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Bottom-up strategies for the synthesis of peptide-based polymers

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Abstract

Thanks to their wide range of biological activities, peptides have been extensively used to afford designed materials with tailored properties. Peptides can be associated to polymers combining the properties of various polymer backbones with those of bioactive peptide sequences. Such conjugates find promising applications in medical devices, tissue engineering, drug targeting and delivery. Improvement of existing polymers by post-modification peptide grafting is achieved through an extensive range of organic reactions, involving the prior preparation of functional polymers displaying suitable anchoring functions. Alternatively, peptides can be used as initiators of polymerization yielding a chimeric molecule bearing a single peptide at the end of macromolecular chains. Finally, novel polymer materials can be designed when the peptide itself is used as a macromonomer. In that case, the unmatched level of repetition of the peptide sequence or/and its self-assembly properties allow to access very high functionalization degree, original structures and bioactivities.

Key words: Peptide, Polymer, Macromonomer, Polymerization, Hybrid Peptide-Polymer

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1. Introduction

Synthetic polymers have been widely developed in life sciences for the design of medical devices, drug delivery systems, implants, biosensors or scaffolds for tissue engineering and regenerative medicine.[1–10] Some of them, like polyethylene glycol (PEG), poly(methylmethacrylate) (PMMA) or polylactic acid (PLA) have been extensively studied and used for medical devices approved by the Food and Drug Administration (FDA).[11]

Polymers can be used as the only component (for example polydimethylsiloxane (PDMS) for the fabrication of soft prosthesis, PLA for stents, and PMMA for dental devices) or associated with other components to add functional (bioactivity, imaging, etc.) or physical properties (mechanical, solubility, stability, etc.) to the desired material. Conjugated to drugs or oligonucleotides, polymers can be used to form particles for gene[12] or drug[13] delivery. They can be grafted on the surface of inorganic nanoparticles (NPs) to yield organic-inorganic hybrid NPs.[14] Polymers associated with cells, tissue inducing molecules and porous three dimensional scaffolds are the main players to prepare hydrogels for tissue engineering.[15,16]

Although biocompatible, most of these synthetic polymers are bioinert, in a sense that they do not exhibit any specific bioactivity by themselves. They have to be functionalized to get additional biological properties. This functionalization can be done by two main ways: either by physisorption (non-covalent) or chemisorption (covalent) modification.

The non-covalent functionalization is usually achieved by adsorption via weak interactions on activated, or non-activated polymers. The adsorption can result from spray coating, dip-coating or incubation. It is noteworthy that the polymer's surface can be treated to enhance the adsorption. For instance, plasma treatment generates hydrophilic moieties on PDMS helping in the establishment of hydrogen bonds with biomolecules of interest.[17] Non-covalent functionalization of polymers by biomolecules is usually mediated through weak and reversible interactions (e.g. electrostatic, hydrogen bonding, aromatic, hydrophobic) or specific affinity interactions (ligand-receptor pairing when the polymers have been previously functionalized by adsorption of a protein).[18] Such specific

non-covalent interactions can be very strong: as the one between biotin-avidin for example.[19] The main advantages of the non-covalent functionalization over the covalent one relies on its simplicity and, in the case of drug delivery systems, the easier release of the active molecules.[20–22]

However, in general, non-covalent attachment leads to less stable functionalized polymers.

On the other hand, covalent functionalization, concerns a large number of polymers and biomolecules. Once conjugated with the desired biomolecules, polymers are then referred in literature as ‘hybrid polymers’ (‘bio hybrid polymers’ if the associated molecule is a biomolecule). For clarity purposes, in this review, the term “hybrid” is dedicated to hybrid inorganic/organic silylated molecules or materials. Biomolecules could be attached to the polymers either directly or via a spacer. The role of the spacer is to ensure an optimal interaction between the bioactive molecule and its target but can also play a part in the final polymer morphology. In the case of the *grafting to* strategy (described below) the biomolecules are connected either by a direct grafting on the active groups present in the polymer, or by a first step of specific activation of the surface followed by the modification (Figure 1).

Several types of biomolecules can be attached to polymer surfaces depending of the desired application. For example, enzymes and antibodies can be conjugated to polymer surfaces in order to design biosensors or hybrid materials used for tissue engineering or the preparation of antimicrobial surfaces.[18,23,24]

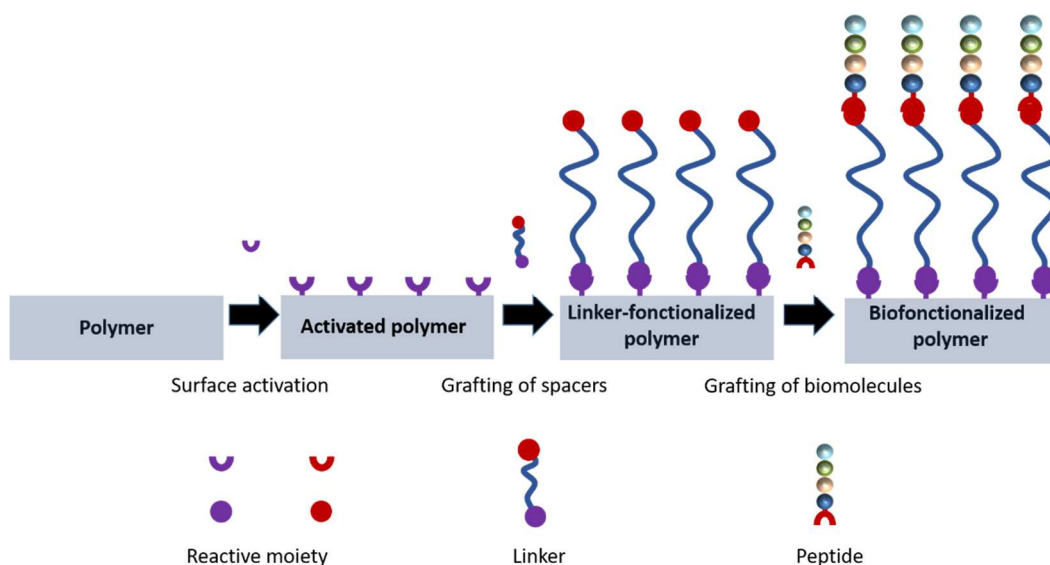


Fig. 1. Multistep covalent biofunctionalization of polymer via (i) activation, (ii) grafting of spacers and (iii) conjugation of the spacers with biomolecules (e.g. peptide).

This review will focus on polymers functionalized covalently with peptides, i.e. peptide-polymers. Peptides are one of the most common biomolecules conjugated to polymers, mainly for their antimicrobial and cell adhesion properties. The preparation of peptides and analogues is well mastered thanks to efficient solid phase peptide synthesis (SPPS) protocols widely spread among many laboratories. Combined to the numerous existing polymers, the large variety of structures and functions of peptides enables the design of an unlimited range of peptide-polymers.[25] The diversity offered by each component combined with the structure of the polymer (i.e. linear, branched or even comb like) offers an additional level of adjustability.

Three different synthetic strategies are described for the preparation of covalent peptide polymer conjugates: *grafting to*, *grafting from* and *grafting through* (Figure 2).[26] The *grafting to* strategy is a multistep process in which the peptide is covalently coupled to the polymer after its synthesis. In the *grafting from* approach, the peptide is the initiator of the polymerization, which can be either Reversible Addition Fragmentation chain-Transfer polymerization (RAFT), Atom Transfer Radical Polymerization (ATRP) or Nitroxide Mediated radical Polymerization (NMP). Lastly, the *grafting through* method is using a Peptide Containing Macromonomer (PCM), leading to homopolymers (resulting from a single monomer), block copolymers (two different monomers polymerized one after the other) or random copolymers (two different monomers polymerized simultaneously).

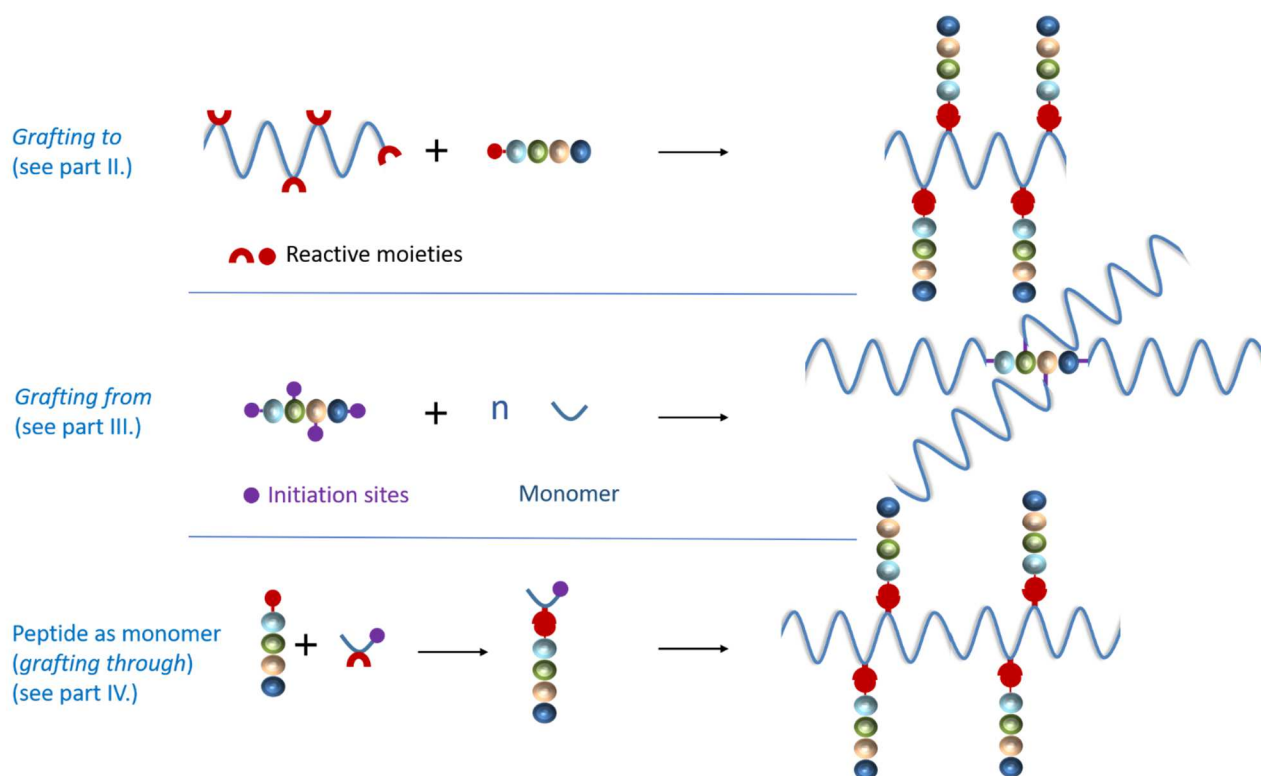


Fig. 2. General strategies to obtain peptide-polymers.

2. Grafting to strategy: Post-functionalized polymers

2.1 Grafting to strategy

2.1.1 General strategy

Grafting to or post-functionalization, to this day is the most common and the most used strategy to prepare peptide-polymers conjugates. This non-direct strategy requires two or more steps, depending on the activation and grafting chemistries applied.

First, the polymer is prepared by conventional polymerization methods, allowing the control of the desired size and structure. Then, the polymer can be isolated, fully characterized and activated for the next step. At this stage, two situations are possible. Either the polymer contains reactive groups (e.g. on its backbone or at its ends) able to react for further grafting. In this case, the grafting is operated straightforwardly after the polymerization, eventually adding reagents for the conjugation with the peptide. If the polymer does not present any suitable reactive functions to attach the peptide, it has first to be modified to display a suitable moiety to react either directly with the

peptide or through a linker that will present the required active group (Figure 1).

In summary, *grafting to* strategy leads to peptide-polymer conjugates based on well-controlled and characterized polymer backbones. Besides, researchers can make a selection among the numerous conjugation strategies described so far that offer a high chemoselectivity when unprotected peptides displaying reactive side-chains are used.[27] Indeed, a peptide may display several types of reactive groups on its lateral side chains and its N and C termini. Therefore, in the case of unprotected peptides, the conjugation chemistry has to be chemoselective in order to avoid any undesired side reactions. Finally, the efficiency of the conjugation in *the grafting to* strategy might be low. Some of the polymer's functions might be difficult to reach by the peptides to be reacted with, especially when they are situated in the core of the polymer.

The overwhelming majority of *grafting to* examples were performed on four major types of polymers: PMMA, PLA, PDMS and PEG.

2.1.2. Examples of common grafted polymers

PMMA was utilized in numerous encapsulation systems, as drug delivery system or in cosmetic applications, thanks to its high chemical stability and mechanical properties adapted to the formation of shells (high Young's modulus and low elongation at break).[28,29] On the other hand, as a biodegradable and nontoxic polymer, PLA is widely used in tissue engineering and in the design of implantable and resorbable medical devices.[30] On the contrary, -PDMS displays remarkable stability and is one of the most used polymers to make soft prosthesis and catheters.[31]

It is also biocompatible and shows stability over thermic and oxidative stress, great gas permeability and very good dielectric property. Additionally, PDMS possess high viscoelasticity enabling it to be easily deformed but rarely in a permanent way.[32–35]

PEG has shown high interest in the synthesis of biomaterials mainly due to its biocompatibility, high hydrophilicity and antifouling properties. In the design of nanocarriers, it is widely used as a stealth

agent against opsonization of delivery systems.[6,36–38] The conjugation of a peptide on a single PEG chain results into PEGylated peptides. In some cases, the PEGylated peptides can be subsequently attached to various polymeric structures depending on the final application (e.g. micelles, nanotubes, hydrogel).[5,39–50] However, PEGylated peptides are closest to *modified peptides* than *peptide-modified polymers* and consequently will not be covered in this review.

The key point of the *grafting to* strategy is the chemical reaction between the polymer and the peptide involving two mutually reactive moieties present on the peptide and on the polymer (Figure 3). If one (or both) of these two types of functions is also present on the peptide (side chains or N or C-terminus), regioselectivity cannot be achieved except if suitable protecting groups are placed on the peptide before conjugation. Unfortunately, the presence of protecting groups might lower the peptide solubility and requires an additional deprotection step after the peptide conjugation. On the contrary, biorthogonal reactions can be performed on unprotected peptides.

The main coupling chemistries used for the synthesis of peptide-polymer by *grafting to* approach are presented in Figure 3, and will be detailed in this chapter.

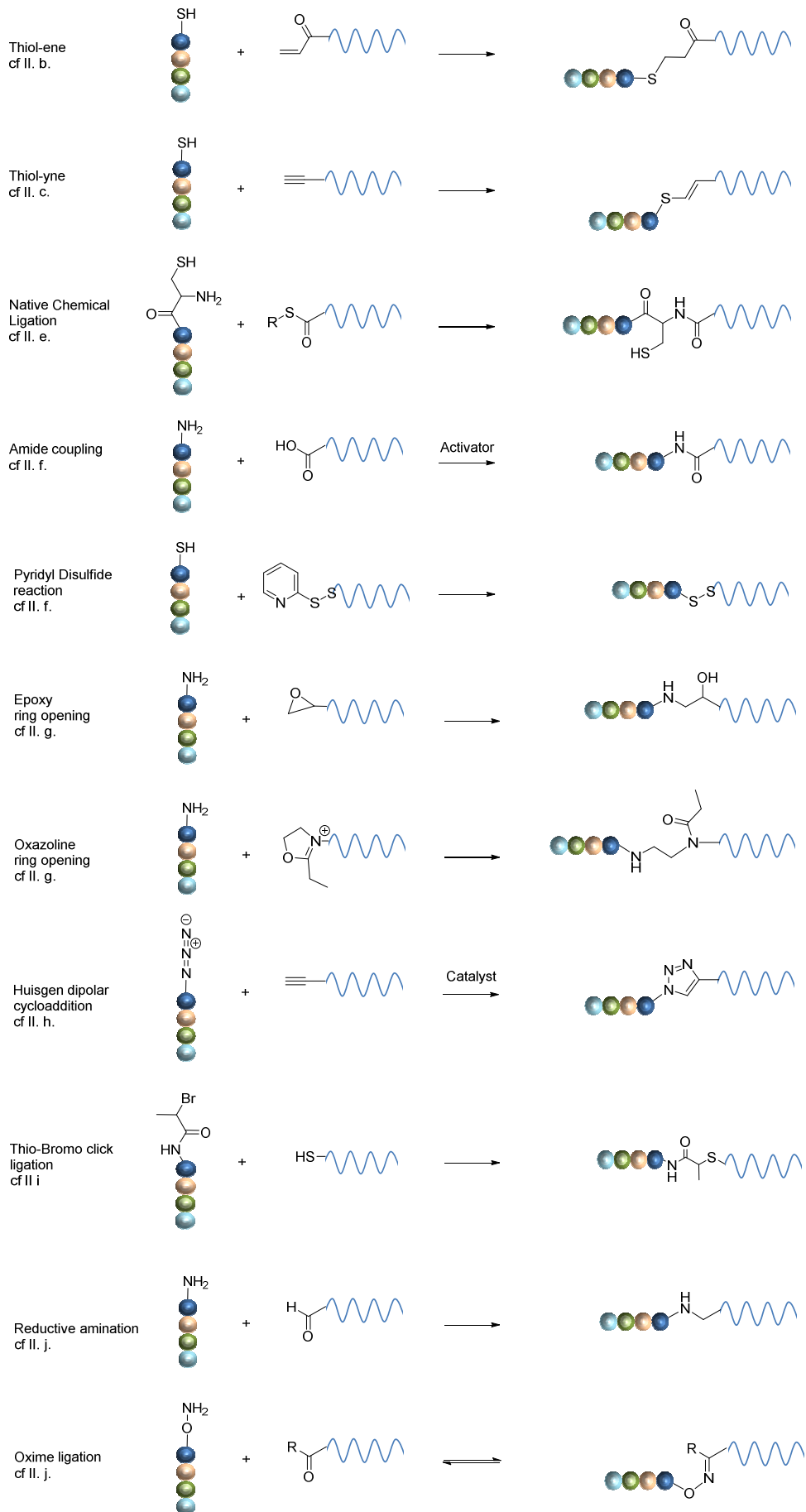


Fig. 3. Examples of chemical reactions applied to peptide-polymer conjugates by grafting-to strategy.

