Advances in Polymer Science 253

Helmut Schlaad *Editor*

Bio-synthetic Polymer Conjugates

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Aims and Scope

The series *Advances in Polymer Science* presents critical reviews of the present and future trends in polymer and biopolymer science. It covers all areas of research in polymer and biopolymer science including chemistry, physical chemistry, physics, material science.

The thematic volumes are addressed to scientists, whether at universities or in industry, who wish to keep abreast of the important advances in the covered topics.

Advances in Polymer Science enjoys a longstanding tradition and good reputation in its community. Each volume is dedicated to a current topic, and each review critically surveys one aspect of that topic, to place it within the context of the volume. The volumes typically summarize the significant developments of the last 5 to 10 years and discuss them critically, presenting selected examples, explaining and illustrating the important principles, and bringing together many important references of primary literature. On that basis, future research directions in the area can be discussed.

Advances in Polymer Science volumes thus are important references for polymer scientists, or scientists interested in polymer science - as an introduction to a neighboring field, or as a compilation of detailed information for the specialist.

Review articles for the individual volumes are invited by the volume editors. Single contributions can be specially commissioned.

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Editor

Bio-synthetic Polymer Conjugates

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Preface

Biosynthetic polymer conjugates (also known as biological-synthetic polymers, biohybrid polymers, or polymer chimeras) are polymers containing biological segments – mostly polypeptides, proteins, polysaccharides, polynucleotides, and terpenes – and synthetic segments. The idea or aim is to synergistically combine the advantageous properties of both components, which include biological function, molecular recognition, chirality, biocompatibility of the biological component and solution properties, and processability of the synthetic component, thereby creating new biomaterials with sophisticated properties and structural features. Conjugate polymers are often designed for life science or biomedical applications (for instance, as smart carrier systems in targeted drug or gene delivery) and also have great potential for materials science (like for the production of bioinspired hierarchical structures or biominerals) and sustainable chemistry (especially polysaccharides and polyterpenes).

The laboratory syntheses of polypeptides and of peptide–polymer conjugates were achieved early in the last century and developed into the sophisticated materials they are today. Peptide sequences, oligonucleotides, and also oligosaccharides can nowadays be readily prepared by automated solid-phase syntheses; however, subsequent conjugation to synthetic polymers is often difficult. Here, the very recent advances in chemoselective coupling strategies, PEGylation, and "click" chemistry have contributed greatly to overcome these problems. Also the synthesis of protein–polymer conjugates has been facilitated by mild and efficient coupling strategies as well as by the development of controlled radical polymerization techniques (conjugation by grafting from). The controlled synthesis of well-defined polyterpenes is least developed, which is attributable to the multifunctionality and also to the limited solubility of terpene monomers.

This volume of *Advances in Polymer Science* is comprised of five chapters summarizing the state of the art in the synthesis of bioorganic–synthetic polymer conjugates based on oligo- and polypeptides (Chap. 1, authored by Henning Menzel), proteins (Chap. 2, Björn Jung and Patrick Theato), carbohydrates (Chap. 3, Ahmed M. Eissa and Neil R. Cameron), nucleotides (Chap. 4, Corinne Vebert-Nardin *et al.*), and terpenes (excluding polyisoprene and natural rubber) (Chap. 5,

Junpeng Zhao and Helmut Schlaad). The main focus is on synthesis, whereas special materials properties and potential applications are not discussed in great detail.

I would like to express my sincere thanks to all the contributors of this volume, authors, and reviewers, for their excellent and stimulating work. I hope that the articles will be an inspiration for new concepts and further developments in the field of biosynthetic polymer conjugates.

Potsdam-Golm Helmut Schlaad
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Contents

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Polypeptide–Polymer Conjugates

Henning Menzel

Abstract Conventional block copolymers are able to self-assemble in bulk, resulting in a microphase separation or formation of aggregates in solution. Polypeptides show secondary structure effects and specific non-covalent interactions, which can cause a specific aggregation behavior and result in interesting supramolecular structures. Conjugation of polypeptides with simple synthetic polymers combines these phenomena with the advantages of synthetic polymers like solubility and processability. Various chemical strategies have been developed for conjugating different synthetic polymers with smaller and larger peptides. Here, we emphasize conjugation methods for peptides prepared by solid phase peptide synthesis having a controlled sequence as well as for polypeptides consisting of only one or two amino acids, which can be prepared by polymerization of the corresponding *N*-carboxyanhydride. By consecutive polymerization using a macroinitiator, block copolymers as conjugates are accessible. Different methods using this approach are highlighted. Furthermore, examples of conjugation of peptides with preformed polymers by "click" chemistry are presented.

Keywords Block copolymer · Click chemistry · Comb-shaped polymers · Controlled radical polymerization · Micelles · NCA polymerization · Polymersomes · Star-shaped polymer

Contents

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Abbreviations

AM	Activated monomer
ATRP	Atom transfer radical polymerization
BLG	γ-Benzyl-L-glutamate
CD	Cyclodextrin
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DVB	Divinylbenzene
GFP	Green fluorescent protein
GPEC	Gradient polymer elution chromatography
HEMA	Hydroxyethylmethacrylate
LC–MS	Liquid chromatography coupled with mass spectroscopy
LCST	Lower critical solution temperature
MALDI-ToF	Matrix-assisted laser desorption ionization-time of flight mass spectroscopy
mRNA	Messenger ribonucleic acid
NCA	*N*-carboxyanhydride
NIPAM	*N*-isopropylacryl amide
NMP	Nitroxide-mediated polymerization
P3HT	Poly(3-hexylthiophene)
PAF	Poly(alanine-*co*-phenylalanine)
PBLG	Poly(γ-benzyl-L-glutamate)
PDMAEMA	Poly[(2-dimethylamino)ethyl methacrylate]
PDMS	Poly(dimethylsiloxane)
PEG	Poly(ethylene glycol)
PEI	Poly(ethylene imine)
PEO	Poly(ethylene oxide)
PGA	Poly(L-glutamic acid)
PI	Poly(isoprene)
PLA	Poly(lactide)
PLL	Poly(L-lysine)
PMeOx	Poly(2-methyl-2-oxazoline)
PMPCS	Poly{2,5-bis[(4-methoxyphenyl)-oxycarbonyl]styrene}
PS	Poly(styrene)

RAFT	Reversible addition fragmentation transfer
SDS	Sodium dodecyl sulfate
SFRP	Stable free-radical polymerization
TEM	Transmission electron microscopy
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
THF	Tetrahydrofuran
ZLL	*N*-benzyloxycarbonyl-L-lysine

1 Introduction

Polypeptides are polymers of α-amino acids and can be of natural or synthetic origin. Typically, polypeptides consist of a defined sequence of different amino acids and have a secondary structure. Polypeptides produced by ribosomal synthesis from an mRNA transcript of a DNA template are usually called proteins, particularly when the secondary structure results in a defined folding. Synthetic polypeptides are typically limited in the number of amino acids; however, larger polypeptides have also been synthesized from just one or two amino acids [1]. After Pauling suggested an α-helical backbone for polypeptides [2], these synthetic polymers have been employed as models for proteins and used to study the secondary structure [3–6]. Doty and colleagues first demonstrated that synthetic polypeptides can undergo helix–coil transitions in solution [3, 7]. The secondary structure effects, the specific non-covalent interactions, as well as the chirality of the polypeptides can cause a very specific aggregation behavior.

Conventional block copolymers are able to self-assemble in bulk (microphase separation) or in solvents (micellization). Due to the specific aggregation behavior of polypeptides, their conjugation with synthetic polymers increases the number of self-assembled structures and may result in new materials [8, 9]. In recent years, several synthetic methods have been developed for the preparation of such polypeptide–polymer conjugates. This review will cover the recent literature on polypeptide–polymer conjugates. The focus will be on the synthesis and properties of homopolypeptide–polymer conjugates, but also some small oligopeptide–polymer conjugates will be discussed. For protein–polymer conjugates, the reader is referred to the review of Jung and Theato [10].

2 Synthetic Strategies

2.1 Synthesis of Polypeptides

Polypeptides can be synthesized by different methods. Solid phase synthesis is the most versatile method regarding the control of the polypeptide sequence and

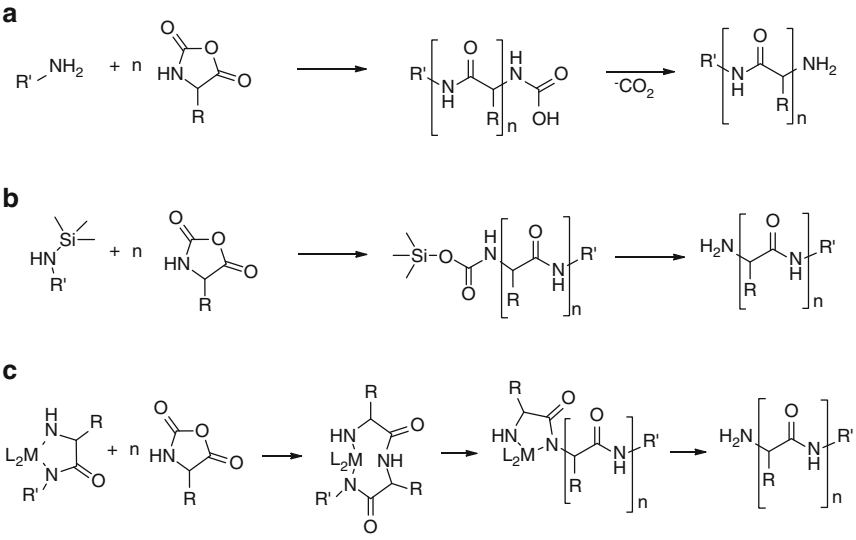


Fig. 1 Methods for synthesis of polypeptides via NCA polymerization: (**a**) amine-initiated [13, 14], (**b**) *N*-trimethylsilyl-initiated [15–17], and (**c**) initiated with transition metal complexes. *M* can be Ni, Co, Fe, Ru, or Ir [18–20]

with that the secondary structure and molecular weight. However, the length of the polypeptide is limited because of the inaccurateness of every single step.

Polymerization of the *N*-carboxyanhydride (NCA) of the corresponding α-amino acid opens the ways to longer polypeptide blocks [11, 12]. The NCA polymerization can be carried out as a ring-opening polymerization with nucleophiles as initiators. There are two mechanisms: the "amine" and the "activated monomer" (AM) mechanisms. The AM mechanism is initiated by deprotonation of an NCA, which then becomes the nucleophile that initiates chain growth. The polymerization according the AM mechanism is a step-growth polymerization in which high molecular weights are obtained only when monomer conversion approaches 100% and does not allow any control over molecular weight and molecular architecture. On the other hand, the amine mechanism is a simple nucleophilic ring-opening chain growth process (see Fig. 1a) in which the polymer grows linearly with monomer conversion providing side reactions are absent [11]. The amine mechanism thus allows the preparation of more complex polymeric architectures. However, there are several side reactions in the ring-opening polymerization of NCAs that result in chain termination or chain transfer [11]. Employing amine initiators therefore typically results in relatively broad molecular weight distributions, and in the synthesis of block copolymers the formation of homopolymers is observed [21].

Several methods have been developed to overcome these problems. Schlaad and coworkers reduced the activity of the end group by employing ammonium salts in which the amine is present in the equilibrium only to a minor extent. Furthermore,

the ammonium group can reprotonate NCA anions and thus prevent a switch towards to the unwanted AM mechanism [22]. This method has been successfully applied for the preparation of block copolymers with poly(L-lysine) [22–24] and was extended for other amino acids [25]. A drawback of this method is that the equilibrium between the ammonium and the free amine groups differs from amino acid to amino acid. Therefore, the method is not all-purpose. A more general method has been developed by Hadjichristidis and coworkers, in which the side reactions are suppressed by using high vacuum techniques and special purification methods [26, 27]. Interestingly Vayaboury et al. were able to show that the side reaction of the amine-initiated NCA polymerization are significantly reduced when the reaction temperature is lowered to 0°C [13]. The effect of temperature on the amine-initiated NCA polymerization was further investigated by Habraken et al. with matix-assisted laser desorption ionization–time of flight mass spectroscopy (MALDI-ToF) methods. The investigations confirm that end-group termination and other side reaction are absent at 0° reaction temperature. Furthermore, a copolymerization of different NCAs is completely random under these conditions [28]. A further investigation was aimed at finding the optimal reaction conditions in terms of temperature and pressure in the amine-initiated polymerization of different NCAs [29]. The investigated NCAs could be divided into two groups: the first group of γ-benzyl-L-glutamate (BLG), protected lysine, and alanine NCAs polymerized faster than the second group of benzyl aspartate, *O*-benzyl serine, and *O*-benzyl threonine NCAs. The latter show several side reaction at 20°C and should be polymerized at 0°C; however, applying high vacuum has no influence. On the other hand, the faster reacting NCAs do not show significant side reactions at 20°C as long as monomer is present. However, after full conversion is reached the end groups undergo side reactions, e.g., the formation of pyroglutamate in the case of poly(γ-benzyl-L-glutamate) (PBLG) [29]. A further improvement in the low-temperature amine-initiated NCA polymerization is possible for polypeptides with a tendency to form β-sheets by the addition of urea [14].

The low temperature NCA polymerization at 0°C has the advantage that it is synthetically less demanding than other controlled NCA polymerization techniques, while offering very good control over the molecular weight and polydispersity. However, polymerization times are relatively long and the molecular weight is somewhat limited.

Cheng and coworkers proposed hexamethyldisilazane as initiator for NCAs. The initial reaction results in a trimethylsilylcarbamate end group, which has a reduced reactivity compared to the amine (see Fig. 1b). In the course of the polymerization, the trimethylsilyl group is transferred from the carbamate to the incoming monomer. In the work-up, the trimethylsilylcarbamate group is hydrolyzed and gives an amine group [15–17, 30].

Deming suggested the use of transition metal complexes as initiators (see Fig. 1c), which result in end groups with reduced activity and very good control over molecular weight and polydispersity. The method allows the preparation of relatively short polypeptide chains, but high molecular weights are also accessible with very good control [18–20, 31].

2.2 Conjugation Strategies

Several strategies are possible for the combination of polypeptides with other polymers. Most of the NCA polymerizations allow the use of functionalized initiators; as depicted in Fig. 1, the R′ group of the initiator is part of the resulting polymer chain. Therefore, polymers with corresponding end groups can be used as macroinitiators in the NCA polymerization to generate block copolymers. The polypeptide can be also used as macroinitiator to start controlled radical or other controlled polymerizations to yield block copolymers. Furthermore, preformed polymers can be used for conjugation, for example by "click" chemistry.

2.3 Peptide Synthesis with Macroinitiators

2.3.1 Amine-Initiated NCA Polymerization for Synthesis of the Polypeptide Block

As already pointed out, the mechanism of the NCA polymerization allows conjugation between a polymer and a polypeptide employing polymers as macroinitiators. Thus, polymers end-functionalized with amino groups have been used to prepare block copolymers [32–34]. The first attempts using this method clearly suffered from the lack of control in the simple amine-initiated NCA polymerization [32]. However, the method has been optimized and used to prepare block copolymers with narrowly distributed poly(styrene) (PS) [35], poly(butadiene) [23], poly(ethylene glycol) (PEG) [36–38], poly(oxazoline)s [39, 40], poly(dimethylsilane) [41], poly(*N*-isopropylacrylamide) (PNIPAM) [42, 43], and others [44, 45]. The influence of the block length and block length distribution on the solid state properties was investigated with very narrowly distributed polymers prepared with ammonium-initiated NCA polymerization [22, 24, 46, 47]. The solid state structure caused by the microphase separation as well as by aggregation of the block copolymers in solution have been investigated in detail and have been reviewed recently [8, 9]. However, some current developments in this field will be presented here.

Vesicles of poly(L-lysine)-*b*-poly(L-glutamic acid) (PLL-*b*-PGA) have been reported to show pH-responsive "inside–out" aggregation. At acid pH, the PLL block forms the corona and the α-helical PGA block forms the core (see Fig. 2). However, at alkaline pH the PLL forms the core of the vesicle wall with the PGA as corona [48]. This principle was further expanded towards pH- and temperature-sensitive block copolymers. At acidic pH and room temperature, PNIPAM-*b*-PGA forms micelles with a PGA core and a PNIPAM corona, whereas at alkaline pH and elevated temperatures PNIPAM forms the core and the PGA the corona. At alkaline pH and room temperature, the block copolymer is moleculary dissolved [42]. PNIPAM-*b*-PLL behaves similarly but forms PLL core micelles at alkaline pH and room temperature [43].

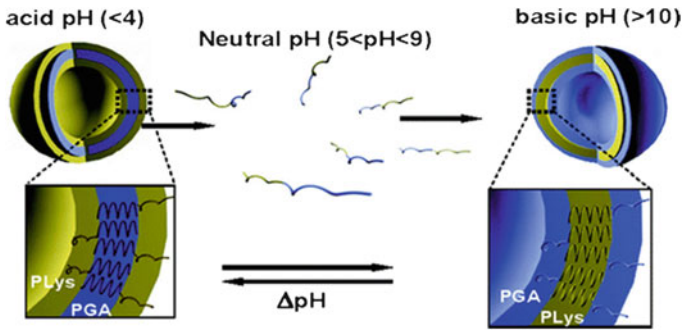


Fig. 2 Self-assembly of the diblock copolymer PGA15-*b*-PLL15 into vesicles. Reprinted with permission from [48]. Copyright (2005) American Chemical Society

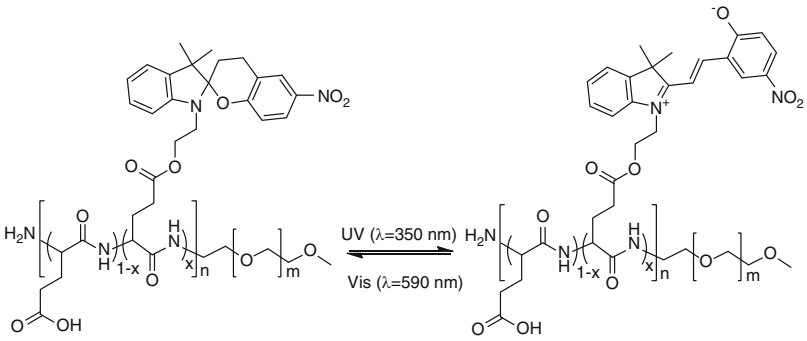


Fig. 3 Spirobenzopyran-modified PEO_{235}-*b*-PGA_{10} diblock copolymer and the photoreaction of the spirobenzopyran/merocyanin moiety [49]

Photoresponsive aggregation and dissolution was observed for block copolymers prepared by polymerization of BLG–NCA with amino-terminated PEG, deprotection, and subsequent introduction of a photochromic dye in the PGA block (see Fig. 3). The degree of substitution was 50%. The block copolymer forms micelles in water. Upon irradiation with UV light, the photochromic spirobenzopyran moiety undergoes a photoreaction that results in the zwitterionic merocyanin form. The transition from the hydrophobic spiropyran group into the more polar merocyanin results in a dissolution of the micelles. Upon irradiation with visible light, the spirobenzopyran and the micelles are restored [49]. The schematic drawing in Fig. 3 does not take into account the fact that the remaining glutamic acid groups are more acidic than the phenol group and are deprotonated at neutral pH.

Other peptide–polymer conjugates with interesting optical properties are rod–rod systems with π-conjugated aromatic polymers. The latter are appealing systems because of their optoelectronic and photoconductive properties, which strongly depend on the solid state morphology. Jenekhe and coworkers reported on triblock copolymers with a polyfluorene middle block and PBLG outer blocks

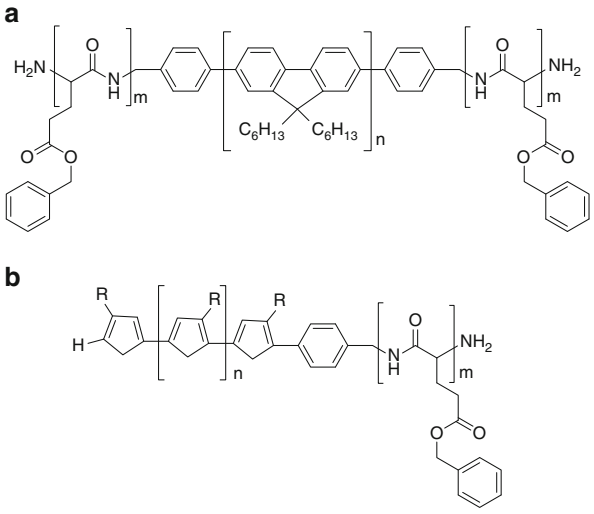


Fig. 4 (**a**) Rod–rod–rod PBLG–poly(fluorene)–PBLG triblock copolymers [50] and (**b**) rod–rod PBLG–poly(hexylthiophene) [44]

(see Fig. 4a), which were prepared by NCA polymerization initiated by amino endgroups [50, 51]. As a function of the copolymer composition and the secondary structure of the PBLG block, the microphase separation results in different nanostructured assemblies. The coil–rod–coil form of the copolymer shows a lamellar ordering with a π-stacking of the polyfluorene blocks, whereas the rod–rod–rod form remains in a cluster-like structure, with more isolated polyfluorene blocks. The photoluminescence of the polyfluorene blocks varies substantially between the two forms [50]. Hundt et al. have prepared block copolymers of regioregular poly(3-hexylthiophene) (P3HT) and PBLG [44]. The semiconducting properties of the P3HT are maintained in the block copolymer. On the other hand, the block copolymers show solvatochromatic behavior in mixed solvents, indicating the influence of the copolymer aggregate structure on the optoelectronic interactions.

PEG and poly(2-methyl-2-oxazoline) (PMeOx) with amine end groups were used to polymerize BLG–NCA and *S*-benzyloxy carbonyl-L-cysteine NCA. The resulting copolymers were compared with regard to their aggregation behavior. There is not very much influence on the aggregation behavior caused by the hydrophilic polymer (PEG or PMeOx), whereas the peptide block has a substantial influence on the size of the micelles and the critical micelle concentration. The authors ascribe this to the different secondary structure and hydrophobicity of the polypeptide blocks [52].

The influence of different secondary structures on the morphology of solid state samples was investigated by Ibarboure and coworkers with triblock copolymers with a poly(dimethylsiloxane) (PDMS) middle block and PBLG outer blocks. The length of the PBLG blocks was adjusted in a way that both longer α-helical blocks and

shorter β-sheet blocks are attached to the PDMS block. The triblock copolymers were then investigated regarding the microphase separation in thin films [41]. When the polypeptide block is relatively short (5–20 units), α-helical and β-sheet secondary structures can be found and a fiber-like morphology is formed independently of the film thickness. However, when the polypeptide block is longer than 20 units and adopts an α-helical conformation, a lamellar morphology is formed for thicker films [41]. Another series of PBLG–PDMS–PBLG triblock copolymers were investigated by Papadopoulus et al. [53]. In this series, the outer PBLG blocks were kept constant but the PDMS block was varied in his length and the effect of the thermodynamic confinement on the persistence length of the PBLG block was investigated.

In an effort to adjust the interface properties of polylactide (PLA) nanoparticles used for drug delivery, copolymers were synthesized having a polypeptide block in between PEG and PLA. One copolymer was prepared by polymerization of O-protected L-serine-NCA with an amino-terminated PEG. Subsequently, the deprotected serine groups were used to polymerize lactide to produce a comb-like copolymer block. This copolymer was compared with a triblock copolymer PEG-*b*-poly(alanine)-*b*-PLA. In this case, the endgroup of the poly(alanine) block was used as initiator [54]. Both types of copolymers form nanoparticles, but the copolymers with the comb-like structure show particularly interesting properties. Zeta-potential measurements indicate that the poly(serine) backbone covers the PLA core.

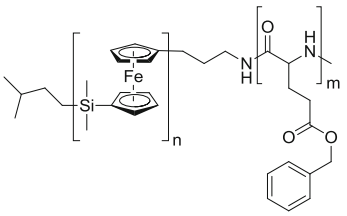
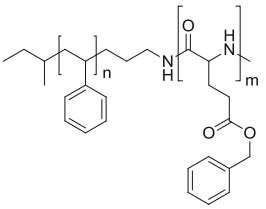
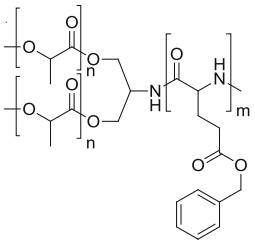
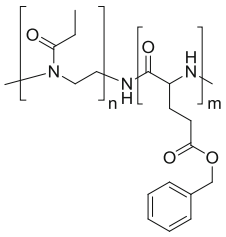
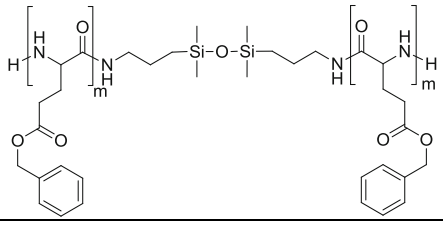
In solution α-helical polypeptide blocks show strong aggregation. This aggregation can result in the formation of gels. Kim at el. first described the formation of thermoreversible gelation of a block copolymer with a PBLG and a flexible coil block in toluene [45] (see Table 1). A similar behavior was found for copolymers with PS or poly(ethylene oxide) (PEO) blocks. The authors suggested the gelation to be a consequence of the formation of nanoribbons with parallel arranged α-helical PBLG blocks fringed by the flexible coil blocks.

Poly(2-ethyl-2-oxazoline)–PBLG block copolymers also show gelation in helicogenic solvents like toluene and benzylalcohol. Gelation is observed for longer PBLG blocks, whereas for shorter blocks other aggregates like vesicles are found [40]. Sun et al. reported on Y-shaped block copolymers with an α-helical PBLG arm and a two-arm PLL as second block [55].

