Morpholine and thiomorpholine derived polymers: Multifunctional platforms for biological applications

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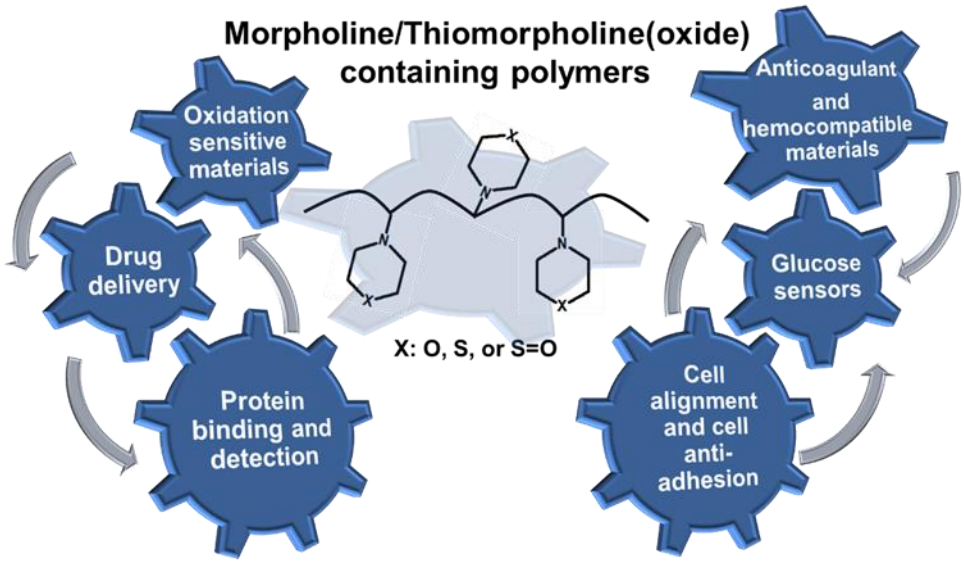
ABSTRACT

Polymers containing morpholine or thiomorpholine (oxide) functions showed an increased interest for biological applications in the recent years. Morpholine containing polymers provide high hydrophilicity and comparable biocompatibility with polyethylene glycol (PEG) which is the gold standard in biology. In particular, poly(*N*-acryloyl) morpholine is one of the most cited polymers containing morpholine, with applications in drug delivery, protein detection or the design of anticoagulant materials. However, there are very few examples of polymers derived from thiomorpholine and thiomorpholine oxide. Compared to morpholine polymers, thiomorpholine containing polymers are less hydrophilic, while thiomorpholine oxide derived polymers are more hydrophilic. Thiomorpholine and thiomorpholine oxide polymers are biocompatible and were recently studied as oxidation sensitive materials and as hemocompatible materials. This review highlights the correlation between the structural characteristics of morpholine and thiomorpholine (oxide) based polymers and their use in various biological applications.



I. Introduction

Heterocycles including piperidine, piperazine, morpholine or thiomorpholine represent structural motifs used to develop various macromolecules with many applications in biological field. Piperidine and piperazine are nitrogen-rich heterocycles which can be easily protonated and lead to structures with high basicity that complex the negatively charged genetic material. Moreover, these two heterocycles can easily be functionalised with polymerisable synthons in order to tune their basicity and to obtain a library of monomers and polymers that were exploited in the field of gene delivery.[1,2,3,4,5] Additional reports indicated that piperidine and piperazine heterocycles were included in the development of antibacterial macromolecules.[6] However, some recent papers indicated that the high basicity of piperidine and piperazine sometimes limit their efficacity in gene delivery applications.[3] For this reason, this review will focus on morpholine and thiomorpholine containing macromolecules. Morpholine and thiomorpholine are multifunctional heterocycles endowed with high water solubility,[7,8] weak base properties due to the secondary amine group,[9] electron-donor behavior[10] and non-cytotoxic properties.[11] These unique properties were exploited to generate various monomer structures, including (meth)acrylates[12] and

(meth)acrylamides.[13,14,15,16] The variety of morpholine/thiomorpholine monomers is a result of the reactivity of the secondary amino group that can be involved in nucleophilic substitution for (meth)acrylate synthesis,[12] or direct amidation to obtain (meth)acrylamides.[16] In addition, the structure of these monomers can be tailored by changing the linker between the polymerisable synthon and the heterocycle. Thanks to this structural variety, the hydrophobic/hydrophilic behaviour, the water solubility and pH responsiveness of the corresponding monomers can be tuned.

The tailored hydrophobic/hydrophilic behaviour of the morpholine monomers inspired scientists to develop a series of homopolymers and amphiphilic copolymers.[17,18,19] Morpholine-derived polymers containing acrylate and acrylamide polymerisable functions present similar hydrophilicity as polyethylene glycol (PEG) derivatives, and were reported to be used in biological applications.[20] In addition, poly(acrylate)/poly(acrylamide) morpholine polymers showed substantially improved biocompatibility and non-cytotoxicity compared to PEG. These properties make them to be considered as valuable alternatives to PEG which is the gold standard.[20] Some articles reported the use of poly(*N*-acryloyl) morpholine for the chemical modification of liposomes.[21,22] According to these studies,[21,22] poly(*N*-acryloyl) morpholine extended the circulation half-lives of liposomes without eliciting the ABC effect upon repeated administration. Furthermore, the hydrophilic poly(*N*-acryloyl)morpholine were used in the fabrication of anticoagulant and hemocompatible membranes. Indeed, the hydrophilic behaviour of morpholine polymers provides a hydration layer which generates repulsion forces between the hydrophobic proteins (found in platelets) and the hydrophilic membrane.[23] Finally, the tertiary amine of morpholine-containing polymers can be protonated, resulting into polycations which have been used for DNA complexation in drug delivery and targeting.[24]

In contrast, only few examples of thiomorpholine and thiomorpholine-oxide derived polymers have been reported.[25,26,27,28,29] Thiomorpholine polymers are less hydrophilic than morpholine polymers, but they are highly biocompatible.[27] Compared to morpholine polymers, thiomorpholine polymers possess interesting redox properties due to the sulphur atom that can be oxidised into sulphur-oxide. Poly(*N*-acryloyl thiomorpholine) and poly(ethyl thiomorpholine oxide methacrylate) were described in the literature. Poly(*N*-acryloyl thiomorpholine) was used in the development of delivery systems sensitive to oxidative stimuli,[28] while poly(ethyl thiomorpholine oxide methacrylate) was reported for promising *in-vitro* hemocompatible properties.[29]

This review intends to underline the correlation between the type of biological application in which these polymers are used and their structural characteristics. The first part of the review will highlight the structural features of individual heterocycles, and of their corresponding monomers ((meth)acrylates and (meth)acrylamides) and polymers. The second and main part of the review will focus on the correlation between the chemical properties of morpholine/thiomorpholine/thiomorpholine oxide polymers and their use in a specific biological application.

II. Structure and reactivity of morpholine, thiomorpholine and the derived polymers

Morpholine, thiomorpholine and their derived polymers have a multifunctional structure which presents a variety of reactive sites involved in the development of distinct properties. This section will briefly discuss the main structure-reactivity properties of the starting organic molecules (morpholine, thiomorpholine), the derived (meth)acrylate and (meth)acrylamide, and some polymers (homo- and/or copolymers) containing these heterocycles (**Figure 1.**).

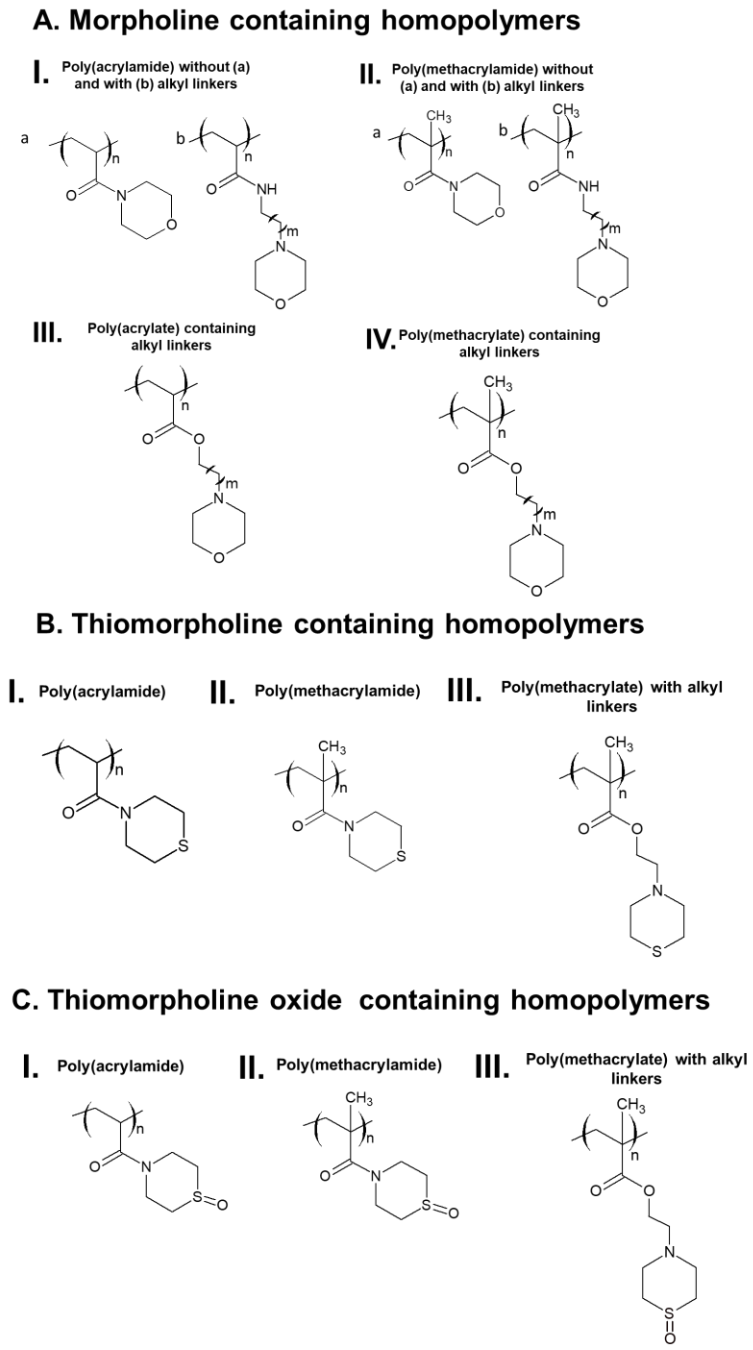


Figure 1. (A) Morpholine-containing homopolymers; (B) thiomorpholine and (C) thiomorpholine-oxide homopolymers

Morpholine is hydrophilic due to the oxygen atoms which forms H-bonds with water molecules. In comparison, thiomorpholine is less hydrophilic since sulphur atom forms weaker H-bonds with water.[30] Both morpholine and thiomorpholine show high water solubility,[13] which makes them

suitable for biological application. Besides their hydrophilicity, morpholine and thiomorpholine are biocompatible and non-cytotoxic.[31] Furthermore, both molecules can act as electron donor molecules (necessary in the formation of H-bonds for example) because of the oxygen or sulphur heteroatoms which possess non-bonding electron pairs.[32] Morpholine and thiomorpholine also present a secondary amine that can be protonated. The pK_a of morpholine is 8.33, while the pK_a of thiomorpholine is 9.13, both values being specific to secondary amino groups.[31,32] Since a tertiary amine is formed when these heterocycles are attached to polymer backbones *via* alkyl linkers, the pK_a values are significantly lowered. For example, poly(ethyl morpholino methacrylate) derivatives feature a pK_a around 4.9, while the thiomorpholine and thiomorpholine oxide analogs show a pK_a around 5.5.[30,33] These acido-basic characteristics were exploited in gene delivery systems, since the protonated morpholine or thiomorpholine cycles can complex with negatively charged DNA molecules and thus deliver DNA in the cell.[34]

The (meth)acrylamide[35] monomers were prepared by direct amidation of the (thio)morpholine. In contrast, acrylates/methacrylates[12] derived from morpholine or thiomorpholine were made by alkylation of the secondary cyclic amine leading to a tertiary amine. Another characteristic of morpholine/thiomorpholine monomers is that various structures can be obtained, by adding short or long alkyl chains between the (meth)acrylamide/(meth)acrylate backbone and the morpholine/thiomorpholine heterocycle. In consequence, the hydrophobic/hydrophilic behaviour of these monomers could be tailored by changing the length of the linker.

The morpholine/thiomorpholine monomers can copolymerise with a range of co-monomers (*i.e.* acrylates, methacrylates, acrylamides, methacrylamides or styrene) with different hydrophilic or hydrophobic behaviour.[36,29] In general, the type of alkyl chain linking the morpholine heterocycle to the polymerisable synthon led to a different reactivity of the monomers during polymerization.

From the series of acrylamide-derived morpholines, *N*-acryloyl morpholine (NAM, also noted as ACMO or AcM) is a highly reactive monomer with fast polymerisation kinetics in RAFT,[8] but less explored in ATRP or NMP.[12] In another work, methacrylamide-derived morpholine was polymerised by RAFT, with slower kinetics compared to NAM.[37] Moreover, morpholino ethyl acrylate- and methacrylate monomers were polymerised by NMP or by RAFT. [12,38,33,39] However, as attested by previous works, (meth)acrylate corresponding monomers were less reactive than NAM.[12] The tailored hydrophilic/hydrophobic properties of morpholine-containing copolymers are important in the development of micelles used for drug delivery systems, especially for drugs which have low water solubility.[40,41,42,43,44,45] In addition, the hydrophilic/hydrophobic behaviour is different for poly(meth)acrylamides and poly(meth)acrylates, both for homopolymers and copolymers. Poly(acrylates)/poly(acrylamides) are less hydrophobic than poly(methacrylates)/poly(methacrylamides); and poly(meth)acrylamides) are less hydrophobic than poly(meth)acrylates as a result of H-bond ability specific to amides.[46]

Compared to morpholine polymers, thiomorpholine polymers were less explored in the literature. In general, these polymers were prepared by RAFT polymerisation of corresponding monomers. As in the case of morpholine derivatives, N-acryloil thiomorpholine (NAS) led to fast polymerisation kinetics in RAFT, while the introduction of intermediary alkyl linkers between the heterocycle and the polymerisable moieties were less reactive than NAS.[28] Thiomorpholine polymers present an interesting redox behavior, since the sulphur atom of thiomorpholine can be oxidised into sulfoxide (S=O) or sulfone (SO_2) group by oxidative agents such as hydrogen peroxide.[28] The redox changes of the sulphur atom of these polymers transform hydrophobic thiomorpholine-containing polymers in hydrophilic thiomorpholine oxide-containing polymers. In addition, the oxidation sensitivity of these polymers (thiomorpholine, thiomorpholine oxide,

thiomorpholine dioxide) is in general interesting for the development of therapies for diseases related to oxidative stress, and especially for anticancer drugs. According to a study published by Sobotta *et al.*, these polymers open interesting perspectives for the use in anticancer therapy since the hydrophobic thiomorpholine polymers can entrap hydrophobic anticancer drugs and, by oxidation of sulphur atom, release the drugs into the tumoral cell.[28]

In summary, the properties of morpholine- and thiomorpholine-derived polymers can finely be tuned by playing with many parameters: hydrophobic/hydrophilic behaviour, nature of the polymerisable group, length of the aliphatic linker between the polymerisable backbone and the heterocycle, the protonation ability of the amine moiety of the heterocycle and lastly, the oxidation ability of the sulphur atom of thiomorpholine. All these parameters, individually or associated, dictate the area of application of these polymers, as it will be described in the next section.