2.2 Michael addition (non-radical thiol-ene chemistry)

The most common chemical reaction for the synthesis of peptide polymer conjugates is the Michael addition, [39,48,51,52] which is based on the creation of a bond between a nucleophile, the donor, and an α,β unsaturated system, which can be an acrylate, vinyl or maleimide group as example, the acceptor.

When applied to peptide polymer conjugation, thiols are generally used as the nucleophilic donor in the Michael addition, and is classified as thiol-ene reaction, albeit with nucleophilic attack mechanism (Figure 4).[53] This reaction is chemoselective and proceed usually between a thiol group on the peptide (unprotected Cys side chain), and an alkene function, displayed by the polymer.

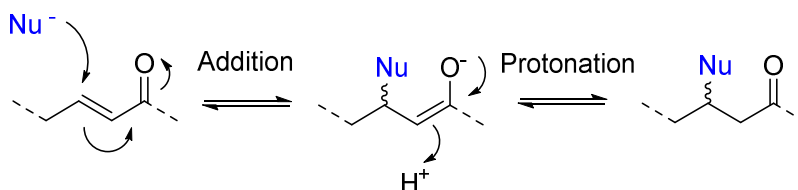


Fig. 4. Mechanism of the thiol-ene (Nu) Michael addition on an α,β unsaturated system.

Michael addition was extensively used to prepare PEG-based peptide polymers. For example, hydrogel network was obtained using Cys-containing peptides with self-assembly properties (i.e. forming coiled coil dimers and tetramers) conjugated to both extremities of PEG chain modified by two maleimide groups as Michael acceptors (Figure 5). [54] This conjugation lead to the formation of a physical viscoelastic hydrogel network.

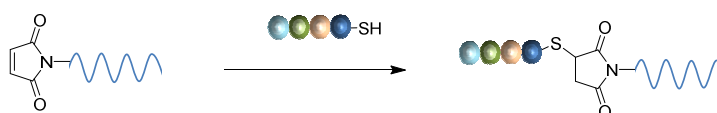


Fig. 5. Thiol-ene Michael addition forming a thioether bond between a Cys-containing peptide and a maleimide functionalized polymer.

A multifunctional hydrogel network with cell adhesion properties and enzyme-triggered degradability was prepared using the same chemistry (Figure 6).[55] It combined two types of polymers : star-PEG

bearing four maleimide at their end of their arms and a heparin bearing pendant maleimide groups to prepare hydrogels. Both were linked through maleimide ligation with cell-adhesion peptides bearing a single cysteine: a laminin derivatized peptide H-SIKVAVGWCG-OH (in green) was coupled on the star-PEG and the fibronectin derivatized peptide H-GCWGRGDSP-OH (in purple) was conjugated on the heparin. Both polymers were crosslinked using the peptide H-GCGGPQGIWQGGCG-NH₂ (in blue) which display a sequence recognized by metalloproteinases flanked by two cysteine residues allowing the reaction with PEG and heparin.

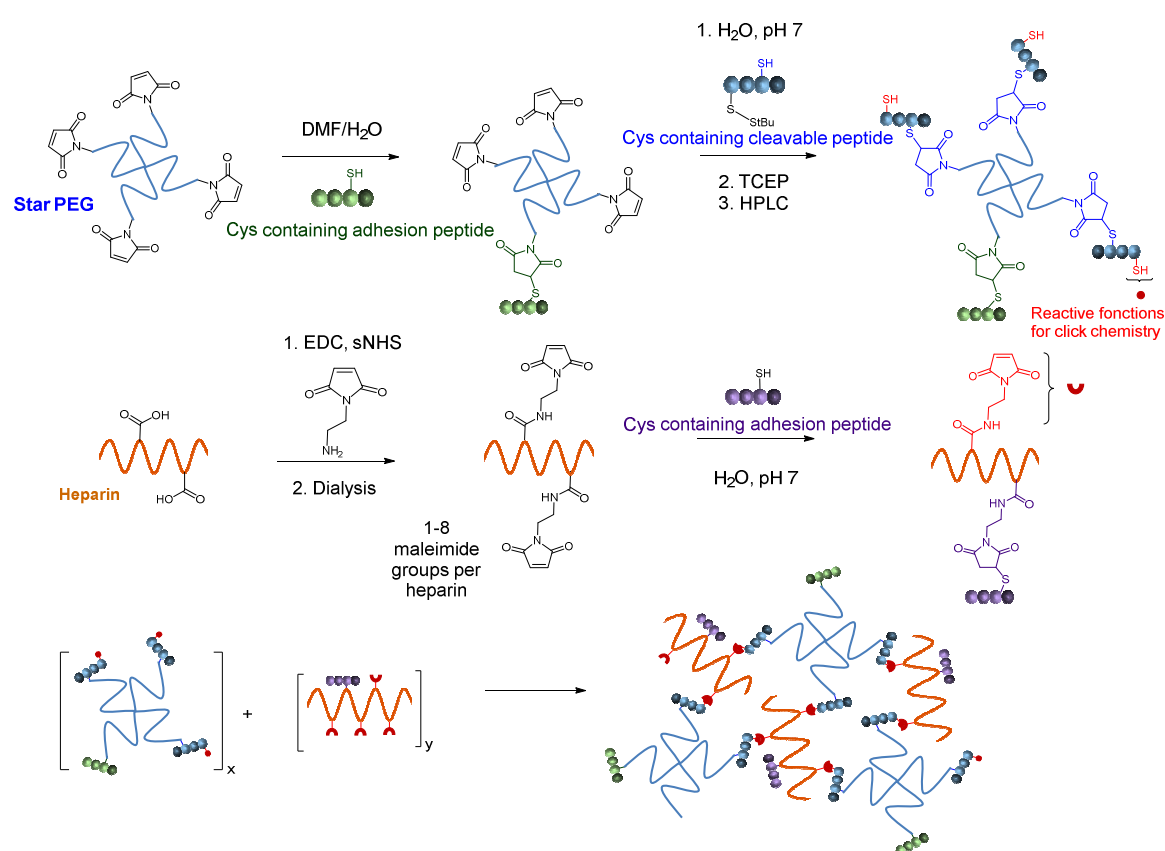


Fig. 6. Cys/maleimide Michael addition strategy to design hydrogel from Star-PEG (blue lines) and heparin (brown lines) using MMP sensitive peptides as cross-linker (blue peptide) and cell adhesion peptides (green and purple peptides).

Besides hydrogels, peptides were also attached to either fully organic polymeric nanoparticles, [56] or nanoparticles with an inorganic core.[51] In the first case, laminin derivatized peptide modified by thiol function at each extremity was able to cross-link polymeric NPs together by thiol-Michael reaction thanks to the acrylate pendant function present in the polymeric NPs. The cross-linking of

these NPs led to the formation of macroscopic gels used for cell encapsulation conferred by the peptides' bioactivity. In another case, polymeric NPs consisted of a biocompatible polyhedral oligomeric silsesquioxane core functionalized by pendant 2-(dimethylamino)ethyl methacrylate and poly(ethylene glycol) monomethacrylate chains polymerized by ATRP. Then, two peptides, H-CAGYGRKKRRRQRRRPKKKRKV-OH and H-CAGW-OH, were grafted to the NPs via cysteine residues present in their sequences, in order to improve the cell penetration and cell adhesion of the NPs, and thus to afford efficient gene delivery systems. [51]

Besides PEG, other polymers have been grafted with peptides thanks to thiol-Michael reaction. RGD based peptide was modified with biotin and Cys residues, i.e. H-CYGGGRGDSK(biotin)-NH₂. It was then coupled to the maleimide function at the extremity of a poly(caprolactone) (PCL).[57] The resulting polymer was 3D printed and possessed cell adhesion property due to the presence of the RGD sequence.

In another example, a thiol-modified peptide, HS-(CH₂)₂CO-YGRGD-OH, was attached to the acrylate group of the pendant chain of the PHEMA block of PMAA-b-PHEMA-b-PMAA copolymer by thiol-acrylate Michael addition. This conjugation was performed to enhance the cell adhesion of the polymer in the field of tissue engineering. [58]

In other studies, Cys containing peptides were conjugated via thiol-ene Michael addition to hyperbranched polymers. For example, an antimicrobial peptide, H-CGLFDIVKKVVGALC-NH₂, was conjugated to a modified hyperbranched polyglycerol (HPG).[59] The HPG was first modified by a N-glycidyl phthalimide via an epoxy function present in its sequence. Then, the N-glycidyl phthalimide group present in the HPG was removed and the obtained amine was conjugate to bifunctional maleimide linker that was then coupled to the Cys-peptide (Figure 7).

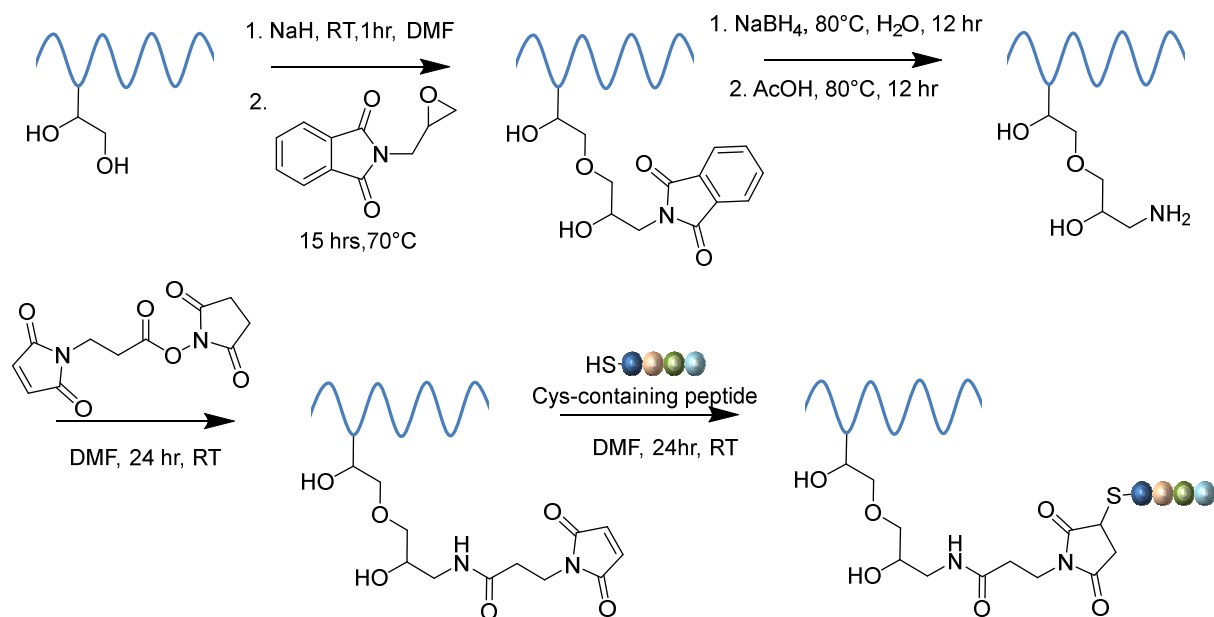


Fig. 7. Coupling of a thiol containing peptide on a maleimide functionalized polymer.

In another example, a Cys-containing proapoptotic peptide H-CGGG(KLAKLAK)₂-OH, and a PEG chain were conjugated to hyperbranched poly(β -thioesters).[44] The resulting peptide-polymer conjugate had the ability to form NPs, to load drugs and contain acid labile linkage on the PPHD part, enabling the release of the drug and the peptide once inside the cells endosomes.

Alternatively, the authors used also peptides which did not contain any Cys residue, neither thiol function and performed the Michael addition through the primary amine at the N-terminus of the peptide sequence.

Some other examples of bioconjugation by Michael addition which do not involve sulfhydryl moieties have been reported. For example, H-RGD-OH peptide was conjugated to vinyl sulfone modified dextran through reaction with the peptide N-terminal amino group, forming a hydrogel for cell delivery.[60] As already stated, most of Michael addition-based conjugations proceeded with the donor on the peptide and the acceptor on the polymer. However, some reverse strategies were also described as the work from Danial et al. which describes polystyrene (PS) –peptide conjugates prepared from a thiol modified PS and a cyclic peptide bearing an allyloxycarbonyl (Alloc) protection on the side chain of a Lysine residue. [61]

decorated NPs were used as fluorescent probes for cancer imaging thanks to the peptide ligand targeting overexpressed integrin receptors on the surface on cancer cells. [64]

2.4 Radical thiol-ene reaction

Non-radical Michael addition is not the only thiol-ene reaction used to prepare peptide polymer conjugates. Radical thiol-ene reactions were also described. The reactions occurs between a thiol and an alkene, most of the time an acrylate derivative, through a radical mechanism.[65] In this case, a photoinitiator is involved, that forms free radical species upon UV irradiation. Then, by reacting with a thiol containing molecule, such as Cys-containing peptides, a thiyl function is created. Then the thiyl reacts with the alkene in order to generate a radical that convert into alkane and generates another thiyl radical which is able to continue the conjugation (Figure 9). This radical reaction stops as soon as

one of the two precursors is consumed.

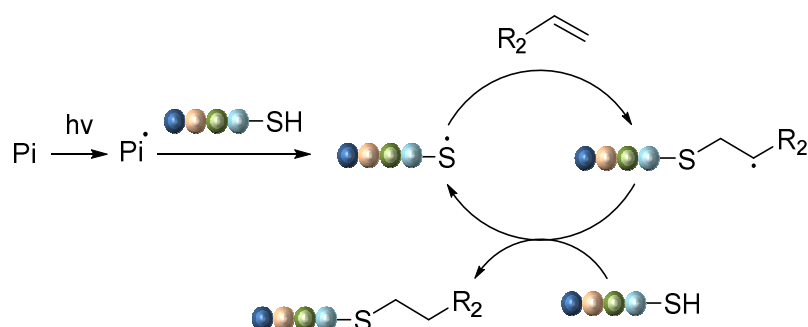


Fig. 9. Radical thiol-ene reaction mechanism applied to a Cys-containing peptide.

The most common example of radical thiol-ene reaction is the cystein lipidation on a peptide or amino acid (CLipPA).[66–68] This reaction has never been applied so far to peptide-polymer conjugate (but only to peptide-lipid conjugates. It is performed between an unprotected Cys-containing peptide and an alkene-containing lipid in order to obtain a lipopeptide. For example, an antimicrobial lipopeptide was synthesized following this technique.[69]

In an elegant study, the radical thiol-ene reaction was applied to a poly(2-alkyl-2-oxazoline) (Pox) based polymer, to conjugate it with a Cys-containing peptide (Figure 10). [70] The Pox analog

polymer was functionalized by a radical thiol-ene reaction on its alkene-containing side chain. Then, an intramolecular reaction afforded an azlactone function on the functionalized side chains. The azlactone function was finally able to conjugate with a Cys-containing peptide by a non-radical ring opening reaction followed by an S-N transfer rearrangement.

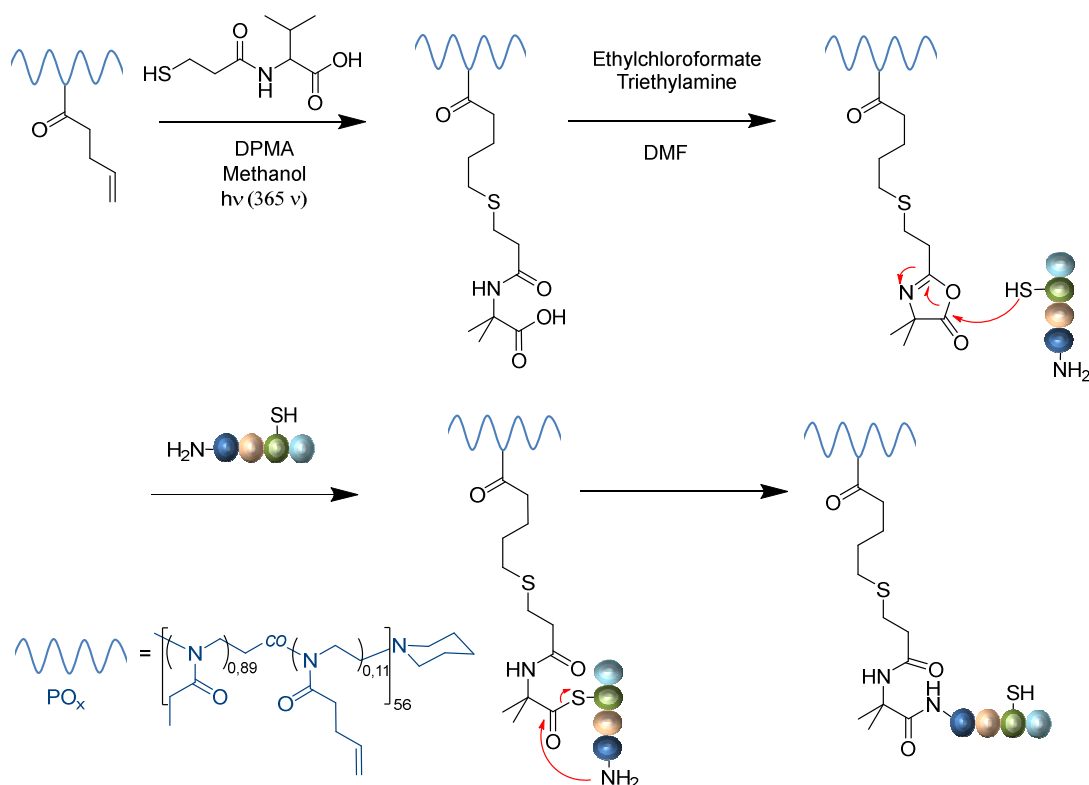


Fig. 10. Conjugation of a Cys-containing peptide on an azlactone functionalized polymer

Another interesting application of radical thiol-ene reaction was reported for the photo-click cross-linking of hydrogels by peptides.[71] A macromonomer modified by a norbornene function (e.g. PEG chain, hyaluronic acid etc.) reacted chemoselectively with Cys-containing peptide linker to provide a hybrid hydrogel. This photoclick orthogonal crosslinking reaction is light-mediated (either UV or visible light), thanks to a low concentration photoinitiator. In most of peptide-polymer hydrogels cross-linked by thiol-ene photopolymerization described in the literature, the norbornene modified macromonomer is a PEG or a star PEG and the peptide is used as cross-linker thanks to its thiol functions, usually afforded by Cys residues. The application of such hybrid hydrogel resides in the possible biodegradability due mainly to the peptide's properties such as chymotrypsin sensitivity,[72]

elastase sensitivity,[73] photolability [74] and other protease sensitivities.[75] Such hydrogels were used for tissue engineering, cell encapsulation, cell culture or differentiation.