Another recent example of an organogelator on the basis of polypeptide blocks are PBLG–PDMS–PBLG triblock copolymers [56]. In this study, the length of the PBLG blocks was varied systematically from 11 to 170 units. When more than 20 BLG units are present, the PBLG blocks adopt an α-helical structure and low critical gelation concentrations were observed. Again, nanofibril formation was suggested as reason for the gelation [45, 56] (see Fig. 5).

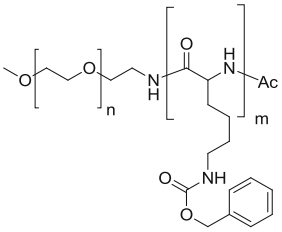
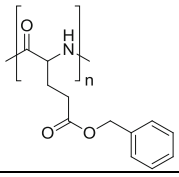
Hermes et al. investigated the influence of secondary structure on the thermoreversible gelation of a PEO-PZLL block copolymer. They varied the secondary structure of the PZLL block by adjusting the stereosequences of the Z-lysine segments in a way that polypeptide block forms a random coil, a β-sheet, or an α-helix [57]. The tendency for organogelation increases for random coil $<$ α-helix $<$ β-sheet.

Table 1 Polypeptide-containing copolymers as organogelators

Type	Formula	Solvent	Ref.
AB		Toluene	[45]
AB		Toluene	[45]
Y-shaped A/B		Benzyl alcohol	[55]
AB		Toluene benzyl alcohol	[40]
ABA		Toluene	[56]

(continued)

Table 1 (continued)

Type	Formula	Solvent	Ref.
AB		THF	[57]
Homopolymer		Toluene	[58–60]

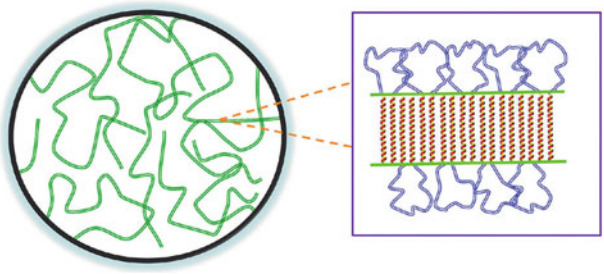


Fig. 5 Self-assembly of the peptide-based PBLG rods during nanofibril formation for PBLG-*b*-PDMS-*b*-PBLG triblock copolymers. Reprinted with permission from [56]. Copyright (2012) American Chemical Society

Thermoreversible gelation has also been observed for α-helical PBLG homopolymers [7, 58–60]. Recently, it was found that at concentrations significantly below the minimum gelation concentration of PBLG in toluene, spherical aggregates composed of PBLG nanofibers can be observed by transmission electron microscopy (TEM) ("fuzzy ball" morphology). At higher concentrations, gels are formed. These gels show a three-dimensional (3D) network structure composed of nanofibers. The proposed self-assembly mechanism leading to the formation of nanofibers is based on a distorted hexagonal packing of PBLG helices parallel to the axis of the nanofiber. The 3D gel network forms due to branching and rejoining of bundles of PBLG nanofibers [60].

Polypeptide-containing polymers can not only gel organic solvents but can also form hydrogels. First examples were polypeptide block copolymers with a hydrophobic polypeptide block with well-defined secondary structure and a charged polypeptide block in the coil form [61, 62]. The assembly mechanism was found to occur via an association of α-helices perpendicular to the long dimensions of the

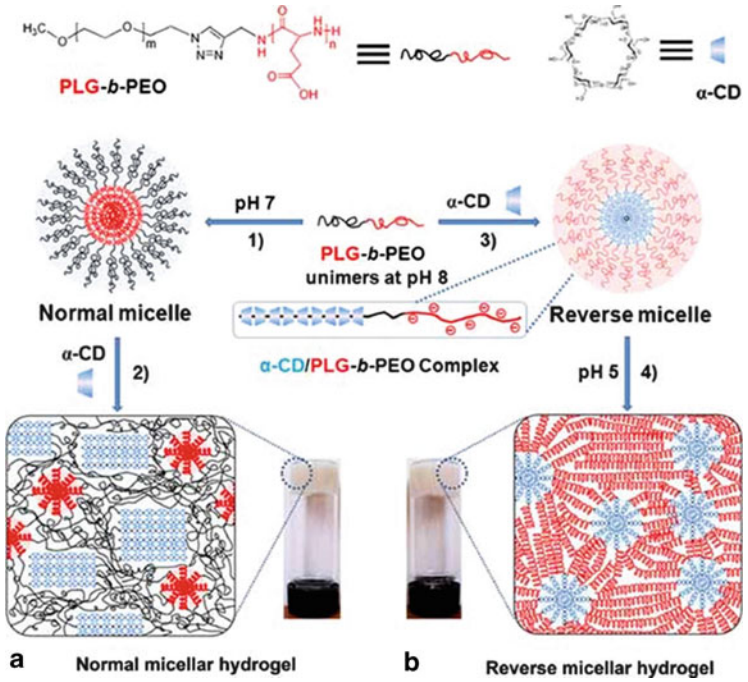


Fig. 6 Proposed structures and gelation mechanism of supramolecular hydrogels: (**a**) normal micellar hydrogel and (**b**) reverse micellar hydrogel. Stages: *1* micellization of copolymer, *2* normal micellar hydrogel induced by supramolecular inclusion complexation, *3* reverse micellization of copolymer, and *4* reverse micellar hydrogelation. Reprinted with permission from [63]. Copyright (2010) Wiley-VCH

fibrils or membranes. This motif is in contrast to helical orientation in coiled-coil fibrils as well as the structures of organogels formed from hydrophobic α-helical polypeptides, as described in the previous paragraph. The assemblies more closely resemble β-sheet fibrils in structure and stability, but without the interstrand hydrogen bonding.

A block copolymer with a PEG and a PGA block, which have been fused by azide–alkyne click chemistry (vide infra) form hydrogels when combined with cyclodextrin (α-CD) [63]. The mechanism for the normal micellar hydrogel is the formation of polypeptide-cored micelles with a PEO corona via hydrogen-bonding-mediated self-assembly, followed by the physical crosslinking of micelles via the supramolecular inclusion complexation of PEO and α-CD. However, a reverse micellar hydrogel is formed by formation of α-CD/PEO poly(pseudorotaxane)-cored micelles with a polypeptide corona (reverse micelles), via supramolecular inclusion complexation-mediated self-assembly, followed by the physical cross-linking of the reverse micelles via the hydrogen-bonding interactions between the polypeptide corona (see Fig. 6). The hydrogels can be loaded with hydrophobic drugs like doxirubicin. The reverse micellar hydrogels show a higher drug loading and better drug-release rates [63].

Marsden et al. prepared copolymers with a homopolypeptide block and a shorter block of peptide with a controlled sequence [64]. The hydrophobic block is PBLG and the hydrophilic block is a coiled-coil-forming peptide. The latter was prepared in solid phase peptide synthesis and the PBLG was polymerized using the N-terminus of the peptide. In this way, the broad size range and structure-inducing characteristics of the homopolypeptide can be combined with the functionality of peptides with a well-defined sequence. The copolymers form nanostructures in water that can be adjusted by choosing the modular building blocks. Because the hydrophilic peptide has a specific sequence, it can be chosen in a way that it acts as a homing device to deliver vesicles to specific targets in the body [64].

Kang et al. studied the influence of stereochemistry of the peptide block on the properties of PEG-*b*-poly(alanine-*co*-phenylalanine) (PEG-L-PAF and PEG-D-PAF) [65]. The block copolymers with similar molecular weights but different stereochemistry were similar in their phase diagrams regarding the sol-to-gel transition of the aqueous solution and temperature-sensitive changes in their self-assemblies.

Segregation of a polymer blend was used to adjust the properties of surfaces. For this purpose, PS-*b*-PGA was used as surface-modifying agent for a PS matrix. The block copolymer was prepared by polymerization of BLG–NCA with an amino end-functionalized polystyrene and subsequent deprotection of the PGA under alkaline conditions. Upon annealing in air the surface of the blend is hydrophobic. However, when the annealing is carried out at elevated temperatures in water vapor, a hydrophilic surface is created showing an migration of the polypeptide block to the surface [66].

2.3.2 NCA Polymerization with Transition Metal Catalysts for Synthesis of the Polypeptide Block

Amine-initiated NCA polymerization has some drawbacks. In particular it is difficult to obtain higher molecular weights with good control and low polydispersity. Therefore, macroinitiators with transition metal complexes as end groups have also been developed and used to prepare polypeptide–polymer conjugates [12, 67, 68]. The basis for this technique is the use of amido-amidate metallacycles as propagating species, which can be prepared from allyloxy carbonyl aminoamides as universal precursors. These simple amino acid derivatives undergo tandem oxidative-additions to nickel(0) to give active NCA polymerization initiators (see Fig. 7) [69]. The residue X can be a functional group, a peptide, or a polymer.

By this chemistry, polymers with one amine end group as well as α,ω-diamine-functionalized polymers can be used to prepare AB or ABA copolymers, respectively. The method gives copolymers with well-controlled polypeptide segments. Furthermore, no unreacted homopolymers or homopolypeptides could be detected. Several examples of the polymer B block have been reported: poly(octenamer) prepared by acylic diene metathesis polymerization [67], poly(methyl acrylate) prepared by atom transfer radical polymerization (ATRP) [70], poly(ethylene glykol) PEG, and PDMS [68]. The method was expanded for the synthesis of

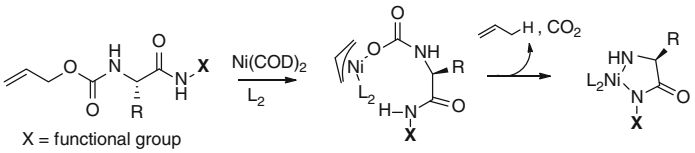


Fig. 7 Formation of amido-amidate nickelacycle form allyloxycarbonylaminoamides [69]; X is a functional group, a polymer chain or another peptide

pentablock copolymers. A central α,ω-diamine-functionalized polymer [poly (octenamer), PEG, or PDMS] is transferred into a ABA triblock copolymer. The living metallacycle end groups are then reacted with isocyanate-capped PEG chains [68].

2.4 Polymer Synthesis with Polypeptide Macroinitiators

In most reports, the peptide–polymer-conjugates are prepared by using a polymeric macroinitiator for the polymerization of the polypeptide; however, the sequence can also be reversed. Polypeptides can be prepared and used as macroinitiators for a polymerization. Particularly suited for this approach are controlled polymerization techniques because they usually allow good end-group control and adjustment of the molecular weight and the molecular weight distribution of the polymer block. There are different mechanisms for a controlled radical polymerization that can be used for this purpose: stable free-radical polymerization (SFRP), ATRP, and reversible addition fragmentation chain transfer (RAFT) polymerization.

2.4.1 Peptide Macroinitiators for SFRP

First examples of using SFRP have been described as a nitroxide-mediated polymerization (NMP) from initiating sites located on the chain termini of a peptide on a solid support [71]. The peptide was constructed on Wang's resin by solid state peptide synthesis. The N-terminus was then converted into a carboxy-functional group by coupling with glutaric acid anhydride and further reacted with an amine-functionalized initiator for the NMP. The initiator was still bound to the resin and was used to initiate the polymerization of *tert*-butyl acrylate and, subsequently, methyl acrylate. The method was further expanded using ATRP (see next paragraph) [72, 73] and other peptides [73]. Becker et al. described the synthesis of amphiphilic block copolymers by this technique, which were capable of forming micelles and having tritrpticin – an antimicrobial peptide – as end group. The antimicrobial peptide bound to the block copolymer showed a slightly lower minimum inhibitory concentration against *Staphylococcus aureus* and *Escherichia coli* and was significantly less toxic for blood cells compared to the free tritrpticin [73].

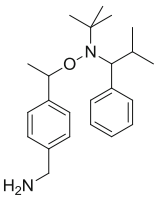
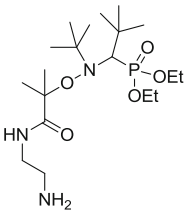
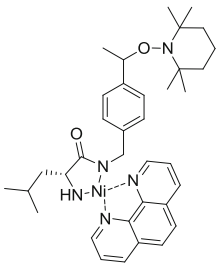
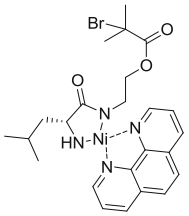
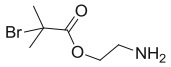
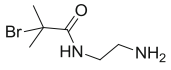
The challenge in applying a route with a polypeptide macroinitiator is the quantitative introduction of the initiating group to the first block, which often requires a modification and purification step prior to the second polymerization. An elegant way to circumvent the modification step and to assure complete functionalization is to start with bifunctional molecules, which have initiating groups for both types of polymerization. A requirement for such a method is that each initiating group is stable in the different polymerization circumstances of the other type of polymerization. This concept was recently reviewed by Bernaerts and Du Prez [74] and adapted by Knoop and coworkers for the preparation of polymer–peptide conjugates employing a nitroxide-mediated polymerization [75, 76]. The initiator used is depicted as entry 1 in Table 2. The amine-initiated NCA polymerization and NMP of styrene do not interfere with each other. Therefore, the polymerization can be carried out as one-pot polymerization without intermediate isolation of the products. The one-pot polymerization can be achieved by the different activation temperatures of the individual polymerizations, i.e., 0°C for the NCA polymerization and 120°C for the NMP. The success of this approach was shown using gradient polymer elution chromatography (GPEC), as there is a trace for the block copolymer with a retention time between those for the two homopolymers, and only a small peak for thermally initiated PS is visible [75].

The technique was taken a step further by crosslinking the block copolymers with divinylbenzene (DVB) to form nanoparticles with a crosslinked PS core and PS-*b*-PBLG arms (see Fig. 8). A clear effect of the block length ratio and the amount of crosslinker in the process was observed. While gel formation occurred even at low block copolymer-to-crosslinker ratios for shorter block copolymers, individual core–shell particles were accessible with longer block copolymers. By debenzylation, PGA blocks are produced and the nanoparticles become water-soluble and pH-sensitive [81].

The combination of amine-initiated NCA polymerization and NMP was also used to prepare amphiphilic peptide–polymer conjugates having copolymers of L-glutamic acid and L-alanine as polypeptide and poly(*n*-butyl acrylate) or PS as polymer block. Micelles and vesicles were prepared from these block copolymers and the effects of peptidases on these particles were tested. It is possible to tune the enzymatic degradation by altering the amino acid composition in the polypeptide block [77].

As mentioned before, the molecular weight of the peptide block is limited when amine-initiated NCA polymerization is used for the preparation of the peptide block. Therefore, a bifunctional initiator was developed to combine the NMP with a transition metal-catalyzed NCA polymerization (entry 3 in Table 2). The NCA polymerization initiated by the nickel-amido-amidate group yields well-defined PBLG macroinitiators with a degree of polymerization $P_n = 80–200$ [78]. The NMP of styrene with the (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) group requires high temperatures of 125°C. At this temperatures and long reactions times in DMF a degradation of the PBLG macroinitiator was found. However up to 5–6 h reaction time no indication of degradation was observed and well defined block copolymers were obtained [78].

Table 2 Bifunctional initiators for the combination of NCA and vinyl polymerization

Entry number	Initiator	NCA polymerization method	Vinyl-polymerization method	Ref.
1		Amine-initiated	NMP	[75]
2		Amine-initiated	NMP	[77]
3		Transition metal	NMP	[78]
4		Transition metal	ATRP	[79]
5	(a)  and (b) 	Amine-initiated	ATRP	[80]

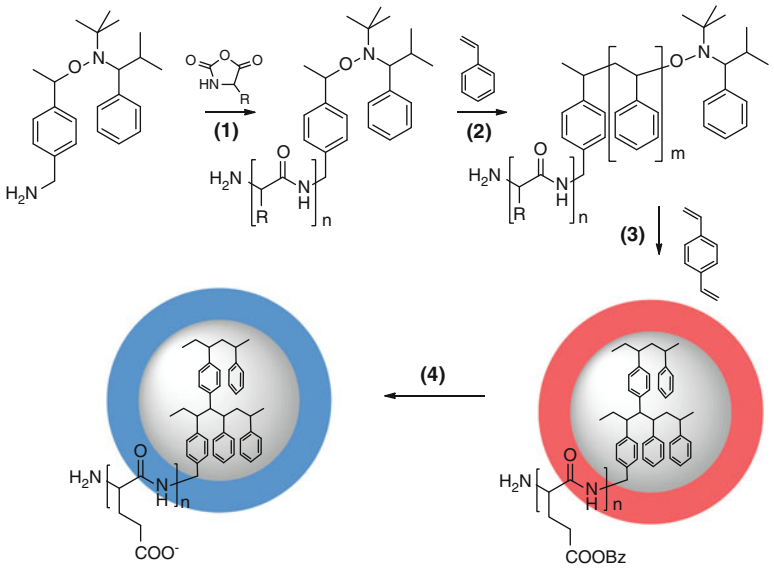


Fig. 8 Synthetic route to PBLG-*b*-PS employing a bifunctional initiator [75] (steps *1* and *2*), formation of nanoparticles (step *3*), and deprotection (step *4*). Redrawn according to [81]

2.4.2 Peptide Macroinitiators for ATRP

ATRP is another important method for preparation of block copolymers and can be used for preparation of polypeptide–polymer conjugates, too. First examples have been described for systems in which the ATRP initiator is at the end of a GRGDS peptide sequence [72, 82]. The GRGDS sequences attached to a poly(hydroxyethyl-methacrylate) [poly(HEMA)] block enabled cell adhesion that was not possible on poly(HEMA) homopolymers [72]. ATRP from a peptide still bound to the solid support is also possible [73]; however, better polydispersities are obtained when the ATRP is carried out in solution [82]. Besides having the ATRP initiating group at the end of the peptide it is also possible to place it at one of the amino acids within the peptide sequence. Functionalization of a peptide at two OH groups of serine residues at both ends with α-bromo ester moieties yielded a peptide with two ATRP initiator sites. After removal of the peptide from the solid support, methyl methacrylate was polymerized with this initiator and an ABA block copolymer was obtained. The block copolymer forms spherical aggregates, but the desired β-hairpin motif of the peptide was not adopted [83]. The ATRP initiators can not only be introduced by a functionalization of the peptide, but it is also possible to use already-modified amino acids to build the peptide [84]. In this case, ATRP initiating sites were introduced into the side chains of tyrosine or serine and the modified amino acids were employed in the solid phase peptide synthesis using Fluorenylmethyloxycarbonyl (Fmoc) protected amino acids. The peptide was then

successfully used as initiator for the polymerization of HEMA and *N*-acetylglucosamine-modified HEMA.

Peptide–polymer hybrid nanotubes have been prepared by modifying cyclic peptides, which can self-organize into tubular structures. Cyclic peptides composed of eight amino acids in alternating D- and L-configuration were modified at distinct side-chain positions to give ATRP initiators [85]. The polymerization of NIPAM in aqueous dispersion was initiated from the surface of the nanotubes formed by self-assembly of the peptide. Investigation of the polymerization kinetics was carried out in isopropanol as solvent and with the use of some sacrificial low molecular weight initiator [86]. The sacrificial initiator was used to increase the number of initiating sites, which is known to reduce early termination reactions of "grafting from" reactions. Furthermore, the sacrificial initiator gives free polymer, which can be isolated and characterized easily and gives a good measure for the molecular weight of the grafted polymer chains. The kinetics of the reaction indicates a loss of active species, while the increase in the molecular weight of the free polymer with the conversion is linear [86]. Better results were obtained with styrene. From the comparison of peptide nanotubes with different polymers as side chains (e.g., PNIPAM, poly(*n*-butyl acrylate) or PS) and with different side-chain lengths, a decrease in the length of the aggregates is found. Independently of the type of polymer attached, the aggregation number is reduced with increasing side chain length [87]. It is also possible to polymerize *n*-butyl acrylate in dimethylsulfoxide (DMSO) as solvent using the aforementioned cyclic peptide with the ATRP initiating sites. The peptide in this case is not self-aggregated but dissolved. After the polymerization of the polymer chain, the self-assembly of the peptide rings can be accomplished by diluting a dimethylformamide (DMF) solution with tetrahydrofuran (THF), the latter being a non-solvent for the peptide [88]. A similar system has been described by ten Cate et al. [89]. In this case, the cyclopeptide was coupled via the lysine amino groups to poly(*n*-butyl acrylate) chains prepared by ATRP, having a carboxyl end group. The aggregates have a structure similar to the aforementioned peptide-polymer hybrid nanotubes. Further examples can be found in some recent reviews [90–93].

Beside small peptides prepared by solid phase synthesis, proteins were also modified with a group containing an ATRP initiator. This can be done by chemically modifying a protein [94, 95] or by genetically engineering proteins to have an unnatural amino acid containing an ATRP initiating group [96, 97]. In this way green fluorescent protein (GFP) was modified with an amino group bearing an α-bromo amide group (see Fig. 9). The genetic engineering allows protection of the protein active site and structurally weak regions and allows precise control of the number of chains attached to the protein. The modified GFP was then used as initiator under standard ATRP conditions for oligo(ethylene oxide) monomethyl ether methacrylate, producing a polymer–GFP conjugate [96]. The system was also used to prepare protein nanogels by an inverse microemulsion and ATRP with activators generated by electron transfer (AGET ATRP) [97].

Homopolypeptides prepared by a NCA polymerization can also be used with ATRP. Qiu et al. have used amine-terminated dendrimers for an amine-initiated

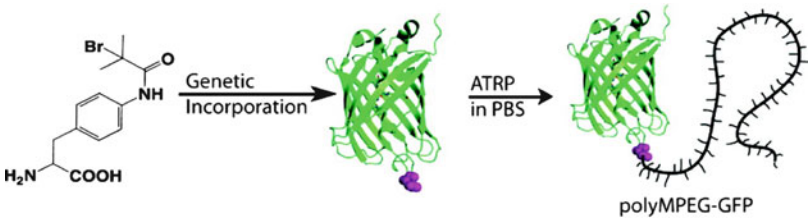


Fig. 9 Representation of how genetically engineered proteins can be used as ATRP initiators. Reprinted with permission from [96]. Copyright (2010) American Chemical Society

NCA polymerization. Subsequently, the N-terminus of the polypeptide chains was converted into an ATRP initiator by reaction with α-bromo isobutyric acid and used to polymerize D-gluconoamidoethyl methacrylate. In this way, star-shaped polypeptide/glycopolymer biohybrids with controlled molecular weights and low polydispersities were synthesized [98].

Besides transforming the N-terminus of the polypeptide chain after the NCA polymerization, bifunctional initiators can be employed (see Table 2). The method can be carried out with amine-initiated NCA polymerization [80] or transition metal-initiated NCA polymerization [79]. In the latter case, it has been shown that the ATRP group is stable in the presence of the nickel catalyst used for the NCA polymerization [79], although nickel complexes can also be used as catalysts in the ATRP polymerization [99].

Both the NCA polymerization and the ATRP are living polymerizations. However, there are some problems associated with the combination of ATRP and NCA polymerization. The first problem is related to the type of linker tethering the ATRP initiator to the peptide. In many investigations, α-isobutyric acid has been used to modify the peptide at the N-terminus or at lysine groups and thus an amide bond is present in the initiator group [72, 73, 82, 85, 95, 96]. Adams and Young have shown in a comparison of peptide based initiators with an ester or an amide bond respectively (see for example Table 2, entries 5a and 5b), that indeed the amide based initiators give higher molecular weights than expected and significantly higher polydispersities [100]. The results are in line with investigations which have shown that significant chain termination occurs during the initiation step [80, 101].

Habraken have proposed a mechanism that involves a disproportionation of the radicals after the transfer of the Br to the copper (see Fig. 10). The disproportionation products have been detected for model initiators by ^{1}H-NMR and liquid chromatography coupled with mass spectrometry (LC–MS) [80]. If macroinitiators are used, this termination reaction results in a substantial amount of macroinitiators remaining unreacted, even after prolonged reaction times. Furthermore, there is no gradual shift of the molecular weight with reaction time, but the macroinitiator trace decreases while the block copolymer trace increases (see Fig. 11) [80].

The second problem is the complexation of copper ions by the peptide chain in a biuret-like reaction [102]. Copper is the transition metal typically used in the ATRP

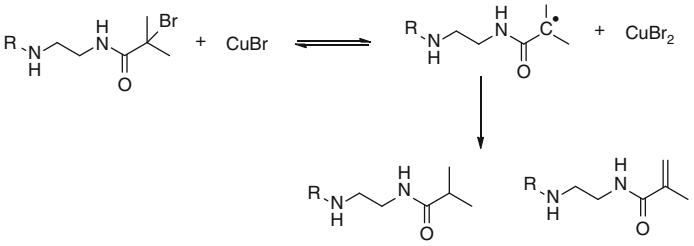


Fig. 10 Proposed termination reaction of amide-based model initiators [80]

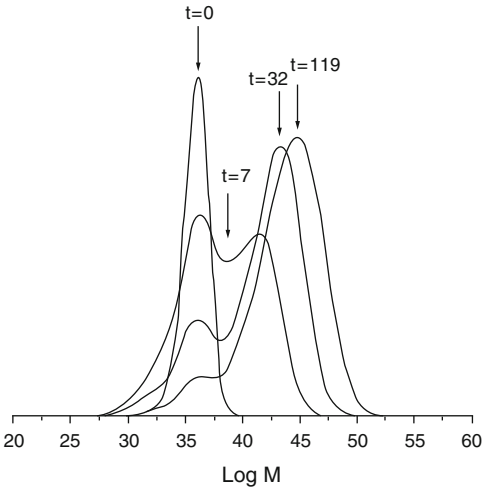


Fig. 11 Evolution of the molecular weight of Methylmetacrylate in ATRP using a PBLG macroinitiator with an amide bond in DMF (CuBr/Me_6TREN at 60°C). *t* is the reaction time in minutes. Reprinted with permission from [80]. Copyright (2009) Wiley-VCH

[99, 103]. Peptides and proteins can effectively complex copper ions, although the extent depends on the structure of the peptide [104]. The copper ion complexation in homopolypeptides is even strong enough to protect the backbone from racemization in the alkaline deprotection of, e.g., PBLG [105]. Therefore, the solvent and the ligand for the ATRP catalyst system have to be chosen accordingly to suppress complexation by the peptide chains [79].

