André Alexandre Cruz de Matos

Development of Bile Acid Sequestrants based on Cationic Hydrogels

Dissertation guided by Jorge F.J. Coelho, PhD, and Arménio C. Serra, PhD, and presented to the Faculty of Sciences and Technology of University of Coimbra to obtain a Master's degree in Biomedical Engineering

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Department of Chemical Engineering
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André Matos

Abstract

Cardiovascular diseases, which are mostly related to high levels of low density lipoproteins (LDL), are currently one of the major causes of death. Currently, there are various polymer based drugs (hydrogels) capable of lowering the cholesterol levels in the blood, through bile acid complexation. However, all of the commercially available bile acid sequestrants have problems in terms of efficiency. Thus, in order to try to create a more effective drug, hydrogels of 2-hydroxyethyl acrylate (HEA) and ((3- acrylamidopropyl)trimethylammonium chloride) (AMPTMA) were prepared using two different polymerization methods: free radical polymerization (FRP) and supplemental activator and reducing agent atom transfer radical polymerization (SARA ATRP). It was found that the new hydrogels were able to bind the sodium cholate (NaCA) (bile acid model molecule), but showing lower binding capacity than the products of the same category that are already present on the market. The effect of the amount of HEA present on the hydrogels on the binding parameters was also investigated. Generally, the results showed that an increase on the amount of HEA lead to an decrease of the binding capacity of the new BAS. The results also showed that BAS produced using SARA ATRP techniques present a higher binding capacity than the ones produced using FRP techniques. Finally, it was observed that copolymers of PAMPTMA-*co*-PHEA showed no sign of degradation in solutions that mimic the stomach and intestine environment.

Keywords: Hydrogels; Bile acid sequestrants; FRP; SARA ATRP; 2-hydroxyethyl acrylate; ((3-acrylamidopropyl)trimethylammonium chloride)

Resumo

As doenças cardiovasculares, que na sua maioria, estão relacionadas com elevados níveis de lipoproteínas de baixa densidade (colesterol LDL), constituem atualmente uma das principais causas de morte. De momento, já existem vários fármacos baseados em polímeros (hidrogéis) capazes de diminuir os níveis de colesterol no sangue, através da complexação de ácidos biliares. Porém, todos os sequestradores de ácidos biliares(BAS) presentes no mercado apresentam problemas ao nível da eficiência. Assim, de modo a tentar criar um fármaco mais eficaz, recorreu-se à preparação de hidrogéis de acrilato de 2-hidroxietil(HEA) e cloreto de (3-acrilamidopropil)trimetil amónio (AMPTMA), usando dois métodos de polimerização distintos: polimerização radicalar livre (FRP) e polimerização radicalar por transferência de átomo com ativador suplementar e agente de redução (SARA ATRP). Verificou-se que os novos hidrogéis são capazes de se ligar ao sal de sódio do ácido cólico (NaCA) (ácido biliar modelo), mas apresentam uma capacidade de ligação inferior aos produtos da mesma categoria que já se encontram no mercado. Foi ainda investigado o efeito que a quantidade de HEA presente no hidrogel tem nos parâmetros de ligação. De um modo geral, verificou-se que a capacidade de ligação diminui com o aumento da quantidade de HEA nos BAS. Os resultados mostraram ainda que os BAS produzidos usando técnicas de SARA ATRP apresentam uma maior capacidade de ligação que os produzidos por técnicas de FRP. Finalmente, foi observado que o copolímero PAMPTMA-co-PHEA não mostrou sinais de degradação pela solução que simulou o ambiente estomacal e intestinal.

List of acronyms

AIBN	2,2'-azobis(2-methylpropionitrile)
AMPTMA	((3- acrylamidopropyl)trimethylammonium chloride)
ATRP	Atom transfer radical polymerization
BAS	Bile acid sequestrants
BDDA	1,4 butanediol diacrylate
DMF	Dimethylformamide
EBiB	Ethyl α-bromoisobutyrate
ECP	Ethyl 2-chloropropionate
EtOH	Ethanol
FRP	Free radical polymerization
FTIR-ATR	Fourier transform infrared attenuated total reflection
GI	Gastrointestinal
HEA	2-hydroxyethyl acrylate
Me_6TREN	Tris[2-(dimethylamino)ethyl]amine
NaCA	Cholic acid sodium salt (sodium cholate)
PRE	Persistent radical effect
PAMPTMA	Poly((3- acrylamidopropyl)trimethylammonium chloride
PHEA	Poly(2-hydroxyethyl acrylate)
PMDETA	*N*,*N*,*N*',*N*'',*N*''-Pentamethyldiethylenetriamine
RDRP	Reversible deactivation radical polymerization
SARA	Supplemental activator and reducing agent
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
TREN	Tris(2-aminoethyl)amine

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Motivation, targets and research significance

Cardiovascular diseases are one of the major causes of death in the industrialized countries. This type of diseases correlates strongly with the levels of plasma cholesterol, mainly the low density lipoproteins (LDL) cholesterol. It is known that the reduction of the levels of LDL cholesterol decreases the risk of cardiovascular related problems.[1]

These facts lead to the development of several substances that are able to reduce the LDL cholesterol levels. The most well-known ones are: statins, that inhibit the HMG-CoA reductase stopping a reaction that occurs on an early stage of the normal cholesterol biosynthesis; ezetimibes, that inhibit the absorption of cholesterol on the brush border of the small intestine; and the bile acid sequestrants (BAS), which can bind the bile acids interrupting the enterohepatic circulation and force the consumption of cholesterol. Both statins and ezetimibes cannot be used by pregnant women or patients with hepatic disorder. Also, these drugs are associated to side effects after long time periods of use. On the other hand, BAS are safe and can be used by everyone. However, they present a lower efficiency than statins which leads to the need of large quantities of BAS to achieve a significant therapeutic performance. This is one of the main problems of the BAS, which can lead to poor patient compliance.

The goal of this work is the development of novel BAS that could show a higher efficiency than the ones already available on the market. This would lead to the decrease of the amount of BAS needed to achieve a significant reduction of the cholesterol level, and consequently a decrease of patient compliance problems

1 - Introduction

1.1 - Polymers

The word polymer derives from the Greek words Poly and Meres, meaning many and parts, respectively, and it is used to describe the macromolecules that are composed by smaller repeating units, known as monomers.[2]

Polymers are obtained by chemical reactions called polymerizations, in which the monomer units are chemically combined to produce chain-like or network-like molecules. This occurs by sequential reaction, as the monomers are chemically bonded together, forming a macromolecule progressively larger.

One of the most common ways to distinguish polymers that are synthesized recurring to the same technique and identical monomers, is the number average molecular weight (M_n), which is defined by the average of the molecular masses of the individual macromolecules that compose the polymer $\left(M_n = \frac{\sum M_i N_i}{\sum N_i}\right)$. Another way to describe the molar mass of a polymer is the mass-average molecular weight (M_w), which is calculated using $M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i}$. From this value it is possible to calculate the degree of polymerization ($DP = \frac{M_n}{M_0}$;where M_0 represents the molecular weight of the monomeric unit), that is defined as the number of monomeric units present on a polymer chain (represents the length of the polymeric chain). In addition, polymers are characterized by their dispersity $\left(Đ = \frac{M_w}{M_n}\right)$, which is a measure of the broadness of the molecular weight distribution.[2]

The properties of the polymers are influenced by their structure, composition and polymerization degree.[3]

1.1.1 - Classification

According to the occurrence, a polymer can be classified as natural (or biopolymer), if the polymer is synthesized by nature; synthetic, if it is synthesized in the laboratory and semi-synthetic, if the polymer is a chemically modified biopolymer.[3]

Depending on the monomers used on the synthesis, a polymer can be classified as (a) homopolymer, in the case of the use of only one kind of monomer, or copolymer, when at least two kinds of monomers are used. In the case of copolymers, there is also a distinction based on the organization of the monomers on the polymeric chain, as seen on Figure 1.1, so that there are (b) alternating copolymers (the monomers alternate in a regular manner), (c) random copolymers (the monomers are randomly arranged), (d) block copolymers (the monomers are arranged on long sequences of one type of monomer linked to another long sequence of the other monomer) and (e) graft copolymers (the chains of one monomer are attached to the main chain of another polymer).[3]

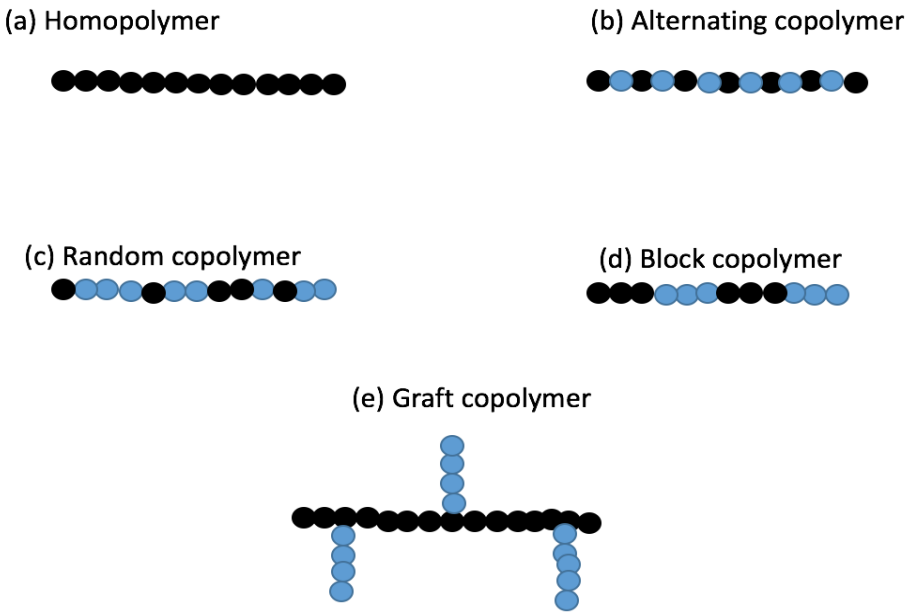


Figure 1.1 - Classification of polymers according to their composition: (a) homopolymer;(b) alternating copolymer; (c)random copolymer; (d) block copolymer; (e) graft copolymer.

Finally depending on the structure of the polymeric chain, that is represented in the Figure 1.2, a polymer can be classified as: (a) linear, when the polymer is composed by long chains of monomers; (b) branched, if the polymer has branches covalently linked to the main chain, and (c) crosslinked, when one of the monomers presents two reactive sites and can thus be linked to two different polymer chains, forming three-dimensional networks (Figure 1.2). Depending on the degree of crosslinking, it is possible to obtain hydrogels, which are insoluble polymer networks.

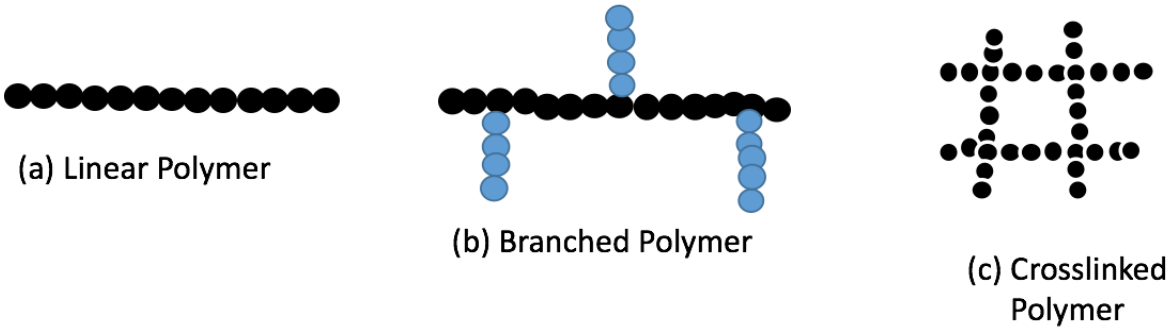


Figure 1.2 –Some of the possible polymeric structures: (a) linear; (b) branched; (c) crosslinked

1.1.2 - Hydrogels

A hydrogel is a three-dimensional crosslinked polymer network. Therefore, any method that can be used to create a crosslinked polymer allows the preparation of hydrogels. One of the most well-known property of the hydrogels is their ability to absorb and hold large amounts of water on their porous structure without dissolving. This characteristic derives from the presence of hydrophilic groups attached to the polymer backbone that allow the retention of high amounts of water.[4]

Due to their properties, hydrogels have a wide range of applications, namely water-treatment additives, membranes for separation of biological compounds, contact lenses, drug-release systems, scaffolds for tissue engineering, implant coatings, and bile acid sequestrants (BAS).[5]

Concerning biomedical applications, an ideal hydrogel should have low price, low soluble content and low residual monomer, high absorption capacity and should be biodegradable resulting in the formation of non-toxic degradation by-products (for specific applications), should

be colorless, odorless, photo-stable, non-toxic, and have a neutral pH after the swelling.[4] As mentioned above, a hydrogel must be biocompatible, meaning that the material should not be mutagenic, cytotoxic and carcinogenic. The biocompatibility of the hydrogels is mainly associated to their hydrophilic nature.[6] Also, due to their hydrophilic surface the hydrogels have a low interfacial free energy, which results in a low tendency of proteins and cells to adhere to the hydrogel surface.[7] Another advantage of hydrogels in respect to biocompatibility is that due to their soft and rubbery nature, the irritation in the surrounding tissue is minimal.[6–8]

Classification

There are several of different ways that can be used to classify hydrogels. However, the most common ones are according to their:[5]

- Source (natural or synthetic);
- Configuration (amorphous, crystalline, semi-crystalline);
- Physical appearance, which depends on the hydrogel preparation process. The most common appearances are matrices, microspheres and films;
- Composition, depends on the number of monomer used, as represented on the Figure 1.3, (a) homopolymers, (b) copolymers or (c) an interpenetrating polymer network (IPN), when a hydrogel is comprised of two independent crosslinked polymer components contained in a network form;
- Electric charge, which is based on the presence or lack of electrical charges on the crosslinked chains. Using this criterion, the hydrogels can be considered ionic, non-ionic or neutral, amphoteric (if the hydrogel contains both acidic and basic groups) or zwitterionic (when each structural unit contains both anionic and cationic groups).

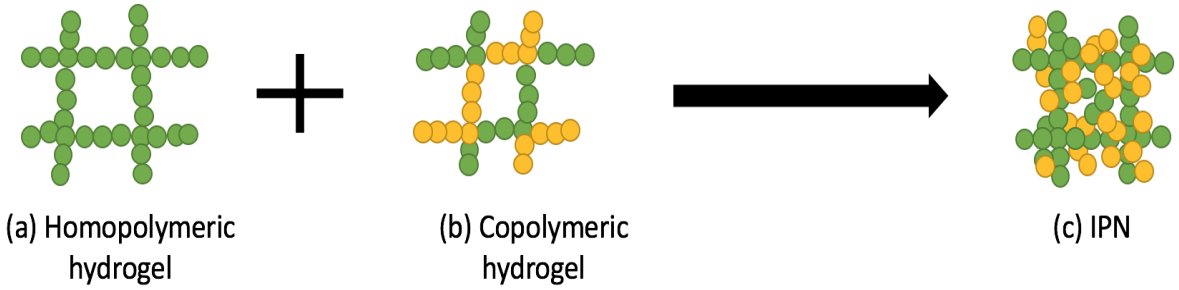


Figure 1.3 - Possible Hydrogel Compositions: (a) homopolymer; (b) copolymer and (c) IPN

Hydrogel formation

As previously mentioned, any technique that has the ability to generate a crosslinked polymer can be used to prepare a hydrogel. Given that, a hydrogel is a crosslinked polymer in which gelation occurs. According to the Flory theory, this occurs in the moment when, on average, each primary chain contains at least one crosslink point.[9, 10]

Although almost all polymerization techniques have the potential to form a hydrogel, there are some that are more commonly used such as the graft polymerization, network formation of water-soluble polymer and radiation crosslinking. [4, 7]

Depending on the crosslinker used, the crosslinking reaction can be chemical (covalent interaction) or physical (non-covalent interaction), which will lead to hydrogels with distinct properties.