III. Biological applications

This section focuses on the biological properties of morpholine and thiomorpholine-containing polymers. These polymers were examined in protein binding systems, drug delivery, glucose sensors, cell alignment and cell adhesion systems, oxidation sensitive materials, as well as hemocompatible and anticoagulant devices. *N*-acryloyl morpholine (NAM) is a commercial monomer, thus poly(*N*-acryloyl morpholine) (PNAM) was one of the most cited polymers in the biological field, after poly(ethylene glycol) PEG which is considered as the gold standard in biology. PNAM is highly hydrophilic and is thus a potential alternative to poly(ethylene glycol) (PEG).

III.1. Protein binding and improvements in protein detection systems

In order to perform high quality *in-vivo* assays of polymers for biological applications, strong interactions between the polymers and the proteins found in cells are required.[29,40,44,45] To reach

specificity towards proteins found in cells, common methods request to chemically modify the polymers (*via* covalent bonds) with biomolecules such as sugars, peptides, proteins, or enzymes. These biomolecules can recognise (*via* host-guest non-covalent interactions and/or electron donor-acceptor interactions) the proteins found in cells. [47,42,48]

In this section, the discussion is organised according to the type of interaction between the modified polymers and the cells: host-guest recognition (case A) based on non-covalent (A.1.) or covalent (A.2.) interactions, or electron donor-acceptor interaction (case B).

- **Host-guest recognition *via* non-covalent interactions (case A.1.)**

The most promising protein binding biomolecules are based on sugar. The interaction between cells protein and sugar follows the host-guest recognition mechanism found in biological systems (**Figure 2. a.**). The traditional method consists in modifying the polymer with a sugar which is a receptor of the targeted cell protein. A classical method consists in the functionalisation of sugar moiety with PEG derivatives.[29,30] However, some reports indicated that statistical or gradient copolymers of NAM and sugar-bearing monomers improved the selectivity and reactivity towards some proteins.[43]

An interesting example of sugar-modified morpholine-containing polymers used for the interaction with proteins was reported in 2017 by Prohl *et al.*[43] Their strategy was to prepare core-shell-corona polymeric micelles composed of a diblock copolymer poly((n-butylacrylate)-*b*-(*N*-acryloylmorpholine) (PBA-*b*-PNAM) and a triblock terpolymer poly((n-butylacrylate)-*b*-(*N*-acryloylmorpholine)-*b*-(α-D-1-S-mannosyl)ethyl acrylate) (PBA-*b*-PNAM-*b*-PManEA). These micelles can interact with Concanavalin A (ConA), a lectin found on pathologic cell surfaces, due to the presence of ManEA units which form non-covalent interactions with ConA. The block copolymer micelles with size varying between 44 nm and 56 nm consisted of an inner core formed

by hydrophobic PBA surrounded by a shell formed by hydrophilic PNAM and PManEA (**Figure 2. b.***)*. The micelles were mixed with ConA to determine the binding affinity between the polymer micelles with the lectin. The increase of the ManEA chains proportion from 30% to 60% (molar %) in the polymeric micelles resulted in a continuous increase of the binding constant with ConA (k_A, from 0.001 to 0.003 s^{-1}). At less than 10% (molar %) of ManEA chains in the polymeric micelles, no clustering effect was detectable, so the lectin binding could not be assessed. Authors concluded that the highest proportion of ManEA chains (60%) led to the best affinity with ConA with a binding constant around 0.003 s^{-1}, 30 times higher than the value reported in the literature for the ConA-mannose binding constant.[44,45]

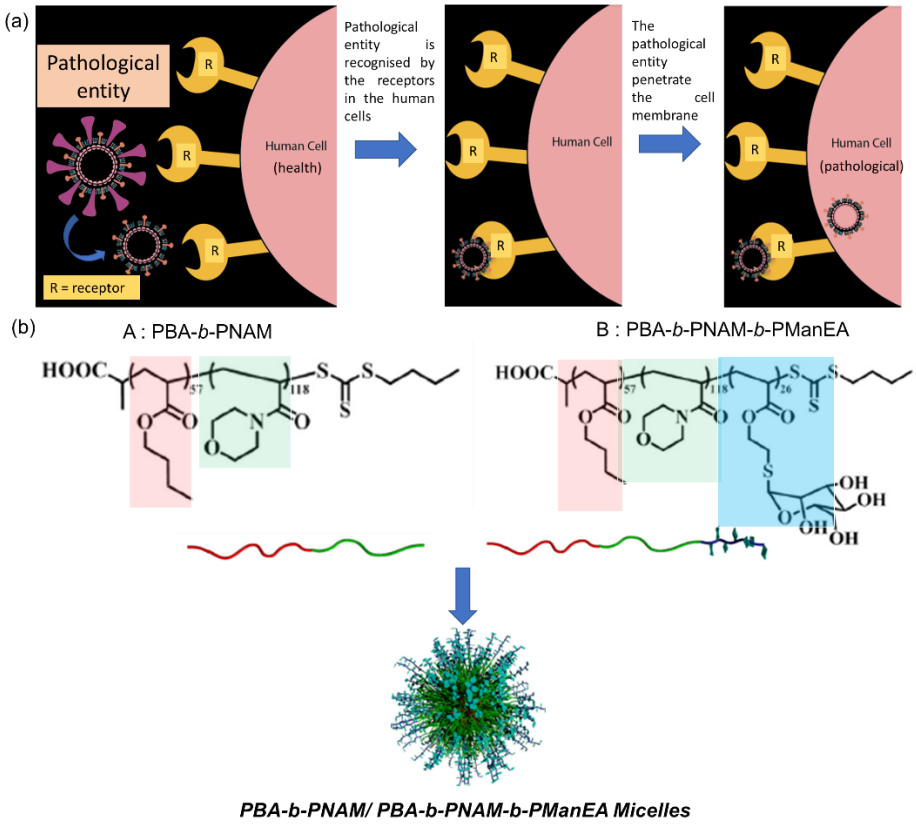


Figure 2. (a) Interaction of cellular receptors and pathological entities. (b) Formation of PBA-*b*-PNAM/ PBA-*b*-PNAM-*b*-PManEA micelles. Adapted from Ref 43, Copyright (2017), with permission from Elsevier.[43]

Another example which emphasizes the advantage of using morpholine-derived polymers for protein recognition applications was presented by Leaver *et al.*[14] They reported the synthesis of telechelic poly(*N*-acryloyl morpholine) (PNAM) bearing galactopyranose chain ends and the use of these copolymers as inhibitors for cholera toxin (CT) (**Figure 3.***)*. This toxin is a protein which binds preferentially the cellular receptors that contain galactose as terminal units *via* non-covalent interactions.[46,49] PEG derivatives linkers were reported previously as inhibitors of CT, but their synthesis was considered to be very challenging because of several protecting/deprotecting steps and the low solubilities of the synthetic intermediates in aqueous medium.[49] To overcome these issues, poly(*N*-acryloyl morpholine) obtained by RAFT was used by the authors as the polymer linker. The authors used a piperazine bifunctionalised chain transfer RAFT agent (containing di- or tri- thiocarbonate groups) to obtain telechelic PNAM by RAFT polymerisation (**Figure 3.**, step a). Then, they incorporated two galactose units by post-modification of the telechelic PNAM (**Figure 3.**, step b and step c). They observed that the affinity of PNAM-galactose modified conjugate to bind the toxin protein was increased compared to individual galactose. Thus, the inhibition of the toxin was more pronounced in the case of PNAM-galactose conjugate. A number of 130 acryloyl morpholine units led to the most efficient toxin inhibitor with an IC_{50} of 1.34 mM.

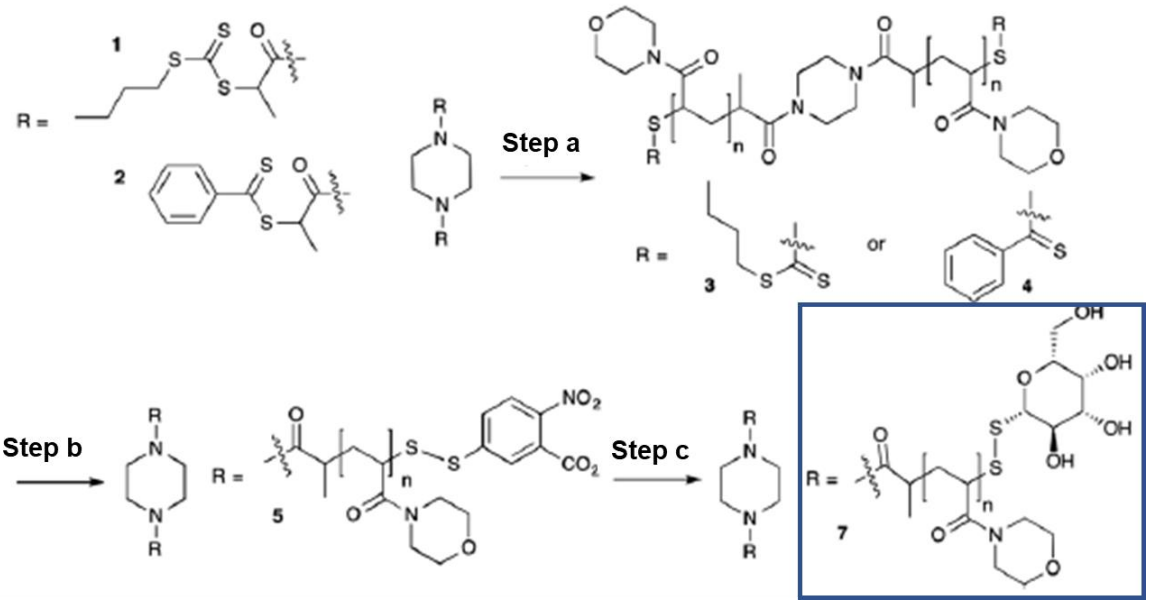


Figure 3. Synthesis of galactose poly(*N*-acryloyl morpholine) polymers (represented in the square): Step a (RAFT polymerisation of *N*-acryloyl morpholine NAM using the bifunctionalised CTA piperazine linker); Steps b and c (Step b: Post-modification of PNAM with 5,5' - dithiobis-(2-nitrobenzoic acid) and Step c: with 1-thio-β-D-galactopyranoside). Reproduced from Ref. 14 with permission from the Royal Society of Chemistry.[14]

Relogio *et al.* also used morpholine-containing polymers for streptavidin-biotin screening applications in 2013.[50] Their system used lucifer yellow (LY) dye labelled P(NAM-*co*-NAS) copolymers covalently conjugated to biotin which was able to recognise the targeted streptavidin protein (by non-covalent host-guest interactions). In order to introduce LY in the polymer structure, the P(NAM-*co*-NAS) copolymer was reacted with amino functionalised LY dye. NAM was considered for its hydrophilicity and biocompatibility, which are important requirements in protein bio-sensing. NAS provided the reactivity of the copolymer with amino-functionalised LY dye. The non-reacted NAS moieties were either hydrolysed in acrylic acid, or were reacted with aminoethyl morpholine (AEM) (**Figure 4. a.**). The final step consisted in the coupling of the thiol

end group of the P(NAM-*co*-NAS) copolymers with polyethylene glycol (PEG) functionalised on one end with maleimide and on the other end with biotin (**Figure 4. b.**). The authors highlighted the reduced fluorescence quenching of the LY-containing copolymers displaying 7- to 43-fold higher brightness than free LY dye. This result was explained by the copolymer chain conformation in water which afford some protection of the fluorophore (thus decreasing deactivation by excited-state proton transfer (ESPT)), even though the water is a good solvent for the copolymer. The study reported the complete complexation between streptavidin (a protein involved in cancer diagnosis) and polymer for a stoichiometry of 1 : 4 (streptavidin : polymer), evaluated by gel electrophoresis. To summarize, this research emphasized the interest in using NAM to develop fluorescent water-soluble polymers for protein detection. As the authors stated, these results are promising alternatives in fluorescent protein sensing detection in cancer diagnosis for example.

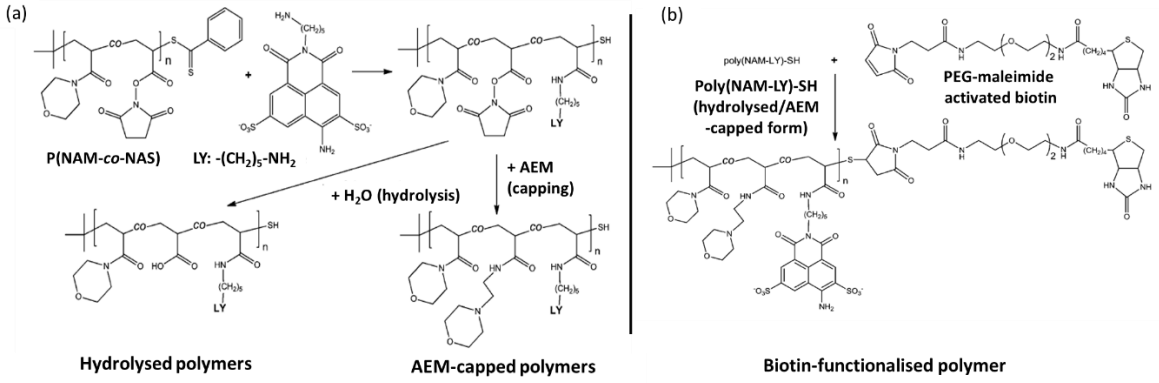


Figure 4. (a) LY Dye functionalisation of P(NAM-*co*-NAS) polymers followed by hydrolysis and/or AEM-capping; (b) Conjugation of hydrolysed/AEM capped forms with PEG-activated biotin resulting in the formation of biotin-functionalised polymers. Adapted from Ref. 50 with permission from the Royal Society of Chemistry.[50]

The use of poly(*N*-acryloyl morpholine-*stat*-*N*-acryloxysuccinimide) P(NAM-*stat*-NAS) copolymers for biotin- streptavidin protein recognition was also shown in 2018 by Duret *et al.*[13]

In this work, the adduct between biotin-streptavidin proteins was achieved by the non-covalent interaction of streptavidin labelled poly(*N*-acryloyl morpholine-*stat*-*N*-acrylamido ethyl morpholine) P(NAM-*stat*-AEM) with biotin-PNAM homopolymer. Streptadivin labelled P(NAM-*stat*-AEM) was prepared in two steps: the controlled coupling of amino-containing chromophore with P(NAM-*stat*-NAS), followed by the capping of the remaining lateral activated ester units by aminoethylmorpholine (AEM). The second step furthermore promotes the aminolysis of the dithiobenzoate chain-end into a thiol group that is then involved into a thiol-ene reaction with NHS, leading to fluorescent polymer probes with an NHS ester chain-end (noted as AEM-capped polymer-chromophore conjugates-NHS Ester) (**Figure 5. a.**). The dye-functionalised polymer was then reacted with streptavidin (**Figure 5. b.***).* This work showed that poly(*N*-acryloyl morpholine-*stat*-*N*-acrylamido ethyl morpholine) copolymers could be successfully involved in an efficient and versatile approach of protein conjugation.