2.4.3 Peptide Macroinitiators for RAFT

RAFT is another controlled radical polymerization, which gives access to interesting polymer structures [106]. The RAFT process offers some advantages because it tolerates a variety of functional groups. However, the RAFT reagents are dithioesters, which are sensitive towards amino groups as they are present in

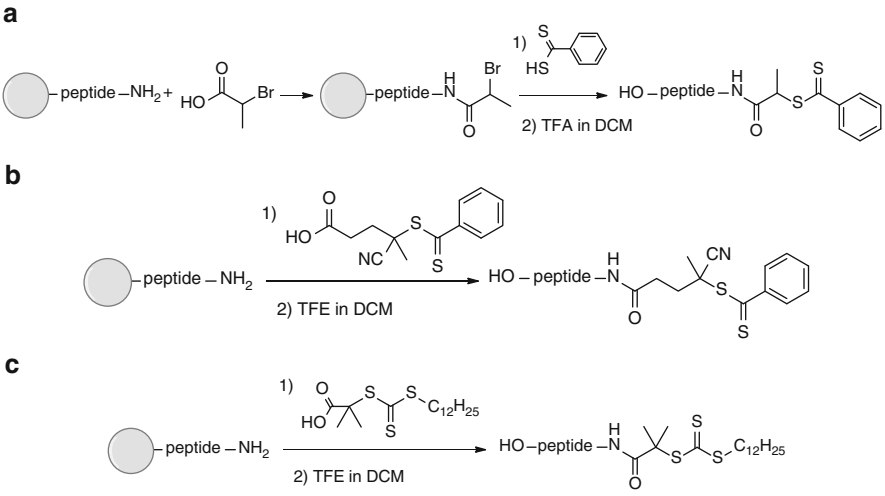


Fig. 12 Modification of the peptide N-terminus into a RAFT group. (**a**) Reaction with α-bromo carboxylic acid and substitution with a dithioester, (**b**) reaction with carboxylic acid, or (**c**) reaction with a trithiocarbonate [107–109]

peptides at the N-terminus and as side chains of, e.g., lysine. Nevertheless, Börner and coworkers presented an approach for the synthesis of polymer–peptide conjugates using RAFT [107–109].

In this approach, the N-terminus of a peptide on a solid phase is reacted with an α-bromo carboxylic acid and, subsequently, the bromine is substituted with a dithioester [107, 108] (see. Fig. 12a) or the N-terminus is reacted with a carboxylic acid already including the chain transfer agent for the RAFT polymerization [107] (see. Fig. 12b). In the latter case, a nucleophilic attack of the peptide amino group on the thioester results is a side reaction yielding a thioamide, which does not interfere with the RAFT polymerization [107]. However, if a trithiocarbonate group is used for the reaction with the N-terminus of the peptide, these side reactions are suppressed because this group shows a higher tolerance against nucleophiles than dithiobenzoates [109]. The kinetic investigations of the RAFT polymerization for such peptide macroinitiators and *n*-butyl acrylate as monomer indicate an efficient control of the polymerization [107–109]. The method was applied to prepare peptide–polymer-conjugates having a high molecular weight polymer and a switchable β-sheet-forming oligopeptide. As long as the temporary structure-breaking units are present in the peptide, it shows good solubility and therefore RAFT polymerization of *n*-butyl acrylate can be carried out with the peptide macroinitiator in a very controlled way (M_n of *n*-butyl acrylate 8,000–38,000 g/mol). After successful synthesis of the polymer block, a pH-controlled rearrangement in the peptide can be triggered and restores the aggregation tendency of the peptide (see Fig. 13). The peptide–polymer-conjugates form a fibrillar microstructure with a left-handed superhelical fine structure [108].

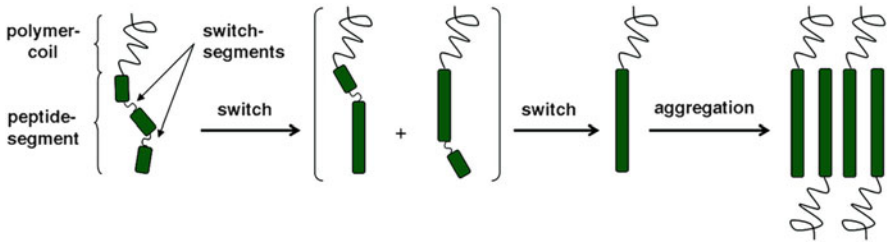


Fig. 13 pH-controlled switch of a polymer–peptide conjugate with two switch defects in the peptide segment: two-step process from the fully disturbed peptide segment (*left*) via two intermediate structures (*middle*) to the undisturbed aggregator (*right*) after RAFT polymerization results in a fibrillar microstructure. Reprinted with permission from [108]. Copyright (2007) American Chemical Society

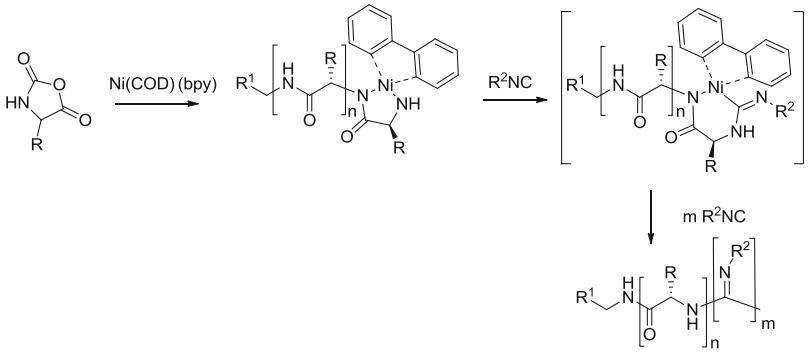


Fig. 14 Synthesis of PBLG-*b*-poly(methylbenzyl isocyanide) (PMBI) by a combination of NCA ring-opening and isocyanide polymerization [111]

Due to the sensitivity of the dithioesters to nucleophilic attack, no bifunctional initiators have yet been designed for combination of an NCA and a subsequent RAFT reaction. However, reversal of the polymerization sequence, i.e., RAFT polymerization employing a Boc-protected amino group-containing RAFT reagent and subsequent deprotection and amine-initiated NCA polymerization is possible [110].

2.4.4 Polypeptide Macroinitiators for Other Polymerization Mechanisms

Kros et al. reported a polymer–peptide conjugate prepared via nickel-mediated NCA polymerization and a subsequent polymerization of an isocyanide, again using the nickel complex as initiator [111]. The active catalyst is attacked by the more electrophilic isocyanide and the coordinated amine reacts with the isocyanide to yield a carbene-like initiator for the isocyanide polymerization (see Fig. 14). The product can be purified from free residual homopolymers by selective solvent

extraction. The block copolymers form hollow capsules when a solution in an organic solvent is dried [111].

2.5 Conjugation of Preformed Polypeptides and Polymers

Proteins and peptide can be conjugated with end-group reactive polymers. There are several methods and reactive groups suitable for this purpose, as pointed out in recent reviews [92, 94, 112]. Therefore, we will focus here on systems in which homopolypeptides prepared by NCA polymerization are conjugated with polymers.

2.5.1 Click Chemistry

Huisgen's 1,3 dipolar cycloaddition (click chemistry) is a particular useful method for combining two preformed and end-functionalized polymer blocks [113], because it combines a fast and quantitative reaction with mild conditions and tolerance of functional groups [114]. Agut et al. reported the preparation of rod–coil block copolymers with a rigid PBLG block and a flexible poly[(2-dimethylamino) ethyl methacrylate] (PDMAEMA) block [115]. The PBLG block was synthesized by NCA polymerization initiated with propargylamine (Fig. 15a) or 1-azido-3-aminopropane (Fig. 15b) to give azide- or alkyne-terminated PBLG, respectively. The flexible PDMAEMA block was synthesized by ATRP using correspondingly functionalized initiators (see Fig. 15). The four buildings blocks were synthesized with adjustable molecular weight. Subsequently, the blocks were reacted in DMF at room temperature using CuBr as catalyst, with a slight excess of the PDMAEMA to drive the coupling to completion. After removal of the excess, pure diblock copolymers were obtained [115].

The PBLG block can be converted into an hydrophilic PGA block by alkaline hydrolysis, resulting in double hydrophilic block copolymers [116]. Close to the isoelectric point, polymersomes are formed by electrostatic interactions developing between the two charged blocks and driving the formation of the hydrophobic membrane of the polymersomes, with the latter being stabilized in water by uncompensated charges. Under basic conditions, PDMAEMA shows lower critical solution temperature (LCST) behavior. Thus at pH = 11 and temperatures below the LCST (40°C), the polymer chains are molecularly dispersed. Above the LCST, the micelles or polymersomes are formed depending on the length of the PGA [116].

The PBLG-*b*-PDMAEMA copolymers also have been used to prepare micelles containing ultrasmall supra-paramagnetic iron oxide nanoparticles. The micelles are formed by nanoparticle-induced transition from bilayers to micelles [117]. This approach - the synthesis of preformed polymer blocks and their combination by click chemistry - can also be transferred to two polypeptide blocks. PBLG and poly (trifluoracetyl-L-lysine) (PTFALL) both can be synthesized by NCA polymerization with the aforementioned propargylamine or 1-azido-3-aminopropane to give azide- or alkyne-terminated polypeptide blocks, which can subsequently be combined

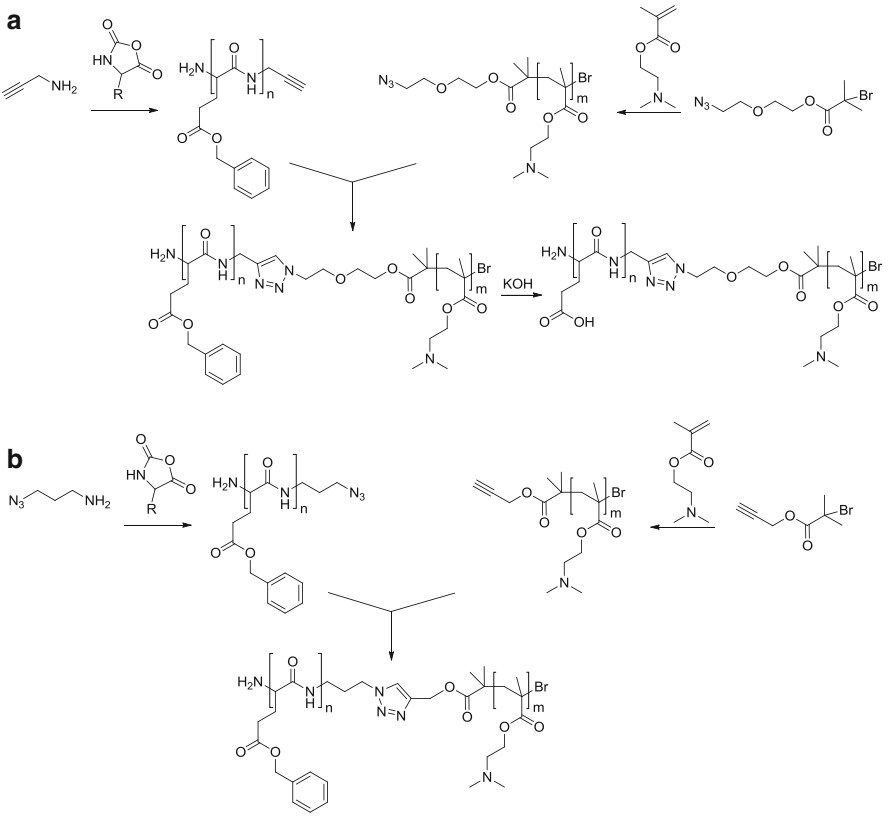


Fig. 15 Synthesis of PBLG-*b*-PDMAEMA by combination of NCA ring-opening and ATR polymerization with (**a**) azide- or (**b**) alkyne-functionalized initiators and subsequent 1,3 dipolar cycloaddition reaction [115]

to the corresponding block copolymers [118]. In a similar approach, PLL was combined with poly[*N*-(*N*′,*N*′-diisopropyl-aminoethyl)aspartamide] in a block copolymer, which is suggested as a potential pH-responsive gene delivery system [119]. Furthermore, it is possible to prepare glycoprotein analogs by coupling the PBLG block with a dextran block. The dextran-*b*-PBLG forms vesicle-like structures in water (see Fig. 16) [120].

An AB_2 Y-shaped polypeptide copolymer was synthesized by click chemistry. *N*-aminoethyl-3,5-(bisprogargyloxy)benzamide was used as initiator for *N*-benzyloxycarbonyl-L-lysine (ZLL)–NCA and 3-azido propylamine for BLG–NCA [121]. After deprotection, the block copolymer forms micelles in water with a PLL core at pH = 12 and with a PGA core at pH = 2.

Zhou et al. prepared a rod–rod block copolymer by click chemistry. Again, 3-azidopropylamine was used as initiator to prepare azido end-functionalized PBLG (Fig. 17). Using an alkyne-functionalized ATRP initiator, an acrylate with a bulky mesogenic side was polymerized. The block copolymers show interesting solid state properties and exhibit liquid crystalline behavior above 135°C [122].

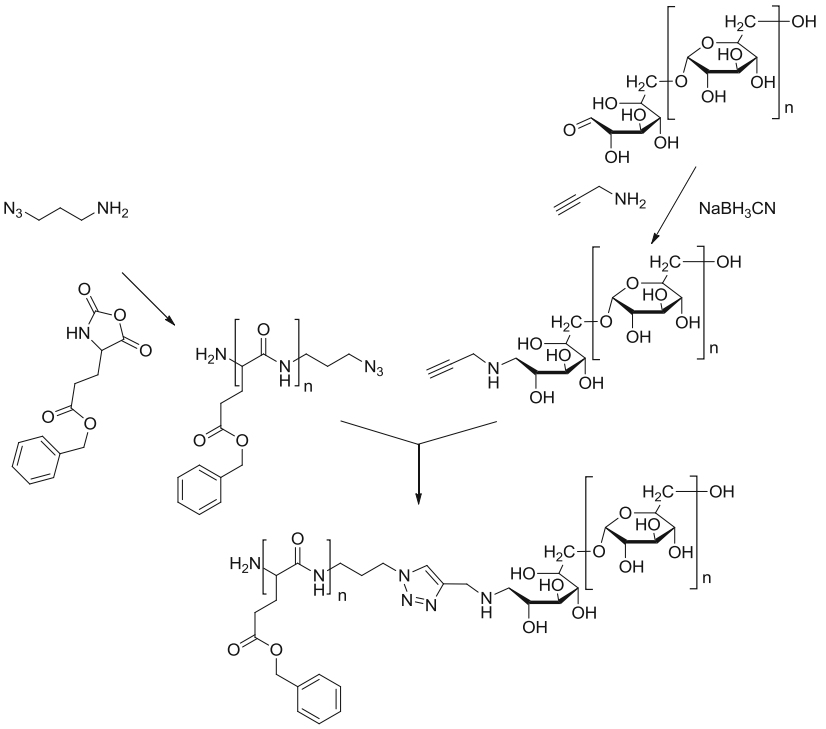


Fig. 16 Synthesis of dextran-*b*-PBLG by a combination of NCA ring-opening polymerization with an azide initiator and end-group functionalization of dextran with an alkyne group and subsequent 1,3 dipolar cycloaddition reaction [120]

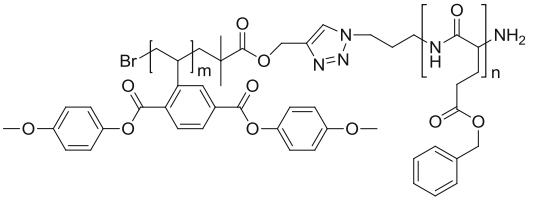


Fig. 17 Rod–rod block copolymer PMPCS-*b*-PBLG prepared by amine-initiated NCA polymerization and ATRP of the second block and subsequent 1,3 cycloaddition [122]

3 Comb-Shaped Polymers with Peptide Blocks

NCA polymerization allows also the preparation of comb-shaped polymers. Lu et al. reported the one-pot synthesis of comb-shaped polymers via ring-opening metathesis polymerization and NCA polymerization (Fig. 18). The authors used a norbornene derivative with a trimethylsilyl-protected amine to prepare a polymer with pendant trimethylsilyl amine groups, which can be used as initiators for the NCA polymerization (see. Fig. 1b) [17].

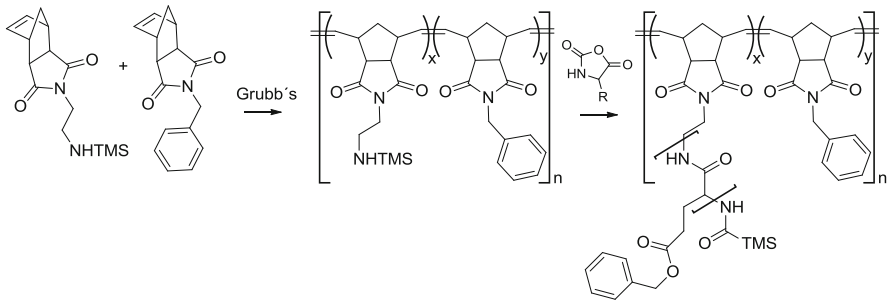


Fig. 18 Polymer brush with a polynorbonene backbone and polypeptide side chains [17]

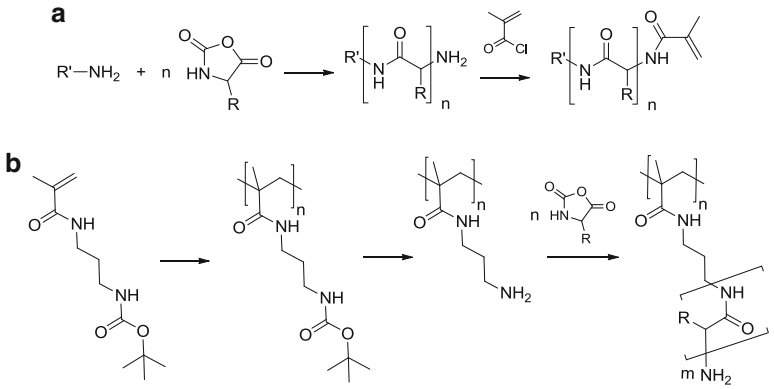


Fig. 19 (**a**) Synthesis of polypeptide macromonomers for grafting through and (**b**) grafting from for the synthesis of polymer brushes with polypeptide side chains [125]

Using 4-vinylbenzylamine hydrochloride as initiator for the NCA polymerization of ZLL–NCA, macromonomers were obtained, which could be copolymerized in a free-radical polymerization with glycidyl methacrylate [123] or *N*-isopropylacrylamide [124]. After deprotection of the PLL, the graft polymers show temperature- and pH-responsiveness and form micellar structures at pH 12 [124].

Schmidt and coworkers have compared a "grafting through" and a "grafting from" approach to prepare polymer brushes with PZLL and PBLG side chains. The macromonomers were prepared by reacting the N-terminus of PZLL and PGLB with methacryloyl chloride (see Fig. 19a) and polymerized by free-radical polymerization. For the grafting from approach, a poly[(2-aminoethyl)methacrylamide] with primary amino groups was synthesized and used as initiator for a NCA polymerization (see Fig. 19b) [125].

Although the degree of polymerization was very low for the polymerization of the macromonomers (grafting through), the grafting from was successful. Analysis of the resulting polymer indicated that every second amino group initiated a peptide

chain with an average length of approximately 10 units. The deprotected polymer with PLL side chains were used to prepare complexes with the surfactant sodium dodecylsulfate (SDS) [126]. The β-sheet formation on the level of a few nanometers, induced by the complexation of the PLL side chains by SDS, forces the achiral main chain into a helical conformation. The helices have a length of several tenths of a nanometer and can be observed by AFM [126]. The self-organization over several length scales for polypeptide–surfactant complexes was also reported by Hammond et al. [127]. They prepared PEG-*b*-PGA block copolymers and complexed them with octyl-, dodecyl-, or octadecylamine. The complexes show hierarchical nanostructures in the solid state. Hanski et al. complexed PBLG-*b*-PLL copolymers with linear SDS or branched dodecyl benzene sulfonic acid [128]. A structural hierarchy was observed as a consequence of an interplay between diblock copolypeptide self-assembly at the tens of nanometer length scale, polyelectrolyte/surfactant self-assembly (which controls the PLL secondary structure at an order of magnitude smaller length scale), and packing of rod-like PBLG helices.

4 Star-Shaped Polymers with Peptide Blocks

The synthesis of star-shaped polymers can be carried out by either a "core first" or an "arm first" approach. Both methods have been described for star-shaped polymers with peptide blocks. Brulc et al. have prepared a four-arm star by amine-initiated polymerization employing a core with four amine groups [129]. Qiu et al. have used a polyamidoamine 0-generation dendrimer with four amino groups as initiators for a NCA polymerization and subsequently transformed the N-terminus into an ATRP initiator [98] (see Sect. 2.2). Thus, a star with four block copolymer arms was obtained. Abraham et al. reversed the sequence and built a star block copolymer with the flexible PS block at the core and a PBLG block outwards. The PS block was prepared by ATRP using an initiator with three sites. The bromine end groups were then transferred into amine groups and subsequently reacted to give nickel amido-amidate groups, which can be used as initiators for a NCA polymerization [130] (see Fig. 20).

A PEG–PEI–PBLG hyperbranched block copolymer was prepared by amine-initiated NCA polymerization from the primary amine groups of poly(ethylene imine) (PEI), which had been coupled to a PEG chain via a diisocyanate. The hyperbranched block copolymers form micelles in aqueous solution with a large hydrophobic core and a cationic corona further stabilized by the PEG chains (see Fig. 21). The micelles can form complexes with DNA via electrostatic interaction, and enzymatic degradation of the micelles was shown in vitro. The copolymers therefore might be useful as new vectors for gene delivery [38].

An arm-first method for the preparation of star-shaped block copolymers and peptide-conjugated polymer nanoparticles was reported by the Heise group. They used nitroxide-functionalized amines for NCA polymerization. The nitroxide

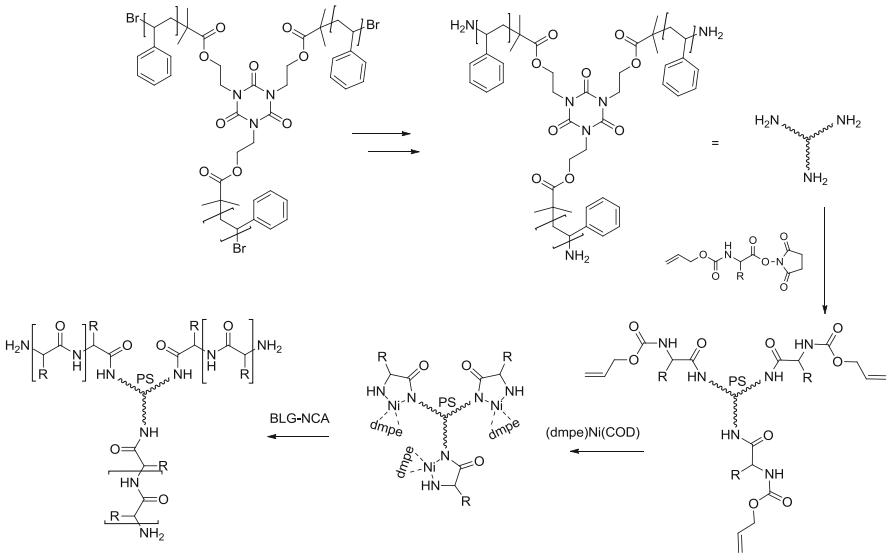


Fig. 20 Synthesis of star-shaped block copolymers by combination of ATRP, tranformation of the end group, and transition metal-catalyzed NCA polymerization [130]

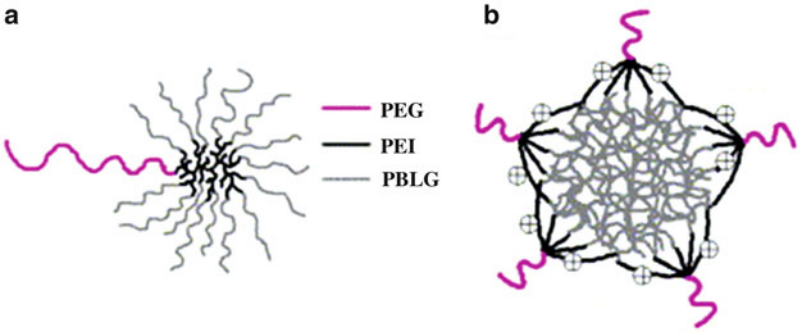


Fig. 21 (**a**) PEG–PEI–PBLG hyperbranched block copolymer and (**b**) cationic micelle. Symbol "*Oplus*" Represents protonated PEI in aqueous condition. Reprinted with permission from [38]. Copyright (2005) Elsevier

function was subsequently used for a controlled radical polymerization of styrene to build a vinyl polymer block. Finally, using the still-present nitroxide end groups, nanoparticles were produced by copolymerization of styrene with DVB [81] (see Fig. 8, p. 13). In another approach to preparation of nanoparticles with polypeptide arms, amphiphilic PS-*b*-PGA block copolymers were used with mixture of styrene and DVB in an aqueous solution. By free-radical polymerization, crosslinked particles were obtained in which the diblock copolymer was encapsulated [131].