The chemical crosslinking ((a) in Figure 1.4) results in permanent covalently linked bonds, and can be obtained using a crosslinking agent that will react with the monomers during the polymerization, or with a polymer that was previously synthesized. These bonds, due to their covalent nature, are stronger than the non-covalent bonds, meaning that the chemically crosslinked hydrogels will be mechanically more stable.[9]

On the other hand, physical crosslinking will bond the polymer chains by non-covalent interactions. In this case, the hydrogels have junction zones that are formed when separate polymer chains interact over a certain length. Commonly, a physical crosslinking ((b) in Figure

1.4) can be obtained by hydrophobic interactions, in which hydrophilic blocks are attached to hydrophobic blocks generating an amphiphilic hydrogel; electrostatic interactions, that occur between two opposite charged polymers or a polymer and a small molecule, and hydrogen bonds. In this case, there is no need to use a crosslinking agent, which may interact with substances, usually drugs, trapped on the hydrogel.[9]

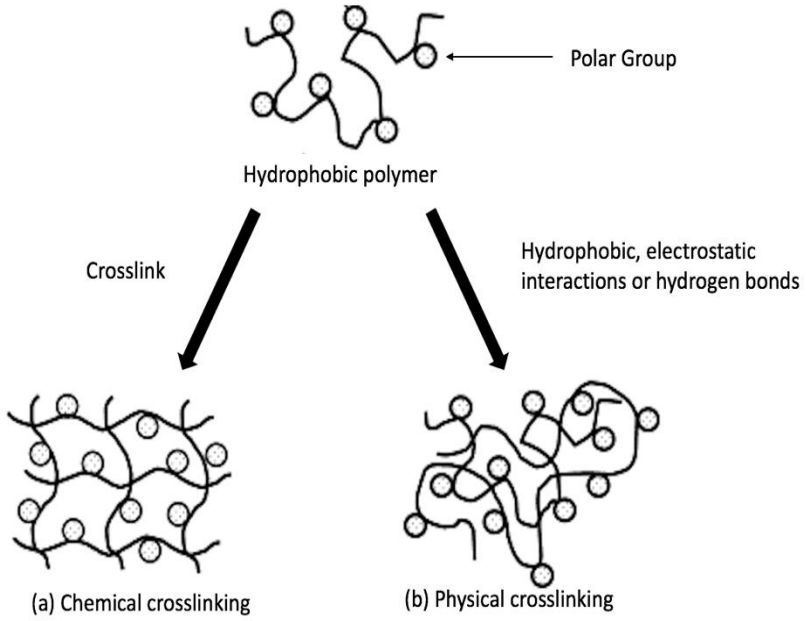


Figure 1.2 – Representation of the network structure resulting from the two different types of crosslinking ((a) chemical and (b) physical).[15]

Swelling

The most important property of a hydrogels is the swelling, which is also named absorption capacity, corresponding to the amount of water that the hydrogel can retain in its structure. Usually, hydrogels can absorb water up to hundred times their dry weight.[11] The swelling capacity of hydrogels turns them into very attractive materials for different applications. Several studies have been conducted to understand the swelling behavior and how this parameter affects the performance of the hydrogel.[12] It has been proposed that the swelling occurs due to the presence

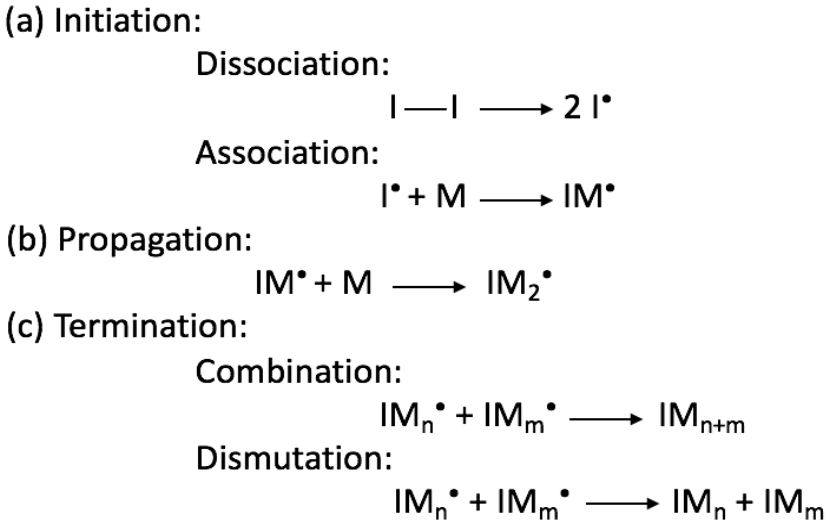
of hydrophilic groups such as the -OH-, CONH-, -$CONH_2$-, and –SO_3H.[11, 13] This conclusion was supported by results showing that the increase of the number of these chemical groups led to an increase of the swelling equilibrium of the hydrogels. In addition, the swelling behavior is also influenced by the crosslinking density. An increase of this parameter leads to the reduction of the amount of water that can be absorbed and also a decrease of the stretchability of the network. As a result, the swelling equilibrium of hydrogels will decrease.[6] Regarding the molecular weight, a higher value will afford hydrogels with a higher swelling ratio (ratio between the weight of the hydrogel in the swollen state and that in the dry state). Network charge, among many other factors can also influence the swelling capacity of hydrogels.[11]

Another factor that sometimes can influence the swelling behavior is the external medium. This can occur when the hydrogel is formed by a smart polymer, which will change its physical-chemical properties as a response to small variations on the surrounding medium.[8]

The swelling mechanism has been described in different studies available in the literatures.[7, 14, 15] When the hydrogel is hydrated by the first time (e.g., in water) the polar hydrophilic groups are also hydrated leading to the formation of “primary bound water”. This causes the network swelling and the exposure of the hydrophobic groups, that are also able to interact with water, leading to the generation of hydrophobically-bound water, also called ‘secondary bound water’. Usually these two types of bonded water are combined in the same term as ‘total bond water’. After the saturation of both hydrophilic and hydrophobic groups with water, the hydrogel will absorb more water, the so-called ‘free water’ or ‘bulk water’. Due to the osmotic pressure, this absorption is opposed by the already formed crosslinks, either physical or covalent, which leads to an elastic network retraction force, meaning that the hydrogel will reach a swelling equilibrium. This additional water will fill the space between the network chains, voids, or macropores. When the hydrogel is degradable, it will disintegrate and/or dissolve, depending on its nature and composition, after the swelling equilibrium. [7, 14, 15]

1.2. - Free radical polymerization

Free radical polymerization (FRP) (Scheme 1.1) is a chain-growth polymerization method, in which the polymer is obtained from the consecutive addition of vinyl monomer units. The polymerization starts with the initiation step (Scheme 1.1 (a)), where the first radicals are created from non-radical species (conventional initiators, which are always needed in this type of polymerization). This reaction is divided in two phases: (i) homolytic scission of the initiator, which can occur by action of many factors (e.g., heat, light or redox reactions), leading to the formation of two radicals per initiator molecule; (ii) addition of one molecule of monomer to the radical that results from the first step of the initiation. The next stage of the polymerization is the propagation (Scheme 1.1 (b)), where several monomer molecules are continuously added to the polymeric chain with an active radical. Finally the termination (Scheme 1.1 (c)) of polymeric growing chains can occur by atom transfer (chain-transfer), atom abstraction (termination by disproportionation) or by combination of two active chains (termination by combination).[16, 17]



Scheme 1.1 - General radical polymerization mechanism

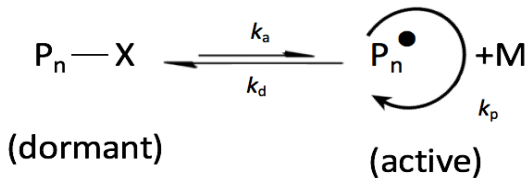
This type of polymerization is an extremely important method for the synthesis of many commercial polymers with high molecular weight, since it can be used with a wide range of monomers. It can also be conducted in different reaction conditions (bulk, suspension and

emulsion) and it is a robust method, meaning that it is less sensitive to solvents, impurities and atmospheric conditions than other methods. However, FRP has a few limitations, being the main one the limited control of certain structural properties of the polymers, such as the molecular weight, the dispersity, the composition and the functionality of the polymeric chains. This cannot be achieved due to the short life-time of the active propagating chains (~1s).[16, 17]

In the last two decades, new polymerization techniques based on reversible-deactivation radical polymerization (RDRP) have been developed in both industrial and academic environments, in order to address the problems associated with the FRP technique.

1.3. – Reversible deactivation radical polymerization

RDRP is a group of polymerization techniques that allow the synthesis of polymers with a narrow molecular weight distribution ($Đ < 1.5$) and several types of chain architectures with well-defined composition.[18] All RDRP techniques are based on the same principle: the maintenance of a very low concentration of radicals during the polymerization by an equilibrium between active (propagating radical) and dormant species (Scheme 1.2).[19]



Scheme 1.2 – General Dormant/Active species equilibrium in RDRP

In these systems, the initiation is very fast, when compared to propagation. Therefore, the initiator species are activated in the beginning of the polymerization, resulting in an apparent growth of all chains at the same time.[18] The propagation step is very similar to that of FRP, with the addition of monomer units to the propagating radicals. However, in the RDRP systems, due to the equilibrium between active and dormant species, the life-time of a propagating radical can be extended to hours (even days), in contrast with the short life-time that the radicals possess in FRP (≈ 1s).[19] Every 0,1-10 ms, propagating radicals are reversibly deactivated in RDRP, which

decreases the probability of radical termination reactions about 90% when compared with FRP systems. As a result, it is possible to control the polymers molecular weight. Nevertheless, termination reactions may also occur in these systems. However, as that the total number of chains is much greater than in FRP, the effect of termination can be considered negligible. As the result of the simultaneous growth of all chains and the absence of termination reactions, the number of propagating polymer chains during the polymerization is constant. Therefore, it is expected that the polymers molecular weight increases linearly with the monomer conversion. Additionally, polymers prepared by RDRP present active chain-ends (dormant chains), which can be reinitiated with further monomer supply to form complex structures, such as block copolymers.

To attain a good control over molecular weight, dispersity and chain architecture on these systems is required a nearly instantaneous initiation, as previously mentioned, and a fast exchange among active and dormant species, meaning that ideally a growing species should interact with a few monomer units before being deactivated.

Another fundamental part of this type of systems is the constitution of a dynamic equilibrium between propagating radicals and dormant species. Depending on the technique, the radicals may either be deactivated by a degenerative exchange process, as it is in the case of reversible addition-fragmentation chain transfer (RAFT), or by an atom transfer, which is the case of stable free radical polymerization (SFRP) and atom transfer (ATRP) radical polymerization.

Processes based on atom transfer rely on the persistent radical effect (PRE), which is a kinetic feature that provides a self-regulating effect in some RDRP systems, inducing a shift towards the dormant species. In these systems, there is an accumulation of the deactivator species (stable radical in SFRP or organometallic complex in ATRP) due to a small fraction of terminated chains at the beginning of the polymerization. These “persistent radicals” can only cross-couple with the growing species. As a consequence of this accumulation the concentration of radicals and the probability of termination decrease with time.[18, 20]

On the other hand, systems that employ degenerative transfer (e.g., RAFT) are not based on PRE, which means that they have slow initiation and a fast termination, as a regular radical polymerization. In this type of system, the concentration of transfer agent is much larger than

that of radical initiators, therefore the transfer agent is considered the dormant species, and the radicals that can terminate or degeneratively exchange with dormant species consume the monomer.[18, 20]

From all RDRP techniques, ATRP is one of the most studied methods and it will be used in this work for the preparation of new BAS.

1.3.1 - Atom transfer radical polymerization

ATRP is a polymerization technique which was developed based on the widely used organic synthesis reaction, atom transfer radical addition (ATRA). This reaction occurs by an atom transfer from an organic halide (P_n-X) to a transition metal complex, forming organic radicals which are rapidly deactivated by back-transfer of the atom from the transition metal to the organic radical species.[18]

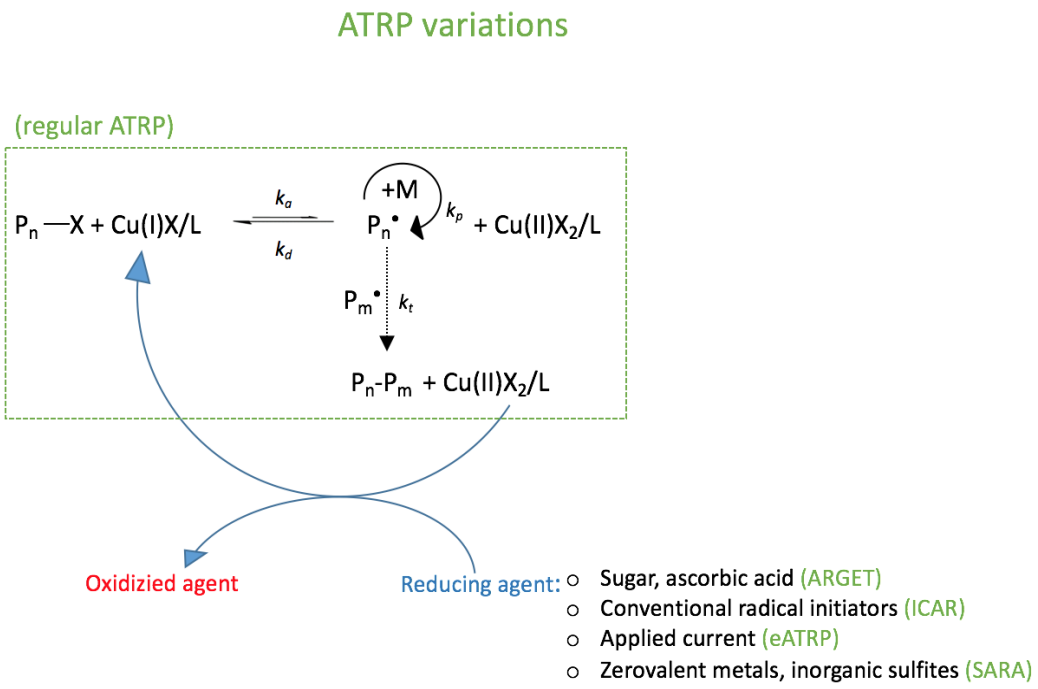
The ATRP involves the use of a transition metal/ligand catalytic complex, and an alkyl halide initiator. The ligand is responsible for the dissolution of the catalyst in the reaction solvent and for the regulation of the catalyst activity. Mecanistically, ATRP (scheme 1.3) proceeds via homolytic cleavage of the initiator, by the catalytic complex in the lower oxidation state, which generates an organic radical and the corresponding higher oxidation state catalytic complex. The radical can then propagate by adding several monomer units, before being reversibly deactivated by the transition metal complex to form a halide-capped dormant polymer chain.[21-23]

ATRP has been the most used RDRP technique due to the ability to polymerize several monomer families, in mild reaction conditions, and the fact that the required reagents are accessible (usually commercially available).[24] The main disadvantage of this technique is the high concentrations of transition-metal catalysts (primarily copper halides) that are required and that will end up in the products. Due to this, the removal of the catalyst after the polymerization becames a necessity.[25] To adress this problem several variations of ATRP were developed (Scheme 1.3), in which the Cu(I) activator species are generated *in situ* due to the combination of Cu(II) with reducing agents, including activators regenerated by electron transfer (ARGET) [26], initiator for continous activator regeneration (ICAR)[26], electrochemically-mediated ATRP

(eATRP)[27] and supplemental activator and reducing agent (SARA)[28] techniques. Recently a metal-free ATRP was also developed.[29] The distinction between methods is the type of reducing agent used, as presented in Scheme 1.3.

On this work, the SARA ATRP was used for the preparation of hydrogels to be used as BAS (Scheme 1.3). This system has the traditional reactions of activation by Cu(I) and deactivation by Cu(II), at the core of the process as a classical ATRP system. In addition Cu(0) is used as a reducing agent for the regeneration of the activator species (through comproportionation with Cu(II)) and as a supplemental activator of alkyl halides.[28]

The SARA ATRP was successfully used on the polymerization of several monomers (e.g., styrene and vinyl chloride)[30] and in eco-friendly solvents (e.g., ethanol/water mixtures or even aqueous medium)[31]



Scheme 1.3 - Different ATRP variation techniques and the reducing agents used on each one of the techniques.

1.3.2 – Reversible-deactivation *vs*. free radical polymerization – differences

FRP and RDRP present analogous chemo-, stereo- and regio-selectivities, being able to polymerize a similar range of monomers using a similar radical mechanism. However, they present six core differences that are listed below: [18]

- The life-time of a growing chain is extended from approximately one second in FRP to more than one hour in RDRP, due to the reversible activation/deactivation of radicals;
- On FRP, the initiation is slow in opposition to the very fast initiation needed in RDRP;
- In FRP, nearly all chains are dead in contrast to the less than 10% of dead chains in RDRP;
- RDRP is usually slower than FRP, but the polymerization rate can be comparable when the target molecular weight is relatively low;
- A steady state of radicals is established in FRP due to similar rates of initiation and termination, whereas in RDRP systems that is due to the balance of activation and deactivation rates;
- In FRP, the termination normally occurs between long chains and constantly generated chains. In RDRP systems based on PRE, the termination rate decreases significantly with time due to the lengthening of the chains, whereas in the systems based on degenerative transfer, the termination is more likely through the reaction due to the constant generation of new chains.