2.5 Native chemical ligation

Originally developed by Kent et al. in 1994 [76] for protein synthesis[77], native chemical ligation (NCL) was more recently applied for peptide-polymer conjugation.

This reaction involves the chemoselective formation of a 'native' peptide bond between a thioester, which can be present at the C-ter of a peptide for example, and a cysteine placed at the N-ter of another peptide, through an intermolecular transthioesterification followed by a S-N acyl shift. A great advantage of the NCL is that it proceeds chemoselectively and can be used with fully unprotected peptides, in water allowing the assembly of large peptides and proteins (Figure 11).

For the moment, NCL was used to prepare peptide-polymer conjugates by reacting peptide C-ter thioesters and Cystein-modified polymers and not the opposite, although the preparation of C-terminal peptide thioesters is not an easy task. Indeed, the instability of thioester in conditions used during common Fmoc/tBu solid phase peptide synthesis represent a synthetic challenge and numerous strategies and dedicated linkers have been developed for that purpose in the recent years as summarized by several reviews.[78–80]

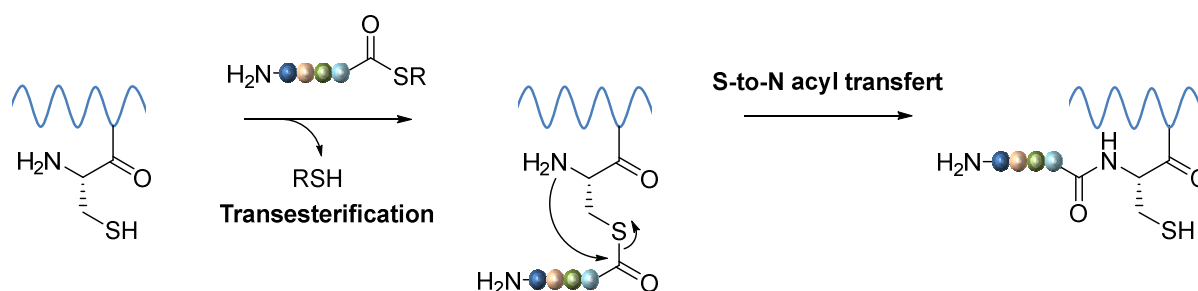


Fig. 11. Principle of native chemical ligation applied on C-terminal thioester peptide and Cysteine modified polymer.

NCL was applied to polyoxazolidine derivatives (i.e. p(MeOx-b-DecOx) and p(MeOx-b-BuOx) synthesized by block copolymerization of a 2-methyl-2-oxazoline (MeOx) with 2-butenyl-2-oxazoline (BuOx) or 2-decenyl-2-oxazoline (DecOx) (Figure 12).[81] The alkene group of the side chain of the

p(DecOx) block was functionalized by a cysteine derivative through the addition of 3-formyl-N-(2-mercaptoethyl)-2,2-dimethylthiazolidine-4-carboxamide. A C-ter mercaptophenylpropionic acid (MPAA) thioester peptide model H-FGGGG-S(C₆H₄)CH₂CO₂H and a biologically relevant C-terminal thioester peptide Thz-EIVYKSPVVSVDTSRHLN-S(CH₂)₂COOBut used in the research area of Alzheimer's disease, were conjugated via NCL to the polymer backbone, in water.

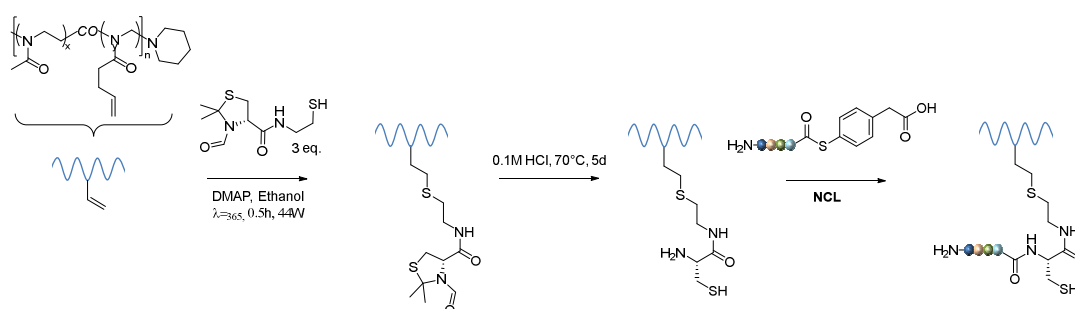


Figure 12: Example of NCL applied to a cysteine-functionalized poly(2-oxazoline)s and peptide thioester [82].

It is worth noting that a cysteine residue is not mandatory to perform NCL conjugation as the β -amino thiol motif is enough to allow the N-S shift. As example, an acrylate-based polymer modified at its extremity by RAFT single unit insertion of N-Boc allylamine moiety was prepared. After aminolysis of the trithiocarbonate, the Boc protecting group was removed to yield the β -amino thiol motif required for ligation with a thioester peptide (Figure 13).[82]

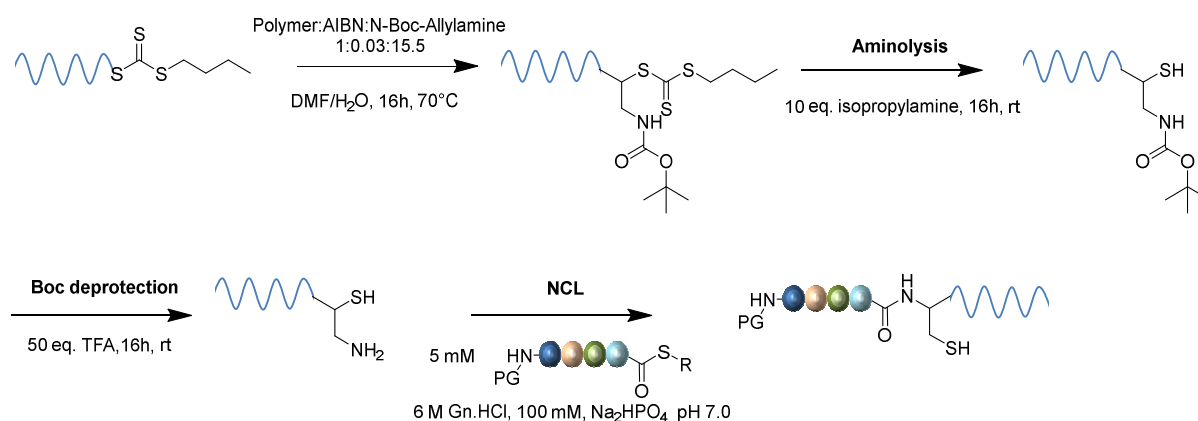


Fig. 13. NCL applied to acrylate-based polymer and peptide modified by ethyl 3-mercaptopropionate.

2.6 Amide coupling

Amide formation, involving primary amine and carboxylic acid functions, is commonly used to link peptides and polymers. This reaction occurs via the carboxylic acid activation, mainly as an active ester, followed by the nucleophilic substitution by an amine. Typically this activation can be done with the combination of a carbodiimide and an auxiliary nucleophile, such as the EDC/NHS combination (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide). This reaction can be performed either in water or in organic solvent. Theoretically, any carboxylic activation method may be employed and the use of peptide coupling reagent such as uroniums (e.g. 3-[Bis(dimethylamino)methyl]urea hexafluorophosphate: HBTU or (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate HATU) in the presence of a tertiary amine (e.g. DIEA, TEA) were reported (Figure 14).

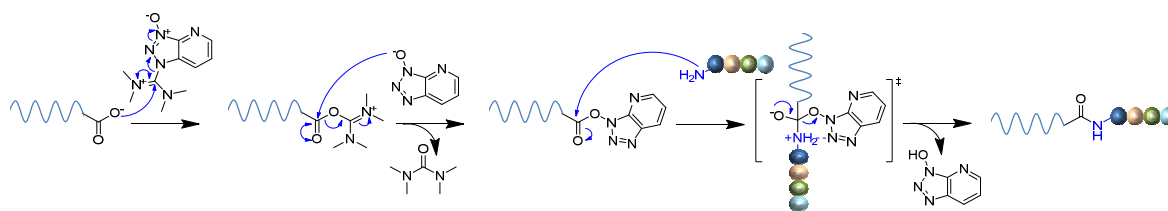


Fig. 14. Amide coupling between a carboxylic acid containing polymer and the primary amine function of a peptide by HATU activation

The amine can be either on the polymer or on the peptide, the carboxylic acid being on the other partner. Despite its apparent simplicity and straightforwardness, amide coupling is not trivial. First, it is worth reminding that C-terminal activation of a peptide results in epimerization and should be considered cautiously.[83] Glu and Asp side chain activation main also lead to unwanted intramolecular cyclization.[83] On top of that, amide coupling is not chemoselective. Peptides may contain several free amino groups, carboxylic acids as well as other reactive groups such as alcohol or thiol functions, leading to unspecific conjugation and a range of unwanted intra and intermolecular reactions. Even if amines are more reactive than alcohols at physiological pH, ester bonds can be formed if the hydroxyl is not protected and the activated acid is in excess. All this means that

potentially reactive function need to be protected during conjugation with then polymer, necessitating addition of deprotection step to insure regioselectivity.

The choice of reactive moieties (i.e. amine or carboxylic acid) enlarges the scope of the possible polymers to be conjugated by an amide formation. As examples, primary amine functions of the polymer were used in chitosan, conjugated to silk fibroin peptide, for the design of wound healing dressings.[84]

HMPA Polyacrylate peptide-polymer conjugates, obtained by amide coupling between the carboxylic acid function of the polymer and the amine function of the peptide, have been described for the design of tumor targeting and drug delivery systems.[85–89] Others examples of acrylate-based peptide-polymers have been reported. The poly(N-vinyl-2-pyrrolidone-co-acrylic acid)-peptide (P(VPcoAA)) conjugated though EDC activation of the carboxylic acid function of the polymer was used as melanoma cancer vaccine prototype[90] or rabies vaccine system[91]. A monolith support for the immobilization of enzymes was prepared by reacting a polyacrylate presenting NHS ester functions with the amino groups of a enzymes, such as the trypsin.[92] PEG and Pox derivatives, activated with NHS on the carboxylic acid function present at the end of the polymer chain, were also coupled to the amine function of the pendant chain of a Lys residue of peptides.[93,94]

For the preparation of polymer/peptide/drug conjugates was used a carboxymethyl dextran presenting carboxylic acid functions as pendant chains. A peptide was then conjugated to the modified dextran after an EDC activation. In this example, the peptide, H-PVGLIG-OH, was used as an enzyme cleavable linker between the two components to design a tumor targeting system: the dextran as polymeric carrier, the peptide as cleavable linker and Methotrexate as the drug. Methotrexate enables first the targeting and then the chemotherapeutic properties once released after the peptide cleavage.[95,96]

Polystyrene (PS) was also conjugated with peptides by amide coupling.[97] As example, PS surface was treated with He plasma to create carboxylic acid functions which were activated by EDC/NHS in order to link the 34-amino acid antibacterial peptide Nisin. It is worth noting that this conjugation

proceeded randomly as Nisin contained several Lysine residues besides its free N terminus. The resulting peptide-modified PS surface shows antimicrobial properties. The same EDC/NHS activation has been described to get an acrylate-based polymer with antimicrobial peptides.[98] The peptide H-TGRAKRRMQYNRR-OH known as Ubiquicidin, was also conjugated with the polymer's end carboxylic acid function through one of its amino groups. The acrylate polymer was a copolymer composed of an hydrophobic part (poly(butyl methacrylate)) and an hydrophilic part (poly(2-(dimethylamino) ethyl methacrylate-co-eosin)) which contains a dye : the eosin Y, also used as a photosensitizer for the photodynamic therapy. The association of the hydrophobic and hydrophilic part enabled the formation of micelles with antimicrobial and photodynamic abilities.

2.7 Pyridyl disulfide reactions

Asymmetric disulfide bridges can be formed between peptides and polymers chemoselectively by using S-pyridylsulfenyl activated disulfides. Resulting disulfide linkage present an attractive feature as it can be cleaved in mild reductive conditions allowing disassembly. As an example, a cyclic peptide presenting both a cysteine residue and a primary amine-containing side chain, was conjugated first, with a PEG carboxylic acid activated by NHS ester, and secondly with a pyridylsulfenyl containing-pPEGA, in order to form an asymmetric hybrid cyclic peptide-polymer.[93] As already stated, the disulfide bridge created between the cyclic peptide and the pPEGA polymer is a reversible covalent bond. Hence, once the disulfide bridge cleaved by reduction, the peptide-PEG conjugate, freed from pPEGA, was able to self-assemble into nanotubes. This strategy allowed the preparation of new smart redox-responsive polymer.

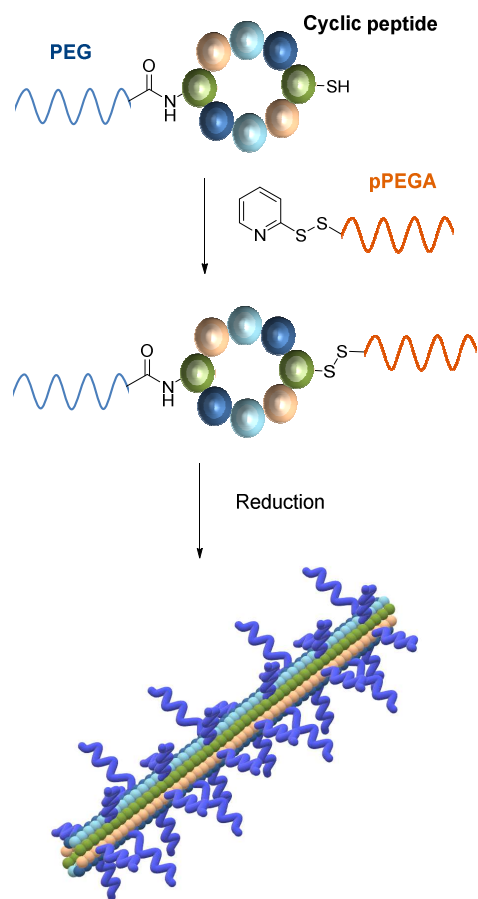


Fig. 15. Self-assembly of cyclic PEG-ylated peptide after reduction.

Chang *et al.* (2010) reported a practical purification method of the enzyme glutathione S-transferase (GST) using a glutathione-poly(N-isopropylacrylamide) (GSH-pNIPAAm) conjugate.[99] This smart peptide-polymer is thermosensitive and precipitates above its lower critical solution temperature (LCST). It was prepared by first synthesizing of a pyridylsulfenyl -pNIPAAm by RAFT polymerization. The substrate of the GST enzyme, glutathione (GSH) was coupled by disulfide bond formation through pyridyl disulfide substitution reaching 95% of conjugation in 30 minutes.[100] The conjugate specifically binds to the GST via its GSH part and the complex is then thermoprecipitated upon heating thanks to the pNIPAAm properties. This purification method is mild and efficient for GST fusion proteins.

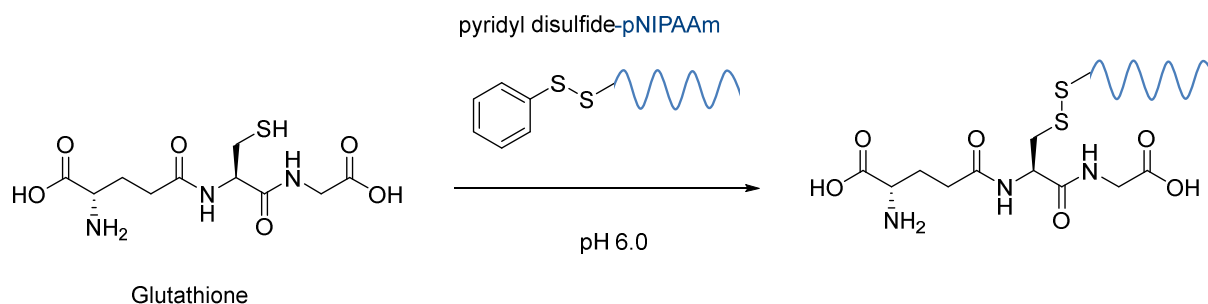


Fig. 16. Conjugation of Glutathione peptide with a pyridylsulfenyl functionalized pNIPAAm by disulfide formation.

2.8 Other nucleophilic substitutions involving amines

Besides the amide formation, other nucleophilic substitutions involving primary amine functions of peptides have been used to modify polymers. Some examples of ring opening reactions can be found in the literature, targeting for example an epoxide moiety displayed by the polymer.[101,102] They are all initiated by a primary amine function on the peptide and are non chemoselective since any free amine group of the peptide may react.[102] Another possibility is the direct conjugation on the terminal group at the extremity of a Pox, the 2-Ethylloxazoline, by the primary amine at the N-terminal amine function of the peptide (Figure 17).[101] No ring opening polymerization (ROP) can be observed in these previous examples, since there is no initiator, catalyst or radical activation/propagation. All these examples used a single conjugation.