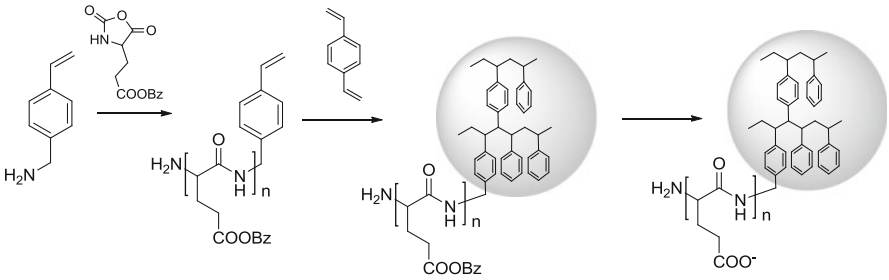


Fig. 22 Synthesis of peptide star-shaped polymers by the macromonomer approach: Stages are: synthesis of PBLG macromonomer with styrene end groups; radical polymerization (SFRP or RAFT) in the presence of DVB by crosslinking of block copolymers; and deprotection of PBLG shell [132]

An alternative approach to synthesis of star-shaped polymers or nanoparticles is the use of macromonomers. The Heise group prepared PBLG macromonomers with a styrene endgroup by NCA polymerization initiated with 4-vinylbenzylamine. The macromonomers were then copolymerized with divinylbenzene by free-radical or RAFT polymerization (see Fig. 22). Finally, the peptide block was deprotected to give PGA blocks and resulted in pH-responsive water-soluble nanoparticles [132].

A very high level of complexity and versatility in molecular structure has been reached by combination of living anionic polymerization with subsequent amine-initiated NCA polymerization under high vacuum conditions [133]. By sophisticated methods, living anionic chain ends were combined and transferred into macroinitiators. In this way, e.g., a linear pentablock copolymer PZLL–PBLG–PS–PBLG–PZLL but also PS–PI–PBLG miktoarm stars were synthesized (PI, polyisoprene). The solid state structure of the latter copolymer was investigated in detail. The α-helical PBLG forms domains with a hexagonal packing, which are arranged around PI cylinders and separated from them by a mixed PS/PI domain [134]. The dependence of the solid state structure on the dimensions and the chemical structure of the of A_2B and A_2B_2 polymer–peptide miktoarm stars (with A being PS and B being PZLL, PLL, or PLL/surfactant complexes with SDS) was reported by Junnila et al. [135]. The side chains of the peptide were observed to have a large effect on the solubility, polypeptide conformation, and self-assembly.

5 Conclusion and Outlook

Interesting new block copolymers are accessible through the combination of peptides prepared by solid phase synthesis or polypeptides prepared by polymerization of the corresponding *N*-carboxyanhydride with other polymers. The different methods for NCA synthesis and their combination with methods for preparing mostly flexible coil polymers have been reviewed. Among the numerous synthetic

methods for the conjugation, the combination of NCA and solid phase polymerization for the peptide block with controlled radical polymerization techniques have been highlighted. In this case, one of the blocks is used as macroinitiator for the other block. The advantage of the methods presented here is that both blocks can be prepared with excellent control over molecular weight and molecular weight distribution. In addition to the conjugation by subsequent polymerizations, the conjugation of polypeptides with preformed polymers, in particular by Huisgen's 1,3 dipolar cycloaddition (click chemistry), have been presented. The versatility of the synthetic method has been further emphasized by reporting some examples of the preparation of comb- and star-shaped polymers. The synthetic methods presented give access to linear, star-, and comb-shaped block copolymers, as well as peptide-conjugated nanoparticles. Due to the specific aggregation behavior of the polypeptide blocks, the polypeptide–polymer conjugates show a plethora of self-assembled structures in the solid state and in solution. Some of the aggregates formed in aqueous solution, like micelles or polymersomes, might find application in drug delivery systems. Recent developments in the synthesis of polypeptide–polymer conjugates offer a tool box that will allow tailoring of conjugates with respect to properties, structures, and applications.

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Chemical Strategies for the Synthesis of Protein–Polymer Conjugates

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Abstract Protein-polymer conjugates have achieved tremendous attention in the last few years. The synergistic combination of properties has led to certain advantages in bio-applications. Over the past few years, numerous chemical strategies have been developed to conjugate different synthetic polymers onto proteins, most of which can be summarized within the scope of click-chemistry. Here we highlight conjugation strategies based on available functional groups present on the synthetic polymer and existing groups of proteins from the natural pool. In particular, the chapter organizes the various possible reactions by classes of functional groups present on protein surfaces, deriving from selected amino acid residues.

Keywords Biomaterials · Click chemistry · Peptides · Polymer conjugate · Protein modification · Proteins

Contents

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1 Introduction

Protein–polymer conjugates are biohybrid materials comprising natural bioorganic polypeptides or proteins and synthetic polymer segments, which can combine or benefit from the advantages of both components and avoid the disadvantages of each separate component [1]. From the point of view of a synthetic polymer chemist, a protein is basically a polymer with a certain chain length, mostly monodisperse, and a definite hierarchical structure. In contrast, the synthetic polymer possesses a molecular weight distribution which depends on the polymerization technique used. Likewise, control of the regional structure is still very limited, even though recent developments on structural control show very promising results [2–4]. For example, this control affects the co-monomer sequence in copolymerization processes. Proteins, of course, feature a unique biorecognition and binding depending on their particular function, while the synthetic counterparts, i.e., synthetic polymers, mostly lack this ability. On one hand, synthetic polymers can be biocompatible, non-toxic, and non-immunogenic, however, their biodegradation is often a problem. On the other hand, their chemical and thermal stability is higher.

The conjugation of proteins to polymers changes the properties of each unit and opens up the avenue to several new applications which the single parts were not able to achieve. This chapter summarizes briefly the main fields of application and gives a short overview of suitable polymers that add a special value when conjugated to a protein. The conjugation chemistry towards protein–polymer conjugates forms the main part of this chapter which aims to highlight conjugation strategies based on available functional groups present on the synthetic polymer and on existing groups of proteins from the natural pool.

2 Applications

Polymer-protein conjugates are suitable for several applications. On one hand, the protein is equipped with new features enabling applications the unmodified protein would not be able to achieve, for example, those requiring a higher stability against enzymatic degradation. On the other hand, a synthetic polymer can be equipped with a unique three-dimensional structure to allow a higher hierarchical order or a biorecognizing structure by covalent conjugation with a protein. Most applications

of polymer-protein conjugates are found in the area of polymeric therapeutics [5, 6], as highlighted below.

2.1 Polymeric Therapeutics

A disease may occur through a mutation or abnormality during the transcription, translation, or post-translational process. A change in the concentration of a protein can also lead, for example, to a malfunctioning metabolism, a signal transduction pathway, or an immune response [7]. Considered from this perspective, proteins provide an excellent opportunity to alleviate or treat diseases. The nature of a protein itself makes it suitable for this application. Due to the complexity of interactions from a protein to its target structure, proteins normally cannot be replaced by small and easily accessible molecules. Using natural proteins, for example, for replacing damaged or absent proteins, are less likely to initiate an immune response. Likewise, through their biological function they are interesting components for regulators or inhibitors of biological processes. Compared to these advantages, several problems generally avoid the direct usage of pure proteins as specific therapeutic agents. The critical points are a low stability in vivo, a short half-life time, and, thus, a rapid clearance from the body. This process is an interaction of several events like proteolysis by enzymes, clearance mechanisms, or protein modifying enzymes, or accumulation or shielding by other proteins [8]. Another challenge is that the immune system may activate a response to a foreign imported protein and negotiate a useful treatment or cause a harmful reaction [9]. This problem is more relevant for proteins of non-human origin or for recombinant proteins [10]. However, a therapeutic effect often only occurs by maintaining a certain concentration of the agent over a period of time [11]. Thus, strategies to prolong the half-life time of therapeutic protein have to be achieved. These strategies include techniques to avoid or to reduce renal clearance, to increase receptor mediated recycling, or to decrease the stability of the interaction of protein-receptor binding during endocytosis [12]. Non-covalent approaches focus on altering the amino-acid sequence or encapsulating proteins into vehicles. Receptor mediated recycling requires linkage to certain other proteins, whereas other concepts use covalent modification of the protein by low molecular weight compounds or polymers. This chapter will focus particularly on the last one. Protein–polymer conjugates implicate the following changes compared to an unmodified protein. The hydrodynamic volume is increased and thus the renal excretion rate decreased if the size of the conjugate is bigger than the glomerular filtration barrier [13]. Additionally, the polymer chains can shield the protein from enzymatic degradation, receptor recognition, or antibodies, which again increases the serum half-life time. Polyethylene glycol is the most used polymer in drug discovery to overcome the above-mentioned problems [14, 15]. Furthermore, the attachment of polyethylene glycol chains onto the protein surface prevents or decreases the immunogenicity and aggregation. Accordingly, PEGylation of

proteins, i.e., the process of attaching a polyethylene glycol chain to a protein, has become a well-established technology for the use of proteins as drugs, especially as anti-cancer agents [16, 17]. Further, another factor of polymeric therapeutics has become very beneficial and motivates the interest in this research field. Macromolecules accumulate in tumor tissues relative to healthy tissue through enhanced vascular permeability. This concept is called the enhanced permeability and retention effect (EPR effect) [18–20]. This effect was initially found with a protein–polymer conjugate called SMANCS of styrene-maleic acid copolymer and neocarzinostatin that features anti-tumoral activity [21]. In addition to the clearance advantages, the EPR-effect pushes the effort to conjugate therapeutic useful proteins to biocompatible polymers.

Examples for successful protein–polymer conjugates that are in clinical use are the above-mentioned SMANCS for hepatocellular carcinoma [22], Oncaspar, a PEG-enzyme conjugate for lymphoblastic leukemia [23], and PEG cytokines like Pegasys [24] and PEG-Intron [25] as antiviral agents against hepatitis [15, 26–28].

In spite of all the benefits from both polymeric parts, a wrong linkage can reduce or inactivate the protein bioactivity, especially by shielding binding pockets or catalytic centers or using necessary amino acid residues for the linkage. The advantage having a monodisperse protein can also be negated by random connection of polymer chains. Consequently, the right linkage strategy is still a challenge and depends strongly on suitable polymers, which is discussed in the following section.

2.2 Smart Polymer Conjugates

The conjugation of proteins to stimuli responsive polymers are used in the field of protein isolation and separation [29, 30]. The ability of some polymers to undergo a reversible change in response to an external physical, chemical, or biochemical stimulus gave them the name smart polymers [31]. Different stimuli have been utilized, such as temperature, light, ionic strength, or electric field for physical factors, or pH and specific ions for chemical triggers or metabolites for biochemical reactions [32, 33].

Responsive polymer-protein conjugates can be used to influence the accessibility of the active site of an enzyme or of the recognition site of a receptor [34–36]. The strategy for controlling the protein activity is based on a reversibly mechanism to block the active site. Upon stimulation, the attached polymer will collapse and hide the active site. An example for a temperature controlled mechanism is poly(*N*,*N*-diethylacrylamide) (PDEAM), which is attached next to the binding site [37]. Below the critical solution temperature of the conjugated polymer, the polymer chain exhibits an extended state and inhibits a binding of target molecules. Above the critical temperature, the polymer is in its shrunken state and unblocks the binding site. An example for light responsive conjugates is a copolymer consisting of *N*,*N*-dimethyl acrylamide and an azobenzene containing acrylate as monomers

[38]. Irradiation with ultraviolet light switched the enzyme activity off. Under visual light the activity is regained.

Another approach consists in using smart polymers for a triggered protein precipitation, which can be used for purification of proteins. For example, the enzyme trypsin was coupled with poly(*N*-isopropylacrylamide) (PNIPAM). After successful conjugation, the protein could be precipitated by heating above the lower critical solution temperature (LCST) of the polymer [39, 40].

2.3 Giant Amphiphiles

Classic amphiphiles or surfactants consist of a hydrophobic and a hydrophilic group. Due to their amphiphilic nature, they self-assemble in aqueous solution to form ordered aggregates depending on their shape [41]. By increasing the size of the amphiphiles from small molecules to macromolecules one obtains block copolymers as supersurfactants. Hence, the combination of proteins as head groups and polymers as tails also leads to the formation of giant amphiphiles. The aggregation behavior is similar compared with their low molecular weight equivalents. However, the giant aggregates usually possess a higher stability, slower exchange dynamics and a lower critical micellar concentration [29, 41, 42]. As an example, an amphiphile consisting of poly(styrene) as the apolar tail and the enzyme horseradish peroxidase as the polar head group is chosen [43]. The hydrophobic tail was connected with the ligand of the apoprotein. The hybrid was prepared by adding the polymer dissolved in an organic solvent to an aqueous solution containing the apoenzyme. These systems have been further improved by using responsive polymers to form giant amphiphiles that lead to stimuli responsive applications. They are considered as interesting candidates for triggered drug release [44].

3 Suitable Polymers

Applicable polymers for therapeutic applications should be water-soluble, non-toxic, and non-immunogenic [45]. They should neither accumulate during a therapy nor remain in the body. Thus, an elimination strategy or possibility for degradation should be available [46]. The body residence time of the conjugate has to be chosen accordingly so that the polymer prolongs the life time and thus allows the distribution through the body to accumulate in the desired tissue [13]. The polymer should be obtainable with a low polydispersity to avoid a broad product mixture. Ideally the polymer should feature only one reactive group to obtain distinct conjugates without cross-linking. Normally a polymer cannot satisfy all requirements and

compromises have to be made. This clearly motivates further intensive research in this area.

The most commonly used polymer is poly(ethylene glycol) (PEG) because it has been approved for human use by FDA and thus finds application as a pharmaceutical excipient resulting from its non-toxic properties. Additionally, its high water solubility and flexibility allow the protein to create a large hydrodynamic radius to enhance the EPR effect and half life [47]. Further, PEG offers only two possibilities for the conjugation: the two chain ends. In the case of the diol, the polymer can react with two groups or, in the case of the methoxy form, only the single hydroxyl group can react. Logically, a broad scope of end group functionalization for PEG has meanwhile been established.

Other suitable polymers are poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) and HPMA copolymers, poly(vinylpyrrolidone) (PVP), poly(ethyleneimine) (PEI), poly(acroloylmorpholine) (PAcM), divinylethermaleic anhydride/acid copolymer (DIVEMA), poly(styrene-*co*-maleic acid/anhydride) (SMA), and poly(vinyl alcohol) (PVA) [13]. These polymers are all of synthetic origin and are based on the polymerization of vinyl monomers, either resulting in a homopolymer or, when using different monomers, resulting in copolymers. They are usually synthesized by free radical polymerization. As the polymers obtained are not biodegradable, their molecular weight has to be lower than the clearance cut-off. Any coupling chemistry can occur via the side chain functionality or by introducing addressable reactive end groups. In contrast, PEG and PEI are synthesized by ring opening polymerization from ethylene oxide or the corresponding amine azeridine, respectively. Alternatively, PEI can also be prepared by polymerization of 2-substituted oxazolines and subsequent polymer hydrolysis.

Poly(glutamic acid), poly(L-lysine), poly(aspartamides), and poly((*N*-hydroxyethyl)-L-glutamine) (PHEG) are examples of poly(amino acids) [13]. An analog polymer is poly(malic acid) that exhibits an ester linkage instead of a polyamide backbone. This is biodegradable in analogy to natural polypeptides. Other natural polymers are normally polysaccharides like dextran or chitosan.

Stimuli-responsive polymers should respond to an external trigger, as mentioned above. Temperature-responsive polymers exhibit a volume phase transition at a certain temperature and undergo a change in solvation state [34]. Certain polymers feature a LCST and become insoluble upon heating, while other polymers may exhibit an upper critical solution temperature (UCST) and become soluble upon heating. Typical temperature-sensitive polymers featuring a LCST are PNIPAM, PDEAM, poly(methylvinylether) (PMVE), and poly(*N*-vinylcaprolactam) (PNVCl) [48–50]. Positive temperature-sensitive polymer systems with a UCST are poly (acrylamide-*co*-acrylic acid) and PEG-*b*-poly(propylene glycol). For completeness, there are also systems with both transition states, so-called shizophrenic polymers [51–53]. pH-sensitive polymers are based on chargeable groups. Anionic polymers often consist of poly(acrylic acid) and accordingly poly(methacrylic acid), while positive charged polymers contain amino groups like poly(ethylene imine) and poly (L-lysine). There are also a few polymeric systems that react upon electric or magnetic stimulus [30]. Apart from the poly(glycol) system, the polymers are of

vinyl origin. Thus, in order for the conjugation to proteins to occur preferentially at the ends of the polymer, certain synthetic criteria have to be met and are discussed in the following sections.

4 Conjugation Strategies

4.1 Natural Pool on Functionalities

If one looks at possible conjugation chemistries, one can choose to tackle this topic from the attachment point of view of synthetic polymers or from the side of the proteins. The chemical groups on proteins available for conjugation reactions are limited and mostly dictated, unless protein engineering methods for the synthesis of artificial protein are employed [54, 55]. From the point of view of polymers, nearly every possible chemical group can be obtained. For this reason, we have structured the main part of this section by means of functional groups available on the single amino acids.

It is essential to have sufficient knowledge of the protein structure to conjugate a synthetic polymer selectively with a certain feature onto the protein surface. Thus, not only the primary sequence matters, but also the proteins three-dimensional structure, i.e., its tertiary structure or, if its exhibits more subunits, its quaternary structure, is important. The desired target amino acid for the conjugation should be freely addressable on the surface and not be hidden in the center. Further, the functionalization must not affect the three-dimensional structure of the protein to maintain its native state and binding pockets or recognition areas. Such changes occur, for example, by alteration of the protein's overall charge or electrostatic and hydrophobic interactions. An exception is the directed deactivation of a function of a protein. Another major challenge is the introduction of polymer chains in a defined quantity. A random attachment of polymer chains usually leads to an undefined product and a main advantage, the monodispersity of the protein, is lost. Especially large proteins present multiple copies of the target amino acid, which can often be as high as 20 amino acids. Thus, the strategy depends on the selected protein and the available information about it. If the information mentioned is not available, the knowledge about the natural amino acid abundance, their average distribution within the three-dimensional structure, and their average appearance on the surface can help to obtain a well-defined protein–polymer conjugate [56]. Furthermore, this illustrates, which amino acids are of particular interest. Noteworthy, the nonpolar amino acids are unimportant for conjugation chemistry. For these amino acids, almost no specific and selective reaction is available to obtain a covalent protein–polymer conjugate provided that the protein maintains its native state. Apart from the five aliphatic nonpolar amino acids,

Table 1 Amino acid composition [61] and their average surface accessibility [62]

Amino acid	Location[a]	Functionality[b]	Natural abundance	ASA[b]
Cysteine	C	Thiol	1.36	0.268
Isoleucine	C	Aliphatic	5.97	0.273
Tryptophan	C	Indole	1.08	0.279
Phenylalanine	C	Benzyl	3.86	0.290
Valine	C	Aliphatic	6.87	0.306
Tyrosine	C	Phenol	2.92	0.319
Leucine	C	Aliphatic	9.66	0.321
Methionine	C	Thioether	2.42	0.364
Alanine	C	Aliphatic	8.26	0.405
Histidine	M	Imidazole	2.27	0.425
Threonine	M	Hydroxy	5.34	0.480
Proline	M	Aliphatic	4.69	0.502
Arginine	M	Guanidine	5.53	0.539
Asparagine	M	Carboxamide	4.06	0.568
Serine	S	Hydroxy	6.55	0.568
Glutamine	S	Carboxamide	3.93	0.573
Glutamic Acid	S	Carboxylic acid	6.75	0.586
Glycine	S	–	7.08	0.588
Lysine	S	Primary amine	5.85	0.607
Aspartic Acid	S	Carboxylic acid	5.46	0.615

[a]Location of the amino acid to their average composition in core (C), intermediate (M) and surface (S)
[b]Average surface accessibility

glycin, which exhibits with its hydrogen residue no functionality at all, the thioether containing methionine, and the aromatic phenylalanine can be excluded. Only phenylalanine has raised minor interest in aromatic chemistry, especially cross-coupling reactions [57]. However a selective coupling should be difficult because of the presence of tryptophan and histidine and is thus only practicable in oligopeptides. Serine and threonine have also less importance because of their weaker nucleophilicity compared with the amino group of lysine. Only at special positions do they offer conjugation chemistry [58]. The same is valid for the two acid amino acids. From the last ten remaining amino acids, asparagine also plays no important role in conjugation chemistry. Thus, it is mainly eight amino acids plus the N- and C-terminus that have to master the conjugation process.

For selective coupling, a less abundant amino acid gives a good first impression for controlled modification for well-defined protein–polymer conjugates [56]. Cysteine and tryptophan are less used in the polypeptide chain. Aiming for a free cysteine is a first approach for a monodisperse product.

Next, the knowledge about different propensities of the location of different amino acids in certain regions of the protein can help to achieve a selective strategy. Examples are the accumulation of certain amino acids in characteristic regions like the N- or C-terminus, catalytic sides, or binding areas [59, 60]. Every amino acid has a preferred location within the protein. Table 1 shows the percentage amino

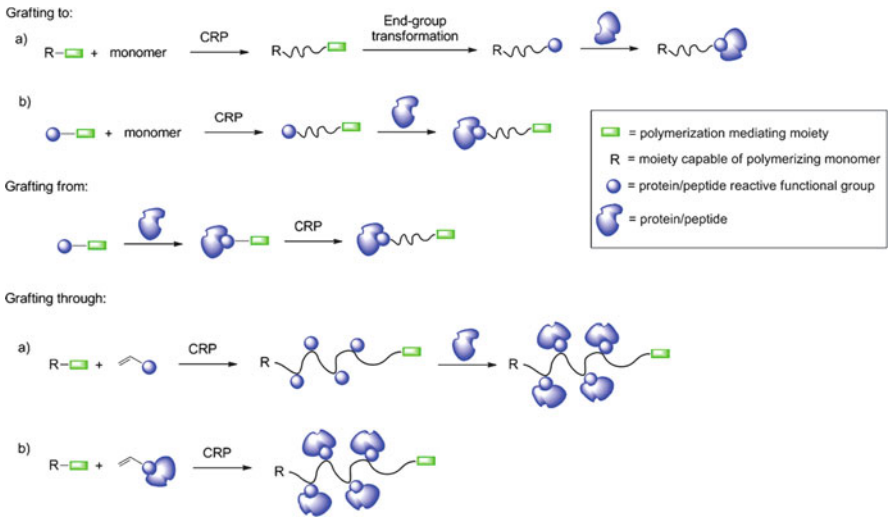


Fig. 1 Combination strategies for protein–polymer conjugates. Reprinted with permission from [65]. Copyright 2011 Royal Society of Chemistry

acid composition in proteins from the SWISS-PROT database [61]. The average surface accessibility indicates whether an amino acid is present more in the core or on the surface. Again cysteine and tryptophan are rare on the surface [62]. Nevertheless, the selective functionalization of tryptophan has been explored [63]. In contrast, lysine with an accessible amine group, which opens a broad range for organic reaction, is a common amino acid on the surface.

4.2 Methods for Protein Functionalizations

In order to synthesize protein–polymer conjugates, three main routes are available [29, 64–66] (see Fig. 1). First, the protein can be directly modified with a preformed polymer. This grafting-to approach is mediated either through covalent attachment of a reactive functional group of the polymer to a corresponding amino acid side-chain, or vice versa, or by a ligand-apoprotein interaction. In the latter case, a cofactor or ligand is covalent linked to a polymer chain. Usually that polymer exists either as an α,ω-telechelic polymer, with a reactive group, allowing conjugation with the polypeptide, on one end and the polymerization initiating group on the other [67]. Alternatively, the reactive group can be introduced by post-polymerization modification of the polymer end group [68]. An indirect protein–polymer conjugation is the grafting-from approach. In this case, a moiety that is able to mediate or initiate a polymerization process is introduced to an amino acid side chain. Consequently, a macro-initiator is formed and the polymer chain can be grown directly from the protein.

The third route follows the grafting-through approach in which various protein reactive groups are incorporated within a growing polymer chain by using monomers that can react either directly or after polymerization via the introduced reactive moieties with peptides or proteins [69]. Noteworthy, this third approach does not necessarily result only in conjugation of one protein/peptide to a polymer chain, but also in conjugation of several protein/peptide to a polymer chain.

PEG conjugates belong to the first mentioned strategy. PEG is characterized by the lack of side chains and the missing possibility to propagate the PEG chain from a macro protein initiator through an anionic polymerization mechanism. In contrast, polymers prepared by radical polymerization lead to a broad range of possible chemical modifications [70]. Established controlled radical polymerization processes are reversible addition-fragmentation chain transfer (RAFT) [71–74], atom transfer radical polymerization (ATRP) [75–77], and nitroxide mediated polymerization (NMP) [78], which all lead to polymers with a low polydispersity and predetermined molecular weight and – most importantly – allow the selective conjugation via end groups to yield well-defined protein–polymer conjugates. In addition, RAFT and ATRP open up the possibility to create grafting-from approaches either by conjugation of chain transfer agents or initiator systems to proteins/peptides, respectively. RAFT-polymerization is enabled by a chain transfer agent that is attached to an amino acid side chain functionality. ATRP takes advantage of attachment of a halogenated moiety from which the polymer chain can grow. After the polymerization process a functional end group at the omega terminus remains, which opens up further reaction possibilities. Further advantages of RAFT and ATRP are the feasibility of the polymerization in a wide range of solvents, reaction conditions, and with various suitable monomers, presuming that the conditions are compatible with the protein/peptide. It should be mentioned that other polymerization processes like cationic and anionic polymerization or polycondensation have not been successfully performed in the presence of proteins.