1.4 - Bile acid sequestrants

1.4.1 – Bile acids

Bile acids are negatively charged amphiphilic end-products of the cholesterol catabolism. These molecules possess several functions, being the most well-known the ability to promote the absorption of lipids in the intestine, due to their action as emulsifiers, aiding in the food digestion. In addition, they also serve as signaling molecules and metabolic regulators, controlling the breakdown of glucose and triglycerides and regulating the cholesterol homeostasis.[32] However, the amphiphilic nature of bile acids which makes them powerful solubilizers of the lipid membrane, is also responsible for their cytotoxic character when in high concentrations. It has also been observed that, in adults, an efficient intestinal conservation of bile acids leads to the down-regulation of both the cholesterol biosynthesis and the LDL-receptor activity, which causes an increase of the plasmatic LDL cholesterol levels.[33]

The synthesis of bile acids, which consists in a series of enzymatic reactions that metabolize cholesterol, accounts for nearly 50% of the daily turnover of cholesterol. This process occurs exclusively on the liver, mainly in the perivenous hepatocytes, which are, the cells surrounding the central hepatic vein.[34] This pathway is indispensable for the regulation of cholesterol homeostasis and for the prevention of the accumulation of cholesterol, triglycerides and toxic metabolites, and for the prevention of injuries in the liver and in other organs.[32] This process leads to the formation of the primary bile acids (Figure 1.5 (a) and (b)), that in humans, due to the action of the intestinal bacterial flora will generate the secondary bile acids.

There are two major bile acids biosynthetic pathways, the neutral and the acidic pathway. On the neutral pathway, the modification of the steroid ring precedes the side-chain cleavage. On the acidic pathway, the side chain cleavage precedes the steroid ring modification. On the neutral pathway, the first and rate-limiting step is the hydroxylation of cholesterol, catalyzed by the enzyme CYP7A1, which will generate bile acid intermediates. These intermediates are converted into the primary bile acids (cholic acid (a) and the chenodeoxycholic acid (b) in Figure 1.5) in a

process governed by the enzyme CYP8B1, without which the liver would only generate chenodeoxycholic acid. On the other hand, the acidic pathway, that is responsible for less than 10% of the total bile acids synthesis, is initiated by the enzyme CYP27A1, that unlike the CYP7A1 is not regulated by the bile acids.[32, 34]

In the distal intestine, these two bile acids are converted in the secondary bile acids, the deoxycholic (Figure 1.5 (c)) and the lithocholic (Figure 1.5 (d)) acids respectively, due to the action of the bacterial 7-α hydroxylase.

Before their secretion into the bile canalicular lumen for storage in the gallbladder as mixed micelles (with cholesterol and phospholipids), the bile acids are amino-conjugated at the carboxyl group with glycine or taurine. This results on the increase of their ionization and solubility at physiological pH, the prevention of the formation of Ca^{2+} precipitates, the minimization of passive absorption and makes the bile acids resistant to cleavage by pancreatic carboxypeptidases. [32, 34]

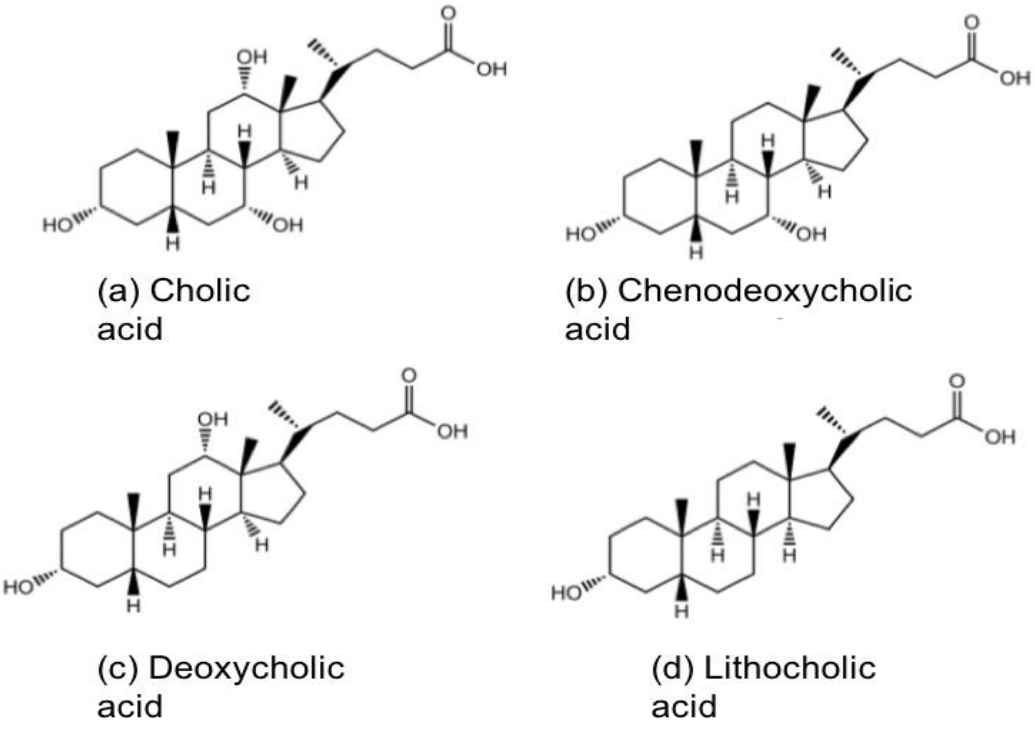


Figure 1.5 - Bile acids produced in the human body: (a) cholic acid; (b) chenodeoxycholic acid; (c) deoxycholic acid; (d) lithocholic acid

1.4.2 – Enterohepatic circulation and BAS mechanism of action

The enterohepatic circulation (Figure 1.6) refers to the circulation of bile acids and other substances from the liver to the gallbladder, followed by their reabsorption on the small intestine, transport across the enterocyte and return to the liver.

After the ingestion of a meal, the gallbladder releases the bile via the bile duct into the gastrointestinal lumen, with the intent of helping the digestion of lipids. Then these bile acids are reabsorbed, a small amount due to passive diffusion in the upper intestine but the most part in the brush border membrane of the terminal ileum. In the later case the bile acids are transdiffused across the enterocyte to the basolateral membrane being secreted on the portal blood circulation to be taken to the liver.[32, 35] In humans, this process is very efficient allowing the reuse of the bile acids between 4 to 12 times a day. However, there is a small amount that is lost through fecal excretion (approximately 0,5 g/day), which is compensated by a new synthesis of bile acids by the liver, in order to maintain a constant concentration of bile acids in the pool.[32]

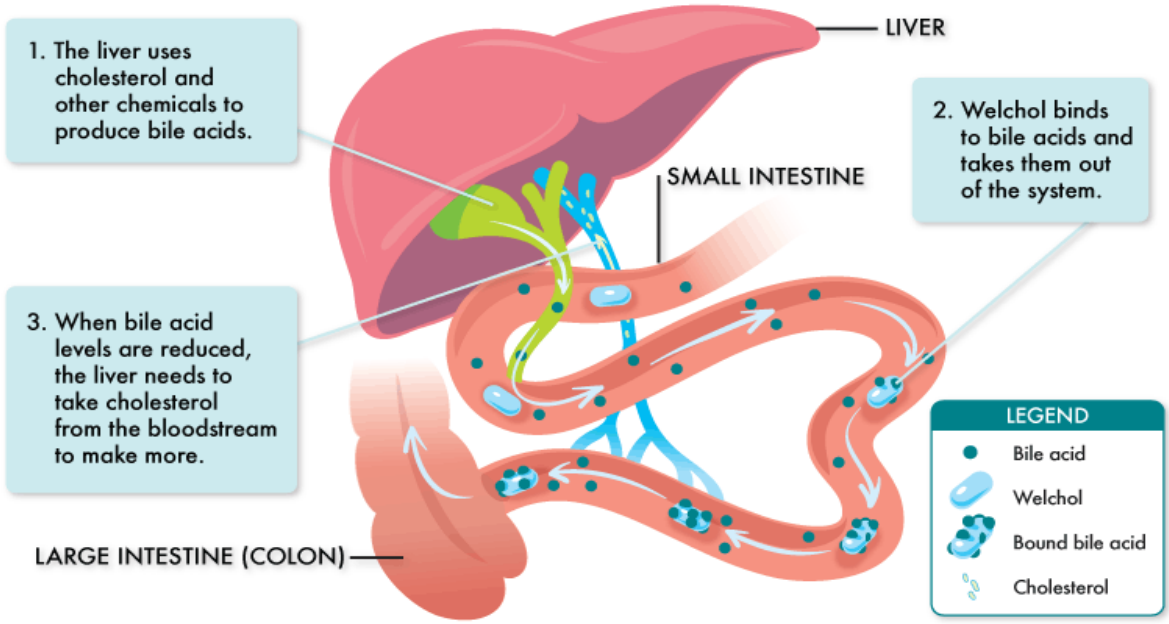


Figure 1.6 – Bile acid sequestrant (Welchol) effect on the enterohepatic circulation[36]

BAS bind the bile acids mainly by both electrostatic and hydrophobic interactions forming an insoluble complex, which leads to the decrease of the absorption of the bile acids on the intestine and the consequent increase of the fecal bile acid excretion. Chemically speaking, a bile acid is constituted by a hydrophobic core and a hydrophilic anionic segment. Therefore, an efficient BAS should have positive charges within its structure and a maximized hydrophobicity to promote the binding process. In addition, BAS should have sufficient hydrophilicity to provide suitable swelling properties in physiological environment. Besides these requirements a BAS, must also have an appropriate density of cationic charges in order to ensure the occurrence of electrostatic interactions with the bile acids, a long aliphatic chain to ensure the occurrence of hydrophobic interactions with the bile acids steroid skeleton. In addition, polymers containing a structure that can complement the bile acids architecture, have shown to favor the binding process.[37]

1.4.3 – Commercial BAS

BAS are a class of polymeric drugs that bind bile acids in the intestine forming a nonabsorbable complex, which leads to the interruption of the enterohepatic circulation.[38] This interruption leads to an increase of the bile acid synthesis through an up-regulation of cholesterol 7-α-hydroxylase hepatic enzyme, decreasing the cholesterol portion of the intrahepatic content, motivating a demand of cholesterol in the hepatocytes. This demand is quelled due to an augment in new synthesis of cholesterol, which means a significant increase of the HMG-CoA reductase activity and of the expression of LDL receptors, thereby decreasing the plasma level of LDL cholesterol.[39]

Due to the presence of negative charges on the bile acids, BAS are usually based on cationic polymeric hydrogels.[40] BAS have been used to reduce LDL-cholesterol since 1970's. Comparatively to other treatments, namely the use of statins, they present a lower efficiency but a higher degree of safety, since they are not absorbed or metabolized on the intestine (no systemic exposure). However, they can cause a decrease on the absorption of some other drugs as well as gastrointestinal disturbances, such as nausea, constipation or abdominal pain.[38]

There are five BAS commercially available at this moment. In the US, there are Cholestyramine (Figure 1.7 (a)), Colestipol (b) and Colesevelam hydrochloride (c), in Japan there is the Colestilan (d) and in Spain and Italy, the Colextran (e).[41]

Cholestyramine and the Colestipol are considered the first generation of BAS. They both bind and remove preferentially dihydroxy bile acids from the bile acids mixture. However, the liver does not produce only this type of bile acid. Indeed, it also produces trihydroxy bile acids, which are mostly left free by these BAS. As a consequence, there is an increase of the quantity of trihydroxy bile acids on the bile acid pool, leading to the decrease of the efficiency of these BAS with the time. Nevertheless this is not the only reason for a low efficiency on the first generation of BAS, being that there is also the competition between the BAS and the active bile acids reuptake transporter system of the gastrointestinal tract.[24]

In order to overcome this limitation, the development of new BAS with a greater binding strength and affinity towards bile acids was needed, which lead to the creation of a second generation of BAS, which includes both Colestilan and Colesevelam hydrochloride.

Colestilan is a BAS with an imidazolium salt on an epoxide polymer skeleton, which has demonstrated more efficient binding of bile salts than Cholestyramine. It has also been shown that Colestilan has a lower rate of adsorption than Cholestyramine for a large number of drugs, meaning that Colestilan has a lesser effect on the absorption of these drugs than Cholestyramine.[42]

The chemical structure of Colesevelam includes spaced long hydrophobic chains with protonated primary amines. These groups are responsible for the establishment of electrostatic interactions between the polymer and the bile acids, while the quaternary amines stabilize the hydrogel. In addition, the crosslinked epichlorohydrin-based structure reduces the chance of systemic absorption, while decreases the interactions with the gastrointestinal tract lining, and improves the water retaining characteristics. As a result, Colesevelam presents less side effects than the first generation BAS, being these mainly minimal constipation and gastrointestinal irritation.[38] Unlike what happens with the first generation of BAS (that has affinity mainly to dihydroxy bile acids), Colesevelam has affinity to both dihydroxy and trihydroxy bile acids, which improves the efficiency in long term treatments.[37, 39, 43]

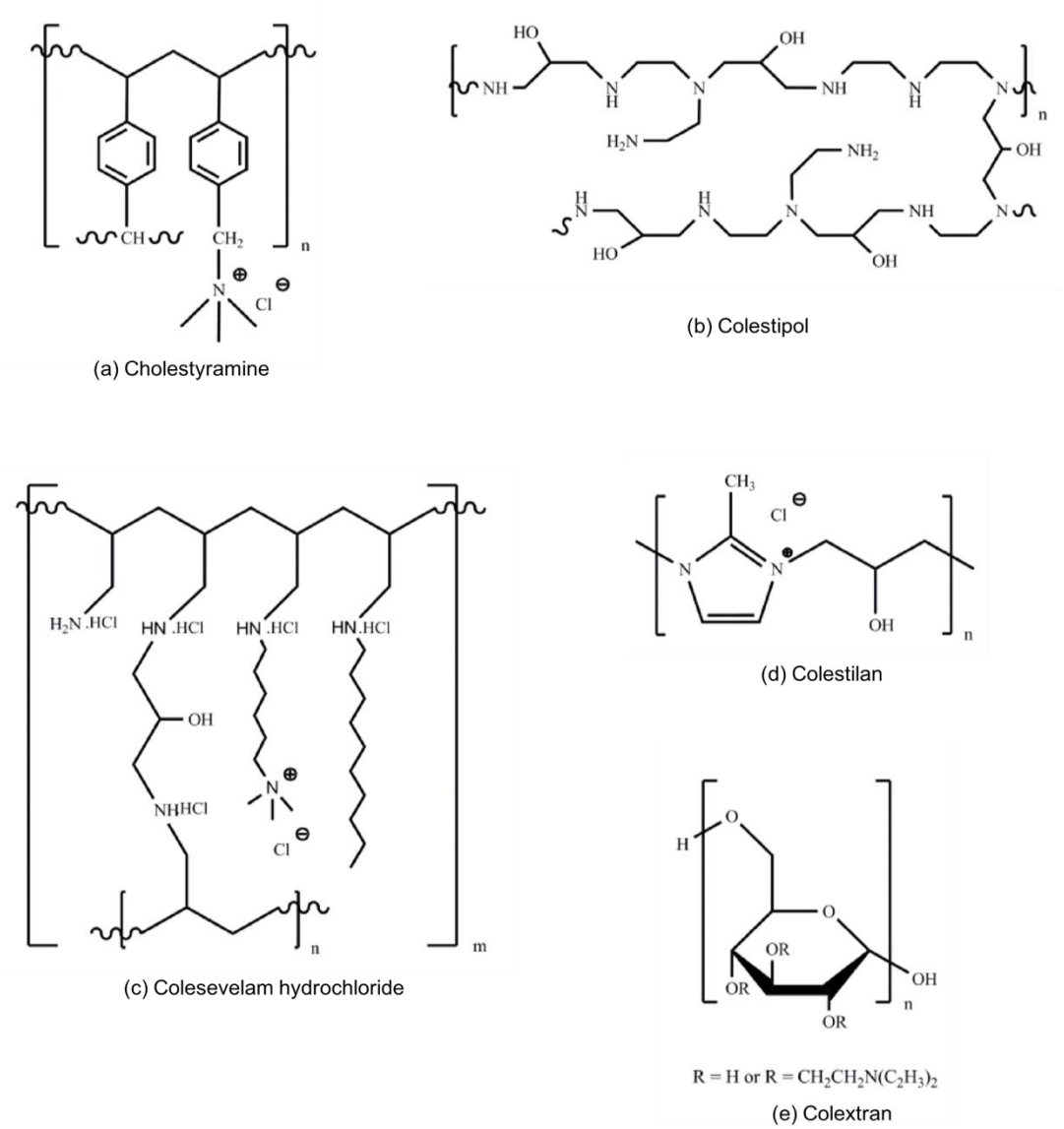


Figure 1.7 - Commercially available BAS: Cholestyramine (a); Colestipol (b); Colesevelam hydrochloride (c); Colestilan (d) and Colextran (e)

Despite the improvement from the first to the second generation of BAS, it is necessary further developments in order to boost the specificity of the BAS towards bile salts, which would lead to a reduction of the undesirable side effects and of the therapeutic dose, while improving the patient compliance and the therapeutic efficiency.