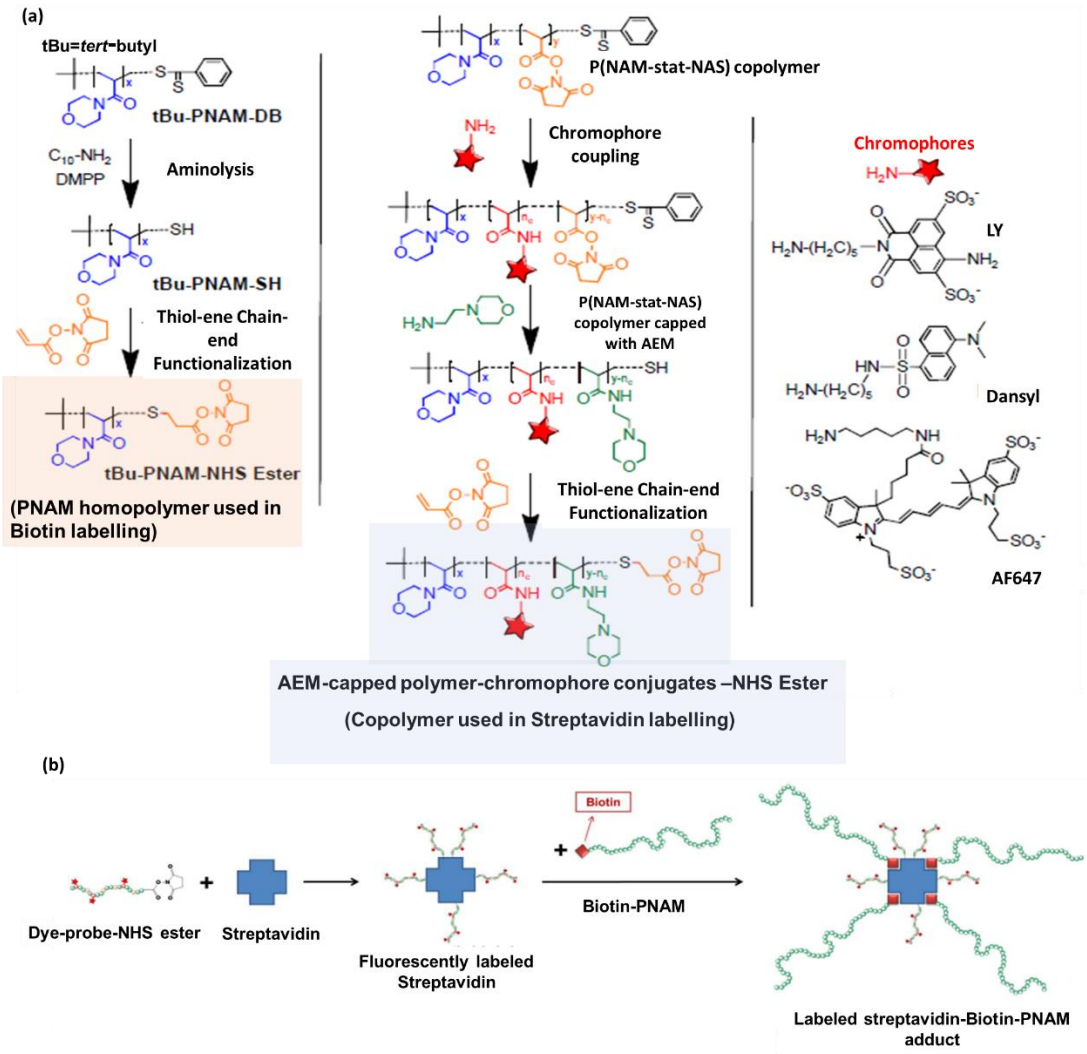


Figure 5. (a) Structural representation of copolymers bearing organic chromophores; (b) Formation of streptavidin-biotin adduct. Adapted from Ref. 13 with permission from the Royal Society of Chemistry.[13]

Poly(NAM-*stat*-NAS) copolymers were also reported by Duret *et al.*[35] as excellent candidates to prepare polymultivalent polymer-peptide ligands used for integrin (the receptor of cRGD peptide) expression (**Figure 6.**). According to the authors, poly(NAM-*stat*-NAS) copolymers were chosen for two reasons: first, because NAM co-monomer provides good water-solubility, biocompatibility, and it limits the non-specific bio-interactions; second, because NAS co-monomer provides reactive esters groups that ensure the conjugation with cRGD peptide (*i.e.*, *via*

the reaction between NAS moieties with free terminal amino groups of cRGD peptide cluster). Then, the study reported that these polymultivalent clusters exhibited a high affinity for integrins (*i.e.*, *via* non covalent interactions between cRGD and integrins) which are actively involved in cell adhesion processes. The authors reported that the best inhibition of cell adhesion (IC_{50} = 8 nM) was observed for a polymultivalent ligand concentration of 4.2 mol% (*i.e.*, expressed as the molar % of the peptide cluster per polymer conjugate). A decrease (to 1.5 mol%) or an increase (to 5.9 mol%) of the peptide molar fraction in the polymer conjugate concentration led to a lower inhibitory effect. According to the authors, the correlation between polymer conjugate molar fraction and inhibitory effect variation was a result of steric hindrance caused by the polymer chains which suppressed the lateral cRGD recognition of integrin, or supplementary crowding effects for high molar fraction of peptide in polymer conjugate.

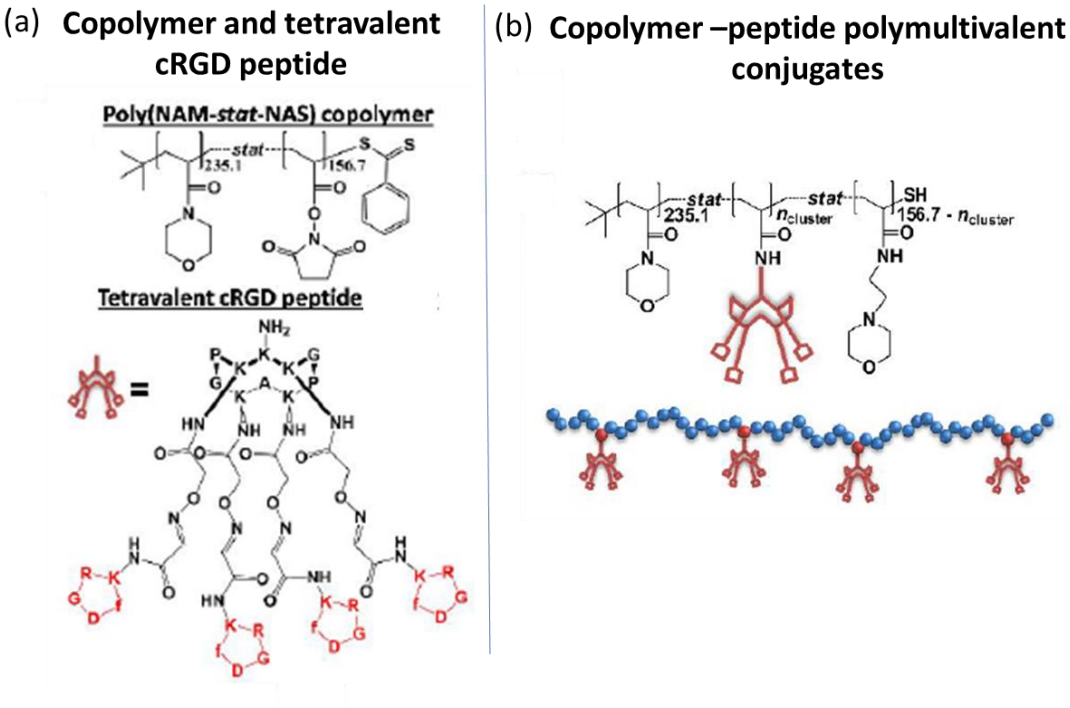


Figure 6. (a) Structural representation of poly(NAM-*stat*-NAS) copolymers and peptide ligand; (b) representation of copolymer-peptide multivalent conjugates. Reprinted with permission from Ref. 35. Copyright 2017 American Chemical Society.[35]

- **Host-guest recognition *via* covalent interactions (case A.2.)**

Poly(*N*-acryloyl morpholine-*co*-*N*-acryloxysuccinimide) P(NAM-*co*-NAS) copolymers were reported in different applications of protein screening. These copolymers contain hydrophilic NAM units, while NAS units are involved in the covalent linking of proteins. For example, Tanaka *et al.*[51] prepared hydrogels made of P(NAM-*co*-NAS) (65:35 molar ratio, 86000 g/mol) used as microarrays in protein A (a specific protein of *Staphylococcus Aureus* and used to detect its infections) screening. The NAS groups of the 3D hydrogel network reacted with the amino terminal groups of the protein A, and the functionalised gels were then dispensed onto a substrate (**Figure 7.**). According to the authors, the interest to use NAM-derived polymers instead of PEG or carboxymethyl dextran for example was that, PNAM showed a unique lack of undesired non-specific protein binding in physiological conditions.

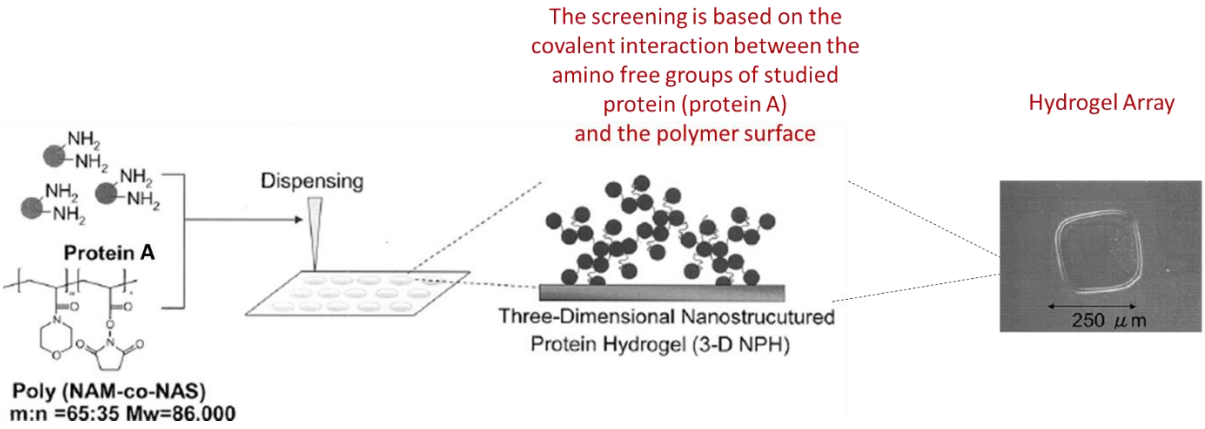


Figure 7. Representation of SPR microarray technology involving P(NAM-*co*-NAS) polymers reacting covalently with amino terminal groups of the protein A. A microarray is a collection of mini spots arranged on a solid substrate which enables the simultaneous screening of proteins in a

quick and efficient manner. The screening is based on the interactions between the studied proteins and host-proteins grafted on the microarray consisting of a polymer gel. Adapted from Ref 51, Copyright (2009), with permission from Elsevier.[51]

Moreover, PNAM showed particular usefulness in the conjugation of enzymes, as reported by Schiavon *et al.*[52] In their work, they investigated the effect of PNAM on the enzymatic stability of uricase. This enzyme regulates the production of uric acid, by lowering the uric acid level in plasma. So, the development of polymers that contribute to the stabilisation of this enzyme in the organism is highly desirable, since high levels of uric acid leads to renal failure.[53] The authors discovered that the conjugation of PNAM (M_w = 6 kDa) on uricase led to a complex hybrid conjugate able to increase the proteolytic stability and blood resistance of uricase *in-vitro*, thus decreasing the content in uric acid for a longer period.

The strategy used by Schiavon *et al.* was based on the covalent reaction between PNAM possessing an NHS activated carboxyl terminal group with the amino group of uricase. The uric acid inhibition and the blood residence time of the PNAM-uricase conjugate were respectively 100 times and 3 times higher than those of the native enzyme. According to the authors, these results were explained to be a consequence of PNAM that masked the enzyme surface. In consequence, the residence time of the enzyme was increased which thus limited the long production of uric acid.

However, the main obstacle when using enzymes such as the uricase to lower the uric acid production in the plasma is that the individual enzyme could entail a severe risk of immunoresponse.[53] Few examples of polymers [42,44] with non-immunogenic properties were reported so far, since parameters such as high molecular weight or repetitive structure altered this property. Caliceti *et al.*[41] developed PNAM-uricase conjugates acting as artificial antigens that

triggered the *in-vivo* immune response at uricase. The conjugation of linear PNAM with uricase proceeded as in the previous reported studies [52] by NHS-ester activation method. As reported by the authors, the immunogenic behaviour of PNAM-uricase linear conjugates was stronger than that of PEG-uricase branched conjugates, which is a known example in the literature for these applications. These results were explained by the authors due to the steric hindrance of the polymers around the enzyme that prevents the antibody approach, as well as by the ability of polymers to coordinate the water molecules. As authors stated, the polymers can be arranged on the enzyme surface in a linear or coiled conformation which emphasises a different degree of hiding of the enzyme. Moreover, the type of polymer conformation (coiled or linear) induces a different capability to of polymers to coordinate water molecules, which is reflected in their accessibility towards the enzyme. A linear conformation (*e.g.*, PNAM) is much more accessible to water molecules than a coiled conformation (*e.g.*, branched PEG). Therefore, the enzyme located in the hydrated polymeric core of linear PNAM or branched PEG is differently accessible to the antibody interaction. To sum up, this work highlighted the advantages of using PNAM for immunogenic enzyme-polymer conjugates.

- **Electron-donor acceptor interactions (case B)**

In this case, interactions between proteins and polymers can take place *via* coordination of a metal. In a paper published by Duret *et al.*[54] the polymer-protein conjugation was achieved by a ligand decorated-morpholine polymer and a metal complexing protein in presence of Ni^{2+} ions. The authors prepared P(NAM-*stat*-NAS) copolymers labelled with a fluorescent Dansyl cadaverin dye moiety and terminated with a nitrilotriacetic (NTA) ligand (**Figure 8. a.**) and used this polymer for site specific labelling of histidine end-tagged streptavidin. As in the previous examples,[35] the P(NAM-*stat*-NAS) copolymer was chosen by the authors due to NAM hydrophilic properties and

NAS reactivity. The recognition and the selective binding between the copolymer backbone and the streptavidin protein was achieved by Ni^{2+} ions coordination of nitrilotriacetic ligand attached to the copolymer and the histidine units of the protein (**Figure 8. b.**).