4.3 Protein–Polymer Conjugates Listed by Amino Acids

The following summarizes chemical ligation strategies to combine selectively proteins with polymer chains based on the ten natural amino acids that are qualified for ligation chemistry and novel non-natural moieties. For every mentioned reaction type, examples from the last few years are given.

4.3.1 Lysine and the N-Terminus of Proteins

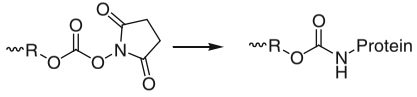
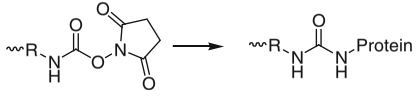
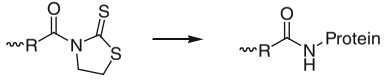
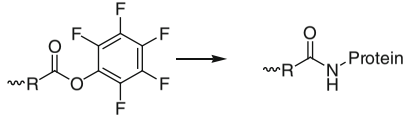
The primary amino group of the lysine side chain and the N-terminus are an attractive target for conjugation chemistry. First, lysine is a common amino acid on the surface and, thus, in mostly every protein a primary amino group should be available. Second, the nucleophilicity of the amine is higher than other nucleophilic groups of other amino acids, in particular the sulfhydryl group of cysteine, hydroxyl

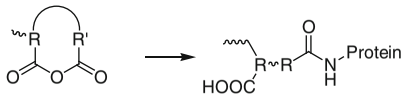
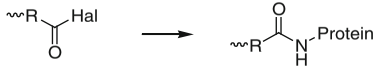
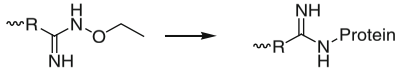
group of serine and threonine, and the imidazole moiety of histidine. Hence, a reaction with an electrophile leads preferably to a linkage with the amino group. A limitation is a possible product distribution consisting of isomers and variable amount of polymer chains per protein if multiple lysine residues are present [79]. Control over the preferred conjugation site, if the conjugation addresses the N-terminus or the ε-amino group of lysine, can be achieved by adjusting pH [80]. The pK_a value of the N-terminal amine group is about 7.6–8.0 and the pK_a of the lysine side chain about 10.0–10.2 [81]. By lowering the pH value from the traditional range for lysine conjugation of about 8.5–9.0, the reaction can be directed to the N-terminus [82]. Noteworthy, amino groups near or at a catalytic center or binding pocket can be blocked by adding a ligand or substrate during the reaction, i.e., competitive inhibition.

Two different conjugate products are possible. In one case the charge of the amino group gets lost and in the other the charge and thus the overall charge of the protein is maintained. Reactions of the first type are acylation like formation of amides and carbamides, or analogous reactions with corresponding thio derivatives. Reactions with aldehydes and ketones with following reduction, i.e., reductive amination, as well as amidination are examples of the second case.

A conventional strategy for the formation of amides from activated carboxylic acids is based on classical organic chemistry. A common method is the use of active esters, such as *N*-hydroxysuccinimide esters, the –OSu group (NHS esters **1**) which is usually prepared from the desired acid, and *N*-hydroxysuccinimide using a coupling agent like dicyclohexylcarbodiimide (DCC) or ethyl(dimethylaminopropyl) carbodiimide (EDC). Protein polymer conjugation can then easily be achieved by reaction of the active ester with an amine under ambient conditions. In addition, several active esters are meanwhile commercially available. The active ester chemistry was also used to attach an initiator for ATRP, such as 2-bromoisobutyric acid, to amino residues of proteins, thereby enabling the grafting of stimuli responsive polymers from the surface of a protein [83]. Alternatively, the opposite way, the grafting-to approach utilizes an active ester bearing ATRP initiator to polymerize the desired monomer and then conjugate the obtained polymer to the protein [84, 85]. In comparison to these examples, the NHS group can also be introduced after the polymerization by end group modification of a free acid and *N*-hydrosuccinimide with DCC [86]. In a similar way, PEG chains can also be functionalized via the NHS route. The hydroxyl end group of PEG can be converted into an acid functionality with succinic anhydride, which is then activated with *N*-hydroxysuccinimide. To overcome the lability of the ester bond, the NHS group can be introduced by spacers containing amide or ether bonds [79, 87]. Ether linked derivatives are formed by the combination of methoxy-PEG (CH_3O-PEG-OH) and an omega functionalized acid like propionic or butanoic acid [88]. Amide linked acid groups are formed by using β-alanine or norleucine. Multiple PEG chains per NHS anchor groups have been realized with spacers having more than one reactive group like lysine, which creates an unsymmetrical branch, or 1,3-diamino-2-hydroxypropane, which creates a symmetric branch [89] (Table 2).

Table 2 Coupling methods for the amino group

	Reaction type	Reaction	Product	Lit.
Acylation				
1	NHS ester		Amide	[83–89]
2	NHS carbonate		Carbamate	[90]
3	NHS carbamate		Carbamide	[91]
4	Thiazolidine-2-thione		Amide	[92]
5	Pentafluorophenyl ester		Amide	[93–95]

6	Anhydride		Amide + free acid	[21, 96–98]
7	Acid halogenide		Amide	[99, 100]
Alkylation				
8	Reductive amination		Secondary amine	[79, 82, 102–105]
9	Amidination		Amidine	[106]

If an acid functionality is not available, hydroxyl groups can be converted by *N*-hydroxysuccinimide, too. Using an oligo ethylene glycol with two accessible hydroxyl end groups, one hydroxyl group can be used to attach an ATRP initiator via an acid bromide to form an ester group. The other hydroxyl group can be converted with *N*,*N*′-disuccinimidyl carbonate to a succinimidyl carbonate compound, which can be reacted with lysine residues to yield carbamate conjugates (**2**) [90]. NHS chemistry can also lead to carbamide linkages (**3**). A system less reactive towards amines results in a conjugation in which only the most nucleophilic amino residue reacts and thus a lower amount of possible isomers are obtained. Such a system is the *β*-alanine-NHCO-OSu group. The carboxylic acid group of *β*-alanine is used for conjugation with the polymer and the amino group is modified with *N*, *N*′-disuccinimidyl carbonate to yield the corresponding carbamate that can react with the most nucleophilic amino group of a protein [91]. Another active ester is based on the thiazolidine-2-thione group (**4**). The carboxylic acid group of a chain transfer agent can react with 2-mercaptothiazoline to form a thiazolidine-2-thione ester group. After RAFT polymerization the polymer can react with amino groups in a grafting-to approach [92]. Other reactive units are amongst others pentafluorophenyl active esters (**5**), benzotriazole carbonates, chlorotriazines, and *p*-nitrophenyl carbonates [79, 87, 93–95].

Another reactive form of a carboxylic acid is the anhydride (**6**). A famous example is the SMANCS conjugate. The antitumor protein neocarzinostatin is linked with its two lysine residues to a poly(styrene-*co*-maleic acid/anhydride) copolymer. The linkage occurs in the side chain by opening the five-membered ring of maleic anhydride. Per linkage one free acid is generated [21]. The copolymer poly(maleic anhydride-*alt*-methyl vinyl ether) can also be conjugated with a protein shell of a virus to immobilize the desired compound. The remaining anhydride repetition units were then used to entrap the conjugate in an amino groups containing film [96]. The conjugation proceeds in both cases in a grafting-through approach within the side chain. The reactive group is introduced through the monomer in the polymerization procedure. An end group modification is not necessary. Using synthetic polypeptides, conjugates with only one linkage per chain are generated. A fully protected polypeptide is coupled with its N-terminal amino group to methacrylic acid anhydride [97, 98]. The resulting monomer can then be copolymerized with biocompatible monomers such as HPMA. Acid halogenides are a similar reactive system (**7**). 2-Bromoisobutyryl bromide reacts in slightly basic buffer solution with lysine residues to yield an ATRP macro protein initiator. The average number of acylated residues depends on the molar ratio of the acid bromine. The protein initiator can be used for a grafting-from polymerization [99, 100].

Free acid can also be used for conjugation chemistry onto a protein's amine group. Poly(acrylic acid) has been coupled under nearly neutral conditions to lysine side chains of hemoglobin using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. A water swollen gel was formed that is slightly cross-linked because of the multiple lysine residues that can react with different polymer chains [101].

Another strategy is to maintain the positive charge of the nitrogen of the amine. The route to form secondary amines via reductive amination from aldehydes and the amine group is especially popular (**8**). In the first step an imine is formed, which has to be reduced to a secondary amine, because of the imine formation is a reversible reaction. This method was devised with PEG-aldehyde and granulocyte colony stimulating factor (G-CSF) [79, 82]. The reaction occurs under slightly acid conditions. The intermediate, the imine, will be directly reduced with sodium cyanoborohydride to result in the secondary amine. The equilibrium is shifted to the product side through the in situ reduction. The advantage of this approach is to obtain a one to one conjugate because of the higher nucleophilicity of the N-terminus and – under acid conditions – the reaction occurs selectively at this position. This conjugate leads to pegfilgrastim, a leukocyte stimulating drug. This conjugation approach can also be adapted to polymers that were prepared by a controlled radical polymerization. Starting from 2-bromoisobutyryl bromide, which is used for ATRP, the aldehyde group is introduced to the initiator system in form of an acetal protected group as 2-(2,2-dimethoxy-ethoxy)-ethanol [102]. After polymerization of the macromonomer methoxyPEG$_{(1100)}$methacrylate, the terminal aldehyde group is recovered by deprotection with trifluoroacetic acid. Again, the N-terminus is addressed under acidic conditions in a one pot reaction with sodium cyanoborohydride as reductant [103]. Recent developments use transition metal mediated catalysis instead of sodium cyanoborohydride. A water-stable iridium complex uses formate as the hydride source [104]. The advantage is a milder reduction of imine groups generated though the coupling process in particular in the presence of disulfide bridges by a longer reaction time [105].

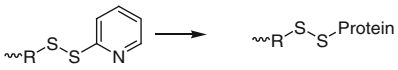
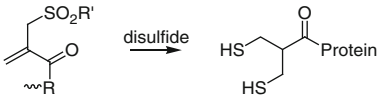
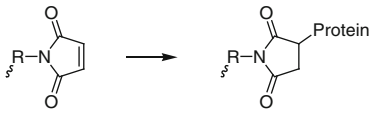
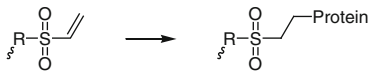
Another route is the amidination of lysine by means of imidoesters and imidothiolanes (**9**). The resulting amidine still carries a positive charge and the overall charge of the protein is recovered [106].

4.3.2 Cysteine

Next to lysine, cysteine is a frequent target in conjugation chemistry. The lower abundance – especially on the surface of a protein – and unique reaction pathways allow a selective addressing and thus result in a lower product distribution (see Sect. 4.1). Cysteine residues may be blocked as internal disulfide bridges. Utilizing dithiothreitol exposes additional free cysteine residues by cleaving the disulfide bond. This reaction may, however, sometimes lead to a loss of the native three-dimensional structure [56]. If the protein lacks a free cysteine residue at the surface, genetic engineering can introduce cysteine moieties for conjugation chemistry [107, 108]. Two main approaches can be applied in protein–polymer conjugates. On the one hand, reactions that create a disulfide bond can be used, adding the opportunity for a cleavable linkage – on the other, many conjugations are achieved by Michael addition that forms an alkylated cysteine.

A common way to introduce polymer chains to cysteine moieties is the use of orthopyridyl disulfides (PDS, **10**). The ATRP initiator 2-bromoisobutyrate can be

Table 3 Coupling methods for cysteine residues

	Reaction type	Reaction	Product	Lit.
10	Disulfide linkage		Disulfide	[109–115]
11	Michael addition of disulfide bonds		Bridged disulfide	[119, 120]
12	Michael addition with maleimides		Thioether	[121–134]
13	Michael addition with vinyl sulfones		Thioether	[135]

built up with the mentioned cysteine reactive residue orthopyridyl disulfides starting from 2,2-dithiopyridine, mercaptopropanol, and 2-bromo-2-methylpropionic acid in two steps [109, 110]. The dithio moiety can react with a free cysteine residue under disulfide formation and in a following grafting-from approach the bromoisobutyrate group initiates the polymerization of, e.g., 2-hydroxyethyl methacrylate [110] or other monomers yielding polymers [111]. Genetically engineered lysozyme with a novel thiol group makes the orthopyridyl disulfide group accessible to the protein [111]. In an analogous way, chain transfer agents can also be attached to this moiety [112]. As an example a water soluble trithiocarbonate was chosen. After coupling to a free cysteine residue and releasing the 2-pyridinethione leaving group, the macro chain transfer agent is able to act in RAFT polymerization [113]. A wide variety of acrylate monomers can be used in this grafting-from approach [114]. Using symmetrical trithiocarbonate chain transfer agents, the possibility to create heterotelechelic protein–polymer conjugates has been explored. Instead of using a non-functional benzyl group for one side of the trithiocarbonate, a second orthopyridyl disulfide group was chosen [115]. First, one PDS group was attached to the protein, while the second one was inaccessible for protein linkage. In a grafting-from approach the protein–polymer conjugate was built with an intact PDS group. The terminal PDS group was then available for further post modifications such as the attachment of dyes. In addition, it is also possible to create chain transfer agents with the PDS group in the middle of a bifunctional chain transfer agent via a side chain. On both sides the orthopyridyl disulfide unit carries

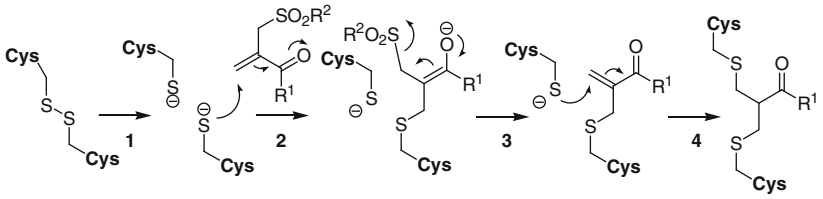


Fig. 2 Mechanism for protein–polymer conjugation through a tree carbon bridged disulfide. *1*: Reduction of the disulfide bond; *2*: addition of the first thiol to the α,β-unsaturated β′-monosulfone; *3*: elimination of the sulfonyl leaving group; *4*: addition of the second thiol

one transfer agent [92]. After polymerization the midchain-functionalized RAFT polymer was conjugated to the protein in a grafting-to approach (Table 3).

Disulfide bonds in proteins have also been utilized to conjugate polymers onto them. For example, bromomaleimides were used to modify selectively and reversibly cysteine residues [116]. Interestingly, dibromomaleimides can be used to re-bridge successfully disulfide bonds following a cleavage with appropriate reducing agents [117, 118].

An alternative interesting approach is the use of an internal disulfide bonds for conjugation chemistry without the loss of the bridge [119, 120]. In the first step the disulfide bridge is cleaved under mild reduction conditions like tris[2-carboxyethyl] phosphine or dithiothreitol. Two nearby thiols are generated. One of these attacks the bis-thiol-specific reagent in a Michael addition. This specific reagent is an α,β-unsaturated β′-monosulfone. A sulfonyl leaving group is released and the vinylogous structure is regained. A second Michael addition completes the new three carbon bridged disulfide. In summary, the alkylating agent starts a sequential addition–elimination reaction cascade (see Fig. 2).

A major route for cysteine modification is the Michael reaction with maleimides (**12**) and vinyl sulfones (**13**). Myriads of examples of different applications and approaches have been presented and only a selection will be discussed in the following to demonstrate the synthetic principle. To graft a polymer from a maleimide moiety to be used within a controlled radical polymerization, trithiocarbonates are well suited [121, 122]. The chain transfer agent consists of a maleimide group that is connected by a glycol linker to the trithiocarbonate unit as the R-group, which can be used in a RAFT polymerization after conjugation to the protein. The grafted polymer still contains the chain transfer moiety at the end group and this macro-CTA can be used for the polymerization of a second monomer resulting in block copolymers.

Grafting-to approaches can be realized in two different ways. Either the maleimide moiety is connected to the initiator system or chain transfer agent or is introduced after the polymerization by end group modification. If the maleimide is introduced before the polymerization, a protecting group for the double bond is necessary to prevent reaction during polymerization [123]. Furan is a suitable protecting group and reacts with maleic anhydride to yield the protected adduct by Diels–Alder reaction [124]. The imide structure is formed by reaction of the anhydride with an amine, generally an α-amino-ω-hydroxy compound. For

example, reaction with 2-bromo-isobutyrylbromide results in the ATRP initiator system. After polymerization furan is removed by a retro Diels–Alder reaction by heating. The free Michael system can then be attacked by a target cysteine residue to obtain the desired protein–polymer conjugate [125]. Complex polymer designs like multiple block copolymers or several post-polymerization steps are accessible following this strategy [126]. RAFT agents are also available through this process. Instead of using an ATRP bromine containing compound, installation of a trithiocarbonate on the protected maleimide is sufficient. For example, a DCC mediated esterification can be used to link the two functionalities [127]. The maleimide moiety can also be introduced after polymerization; however, another functionality at the end group of the polymer is required for this approach. Using 2-aminoethanethiol hydrochloride as a simple chain-transfer agent leads to an amino functionalized polymer. A maleimide with a succinimide moiety results in the polymer that can be reacted with a cysteine unit [128]. If a RAFT polymerization was chosen, the trithiocarbonate end group can be replaced with an azo compound in a radical mechanism, following a method developed by Perrier [129]. Derivatives of 4,4′-azobis(4-cyanovaleric acid) are well-suited because they result in an acid functionality that can be used for coupling with a maleimide residue. In this case the double bond of the maleimide has to be protected with furan again. Another grafting-to approach takes advantage of mPEG-maleimide, which can be coupled directly to a cysteine moiety [130]. Depending on the starting material, homofunctionalized, homodimeric, or star polymers can be synthesized. Using a symmetrical RAFT agent with a trithiocarbonate moiety on each side, telechelic polymers are accessible [131]. After end group replacement with the azo compound the resulting polymers have the maleimide group on both sides, to which two identical proteins can be attached. When an α-ω functionalized RAFT agent is used instead, two different groups are available for a post-polymerization conjugation to two different proteins [132]. Using a tetrameric CTA with four identical trithiocarbonate moieties, star polymers with at least four maleimide groups are possible. In this case four identical proteins are linked together [133]. Instead of using azoinitiator based chemistry, the trithiocarbonate structure can be cleaved by using a mixture of hexylamine and tributylphospine. A thiol terminated polymer is obtained that reacts with a bis-maleimide in excess to yield a maleimide terminated polymer [134].

A polymer made by the RAFT process with a dithioester results in a dithioester end group. This ester can also be cleaved by aminolysis, resulting in a thiol terminated polymer. In an analogous way to the bis-maleimide compound, divinyl sulfone can be used, thereby creating a vinyl sulfone end group. This group is then able to react with cysteine groups by Michael addition (**13**) [135].

If no free cysteine is available, a one-pot approach enabling the breaking of a disulfide bond and conjugation by Michael Addition may overcome this circumstance. For example, a phosphine was used to reduce the disulfide bridge and the

two free thiols were reacted readily with an acrylate terminated mPEG in a Michael reaction [136].

A completely different approach uses the thiol group of cysteine as a chain transfer agent [137]. The polymerization is photo-induced and the polymer chain grows from the cysteine residue.

4.3.3 Tyrosine

Reactions on tyrosine occur either at the oxygen atom of the phenol unit or at the aromatic ring through electrophilic aromatic substitution (EAS). Thus, the reactions can be divided into *O*-alkylated and *C*-alkylated products next to aromatic substitution products.

A π-allyl species like allylic acetates, carbonates, and carbamates are inert towards amino acid functionalities until they are activated with a palladium catalyst like palladium acetate and triphenylphosphine tris(sulfonate) as a water-soluble ligand [138, 139]. With a palladium catalyst, the phenolate oxygen of tyrosine will be alkylated. The conjugate is an allyl aryl ether (**14**) (Table 4).

Instead of using an *O*-alkylation, several approaches use an EAS reaction. A three-component Mannich-type coupling forms a *C*-alkylated product [140]. At first an imine is generated in situ from an aldehyde and an electron-rich aniline. Then the imine acts as an electrophile and gets attacked by the aromatic tyrosine residue to yield the resulting secondary amine (**15**). An alternative route is based on a diazonium coupling [141, 142]. A diazonium salt is prepared by the reaction of an aromatic amine and sodium nitrite under acidic conditions and is then reacted with the tyrosine residue to result in an azo compound (**16**). This approach can also be used to add a small molecule with a new function to the protein if a hetero bis-functional diazonium salt is utilized. In addition, tyrosine can react with highly reactive electrophiles such acyclic diazodicarboxylate to yield the corresponding triazolidine compounds (**17**) [143].

In summary, the reactions mentioned are rarely used for polymer ligation at present but rather for small molecule conjugation. The reactions take place preferentially at the aromatic ring of tyrosine instead of tryptophan and phenylalanine.

4.3.4 Glutamine

The amide structure is not accessible for organic chemical reactions. Instead, an enzymatic approach is available. Transglutaminase (TGase) catalyze the acyl transfer between the γ-carboxamide of protein-bound glutamine and a primary amine resulting in the formation of a γ-amide of glutamic acid and ammonia (**18**) [144]. Unbranched primary amines act as acyl acceptors and are usually the ε-amino group of natural lysine [145]. Transglutaminases are a large family of enzymes and they were found in numerous organisms including mammals. Certain TGase species accept a wide variety of primary amines and thereby allow the possibility to use polymers with an amine end group [146]. A microbial TGase

Table 4 Coupling methods for tyrosine residues

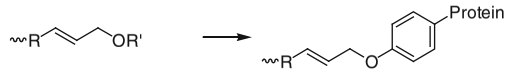
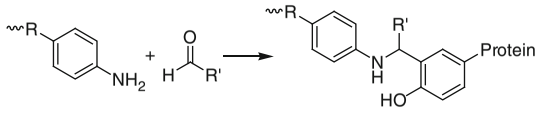
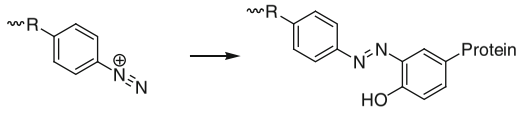
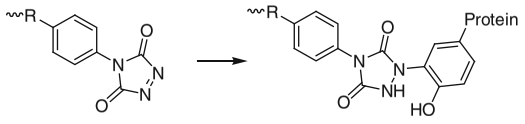
	Reaction type	Reaction	Product	Lit.
14	*O*-Alkylation		Aryl-allyl ether	[138, 139]
15	Mannich		Secondary amine	[140]
16	Azo-coupling		Azo compound	[141, 142]
17	EAS		Addition product	[143]

Table 5 Coupling method for the glutamine group

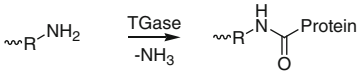
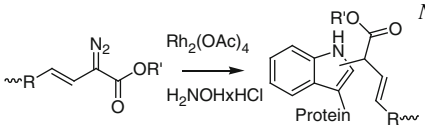
	Reaction type	Reaction	Product	Lit.
18	Enzymatic		Amide	[145, 148]

Table 6 Coupling method for the tryptophan group

	Reaction type	Reaction	Product	Lit.
19	Rhodium carbenoid reaction		*N*-/*C*-Alkylation	[105, 139]

from *Streptomyces mobaraense* works independently of a cofactor and has a higher reaction rate; thus it is well suited as a catalyst for ligation [147]. An ideal candidate as a polymer is mono amino functionalized poly(ethylene glycol) and, accordingly, several protein-PEG conjugates have been reported in the literature [145, 148]. Normally only a few glutamine residues act as substrates for TGase. In addition, the selectivity can be increased by adding co-solvents [148] (Table 5).

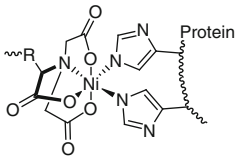
4.3.5 Tryptophan

Tryptophan offers an indole side chain that can be used for ligation chemistry. A water-compatible rhodium carbene can be added to the indole ring (**19**) [105, 139]. The reactive species is generated in situ by a conjugated diazo compound by a rhodium catalyst like rhodium(II) acetate [63, 139, 149]. The reaction takes place in the two- and three-position of indole. Thus, a mixture of *N*-alkylated and *C*-alkylated product is obtained. It is necessary to add hydroxylamine hydrochloride as an additive to bind to the distal rhodium carbenoid complex. The usage of this salt lowers the pH value below 3.5 and therefore limits the scope of this methodology. As a side reaction, the carbene inserts into the O–H bond of water (Table 6).

4.3.6 Histidine

The imidazole side group is able to form stable complexes with transition metal ions. These metals ions are generally divalent ions like Zn^{2+}, Ni^{2+}, Co^{2+}, and Cu^{2+}, analogous to natural metalloproteins. Several histidines in a repetition motif increase the affinity. Proteins without a binding motive for metal ions can be fitted with a polyhistidine-tag (His-tag) that consists of six histidines in a row. The His-tag is added by a vector technique or during the PCR reaction using primers

Table 7 Coupling method for the histidine group

	Reaction type	Complex structure	Product	Lit.
19	Coordination reaction with transition metals such as nickel		Metal complex	[152–157]

containing the motif. This approach has been adopted from protein purification using metal ion affinity chromatography [150, 151] (Table 7).