Besides their properties relative to the control of the cholesterol levels, BAS possess other benefits for the health, being one of the more significant their ability to reduce the fasting blood glucose and to decrease hemoglobin A1c in diabetic patients.[44]

1.4.4 – Case-studies reported on literature

In addition to the five polymeric BASs that are already commercially available, there are several others that are being developed and tested, both *in vitro* and *in vivo,* for the binding of bile acids molecules. From all the proposed materials (dietary fibers, antacids, charcoal and various polymers)[37], the most studied ones are the polymers due to their large range of properties, specially the cationic hydrogels considering the anionic nature of the bile acids.

There are three major approaches for the synthesis of novel BAS: the synthesis or modification of polymers based on commercial-like BAS; the bio-conjugation of polymers with bile acids and the molecular imprinting.

The most commonly used approach is the synthesis or modification of commercial-like BAS polymer backbones. Using this approach, Figuly and colleagues[45] synthesized several poly(alkylamine)-based polymer networks, prepared by the reaction of numerous (di)amines with dihalo compounds or diepoxides, in different solvents. The preliminary *in vitro* binding experiments showed that the hydrogels created by diamines and dibromoalkanes were more efficient than Cholestyramine in the binding of methyl cholate. Based on the same approach, Mendonça *et al.*[46]*,* prepared poly(((3- acrylamidopropyl)trimethylammonium chloride)-based hydrogels and star block copolymers by SARA ATRP, to study the effect of several polymeric characteristics (e.g., length of the cationic segment) on the BAS binding efficiency. The *in vitro* experiments showed that an increase of the cationic density of the polymers lead to an increase of the binding efficiency, indicating that the electrostatic interactions play an important role on the binding process. Regarding the structure of the polymers both hydrogels and block copolymers present similar cooperative binding isotherms, yet the hydrogels showed a higher binding capacity. The results also showed that, probably due to the high density of positive

charges (derived from the PAMPTMA segment), the materials exhibited higher affinity towards sodium cholate (NaCA) micelles ($[NaCA]_0 > 7$ mM), rather than unimers.

Zhang *et al.*[47] used an approach based on the bio-conjugation of the polymer with the bile acids, which according to the authors would lead to an enhanced binding capacity due to Van der Waals and/or hydrogen bonding interactions with the nearest neighbors, meaning that the bile acids were either attached to the polymer backbone or chemically modified in order to produce polymerizable compounds. In this case particles of poly(styrene) were crosslinked with divinylbenzene, then 0-15% of the choloromethylene groups were quaternized by the aminated derivatives of cholic acid and the remaining were quaternized as ammonium groups, by using triethylamine. The binding capacity of the BAS was evaluated in the presence of sodium taurocholate recurring to ultraviolet spectroscopy. It was found that the binding capacity increased with the increase of the content of tertiary amine derivative of cholic acid in the polymer matrix.

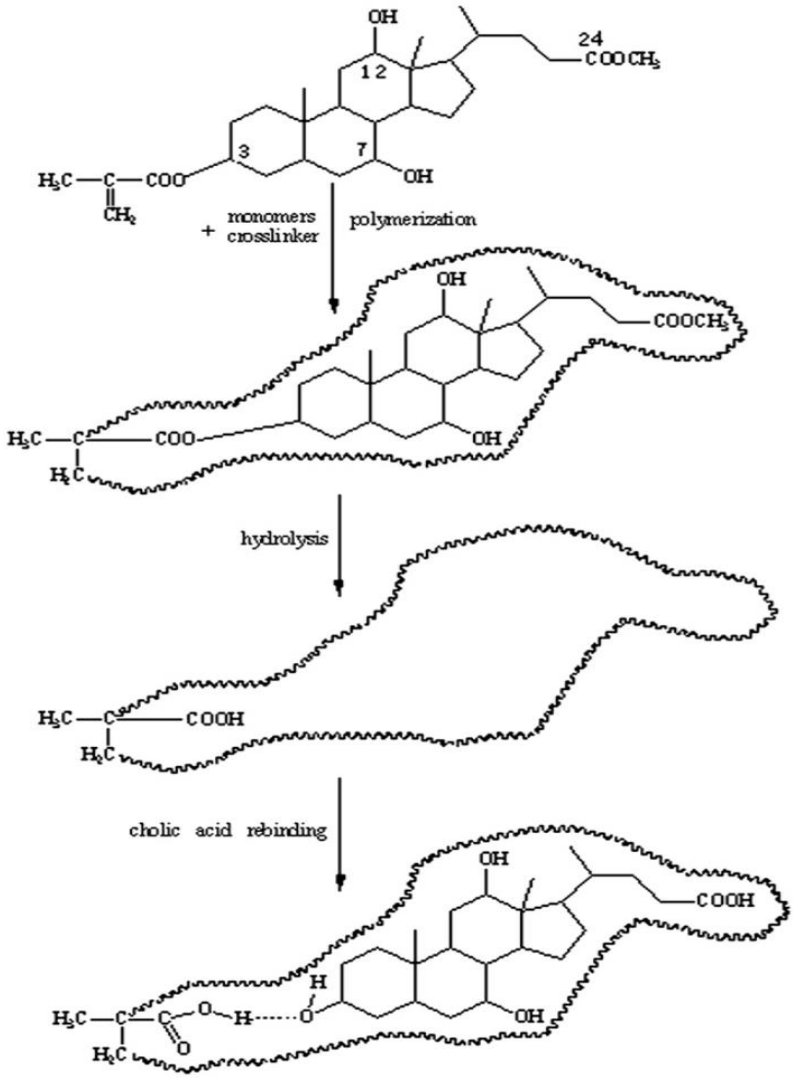
The molecular imprinting is the most recent approach followed for the preparation of polymeric BAS. This technique allows the connection of functional monomers to a polymer backbone in the presence of template molecules. From the interaction between the functional groups or chemical structures present on the monomer and the template molecules a pre-polymerization complex can be generated. The type of interactions (covalent or non-covalent) established during this process is the main difference between the two molecular imprinting techniques. After the reaction, the template molecules must be removed from the polymer, which leads to the formation of specific cavities. The removal of the molecules may occur by chemical cleavage or by solvent extraction via diffusion, depending on the type of interaction established between the monomer and the template. Due to the complexity of the first method the non-covalent systems are the more widely used, even showing lower efficacy when compared with the covalent systems.[48] One possible method to increase the BAS efficiency, is the use of bile acids as template molecules. This would create artificial recognition cavities in the BAS, which would enhance the binding forces during the sequestration process, allowing the BAS to compete with the desorbing forces that exist in the GI tract and thus preserving the bile acid molecules in the polymer matrix. Wang and co-workers[49] used a hybrid imprinting method

(Scheme 1.4) (a covalent template structure was used but the binding was entirely non-covalent) to prepare polymers that bind specifically the cholic acid, due to the creation of cavities with well-positioned carboxylic groups for the binding of cholic acid via hydrogen bonding. They prepared several polymers based on 3α-methacryloyl cholic acid methyl ester (a template-containing monomer) crosslinked with ethylene glycol dimethacrylate. The *in vitro* experiments showed that the BAS produced using this method possessed a slightly lower binding capacity towards cholic acid in aqueous medium, comparatively to BAS produced without template. However, when ethyl acetate was used as solvent the imprinted BAS presented a much higher binding capacity, than the control BAS. It was also reported that the binding capacity increased with the decrease of the polarity of the solvent used on the binding tests. In another work, Huval and co-workers[50] used a non-covalent molecular imprinting approach to enhance the BAS binding capacity, due to the creation of complementary binding sites to the carboxyl groups present on the bile acids. The authors prepared several polymer networks based on poly(allylammonium chloride) crosslinked with epichlorohydrin, using NaCA as the template molecule. Both *in vitro* and *in vivo* experiments were in agreement with previous results found in the literature, showing that the imprinting technique could be used for the preparation of polymers with increased binding capacity. In addition, the results showed that the binding capacity increased with the increase of the cholic acid template content used for the imprinting. Even though there are several papers that report that the use of molecular imprinting increases the BAS efficiency, there are some studies that show that this approach is not useful in some circumstances. Using a computational modelling of the affinity of imprinted polymers towards bile acids, also supported by experimental work, Yañez and colleagues[51] showed that the molecular imprinting technique is not very effective for polymers that already possess a high affinity towards the template molecules.

The studies reported in the literature provided valuable information concerning the variables that influence the BAS binding capacity, and thus must be taken into account for the BAS design:

- Cationic groups: the presence of positive charges on the polymer structure ensures the occurrence of electrostatic interactions, which are the main mechanism of the binding process involving ionized bile salts;

- Degree of crosslinking: lower crosslinking densities lead to enhanced swelling ratios, which typically leads to higher binding capacities;
- Polymers shape: the use of appropriate polymer structures that complement the bile salts architecture can favor the binding process.



Scheme 1.4 - Preparation of the cholic acid-imprinted polymer based on poly(3α-methacryloyl methyl ester).[49]

1.5 – Strategy followed on this work

The aim of this work was the development of polymeric BAS that possess a similar or better efficacy than the most efficient BAS commercially available (Colesevelam). This work was also envisaged to enable the study of the effects of different polymerization techniques on the BAS efficacy.

The project was designed based on the work developed in the research group, in which PAMPTMA-based hydrogels showed a maximum binding capacity close to that of Colesevelam.[46] Aiming to increase the binding capacity of these materials and based on the information found in the literature[47,49], it was hypothesized that the addition of poly(hydroxyethyl acrylate) (PHEA) to the mentioned hydrogels, could provide additional hydrogen bonding between the bile acids and the BAS.

A bile acid sequestrant is considered commercially viable if the production is reproducible at an industrial scale. Taking this in consideration, the polymerization techniques employed on this work should be relatively simple and easily applied at large scale production. Therefore, it was decided to use FRP for the preparation of the BAS. The SARA ATRP was also used for comparison purposes, since the original PAMPTMA-based hydrogels were synthesized by this technique.[46]

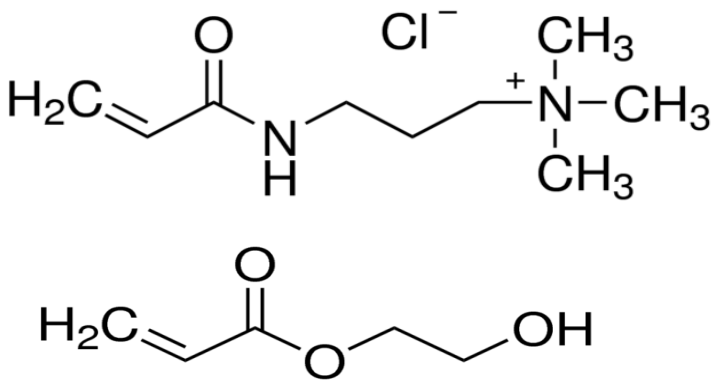


Figure 1.8 - Chemical structure of the monomers used on this work: AMPTMA (top) and HEA (bottom).

2 – Materials and methods

2.1 – Materials

Acetonitrile (high-performance liquid chromatography (HPLC) grade, Fisher Chemical), AMPTMA (solution 75 wt. % in H_2O, Aldrich), alumina (basic, Fisher Scientific), 1,4 butanediol diacrylate (BDDA, 99+%, Alfa Aesar), $CuBr_2$ (Acros, 99+% extra pure, anhydrous), $CuCl_2$ (99% Sigma Aldrich), NaCA (99% Acros Organics), deuterium oxide (99.9%, Cambridge Isotope Laboratories), dimethylformamide (DMF, Sigma-Aldrich, +99.8%), ethyl α-bromoisobutyrate (EBiB) (98%, Sigma-Aldrich), potassium dihydrogen phosphate (Merck), ethanol (99.5%, Panreac), ethyl 2-chloropropionate (ECP) (97%, Aldrich), hydrochloric acid (37%, Fisher Scientific), *N*,*N*,*N*′,*N*′′,*N*′′-Pentamethyldiethylenetriamine (PMDETA) (Aldrich, 99%), pancreatin from porcine pancreas (Sigma), pepsin (Sigma Aldrich), phosphoric acid (85 % Fisher Scientific), potassium hydrogen phosphate (Merck), sodium chloride (99.5%, Acros Organics), tetrabutylammonium hydroxide 30-hydrate (≥ 99.0%, Sigma-Aldrich), tris(2-aminoethyl)amine (TREN) (96%, Aldrich) and 2,2′-azobis(2-methylpropionitrile) (AIBN) (98%, Sigma-Aldrich) were used as received.

Colesevelam hydrochloride was kindly supplied by Bluepharma and it was used as received.

HEA (Sigma-Aldrich; ≥99%) was purified by first dissolving the monomer in water (25% by volume). The solution was extracted with hexane (6 times) to remove the diacrylates. The aqueous solution was salted (250 g/L NaCl) and the monomer was then separated from the aqueous phase by ether extraction (4 times). Hydroquinone (200 ppm) was as stabilizing agent to inhibit thermal polymerization. The organic solvent was removed under reduced pressure The purified monomer was kept in a fridge and immediately prior to use, it was passed through a sand/alumina column, in order to remove the radical inhibitor.

Tris[2-(dimethylamino)ethyl]amine (Me_6TREN) was synthesized according the procedures described in the literature.[52]

Metallic copper (Cu(0), *d* = 1 mm, Sigma Aldrich) was washed with HCl in methanol and subsequently rinsed with methanol and dried under a stream of nitrogen following the literature procedure.[53]

Purified water (Milli-Q®, Millipore, resistivity >18 MΩ.cm) was obtained by reverse osmosis.

Simulated gastric fluid (SGF) was prepared by dissolving sodium chloride (50 mg) and pepsin (80 mg) in hydrochloric acid (175 µL). The volume was adjusted to 25 mL with water to give a solution with pH = 1.2.

Simulated intestinal fluid (SIF) was prepared by dissolving potassium hydrogen phosphate (101.5 mg), potassium dihydrogen phosphate (91 mg) and pancreatin (250 mg) in water (25 ml). The pH was adjusted with 0.2 N NaOH or 0.2 N HCl to 6.8.

2.2 – Equipment

PAMPTMA and PAMPTMA-*co*-PHEA samples were analyzed by a size exclusion chromatography (SEC) system equipped with an online degasser, a refractive index (RI) detector and a set of columns: Shodex OHpak SB-G guard column, OHpak SB-804HQ and OHpak SB-802.5HQ columns. The polymers were eluted at a flow rate of 0.5 mL/min with 0.1 M Na_2SO_4 (aq)/1 wt% acetic acid/0.02% NaN_3 at 40 °C. Before the injection, the samples were filtered through a polytetrafluoroethylene (PTFE) membrane with 0.45 µm pore. The system was calibrated with five narrow poly(ethylene glycol) standards and the polymers molecular weights (M_n^{SEC}) and *Đ* (M_w/M_n) were determined by conventional calibration using the Clarity software version 2.8.2.648.

400 MHz ^{1}H NMR spectra of reaction mixture samples were recorded on a Bruker Avance III 400 MHz spectrometer, with a 5-mm TIX triple resonance detection probe, in D_2O. Conversion of monomers was determined by integration of monomer NMR signals obtained before and after the reaction as occurred using the MestRenova software version: 10.0.1-14719.

^{13}C cross polarization/magic angle spinning (CP/MAS) solid state NMR spectra of the

hydrogels were recorded on a Bruker Avance 400 spectrometer operating at a carbon frequency of 400 MHz, using a 4 mm double-bearing MAS probe with proton 90° pulses of 3.5 µs, a contact time of 2 ms and recycle delays of 3 s. Chemical shifts are given in ppm from tetramethylsilane.

