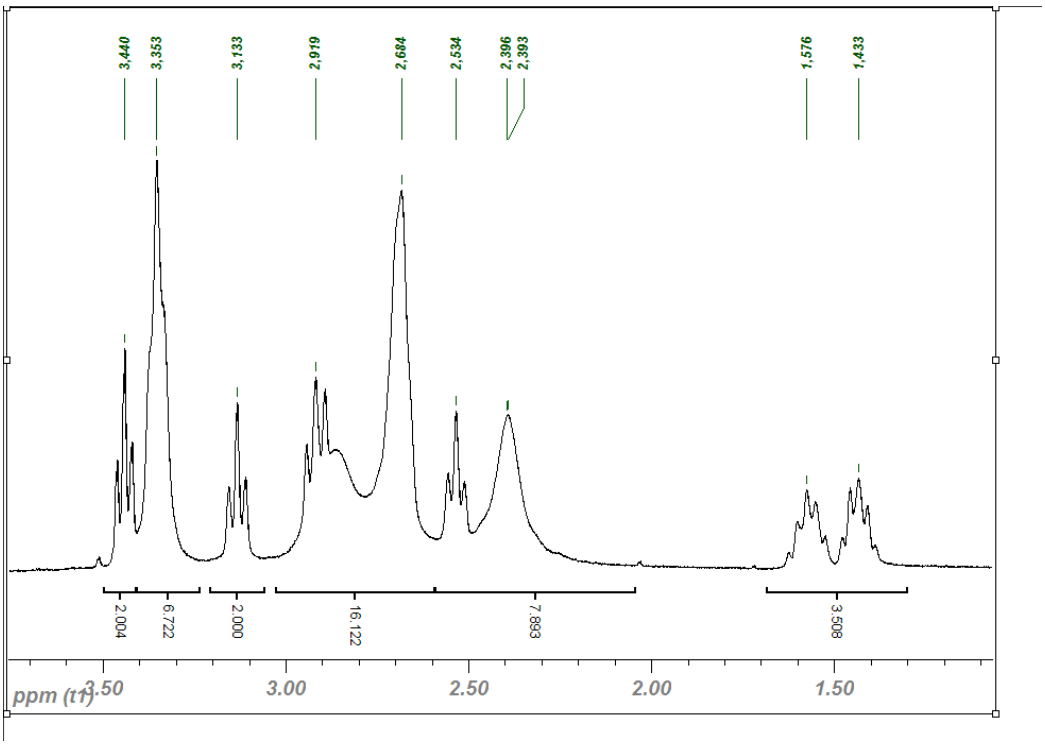
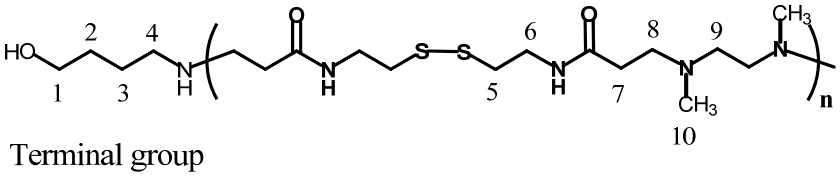


Figure 6.10. ^{1}HNMR spectrum of HB p(CBA-EDA) (ABOL-t) in D_2O at 25 °C.

L-p(CBA-DMEDA): this polymer was prepared as L-p(BAP-DMC) starting from 4.34 ml of a solution MeOH/bidistilled water 4.636/1, N,N'-dimethylethylenediamine (DMEDA) (purity: 99 %; 508.9 mg; 5.715 mmol) and N,N'-bisacryloylcystamine (CBA) (1488.2 mg; 5.715). The reaction was carried out at 40 °C for 5 days. End-capping of the terminal double bonds and purification were performed as L-p(BAP-DMC). $\overline{M_n}$: 12500; PD: 1.46 (by GPC System 3). Yield 75% w/w.^{1}H NMR (D_2O, all broad peaks): δ = 2.60 (H10) (6H, b); 3.21 (H9) (4H, b); 2.68 (H8) (4H, t); 3.15 (H6) (4H, b); 2.56 (H5) (4H, b); 3.36 (H7) (8H, b).



Scheme 6.12. Structure of L-p(CBA-DMEDA).