Necessarily, the polymer has to be fitted with a metal binding group. A requirement is that the chelation group in the polymer remains with some free chelating sites for binding to the protein. As a ligand, the imidazole group itself is suitable for polymer binding. It can be introduced as vinylimidazole, resulting in copolymers. A stimuli responsive polymer based on *N*-isopropylacrylamide is copolymerized with vinylimidazole [152]. The copolymer is loaded with copper(II) ions in form of copper sulfate that initialize the complex. Alternatively, the ion binding group can be introduced in the polymer by post-polymerization modification techniques. Active ester monomers like *n*-acryloxysuccinimide or pentafluorophenylacrylate have been homo- [153, 154] or copolymerized [155] and converted in a post-polymerization reaction into a chelation ligand. A suitable compound is nitrilotriacetic acid with an anchor group attached to the backbone. Nitrilotriacetic acid offers four chelating sites and leaves two sites open for a nickel(II) central atom. As an example, His-tagged silicatein had been immobilized onto a polymer coated γ-Fe_2O_3 nanoparticle [156, 157].

4.3.7 Aspartic Acid, Glutamic Acid and C-Terminus

A contrary approach to conjugation to lysine and the N-terminus would be the conversion of the carboxylic acid group with amines and coupling agents like carbodiimides and *N*-hydroxybenzotriazole. Because of the nucleophilicity of lysine, inter- and intramolecular side reactions take place. Thus a selective pathway via carbonyl chemistry is absent and other direct chemical approaches are rare. Instead, enzymatic and biosynthetic methods were developed to enable single site modification at the C-terminus. New functional groups can be added at the C-terminus using intein-mediated protein splicing. In principle, the carboxylic acid can be converted into every chemical group and some examples are summarized below. A unique thioester group can be exposed with this procedure [158]. The soft nucleophile reacts with electron-deficient azides like sulfonazides. The reaction proceeds through the formation of a thiatriazoline intermediate that decomposes yielding an amide product under elimination of nitrogen gas and sulfur. PEG sulfonazide can be synthesized from amino-PEG and 4-carboxybenzensulphonylazide

following routine coupling methods. Adding a phosphinothioester through intein-mediated protein splicing affords a Staudinger ligation [159]. Azides are the corresponding reacting agents. Also the grafting-from route is possible by adding an ATRP initiator via the intein route [160]. Thereby only a one-to-one conjugate is achieved.

4.3.8 Arginine

The difficulty for residue-selective modification of the guanidinium group in arginine is the lower reactivity compared to the ε-amino group of lysine. Thus, conventional reactions like the conversion with active esters and Michael addition cannot be realizable [161]. Instead, by taking advantage of a kinetic selectivity, a thermodynamic pathway is available. MPEG chains bearing an α-oxo-aldehyde end group react with arginine under mild conditions [161]. Adduct products formed from lysine and polymer as minor byproducts could be cleaved with hydroxylamine buffer at neutral pH value. Cysteine can also act as a nucleophile, but the formed adduct is unstable and will get cleaved. Further improvements are clearly needed, such as the elimination of possible branched structures, but it is an interesting approach to use an amino acid that could not been used for ligation reactions yet.

4.3.9 Phenylalanine

A selective addressing of the phenyl group of phenylalanine like organometallic cross coupling reactions is not possible through the coexistent occurrence of the other aromatic side chains. Pathways for introducing functional groups like iodination exist, but are only of interest for oligopeptide conjugation. Iodated side chains enable access to these groups by palladium coupling reactions [57].

4.3.10 Non-natural Amino Acids

The functional pool of organic groups can be expanded by introducing new groups with non-coded amino acids. Selectively a certain amount of ligation sites are incorporated in the polypeptide chain and, thus, they directly correlate with the desired amount of synthetic polymers per protein unit. Different techniques can be used to introduce non-natural amino acids [162]. The main approaches are translational incorporation by using analogous amino acids, new tRNA for a 21st amino acid, replacing a stop codon with a novel tRNA loaded with a new amino acid, expanding the size of codons for more different codes and non-natural base pairs [162]. Further semi-synthetic approaches are available with solid-phase peptide synthesis in combination with native chemical ligation.

The synthetic erythropoiesis protein (SEP), a permitted therapeutic agent, is an example for this technique. Two branched polymers with negative charged end

group are attached at the polypeptide chain [163]. The peptide chain was split into four segments, synthesized by solid-phase peptide synthesis, and ligated afterwards. Thus two non-natural derivatives of lysine could be integrated. The Nε-levulinyl modified lysine group bears a keto group for ligation chemistry. The keto group can react with aminooxy groups yielding the respective oximes. This route will be discussed in Sect. 4.4.

An amino acid bearing an azide group can be incorporated with para-azidophenylalanine muting a codon from an amino acid located on the surface to a stop codon and the corresponding tRNA with the novel amino acid [164]. Alkyne terminated PEG was then coupled by the [3+2] cycloaddition reaction to the protein.

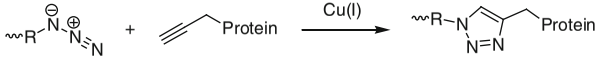
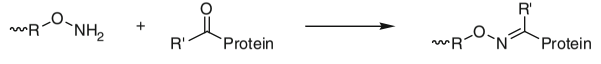
A grafting-from approach is also possible by introducing an amino acid bearing an ATRP initiator moiety [165]. In this approach the non-natural amino acid was incorporated through translational transformation. As ATRP initiator, 2-bromo-isobutyric acid was used, which was attached to the 4-aminophenylalanine.

4.4 Protein–Polymer Ligation via the Indirect Pathway

In this two-step pathway, an amino residue is first converted with a low molecular weight compound to result in a new functionality. This reaction normally uses methods from the previous sections. In a second step the actual ligation reaction is conducted, resulting in the protein–polymer conjugate. Thus, new functional groups are available via chemical synthesis. Common groups for this category are azides and alkynes for click chemistry and carbonyl groups and aminooxy for oxime ligation. The Huisgen 1,3-dipolar cycloaddition uses azides and alkynes with copper (I) as a catalyst (**20**) [166]. Using the classical condition consisting of copper(II) sulfate and sodium ascorbate may lead to degradation of the protein component [167]. This is caused by the generation of reactive oxygen species like the hydroxyl radical by oxidation of the catalytic reactive species to the copper(II) state. Further, the reducing agent may influence the bioactivity of the protein. Thus, reaction conditions have to be chosen that avoid these problems. The ligand should stabilize the oxidation state of the catalytic copper(I) state, sequester the metal ion, prevent the protein for damage, and should not constrain the reaction rate. Such ligands are tris(triazolylmethyl)amine and bathophenanthroline disulfonate. Polymers with a group for click reaction are easily obtainable for ATRP [168]. The bromide end group from the ATRP initiator can be replaced by azide with post polymerization end group modification. RAFT polymers can be fitted with an azide group using a RAFT agent that bears the azide group [169]. In this case, the polymerization temperature should be kept strictly below a certain temperature to maintain the azide group. A protein can be equipped with an alkyne group by using an *N*-alkyne functionalized maleimide, which reacts with cysteine groups; see Sect. 4.3.2.

Another common method is the oxime formation from aldehydes or ketones and aminooxy compounds (**21**). The functionalities are orthogonal to the natural amino acid residues. Thereby, the aminooxy group can be located on the polymer or protein and

Table 8 Reaction ways via the indirect route

	Reaction type	Reaction	Product	Lit.
20	1,3-Dipolar cycloaddition		Triazole	[168, 169]
21	Carbonyl reaction		Oxime	[170–172]

Functionalities could be also vice versa

grafting-to as well as grafting-from approaches are possible. The amino group of lysine can be converted with isopropylidene aminooxyacetic acid [170]. Thus, the aminooxy group is protected with acetone in the form of the corresponding isopropylidene compound. Deprotection is achieved by treatment with methoxylamine. The free aminooxy group reacts with an aldehyde-terminated PEG to yield the oxime conjugate. In the opposite way, the aminooxy group can be attached to the polymer. Equipping an ATRP initiator with a BOC protected aminooxy group leads to a polymer whose protecting group can be removed with trifluoroacetic acid, resulting in an aminooxy end functionalized polymer [171]. The protein is fitted with a keto group using the Nε-levulinyl lysine route. The lysine side chain is converted with *N*-hydroxysuccinimidyl ester levulinate to the levulinyl-modified protein. Both components react again to form an oxime linked conjugate. Another way for the oxime formation is the conversion of the N-terminal amine into an aldehyde using the enzyme pyridoxal-5-phosphate [172]. This aldehyde can then react with an aminooxy functionalized ATRP initiator. The resulting macro initiator system is able to be used in a grafting-from polymerization. This method allows formation of a one-to-one conjugate (Table 8).

5 Conclusions and Outlook

This review highlighted different synthetic routes towards protein–polymer conjugates. Even though there have been numerous chemical strategies described to conjugate different synthetic polymers onto proteins, it requires a careful selection of the right chemistry that is most suitable for a respective conjugation. As such, we have divided the various possible reactions into classes of functional groups present on protein surfaces, deriving from selected amino acid residues. It should therefore allow interested scientists to choose the right chemistry for their particular scientific problem.

Advances in this area are twofold. Suitable ligation chemistries must be compatible with both proteins and polymers. As such, scientists are encouraged to look beyond and receive inspiration from either scientific community. Development of further chemistries is continuously needed to meet the demand for the synthesis of highly defined protein–polymer conjugates. Clearly there are still limitations in the conjugation chemistry to differentiate between various accessible groups available on the surface of proteins, which are of particular importance when one-to-one conjugates are targeted. Given recent developments in the area, it can be concluded that we will surely see further new conjugation chemistries in the near future.

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Glycopolymer Conjugates

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Abstract This review describes recent developments in the field of glycopolymer (polymer–carbohydrate conjugate) synthesis. The interest in this class of polymers that have a wide range of biological recognition properties has grown rapidly in the last decade due to their application in the areas of biotechnology and medicinal chemistry. Modern synthetic methodologies such as controlled radical polymerization, ionic polymerization, ring-opening polymerization (ROP), ring-opening metathesis polymerization (ROMP) and Click chemistry have recently been proven to be extremely efficient and versatile tools for building tailor-made functional polymers with different molecular architectures. The use of these synthetic methods to prepare glycopolymer conjugates is outlined and discussed in detail. The self-assembling behavior of these glycopolymer designs and their interactions with their corresponding lectins (cell surface receptor proteins) are also presented in this review.

Keywords Binding interactions • Biological functions • Biopolymers • Glycopolymers • Glycosides • Living polymerization • Polymer conjugates • Self-assembly

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Contents

Abbreviations

AcGalEMA	2-(2′,3′,4′,6′-Tetra-*O*-acetyl-β-D-galactosyloxy)ethyl methacrylate
AcGEA	2-(2′,3′,4′,6′-Tetra-*O*-acetyl-β-D-glucopyranosyloxy)-ethyl acrylate
ACPA	4,4′-Azobis(4-cyanopentanoic acid)
AFM	Atomic force microscopy
ARGET	Activators regenerated by electron transfer
ATRP	Atom transfer radical polymerization
BLG	Benzyl L-glutamate
BSA	Bovine serum albumin
Con-A	Concanavalin A
CRP	Controlled radical polymerization
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
DEGMA	Di(ethylene glycol) methyl ether methacrylate
DIPEA	*N*,*N*-diisopropylethylamine
DLS	Dynamic light scattering
ELISA	Enzyme-linked immunosorbent assay
FRP	Free radical polymerization
GalEMA	2-(β-D-Galactosyloxy)ethyl methacrylate
GAMA	2-Gluconamidoethyl methacrylate
GluEMA	2-(β-D-Glucosyloxy)ethyl methacrylate
HEMA	2-Hydroxyethyl methacrylate
HIA	Hemagglutination inhibition assay
ICAR	Initiators for continuous activator regeneration
ITC	Isothermal titration microcalorimetry
LAMA	2-Lactobionamidoethyl methacrylate
MAGlu	2-Methacryloxyethyl glucoside
MAIpGlc	3-*O*-methacryloyl-1,2:5,6-di-*O*-isopropylidene-D-glucofuranose
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MCDO	5-Methyl-5-carboxyl-1,3-dioxan-2-one
MS	Mass spectrometry
NCA	*N*-carboxyanhydride
NHS	*N*-hydroxysuccinimide

NIPAM	*N*-isopropylacrylamide
NMP	Nitroxide-mediated polymerization
PCL	Poly(ε-caprolactone)
PDEA	Poly[2-(diethylamino)ethyl methacrylate]
PDEGMA	Poly(diethyleneglycol methacrylate)
PDI	Polydispersity index
PDPA	Poly[2-(diisopropylaminoethyl methacrylate)]
PET	Poly(ethylene terephthalate)
PG	Propargylglycine
PGAMA	Poly(glucosamidoethyl methacrylate)
PGMMA	Poly(glycerol monomethacrylate)
PLA	Poly(lactide)
PLAMA	Poly(2-lactobionamidoethyl methacrylate)
PLG	Poly(L-glutamate)
PNA	Peanut agglutinin
PNIPAM	Poly(*N*-isopropylacrylamide)
PVA	Poly(vinyl alcohol)
Pβ-BLG	Poly(β-benzyl L-glutamate)
Pγ-BLG	Poly(γ-benzyl L-glutamate)
RAFT	Reversible addition–fragmentation chain transfer
RCA_{120}	*Ricinus communis* agglutinin
ROMP	Ring-opening metathesis polymerization
ROP	Ring-opening polymerization
SEC	Size exclusion chromatography
SPR	Surface plasmon resonance
TEM	Transmission electron microscopy
TEMPO	2,2,6,6-Tetramethylpiperidinyloxy
TMC	Trimethylene carbonate
VLA	*N*-(*p*-vinylbenzyl)-[*O*-β-D-galactopyranosyl-(1-4)]-D-gluconamide

1 Introduction

The study of carbohydrates began in the late nineteenth century with the work of Emil Fischer. Carbohydrate ring structure was elucidated in the 1930s by Haworth and colleagues. Soon after, polysaccharides were discovered and appeared to be present in every living organism; vegetable and animal. The importance of the role of carbohydrates in biological events has given rise to a burgeoning new branch of biology known as glycobiology [1, 2]. Carbohydrates express what is known as the "glycocode" and are unsurpassed in the amount of information they can communicate [3]. Information is encoded in the anomeric stereochemistry, ring size, and the ring substituent functionality, whereas in peptides and oligonucleotides, information is only based on the number of amino acids or nucleosides present and their sequences.

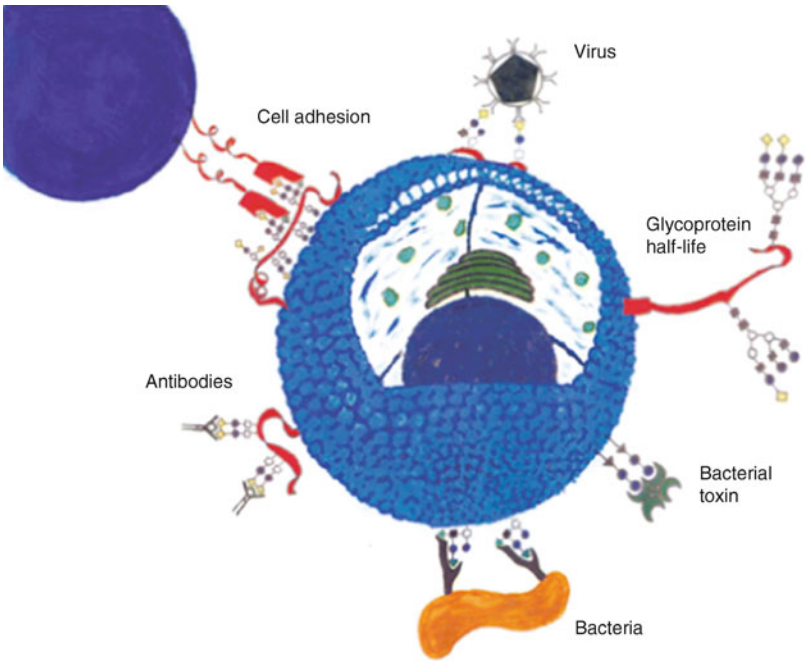


Fig. 1 Protein–carbohydrate interactions at the cell surface mediating cell–cell binding, cell–microbe (bacterial, viral, and bacterial toxin) adhesion and cell–antibody binding. The sugar chains can be linked to proteins (*ribbons*) or anchored in the plasma membrane via a lipid. Reprinted from [6] with permission. Copyright 2005, Macmillan Publishers Ltd

Hence, the density of structural information for glycosylated macromolecules is extremely high and easily surpasses that of nucleic acids and proteins.

The surface of every cell in animals and plants contains carbohydrates in the form of polysaccharides, glycoproteins, glycolipids, and/or other glycoconjugates. Through their carbohydrate moieties, naturally occurring glycoconjugates have been found to play essential roles as recognition sites involved in biological functions [4]. Recognition is key to a variety of biological processes and the first step in numerous phenomena based on cell–cell interactions, such as fertilization, embryogenesis, cell migration, organ formation, immune defence, microbial and viral infection, inflammation, and cancer metastasis [2, 5]. These recognition processes proceed by specific carbohydrate–protein interactions (Fig. 1) [6]. The proteins involved, generically named lectins, are most frequently found on cell surfaces. They have the ability to bind specifically and non-covalently to carbohydrates [7]. The mechanism of the carbohydrate–lectin interaction and the structures of the glycosylated molecules involved in these recognition processes are the subject of intense investigation. Recently, polymer chemists offered a biomimetic approach based on new polymeric materials having sophisticated functions similar to (or even superior to) those of natural glycoconjugates. These materials

are very promising candidates as drug carriers, hydrogels, biodegradable plastics, immunodiagnostic reagents, high affinity anti-adhesins, targeting devices, immunohistochemical tools, and anti-inflammatory agents [8–10]. Indeed, these carbohydrate-containing synthetic polymer (glycopolymer) conjugates can offer numerous practical and financial advantages over those of natural glycoconjugates. According to the broad definition of glycopolymers, chemically modified natural polymers such as cellulose and chitin grafted to synthetic polymers may be included. However, in this review, glycopolymers will be referred to in a rather narrower sense as synthetic polymers containing pendant carbohydrate moieties that act as specific biological functional groups similar to those of naturally occurring glycoconjugates [11]. More emphasis will be given to multi-branched polymers than to functionalized linear polymers.

Modern developments in precision polymerization have made it possible to construct glycopolymers with controlled structure, which can be made with almost any desired carbohydrate densities and added functionalities. Many polymer carriers have been found to be nontoxic and non-immunogenic and stable at a wide range of pH. They can feature homogeneous glycan structures [8]. Consequently, synthetically complex carbohydrates and carbohydrate-based polymers, "glycomimics," are emerging as an important well-defined tool for investigating carbohydrate–protein interactions [12, 13]. However, from the molecular recognition sense, it is very important to develop suitable and facile synthetic methods to attach carbohydrate groups to polymeric carriers without affecting the carbohydrates' ability to bind specifically with lectins. It is therefore quite natural that studies aimed at developing glycopolymer conjugates based on highly precise molecular structure and cell recognition abilities have been accordingly fostered. A number of research collaborations have begun to develop carbohydrate-containing polymers targeting a wide range of applications, such as surfactants [14], detergents [15], texture-enhancing food additives [16], drug release systems [17, 18], scaffolds for tissue engineering [19–21], inhibitors to avoid rejection in xenotransplantation [22], treatment of infectious disease [23], and treatment of HIV [24].

Amphiphilic glycopolymer conjugates are capable of assembling into well-defined nanostructures such as micelles. Nanoscale polymeric carriers are of increasing interest as a means for drug transport and release. Polymeric vesicles (also known as polymersomes), which are capsules with a bilayered membrane, are another interesting example of self-assembled morphologies and have many potential applications in nanomedicine, in vivo imaging, and drug delivery. Although some research has focused on the synthesis of amphiphilic glycopolymers, not many examples have been reported that actually form polymersomes [25, 26]. However, it is believed that glycopolymers capable of assembling into capsule-like structures with multiple copies of a glycoligand presented into solution constitute a potent mimic of eukaryotic cell surfaces [27]. The dimensions of a self-assembled structure, such as size and shape, can be adjusted by changing the hydrophilic/hydrophobic ratio in the glycopolymer and/or the method used for inducing assembly. The choice of block copolymers in the building blocks of a glycopolymer conjugate can introduce features like biocompatibility and targeted release.

The field of design and synthesis of glycopolymer conjugates has evolved so rapidly that there are already a few reviews worth consulting [28–38]. Herein, we provide an overview of the most recent advances in the various synthetic strategies for achieving well-defined glycopolymer conjugates, focusing on the controlled polymerization of glycomonomers together with postpolymerization modification of reactive precursor polymers. Some binding studies between glycopolymers and lectins are also discussed. Furthermore, successful examples of self-assembled nanostructures from glycopolymer conjugates and their biological behavior are highlighted.

2 Lectin–Carbohydrate Interactions and the "Cluster Glycoside Effect"

Carbohydrate-binding proteins, known as lectins, are found in all biological systems; plants, animals and microorganisms. They are very diverse in terms of their structure, size and function. The interaction between carbohydrates and lectins is reversible but highly specific which justifies their pivotal role in many biological recognition events such as some cell adhesion processes. Cell agglutination (hemagglutination) is based on the binding between lectins and carbohydrates [39, 40]. Lectins have been presented as tools for the molecular understanding of the glycocode. Efforts have been made to investigate the mechanism of the binding interactions between lectins and carbohydrates [1]. Many challenges hinder the understanding of the nature of these interactions [41]. The three-dimensional nature of the recognition between carbohydrate and lectin has been intensely investigated [42, 43]. Accordingly, lectins were divided into two major categories: (1) lectins that completely entrap the carbohydrate ligands in deep binding pockets and (2) lectins that bind their ligands in shallow pockets or grooves on the protein surface [34].

Carbohydrates bind weakly to their corresponding lectins; dissociation constants, K_d, are typically 10^{-3}–10^{-6} M, which does not explain the selectivity observed in the in vivo recognition events mediated by lectin–carbohydrate binding. Nature has overcome this binding limitation through multivalency. The enhancement in the binding interactions, on a per-sugar basis, of multivalent glycoconjugates compared to monovalent ligands is known as the "cluster glycoside effect" [12, 44]. It has been observed that the activity towards Concanavalin A (Con-A) increases by increasing the amount of sugar molecules along the polymer backbone (the epitope density) up to a certain point where the glycopolymer becomes too crowded, limiting further access to the lectin (Fig. 2) [45]. The optimum epitope density required to achieve the maximum multivalent interactions between glycopolymers and Con-A was investigated using turbidimetric assays and quantitative precipitation [45]. It is also believed that the cluster glycoside effect is based on aggregation [41]. Nevertheless, further investigations on the cluster glycoside effect are desired. This requires access to multivalent ligands. Different

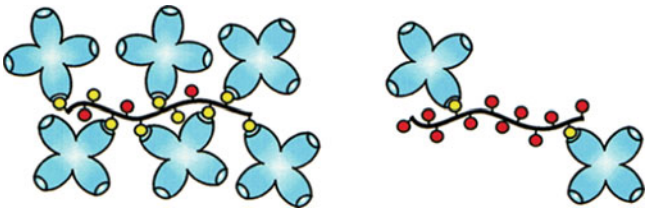


Fig. 2 Con-A clustering by multivalent ligands. *Left*: high-density polymers can recruit many receptors to a single molecule; however, steric effects prevent binding of every residue. *Right*: low-density polymers bind fewer total receptors per molecule. Increasing spacing between residues allows for more efficient binding. Reprinted from [45] with permission. Copyright 2002, American Chemical Society

multivalent glycoconjugates, such as dendrimers and nanoparticles, have been employed to investigate their lectin binding. Dendrimers are challenging to synthesize and the structure of nanoparticles can be ill-defined making it very difficult to elucidate structure–activity relationships [46]. Therefore, there is increasing interest in the synthesis of glycoconjugates based on synthetic polymer backbones with multiple copies of carbohydrate moieties to act as ligands for carbohydrate-binding proteins. Complex polyvalent glycopolymers of defined valency and architecture have been studied extensively with regard to lectin binding and consequently have been used as vehicles for therapeutics or as therapeutics themselves [47].

Carbohydrate–lectin binding has been evaluated by a wide variety of techniques based on the formation of isolated complexes between lectins and their ligands [48]. The most commonly used techniques for lectin binding assays include hemagglutination inhibition assay (HIA), enzyme-linked immunosorbent assay (ELISA), isothermal titration microcalorimetry (ITC), surface plasmon resonance (SPR), and turbidimetry. HIA is one of the oldest techniques used for measuring the interactions between viruses/viral antigens and their corresponding ligands. A soluble lectin is added into microwells containing solutions of different concentrations of carbohydrate (ligand), causing precipitation of aggregates. The minimum concentration of ligand that inhibits this precipitation (hemagglutination reaction) is reported [41]. McCoy and coworkers developed an enzyme-linked lectin assay to detect specific carbohydrate units on the surface of unfixed cells. This assay can be read in standard ELISA plate readers but allows better detection and relative quantification of specific surface carbohydrate units than is possible by standard immunofluorescence with fluorescein-conjugated lectins [49]. ITC is a technique that determines binding energies through classical thermodynamics. It relies on quantifying the heat generated (enthalpy) from the carbohydrate–lectin binding [50]. The strength of binding events can also be determined by probing the change in the refractive index of a gold-coated chip with immobilized ligands when a flow of lectin solution is allowed to pass over the surface [51]. This technique is known as SPR and has been previously utilized to evaluate glycopolymers [52–55]. Turbidimetric assay coupled with UV–vis spectroscopy is another successful method for determining the binding of glycopolymers with lectins [45]. Not only the choice of the binding assay but also

the solution used for conducting the binding assay is important for evaluating the binding constants. It has been found that when certain concentrations of Ca^{2+} and Mn^{2+} salts are used with the same anion (Cl^{-}), the aggregation of the glycopolymer during their interaction with lectins is significantly enhanced [56, 57].