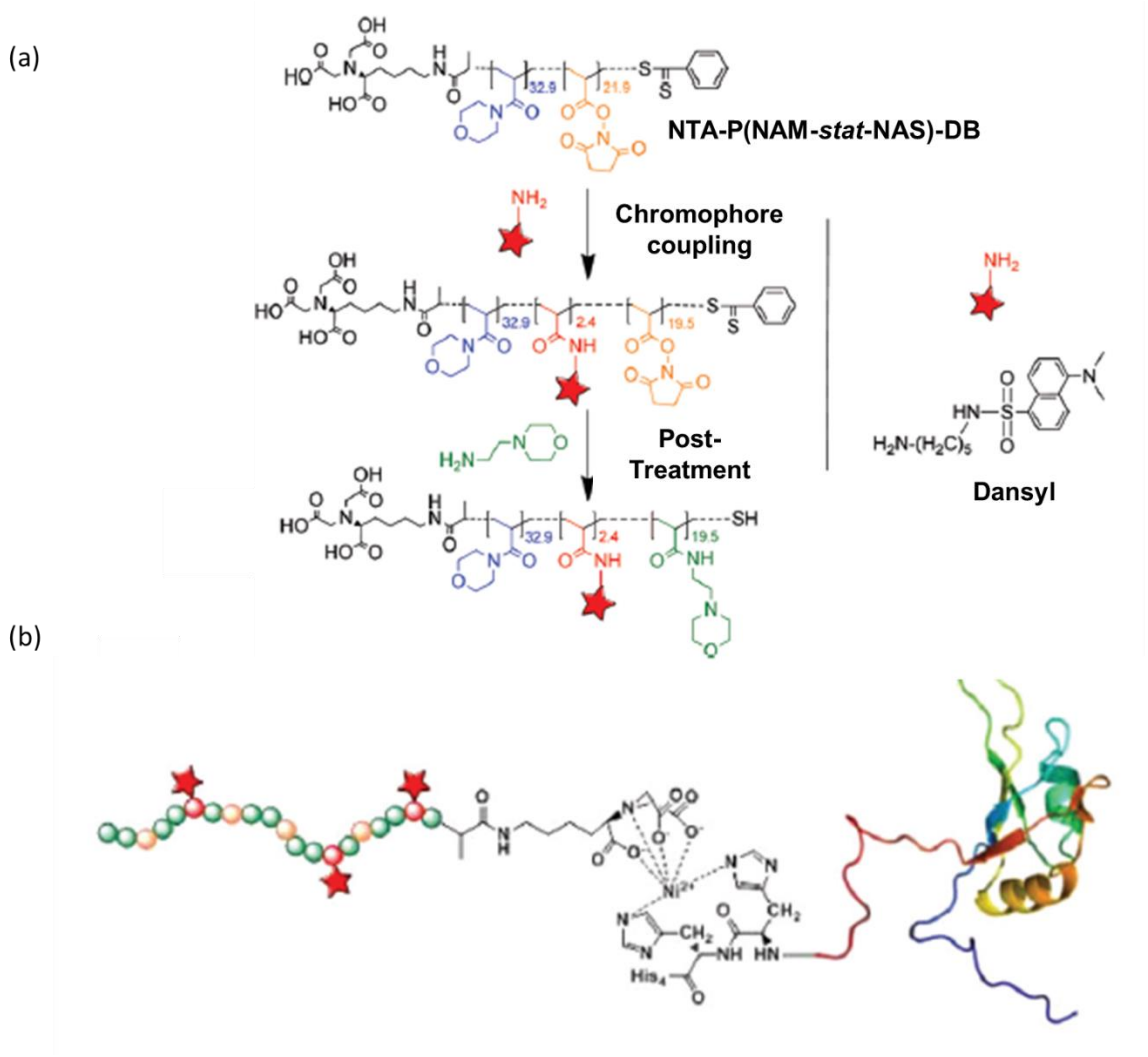


Figure 8. (a) Structural representation of P(NAM-*stat*-NAS) polymers containing NTA ligand and organic chromophore (Dansyl) (b) Graphical representation of coordination process: the polymer-protein conjugate is formed by the coordination between NTA-polymer macro ligand and His-containing protein by Ni^{2+} ions. Adapted from Ref. 54 with permission from the Royal Society of Chemistry.[54]

To conclude, NAM containing polymers showed a high interest in the conjugation with various proteins for applications including protein or enzyme detection. As mentioned by the different authors, the interest of using NAM containing polymers was their high hydrophilicity as well as non-immunogenic properties, which may open their use as new alternative to traditional PEG.

III.2. Drug delivery systems

Morpholine-derived polymers were used in the delivery of a range of small molecules (anti-cancer or anti-viral drugs, antibiotics, anti-inflammatories, nitrogen monoxide NO) and biomacromolecules (hormones and DNA). For drug encapsulation, morpholine-containing polymers were used as micelles/core-shell particles able to entrap and deliver the drug. The micelles result from the self-assembly of morpholine-containing amphiphilic block copolymers containing a hydrophilic block of morpholine and hydrophobic block. As a general strategy,[55] a hydrophobic drug is entrapped in a hydrophobic core surrounded by a hydrophilic shell; in this case, the hydrophilic shell is composed by morpholine-derived blocks. The morpholine-containing blocks enable the transportation of the drug into the organism in order to produce the therapeutic effect. Hydrophilic drugs (or slightly hydrophobic), are generally adsorbed on the hydrophilic morpholine chains surface (the shell) and delivered then to the specific site. However, each drug present distinctive particularities that require a particular design of the polymer delivery system.

Morpholine-derived polymers were used to deliver a range of anticancer drugs, particularly paclitaxel (PTX), doxorubicin (DOX), 4-hydroxy-2-nonenal (HNE), Everolimus, or anethole-dithiolethione (ADT-OH). Xu *et al.* reported in 2017 the preparation of H_2O_2-redox responsive core-shell micelles from $PNAM_{95}$-*b*-$PAEFC_{25}$ (poly (*N*-acryloylmorpholine)$_{95}$-*b*-poly(2-acryloyloxyethyl ferrocene carboxylate)$_{25}$) block copolymers and their use for encapsulation and delivery of paclitaxel (PTX).[56] PNAM block forms the hydrophilic shell of the micelles, while

PAEFC block, an oxidation sensitive polymer, forms the core (**Figure 9.**). The higher concentration in H_2O_2 in tumoral cells than in normal cells induces an oxidation of the ferrocene moieties of the PAEFC core and therefore causes the disruption of the micelles and the drug release. In the presence of H_2O_2 and in acidic medium (pH 5.8) specific to tumoral cells, after 64h, these micelles released 38.2% of drug, while in neutral conditions (pH 7.4), the cumulative drug release amount was less than 12.2%.

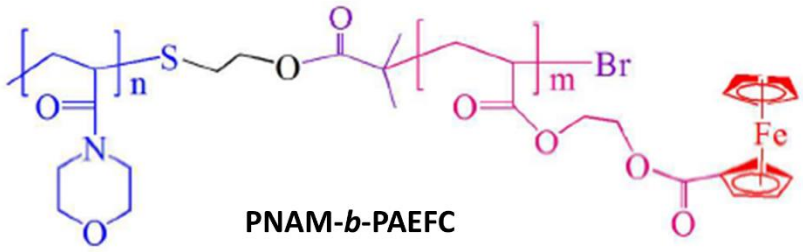


Figure 9. General structure of PNAM-*b*-PAEFC block copolymers. Adapted with permission from Ref. 56. Copyright 2017 American Chemical Society.[56]

Another example where PNAM was used in anticancer drug encapsulation was illustrated by Ramesh *et al.*[57] They prepared doxorubicin (DOX) delivery systems based on AB_2 miktoarm star block copolymers formed from PLA (as A arm) and two poly(*N*-acryloyl morpholine) (PNAM) as B arms (**Figure 10. a.**). They synthesized the mikto arms by ROP polymerisation of L-lactide using a mikto-initiator, resulting in a PLA macroinitiator, followed by RAFT polymerisation of NAM. The authors prepared a range of star block copolymers containing one arm of PLA (composed by 20 LA units) and two arms of PNAM blocks with different DP (13, 40 or 70). The self-assembly of the star copolymers resulted in micelles comprising of a PLA core and PNAM shell. After DOX was encapsulated into the micelles, the absorbance (λ_{max}) showed a red shift to 497 nm due to π-π stacking, which was explained by the authors according to the fact that DOX was encapsulated within the hydrophobic PLA core of the copolymer micelles. Moreover,

increasing the DP of the PNAM arms from 13, to 40 and 70, increased the DOX loading efficiency at neutral pH (*i.e.*, defined as [weight of loaded drug/weight in feed] x 100) from 55.6% to 66.8% and 78.7% respectively (**Figure 10. b.**). As stated by authors, these results were reasonable with the fact that the core-shell structure alteration of the micelles took place. This morphology is dependent on the DP of hydrophilic PNAM, since hydrophobic DOX was physically encapsulated in the hydrophobic core. In addition, to study the ability of DOX release in the specific conditions of cancer cells, the authors studied the release profiles of DOX loaded micelles in slightly acidic conditions (pH 6.4). The three samples of the micelles exhibited a much higher cumulative drug release (between 42% and 67%) under these conditions than at neutral pH (between 31% and 50%) suggesting an increased efficiency of this delivery system in tumoral environment. The higher amounts of DOX released in acidic conditions were explained by the protonation of the primary amine ($pK_a = 8.3$) of DOX which increases its solubility in aqueous medium. This higher solubility facilitated the drug expulsion out of the polymer micelle. These results were comparable to those obtained when PEG was used as the hydrophilic component, confirming according to the authors the high potential of PNAM for biological use.

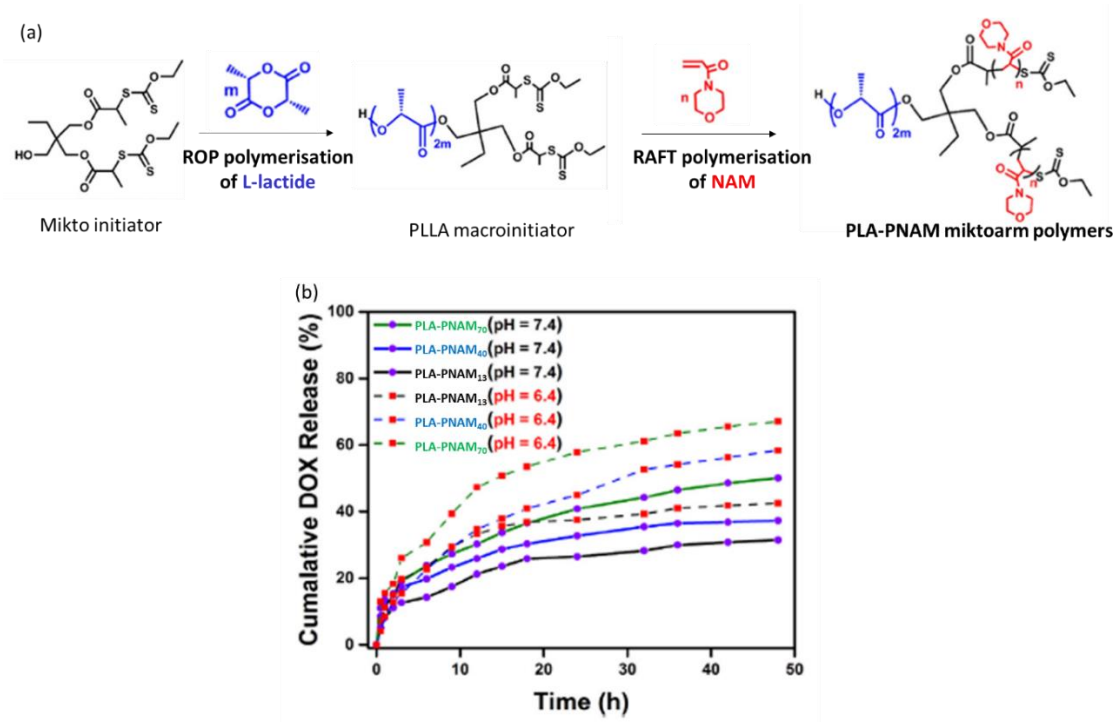


Figure 10. (a) Structure of PLA-PNAM mikto-arm polymers; (b) Doxorubicin (DOX) cumulative release profiles in acid and neutral medium. Adapted from Ref 57, Copyright (2018), with permission from Elsevier.[57]

Nevertheless, despite excellent properties shown *in-vitro*, none of the previous PNAM derived polymers were tested for *in-vivo* applications. Pizzimenti *et al.* made a significant contribution to the field of polymer complexes containing PNAM for the *in-vivo* delivery of HNE (4-hydroxynenal, an anti-tumoral agent).[58,59] HNE is known for its preference to be internalised by melanoma cancer cells and for his strong antiproliferative effect.[58] However, HNE presents poor water solubility which is an obstacle for *in-vivo* applications. The authors demonstrated that an amphiphilic PNAM-βCD conjugate (*i.e.*, βCD: β-cyclodextrin) led to a supramolecular complex able to entrap HNE. The PNAM-βCD conjugate consisted of a single βCD moiety at one terminus which contains a lipophilic inner cavity able to entrap HNE. The hydrophilic outer surface of the conjugate was formed by the free hydroxyl groups of βCD, and by the PNAM hydrophilic chain.

According to the authors, the presence of PNAM improved the overall hydrophilic properties of the conjugates and thus enhanced the aqueous solubility of HNE. HNE encapsulation by PNAM-βCD conjugates reached values above 83.5%. The *in-vitro* release results in PBS (pH 7.4) indicated a very slow-release rate for HNE (6% in 2h), demonstrating a stable host–guest complex based on noncovalent interactions. At the end, the *in-vivo* evaluation on melanoma cells showed that after 8 days in contact with the HNE/ βCD-PNAM-complex, the cells had smaller tumour nodules and remained closer to the epidermis with lower invasion of the dermal structures (**Figure 11.**). This result was explained by the addition of PNAM in the HNE-complex which acted as a protective hydrophilic shell avoiding the degradation of HNE. This study reported the first promising *in-vivo* drug delivery results involving PNAM-derived structures.

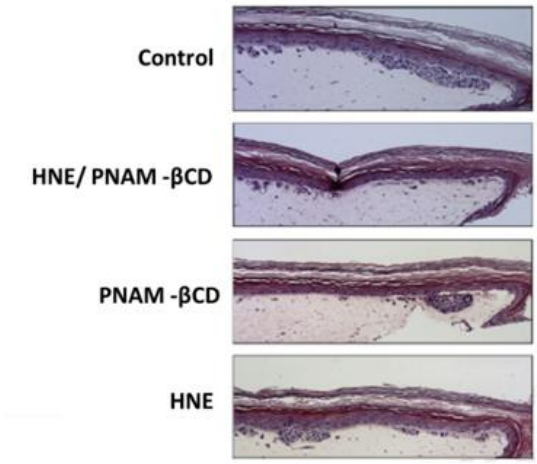


Figure 11. HNE/PNAM-βCD effect after 8 days on A375 melanoma invasion in a 3D tissue model. The top bright red layer represents the epidermis; the next layer of cells with dark blue nuclei represents the melanocyte layer; and the bottom largely unstained area represents the fibroblast-contracted collagen dermal substrate. Reprinted from Ref 59, Copyright (2013), with permission from Elsevier.[59]

The same PNAM-βCD conjugate[59] was employed by Bencini *et al.*[36] in the delivery of acyclovir (an antiviral drug). The authors stated that this system released 70% (w/w) of acyclovir in only 2h. According to them, the hydrophilic character of morpholine blocks changed the cyclodextrin behaviour from highly hydrophobic to amphiphilic, allowing solubilisation of acyclovir up to 13.3% (%wt) in the cyclodextrin cavity.