Another example of such conjugation can be given with the N-terminal amine of a peptide that substituted halogen atoms of poly(dichlorophosphazene) to lead to a biodegradable polyphosphazene functionalized peptides.[103]

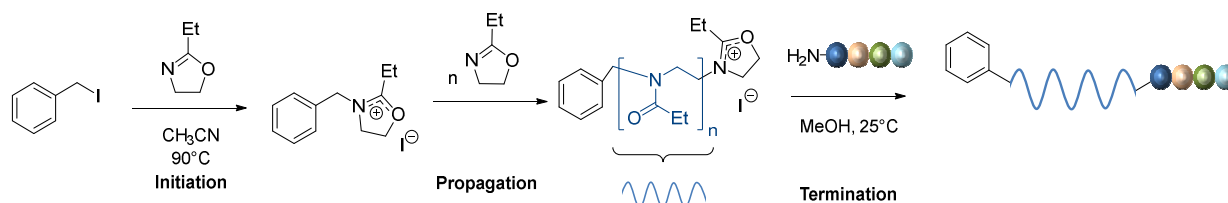


Fig. 17. Peptide polymer coupling via termination step of the polymerization

2.9 Huisgen dipolar cycloaddition

Huisgen dipolar cycloaddition is a widely used click chemistry reaction for post-functionalization of polymers by peptides. Usually the polymer bears the alkyne function while the peptide displays the

azide one. It is a chemoselective reaction forming a 1,2,3 triazole linking the two components (Figure 18). [65] In contrast to copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC), the thermal Huisgen 1,3-dipolar cycloaddition leads to the formation of regioisomers (Figure 19). The copper catalysis prevents this side reaction therefore leads to biorthogonal coupling but unfortunately copper presence in the final polymers might lead to cytotoxicity. Interestingly, this type of ligation may proceed in water, at neutral pH, in physiological conditions.

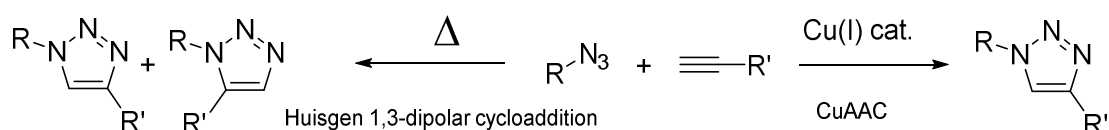


Fig. 18. Comparison of catalyst for Huisgen cycloaddition.

CuAAC has been done on diverse polymers, mainly acrylate-based ones. As example, cyclic peptides bearing azide moiety at their side chain, usually due to the incorporation of Lys(N₃) residue, and displaying self-assembly properties were conjugated with the alkyne functions of a poly(*n*-butyl acrylate) or poly(hydroxy ethyl acrylate) in order to self-assemble into well-structured nanotubes.[104–107]

Poly(2-hydroxyethyl methacrylate) (HEMA) was copolymerized with propargyl acrylate leading to p(HEMA-co-PgA), a copolymer wearing an alkyne function on its pendant chain. This alkyne function was reacted by CuAAC with the azide function of a fluorescent RGD peptide.[108] Interestingly, the azido moiety was separated from the peptide sequence by a photocleavable linker allowing a photo responsiveness of the cell adhesion properties by UV irradiation: the photocleavable linker cut the peptide in two parts when irradiated and so turns off the cell adhesion ability of the polymer.

This technique was also applied to Pox. As example, two alkyne modified Pox were conjugated with azide-modified peptides, such as a polyTyrosine azide leading to aqueous and non-aqueous stable micelles;[109] and a cRGD derivative, providing a radiotherapy targeting system.[110] In a similar way, superparamagnetic NPs were functionalized by hydroxymethylenebisphosphonates (HMBP) bearing

an alkyne function. Then an azido cRGD peptide was conjugated to the polymer yielding integrin targeting NPs.[111]

Alternatively, the azide function can also be present on the polymer and the alkyne function on the peptide. For example, an acrylate-based polymer with azide functions was conjugated to alkyne-modified gramicidin S peptide by CuAAC.[112] This reaction has also been performed directly on solid support after the SPPS synthesis of a peptide.[113] A 8-mer peptide, (D-Val-L-Val)₄ has been synthesized on support by SPPS and then directly modified by 4-pentynoic acid before being conjugated to azide end-functionalized poly(dimethylaminoethyl methacrylate). This peptide-polymer conjugate is able to self-assembly into nanotubes after cleavage from the resin. These nanotubes can have various length and are pH and thermo-responsive.

In another example, an alkyne end-modified block copolymer PLA-PEG was coupled to an azido cRGD derivative.[114] The resulting peptide-polymer was able to form polymersomes with targeting and drug loading properties.

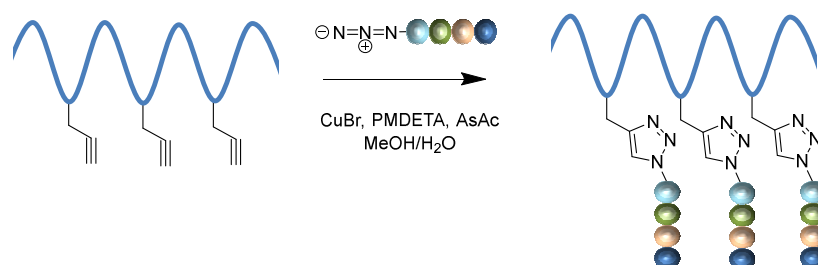


Fig. 19. Huisgen dipolar copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) of a alkyne-functionalized polymer and an azido peptide.

An alternative to the CuAAC is the strain-promoted azide-alkyne cycloaddition (SPAAC), avoiding the use of cytotoxic copper catalyst. This strategy presents a high reaction speed and conversion efficiency.

Canalle et al. reported the use of cyclooctyne to replace the simple alkyne function, which enables a copper-free coupling by SPAAC.[112] This technique was applied to a cyclooctyne derivatized

gramicidin S peptide, a PEG chain and a coumarin in order to conjugate them to an azide modified acrylate-based polymer. However, due to the steric hindrance of the cyclooctyne, lower degree of grafting was observed with the copper-free strategy.

Also by SPAAC, an azide modified peptide, JR2EK-Az at position 22, was conjugated to a cyclooctyne functionalized PEG anchored on hyaluronic acid biopolymer leading to peptide-hyaluronan hydrogel (Figure 20).[115]

Liu et al. showed that azadibenzocyclooctyne (DBCO) functionalized microbubbles, made from human serum albumin (HAS) were functionalized by SPAAC reaction with fluorescent rGFP-PEG-azide protein in 5 minutes at 37°C.[116] Such microbubbles can be useful for real-time imaging or as a drug-delivery system. This cargo strategy is adaptable to many different self-assembled systems (liposome[117] or micelle[118]) and different grafted peptides/proteins (RGD or antibody) as targeting ligand.

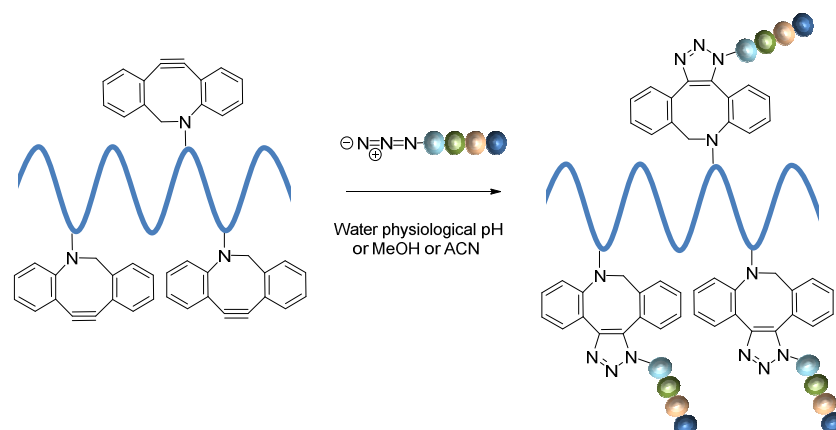


Fig. 20. SPAAC coupling of an azide-peptide on a cyclooctyne modified polymer.

2.10 Thio-Bromo click reaction

The thio-bromo click reaction is another strategy used for peptide-polymer conjugation. This reaction is based on a thiol containing polymer and a bromo functionalized peptide. An amyloid bromo modified peptide Br-CH(CH₃)CO-LVFF-OMe, was conjugated to two type of polymers : a poly(diethylene glycol methyl ether acrylate) obtained by RAFT[119] or a monomethyl ether PEG (Figure 21).[120] In both cases, the peptide was synthesized in solution and functionalized by 2-

bromopropionyl bromide (Figure 18). In the first example, the thiol function was provided by the thiocarbonylthio group that was used as chain transfer agent. The final peptide-polymer system was thermo-responsive and able to self-assembly into micelles. In the second example, the thiol function was present at the end of the mPEG chain. This functionalization was operated by three successive reactions: the initial hydroxyl termination was mesylated, then turned into a thioacetate-cap and finally the hydrolysis of the acetyl group released a thiol function. The peptide-polymer conjugate obtained was able to self-assembly into fibrillary nanostructure.

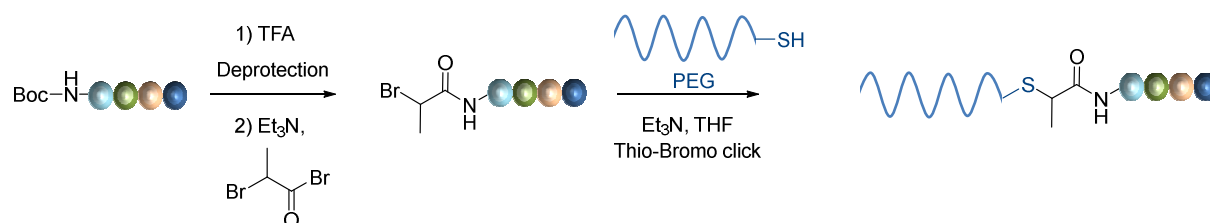


Fig. 21. Thio-Bromo click reaction of a Bromo-modified peptide on a thiol-polymer.

2.11 Reductive amination

Imine formation is extensively used to prepare peptide-polymer conjugates (see next chapter). However, this reaction is reversible, and hydrolysis gives back starting materials. To achieve an irreversible linkage between an aldehyde polymer and peptide presenting one primary amine function, a reductive amination reaction can be performed. Typically, after the imine formation, NaBH_3CN mediated reduction in water affords secondary amine. This reaction was performed to conjugate the peptide hormone oxytocin $\text{H-c}[\text{CYIQNC}]\text{PLG-NH}_2$, to acrylate-based polymer containing Pox pendant chain: $\text{p}(\text{OEtOxMA})$ (Figure 22) and presenting an aldehyde group at one extremity of the backbone.[121] The aldehyde function was obtained after $\text{TFA}/\text{H}_2\text{O}$ (1/1, v/v) removal of the acetal of the initial Acetal- $\text{p}(\text{OEtOxMA})$.

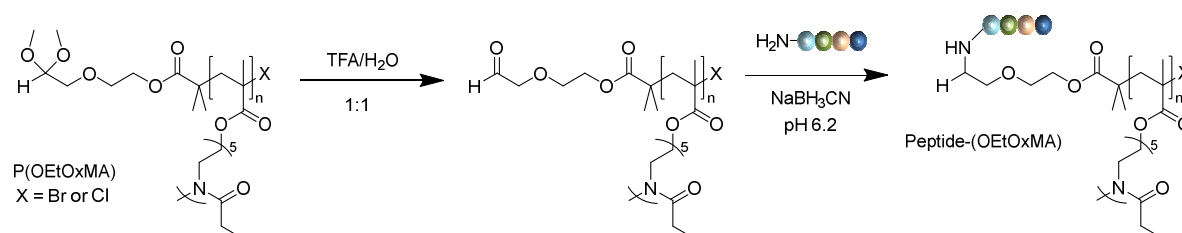


Fig. 22. Reductive amination coupling of a peptide at the extremity of p(OEtMA).

2.12 Schiff-base and boronate linkages: dynamic covalent ligations

The last examples of peptide-polymer conjugation take advantage of the specific reactivity of aldehydes and ketones; and boronates. Imine, oxime and hydrazone ligations involve an amino, aminoxy and a hydrazine moieties respectively, and allow chemoselective reaction in water with aldehydes and ketones. Imine, oxime and hydrazone ligation are reversible dynamic covalent bonds and submitted to an equilibrium. Their formation is possible at acidic or neutral pH (Figure 23) and rate-determined by the dehydration step of the tetrahedral intermediate. However, if the medium pH is too low, the nucleophilic group can be protonated leading to an oxynium or hydrazinium ion, thus decreasing its reactivity and disfavoring the oxime/hydrazone formation. These conjugates gain in stability with the electronegativity of the β -heteroatom and the steric hindrance of the carbonyl group (stability: oxime > hydrazone and ketone > aldehyde). The reverse-reaction (hydrolysis) is acid catalyzed and initiated by the protonation of the imine nitrogen. However, the oxime and hydrazone formation can be promoted with an excess of a nucleophile.

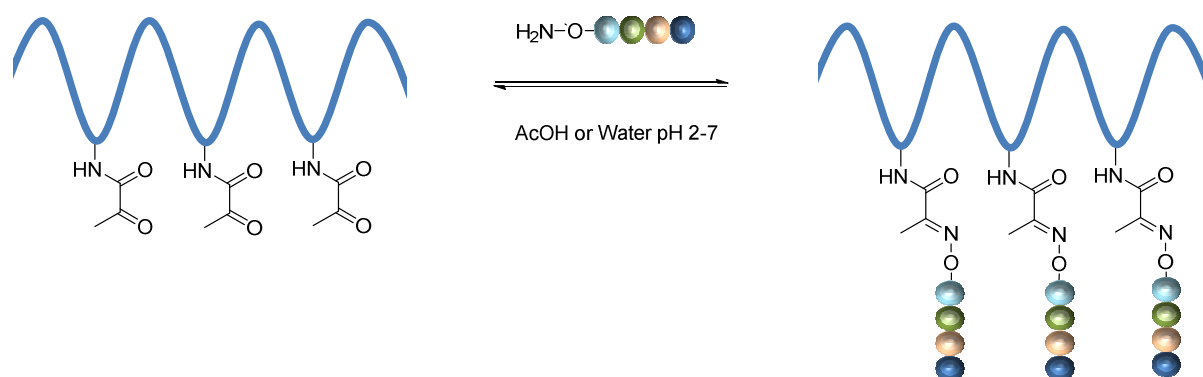


Fig. 23. Conjugation of an amine terminated peptide on an aminoxyacetyl functionalized polymer by oxime ligation.

For example, oxime ligation was performed with different peptide vectors, functionalized at their N terminus by aminoxyacetyl (Aoa) group (e.g. Aoa-LEEEEEAYGWMDF-NH₂). [122] All these Aoa-peptides were conjugated to two different polyvinylpyrrolidone block copolymers, bearing various ratio of pyruvoyl groups. The oxime ligation kinetic was studied and showed a rapid and complete conversion: up to 95% ligation after 2h. Interestingly, all these reactions are reversible and the

formed linkage is still sensitive to hydrolysis in acidic conditions

An elegant example of dynamic hydrazone linkage was reported by Wang et al. with the preparation of a thermosensitive cross-linked hydrogel.[123] A hydrazone-modified elastin-like protein (ELP-HYD) comprising cell-adhesive domain (RGD) was linked to an aldehyde-modified hyaluronic acid (HA-ALD) via a dynamic covalent hydrazone bond, allowing a rapid gelation at room temperature (Figure 24). This reversible hydrazone strategy allows to automatically back cross-link after a mechanical cleavage of the system. This hydrogel enables the protection of stem cell during syringe injection and its self-healing property provided by the reversible hydrazone bond allowed the conservation of its structural integrity and homogenous repartition of the stem cells incorporated in its network. Above 37°C, the thermoresponsive ELP aggregates itself leading to the thickening of the hydrogel network. This hydrogel reinforcement provides a tenfold higher erosion resistance permitting the survival and proliferation of cells inside the gel 3 weeks after injection. Thus, this hydrogel is promising for stem cell transplantation and tissue regeneration.

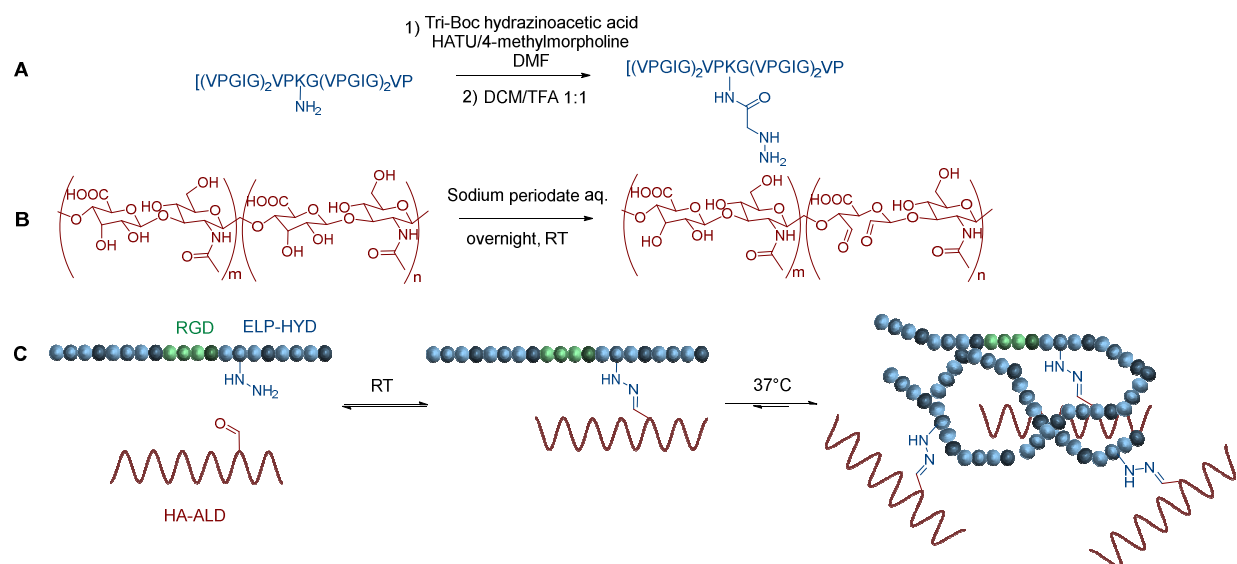


Fig. 24. Hydrazone dynamic ligation A. Synthesis of hydrazone-peptide B. Synthesis of Hyaluronic acid aldehyde C. Crosslinking of the hydrogel and its thermal assembling

Imine bond, is widely used for dynamic polymers. Its imine/hydrolyzed state equilibrium is pH-controlled with the imine being more pH-sensitive than oxime and hydrazone. Wei et al. developed a

dual cross-link bifunctional and self-healing network to generate modular gradient hydrogel.[124] The bifunctional network is crosslinked by thiol-ene Michael addition of the dithiol matrix metalloprotein (MMP)-sensitive peptide and the thiol function of RGD peptide on the acrylate function of oxidized acrylated hyaluronic acid (OAHA). Then, the imination of the aldehyde part of the OAHA by amino group of N-carboxyethyl chitosan (CEC) led to the self-healing network CEC-OAHA-MMP (Figure 25). Some modules of this CEC-OAHA-MMP with different ratio of MMP-sensitive crosslinker and RGD were conjugated together for 20 minutes at 37°C (leading to the gradient). The self-healing property led to robust gradient hydrogel , which can be handled and lifted without fractures and is injectable at 37°C. Thanks to its injectable and self-healing properties, this customizable gradient hydrogel can mimic spatiotemporal gradient cues of tissues to study and guide cellular response to their microenvironment.