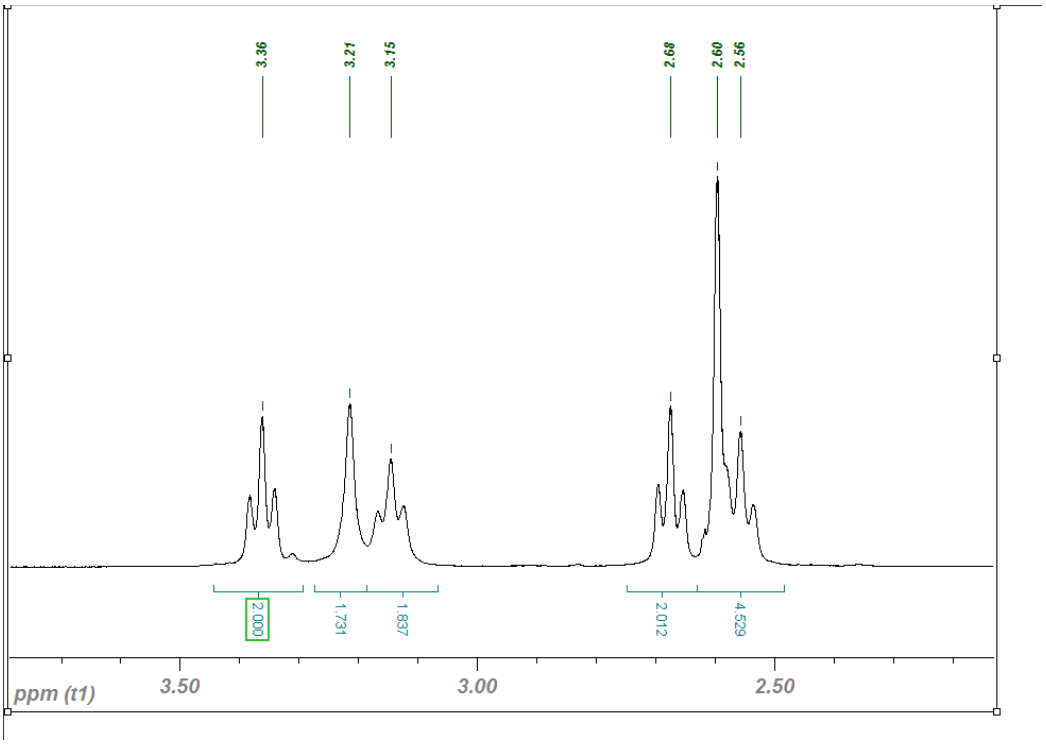


Figure 6.11. [1]HNMR spectrum of L-p(CBA-DMEDA) (ABOL-t) in D_2O at 25 °C.

The [1]HNMR spectra of ETA terminated polymers are qualitatively the same of ABOL terminated polymers, but in those of ETA terminated all ABOL peaks ([1]H: 1.60, 1.40 ppm) are not present, whereas peak at 3.50 (2H) and 2.80 (2H) ppm are observed.

All the ^{1}H NMR spectra of the PAAs showed above display many broad or overlapped signals (between 2.5 and 4.5 ppm). This is due to the coupling of nuclear spins of several di-methylene groups. This is not unexpected. In NMR spectra of small molecules, these interactions are averaged to zero by rapid, isotropic tumbling. However, in polymeric solutions, the molecular motion is slow enough that this coupling can contribute significantly to the peaks definition.

References

[1] C.E. Thomas, A. Ehrhardt, M.A. Kay, Progress and problems with the use of viral vectors for gene therapy, Nat. Rev. Genet., (4), 346–358, **2003.**

[2] D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, Nat. Rev. Drug Discov., (4), 581–593, **2005.**

[3] S.C. De Smedt, J. Demeester,W.E. Hennink, Cationic polymer based gene delivery systems, Pharm. Res., (17), 113–126, **2000.**

[4] R. B. Merrifield, *J. Am. Chem. Soc.,* (85), 2149, **1963.**

[5] P. Ferruti, *Macromol. Synth.*, (9), 25, **1985.**

[6] P. Ferruti, E. Ranucci, F. Trotta, E. Gianasi, G. E. Evagorou, M. Wasil, G. Wilson, R. Duncan, *Macromol. Chem. Phys.*, (200), 1644, **1999.**

[7] A. Wahl, I. Schnell, U. Pyell, *Journal of Chromatography* A, (1044), 211–222, **2004.**

[8] B. Malgesini et al., *Macromol. Biosci*., (3), 59–66, **2003.**

[9] S. Richardson, P. Ferruti, R. Duncan, *J. Drug Targeting,* (6), 391-404, **1999.**

[10] P. Ferruti, E. Ranucci, F. Trotta, E. G. Gianasi, M. W. Evagorou, G. Wilson, R. Duncan, *Materials Sciences Bullettin*, pag.49, **1999.**

[11] Richardson, S.; Ferruti, P.; Duncan, R. *J. Drug Targeting,* (6), 391-404, **1999.**

[12] E. Emilitri, P. Ferruti, R. Annunziata, E. Ranucci, *Macromolecules*, (40), 4785-4793, **2007.**

Acknowledgements

This Ph.D. course has been for me a unique experience in which not only I learned how to work in laboratory, but also where I have found lots of friends and people who has believed in my skills.

First of all, I would like to thank my promotors, Prof. Paolo Ferruti and Prof. Elisabetta Ranucci for convincing me to begin the PhD course, and for giving me the possibility of work in their group. I also wish to express my gratitude to Dr. Amedea Manfredi, who supported me in lab and for writing this thesis. Moreover, I would like to thank Prof. Johan F. J. Engbersen for giving me the opportunity to work at the PBM group (University of Twente), that was a really nice and unforgettable experience.

Special thanks are due to Marco Bencini, who supervised me during my Master thesis, taught me how to work hard and how to approach to research problems.

I am very grateful to all the colleagues and friends that contributed to the work presented in this thesis, Irini, Severine, Elisa, Paola R., Loredana, Marco S., Jacopo, Ettore, Fabio, Roberto M., and especially Valeria, who patiently waited for me six months! Thank you! But I cannot forget all the friends I worked with in these years, Giusy, Manuela, Raffaella, Silvia C., Sara, Silvietta, Claudia, Federica, Stefano, Beppe I., Marco R., Luca, Andrea, Davide, Davidino, Ivan and “i Pippi”. Thank you guys!

I am really grateful to all the people at the PBM. During my experience in this group, I had a nice time and I found lots of nice people. I especially thank my colleagues Greg and Martin that showed me much more than the laboratory! I will not forget all the nights at the Vestingbar and in the city center! Thanks to Karin, who gave me a great help before my arrival in Enschede and even later. Thanks also to my roommates, Jung Seok, Chao, Niels and Gert Jan, and moreover Hans, Rong, Sytze, Hongzi, Andries, Marloes, Yan, Sigrid, Janine, Ferry, Erhan and all the Master and Bachelor students, thank you for the help, the support and the nice time we had together, Thank you all!

Federico