Jo *et al.*, studied micelles formed *via* the self-assembly of poly(*N*-acryloyl morpholine)-*b*-poly(*N*-acryloyl azocane) copolymers (PNAM-*b*-PAH) (15% PNAM and 85% PAH, wt %) for the delivery of Everolimus, an anticancer hydrophobic drug also used for the treatment of cardiovascular diseases (**Figure 12. a.** and **Figure 12. b.**).[17] The system afforded an encapsulation efficiency of 60% in the hydrophobic PAH core of the self-assembled micelles. This polymeric system showed that in 3 weeks, 87% of Everolimus loaded was released. However, no evidence to explain this behaviour was provided by the authors. A hypothesis was that the non-covalent interactions between the PAH blocks of the copolymer and the cavity of the drug contributed to maintain it in the polymer matrix.

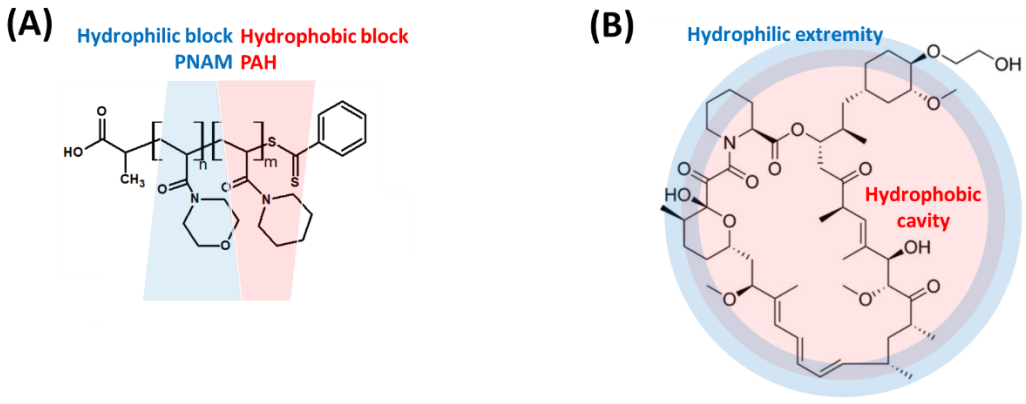


Figure 12. (A)Structure of PNAM-*b*-PAH polymers; (B) Structure of Everolimus drug [17]

Hasegawa *et al.*[60] reported in 2015 the formation of polymeric micelles *via* the self-assembly of amphiphilic block copolymers used for the delivery of an anticancer drug, (5-(4-hydroxyphenyl)-

3H-1,2-dithiole-3-thione) (abbreviated as ADT-OH). The block copolymers are composed of hydrophilic PNAM blocks and poly(N-acrylamide) hydrophobic blocks containing glycine (Gly) or isoleucine (Ile) and bearing ADT-OH units (abbreviated as PIleADT or PGlyADT). In the hydrophobic block, ADT-OH was linked by ester bonds to different acrylamide derived linkers made either of glycine (Gly) or of isoleucine (Ile) (**Figure 13.**). The hydrolysis of the ester bonds linking the ADT-OH to the amino acid-containing linkers led to the release of ADT-OH from the micelles. The ADT-OH *in-vitro* release studies showed that the drug was released faster from the PNAM-PGlyADT micelles (up to 80%) than from the PNAM-PIleADT micelles (only 10%). These differences in release percentage were attributed to the presence of sec-butyl hydrophobic group in Ile which slowed down the hydrolysis. Hence, the use of amino acids as linkers allowed to tailor the hydrolysis rate of the drug-loaded micelles, thus controlling the drug release. Cellular viability tests on three human cancer cell lines (Hep3B, MCF7 and HT29) showed that PNAM-PGlyADT micelles decreased the metabolic activity of cancer cells (*i.e.*, cellular viability below 60% which indicates cytotoxicity) as a result of fast ADT-OH release. Whereas PNAM-PIleADT micelles did not show any obvious toxicity up to 400mM due to the slow release of ADT-OH from polymer matrix. The authors also showed that the PNAM-P(Gly/Ile)ADT performed better than PEG-ADT conjugates where the ADT-OH was linked to PEG by ester bonds. This was ascribed to the hydrophilic PNAM that improved the swelling properties of the micelles and their drug release properties.

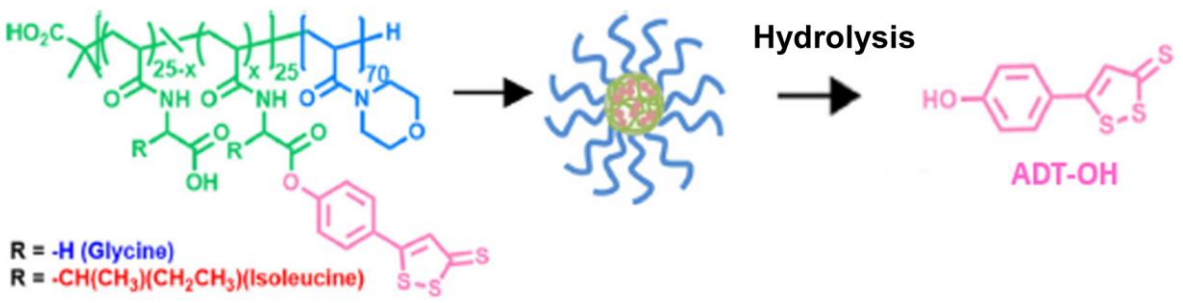


Figure 13. Structure of N-acryloyl morpholine derived polymers containing Ile/Gly groups and ADT-OH and the release of ADT-OH. Adapted with permission from Ref. 60 Copyright (2015) Wiley.[60]

Efe and colaborators[61] reported the preparation of PNAM-containing hydrogels (**Figure 14.**) as matrix to deliver ciprofloxacin (an antibiotic drug). The hydrogels were obtained by UV photo-copolymerisation of NAM and HEMA (2-hydroxyethyl methacrylate) with poly(ethylene glycol)-diacrylate (PEG-DA) as cross-linking agent. As stated by the authors, an increase in NAM content (from 0 to 30 mol%) led to a higher drug release (from 73% to 98% after 7h) due to an increased swelling rate of the hydrogel in water, generating a channel like morphology appropriate for drug diffusion through the hydrogel.

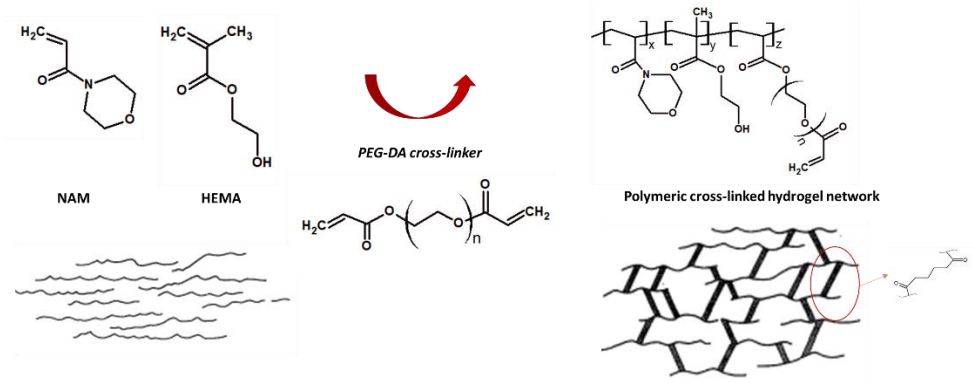


Figure 14. Representation of PNAM-containing hydrogels for ciprofloxacin delivery[61]

In addition to applications in drug delivery, morpholine-derived polymers were studied for the development of nitric oxide (NO) release systems (**Figure 15. a.**) by Seabra *et al.*[62] A lack or a

disequilibrium in NO, a key modulator of the cardiovascular system, can lead to serious pathologic disfunctions such as hypertension or thrombosis. NO release capable polymers were intensively studied to prevent the thrombus formation after cardiological stent implantation.[63] The big challenges of NO-releasing polymers are the short NO half-life (1.5 s *in vivo*) and its sensitivity to oxidation. Polymers able to protect NO from oxidative degradation and to release NO over a prolonged time are thus highly desirable. Poly(*N*-acryloylmorpholine)-*b*-poly(N-acryloyl-2,5-dimethylpiperazine) (PNAM-*b*-PAZd) block copolymers were synthesized and their self-assembly led to core-shell micelles with an average size of 50 nm. (**Figure 15. b.***)*. The amphiphilic copolymers contained a hydrophilic PNAM block (shell) and a PAZd hydrophobic block (core). The reaction of hydrophobic PAZd block with NO resulted in a NONOate fragment that preserved the NO in the amphiphilic polymer system. The hydrophobic PAZd core of the micelles protected the NONOate from degradation, while the hydrophilic PNAM conferred adequate solubility in physiological medium. The micelles prolonged the NO release in aqueous solution (half-life of 7 days, NO release over 3 weeks in aqueous solution under physiological conditions). As stated by the authors, these results were influenced by the hydrophilic behaviour of PNAM shell that protected the hydrophobic core from quick degradation and release of NO.

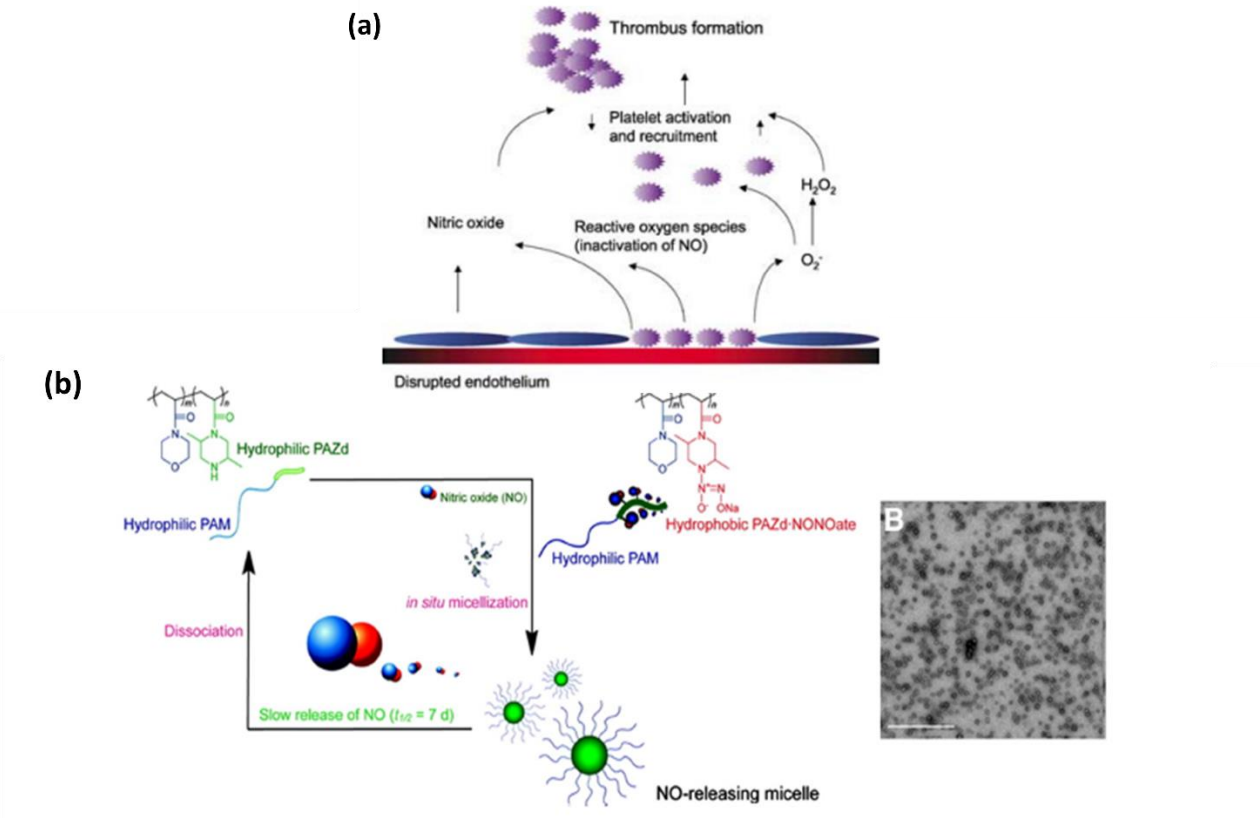


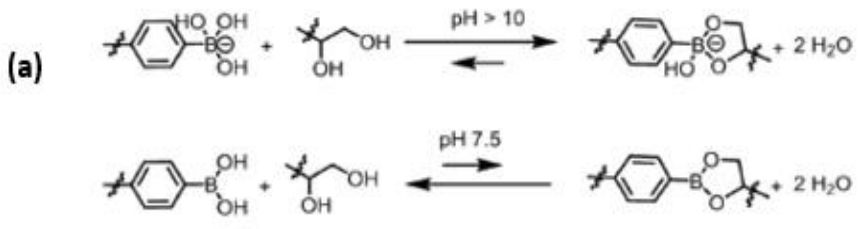
Figure 15. (a) Importance of NO in preventing thrombus formation; (b) Representation of P(NAM-AZdNONOate) polymer micelles for NO release. Reprinted from Ref 62, Copyright (2009), with permission from Elsevier.[62]

To sum up, the hydrophilic properties of morpholine containing polymers were the main reason for using PNAM in order to improve the solubility and the delivery of hydrophobic and amphiphilic drugs with multiple destinations (*i.e.,* anticancer therapy, antivirals, antibiotics, anti-inflammatory agents and NO-containing molecules). Overall, the drug delivery properties of PNAM (*i.e.,* encapsulation, cumulative drug release, etc.) were reported to be comparable to those of PEG conjugates which were already developed for this application. Compared to PEG, PNAM presented the advantage to be synthesised by (controlled) radical polymerisation in a various range

of molar weights (which are crucial to develop specific applications), which is sometimes difficult for PEG when low molar weights are required.

III.3. Glucose sensors

Another area of biological applications of morpholine-containing polymers is the development of glucose sensors. Boronic acid derivatives are involved in reversible reactions with diols, this property being used to identify and recognise sugar fragments (*via* reaction between two neighbouring hydroxyl units of sugar with the boronic acid fragment- **Figure 16. a.**).[64] Zhang *et al*.[65] have developed an interesting biomimetic polymeric platform able to complete unfolding-folding cycles in the presence of glucose, and therefore act as a sensor system. This artificial platform consisted in poly(*N*-acryloyl morpholine)-*b*-poly(*N*-acryloyl-morpholine-*stat*-glycerol acrylate) (PNAM-*b*-P(NAM-*stat*-GLA)) diblock copolymers. The diblock copolymer was cross-linked with a phenyl diboronic acid (by covalent reaction between the hydroxyl units of GLA boronic acid). As a result of this recognition between the boronic acid and the glycerol moieties of the copolymers, the system self-assembled into a folded structure, as shown by the DLS data which showed aggregates with a size ~ 111 nm (**Figure 16. b.**). After the addition of glucose, the system unfolded (according to DLS experiments showing unimers ~ 8 nm), as a result of a transesterification competition reaction between glucose and the boronic ester on one side and PNAM-*b*-P(NAM-*stat*-GLA) and boronic ester on the other side.