Fourier transform infrared attenuated total reflection (FTIR-ATR) spectroscopy was performed using a Jasco, model 4000 UK spectrometer. The samples were analyzed with 120 or 150 scans and 4 cm^{-1} resolution, between 600 and 4000 cm^{-1}.

The concentration of free NaCA remaining after the equilibrium binding and kinetic binding experiments was determined by high performance liquid chromatography (HPLC). The analyses were conducted at 25 °C using an Agilent Zorbax ODS, 5µm, 4.6 x 250 mm column, under isocratic elution. The mobile phase used was 20 mM tetrabutylammonium hydroxide 30-hydrate (pH adjusted to 7.5 with phosphoric acid)/acetonitrile = 55/45 (v/v), with a flow rate of 0.7 mL/min. The NaCA was detected by ultraviolet light at 210 nm. A NaCA standard calibration curve (R^2 > 0.99) was constructed for the quantification of the NaCA present in the injected sample. For the quantification of low concentrations (0.1–7 mM), a 200 µL injection loop was used. For concentrations above 7 mM, a 20 µL injection loop was used.

2.3 – Procedures

Typical procedure for the preparation of hydrogels by FRP

Several PAMPTMA-*co*-PHEA hydrogels were synthesized by FRP, using BDDA as the crosslinker. Generally, the FRP followed this procedure: a solution of crosslinker (BDDA – 683.6 µL, 3.62 mmol) and monomers (AMPTMA – 3.0 mL, 12.08 mmol and HEA – 0.2 mL, 2.41 mmol) in water (10.21 mL) and ethanol (10.96 mL) was added to a round-bottom flask equipped with a magnetic stirrer bar. DMF (58.33 µL) was added to the mixture to serve as the internal standard for the determination of the monomers conversion by ^{1}H NMR spectroscopy. Before the addition of the initiator (AIBN – 51.6mg, 0.31 mmol), the flask was sealed with a stopper and bubbled with nitrogen for 15 minutes. The reaction was allowed to proceed with stirring at 60ºC during 24 h.

A sample for NMR was collected after the polymerization, if a liquid phase was available. Finally, the reaction mixture was washed several times with ethanol and water and the hydrogel was recovered by freeze drying.

Homopolymerization of AMPTMA by SARA ATRP

Two PAMPTMA polymers were synthesized by SARA ATRP, using two different ligands (TREN and PMDETA), to verify the possibility of using a ligand that is not the Me_6TREN, the most used and efficient, but also one of the most expensive ligands. These experiments followed this procedure: a solution of the catalyst ($CuBr_2$ – 2.4 mg, 18 μmol), ligand (PMDETA – 7.6μL, 36 μmol), initiator (ECP – 4.6 μL, 36 μmol) and monomer (AMPTMA – 0.9 mL, 3.62 mmol) in water (1.0 mL) and ethanol (1.2 mL) was added to a 10 mL Schlenk tube equipped with a magnetic stirrer bar. Next, Cu(0) wire (*l* = 10 cm; *d* = 1 mm) was added to the Schlenk tube, which was sealed with a glass stopper, frozen in liquid nitrogen, deoxygenated with, at least, three freeze-vacuum-thaw cycles and purged with nitrogen. The reaction was allowed to proceed with stirring at room temperature during 1 h.

Copolymerization of HEA and AMPTMA by SARA ATRP

A PAMPTMA-*co*-PHEA polymer was synthesized by SARA ATRP, to see if the SARA ATRP method was adequate to produce PAMPTMA-*co*-PHEA hydrogels. This experiment followed this procedure: a solution of the catalyst ($CuBr_2$ – 3.6 mg, 16 μmol), ligand (Me_6TREN – 8.6μL, 32 μmol), initiator (EBiB – 11.8 μL, 80.54 μmol) and monomers (AMPTMA – 2 mL, 8.05 mmol and HEA – 100 μL, 0.805 mmol) in water (0.76 mL) and ethanol (1.26 mL) was added to a 10 mL Schlenk tube equipped with a magnetic stirrer bar. DMF (30 μL) was added to the mixture to serve as the internal standard for the determination of the monomers conversion by ^{1}H NMR spectroscopy. Next, Cu(0) wire (*l* = 5 cm; *d* = 1 mm) was added to the Schlenk tube, which was sealed with a glass stopper, frozen in liquid nitrogen, deoxygenated with at least three freeze-vacuum-thaw cycles and purged with nitrogen. The reaction was allowed to proceed with stirring at room temperature during 24 h. The molecular weight of the copolymer was determined by SEC and the monomer conversion was accessed by ^{1}H NMR spectroscopy.

Typical procedure for the preparation of hydrogels by SARA ATRP

Several PAMPTMA-*co*-PHEA hydrogels were synthesized by SARA ATRP, using BDDA as crosslinker. In this work, two different catalysts ($CuCl_2$ or $CuBr_2$) and initiators (ECP or EBiB) were used. Typically, the SARA ATRP followed this procedure: a solution of the catalyst ($CuBr_2$ – 3.6 mg, 16 μmol), ligand (Me_6TREN – 8.6μL, 32 μmol), initiator (EBiB – 11.8 μL, 80.54 μmol), crosslinker (BDDA– 151.9 μL, 0.805 mmol) and monomers (AMPTMA – 2 mL, 8.05 mmol and HEA – 100 μL, 0.805 mmol) in water (0.76 mL) and ethanol (1.26 mL) was added to a 10 mL Schlenk tube equipped with a magnetic stirrer bar. DMF (30 μL) was added to the mixture to serve as the internal standard for the determination of the monomers conversion by ^{1}H NMR spectroscopy. Next, Cu(0) wire (*l* = 5 cm; *d* = 1 mm) was added to the Schlenk tube, which was sealed with a glass stopper, frozen in liquid nitrogen, deoxygenated with at least three freeze-vacuum-thaw cycles and purged with nitrogen. The reaction was allowed to proceed with stirring at room temperature during 24 h. Finally, the reaction mixture was washed several times with ethanol and water and the hydrogel was recovered by freeze drying.

NaCA salt kinetics of binding

The kinetic studies of the binding were performed by varying the time that a NaCA solution of 30 mM was in contact with the hydrogels. For each polymer, eight incubation flasks containing a 10 mg sample were set up. Next, 2 mL of SIF were added to the flasks and the polymers were allowed to soak at the regular temperature of the human body (37ºC) overnight. In the following day, predetermined volumes of SIF (0.5 mL) and 40 mM stock solution of NaCA (7.5 mL) were added to the flasks to make the NaCA concentration of 30 mM. The eight flasks were incubated at 37 °C (100 rpm), each one for a distinct period of time, from 3 minutes to 3 hours. The hydrogel samples were filtered with polyvinylidene fluoride (PVDF) syringe filters (0.2 μm). The first 5 mL of filtrate were discarded and then an aliquot of 2.5 mL was collected for further analysis. The filtrates were analyzed by HPLC in order to determine the free concentration of NaCA. The amount of NaCA bonded to the polymers was calculated from the difference between the initial amount of NaCA introduced in the binding assays and the amount of free NaCA in the filtrates.

NaCA equilibrium binding experiments

The equilibrium binding studies were performed by varying the initial concentration of NaCA ($[NaCA]_0$) in the SIF (without pancreatin). For each polymer, nine incubation flasks containing a 10 mg sample were set up. Next, 2 mL of SIF were added to the flasks and the polymers were allowed to soak at 37ºC overnight. In the following day, predetermined volumes of SIF and 40 mM or 50 mM stock solution of NaCA were added to the flasks to make the final volume of the solvent mixture 10 mL with target concentrations of 0.3, 1, 3, 7, 10, 12.5, 20, 30 and 40 mM. A blank incubation tube containing just 10 mL of SIF (control) was also prepared for each polymer. The nine flasks were incubated at 37 °C (100 rpm) for 3 h and then filtered. The hydrogel samples were filtered with polyvinylidene fluoride (PVDF) syringe filters (0.2 µm). The first 5 mL of filtrate were discarded and then an aliquot of 2.5 mL was collected for further analysis. The filtrates were analyzed by HPLC in order to determine the free concentration of NaCA. The amount of NaCA bonded to the polymers was calculated from the difference between the initial amount of NaCA introduced in the binding assays and the amount of free NaCA in the filtrates. The experiments were done in triplicate, being that the mean of the values obtained for each experiment was considered the amount of NaCA that was bonded to the hydrogel. The Hill equation (Equation 1) was used to fit the experimental data and to determine the binding parameters for each polymer, using the MatLab software.

$$q_e = \frac{q_{max} \times K^n \times C_e^n}{1 + K^n \times C_e^n} \ (Equation\ 1)$$

In the equation, q_e is the amount of NaCA bonded to the polymer in mg/g polymer, q_{max} is the apparent maximum amount of NaCA binded to the polymer (binding capacity) in mg/g polymer, *K* is the intrinsic binding constant (relative to the strength of binding) in $L \cdot mg^{-1}$, C_e is the free NaCA concentration in mg/L and *n* is the cooperative parameter (measure of the cooperativity of binding). For *n* = 1, the equation corresponds to the Langmuir isotherm (non-cooperative binding).

Swelling tests

The hydrogel samples were soaked in SIF (without pancreatin) for 24 h at the physiological temperature (37 °C) (100 rpm), and then centrifuged sufficiently to pellet the swollen polymer. The supernatant was discarded and the remaining water was gently removed using filter paper. The swelling capacity of the hydrogels was determined from Equation 2:

$$Swelling\ Capacity(\%) = \frac{w_d - w_s}{w_s} \times 100\ (Equation\ 2)$$

In the equation presented above, w_s is the weight of the swollen sample after immersion and w_d is the weight of the dry sample before immersion. The samples were analyzed in duplicate.

***In vitro* degradation studies**

The degradation of linear PAMPTMA-*co*-PHEA was evaluated in both SGF and SIF solutions. Briefly, 30 mg of purified polymer was immersed in 3 mL of each solution in 10 mL screw-cap glass tubes. The samples were incubated at 37 °C under continuous shaking at 100 rpm. After a predetermined incubation time (2 h for SGF and 3 h for SIF), the samples were analyzed by SEC and ^{1}H NMR to evaluate the extent of degradation. The experiments were done in triplicate.

3 - Results and discussion

3.1 – Preparation of BAS hydrogels by FRP

The FRP offers a simple and inexpensive way to synthesize polymers, which is something very important in the production of commercially viable BAS. Therefore, FRP was used to prepare novel BAS hydrogels based on cationic copolymers. These hydrogels were designed to contain cationic PAMPTMA segments, responsible for the electrostatic binding of bile salts, and hydrophilic PHEA segments, that could be able to bind bile salts due the formation of hydrogen bonds. It was expected that the binding of bile acids through electrostatic interactions should not be affected by the changes in the pH value along the intestine environment, due to the presence of permanent positive charges in the hydrogel. However, the same cannot be said about the binding through hydrogen bonds, due to the contribution that hydrogen bonds provide the stabilization of the micelles and the fact that the critical micellar concentration is variable with the pH value. [46, 54]

The first step of this process was the synthesis of two homopolymers of PHEA and PAMPTMA, to verify the ability of the chosen system to polymerize these monomers and to prepare homopolymer samples that could be used as reference materials for further characterization of the hydrogels by FTIR. This process was followed by the optimization of the reaction conditions, which are summarized on the Table 3.1. The optimization allowed the definition of the reaction conditions, which conducted to the formation of a hydrogel, while maintaining a liquid phase on the reaction mixture, that could be used to estimate the monomer conversion by ^{1}H NMR.

Table 3.1 - Experimental conditions used in the optimization of the synthesis of PHEA/PAMPTMA hydrogels by FRP. Reaction conditions: $[AMPTMA]_0/[HEA]_0=5$; $H_20/EtOH=50/50(v/v)$; T=60ºC.

Entry	Experiment code	Crosslinker(BDDA)[(a)]	Initiator%(AIBN)[(a)]	$[AMPTMA]_0$ (M)	Gelation Time (min)
1	AH 1	0.2	3	2	(b)
2	AH 5	0.4	3	2	120
3	AH 6	0.4	3	1	18
4	AH 7	0.4	3	0.5	13
5	AH 8	0.4	2	0.5	21
6	AH 9	0.4	1	0.5	(b)
7	AH 10	0.3	2	0.5	81

(a) – Molar ratio relative to the cationic monomer
(b) – No macroscopic gelation was verified

As previously mentioned, macroscopic gelation should occur when, on average, each primary chain possess at least one crosslink point, which means that the ratio between [reacted crosslinker]/[initiator] should be at least one.[10] This ratio ,and consequently the gelation, is influenced by the amount of crosslinker, initiator and the monomer concentration. Therefore, in order to evaluate the optimal conditions for the production of hydrogels by FRP, these parameters were varied. The results showed that for the range of AMPTMA concentrations that were investigated (0.5 to 2 M), there was always enough reacted crosslinker in relation to the initiator concentration to allow the macroscopic gelation (Entry 2 to 4). Regarding to the initiator (Entry 4 to 6), it was verified that a percentage lower than 2% was not enough to obtain macroscopic gelation. Finally, the amount of crosslinker needed to obtain a macroscopic gelation was also investigated (Entries 1,2 and 7), in this case the results showed that a molar ratio relative to AMPTMA of 0.3 generates enough crosslink points per primary chain to enable the macroscopic gelation, which does not happen when this ratio decreases to 0.2.

Based on the results obtained, the molar ratio was defined as $[AMPTMA]_0/[BDDA]_0/[AIBN]_0$=1/0.3/0.02, with $[Monomers]_0$=0.5 M, for the preparation of the BAS hydrogels. It was also concluded that the measure of the gelation time (time at which the transition from a viscous liquid to an elastic gel occurs), is very imprecise, meaning that this value is merely indicative.

Because one of the aims of this work was to evaluate the influence of the formation of hydrogen bonds in the binding of bile acids by the BAS, several hydrogels with different molar ratios of HEA were prepared (reaction conditions are summarized on Table 3.2). In addition, a PHEA hydrogel and a PAMPTMA hydrogel were synthesized as control materials.

Table 3.2 – Reaction conditions for the preparation of cationic hydrogels by FRP. Reaction conditions: $[AIBN]_0/[BDDA]_0$=0.02/0.3; H_2O/EtOH=50/50; T=60ºC; t=24h

Entry	Code	AMPTMA molar ratio[(b)]	HEA molar ratio[(b)]	$[AMPTMA]_0$ (M)
1	AH 10	1	0.2	0.5
2	AH 11	1	0.5	0.5
3	AH 12	1	1	0.5
4	AH 15	1	1.5	0.5
5	AH 16	0	1	1
6	AH 17[(c)]	1	0	0.5
7	AH 18[(c)]	1	0	1
8	AH 19	1	0	2

(a) – Data not available
(b) – Molar ratio in comparison with AMPTMA
(c) – No macroscopic gelation was verified

It has been reported that the monomer concentration affects the gelation point.[55] For example in diluted conditions, there is an increase of the intramolecular cyclization reactions, leading to the delay or ultimately to the prevention of the occurrence of gelation. As previously mentioned, the monomer (AMPTMA) concentration of 0.5 M proved to be enough to allow macroscopic gelation, for $[BDDA]_0/[AIBN]_0$=0.3/0.02. However, in the production of the homopolymeric hydrogels (control materials) the same concentration of monomers, for the same

crosslinker/initiator ratio, was not enough to allow macroscopic gelation (Entries 6 and 7), probably due to a low conversion of crosslinker that was achieved (data not available).

In the majority of the synthesis, it was impossible to determine the monomer and crosslinker conversion, due to the lack of a liquid phase at the end of the reaction. Therefore, the solvents recovered from the washing of the hydrogels were analyzed by ^{1}H NMR to verify the existence of unreacted monomers and crosslinker molecules. These tests showed that the residues not contain HEA or BDDA, but contain AMPTMA (example in Figure A2). This showed that not all of the cationic monomer (AMPTMA) was reacted, meaning that the conversion of this monomer did not reach 100%. However, since this analysis is qualitative, it was not possible to determine the conversion value. In addition, the lack of HEA and BDDA molecules in the NMR spectrum, cannot be used as proof that both molecules have reacted completely, since some residual monomer could be trapped inside the hydrogel network. Since it was not possible to determine the conversion of the monomers in the hydrogel by ^{1}H NMR analysis, the hydrogels were analyzed by solid state NMR (SSNMR), which could be used to determine the ratio between the PAMPTMA and PHEA segments. Usually, the results obtained from ^{13}C SSNMR are qualitative, meaning that the intensity of the signals is not proportional to the number of the carbon atoms present in the polymer structure. However, it is also possible to perform a quantitative analysis. Unfortunately, due to time constraints, it was not possible to analyze the hydrogels in the quantitative mode. Nevertheless, Figure 3.1 shows the ^{13}C SSNMR spectrum of a representative hydrogel (AH 11), in the qualitative method. It is possible to observe that the ratio between PAMPTMA/PHEA could be determined (if a quantitative analysis could be performed), since the methyl (CH_3) and hydroxyl (C-OH) signals are isolated. This would be critical to confirm the composition of the hydrogels.