In another study, Stair *et al.* developed a sensor material for the detection of protease by using a diamine peptide (H-AAPVAAK-OH) as crosslinking agent by imine linkage with aldehyde functionalized dextran.[125]

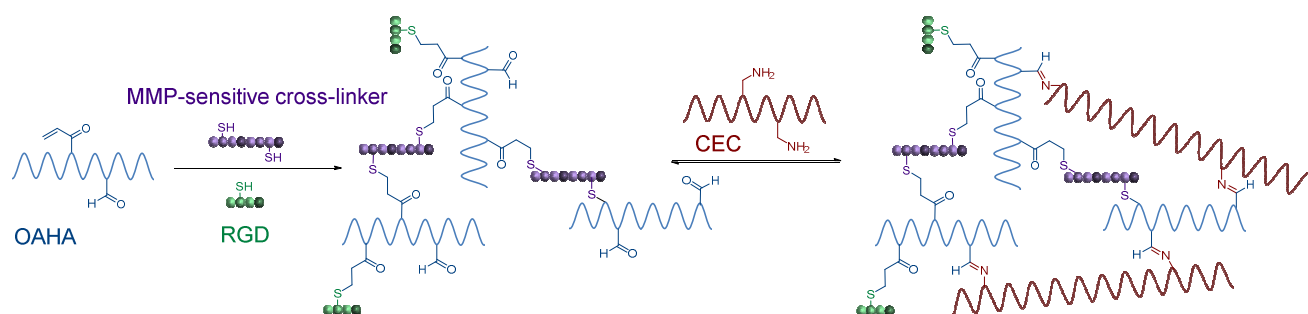


Fig. 25. Imine ligation as reversible crosslinking strategy.

As previously introduced, dynamic covalent ligations can provide some interesting properties such as self-healing. Moreover, dynamic covalent bonds can be used to develop some stimulus responsive materials (pH responsive, thermosensitive, photosensitive, etc.) leading to intelligent polymer/peptide materials.

For example, an acido-labile hydrazone bond was used by Quader *et al.* to develop pH-sensitive

polymeric micelles to deliver a peptidyl proteasome inhibitor MG132 (Z-Leu-Leu-Leu-H).[126] The peptidyl aldehyde was linked to a copolymer PEG-b-poly(hydrazine-aspartamide) through an hydrazone reversible bond. After conjugation, the loaded copolymer self-assemble in water to form stable micelles with a PEG shell and MG132-loaded core. *In vivo*, this loaded polymeric micelle was dissociated in the late endosomal compartment of tumoral cells and the hydrazine linkage was then hydrolyzed in the endosomes acid medium to regenerate the bioactive peptidyl aldehyde. In addition, this method avoided drug leakage and protect the aldehyde functional group until reaching the targeted cancer cells.

A similar strategy was applied for the delivery of bortezomib, a peptidyl boronic acid proteasome inhibitor for cancer bone-metastasis therapy.[127] An acido-labile aryl boronate reversible linkage was used to introduce the peptidyl drug on a functionalized PEG 1,2-benzendiol (catechol) via condensation of the aryl diol with-the boronic acid group. Associated with alendronate (a bone targeting material), a mixed micelle was obtained. In the acid medium of bone metastasis, the micelle was disassembled and the dynamic aryl boronic ester released the original peptidyl boronic acid.

Dynamic covalent ligations are thus applicable in different manners for vectorization, targeting and drug delivery, and promise some more incoming application. [128]

In summary, all the *grafting to* conjugation techniques lead to covalent attachment between polymers and peptides. Most of them do not allow the use of unprotected peptides. As example, even in thiol-ene and thiol-yne reactions, other nucleophiles than thiol groups, such as primary amines, may also react on unsaturated moieties.[129] Thus, it is important to pay attention to the use of excess of thiol or to keep primary amines of the peptide protected. On the contrary, some reactions such as NCL or Thio-Bromo click proceed chemoselectively with unprotected peptides. Only one function of the peptide can react with the associated function of the polymer: a thioester and a cysteine for the former; a thiol and Bromo functionalized peptide for the later. This avoids tedious

deprotection steps and purification after the conjugation.

Besides chemoselectivity, another criterion has to be considered to choose the appropriate conjugation technique: the toxicity of the reagents, catalysts or initiators. Indeed, for biological applications, biocompatible conjugations are preferred and toxic components, such as copper catalyst for the Huisgen dipolar cycloaddition, have to be avoided. Additionally, the reaction conditions, such as the solvent and the pH, could be of uppermost importance in the case cell encapsulation. Biorthogonal reactions, able to be performed in water at physiological pH without any toxic reactant, such as NCL or SPAAC, are required in this case. The steric hindrance is also a parameter to be taken in account. For example, the cyclooctyne functionalization leads to higher steric issue than the amide coupling.

Finally, the ease of introduction of the functional group on the polymer or the peptide is often a limiting factor. Ideally, the introduction of the reactive groups should be straightforward, cost-effective and compatible with certain purifications steps. The conjugation step itself has to be extremely efficient in order to allow the fine control of the desired grafting density. Indeed, some of the functional groups are particularly difficult to obtain and/or purify, as the azlactone function for example. Therefore, simpler moieties such as carboxylic acids or primary amines, are favored since they are easier to display on both peptides and polymers.

No doubt that the *grafting to* strategy will benefit from the novel biorthogonal reactions developed in the field of chemical biology. These should be chemo-selective, proceeding in water in biocompatible conditions at neutral pH and allowing the use of unprotected peptides, with non-toxic components. Enzyme-based reaction, such as sortase ligation could be also investigated to prepare peptide-polymer conjugates.[130]

3. *Grafting from* strategy: Peptide as initiator of the polymerization

3.1 *Grafting from* strategy

Instead of being post-functionalized with peptides, the polymers can be generated *from* peptides, which take part in the polymerization as initiators. In other words, the difference between “*grafted to*” and “*grafted from*” techniques resides in the fact that the peptide is involved in the polymerization and is not added afterwards.[26] In this approach, as the conjugation is done during the polymerization process, the polymer characteristics (e.g. size, composition) cannot be modulated as easily as in *grafting to* approach. However, the significant size difference between the peptide, the residual monomer and the peptide-polymer allows the later recovery of the desired conjugate.

Polymerizations that can be initiated by a peptide include ATRP, RAFT and NMP; all of them being classified as Reversible Deactivation Radical Polymerizations (RDRP). RDRPs have the advantage to enable a good control of the final molar mass as well as the chain functionalities of the polymer and will be summarized in the following section of this review.[131]

3.2 *Atom Transfer Radical Polymerization (ATRP)*

ATRP is a living radical polymerization catalyzed by a transition metal complex, which generates radicals from an initiator molecule containing one or several halogen atoms: the initiator is usually an alkyl halide (Figure 26). Each halogen in the initiator structure is the seat of a polymer chain elongation. The crucial factor of this polymerization process is the equilibrium between propagating radical and dormant species. This equilibrium is based on the formation of the radical through the dormant species and then the exchange of the radical with the transition metal complex. ATRP is well controlled and thus leading to polymers with low polydispersity index (\mathcal{D}) meaning that the polymer chains obtained are about the same size. This polymerization method also gives access to high molar mass polymers.[132]

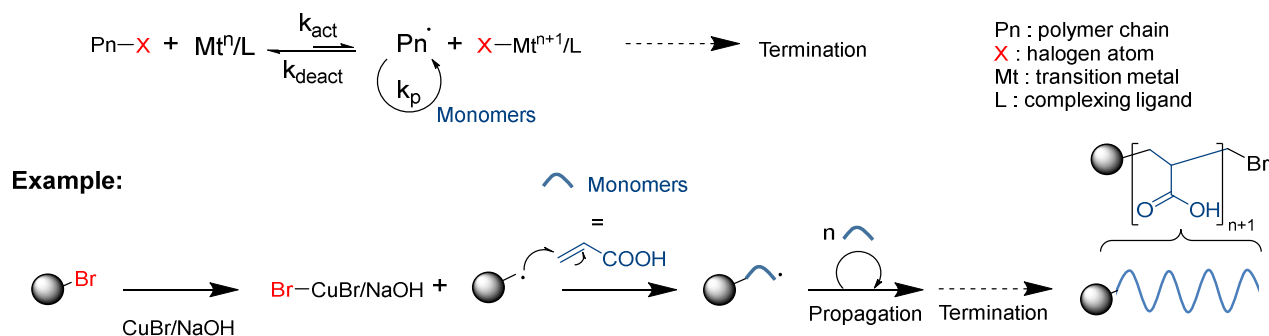


Fig. 26. Equilibrium established during ATRP and example of supported synthesis of polyacrylate by ATRP.

The way for a peptide to take part in this polymerization is to play the role of the initiator. Indeed, modified peptides bearing halogen at one or several positions (e.g. Br functionalization by 2-bromoisobutyric acid coupled on Lys side chains, Figure 27) have been successfully used as initiators for ATRP.[133–136] The geometry and the number of halogen atoms on the functionalized peptide control the structure of the resulting polymer.

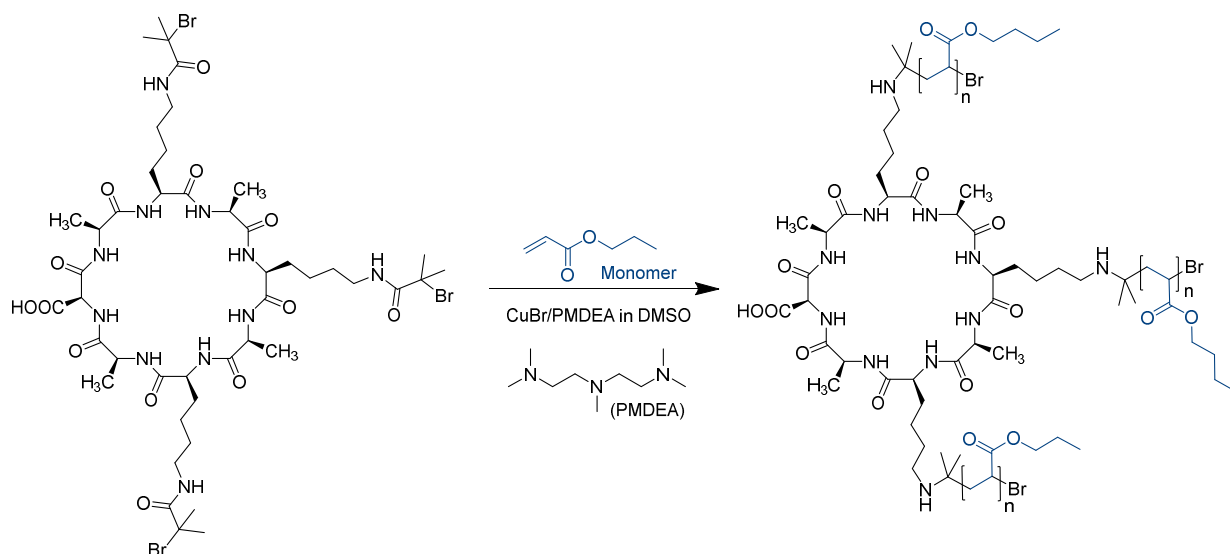


Fig. 27. Example of cyclic peptide used as initiator for ATRP polymerization of acrylate based polymer.

3.3 Reversible Addition-Fragmentation chain Transfer polymerization (RAFT)

RAFT is another radical polymerization method, which enables the synthesis of well controlled polymers in term of size (Mw), polydispersity Đ and even architecture.[137] RAFT polymerization needs a radical source, such as azobisisobutyronitrile (AIBN) and a chain transfer agent, such as

thiocarbonylthio or thiocarbamate groups. The radical initiates the polymerization by activating the monomer and thus enabling the growth of the chain by transfer of the radical from the chain to another monomer. The chain transfer agent plays an important role in this process, as it ensures the propagation of the radical to activate another monomer (Figure 28). Peptides can be coupled to the chain transfer agent thus playing the role of initiator for the RAFT polymerization.[138–140]

As an example, two cyclic peptides were modified by thiocarbonylthio groups in order to become RAFT initiators. Then, they enabled a *grafting from* polymerization on its modified side chain (Figure 29). However, cyclic peptides are not the only possibility; H-GGRGDS-OH was also modified at its N-terminus with S-1-dodecyl-S'-(R,R'-dimethyl-R''-acetic acid) trithiocarbonate to initiate RAFT polymerization (Figure 29) and provided a linear peptide-polymer conjugate.[138] This example lead to a peptide-polymer conjugate with cell adhesion properties. In addition, this technique has been applied to cell penetrating peptides, such as Transportan 10, modified by 4-Cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid .[141] Once polymerized with acrylate-based amphiphilic block, the resulting copolymer was able to self-assemble into vesicles or rod-like structure showing enhanced cellular internalization properties. It is noteworthy that the thiocarbonylthio group of the RAFT agent is conserved in the final polymers.

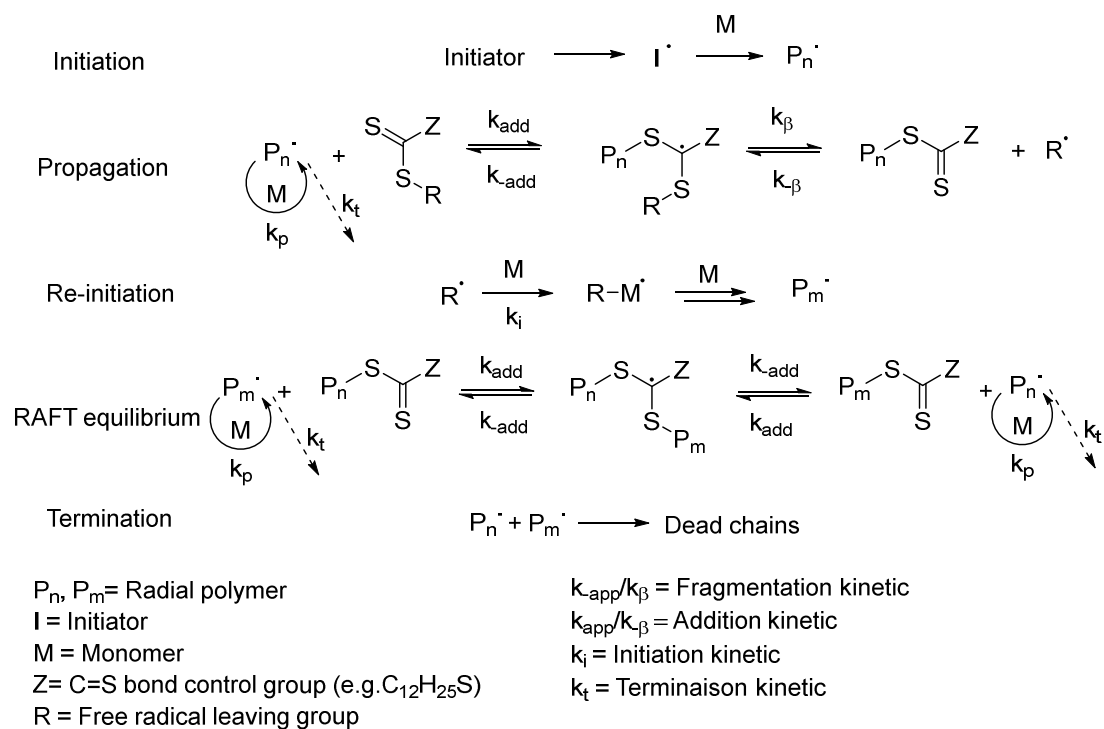


Fig. 28. Mechanism of RAFT polymerization.

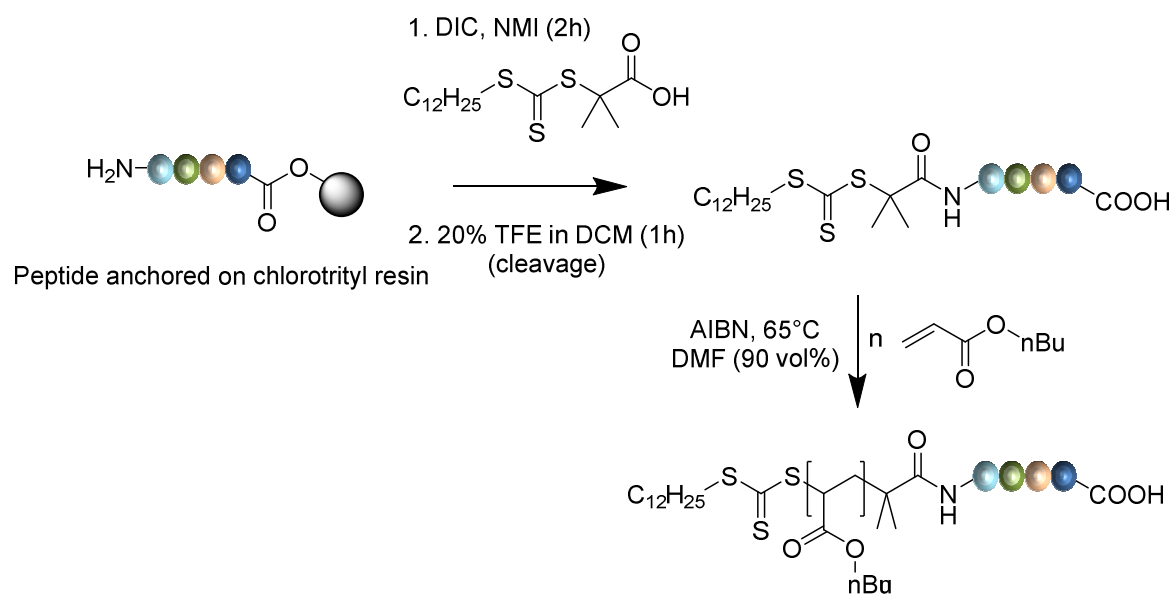


Fig. 29. Example of peptide functionalization with *S*-1-dodecyl-*S*-(*R,R*-dimethyl-*R*-acetic acid) trithiocarbonate initiator and RAFT polymerization by grafting from strategy.