The architecture of the glycopolymer plays an important role in its ability to bind to lectins. Due to their structure, linear glycopolymers can produce intra- and intermolecular clusters caused by the hydrogen bonding of the hydroxyl groups presented on the carbohydrate moieties and/or hydrophobic interactions from the polymer backbone. Many of the publications reporting glycopolymer synthesis also describe the bioactivity of the synthesized glycopolymers (Tables 1, 2, and 3). For instance, it was found that the spacing and orientation of carbohydrate along a glycopolymer chain affects recognition by lectins [116]. The absence of bioactivity was reported in a binding study between Con-A and a glycopolymer with pendant mannose residues attached via the sugar 6-position [58]. Therefore, lectin-reactive glycopolymers have been synthesized by conjugating the carbohydrate moiety to the polymer backbone at the anomeric carbon (C_1). The interaction of β-D-mannopyranoside with Con-A was found to be four times weaker than the anomeric counterpart, α-D-mannopyranoside, which was attributed to the steric hindrance of the glycosyl bond in the binding site. Nevertheless, glycopolymers with C_2-fluoro-substituted β-D-mannopyranoside showed enhanced binding affinity with Con-A compared to the non-fluorinated analogs. This was believed to be due to the strong hydrogen bond between the fluorine atom and the amino acid at the binding site of the Con-A [59].

Although the mechanism by which multivalent ligands act has not yet been fully understood, it is increasingly accepted that there are certain parameters that seem to influence the binding. One such parameter is the distance between two ligands relative to that between two binding sites. It has been observed that an optimum binding can be achieved if the two distances are equivalent [117]. In this sense, flexible polymers are much more preferred than stiff polymers. Very stiff polymers can only bind if their geometry exactly matches that of the receptor, which is usually unlikely. It was observed that the rigid helical poly(glycosyl phenyl isocyanate) has very little binding with lectins, whereas the equivalent polymer with a flexible phenylacrylamide backbone showed strong binding [116]. Introducing a spacer between the carbohydrate and the polymer backbone was also found to enhance the binding with lectins as a result of an increase in the flexibility of the ligand [118]. Furthermore, an increase in the molecular weight of the polymer was noticed to promote the binding [119]. These findings allow the conclusion that not all carbohydrate molecules are involved in the binding process, i.e., a high carbohydrate density on the polymer backbone is not always required [45, 120].

3 Synthesis of Glycopolymers

Glycopolymers can generally be synthesized either by polymerization of monomeric glycosides (glycomonomers) or by functionalization of a suitably reactive precursor polymer with carbohydrate-containing reagents. Although the synthesis

Table 1 Selected examples of the polymerization of glycomonomers, the lectin binding of the glycopolymers, and the self-assembled architecture

Glycomonomer	Polymerization method	Lectin binding	Self-assembled architecture	Refs.
6-*O*-methacryloyl mannose	RAFT	Con-A	None (linear polymer)	[58]
Styryl monomers containing β-D-mannopyranose, 2-acetamido-2-deoxy-β-D-mannopyranose, 2-deoxy-2-fluoro-β-D-mannopyranose, and 2-deoxy-β-D-arabino-hexopyranose	FRP	Con-A	None (linear polymer)	[59]
GEMA	ATRP	Con-A	Vesicles [PGEMA-*b*-poly(diethyleneglycol methacrylate)]	[27]
Glucosyloxyethyl methacrylate	FRP	Con-A	Vesicles [poly(glucosyloxyethyl methacrylate)-*b*-PS]	[60]
6-*O*-acryloyl-α-D-galactopyranose	RAFT	–	Micelles and nanospheres [poly(6-*O*-acryloyl-α-D-galactopyranose)-*b*-PLA]	[61]
AcGalEMA	NMP	PNA	Micellar structures (PGalEMA-*b*-PS)	[62]
GalEMA	FRP	PNA	None (linear polymer)	[40]
3-*O*-methacryloyl-1,2:5,6-di-*O*-isopropylidene-D-glucofuranose (MAIpGlc)	ATRP	–	None (linear polymer)	[63]
VLA	NMP	RCA_{120}	Cylindrical structure and helical conformation	[64, 65]
2,2,6,6-Tetramethylpiperidinyloxy-terminated polystyrene and 4-vinylbenzyl glucoside peracetate and 4-vinylbenzyl maltohexaoside peracetate	NMP	–	Core-glycoconjugated star-shaped polymers	[66]
2,3;4,5-Di-*O*-isopropylidene-1-(4-vinylphenyl)-D-gluco(D-manno)pentitol, 2,3-isopropylidene-1-(4-vinylphenyl)-D-threo(D-erythro)triol, 1,2;3,4-di-*O*-isopropylidene-1-(4-vinylphenyl)-D-glyero(L-glycero)-α-D-galactopyranose and 2,3;4,5-di-*O*-isopropylidene-1-(4-vinylphenyl)-D-manno(D-gluco)-hexulo-2,6-pyranose	TEMPO	–	None (linear polymer)	[67]
1,2,5,6-Di(isopropylidene)-D-glucose-2-propenoate	NMP	–	None (linear polymer)	[68]

(continued)

Table 1 (continued)

Glycomonomer	Polymerization method	Lectin binding	Self-assembled architecture	Refs.
α- and β-anomeric non-sulfated or sulfated *N*-acetyl-D-glucosamine-carrying alkene- and acrylate-derivatized unprotected glycomonomers	Cyanoxyl-mediated polymerization	–	None (linear polymer)	[69–71]
AcGEA	ATRP	–	None (linear polymer)	[72]
GAMA and LAMA	ATRP	–	Micellar structures (blocks of PEO, PPO, PCL, PDEA, PDPA, or PGMMA)	[73–75]
GAMA	ATRP and NMP	Con-A	Spherical micelles and worm-like aggregates (star-shaped PCL-*b*-PGAMA)	[76]
GAMA	ROP and ATRP	Con-A and BSA	Spherical micelles and vesicles (PGAMA-PCL-PGAMA triblock copolymer)	[77]
GAMA and LAMA	ATRP	Con-A and RCA_{120}	Polymer brush on a colloidal gold monolayer	[78, 79]
2-Acryloylethyl octaacetyllactoside and 2-acryloyloxyethyllactoside	ATRP and ROP	RCA_{120}	Micelles [PLG-poly(2-acryloyloxyethyllactoside)-PLG] triblock copolymer	[80, 81]
MAGlu	RAFT	–	None (linear polymer)	[82]
Methyl 6-*O*-methacryloyl-α-D-glucoside	RAFT	–	None (linear polymer)	[83, 84]
6-*O*-vinyladipoyl-D-glucopyranose	Lipase-catalyzed transesterification	–	None (linear polymer)	[85]
6-*O*-vinyladipoyl-D-glucopyranose	RAFT	–	None (star polymer)	[86]
GluEMA and GalEMA	RAFT	–	None (linear polymer)	[87, 88]
p-Acrylamidophenyl α-mannoside and *p*-acrylamidophenyl *N*-acetyl-β-glucosamine	RAFT	*Escherichia coli*	Gold nanoparticle	[89]
2-[(+/−)-*exo*-5-Norbornene-2-carboxamido]-2-deoxy-D-glucopyranose and the protected sugar derivatives based on this monomer, 2-[(+/−)-*exo*-5-norbornene-2-carboxamido]-2-deoxy-1,3,4,6-tetra-*O*-acetyl-D-glucopyranose, 2-[(+/−)-*exo*-5-norbornene-2-	ROMP	–	None (linear polymer)	[90]

carboxamido]-2-deoxy-1,3,4,6-tetra-*O*-benzyl-D-glucopyranose, 2-[(+/−)-*exo*-5-norbornene-2-carboxamido]-2-deoxy-1,3,4,6-tetra-*O*-triethylsilyl-D-glucopyranose, and 2-[(+/−)-*exo*-5-norbornene-2-carboxamido]-2-deoxy-6-*O*-trityl-D-glucopyranose				
7-Oxanorbornene containing α-*O*-glucoside and α-*O*-mannoside	ROMP	Con-A	None (linear polymer)	[91]
Norbornene containing 3-sulfated galactose	ROMP	L- and P-selectin	None (linear polymer)	[92]
Glycosylated NCAs	ROP	–	None (linear polymer)	[93–96]
1,2;5,6-Di-*O*-isopropylidene-3-*O*-MCDO-D-glucofuranose (IGFC), 2,3;5,6-di-*O*-isopropylidene-3-*O*-MCDO-D-mannofuranose (IMFC), and 1,2;3,4-di-*O*-isopropylidene-3-*O*-MCDO-D-galactopyranose (IGPC)	ROP	–	Micelles (PTMC-*b*-PIGFC, PTMC-*b*-PIMFC, and PTMC-*b*-PIGPC)	[97]
2′-(4-Vinyl-[1,2,3]-triazol-1-yl)ethyl-*O*-alpha-D-mannopyranoside	RAFT	Con-A	Thermoresponsive micelles (poly{2′-(4-vinyl-[1,2,3]-triazol-1-yl)ethyl-*O*-alpha-D-mannopyranoside}-*b*-PNIPAAM)	[98]

Table 2 Selected examples of glycopolymers made by post-polymerization functionalization of preformed polymers and their self-assembly and binding to corresponding lectins

Polymerization method	Monomer	Carbohydrate epitope	Post-polymerization functionalization method	Lectin binding	Self-assembled architecture	Refs.
ATRP	Trimethylsilyl-protected propargyl methacrylate	Mannopyranoside and galactopyranoside azides	CuAAC Click reaction	Con-A and RCA I	None (linear polymer)	[99]
NMP	Pentafluorostyrene and styrene	Glucothiose	Thiol-*para* fluoro Click reaction	–	Nanoparticles	[100]
RAFT	Pentafluorophenyl acrylate	Glucose and galactose amines	Amidation reaction	Con-A	None (linear polymer)	[101]
RAFT	Alkene functional HEMA scaffolds and ethylene glycol methacrylate	Glucothiose	Thiol–ene Click reaction	Con-A	Micelles	[102]
Suzuki coupling polymerization	Bromo-alkane functionalized polymers	Glucothiose	Thioetherification reaction	–	None (linear polymer)	[103]
ROMP	NHS-substituted norbornene	Mannose-amine	Carbodiimide coupling	Con-A	None (linear polymer)	[104]
ATRP	*N*-Acryloxysuccinimide	Galactose-amine	Substitution reaction	–	None (linear polymer)	[105]
FRP [charge transfer complex (CTC) mechanism]	VP and MA	*N*-(4-Aminobutyl)-*O*-β-D-galactopyranosyl-(1-4)-D-gluconamide	Amidation reaction	RCA_{120}	Aggregates	[106]
NCA ROP	BLG and propargylglycine NCAs	Azide-functionalized galactose	CuAAC Click reaction	RCA_{120}	Vesicles	[107]
Anionic polymerization	1,3-Butadiene and styrene or ethylene oxide	Protected glucothiose	Photo-addition	–	Vesicles	[108, 109]

Table 3 Selected examples of polymer architectures end-functionalized with a single carbohydrate group

Polymer composition	End group	Functionalization method	Lectin binding	Self-assembled architecture	Refs.
PEO-*b*-PLA	Glucose, galactose, lactose and mannose	Anionic polymerization initiation/ reductive amination	Con-A and RCA-1	Micelles	[110]
Tetra (*p*-phenylene)-oligo(ethylene oxide)	Mannose	Glycosylation	–	Vesicles	[111]
PS	β-Cyclodextrin	CuAAC Click reaction	–	Vesicles	[112]
PNIPAM	Maltoheptaose	CuAAC Click reaction	–	Vesicles	[113]
PBLG	Dextran and hyaluronan	CuAAC Click reaction	–	Vesicles	[114, 115]

of glycomonomers often requires tedious multistep reactions, most attempts to synthesize glycopolymers have been based on the polymerization of glycomonomers due to the better defined molecular architecture of the resulting polymer (assurance of complete functionalization, i.e., one carbohydrate group per repeating unit) [28, 63, 121–123]. Different polymerization techniques have been employed including free radical, controlled radical, anionic, cationic, ring-opening and ring-opening metathesis [8, 28, 29]. On the other hand, the functionalization of preformed polymers can often result in glycopolymers that suffer from a lack of a regular structure because of incomplete functionalization caused by steric hindrance [124]. However, this method does have advantages because the prepolymer is often more easy to characterize structurally than the glycopolymer.

Despite the fact that glycopolymers could be synthesized by modification of natural sugar-based polymers or by chemo-enzymatic methods, the authors hesitate to classify these synthetic pathways as main routes for making synthetic glycopolymers. In this review, a narrower definition of glycopolymers is used (see above). Nevertheless, a particularly important class of glycosylated substrates is glycopolypeptides, which have the added features of defined secondary structures, biocompatibility, and biodegradability. These glycopolypeptides are most conveniently prepared by controlled polymerization of carbohydrate-bearing *N*-carboxyanhydrides (NCA) using transition metal complexes, or in strictly inert polymerization environments [107, 114, 115, 125, 126].

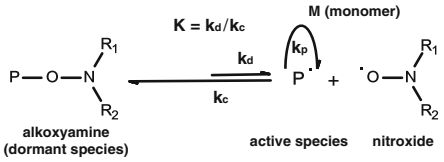
The rest of this section covers the main strategies used for the synthesis of glycopolymers, particular highlighting recent selected examples.

3.1 Polymerization of Glycomonomers

From the biological activity perspective, it is highly desired that glycopolymers are synthesized from anomerically pure glycosylated monomers since most lectins bind one anomer orders of magnitude stronger than the other [127]. Not only the attachment position of the carbohydrate residues influences the binding but also the length and nature of the spacer between the carbohydrate units and the polymer backbone. For instance, it has been reported that glycopolymers with longer spacers bind more efficiently compared to shorter spacers as a result of the greater ligand–receptor accessibility and an increase in flexibility of the ligand [128, 129]. Moreover, it has been shown that binding is more pronounced in spacer-*N*-linked glycopolymers than that of spacer-*O*-linked glycopolymers [130]. Stereo-selective synthesis of glycomonomers has previously been achieved by both enzymatic and well-established carbohydrate chemistry routes. For example, a β-D-galactosidase was used for trans-glycosylation of 2-hydroxyethyl methacrylate (HEMA) with either *p*-nitrophenyl-β-D-galactose or lactose as the glycosyl donor [131, 132]. Although these syntheses result in β-anomer selectivity, and remove the need for protecting group chemistry, the extended reaction times (up to 8 days) coupled with low conversions (~40%) and exotic enzymes makes enzymatic synthesis less desirable. Chemically, β-stereoselective products were obtained by glycosylation reactions of β-D-galactose pentaacetate with HEMA using boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$) as a catalyst, but yields were typically low ($\leq$45%) [133]. Moreover, the esterification of acryloyl chloride with 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose has been also reported [61].

Recently, the copper-catalyzed azide-alkyne cycloaddition (CuAAC) Click reaction has been utilized by Haddleton et al. and Stenzel et al. to synthesize different glycomonomers [98, 99, 134, 135]. The synthetic approach involves introduction of an azide functionality to sugars followed by CuAAC reaction with polymerizable alkynes or preformed polyalkynes. The typical synthesis of azidoalkyl glycosides includes protection of the hydroxyl groups, activation of the sugar, glycosylation with the alcohol, displacement of the leaving group by the azide, and deprotection [136]. A simple and practical methodology was recently developed to access both the α- and β-anomers of a variety of free sugars using a strong acid cation exchanger resin, without the need to protect the hydroxyl groups [137]. Shoda et al. reported a one-pot direct synthesis of various β-glycosyl azides in water using unprotected sugars and sodium azide mediated by 2-chloro-1,3-dimethylimidazolinium chloride [138]. The developments in the production of glycosyl azides have dramatically increased their potential as precursors of glycoarrays and glycoconjugates.

The thiol-*para*-fluorine Click reaction was demonstrated to be another facile route for glycosylated monomer synthesis with very high yields and purity. A glycomonomer with an overall yield of 84% was provided by coupling acetylated β-D-thioglucopyranose to pentafluorostyrene and subsequent deacetylation with sodium methoxide [139].



Scheme 1 Mechanism of NMP

Table 1 lists some examples of glycomonomers used for synthesizing glycopolymers using different polymerization techniques.

3.1.1 Controlled Free Radical Polymerization

A large number of glycopolymers synthesized by conventional free radical polymerization (FRP) of carbohydrate-containing vinyl monomers have been reported in the literature [28, 29, 34]. For instance, Cameron and coworkers investigated the polymerization of a protected carbohydrate-containing monomer, 2-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-galactosyloxy)ethyl methacrylate (AcGalEMA), followed by deacetylation. An alternative route was developed by polymerizing the deacetylated AcGalEMA monomer, 2-(β-D-galactosyloxy)ethyl methacrylate (GalEMA), in a water–methanol mixture. Interestingly, it was found that the latter route leads to much better defined materials, as evidenced by NMR spectroscopy and elemental analysis. The binding of poly(GalEMA) with peanut agglutinin (PNA) was also investigated [40, 87]. However, the molecular weights and molecular weight distributions of glycopolymers synthesized by FRP of carbohydrate-containing monomers were not sufficiently controlled until "living" radical polymerization techniques were successfully implemented for glycopolymer synthesis. This section reviews recent advances in the synthesis of glycopolymers having controlled molecular weights via various types of living polymerization methods.

In recent years, controlled radical polymerization (CRP) techniques such as nitroxide-mediated polymerization (NMP) [140], atom-transfer radical polymerization (ATRP) [141, 142], and reversible addition fragmentation transfer polymerization (RAFT) [143–145], have been proven to be very efficient for the preparation of well-defined functional polymers.

Nitroxide-Mediated Polymerization

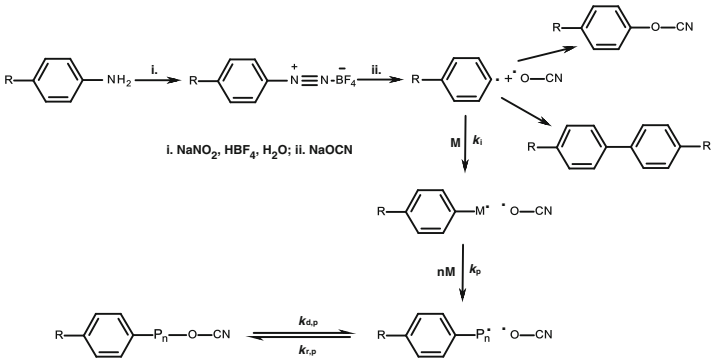
NMP is a metal-free polymerization technique that relies on the reversible capture of the propagating species by nitroxides with the formation of dormant chains (alkoxyamines) (Scheme 1). Whenever this equilibrium is shifted toward the dormant form, the stationary concentration of the active species is low and the irreversible chain termination is limited [140, 146].

NMP has found some application in the synthesis of glycopolymers. However, in most cases, protection of the carbohydrate hydroxyl groups was required, especially when higher degrees of polymerization (DP) were targeted (DP $\geq$ 100) [64, 66–68, 147–151]. The polymerization of acrylate and styrenic-based glycomonomers using a di-*tert*-butyl nitroxide-based alkoxyamine initiator has been reported [64, 147, 148]. The polymerizations of styryl monomers bearing four different isopropylidene-protected mono-sugars using 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) as a mediator were reported [67]. TEMPO-terminated polystyrene macroinitiator was utilized to chain extend two styrenic-based glucoside peracetate and maltohexaoside peracetate glycomonomers with a reaction temperature of 138°C [66]. Hawker et al. reported the polymerization of 1,2,5,6-di-(isopropylidene)-D-glucose-2-propenoate with an α-hydrido alkoxyamine initiator functionalized with a lipophilic *N*,*N*-di(octadecyl) amine group at a reaction temperature of 105°C [68]. A well-defined glycopolymer was obtained with a narrow polydispersity by nitroxide-mediated radical polymerization of an acetylated lactose-substituted styrene monomer followed by deprotection [65]. Using a methacrylic acid-based alkoxyamine with *N*-*tert*-butyl-*N*-(1-diethylphosphono-2,2-dimethylpropyl) nitroxide as mediating agent, Stenzel and coworkers employed styrene as a comonomer (10%) in the copolymerization of a methacrylate-based glycomonomer, AcGalEMA, which lowered the required reaction temperature to 85°C [62].

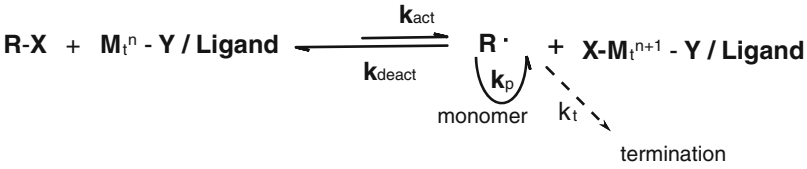
Cyanoxyl-Mediated Polymerization

Polymerization mediated by cyanoxyl radical (NC–O$^{\bullet}$) has been utilized as an alternative CRP technique for the polymerization of unprotected glycosylated species. This technique has been reported to have some major advantages over NMP. It requires lower reaction temperatures (ambient to 70°C), which consequently reduces problems associated with thermal stability [152, 153]. Chaikof et al. have reported the synthesis of several biomimetic glycopolymer conjugates from alkenyl, acryloyl, and acrylamide glycomonomers, often in their sulfated form, using cyanoxyl-mediated polymerization [69, 70, 154, 155]. Some of these glycopolymers have been tested as mimetics of heparin or heparan sulfates [154]. It has been found that polymers featuring β-*N*-acetyl glucosamine residues bind with fibroblast growth factor 2, but weakly compared with heparan sulfate [156]. It has also been found that binding is independent of the linker length between carbohydrate and polymer backbone. Further investigation revealed that sulfated analogs of polymers with pendant lactose groups bound more strongly than the non-sulfated ones [71].

End-group functionality can be introduced to glycopolymers synthesized by cyanoxyl-mediated polymerization. This has been achieved by the use of functional amines to produce diazonium cations and thus the aryl radical initiation fragment (Scheme 2). The cyanoxyl chain-end of the resulting polymers can be converted from a cyanate to a free hydroxyl group on reaction with pyridine. Chaikof et al. reported the synthesis of a series of acrylamide-based copolymers using initiating fragments with different functionalities including alkoxy, amino, carboxyl, hydrazido, and biotinyl [71, 157].



Scheme 2 Formation of cyanoxyl radicals and cyanoxyl-mediated polymerization mechanism



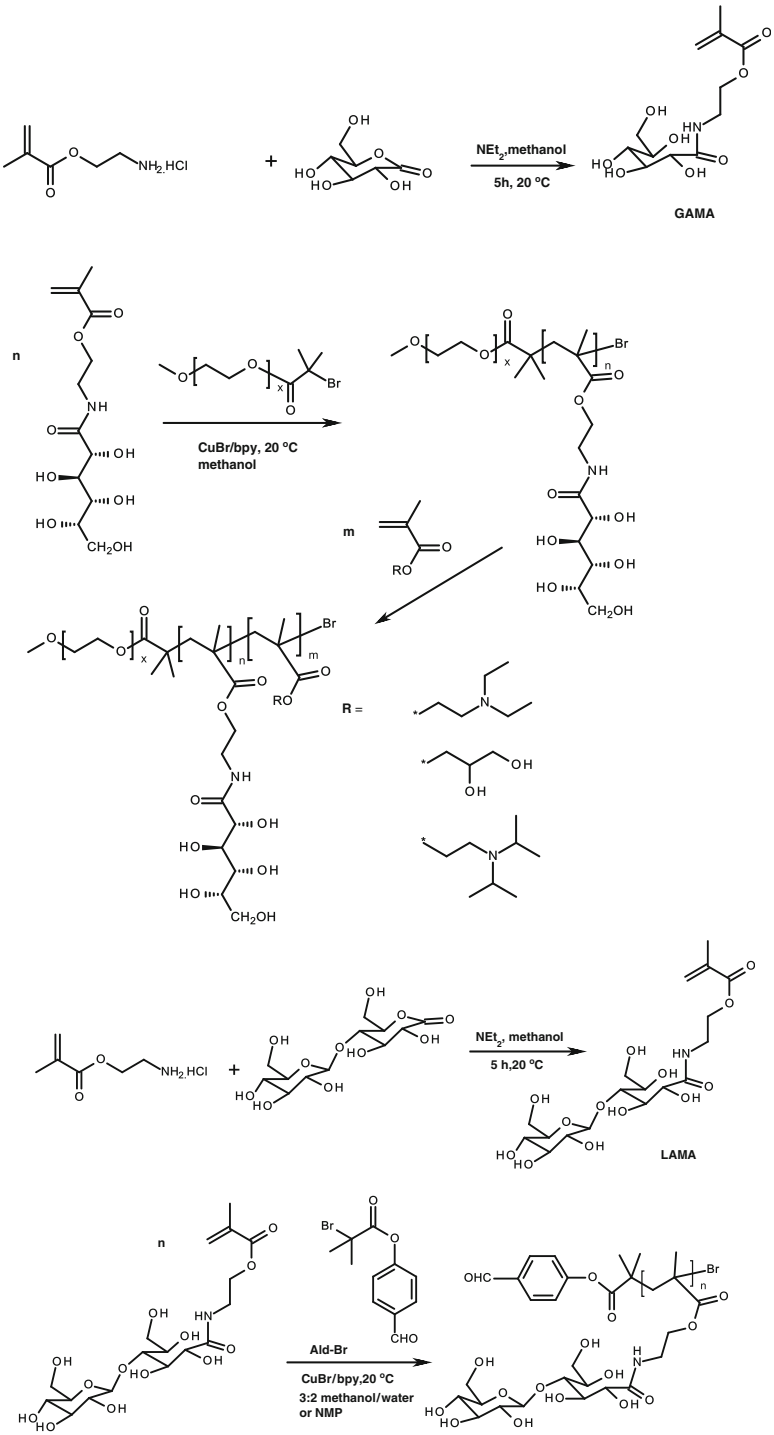
Scheme 3 Transition metal-catalyzed ATRP

Atom-Transfer Radical Polymerization

Atom-transfer radical polymerization (ATRP) is another example of a CRP technique. The general mechanism for ATRP is shown in Scheme 3. The radicals, or the active species, are generated through a reversible redox process catalyzed by a transition metal complex (M_t^n–Y/ligand, where Y may be another ligand or the counterion), which undergoes a one electron oxidation followed by abstraction of a halogen atom, X, from a dormant species, R–X. Several transition metals can catalyze the process, but Cu(I) complexes have received the most attention [141].