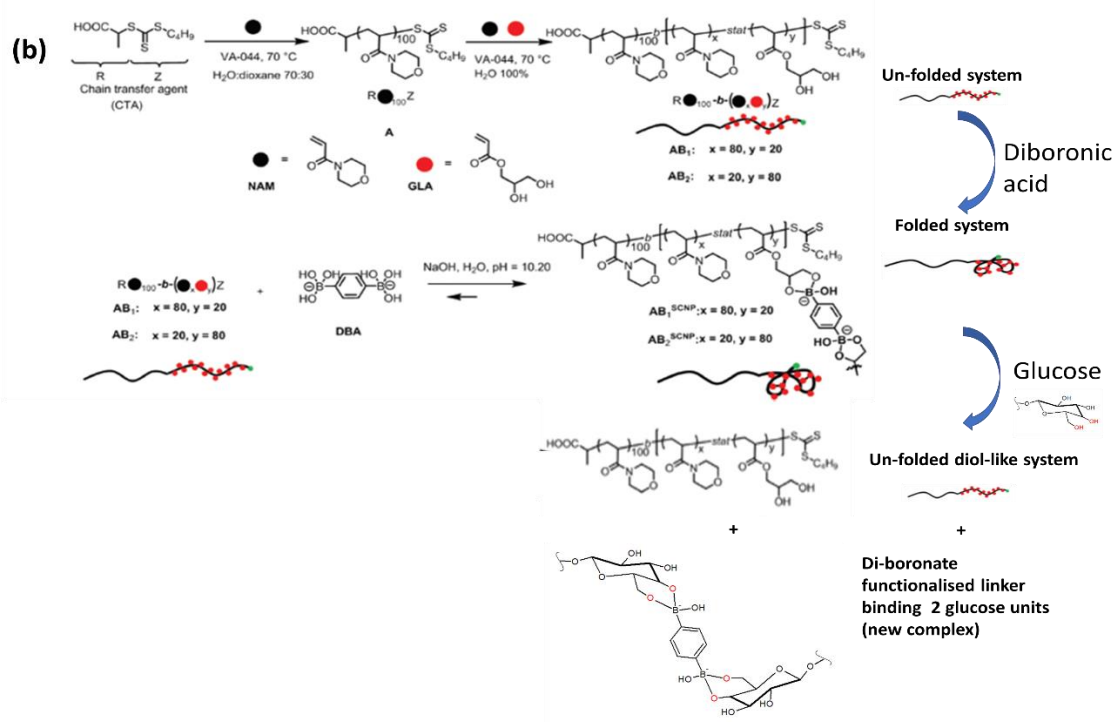


Figure 16. (a) Reversible reaction of boronate esters with diol fragments (at pH 10) and reversible reaction of diboronic acid with diols (at pH 7.5); (b) Representation of the reaction of PNAM-b-P(NAM-co-GLA) diblock copolymers with diboronic acid and the influence of glucose in the polymer folding process. Adapted from Ref. 65 with permission from the Royal Society of Chemistry.[65]

However, a serious issue in the development of boronic acid-based polymers for glucose recognition is that the glucose responsiveness takes place only for pH values higher than 10 because of the high pK_a of boronic acid polymers (**Figure 16. a.**), which is very limiting for *in-vivo* applications. A strategy was to lower the pK_a of boronic acid polymers by adding electron-withdrawing groups such as carboxyl or amino into phenyl boronic acids structures. The decrease in pK_a resulted from the coordination between B atoms and O or N atoms.[66,67] For example, Gaballa *et al.*[68] used acryloyl morpholine monomers since it contains N as electron-withdrawing atom. Their study dealt with poly[(*N*-acryloylmorpholine-*b*-(*N*-acryloylmorpholine-*co*-

pentafluorophenyl acrylate)] block copolymers, (PNAM-*b*-(PNAM-*co*-PFPA)), obtained by RAFT polymerisation of NAM and FPA from a macromolecular PNAM chain transfer agent. The reaction of PNAM-*b*-(PNAM-*co*-PFPA) with 3-amino phenyl boronic acid led to PNAM-*b*-(PNAM-*co*-PBA) block copolymers. The self-assembly of PNAM-*b*-(PNAM-*co*-PBA) led to core-shell micelles composed by a hydrophilic PNAM shell and a hydrophobic PBA containing core (**Figure 17.***)*. According to the authors, the prepared micelles were sensitive to a glucose concentration level specific to hyperglycemia (*i.e.*, glucose concentration above 2 g/L), similar to PEG derived systems reported by Wang *et al.*[67] This result was ascribed by the authors to two reasons: the hydrophilicity of the systems thanks to the use of PNAM, as well as the decreased pK_a of the polymer through the formation of boronate ester bonding, which enhanced in consequence the glucose-responsiveness of the polymers in physiological medium.

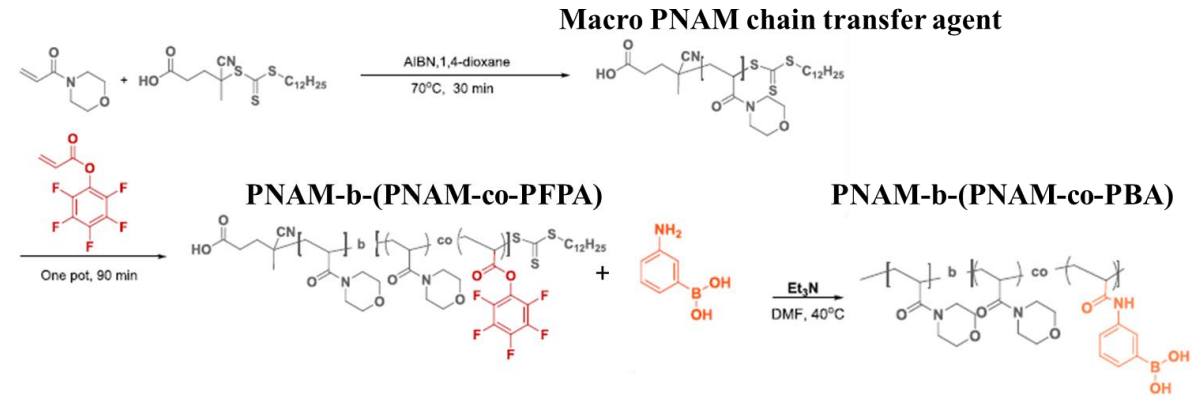


Figure 17. Structure of PNAM-*b*-(PNAM-*co*-PBA) copolymers. Adapted with permission from Ref. 68. Copyright 2019 American Chemical Society.[68]

III.4. Biomaterials for cell alignment and cell anti-adhesion

Another field of application of morpholine-containing polymers is the development of biomaterials for cell alignment or cellular anti-adhesion devices. After a cardiovascular attack or a thrombosis, the arterial reconstruction demands materials able to promote the regeneration of the

tissues, a process which requires to avoid the adhesion of lipophilic substances. In addition, the materials used to prevent such adhesion have to be efficient in physiological medium.[69] Another requirement of arterial reconstruction materials is that the design of the material should mimic the extracellular matrix (ECM). Micropatterned surfaces are substrates onto which adhesion can be tuned so as to obtain regular arrays used for cell immobilisation and/or alignment. Such materials were reported by Takahashi *et al.*[70,71] who developed PNAM containing micropatterned polymer brush surfaces by combining surface-initiated RAFT polymerization and photolithography techniques. They synthesised by RAFT poly(*N*-acryloyl morpholine)-*b*-poly(*N*-isopropylacrylamide) (PNAM-*b*-PNIPAM) block copolymer brushes on micropatterned surfaces where NHDF cells (normal human dermal fibroblast cells) were seeded and started to align as in a natural tissue (**Figure 18. a.**). First, PNIPAM brush was prepared from initiator-immobilized glass substrates by a surface-initiated RAFT polymerisation process. Then, some living terminal groups of PNIPAM were removed by photopatterning. Finally, NAM was polymerised to grow the second block, which results in the formation of PNIPAM-*b*-PNAM . According to the study, PNIPAM surface enabled hydrophobic and cell-adhesive properties at 37°C because cell-adhesive proteins such as fibronectin from the physiological medium adhere to the hydrophobic PNIPAM surface. The authors observed that compared to PNIPAM polymers alone or to the regions rich in PNIPAM, the introduction of PNAM suppressed the NHDF (normal human dermal fibroblasts) adhesion on the polymer surfaces. Moreover, they reported that in the presence of NHDF, the patterned PNAM-*b*-PNIPAM regions enabled the cell to migrate and proliferate, but not to adhere (**Figure 18. b.**). After immunostaining the cell layers, fluorescence microscopy showed the orientation of actin filaments and fibronectin which regulate the reformation of ECM (extracellular matrix). These results were due, according to the authors, to the cell-repellent property of

hydrophilic PNAM that allow the cells to migrate and proliferate, but not to adhere, which further led to a specific filament-like orientation as in the case of ECM (**Figure 18. c.**).

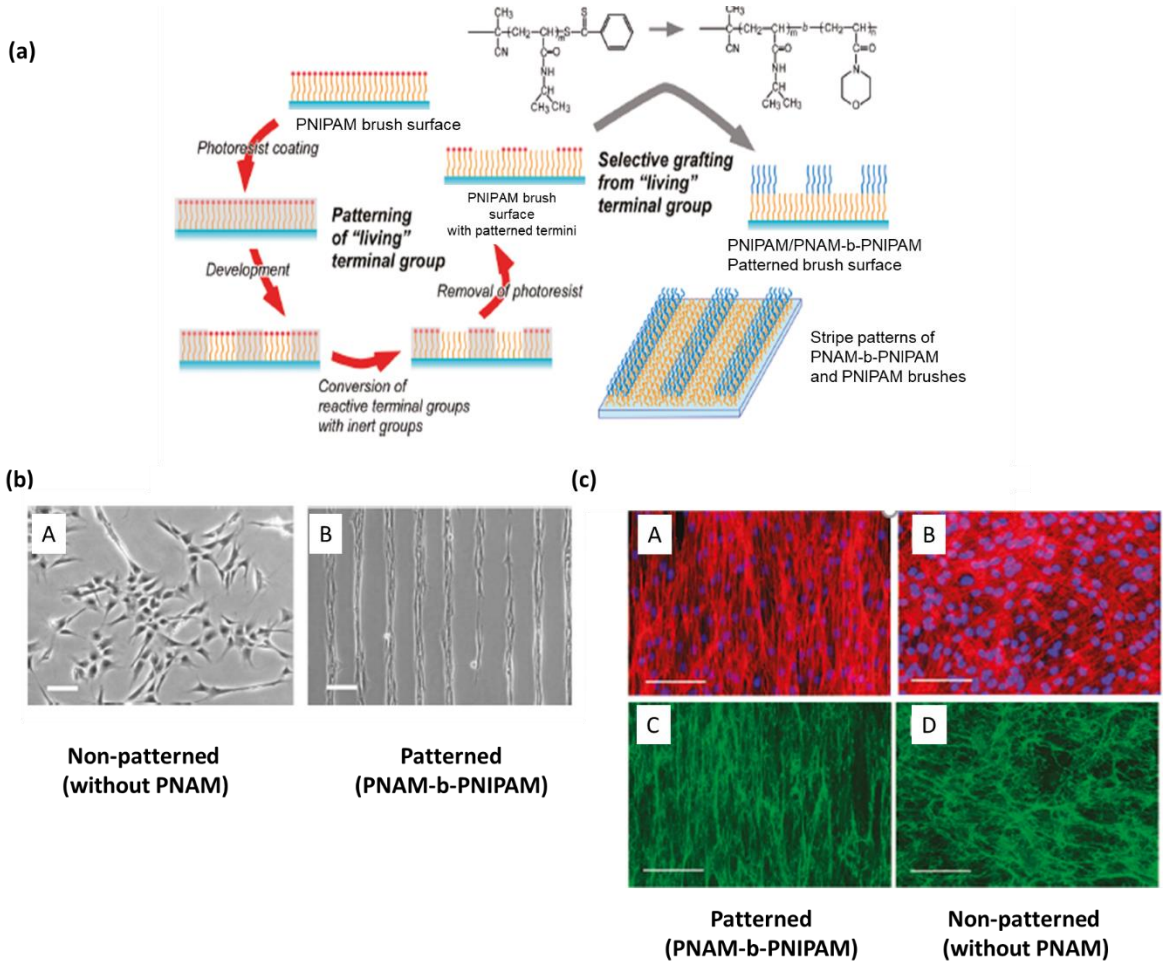


Figure 18. (a) Representation of the process involved in the design of PNAM-*b*-PNIPAM brush polymers; (b) Behaviour of non-patterned (A) and patterned (B) polymer brushes for cell migration: for the non-patterned surfaces (without PNAM), the fibroblasts adhered but did not migrate; for patterned surfaces (containing PNAM-*b*-PNIPAM copolymer brushes, the fibroblasts did not adhere, but they migrated and proliferated;(c)The fluorescence images of adherent fibroblasts of patterned (A, C) and non-patterned (B, D) surfaces. The fibrolectin is aligned to reproduce ECM in the case of patterned surfaces (containing PNAM-*b*-PNIPAM copolymers brushes), while for non-patterned surfaces (without PNAM) the fibers are not aligned. Globally,

the patterned surfaces using PNAM improved the cell migration and alignment to develop ECM biomimetic materials. Adapted with permission from Ref. 70. Copyright 2011 American Chemical Society.[70]

Other important contributions of PNAM-*b*-PNIPAM copolymers in this field were reported by Kobayashi *et al.*[72] Following the work of Takahashi *et al.*,[71] they achieved the alignment of myoblasts on the stripe micropatterns of the PNIPAM/PNIPAM-*b*-PNAM brush domains over a surface of 50/50 μm at 37 °C. At 20°C the aligned cells were detached from the copolymer brush domains as a contiguous sheet from the surface. This result was explained as a result of the LCST properties of PNIPAM (*i.e.*, with an LCST at 37 °C) which becomes hydrophilic upon lowering the temperature to 20°C, but also a consequence of the hydrophilic non LCST behaviour of the PNAM chains.

Another study showing the anti-adhesion action of PNAM was published by Gorman and collaborators.[73] In their study, PNAM used as cell spatial barrier. Photopolymerization of NAM was performed in the presence of a photoinitiator (*i.e.*, 2,2-dimethoxy-2-phenylacetophenone), without any organic solvent, leading to a gel. As stated by the authors, the PNAM gel acted as a cell spatial barrier for 38 h, without cytoskeleton formation. After 80h, the cells filled up the gel cavities and regenerated the natural tissue. This result was explained by the authors to be a consequence of quick dissolution of PNAM over short timeframes in water. Therefore, the polymer undergoes dissolution from water exposed cell surface to bulk, gradually decreasing in weight and surface area until a small quantity of polymer remains prior to full dissolution. In this study, the authors showed that PNAM is a non-adhesive material to cells, where deposited and dissolved at a predictable rate, it acted to delay cell adhesion over the covered area without affecting or harming cells adjacent to the boundary, proving no cytotoxicity.