3.4 Nitroxide-Mediated radical Polymerization (NMP)

The last example of RDRP polymerization is the NMP polymerization. It is also based on propagating and dormant species, as in ATRP. However in this case, initiators are alkoxyamines that undergo

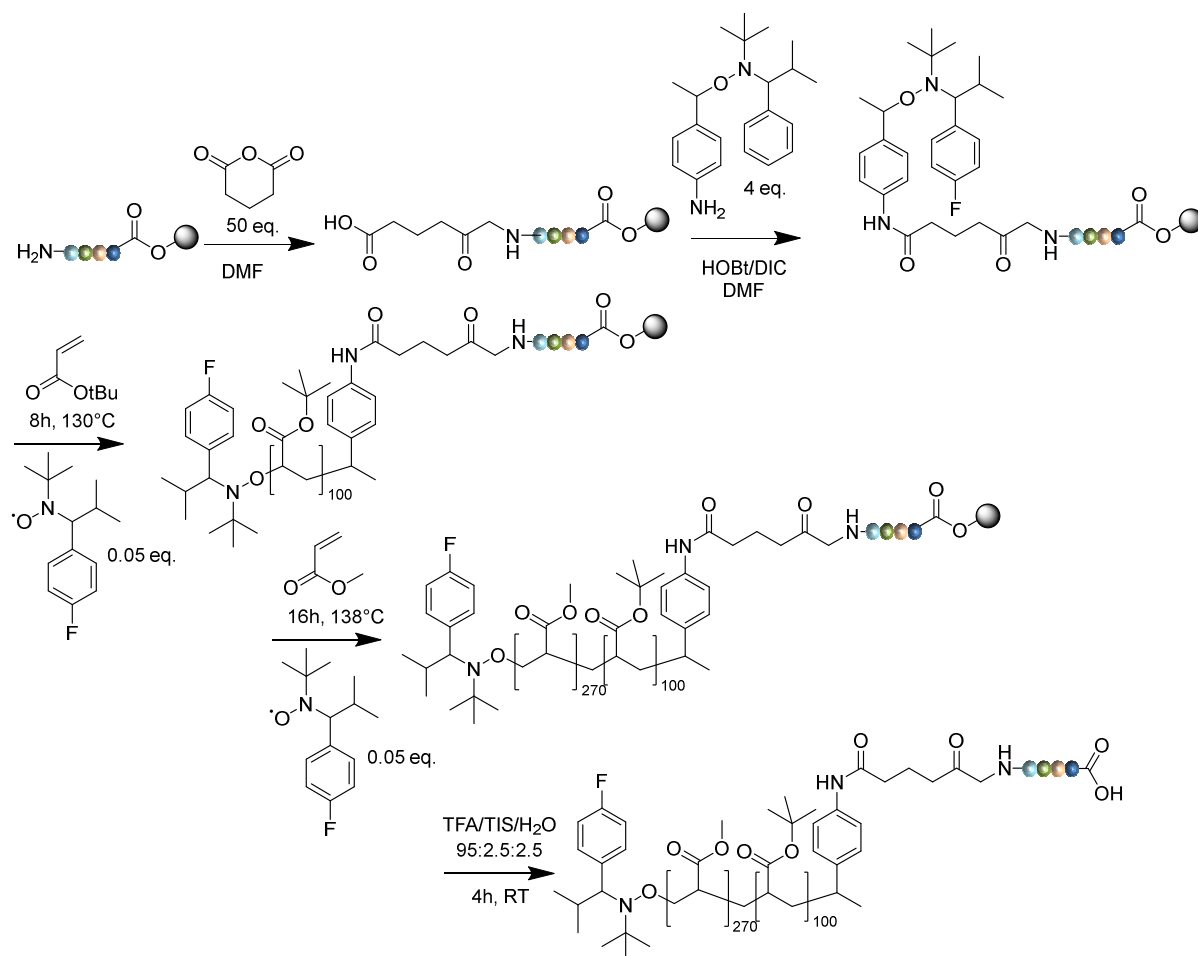


Fig. 31. Peptide functionalization with fluorine-labeled alkoxyamine initiator and NMP polymerization by grafting from strategy.

In all the cases, these three types of polymerization afforded polymers (or copolymers) conjugated with a single peptide sequence. Interestingly, the peptides can contribute to the structural organization of the resulting hybrid polymer. Indeed, even when simple single peptide-polymer blocks are obtained, the physicochemical properties of the peptides (ex: charge, polarity) or their inner self-assembling properties can yield to supramolecular arrangements such as core-shell cylinders, micelles or nanotubes.[135,136,139] In some cases, the cyclic peptide self-assembles, driving the global supramolecular arrangements into core-shell cylinder instead of polymeric micelles, that is the result of the basic block copolymer assembly (Figure 32).[139]

In a very elegant study, the group of S. Perrier used a diamine functionalized cyclic hydrophobic self-assembling peptide (SAP), c[KLWLKLWL], as the initiator for RAFT polymerization.[145] First, a

hydrophobic butyl acrylate polymer was attached to the SAP to form the hydrophobic core followed by RAFT addition of the hydrophilic dimethyl acrylamide (DMA) to provide hydrophilic shell. The obtained peptide-polymer conjugates self-assembled in well-defined nanotubes with remarkable stability combined with solubility in water, biocompatibility and ease of derivatization. It is important to note that in this example the peptide was the driving force that determined the final morphologies of the nano-objects.

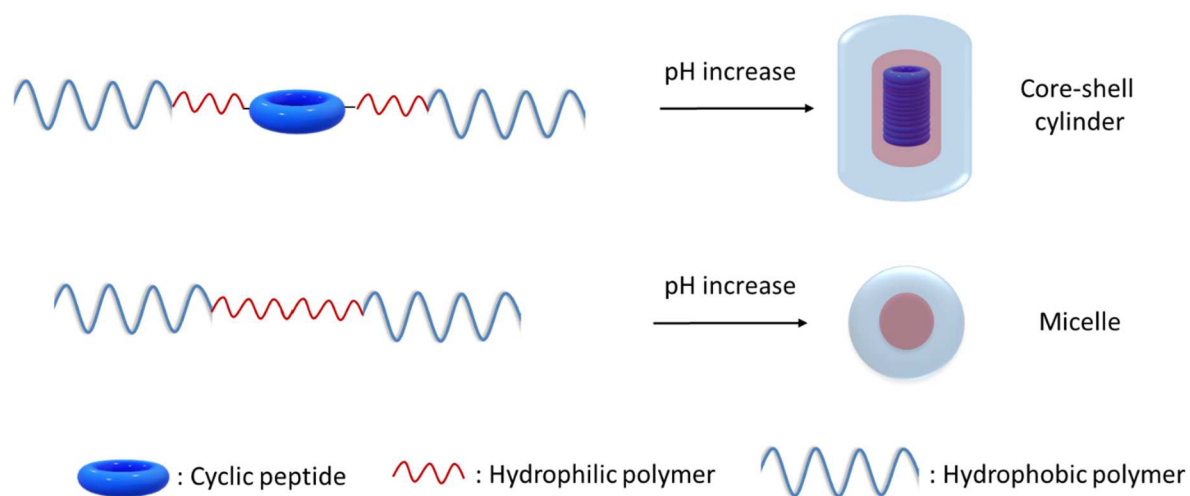


Fig. 32. Example of supramolecular arrangement issued from peptide-polymer based of grafting from strategy.

In summary, using peptides as initiators change the paradigm of peptide polymers by letting the peptide part playing the first role during the polymerization process, even having impact on the final polymer structure. Indeed, the peptide can also be the templating element that manages the final macro-architecture. For example, in the case of the ARTP, the number of halogen functions present on the peptide will drive the number of polymer chains and in some cases their spatial arrangement. As seen in some examples of this polymerization strategy, there is a complementary interplay between peptide's and polymer's physical properties that can define final morphologies such as nano-tubes. Amazingly, in this case a single peptide sequence goes beyond simple derivatization of the polymer and becomes a instrumental structuring factor.

The three most used techniques for grafting from strategy, ATRP, RAFT and NMP, are applicable to a

wide range of monomers. Depending on the desired applications, each technique could present an advantage. The most versatile one is the ATRP, thanks to its catalyst, which can be applied to almost every monomer whereas for the RAFT and the NMP, each polymerization agent is applicable to only a small set of monomers. However, their catalyst, copper halide or amine-based ligand, is usually present in high concentration and hard to wash off. This factor is implying toxicity and so limiting commercial and biological applications.[146] Research is actually ongoing to limit the quantity and thus the toxicity of such catalysts. Besides the catalyst problem, the ATRP is often air sensitive, while RAFT and NMP are not, and not applicable to aqueous media. The main advantage of ATRP is the easy preparation of the peptide initiator, the good control of the resulting architecture as well as the possibility to manage the final end chain functionalization thanks to the presence of the halogen. Also the ATRP is highly responsive to external light stimulation and has a good tolerance over functional groups, and thus does not need protections on the peptide initiator.[147]

The RAFT polymerization is also widely used in the case of peptide-polymer conjugations. However, RAFT agents are not as universal as ATRP ones and have to be chosen in function of the monomers. These agents can be quite complex to synthesize and purify, thus leading to residual sulfur traces as well as unwanted color in the resulting polymer.[148] Fortunately, they can both be eliminated by purification steps.[149] The advantages of the RAFT technique is that it is metal free and can be performed in water, which allows the use of this technique with hydrophilic peptides as initiators and its use for biological applications. In addition, this technique allows a good control over the end chain polymer function, which is useful if any post-functionalization is planned. Finally, the NMP is the less versatile of the three techniques described here. Indeed, the alkoxyamine and nitroxide initiating system are applicable only to a small range of monomers. Research is currently ongoing to bypass this limitation and obtain a reversibly modified alkoxyamine or nitroxide system, either covalently or by supramolecular interaction in order to obtain an universal initiator.[150] Still the NMP polymerization has one main advantage over ATRP and RAFT, it is free of purification.[151]

4. *Grafting through* strategy: peptide as macromonomer (PCM)

Using a peptide as monomer is undoubtedly much more demanding in terms of chemical requirements than 'grafting' strategies. Indeed, the peptide needs to be functionalized with at least one 'polymerizable' moiety. This reactive group has to be introduced in the peptide sequence in order to (co)polymerize according to the chosen polymerization method without interfering with the other functional groups of the biomolecules. PCM (peptide-containing macromonomer) are usually called "macro-monomers" due to their relatively high molar mass. The use of PCM allows the synthesis of polymers with a higher level of peptide repetitions than *grafting from* approaches, yielding completely new characteristics to the final polymer deriving from the peptide properties (i.e. structure, self-assembly, biological properties). The backbone of the final polymer, on the other hand, is the result of the type of polymerizable moieties.

4.1 *Chain growth polymerization of acrylate-peptide macromonomer*

The most common reaction used with PCM is the poly-addition of acrylate or PCM, which is also the most common strategy for the *grafting to* method. Numerous research projects utilize biomolecules such as saccharides[152–155], nucleotides[156–158] or peptides that are modified with a methacrylate or acrylate group.[159]

The acrylate or methacrylate moiety can be introduced in solution or on solid support either at the C-terminal COOH function or at the N-terminal NH₂ function (Figure 33 and Figure 34) in order to generate an acrylate-based polymers with amino acids as pendant chains.[160] It is noteworthy that the acrylate or methacrylate functionalization of peptides has to be done on protected peptides, otherwise more than one acrylate group would be anchored on the macromonomer and the polymerization would lead to a 3D network instead of linear polymers.

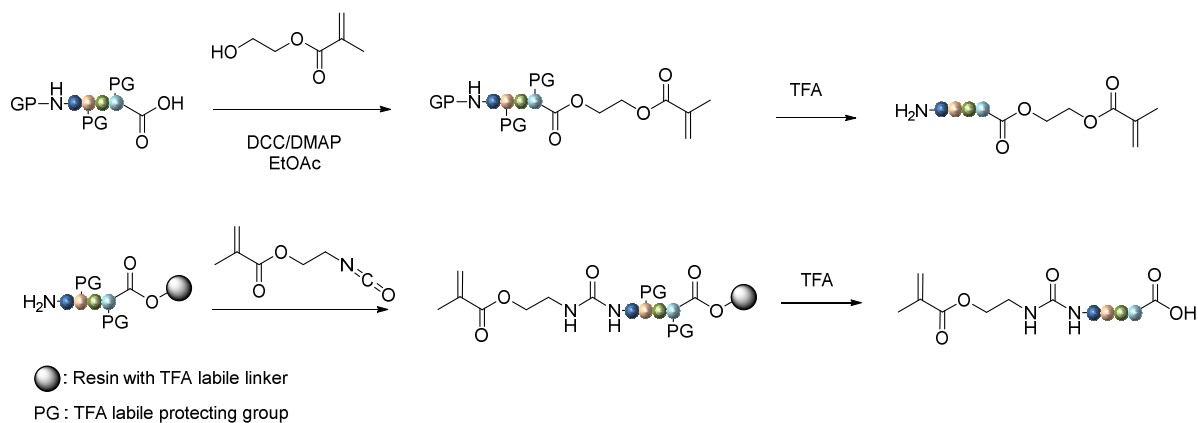


Fig. 33. Example of peptide modification at the C-terminus and N-terminus: synthesis of methacrylate functionalized protected peptide. A) Synthesis of COO-methacrylate-peptide monomers B) Synthesis of N-methacrylate-peptide monomers.

Then the acrylate/methacrylate-peptide monomers are able to react in the same way as methyl methacrylate (MMA) [161] by RAFT or ATRP polymerization. Peptides are then incorporated as pendant chains along an aliphatic backbone (Figure 34).

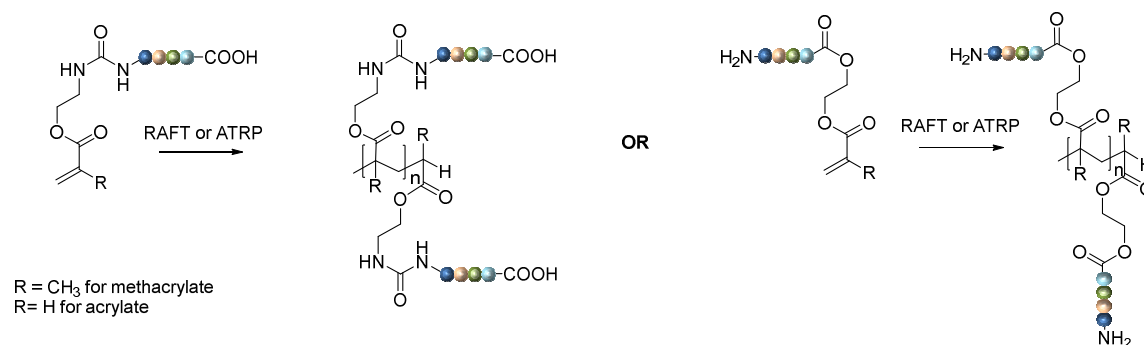


Fig. 34. Polymerization by RAFT or ATRP of acrylate or methacrylate functionalized peptide macromonomer at its N (left) or C-terminus (right).

Noteworthy, this strategy is particularly useful to set up polymer-induced self-assembly (PISA) approaches (Figure 35).[162,163] Methacrylate or acrylate modified amino acids, (functionalized at the C-ter, e.g. cysteine, alanine[164]), and short peptides, such as cationic antimicrobial peptides, have been copolymerized with 2-hydroxypropyl methacrylate (HPMA) by RAFT.[165] The resulting block copolymers comported a hydrophilic (the peptide) and hydrophobic (pHPMA) part. This amphiphilicity and the hydrophobic/hydrophilic balance was the driving force leading to supramolecular self-assembly into spheres, worms or vesicles as a function of the ratio of both parts

of the resulting copolymer. Noteworthy, a linker is often introduced between the peptide and the polymerizable moiety that brings additional properties to the resulting material, favoring the self-assembly of the peptides[166] or bearing a cleavable bond (e.g. disulfide bridge) for potential peptide delivery applications. [167]

Recently, Dao et al. reported for the first time the RAFT PISA polymerization of self-assembling peptides (SAP) containing monomers. The so-called SAPPISA methodology combined the self-assembling properties of SAP and the PISA process to get novel hybrid organized polymer-peptide structures.[168] In a first study, the authors synthesized the short but extensively reported Phe-Phe SAP sequence[169] connected to methacrylic acid via a glycine linker providing the MA-Gly-Phe-Phe (MA-GFF) monomer. The ability of this peptide to still self-assemble was first confirmed followed by its statistical RAFT copolymerization with the globally hydrophilic monomer glycerol monomethacrylate (GMA) to provide the targeted hydrophilic SAP-containing macromolecular chain transfer agent (macro-CTA). This hydrophilic polymer was then chain extended with the hydrophobic 2-hydroxypropyl methacrylate (HPMA) under aqueous dispersion PISA to afford the P(GMA₆₅-stat-(MAm-GFF)₇)-b-PPHMA₂₈ polymer that self-assembled into fibrous structures not reported previously for PISA-prepared nano-objects. It was impressive that the PISA self-assembly was influenced by the introduction of only about six SAP moieties. In addition, these novel polymers showed thermosensitivity in both size and morphology.

In a subsequent study, the SAP monomers MA-Gly-Phe-Phe and MA-Phe-Gly-Asp were introduced in the hydrophobic core of PISA polymers to afford a wide range of novel morphologies.[170] As in the previous work from the same group, the SAP sequences played a pivotal role in the determination of the macromolecular architectures.