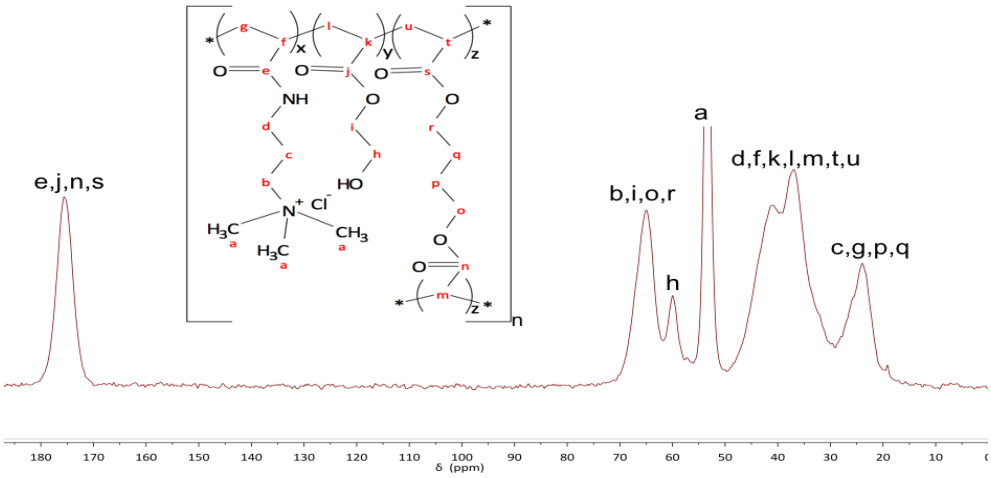


Figure 3.1 -^{13}C solid state NMR spectrum, of a representative hydrogel (AH 11).

The structure of the hydrogels was also confirmed by FTIR analysis, by the appearance of the characteristic absorption bands of both PAMPTMA and PHEA (Figure 3.2): quaternary ammonium group at 1470 cm^{-1}; N–H stretching at 1540 cm^{-1} and C=O stretching at 1630 cm^{-1} for PAMPTMA[56] and C=O stretching at 1712 cm^{-1}, the CH_2 peak at 1410 cm^{-1} and O–H bending at 1298 cm^{-1}, and the ester peak (C=O stretching) at 1200 cm^{-1} for HEA.[57]

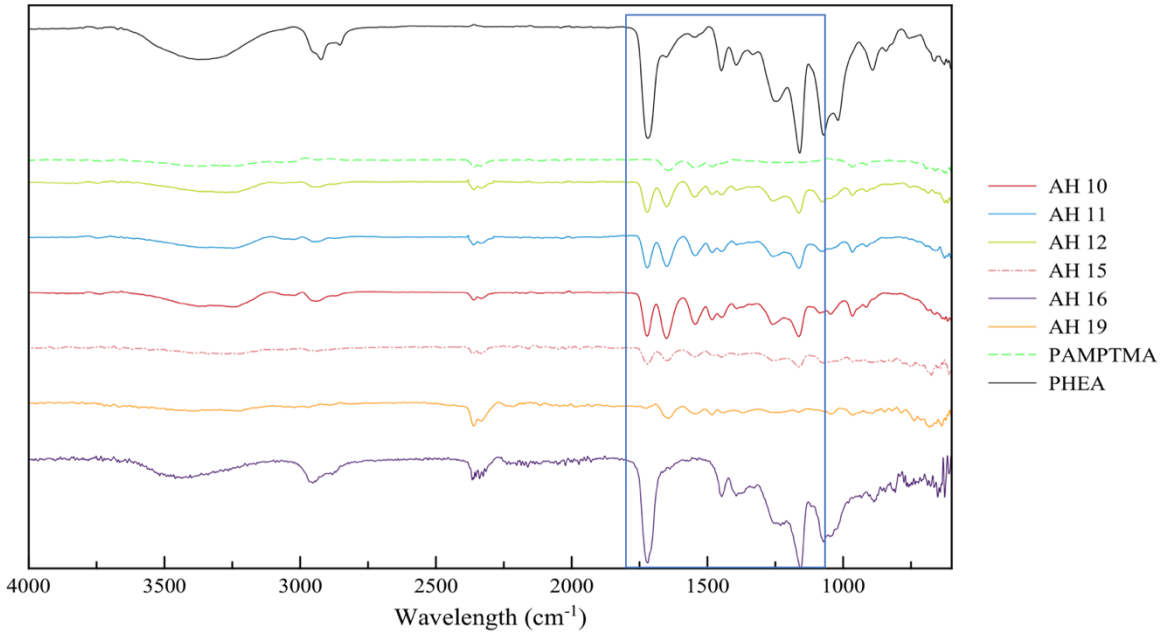


Figure 3.2 - FTIR spectra of PHEA, PAMPTMA and copolymeric hydrogels (PHEA-co-PAMPTMA) samples prepared by FRP.

3.2 – Preparation of BAS hydrogels by SARA ATRP

Besides the development of novel BAS based on cationic hydrogels, this work had the objective of studying the effect that different polymerization methods could have on the binding capacity of the BAS. For this purpose, analogous hydrogels to those produced by FRP were produced by SARA ATRP, which is a method that offers a better control over the polymeric structures, and generates gels with a more homogeneous network structure and a higher swelling ratio.[58] This method has been previously used for the preparation of PAMPTMA hydrogels to act as BAS (starting point of this work).[46]

In the referred SARA ATRP system, Me_6TREN was used as the ligand. This ligand is expensive (1 ml ≈ 75 €), which is a disadvantage considering a future industrial application of the method. Therefore, the use of two cheaper ligands (TREN and PMDETA) was investigated for the polymerization of PAMPTMA. The results are summarized on the Table 3.3.

Table 3.3 - SARA ATRP of AMPTMA initiated by ECP, using different ligands. Conditions: $[AMPTMA]_0/[ECP]_0/[CuCl_2]_0/[Ligand]_0$ = 100/1/0.5/1; EtOH/H_2O = 50/50 (v/v); T = 25 °C.; $[AMPTMA]_0$ = 1.45 M; t = 90 min.

Code	Ligand	$M_n^{SEC} \times 10^{-3}$	$Đ$	AMPTMA conv.(%)
1	TREN	—	—	0
2	PMDETA	12.7	1.28	5

Comparing these result with the ones reported in the literature using Me_6TREN as the ligand[31], it is possible to observe that both ligands were not a suitable replacement for the Me_6TREN, since a very low monomer conversion was obtained after 90 min of reaction (see Table 3.3 and compare with ≈ 95% using Me_6TREN).[31]

After this result, it was necessary to confirm that it was possible to copolymerize HEA and AMPTMA, using the SARA ATRP method, with Me_6TREN. To this purpose a PAMPTMA-*co*-PHEA polymer was synthesized using EBiB as initiator, water (80% in volume) and ethanol (20% in volume) as the solvents, Cu (0) wire and $CuBr_2$ as the metal catalyst, and Me_6TREN as the ligand,

with a monomer concentration of 1.45 M and $[AMPTMA]_0/[HEA]_0/[Ligand]_0/[Catalyst]_0/[Initiator]_0$ =100/20/0.4/0.2/1. The mixture was then allowed to react for a day at 30°C. The resulting polymer had a $M_n^{SEC} = 16.2 \times 10^3$ and $Đ = 1.19$. The AMPTMA conversion was determined to be 68% and the HEA reached 89% of conversion, which proved that the SARA ATRP method is suitable for the preparation of the hydrogels.

Several DP's of HEA were then investigated, while maintaining the rest of the reaction conditions, in order to prepare hydrogels similar to the ones prepared by FRP. The main results are summarized on Table 3.4.

Table 3.4 - Targeted DP of HEA and conversion achieved of both monomers and crosslinker in the preparation of cationic hydrogels by SARA ATRP. Conditions: $[AMPTMA]_0/[BDDA]_0/[EBiB]_0/[CuBr2]_0/[Ligand]_0$ = 100/10/1/0.5/1; EtOH/H_2O = 50/50 (v/v); T = 30 °C.; [AMPTMA]0 = 2.0 M; t = 24h

Entry	Code	HEA DP[(a)]	AMPTMA conv. (%)	HEA conv.(%)	BDDA conv.(%)
1	PM 01	10	64	66	63
2	PM 02	50	81	75	75
3	PM 03	100	66	65	70

(a) – Molar ratio in comparison with the initiator

The results showed that the hydrogels could be produced by SARA ATRP using a lower concentration of crosslinker than in FRP. This could be probably due to the differences in the hydrogel formation: In the FRP reactions, due to a low polymer concentration at the beginning of the reaction, most of the pendant vinyl groups can be consumed via intramolecular cyclization, ultimately producing highly crosslinked nanogels, that later on the reaction are connected to form a heterogeneous gel.[59] In contrast, in the SARA ATRP reactions, due to a fast initiation all the initiator molecules are rapidly converted on primary chains, which leads to a higher number of propagating chains and consequent higher concentration of polymer. Also, since the reaction is controlled, it is possible to make sure the crosslinker is in excess in comparison to the initiator,

in order to promote macroscopic gelation. It was observed that the monomer concentration of 2.0 M was enough to allow macroscopic gelation with $[BDDA]_0/[EBiB]_0$=10/1.

In opposition to the synthesis by FRP, all the hydrogels produced by SARA ATRP presented a liquid phase allowing the determination of the monomer and crosslinker conversion. The values obtained were relatively high, although the HEA conversion presented a value lower than the expected (89%). This could mean that the reaction was not kept long enough to allow a full conversion, since the diffusion limitations experienced by monomers and catalytic complex in the polymer network after the gelation point were not taken into account.[60]

Even though the liquid phases were analyzed with resource to ^{1}H NMR, it is also possible that some monomer molecules could be entrapped in the hydrogels network. Therefore, the results should be interpreted with caution, as the conversions obtained could be an approximation of the real value. To confirm the chemical structure of the hydrogels prepared by SARA ATRP, samples were also analyzed by FTIR. The presence of the expected monomers in the hydrogel was confirmed, due to the presence of the already mentioned characteristic absorption bands of both the monomers (HEA and AMPTMA) in the FTIR spectrum (Figure 3.3) of the hydrogels.

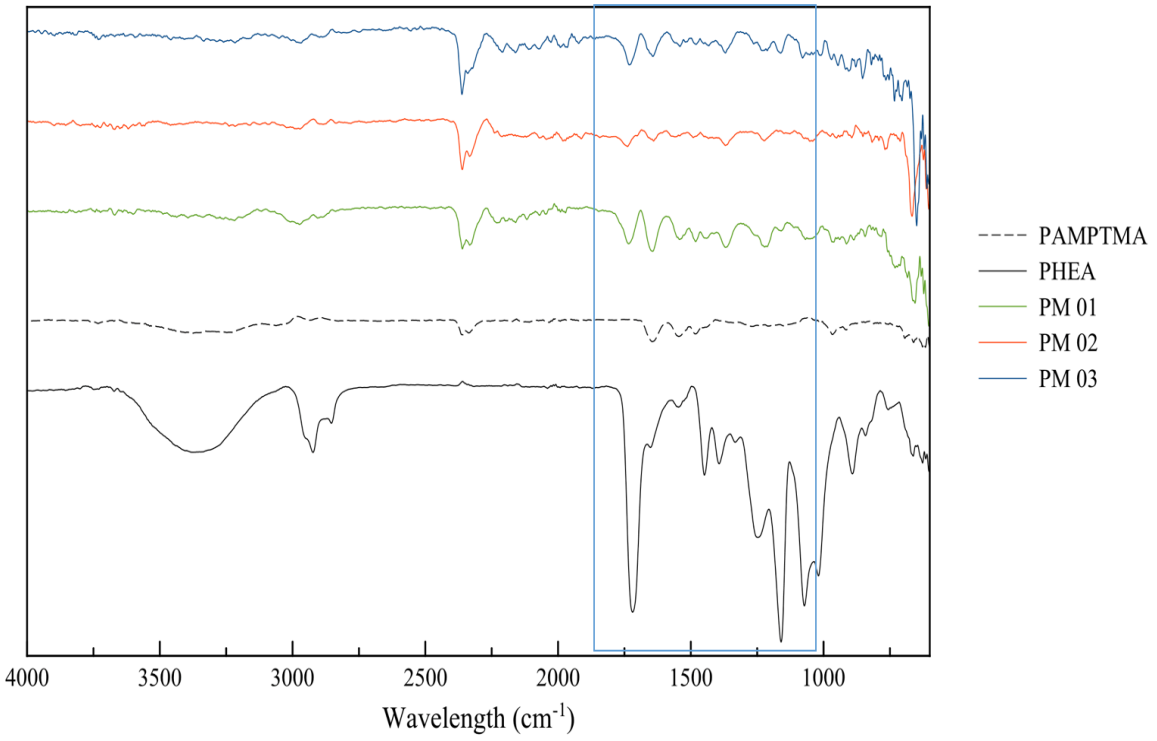


Figure 3.3- FTIR spectra of PHEA, PAMPTMA and copolymeric hydrogels (PHEA-co- PAMPTMA) samples prepared by SARA ATRP.

3.3 – Kinetics of sodium cholate binding

Usually in the BAS binding tests provided by pharmaceutical guidelines, the BAS are left to incubate with the bile salts during 24 hours, in order to allow the system to reach the equilibrium. However, this incubation time does not correspond to the time that the BAS are in contact with the bile salts in physiological conditions (≈3h). Therefore, before the realization of the binding tests, it was necessary to verify the time required for the achievement of the binding equilibrium.

Since cholic acid-based bile salts represent a large portion (30-40%) of the bile salts present in the human bile[61], the use of cholic acid could be a good indicator of the binding capacity of the BAS candidates. Therefore, NaCA was used as the model bile salt for the binding experiments.

The kinetic experiments were realized in a solution of NaCA with a pH=7.6, since at this pH the bile salt is completely ionized[46]. To determine the NaCA concentration on solution, after a determined incubation time (from three minutes to three hours in the presence of the hydrogel), the filtrate was analyzed by HPLC. The kinetic results obtained for the hydrogels produced by FRP are presented on Figure 3.4.

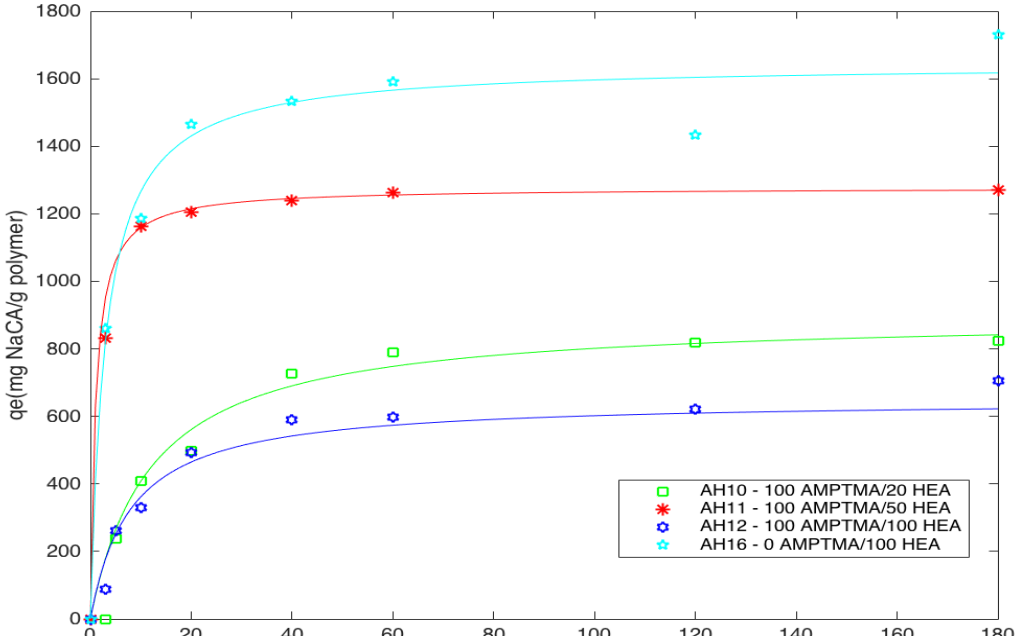


Figure 3.4 - Binding kinetics for the several hydrogels produced by FRP. Binding conditions: 30 mM NaCA in phosphate buffer (pH=7.6) at 37 °C.