III.5. Oxydation sensitive materials

H_2O_2-sensitive polymeric systems are of high interest for cancer diagnosis and therapies because the tumoral regions are rich in hydrogen peroxide. Thus, the development of micelles sensitive to oxidative agents such as H_2O_2 is very promising. Moreover, oxidation sensitive polymeric micelles could destroy tumours when anticancer drugs are entrapped in the polymeric matrix cavity and then released under oxidation stimuli. Inspired by these properties of oxidation-sensitive materials, Sobotta *et al.*[28] designed poly(*N*-acryloyl morpholine)-*b*-poly(*N*-acryloyl thiomorpholine) (PNAM-*b*-PNAT) block copolymers. These polymers self-assembled in micelles containing a PNAT hydrophobic core covered by a hydrophilic PNAM shell. The PNAM-*b*-PNAT polymer micelles presented a high sensitivity towards low concentrations of hydrogen peroxide (10 mM) that provoked their disintegration (**Figure 19.**). The micelles degradation took place by oxidation of the thioether groups of PNAT hydrophobic block, resulting in hydrophilic sulfoxide-containing PNATOx block.

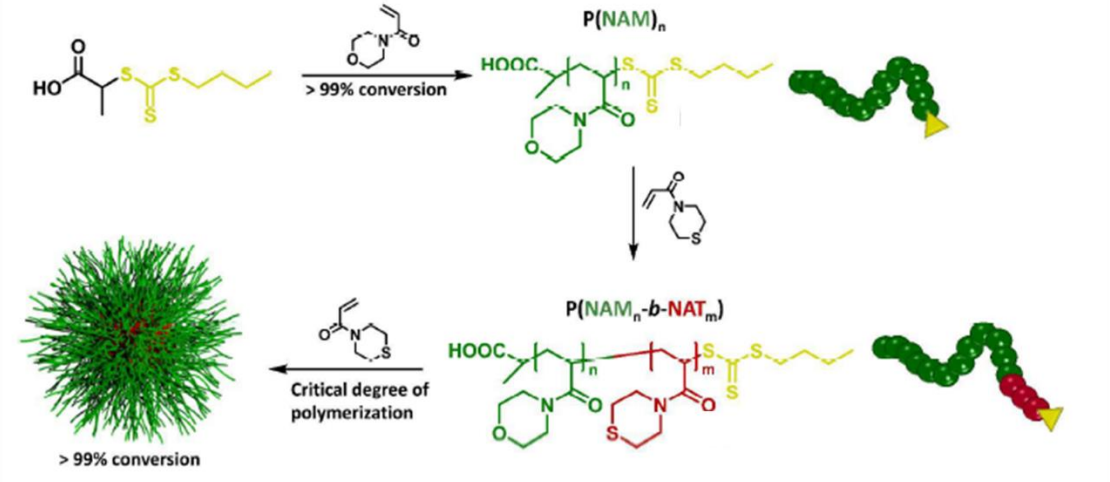


Figure 19. Representation of P(NAM-*b*-NAT) copolymer oxidation sensitive micelles. Reproduced from Ref. 28 with permission from the Royal Society of Chemistry.[28]

Another significant contribution was recently reported by Sobotta and collaborators (**Figure 20.**).[74] They prepared polymer-based vesicles (named as polymersomes) sensitives to reactive oxygen species (ROS). To this, they applied aqueous RAFT dispersion polymerisation starting from a hydrophilic macro-chain transfer agent containing 25 units of *N*-acryloyl morpholine (mCTA, noted $PNAM_{25}$) used to polymerise *N*-acryloyl thiomorpholine (NAT), in order to obtain $PNAM_{25}$-*b*-$PNAT_n$ block copolymers (n = 25, 50 and 70). Then, the authors monitored by DLS and cryo-TEM the oxidation of amphiphilic $PNAM_{25}$-*b*-$PNAT_n$ in the presence of H_2O_2, which gives the corresponding hydrophilic $PNAM_{25}$-*b*-$PNATOX_n$. As reported by the authors, the $PNAM_{25}$-*b*-$PNAT_n$ vesicles are stable even after 7h of oxidation, while after 8h of oxidation the vesicles are completely degraded and only membrane fragments are observed.

Figure 20. Representation of the oxidation steps of PNAM-*b*-PNAT-based micelles. Adapted with permission from Ref. 74 Copyright (2021) Wiley.[74]

In another study, ROS-sensitive $PNAM_{25}$-*b*-$PNAT_{30}$ micelles were investigated by Gardey *et al.*[64] (**Figure 21.**) for the treatment of inflammatory bowel disease (IBD). The block copolymer were synthesised using the same method described by Sobotta *et al.*[74] IBD is characterised by increased levels of ROS species in inflamed areas of the gastrointestinal tract and in circulating immune cells. The immune cells of people affected by IBD are known for an enhanced ROS

production compared with immune cells of healthy persons. The authors were interested to investigate if such a disease related activation is sufficient to induce a degradation of ROS-sensitive micelles. To this, they incubated the Nile Red loaded micelles for 1 h and 6 h with isolated monocytes from IBD donor persons, and performed control experiments with monocytes from healthy donors. They noted that after 1 h of incubation, the fluorescence intensity of the Nile Red loaded micelles was significantly decreased for monocytes isolated from patients with active IBD, compared with the cells from healthy donors. After 6h of incubation, the cells isolated from the patients with IBD still revealed a decreased fluorescence intensity compared to healthy donors. As stated by Gardey *et al.*, this study showed that disintegration of ROS-sensitive NAT based micelles can be selectively triggered by monocytes isolated from patients with IBD, which renders these materials promising carriers for drug delivery.

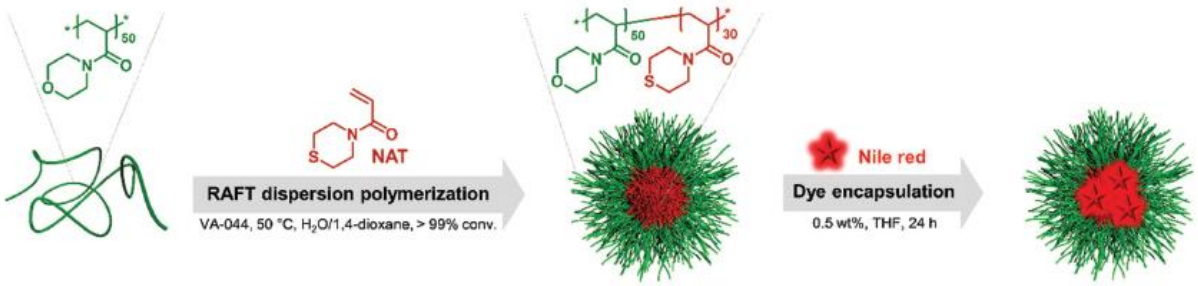


Figure 21. Schematic illustration of synthesis of block copolymer-based micelles *via* aqueous reversible addition fragmentation chain transfer (RAFT) dispersion polymerisation and subsequent dye loading. Reproduced with permission from Ref. 64 Copyright (2021) Wiley.[64]

Other important contributions about oxidation-responsive morpholine-based polymers and their use as anti-angiogenic compounds were provided by Hasegawa *et al.*[75] Polymers providing an anti-oxidant behaviour (*i.e.*, that inhibit the oxidation process by scavenging the dangerous reactive oxidative radicals derived from H_2O_2) are known to be anti-angiogenic. Angiogenesis (*i.e.*, blood

vessel growth) becomes uncontrolled in cancerous tissues and manifests by the formation of tubes in the endothelial cells caused by undesired oxidation of cells. The authors reported the synthesis of poly(*N*-acryloyl morpholine-*b*-poly(*N*-acryloyl glycyl modified catechol) (PNAM-*b*-PDA) copolymers by RAFT polymerisation. PNAM is the hydrophilic component, while PDA is the hydrophobic component providing anti-oxidant activity. A macro-chain transfer agent containing Poly(*N*-acryloyl glycine tert-butyl ester) PAG was prepared (1), and then the *N*-acryloyl morpholine was further polymerized to yield the PNAM-PAG block copolymers (2), as presented in the **Figure 22**. After the removal of the chain transfer agent end group (3), the carboxyl group-bearing blocks (4) were modified with dopamine (DA) by DCC/NHS coupling to give PNAM-*b*-PDA_x (5) block copolymers (where "x" represented the degree of modification, *i.e.* the number of DA groups per polymer chain). PNAM-*b*-PDA_x block copolymers self-assembled in water into micelles with high stability against auto-oxidation (*i.e.* high antioxidant effect), especially with increasing the degree of modification of PDA from 18 to 38. (**Figure 22.**).

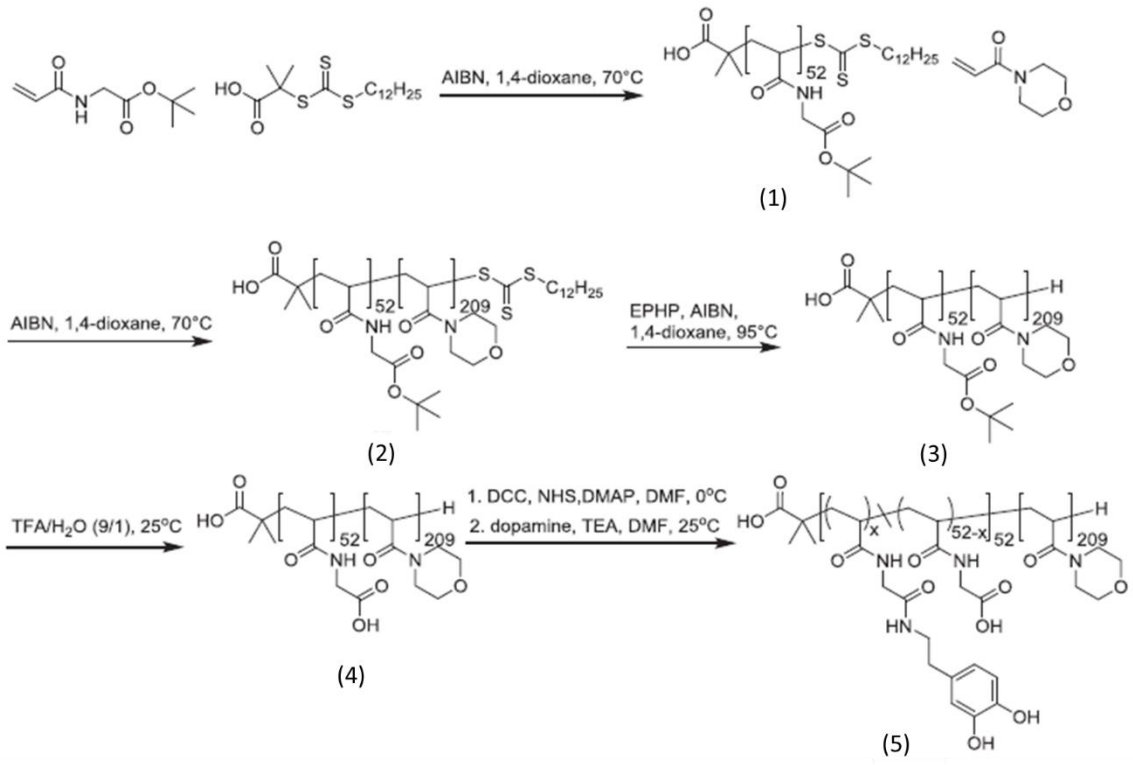


Figure 22. Synthesis of PNAM-*b*-PDAx block copolymers. Reprinted from Ref. 75, Copyright (2015), with permission from Elsevier.[75]

III.6. Anticoagulant and hemocompatible materials

Anticoagulant and hemocompatible materials represent another area of application of morpholine-containing polymers which is discussed in this review. Classical anticoagulant materials are in general hydrophobic polymer membranes that inhibit platelet aggregation during the coagulation process. Therefore, they interfere with the formation of fibrin and stable aggregated platelet products (**Figure 23. a.**).[76] For example, the anticoagulant properties of such materials can be improved by the functionalisation with hydrophilic polymers such as PEG. The use of hydrophilic polymers reduces the interactions between membrane surface and blood protein, preventing the blood coagulation on the membrane. However, a disadvantage of the PEG-modified anticoagulant membranes is that non-desired oxidation processes of the PEG ether bonds occur,

which results in reactive aldehyde moieties that are toxic for the organism. To overcome the non-desired oxidation in contact with biological medium, Liu *et al*. (2016)[78] used PNAM in the development of anticoagulant polymeric membranes instead of PEG, since PNAM showed similar hydrophilic properties as PEG, but it does not contain ether bonds sensitive to the oxidation. They prepared membranes composed of poly(vinylidene fluoride)-*g*-poly(*N*-acryloyl morpholine) PVDF-*g*-PNAM copolymers prepared *via* immersed phase inversion method (**Figure 23. b.**). The resulting polymeric membranes presented high hydrophilicity, high resistance to protein adsorption and decreased haemolysis ratio.

Respectively, the authors reported that the hydrophilicity of the membrane was slightly increased when using PNAM, the water contact angles varying from 90.8° (for PVDF) to 78.8° for PVDF-*g*-PNAM. These results were then exploited to study the resistance of membranes to blood proteins, which is a requisite for anticoagulant materials production. According to their study, increasing the grafting degree (GD) of PNAM (*e.g*., expressed in %, as the number of NAM repeat units per PVDF repeat unit) in PNAM-*g*-PVDF membranes from 5.22% to 13.52%, decreased the amount of adsorbed BSA (bovin serum albumin) from 60 µg/cm^2 to 40 µg/cm^2. This result was explained by the authors to be due to the hydrophilic PNAM that forms a hydration layer after binding of water molecules, thus avoiding the direct contact between the membrane and the proteins which eventually suppresses nonspecific protein adsorption.