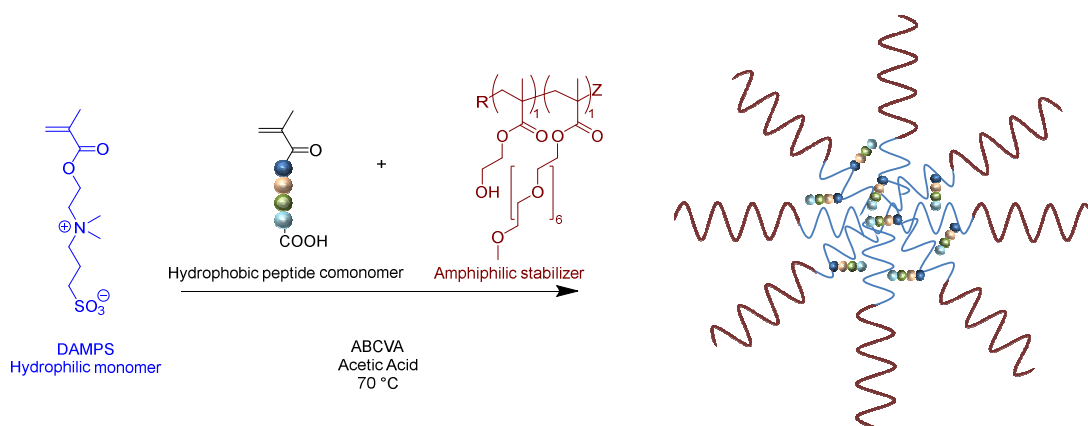


Fig. 35. Example of acrylate functionalized macromonomers polymerized by RAFT and self-assembled into polymeric micelles by PISA: Copolymerisation of hydrophilic DMAPS with hydrophobic peptide monomers and amphiphilic stabilizer of the micelle.

4.2 Ring Opening Polymerization (ROP) of peptide macromonomers (NCA)

Besides ATRP and RAFT on acrylate PCM, ring-opening polymerization (ROP) was also used to polymerize peptide sequences. ROP was initiated by a nucleophilic attack (e.g. amino group or primary alcohol) which in turn, unmask another nucleophilic function suitable for further chain elongation. It implies that this strategy is not suitable with some unprotected amino acid having nucleophilic groups as side chains or at the peptide's extremities.

Noteworthy, homopolymers obtained from ROP of amino acid lactones [171] or amino acid N-carboxyanhydrides (NCA) [172–174] were reported in the literature. These are not peptide polymer as defined in this review as they are not composed of well-defined peptide sequences. For example, Serine lactones were applied to the synthesis of Ser and homologated Ser homopolymers. [175] It is noteworthy that the obtained polyesters can be converted into polyamides via a multi O-N acyl transfer reaction.

NCA of Lys and Met were extensively used to obtain homo-oligomers as polyLys or polyMet.[176,177] Derivatives of polyglutamic acid such as poly(benzyl-L-glutamate) (pBLG) or poly(stearyl-L-glutamate) (pSLG) were also obtained by polymerization of side-chain-modified Glu NCA (Figure 36).[178–180] ROP of two NCA modified amino acids, Lys and Val, have led to a direct grafting on hyperbranched polyamide amine NPs.[181]

The ROP of a Pox monomer lead to a polyamide whose structure was close to a peptide backbone. In order to obtain peptide-like polymer with amino acids as pendant chain, peptide sequences were grafted on the amine-modified polyoxazoline from the N or C-terminal peptide's extremities, while all other reactive group remained protected.[182,183]

The only example reported so far of PCM associated with Pox is issued from the ROP of NCA functionalized peptide or amino acids, copolymerized with oxazoline monomer. Indeed, different NCA amino acids (e.g. Phenylalanine, protected Glutamic acid, glycosylated Serine) were copolymerized with Pox monomer to obtain block copolymer including a block with a peptide as pendant chain.[184,185] All the previously cited examples do not correspond, strictly speaking, to peptide-polymers as defined in this introduction. Indeed, the peptide part is not a well-defined sequence but a polyamide formed by the random repetition of amino acids.

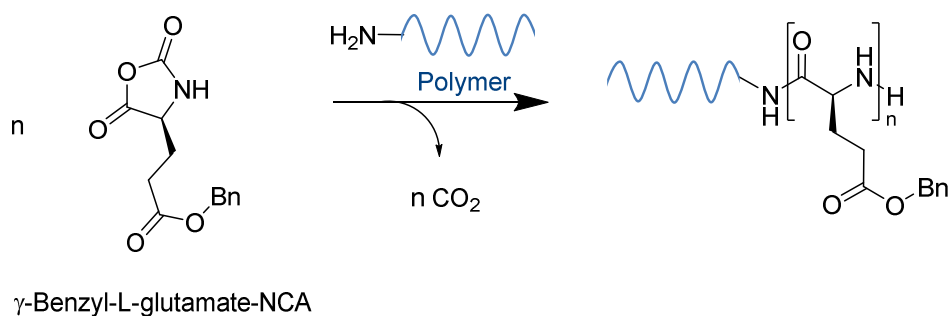


Fig. 36. Example of ROP of BLG: synthesis of polymer-poly(benzyl-L-glutamate) copolymer.

In contrast to ROP of lactones or Pox which was never used to polymerize well defined peptide sequences, peptide-NCA derivatives were designed and engaged in polymerization to yield to well defined comb-like peptide-polymers with a pendant peptide sequence (Figure 37).[186]. In the described strategy, the NCA ring was directly generated on solid support at the end of the SPPS thanks to the introduction of a glutamic acid coupled through its side chain on the N-terminus of the peptide sequence. In order to ensure the selective formation of the NCA of the glutamic acid, performed by cyanuric chloride, the side chain protections of the peptide have to be kept. After cleavage from the resin, the NCA-peptide macromonomer was then ready to polymerize. It is

noteworthy that unprotected Lys residues could not be used in this strategy as they would also have initiated the ROP, leading to side reactions.

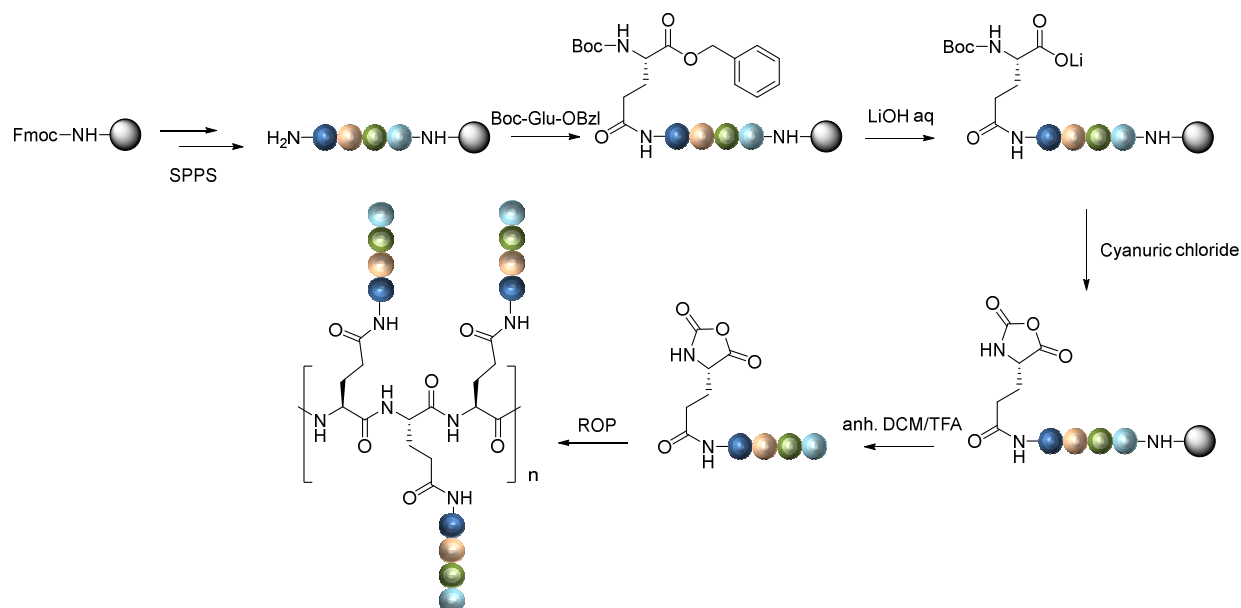


Fig. 37. Synthesis and polymerization of an NCA modified peptide macromonomers.

4.3 Ring Opening Metathesis Polymerization (ROMP) of norbornene-peptide macromonomers

The only reported example of Ring Opening Metathesis Polymerization (ROMP) relies on norbornene-containing monomers whose alkene function undergo a ring opening catalyzed by ruthenium based Grubb's catalyst. Interestingly Norbornene derivatives can be obtained from precursors displaying a primary amine function after oxalyl chloride activation of the norbornene group (Figure 38).[187] Prior to polymerization, norbornenyl-amino acid and esters, have to be synthesized. Consequently, and as it was the case for NCA, norbornenyl-amino acid, such as Gly, Ala, Phe and Asp, were first polymerized this way in the 90's leading to homooligomers.[188]

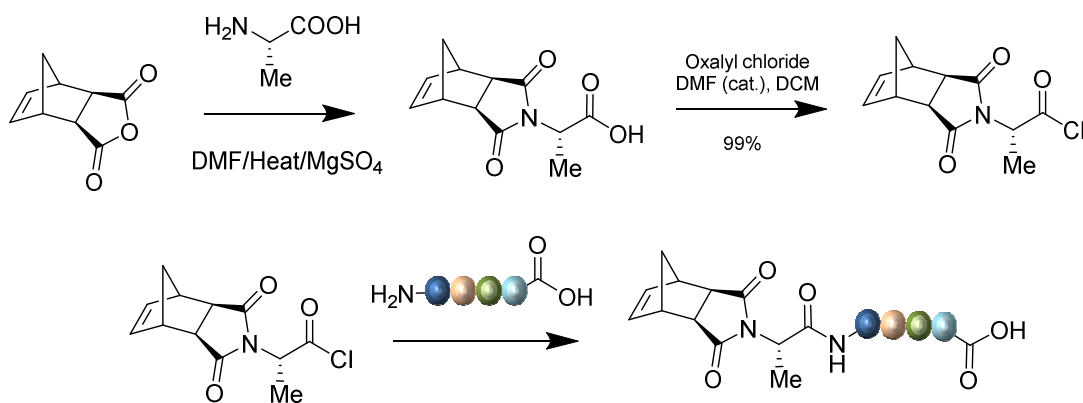


Fig. 38. Example of preparation of the activated norbornene reactive block to be coupled on an amino group of a peptide.

Interestingly, the chemoselectivity of the metathesis reaction enabled the polymerization of well-defined unprotected peptide sequences, conjugated at the N-terminus to a norbornene moiety. The polymerization of norbornene resulted in a comb-like hybrid peptide polymer (Figure 39).[189] As example, the polymerization of such macromonomer led to a norbornene-based polymer bearing as pendant chains, elastin-like cyclic peptides (H-c[KVPGGGVPLG]), the peptide sequence being cyclized between the side chain of the Lys and the C-ter COOH). This peptide exhibits thermal-responsive properties responsible of its crystallization ability. Enzymatic treatment of endopeptidase thermolysin opened the peptide cycle thus decreasing the thermal response and the polymer crystallization ability.[190] Other N-terminus norbornene-modified peptides such as Nor-KGKGK-OH, Nor-GPLGLAGGWGERDGS-OH or Nor-KLAKLAKLAKLA-OH were copolymerized to obtain amphiphilic peptide-polymer with self-assembly properties.[191]

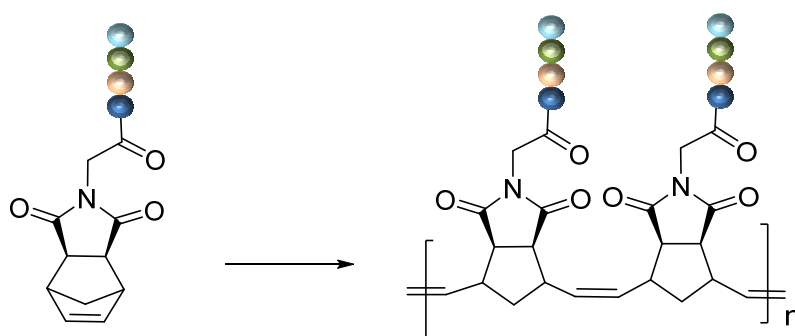


Fig. 39. Norbornene functionalized peptide macromonomer polymerized by ROMP.

Copolymerization with norbornene-modified PEG monomers lead to interesting PEG-peptide-copolymer with high water solubility.[192,193] Specific structures such as spherical or cylindrical micelles, were obtained by copolymerization of norbornene PEGylated peptides with hydrophobic norbornene monomers.[194] These results paved the way to apply ROMP in PISA protocols (ROMPISA) to obtain amphiphilic polymer nanostructures such as micelles, worms or vesicles in function of the ratio of the peptide block over the PEG one [195] or enzyme-responsive polymer NPs by action of the thermolysin on the GPLGLAGGWGERDGS sequence thanks to the presence of the recognition sequence PLGLAG.[196]

4.4 Poly-condensation of diacid chloride with diamino-peptide macromonomer ('peptide-nylons')

Despite the wide utilization of nylons in industry and in every-day life, the covalent introduction of peptide sequences in a polyamide polymer remained nearly unexplored. Introduction of unnatural amino acids in nylon 3 was reported in 2009 by the group of S. Gellman leading to antibacterial materials,[197] but the synthesis of nylon 6,6 analogues with peptide sequences was only disclosed very recently by our group.[198] They were obtained by substituting the classical aliphatic diamine (e.g. diaminohexanoic) by peptides bearing two primary amino groups; one of them being the N-terminal amine and the other one afforded by a Lys side chain or by a diamino spacer placed on the C-terminus (Figure 40).[199] This strategy allowed the design of customizable peptide nylons, resulting in either linear or comb-like polymers in function of the structure of the peptide macromonomer. Thus, the composition of the polymer is controlled by the quantity of peptide macromonomer incorporated related to the diaminohexane. Polymerization proceeded at the interface of DCM and KOH solution leading to Cell-adherent or antibacterial nylons depending on the peptide sequence chosen (i.e. Arg-Arg or Arg-Gly-Asp-Ser, respectively). Noteworthy, this strategy was not suitable for peptides bearing other unprotected amino groups than the two involved in the polymerization.

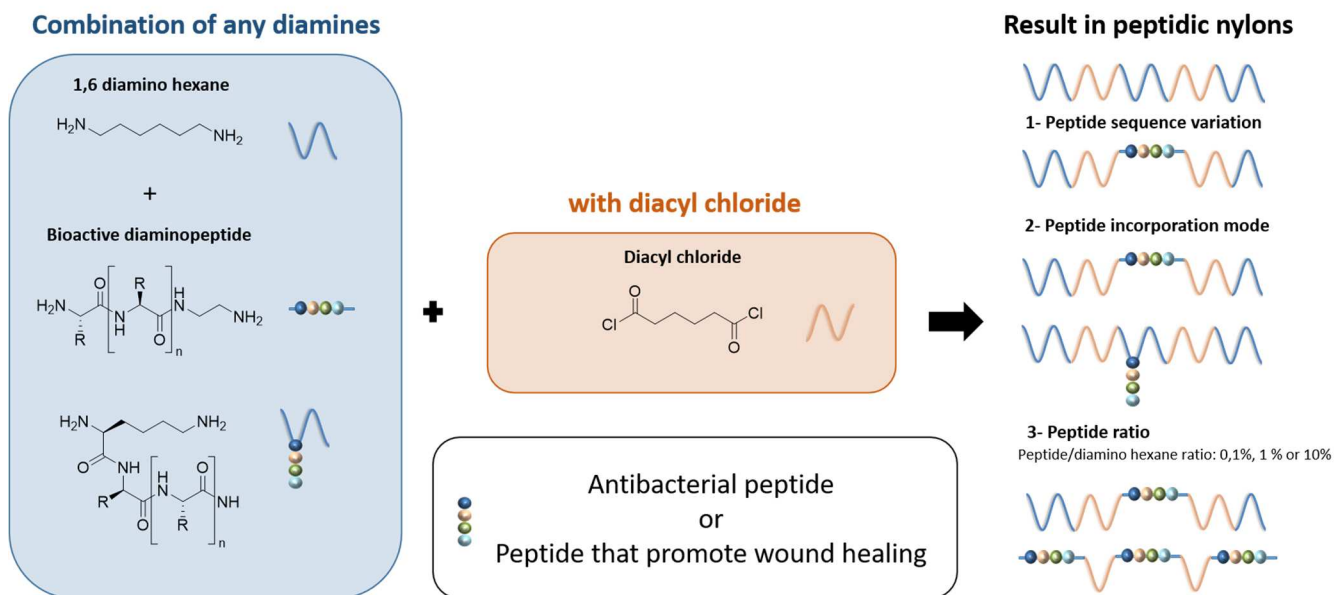


Fig. 40. Poly-condensation of diacid chloride with diamino-peptide macromonomer.

4.5 Nitroxide Mediated Polymerization (NMP) of peptide macromonomers

NMP was performed with a peptide macromonomer.[200] A cyclic peptide, opened by heat, leads to both a reactive carbon radical and a nitroxide radical respectively at the N-terminus and the C-terminus of the peptide (Figure 41). The reactive carbon radical was able to initiate NMP while the nitroxide one was trapping radicals of growing polymer chain to terminate it but in a reversible way. Usually used as an additive, TEMPO was here directly incorporated into the peptide chain thanks to Fmoc/tBu SPPS, using Fmoc-TEMPO-COOH. A copolymerization by NMP with vinyl monomers yielded a hybrid block peptide-polymer.