The results showed that the tested hydrogels reached the binding equilibrium before the three hours, more specifically after 1h. It was also possible to observe that the hydrogel composition did not influence the time needed to reach the equilibrium.

The same experiments were then conducted using the hydrogels produced by SARA ATRP. Colesevelam, which is the most efficient BAS that is commercially available, was used as the reference. The results presented on the Figure 3.5.

Similarly, to what was observed for the hydrogels produced by FRP, the hydrogel BAS

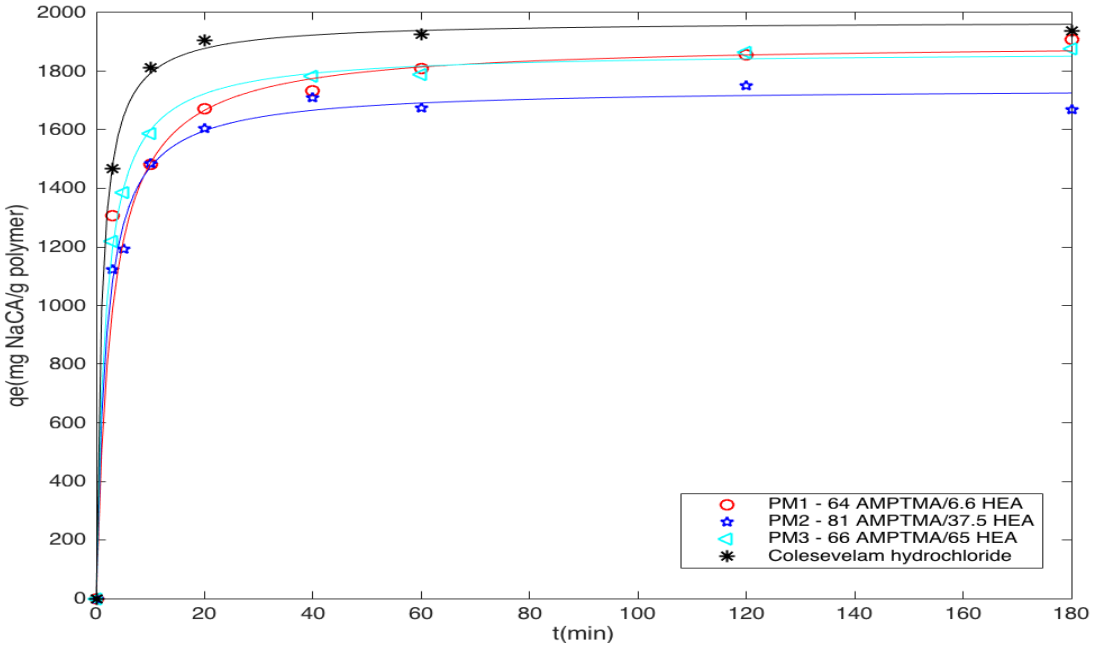


Figure 3.5 - Binding kinetics of the several hydrogels produced by SARA ATRP and Colesevelam. Binding conditions: 30 mM NaCA in phosphate buffer (pH=7.6) at 37 °C.

prepared by SARA ATRP reached the binding equilibrium after approximately 1h and that the behavior is not affected by the composition of the hydrogel. Results also showed that the commercial BAS (Colesevelam) reached the binding equilibrium after 20 min. The fact that the hydrogel BAS reached the maximum binding capacity before the 3h is a good indicator of their viability, since the intestinal transit takes about 3h.

Since the BAS hydrogels reached the binding equilibrium after approximately 1h, the binding tests were designed to have an incubation period of 3h.

3.4 – Sodium cholate equilibrium binding

As already mentioned, bile acids are amphiphilic molecules that are usually conjugated with taurine or glycine in the human bile.[32] Under the small intestine conditions, these complexes are in their ionized form, being called bile salts due to this fact. The binding experiments were performed using NaCA solutions with concentration in the range of 0.3 to 40 mM at pH=7.6. From the analysis of the binding isotherms (Figure 3.6), for the binding of NaCA by the hydrogels prepared by FRP, it is possible to observe that the curves exhibited a sigmoidal shape.

The sigmoidal isotherms suggest the existence of a cooperative binding, which means that the affinity of the hydrogel for the bile salts varies with the amount of bile salts already bonded. This was also observed for the PAMPTMA-based hydrogels described in the literature.[46]

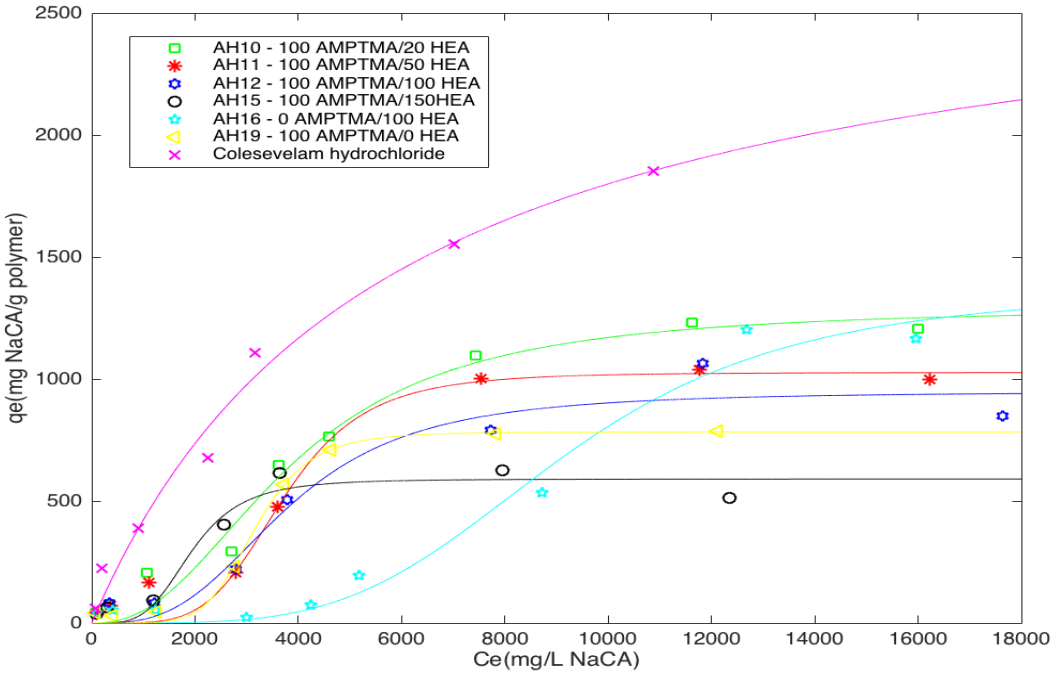


Figure 3.6 - Isotherms for the binding of NaCA by the Colesevelam and the hydrogels prepared by FRP. Binding conditions: 50 mM phosphate buffer (pH = 7.6) at 37 °C. The lines represent the fitting to Langmuir or Hill models for the Colesevelam and hydrogels, respectively.

On the PHEA hydrogel isotherm (cyan pentagrams in Figure 3.6), it was possible to observe that the hydrogel starts to bind for higher concentrations of NaCA, in comparison to the other

hydrogels. Since the PHEA hydrogel does not possess cationic segments, the formation of hydrogen bonds and hydrophobic interactions, between the hydrogel and the bile salts, should be the only forces responsible for the binding. It has been suggested that the binding of bile salts micelles is mostly due to electrostatic interactions.[46] This would mean that a hydrogel without cationic segments, like the PHEA hydrogel, would bind a small amount of bile salt micelles, which can explain the behavior observed for the PHEA hydrogel.

From the analysis of the PAMPTMA hydrogel isotherm (yellow triangles in Figure 3.6), it was possible to verify that the binding followed the behavior already described for similar hydrogels, produced with this cationic monomer.[46] This behavior was attributed to a stronger interaction between the hydrogel and the NaCA micelles (electrostatic interactions) than with NaCA unimers (hydrophobic and electrostatic interactions), instead of a cooperative binding process. However, in this case (AH 19 sample in Figure 3.6), the maximum binding capacity was lower than the one reported.[46] Considering that the hydrogels used on this experiment were produced using a different polymerization method, and a higher amount of crosslinker, it was hypothesized that this could be responsible by the differences on the binding values. Most probably the hydrogel produced by FRP presented a lower swelling ratio than the PAMPTMA hydrogel described in the literature (produced by SARA ATRP). Usually, a higher swelling ratio provides a higher binding capacity.[62] Thus it was necessary to determine the swelling ratio of this hydrogel, through swelling tests, so it could be compared to the swelling ratio of the hydrogels reported on the literature. The results of the swelling tests, showed that this hydrogel (Table A1) had a swelling ratio lower than the hydrogels reported on the literature[46], which could explain the difference on the maximum binding capacity.

The equilibrium binding of NaCA by Colesevelam (magenta crosses in the Figure 3.6) followed a typical Langmuir isotherm, meaning that the cooperative parameter on the Hill equation takes the value of the unity (n=1), which indicates that cooperativity does not exist, as already reported by other authors.[63]

The data regarding the hydrogels prepared in this work were adjusted to the Hill equation (the corresponding R^2 values are shown on Table 3.5), which is commonly used to describe

cooperative binding processes.[50] The values of the overall binding constant (*K*), the cooperative parameter (*n*) and the maximum binding capacity (q_{max}) are presented in Table 3.5.

Table 3.5 - Binding parameters of the cationic hydrogels prepared by FRP and the commercial BAS Colesevelam.

Entry	Code	R^2	*K* (L/mg)*10⁴	*n*	q_{max} (mg/g)[a] * 10^{-3}	K_d (mM)	HEA %[b]
1	Colesevelam	0.985	1.76±0.41	1	2.81±0.31	13.19±2.49	0
2	AH 10	0.976	2.58±0.28	2.3±0.6	1.29±0.10	9.00±0.88	17
3	AH 11	0.975	2.68±0.22	4.6±2.0	1.02±0.05	8.66±0.65	33
4	AH 12	0.959	2.63±0.37	3.0±1.4	0.94±0.08	8.83±0.82	50
5	AH 15	0.953	5.15±0.80	3.9±1.5	0.59±0.04	4.51±0.60	66
6	AH 16	0.979	1.09±0.11	4.0±1.1	1.36±0.18	21.30±1.95	100
7	AH 19	0.994	3.14±0.79	6.0±0.8	0.78±0.02	7.39±1.48	0

(a) - mg NaCA/g hydrogel
(b) - Theoretical value

The cooperative parameter was always higher than the unity, which means the occurrence of positive cooperativity in the binding. This parameter was in the same range for both the copolymeric hydrogels (entries 2 to 5 in Table 3.5) and the PHEA and PAMPTMA hydrogels (entries 6 and 7 in Table 3.5). In addition, it was possible to observe that the strength of the binding of NaCA by the copolymeric hydrogels (entries 2 to 5 in Table 3.5) prepared by FRP was comparable to that of the Colesevelam, as seen by the similar or even higher *K* values.

In order to assess the overall efficiency of the prepared hydrogels, and gather information about the ability of the hydrogels to bind NaCA molecules under the physiological conditions, there are two parameters that must be taken into account: the binding capacity (q_{max}) and the dissociation constant $\left(K_d = \frac{1}{K}\right)$, which represents the concentration of bile salt at which the hydrogel is half-saturated. In order to provide an effective binding, K_d should be lower than the concentration of bile salts in the intestine (≈15 mM)[64]. The hydrogels PAMPTMA-*co*-PHEA as well as PAMPTMA hydrogel presented dissociation constants between 4.51 and 9.0 mM (Table 5),

which means that the materials prepared could be promising BAS candidates. The dissociation constant of the PHEA hydrogel revealed that the material is not suitable for the targeted application. This result shows the importance of the electrostatic interactions on the binding process, as described on the literature[38,43]. Besides this, the binding capacity (Table 3.5) was much lower than that of the Colesevelam but, at the same time was slightly higher than the one reported on the literature for Cholestyramine and other BAS candidates.[65]

Since it was not possible to determine exactly the monomer conversion during synthesis of the hydrogels, as previously mentioned, for discussion purposes it was considered that the monomers achieved similar conversions during the preparation of all the hydrogels. Therefore, the relative ratios PAMPTMA/PHEA between samples was considered the same as the targeted one. From the analysis of the Table 3.5, it is possible to observe that the binding capacity of the copolymeric hydrogels decreased with the increase of the percentage of PHEA. Although this observation was unexpected, due to the fact that the presence of HEA should lead to the formation of hydrogen bonds between the polymer and the bile salts, which would increase the binding efficiency, similar results were previously reported.[66] In another work,[49] it was also observed that the binding capacity of a polymer, with a binding mechanism based on hydrogen bonding, decreased with the increase of the solvent polarity. This could mean that in polar media, like water, the hydrogen bonds are formed preferably between the polymer and polar molecules, which would explain the reason for the results obtained, since the SIF is a polar media. In addition, the increase of the percentage of PHEA in the hydrogels leads to a decrease of the density of positive charges and a consequent decrease of the electrostatic interactions between the polymer and the bile salt micelles, which are the main attractive forces involved in the binding process.

Taking into account these results, it was expected that the PHEA hydrogel would have the lowest binding capacity of all the hydrogels tested, however, it presented the highest value. This could be probably related to a lower degree of crosslinking and the consequent higher swelling ratio, which according to several works leads to a higher binding capacity.[62, 67] Since it was not possible to determine the crosslinker conversion, it was necessary to determine the swelling ratio, in order to verify this hypothesis. The tests showed that this hydrogel had the lowest

swelling ratio (around 160%) of the all the tested hydrogels, which contradicts the hypothesis that the high binding capacity of the hydrogels was the result of a high swelling ratio. More studies will then be needed to clarify this behavior, since it was expected that the PHEA hydrogel would show the lowest binding capacity from all the samples.

To study the effect that different polymerization methods have on the binding of bile acids, the equilibrium binding of NaCA by the hydrogels produced by SARA ATRP was investigated using HPLC. In order to maintain the consistency of the experiments, these were conducted under the same conditions used for the hydrogels produced by FRP. From the analysis of the binding isotherms, it was possible to observe that the hydrogels produced by SARA ATRP have a higher NaCA binding capacity than the ones produced by FRP (compare Figure 3.6 with Figure 3.7).

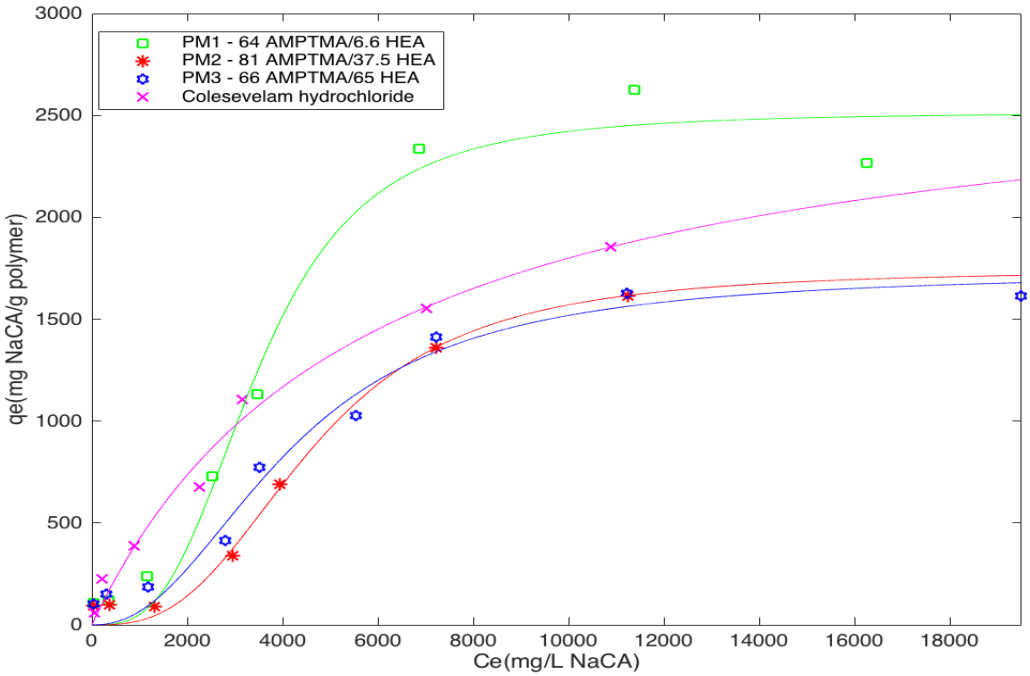


Figure 3.7 - Isotherms for the binding of NaCA by the Colesevelam and the hydrogels prepared by SARA ATRP. Binding conditions: 50 mM phosphate buffer (pH = 7.6) at 37 °C. The lines represent the fitting to Langmuir or Hill models for the Colesevelam and hydrogels, respectively

Remarkably, the PM 01 hydrogel presented a higher binding capacity than the Colesevelam (compare green circles with the magenta crosses in Figure 3.7), it was also verified that the isotherms for the hydrogels PM 02 and PM 03 were very similar, which was expected since both

hydrogels have similar compositions, as a result of the different monomer conversion achieved (see Table 4) achieved similar conversions for the monomers used.