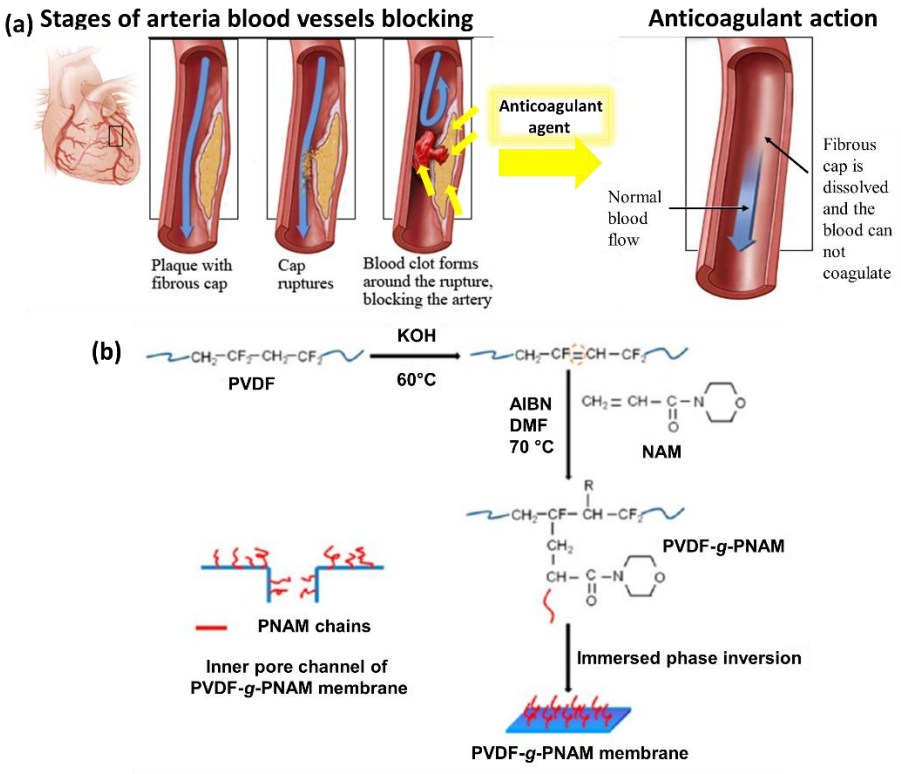


Figure 23. (a) Anticoagulant drug action in the prevention of arteria blood vessels blockage; (b) The preparation of PVDF-*g*-PNAM membranes. Adapted with permission from Ref. 78. Copyright 2013 American Chemical Society.[78]

Other contributions in this field were done by Shen *et al.*[23] who designed poly(vinylidene fluoride-*g*-*N*-acryloyl morpholine) PVDF-*g*-PNAM copolymers membranes as hemocompatible materials to suppress protein adsorption and platelet adhesion (**Figure 24.**). The PVDF-*g*-PNAM copolymers were prepared by ATRP. Three phenomena occur when a material surface is put in contact with blood: protein adsorption, platelet adhesion and thrombus formation. BSA (bovin serum protein) and BFG (blood protein) were selected as model proteins to evaluate the protein adsorption resistance of the membranes. The authors reported that an increase of PNAM concentration in the membrane from 0.028 to 0.224 g/mL led to a significant decrease of the protein adsorption from 119 μg/cm^2 to 30 μg/cm^2 respectively (**Figure 24.**). This decrease of

protein adsorption was ascribed to the hydrophilic nature of PNAM which diminished the hydrophobic interactions between the protein and the hydrophobic PVDF. PNAM generated the formation of a hydration layer which disabled the adsorption of protein on the membrane surface, further suppressing the platelet adhesion. In addition, the study showed that the hydrophilicity of PNAM had a strong influence on the evolution of hemolysis rate which is important in the evaluation of hemocompatible materials. An increase in PNAM concentration led to a decrease of hemolysis rate from 4.8% (0 g/ mL PNAM) to 0.4% (0.224 g/mL PNAM) in PVDF-*g*-PNAM copolymer membrane. These results demonstrated that PNAM is a good candidate to improve the hemocompatibility of anticoagulant materials.

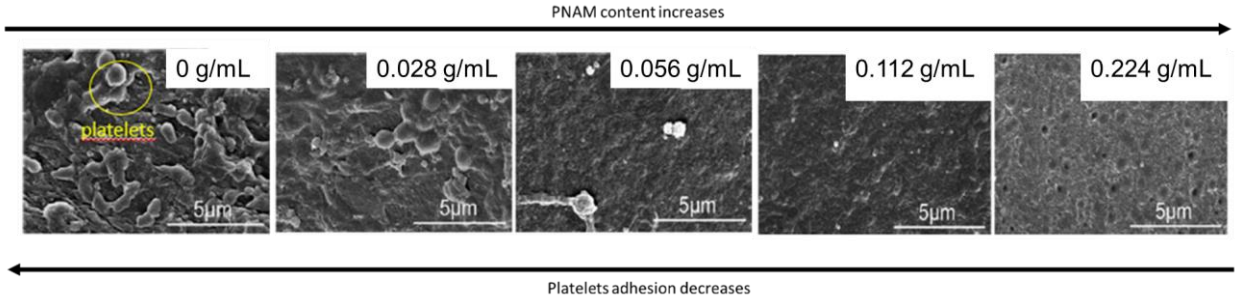


Figure 24. FESEM images of platelets adhesion for simple PVDF membranes and PVDF-g-PNAM membranes prepared with different contents of PNAM. Adapted with permission from Ref. 23 Copyright (2014) Wiley.[23]

Mochizuki *et al.*[77] studied the hemocompatibility performance of poly(*N*-acryloyl morpholine -*stat*-butyl methacrylate) P(NAM-*stat*-BMA) copolymers that were used as substrates spin-coated on PET surfaces. The P(NAM-*stat*-BMA) films exhibited excellent blood platelet compatibility for NAM content ranging from 45 to 80% (mol %). This result was correlated with the fact that platelets adhesion is lower on highly hydrophilic surfaces.

Lastly, the thiomorpholine-oxide containing polymers showed an emerging potential in the field of hemocompatible materials. In this regard, a previous work of our group reported the synthesis

of a new hydrophilic, non-cytotoxic and hemocompatible homopolymer named poly(ethyl thiomorpholine oxide methacrylate) PTHOXMA.[30] This homopolymer provided a double stimuli sensitivity to the pH and to the temperature, providing a pK_a around 5.5 and a LCST at physiological pH (pH 7.4) around 65°C. Moreover, the authors reported that PTHOXMA shows non-hemolytic behaviour at pH 7.4, since the hemoglobin release was very low (below 1%). As stated in this study, these results were explained due to the non-charged behaviour of the polymer at physiological pH which avoided the damage of cellular membrane of the erythrocites (**Figure 25.**).

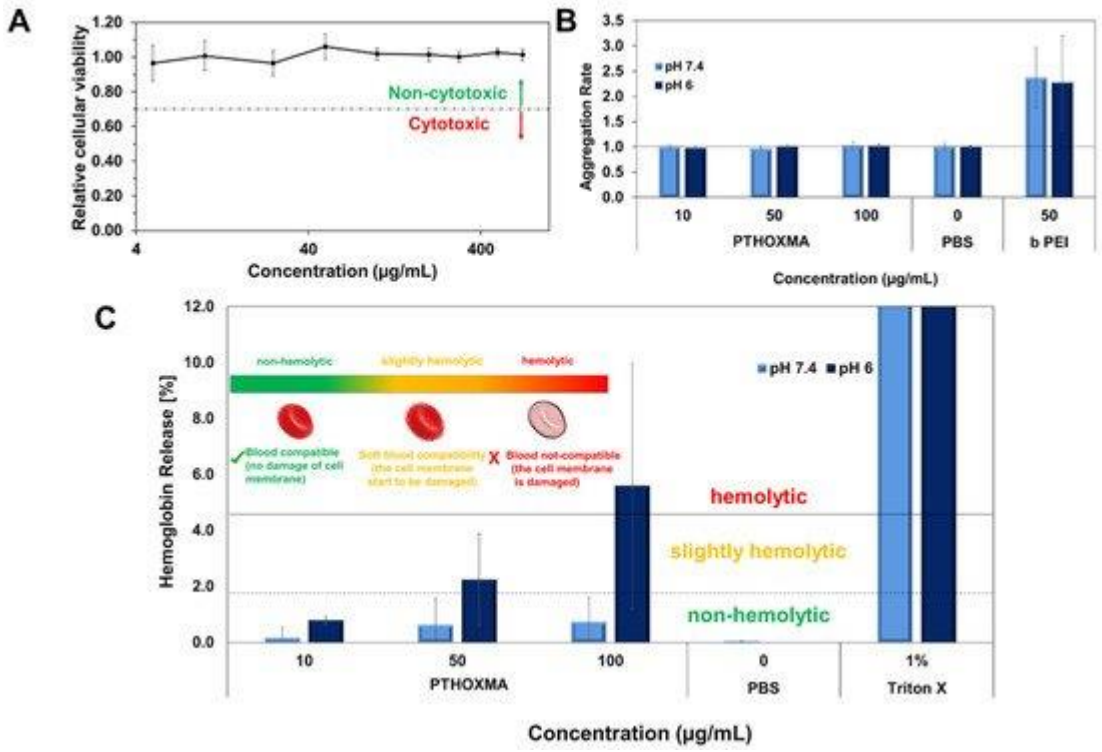


Figure 25. (a) Cellular viability results of PTHOXMA on fibroblast cell line L929 (at pH 7.4); (b): Aggregation activity of PTHOXMA; (c): Haemolytic activity of PTHOXMA evaluated by the release of haemoglobin from sheep blood erythrocytes. Reprinted with permission from Ref. 30.[30]

IV. Conclusions and Future Perspectives

This review emphasises the correlation between the structural-chemical properties of morpholine and thiomorpholine derived polymers containing (meth)acrylate/(meth)acrylamide polymerizable motifs, and their use in biological applications (**Figure 26**). Morpholine containing polymers are hydrophilic and non-immunogenic, which make them interesting alternatives to traditional PEG gold standard. Contrary to morpholine-based polymers that are predominantly hydrophilic, the thiomorpholine derived polymers showed a global hydrophobic behaviour. However it can easily be tailored to hydrophilic materials by oxidation of sulphur atom of thiomorpholine. In addition, the pH responsiveness of morpholine and thiomorpholine oxide derived polymers due to protonable secondary amino group, non-cytotoxicity and biocompatibility were other significant properties which explain the development of such materials for biological applications.

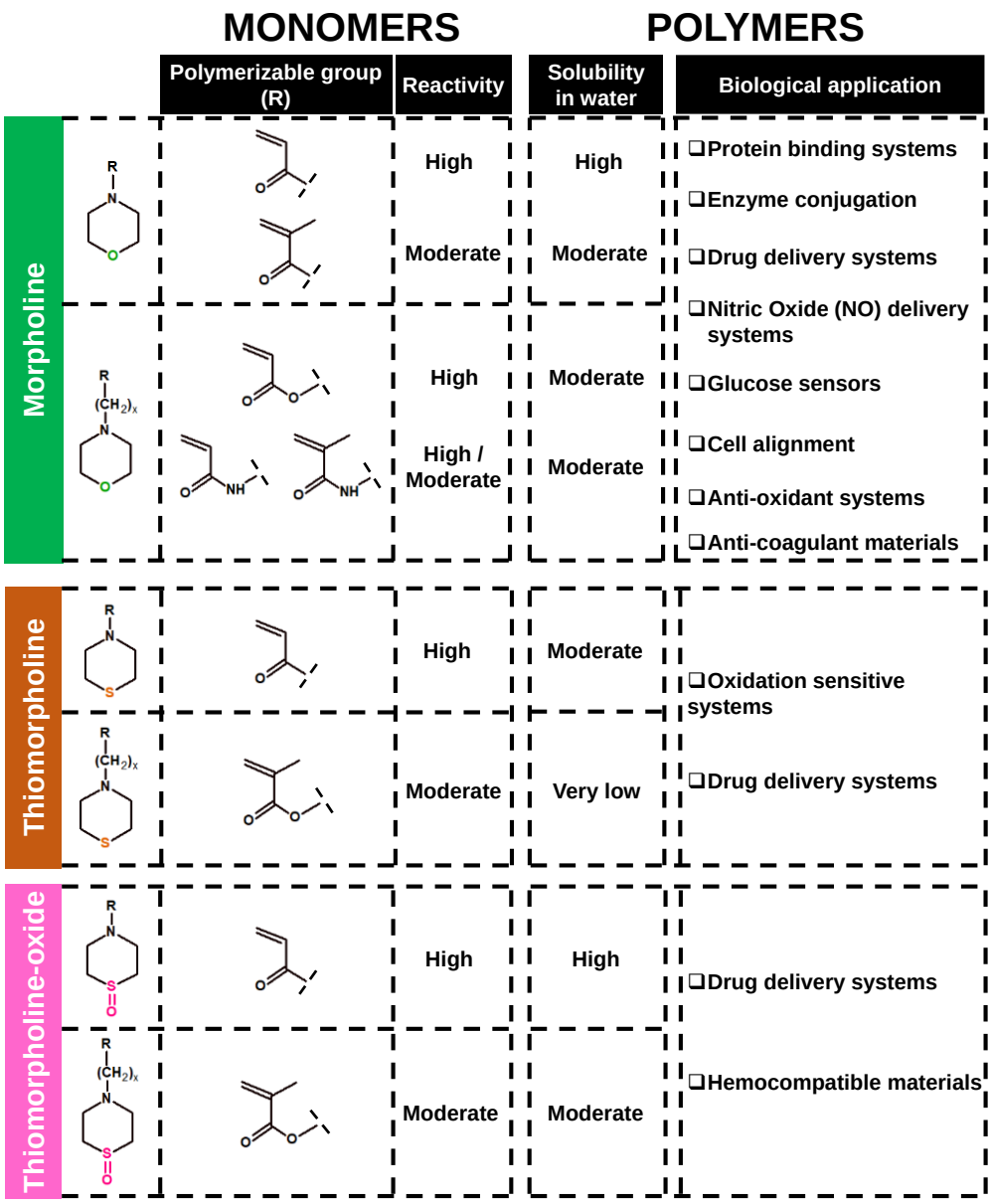


Figure 26. Summary of the chemical and biological properties of morpholine/thiomorpholine containing monomers and polymers

As underlined by most of the papers, morpholine containing polymers show comparable biological properties with those of PEG. However, PNAM polymers were reported to be more effective for *in-vivo* applications in the field of protein sensing than high molar mass PEG derivatives. In addition, PNAM could easily be synthesised by controlled polymerisation

techniques which render them more flexible and versatile to study. These advantages of PNAM were used to prepare peptide or protein-PNAM conjugates (*e.g.*, with cRGD peptide receptors, cholera toxin, biotin and streptavidin). The resulting polymeric micelles found their application as efficient markers for some therapeutic proteins responsible for inflammation or immunological reactions. Also, PNAM was reported in the conjugation with enzymes (*e.g.*, uricase) where it improved the enzymatic activity compared to the simple enzyme. Additionally, poly(*N*-acryloyl/) poly(methacryloyl) morpholine polymers were used as carriers for anticancer (*e.g.*, Paclitaxel, Doxorubicin, Everolimus, etc), anti-inflammatory (*e.g.*, Ibuprofen), or antiviral drugs (*e.g.*, Aciclovir), etc. Herein, the role of morpholine polymers was to create a hydrophilic capsule able to protect the drug and to transport it through the cell. In addition, they were involved in the delivery of small instable molecules such as NO which are important in the prevention of blood platelet adhesion on the capillaries. Also, significant researches including PNAM derived polymers were done in the field of anticoagulant and hemocompatible membranes. In this case, PNAM was used as repellent due to the hydrophilic protective layer formed on the membrane that prevented the platelet adhesion.

While PNAM was highly explored for biological applications, the thiomorpholine polymers were less investigated. Even if thiomorpholine polymers are less hydrophilic than morpholine ones, they present an interesting redox behaviour of the sulphur atom. Practically, this oxidation sensitivity is the key to transform hydrophobic thiomorpholine polymers in highly hydrophilic thiomorpholine oxide polymers. The oxidative reactivity of the thiomorpholine-polyacrylate polymers to the corresponding sulfoxides was the key to explain their use as stimuli responsive systems in oxidative sensitive drug therapies.

In summary, the morpholine and thiomorpholine related polymers represent artificial platforms with multiple uses in biology. Their particular structural features and versatile amphiphilic behaviour rests to be an interesting area of investigation in the development of smart materials with multiple functions.

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Conceptualization, L. V. Arsenie, S. Catrouillet and V. Ladmiral; writing—original draft preparation, L. V. Arsenie; writing—review and editing, L. V. Arsenie, P. Lacroix-Desmazes, V. Ladmiral, S. Catrouillet. All authors have read and agreed to the published version of the manuscript.

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