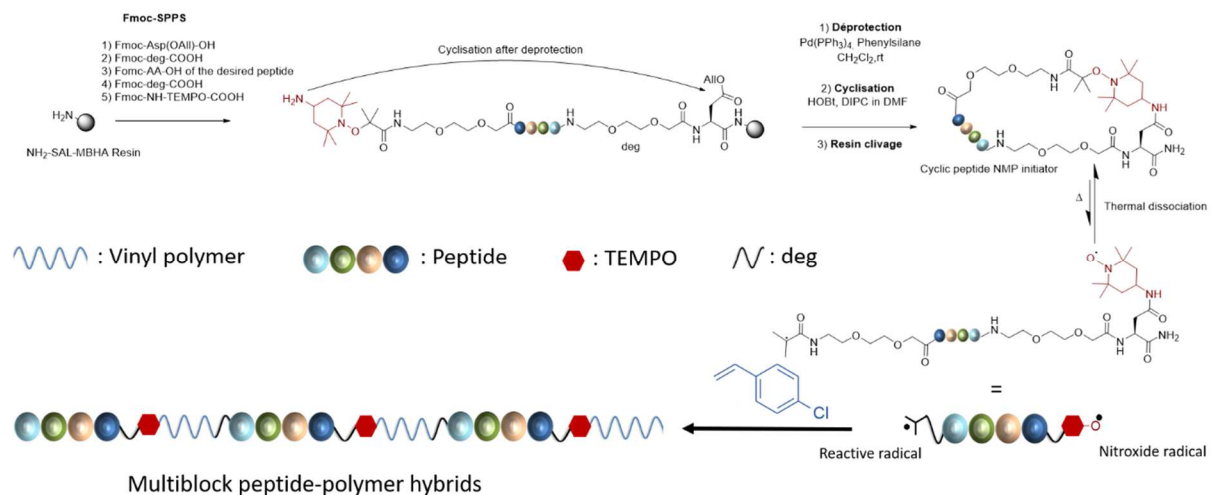


Fig. 41. Example of NMP polymerization of a cyclic peptide macromonomer.

4.6 Poly-condensation of silylated peptide macromonomers ('peptide-silicone')

Silylated peptide macromonomers were developed for many applications ranging from the synthesis of hybrid mesoporous silica catalyst[201] to hydrogels for cell encapsulation[202] and 3D printing[15]. Versatility of silicon chemistry was also utilized to prepare silicone containing peptides by several pathways.

First, monosilylated peptides were successfully used in *grafting to* approach. As example, antibacterial peptides were immobilized on silicone catheter after plasma activation in order to get antimicrobial properties [203] and wound healing peptides were grafted on silicone dressing.[204]

Such kind of peptide macromonomers as well as the resulting materials are qualified as 'hybrid' because they presented a silicone atom acting in inorganic sol-gel polymerization.

As example, peptide macro monomers bearing a silyl groups for grafting to approach were prepared by reacting an isocyanate derivative with the N-terminus of a peptide, in solution or on solid support (Figure 42). Of course, other reactive moieties should be protected at this stage.

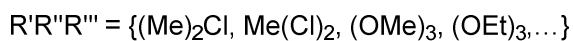
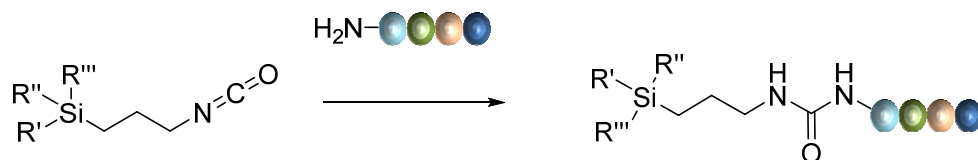


Fig. 42. N-terminus peptide silylation by reaction with an isocyanate.

Grafting through strategy was also explored using hybrid peptides-silicon macromonomers. The first reported example concerned the hydrolysis and condensation of bi-functionalized dimethylchlorosilane peptides that yielded Si-O-Si bridged polymerized peptide sequences (Figure 43-A).[205] This condensation proceeded chemoselectively towards AA side chains, thus enabling the use of unprotected peptides. Another advantage of this methodology is that the polymerization can be performed in water at very mild conditions. This strategy provided not only linear peptide polymers but also comb-like peptide polymers when using an N-terminus bi-functionalized Lysine residue (Figure 43-B).[131] On the other hand, by using mono functionalized dichloromethylsilane peptides, comb-like polymers were obtained along a PDMS-like backbone (Figure 43-C).[205,206]

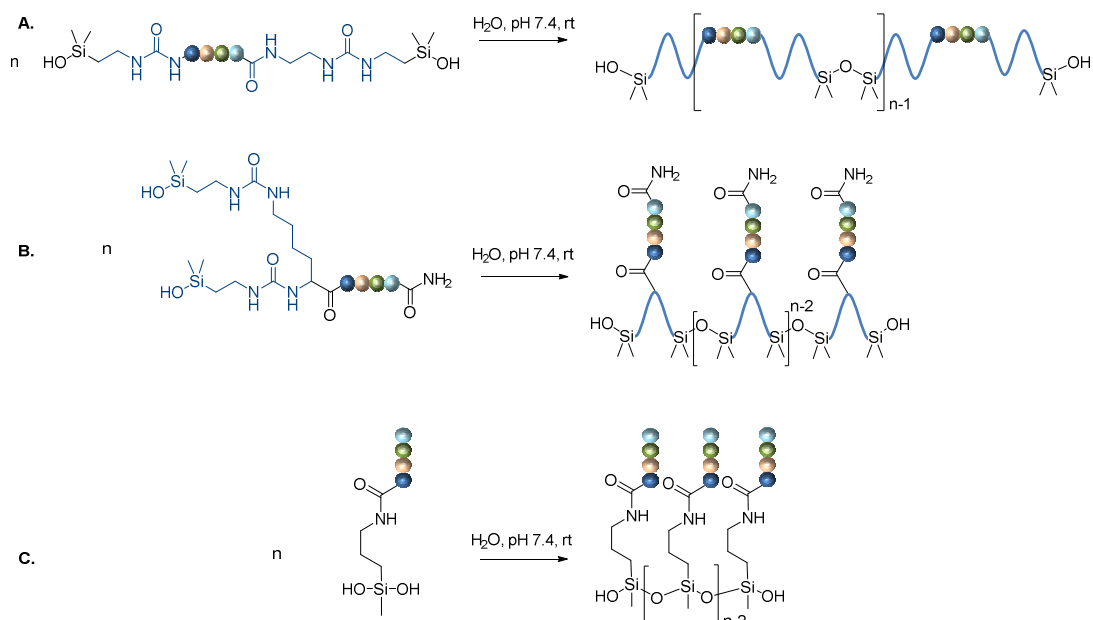


Fig. 43. Inorganic polymerization of silylated peptide macromonomers. A: Condensation of bi-functionalized dimethylchlorosilane peptides after hydrolysis. B: Condensation of N-terminus bi-functionalized Lysin-peptide after hydrolysis. C: Condensation of mono functionalized dichloromethylsilane peptides after hydrolysis.

Thanks to the versatility of silane chemistry, different hybrid macromonomers can be copolymerized in a single step to yield multifunctional PDMS-analogues. For example, mono functionalized dichloromethylsilane bioactive peptides, Ahx-Arg-Arg for antimicrobial purposes and RGD peptide for cell adhesion, were incorporated into a PDMS oil by copolymerization with dichlorodimethylsilane to obtain a bioactive PDMS.[207] Therefore, two different hybrid monomers, dichloromethylvinylsilane or dichloromethylsilane, at various ratio, were also copolymerized with dichlorodimethylsilane, to get modified PDMS multifunctional material by a cross-linking that followed a Karstedt catalyst hydrosilylation (Figure 44). One PDMS oil contained the bioactive peptide, an antimicrobial or a cell adhesive peptide along with a vinyl-silane moiety afforded by dichloromethylvinylsilane monomer. The other PDMS oil contained a H-silane function afforded by dichloromethylsilane monomer. The hydrosilylation of these two oils together lead to solid PDMS material, still demonstrating the bioactivity of the initial peptide: either antimicrobial or enhanced cell-adhesion property.

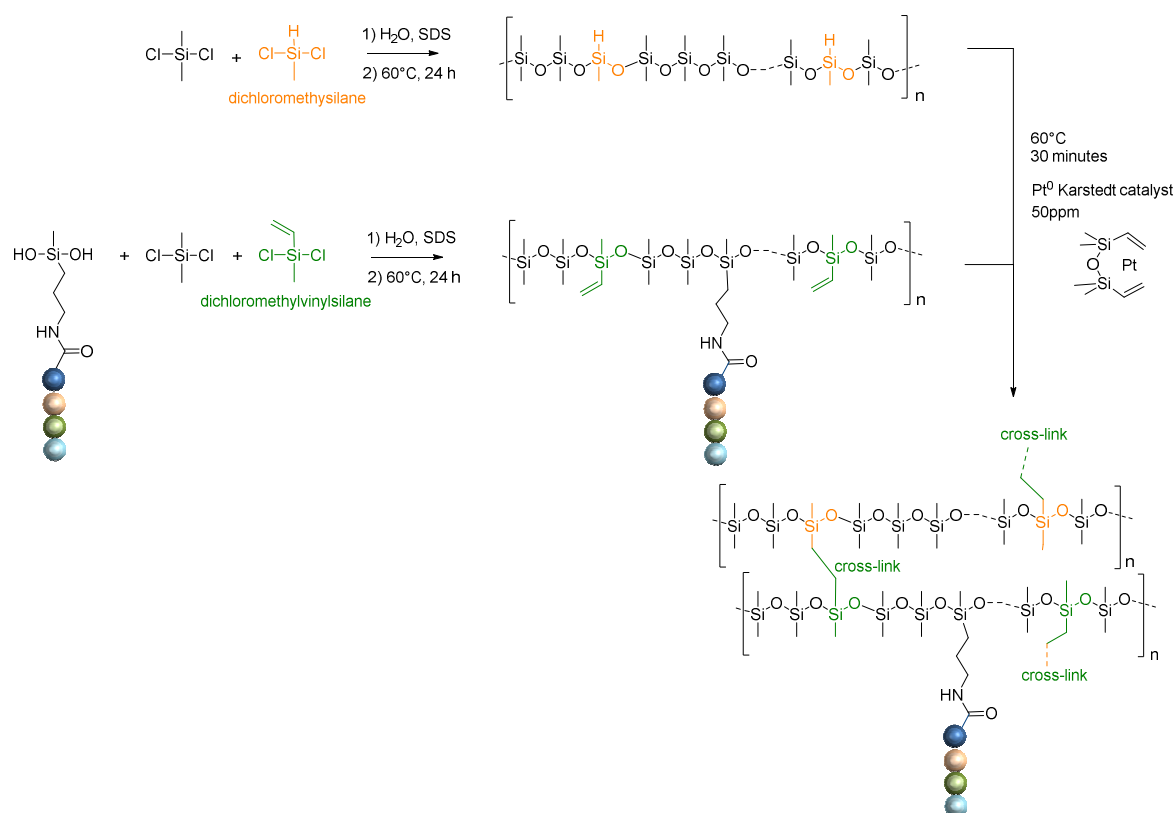


Fig. 44. Copolymerization and cross-linking by hydrosilylation of bioactive peptide containing PDMS.

In summary, the polymerization of a peptide macromonomer is undoubtedly highly attractive in a first sight. This strategy is theoretically customizable as since different peptides bearing a polymerisable moiety can be combined in a desired ratio and copolymerized altogether with the same chemistry. It enables, the combination of the properties of several different peptides involved in the polymerization process. However, in function of the desired application, some strategies are favored over others.

Those polymerizations based on a radical activation impose some limitation for the polymerization conditions. Indeed, the presence of the radical can lead to side reactions and restrain the possibility for the peptide to bear any other alkene function in the case of acrylate-based macromonomers. Also to our experience, peptides cannot contain Tyrosine residues as they behave as radical traps. In the same way, most of the presented polymerization strategies require that peptide sequences stay protected. Alternatively, some amino acids have to be avoided such as the Lys for the NCA and the 'peptide-nylons'. This impairs the customizable aspect of these techniques. Moreover, purification, can be troublesome as not all monomers are stable during HPLC preparative isolation. As examples, NCA peptide monomers hydrolyze very quickly and hybrid alkoxysilane peptides have the tendency to react covalently with silica-based stationary phase of columns. Hopefully, most peptide-containing monomers can be prepared on solid support and thus are subject of easy washings steps, which can possibly help to skip the final purification step. As already discussed for *the grafting to or grafting through* strategies, toxic catalysts and reagents have to be avoided if a biological application is envisioned. For example, the use of ruthenium catalyst in ROMP implies a careful purification to remove any traces of metal in the final peptide-polymer.

In contrast, some biorthogonal strategies do not require the use of toxic catalyst or neither present limitations about the choice of the peptide sequence to be polymerized. This is the case for the sol-gel hydrolysis and condensation between silylated macromonomers. Proceeding in physiological

conditions[202,208], it allows the direct incorporation of sensitive biological compounds, such as proteins, nucleic acids, or even living cells, in the resulting hybrid system (i.e. hydrogel[202,209], bioink[208,209], particle[210]).

Interestingly, ROP of lactones or Pox peptide macromonomers, have never been described so far although examples of polymerization of amino acids by these strategies can be found in literature. In the same way, other conjugation techniques, such as NCL and SPAAC, could be used as an inspiration to develop new polymerization strategy for peptide-macromonomers in soft and biocompatible conditions. However, the design of a photo-polymerizable systems should favor more straightforward biological applications in particular in bioprinting. It could be applied *in vivo* for the synthesis of implants that could be formed directly where needed by polymerization following a light-written pattern.

Noteably, the establishment of dynamic covalent bonds between peptide macromonomers could be another promising strategy to obtain smart peptide-based materials. In a seminal example, Dynamic disulfide bonds have already been used to induce reversible self-assembling of a peptide. A cysteine containing amphiphilic peptide n-pentadecane-CO-NH-CCCCGGGS(H₂PO₃)RGD-OH has been designed by Hartgerink et al. to form nanostructured fibrous scaffold under oxidative condition.[212] The reversible intermolecular disulfide bonds can regenerate free thiol cysteine by reduction and induce the disassembling.

In all these bottom up approaches, peptides take even more critical part in the control of the final morphologies as illustrated by the recent PISA polymerizations of self-assembling peptides. The obvious advantage of the grafting through strategy is the ability to position the peptide sequences either in the core or in the shell of the obtained polymer architectures with very high efficiency, which could be very challenging for the other two reported strategies. Once again, the peptides can play a dual role in either providing a function to the polymer or driving the template of its structural organization. In another example, the role of the peptide in the structural organization is also

highlighted in the self-mineralization concept where bis silylated PhePhe pseudodipeptide undergoes inorganic polymerization. The morphology of the resulting peptide-based material is driven by the controlled self-assembly of the peptide, during the polymerization.[213]

5. Conclusion

A large choice of strategies to obtain peptide-polymers has been described in recent years. First, often following a polymer modification, peptides can be grafted on the polymer by diverse conjugation techniques. On the other hand, peptides can take part of the polymerization as an initiator. In that case, only a single peptide sequence playing the role of initiator is incorporated in the polymer. At last, the peptide may play an even more important role as a macromonomer, leading to polymers incorporating several copies of well-defined peptides along their backbone.

For a long time, peptides have been relegated to a second role in peptide-polymer conjugates, only considered as a way to improve the polymer properties, by post-functionalization for example. If the biological activity and bioavailability of peptides was extensively used to provide polymers with functions, the exploitation of their structural and recognition features is in development. The recent literature demonstrates that peptides have to be considered as attractive starting blocks for the design of novel macromolecular and biomimetic materials. They can drive and determine the final morphologies of the peptide containing polymers while also conferring them thermo-sensitivity, pH sensitivity and self-healing properties. Inspired by protein structural features and predictive weak interactions networks, the tremendous progress performed in the field self-assembling peptides will certainly fuel the research of well-defined functional peptide polymers.

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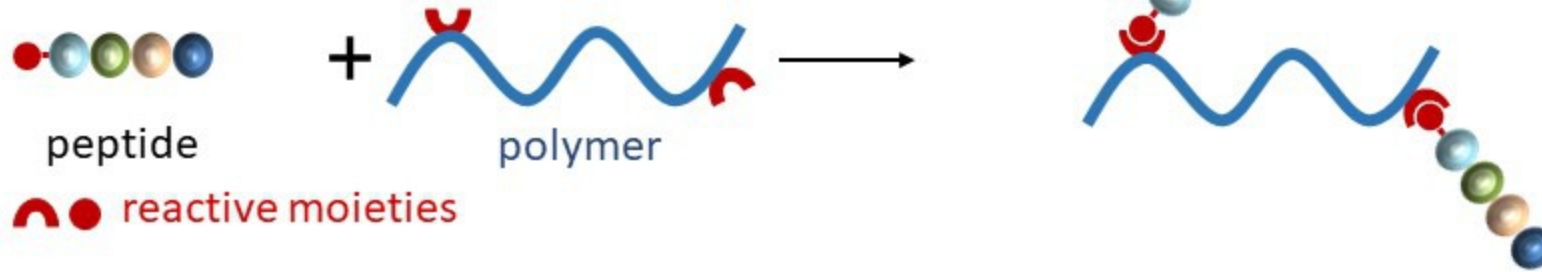
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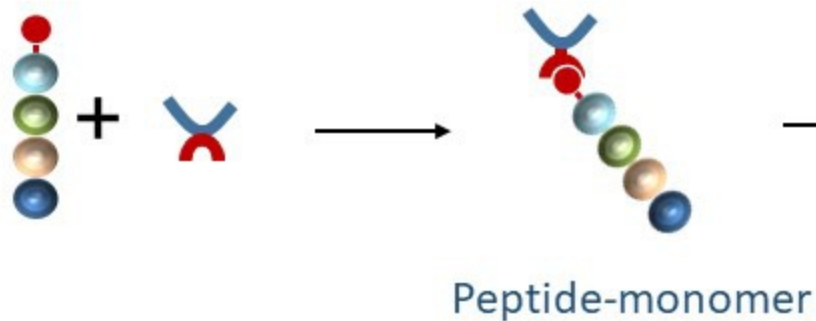
Grafting to



Grafting from



*Bottom-up strategy using peptide as macromonomer
(Grafting through)*



- High functionalization degree
- Novel properties afforded by the peptide sequence (self assembly, bioactivity)