The data were adjusted to the Hill equation, with good fitting for all the samples investigated ($R^2 \geq 0.98$ in Table 3.6). It was verified that similarly to the hydrogels produced by FRP, the cooperative parameter (Table 3.6) was always higher than one, meaning that there was a positive cooperative binding.

Table 3.6 - Binding parameters of the cationic hydrogels prepared by SARA ATRP and the commercial BAS Colesevelam

Entry	Code	R^2	$K\ (L/mg)*10^4$	n	q_{max} (mg/g)[a] * 10^{-3}
1	Colesevelam	0.985	1.76±0.41	1	2.81±0.31
2	PM 01	0.980	2.85±0.27	3.0±0.9	2.51±0.15
3	PM 02	0.991	2.15±0.22	2.8±0.6	1.74±0.15
4	PM 03	0.976	2.39±0.27	2.2±0.5	1.73±0.13

(a)– mg NaCA/g hydrogel

This could indicate that a more homogeneous network could lead to hydrogels with a higher binding capacity. In addition, the hydrogels produced using this method have a lower amount of crosslinker, which could mean that they possess a higher swelling ratio, which in turn could also lead to a higher binding capacity. In order to verify which one of the differences (polymerization technique or amount of crosslinker) induced the increase of the binding capacity, the swelling ratio of hydrogels with similar composition was determined (Table A1).

It was observed that both copolymeric hydrogels, with similar composition, produced by either FRP or SARA ATRP, presented similar swelling ratios (around 2000%, see entries 2 and 3 in Table A1), which suggests that the homogeneous network could be the factor responsible for the higher binding capacity of the hydrogels. This is an extremely interesting finding, which points out to the potential of the SARA ATRP technique for the preparation of high-performance materials. However, the results have to be interpreted with caution, since the real composition

of both samples could be an estimate. More investigation on this topic will be needed, namely the interpretation of future solid state NMR results.

From the analysis of the binding capacity (q_{max} on the Table 6) of the hydrogels produced by SARA ATRP, it was possible to observe that, like in the hydrogels produced by FRP, an increase in the amount of PHEA present on the hydrogel led to a decrease in the binding capacity, which is possibly related with the polarity of the SIF, as previously explained.

The remaining binding parameters (intrinsic binding constant and cooperative parameter), were in the same range as the ones obtained for the FRP-based hydrogels.

3.5 – *In vitro* degradation studies

Since the polymeric BAS are administered orally, it is necessary to ensure that the BAS reach the intestine with the same properties that possess initially. To confirm this, the stability of the synthesized materials, under conditions that simulate the gastrointestinal tract, was evaluated.

The determination of the molecular weight and the confirmation of the polymers chemical structure, after being subjected to the mentioned conditions, are effective tools to quantify any possible degradation. Since it is not possible to determine the molecular weight of a hydrogel by either SEC or ^{1}H NMR spectroscopy, the degradation of the materials was evaluated by analyzing a linear PAMPTMA-co-PHEA copolymer. Therefore, it is assumed that the degradation mechanism of the hydrogel will be similar to that of the linear copolymer. The degradation studies were carried out in triplicate at 37 °C in SGF at pH=1.2 for2 hours and in SIF at pH=6.8 for 3 hours, according to procedures described on the literature[68] Table 3.7 summarizes the molecular weights and dispersity of the polymers before and after degradation.

Table 3.7 - Molecular weight and dispersity of linear PAMPTMA-co-PHEA before and after exposure to the degradation solutions at 37 °C.

Sample	Medium	Degradation time (h)	$M_n^{SEC} \times 10^{-3}$	$Đ$
PAMPTMA-*co*-PHEA	None	0	84.3	2.60
	SGF (pH=1.2)	2	84.3±0.2	2.93±0.13
	SIF (pH=6.8)	3	82.0±0.8	3.04±0.06

The results obtained by SEC, suggest that there was no degradation of the polymer backbone in both the SGF and SIF, since there was no significant variation of the molecular weight values. Nevertheless, these results cannot be considered as a guarantee that there was not degradation of the polymer, since the degradation of the polymer side chains could have a residual effect on the polymers molecular weight value. In order to evaluate a potential side chain degradation, additional ^{1}H NMR spectroscopy analysis were conducted. The results showed that there was no degradation of the copolymer in the SGF, since the ratio between the integral of the protons of the backbone and the protons of the side chains, remained constant for the samples studied. It appears that the SIF degraded the AMPTMA side-chain, since the ratio between the signal of the AMPTMA side-chain and the methylene bridge (signal i) of the PHEA segment decreased (Figure 3.8). However, it is important to note that, in a previous works, it was confirmed that the PAMPTMA polymer is stable in SIF, under the same conditions investigated in this work.[46]

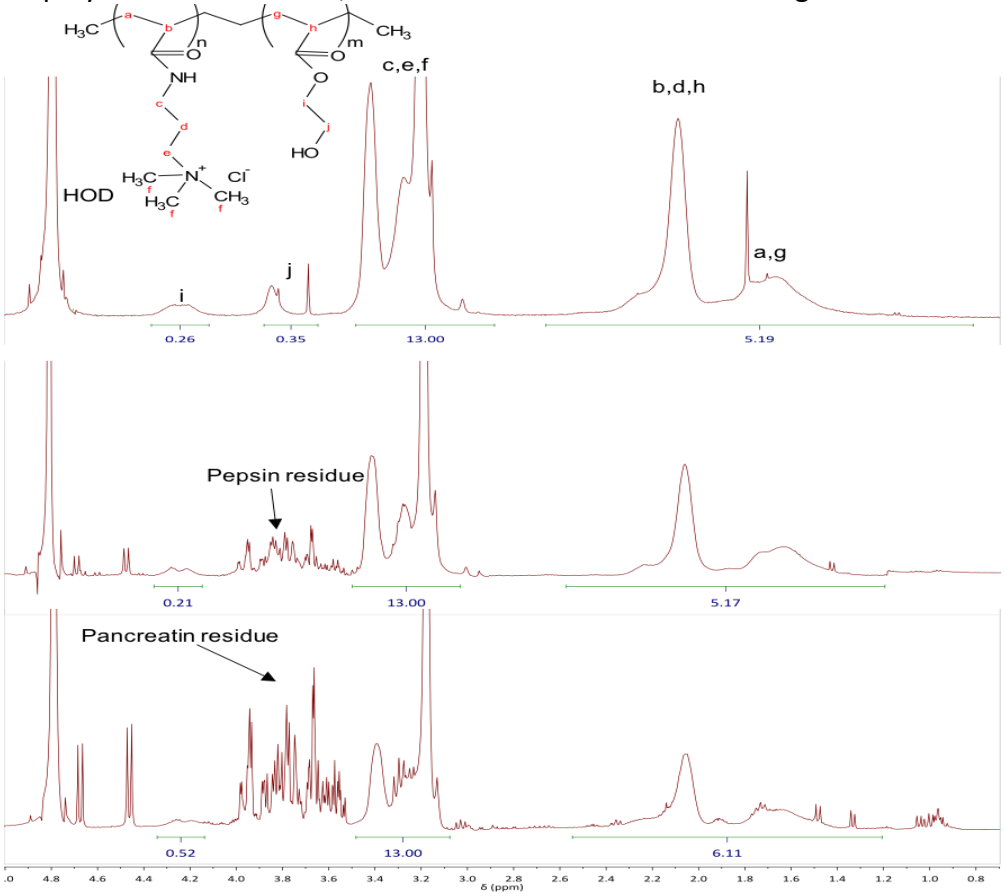


Figure 3.8 - ^{1}H NMR spectra, in D_2O, of the linear PAMPTMA-co-PHEA before (top) and after degradation in SGF (middle) or SIF (bottom) at 37 ºC

In addition, from Figure 3.8, it is possible to observe that the signal of PHEA (signal i) is very small and it is also affected by the signal of the pancreatin residue. Therefore, the value of the integrals determined could be influenced by the previous mentioned conditions, leading to a misleading interpretation of the results. To verify this hypothesis, the same experiments should be conducted using a copolymer with a higher amount of HEA, to increase the intensity of the NMR signal.

4 – Conclusion and future work

4.1 – Conclusion

The SARA ATRP and FRP methods were used to synthesize novel BAS candidates based on PAMPTMA-*co*-PHEA hydrogels. NaCA was used as the model bile salt for the study of the *in vitro* equilibrium binding. Both types of hydrogels, produced by FRP and SARA ATRP, exhibited a higher affinity towards NaCA micelles rather than NaCA unimers. In addition, the hydrogels showed to have a similar performance to the commercial BAS Colesevelam hydrochloride regarding the binding affinity. However, the maximum binding capacity, presented a lower value than the Colesevelam, mostly for the hydrogels produced by FRP. It was shown that the SARA ATRP method has the ability to produce hydrogels with a higher maximum binding capacity, when compared to the ones produced by FRP. Also, regarding this binding parameter, it was possible to conclude that it could be adjusted by changes on the polymers targeted composition. Typically, increasing amounts of PHEA in the hydrogel lead to a lower binding capacity, probably due to the polarity of the medium, which could prevent the formation of hydrogen bonds between the hydrogel and the bile salts. The hydrogels prepared proved to be stable in the degradation solution that mimicked the stomach and intestine conditions.

4.2 – Recommendations for future work

This work has shown that it is possible to use several polymerization techniques to produce hydrogels that can act as BAS. However, it has also shown that the characterization techniques that were employed were insufficient, since it was not possible to determine the exact

composition of the produced hydrogels. A more effective analysis of the hydrogels, namely by quantitative SSNMR should be considered as future work.

The results obtained considering the synthesis of novel PAMPTMA-*co*-PHEA based hydrogels by SARA ATRP showed that this technique allowed preparation of BAS with improved binding capacity. On this matter, there are several *in vitro* experiments that could be suggested as future work, including:

- Competitive equilibrium binding using different conjugated bile salts in the initial solution. This study is extremely important since it will provide a more precise information about the efficiency of the BAS in physiological conditions;
- Equilibrium binding studies with acidic pre-treatment of the polymers. This study will provide information about the effect that a stomach mimicking environment has over the binding capacity of the hydrogels;
- Equilibrium binding studies using polymers with different amounts of PHEA that were not tested on this work. This study will provide a more precise information about the effect that the amount of PHEA has on the binding capacity;
- Equilibrium binding studies using solvents with different polarities. This study will provide information about the effect that the polarity of the medium has on binding capacity of the hydrogel and more specifically on the binding through the formation of hydrogen bonds;

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Appendix A

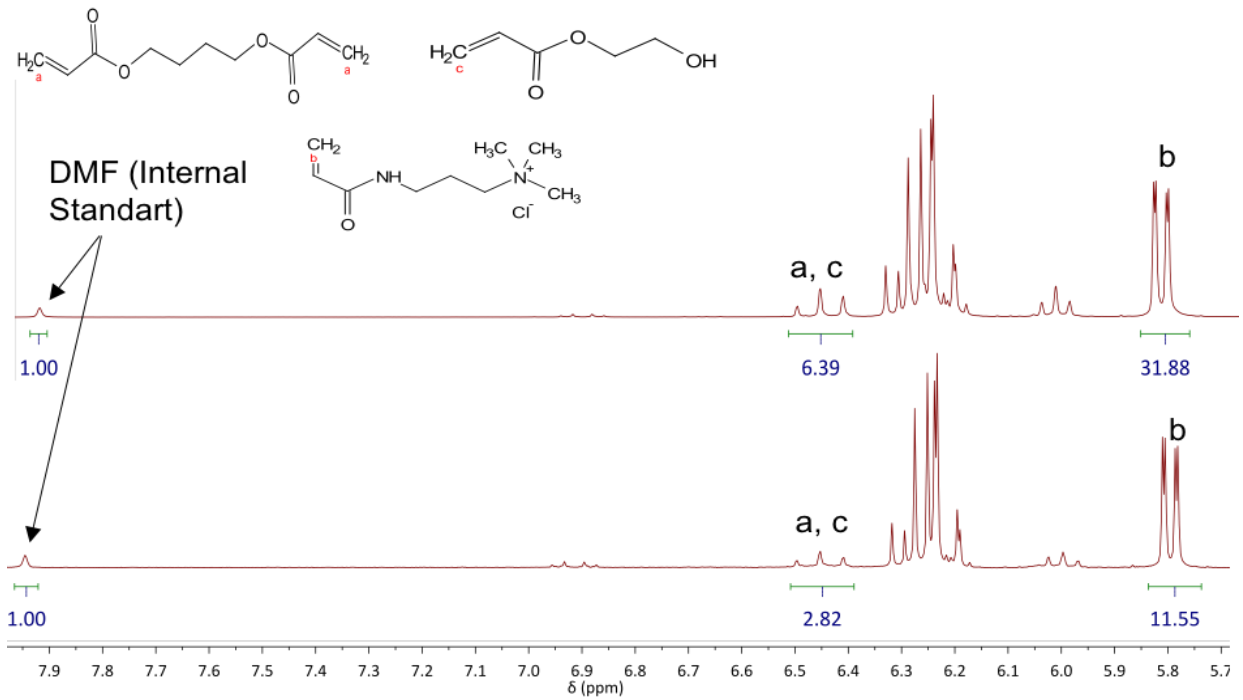

Figure A1 - ^{1}H NMR spectra, in D_2O, of the reaction mixture (liquid phase) used on the synthesis of a PAMPTMA-co-PHEA hydrogel: before (top) and after reaction (bottom).

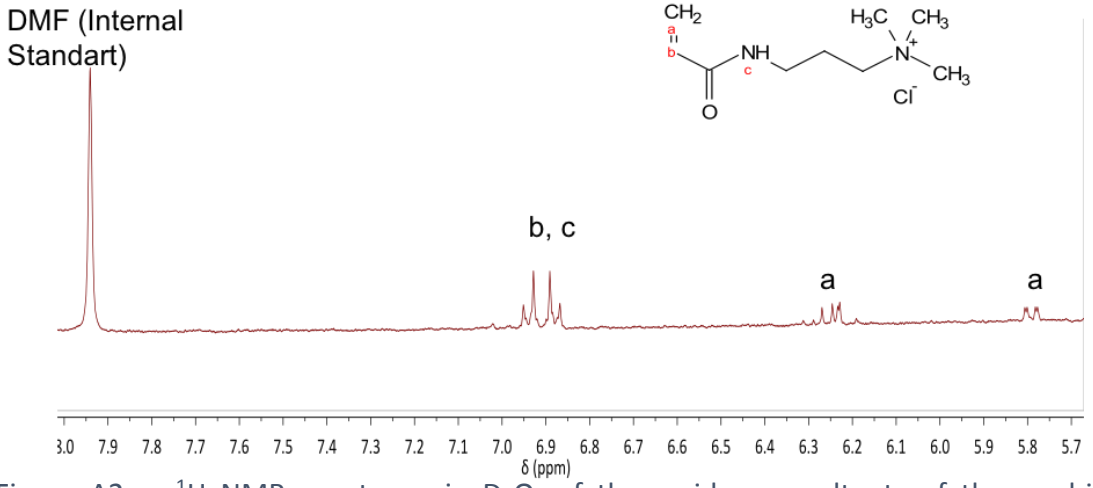

Figure A2 - ^{1}H NMR spectrum, in D_2O, of the residues resultants of the washing of a PAMPTMA-*co*-PHEA (AH 12) hydrogel.

Table A1 – Composition and swelling capacity of some of the hydrogels used on the binding experiments.

Entry	Code	AMPTMA DP	HEA DP	BDDA DP	Swelling ratio$\times 10^{-3}$(%)
1	Colesevelam	-	-	-	0.636±0.153
2	AH 11	100[(a)]	50[(a)]	30[(a)]	2.007±0.081
3	PM 02	81[(b)]	37.5[(b)]	7.5[(b)]	2.400
4	AH 16	0	100[(a)]	30[(a)]	0.162±0.059
5	AH 19	100[(a)]	0	30[(a)]	1.099±0.223

(a) – Theoretical value
(b) - Value obtained by ^{1}H NMR analysis