As with NMP, the majority of glycopolymer syntheses via ATRP involve the use of protected glycomonomers [63, 72, 80, 158–171]. However, Narain and Armes elegantly reported the ATRP of unprotected glycomonomers synthesized by ring-opening of either glucono- or lactobiono-lactone with 2-aminoethyl methacrylate. The polymerization was carried out using a CuBr/2,2′-bipyridine catalytic system with either a PEGylated or aldehyde-functionalized initiator in different ratios of MeOH to H_2O (Scheme 4). The polymerization gave high conversions to polymer with controlled number-average molecular weight (M_n) and low polydispersity index (PDI). They also found that the polymerization time can be reduced, at the expense of some control, from 15 h to under 1 h by using water alone as a solvent [73–75].

The main advantage of ATRP over NMP is the lower polymerization temperature, which is particularly crucial because glycomonomers are usually unstable at high temperatures ($\geq$120°C) [64]. On the other hand, a potential limitation of the



Scheme 4 Synthesis of GAMA and LAMA followed by ATRP. Redrawn from [75]

application of polymers made by ATRP as biomaterials is the copper contamination of the final product. Traditional ATRP techniques require relatively large amounts of copper catalyst (typically 0.1–1 mol% versus monomer). Recently, considerable effort has been devoted to decrease the amount of copper catalyst used in ATRP systems [172]. The activators regenerated by electron transfer (ARGET) ATRP process [173], which involves an excess of reducing agent [such as tin octoate, ascorbic acid, or copper(0)] relative to the catalyst, is reported to continuously regenerate the activators by reduction of the copper(II) that accumulates because of unavoidable radical termination. In initiators for continuous activator regeneration (ICAR) ATRP [174], a source of organic free radicals is employed to continuously regenerate the copper(I) activator, which is otherwise consumed in termination reactions when catalysts are used at very low concentrations. These techniques have been coupled with different purification strategies such as passing through a column filled with neutral alumina, stirring with an ion exchange resin, or a reprecipitation method to remove/reduce the copper contamination to parts per million levels in the polymeric material [175].

There are many reports in the literature of the lectin-binding properties of glycopolymers prepared via ATRP. A system for targeted peptide delivery based on four-armed star-shaped poly(ε-caprolactone)-β-poly(glucosamidoethyl methacrylate) (PCL-β-PGAMA) copolymers was developed and the binding of these polymers with a glucose/mannose-specific lectin (Con-A) was investigated by Dong and Dai [76]. The polymers were synthesized by ROP and ATRP and the work was extended to prepare a polypseudorotaxane-centered triblock copolymer by inclusion of α-cyclodextrin into the PCL block. The recognition between these polypseudorotaxane/glycopolymer biohybrids and Con-A was also established [77]. Kitano et al. reported the synthesis by ATRP of a polymer with many pendant galactose residues from galactose-carrying vinyl monomer, 2-lactobionamidoethyl methacrylate (LAMA), with a disulfide-carrying ATRP initiator, 2-(2′-bromoisobutyroyl)ethyl disulfide (DT-Br). The obtained glycopolymer (DT-PLAMA) was conjugated with a colloidal gold monolayer deposited on a cover glass, forming a polymer brush. SPR was used to investigate the association and dissociation processes of galactose residues on the colloidal gold with a galactose-specific lectin, *Ricinus communis* agglutinin (RCA_{120}). The association constant of the lectin with the galactose residues in the DT-PLAMA brush was twofold larger than that for free galactose. Moreover, the ability of this DT-PLAMA brush to associate with a HepG2 cell having galactose receptors was investigated microscopically [78]. Similarly, Vamvakaki et al. used ATRP to synthesize homogeneous glycopolymer brushes based on LAMA and D-gluconamidoethyl methacrylate (GAMA) from a surface-attached initiator on gold substrates. AFM was used to image the surface morphology of the polymer brushes. The binding of the prepared glycopolymers with Con-A and RCA_{120} was studied by SPR [79].

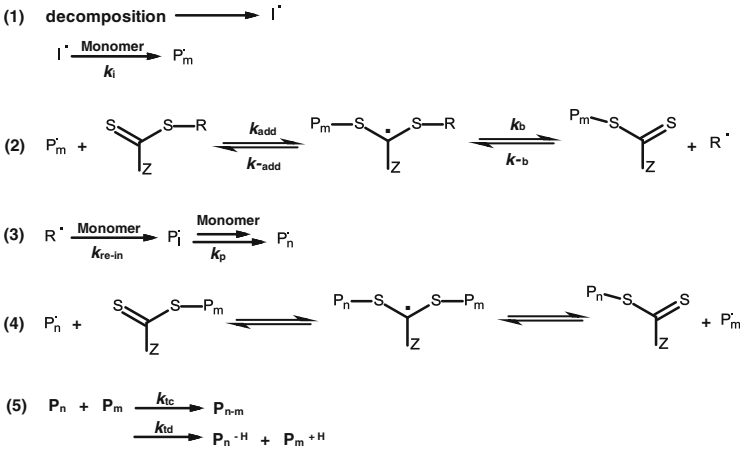
A–B–A triblock copolymers composed of a lactose-containing glycopolymer B block conjugated to PLG A blocks were synthesized by ATRP followed by the ROP of β-BLG NCA. It was reported that these triblock glycopolymer conjugates self-assemble in water into lactose-containing polymeric aggregates that show specific interactions with RCA_{120} lectin [80, 81].

Reversible Addition–Fragmentation Chain Transfer Polymerization

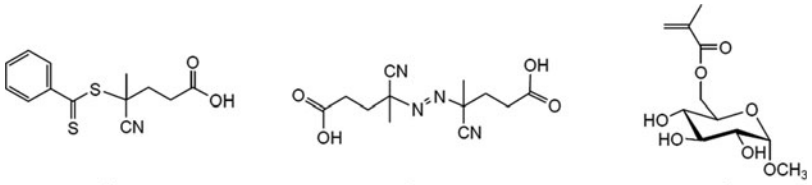
RAFT polymerizations were first reported in 1998 by the CSIRO group [176]. The general mechanism for RAFT polymerization is shown in Scheme 5 [145]. The key step in the RAFT process is the rapid and reversible exchange between growing chains (step 4 in Scheme 5). This ensures that irreversible termination is minimized and chain growth is controlled.

RAFT is the most popular recent route for synthesizing glycopolymers from glycomonomers. Its tolerance to a wide range of reaction conditions and functionalities makes it suitable for the polymerization of an almost limitless range of monomers. RAFT yields polymers with precisely controlled structures including random, block, gradient, grafted, and star copolymers [143, 177–179]. RAFT polymerizations are typically carried out between 60 and 70°C; however, polymerization at room temperature is possible. In 2003, the first example of a glycopolymer made by RAFT polymerization was reported by Lowe et al. [82]. The RAFT polymerization of 2-methacryloxyethyl glucoside (MAGlu) was conducted in aqueous media using 4-cyanopentanoic acid-4-dithiobenzoate as a chain transfer agent (CTA), due to its inherent water-solubility and its applicability for methacrylic monomers, and 4,4′-azobis(4-cyanopentanoic acid) (ACPA) as an initiator. The polymerization proceeded in a controlled manner with PDI of as low as 1.07. They also investigated the formation of block copolymer by chain extension of the homopolymer (PMAGlu) as a macro-CTA with 3-sulfopropyl methacrylate. The chain extension progressed quantitatively with no detectable low molecular weight peak in the SEC; however, the PDI increased. Using the same CTA (RAFT agent) (1 in Scheme 6) and initiator (2 in Scheme 6) combination as Lowe et al. [82], the effect of an added base on the aqueous polymerization of a monomer featuring a methacrylate attached to the 6-position of the sugar, methyl 6-*O*-methacryloyl-α-D-glucoside (3 in Scheme 6), was investigated [83]. The RAFT agent was dissolved in the aqueous solution using sodium carbonate or sodium bicarbonate. An inhibition period of 60–90 min was observed at the beginning of the polymerization. Better results were obtained with sodium bicarbonate, in which case the molecular weight distribution remained narrow and unimodal up to 81% conversion. Improved results were obtained when ~10% ethanol was added to the polymerization mixture. It was believed that the use of EtOH aids the solubility of the CTA and initiator without resorting to the use of a base and thus reduces the rate of CTA hydrolysis.

Furthermore, Cameron and Albertin [84] reported a detailed kinetic study of the RAFT polymerization of methyl 6-*O*-methacryloyl-α-D-glucoside with the same CTA and initiator in homogeneous aqueous media. The influence of temperature, initiator and CTA concentration, molar mass of the CTA radical leaving group, and the presence of residual oxygen on the polymerization kinetics were investigated in comparison with corresponding conventional FRPs (i.e., with no CTA present). RAFT processes were characterized by an initial non-steady-state period, the length of which depended inversely on the radical flux in the system, and were found to proceed at a significantly slower rate than the corresponding conventional FRPs.



Scheme 5 General mechanism for RAFT polymerization



Scheme 6 Structure of the CTA (RAFT agent) (*1*), initiator (*2*), and methyl 6-*O*-methacryloyl-α-D-glucoside monomer (*3*) used to prepare glycopolymers by RAFT

The results of this investigation were used as a guide for the preparation of a series of well-defined living glyco-oligomers (DP_n = 15–66, PDI = 1.05–1.12) in high yield.

Stenzel et al. [86] reported the synthesis of glycostars by polymerization of 6-*O*-vinyladipoyl-D-glucopyranose, which had been previously prepared via lipase-catalyzed transesterification of divinyladipate with α-D-glucopyranose [85]. The polymerization was carried out using a tetrafunctional xanthate CTA. As was seen with linear polymers [85], conversions were low, reaching only 35% after 4 h and a limiting conversion of 50% after 9 h. Molecular weights were seen to increase with conversion but were higher than predicted for any given conversion. This could be attributed either to the use of SEC with inappropriate calibration or to the occurrence of side reactions. The same research group also demonstrated the synthesis of block copolymers via RAFT polymerization; one synthesis was based on glucosyl methacrylate and HEMA [180, 181] and the other involved *N*-acryloyl glucosamine with *N*-isopropylacrylamide (NIPAM), affording narrow dispersed thermosensitive diblocks [182]. The work was then extended to produce thermoresponsive glycopolymer brushes. Homopolymers and block copolymers of NIPAM and *N*-acryloyl glucosamine were synthesized. A trithiocarbonate CTA was first

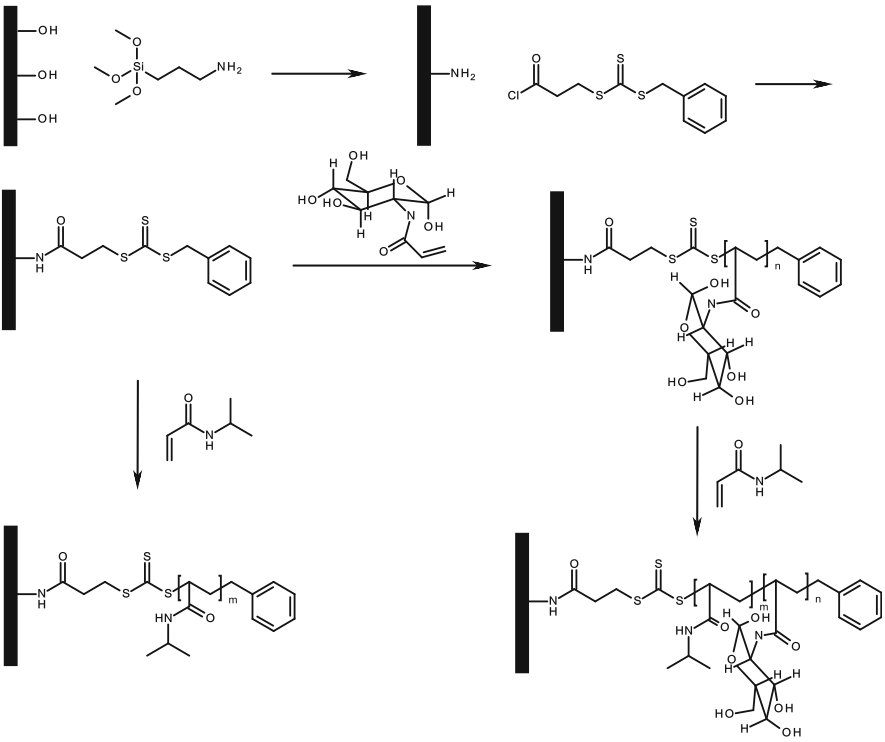


Fig. 3 Synthesis of stimuli-responsive glycopolymer brushes using RAFT polymerization via Z-group approach. Redrawn from [183]

immobilized on a silica substrate via the Z-group. Polymerizations were carried out by submerging the trithiocarbonate-functionalized substrate in a solution of the appropriate monomer and ACPA (Fig. 3). A greater degree of control was obtained when a small quantity of free trithiocarbonate was used. The free RAFT agent was believed to suppress termination reactions when the polymers are unattached from the surface. The brush thicknesses increased with monomer conversion as revealed by ellipsometry [183].

Polymers prepared by RAFT have the advantage that chain end groups can be readily converted to thiols and thus anchored to organic or inorganic substrates such as gold via Au–S covalent bonds [88, 89, 184]. These glycopolymer hybrids are of great interest because of their potential use in many applications including chemical sensing, responsive surfaces, and affinity chromatography. Cameron et al. established the synthesis of glycosylated nanoparticles [88]. They used RAFT to polymerize an anomerically pure glycomonomer, GalEMA, in a manner similar to that reported by Davis et al. [87]. The produced glycopolymers had predictable molecular weights with low polydispersity (<1.2). Recently, Toyoshima and Miura described the RAFT polymerization of random copolymers from *p*-acrylamidophenyl α-mannoside, *p*-acrylamidophenyl *N*-acetyl-β-glucosamine, and acrylamide using

(thiobenzoyl)thioglycolic acid as RAFT agent. The produced glycopolymers were treated with $NaBH_4$ and the thiol end-functionality was then reacted with gold nanoparticles forming Au–S bonds. The bimolecular recognition of the pendant mannose and glucose moieties on the gold particles with Con-A was demonstrated [89].

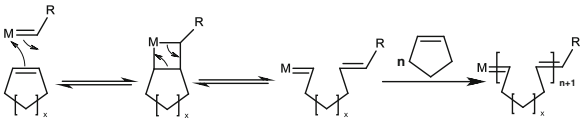
3.1.2 Non-Free-Radical Polymerization

Prior to CRP, the only potential synthetic pathways to glycopolymers of controlled architecture were either ionic or metal-catalyzed polymerizations such as ROMP. ROMP of strained cyclic, bicyclic, or multicyclic monomers results in ring scission and the formation of unsaturated linear polymers. Productive cleavage of the metallacyclobutane species formed when cyclic olefins undergo [2+2] cycloaddition with metal-alkylidenes, leading to ring opening of the olefin (Scheme 7).

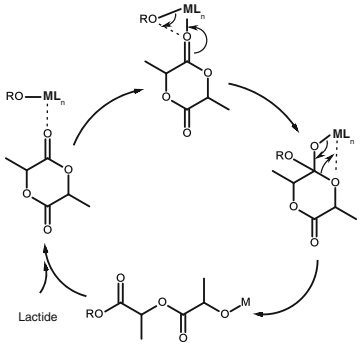
Ionic polymerizations are known to be highly sensitive to monomer functionality and impurities due to the nature of the propagating species. Therefore, the necessity for protected species and harsh polymerization conditions have limited the use of this method in synthesis of glycopolymers [185–188]. After the development of ruthenium-based catalysts, which are tolerant to many functional groups, the synthesis of glycopolymers from unprotected glycomonomers via ROMP became permitted [90]. ROMP has been used in the synthesis of polymers with glucose and mannose moieties [45, 91, 189, 190]. Sulfated glycopolymers that recognized L- and P-selectin, which facilitate leukocyte trafficking to sites of inflammation, have also been synthesized via ROMP [92, 191]. Recently, ROMP has been utilized to generate a new class of chondroitin sulfate glycomimetic polymers that display defined sulfation motifs, while mimicking the multivalent architecture of native glycosaminoglycan chains [192]. Controlled/living ROMP of glycomonomers allows the synthesis of block copolymers. However, removal of the heavy metal catalyst residue is required if the products are to be used in biological applications. Kiessling et al. suggested that the use of a preformed ruthenium catalyst by treating a small quantity of the monomer with $RuCl_3$ for the ROMP of 7-oxanorbornene bearing glucose or mannose side groups reduces the metal contamination in the products and subsequently produces consistent protein binding assays [189].

Alternatively, highly ring-strained heterocyclic compounds may undergo cationic or anionic ROP to yield polymers of controlled, high molecular weights and block copolymers are possible. Only a narrow range of highly specific monomers are available for ROP such as lactones, lactides, and NCAs (Scheme 8). ROP of these monomers leads to readily hydrolyzable ester or amide groups in the polymer backbone, which may result in biodegradation under physiological conditions.

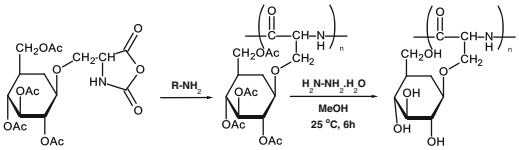
Aoi et al. reported the synthesis of different glycopeptides from glycosylated NCAs using anionic ROP (Scheme 9) [93–95]. Recently, Deming and Kramer described the preparation of new glycosylated lysine NCA monomers that undergo living polymerization to give well-defined, high molecular weight



Scheme 7 Mechanism of ROMP of strained cyclic olefins



Scheme 8 Coordination–insertion mechanism for ROP of lactide



Scheme 9 Synthesis of glycopeptides by ROP of NCAs

homoglycopolypeptides and block and statistical glycocopolypeptides [96]. This system was believed to solve many long-standing problems in the direct synthesis of glycopolypeptides from NCAs relating to monomer synthesis, purification, and polymerization. The produced water-soluble glycopolypeptides were claimed to have the potential to impart functionality and improve biocompatibility in copolypeptide materials such as hydrogels for tissue engineering and vesicles for drug delivery. Moreover, the synthesis of a family of amphiphilic block glycopolymers containing D-glucose, D-galactose, and D-mannose via metal-free organo-catalyzed ROP of functional cyclic carbonates was reported. The used method was shown to generate narrowly dispersed glycopolymers of controlled molecular weight and end-group fidelity [97].

3.2 Post-polymerization Glycosylation

As discussed previously [87], the most common method for producing well-defined glycopolymers is by the polymerization of sugar-functionalized monomers.

Nevertheless, there are reports of post-polymerization functionalization methodology applied to the synthesis of glycopolymers. Unlike carbohydrate monomer synthesis, the post-polymerization modification approach offers simple synthetic and purification routes to produce libraries of glycopolymers by attaching different sugar moieties to pre-formed polymer backbones. However, conducting reactions on a pre-synthesized polymer backbone tends not to lead to comprehensive functionalization, giving an inhomogeneous sequence within the chain that would probably affect the precise recognition processes. Highly efficient methods for post-polymerization functionalization of a reactive polymer scaffold are therefore a desired target. This section presents some recent synthetic strategies employed for this purpose (Table 2). A review on the synthesis of functional polymers by post-polymerization modification was recently reported [193].

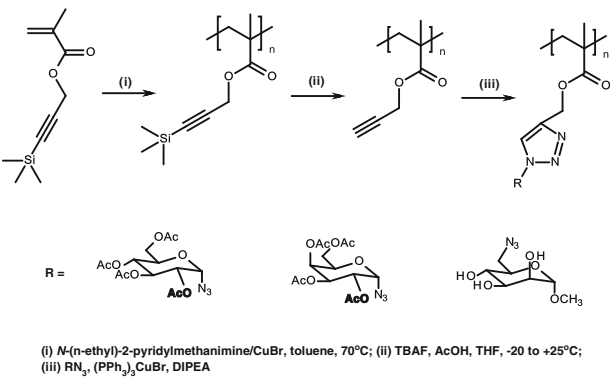
3.2.1 Post-polymerization Functionalization Using Click Reactions

The introduction of the concept of Click chemistry, as a family of organic reactions that fulfil certain criteria drawn by Sharpless and coworkers in 2001 [194], has indeed captured the attention of synthetic chemists in the field of post-polymerization modification towards glycopolymer synthesis [32]. The most widely employed Click reaction is the CuAAC reaction. ATRP has been used extensively in conjunction with CuAAC Click chemistry. This is probably because both techniques are mediated by Cu(I). Moreover, the halogen chain ends of polymers prepared using ATRP can easily be transformed into azides to form what is known as azido-telechelic polymers. Many examples of glycopolymers prepared by the combination of Click chemistry and ATRP have been reported [32, 99].

Due to the instability of azides, it was believed that the preparation of alkyne-functionalized polymers from alkynyl monomers is more likely to be successful. However, the ATRP of propargyl methacrylate [40] was reported to result in polymers of PDIs >3 at 50% conversion. This was attributed to transfer of the radical to the alkynyl moiety. Therefore, the polymerization of the azide-functionalized monomer was investigated instead. The azide-functionalized polymethacrylate was subsequently reacted with alkyne substrates [195]. Haddleton and coworkers [99] tackled the intolerance of the alkyne functionality towards ATRP condition by protection using the trimethylsilyl (TMS) group. They also reported the successful synthesis of well-defined glycopolymers containing galactosyl and mannosyl chain functionality in a quantitative yield from the corresponding protected or unprotected glycosyl azides (Scheme 10). Moreover, the binding assays of these glycopolymers with Con-A were reported to be positive.

The efficiency of Click coupling reactions was shown by the work of Hawker and coworkers [196] on the synthesis of asymmetric dendrimers from unprotected azido-sugars and alkyne-terminated dendrimers. The produced materials contained multiple functionalities (carbohydrate and fluorophore).

Riguera et al. [197] reported the conjugation of unprotected alkyne-derived carbohydrates to dendritic systems incorporating terminal azides using Click

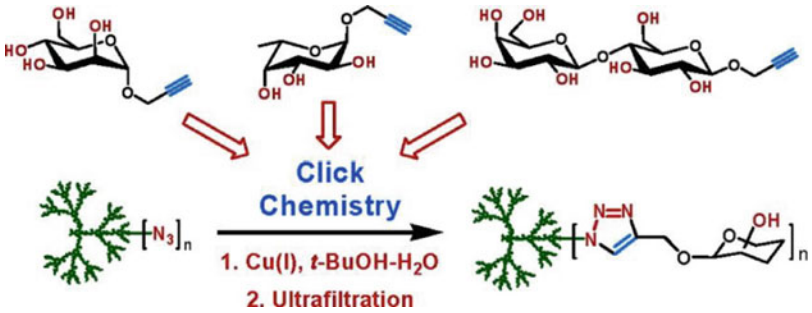


Scheme 10 Polymerization and Click glycosylation of TMS-protected propargyl methacrylate as demonstrated by Haddleton et al. [99]

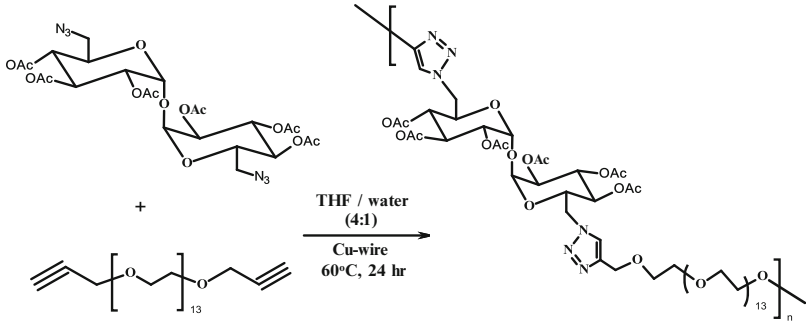
chemistry in combination with ultrafiltration, as opposed to the lengthier process of employing protected glycosides or introducing the Click functionality on each generation of previously synthesized dendrimers (Scheme 11). It was also shown that the reported procedure is quick, efficient, and reliable, allowing the incorporation of up to 27 unprotected fucose, mannose, and lactose residues in reproducible high yields (up to 92%), requiring only catalytic amounts of Cu. Both ^{1}H-NMR and MALDI-TOF MS were used to establish the completion of the conjugation process [197].

Recently, a Click polymerization strategy has been utilized to produce glycopolymers in which carbohydrates are incorporated into the backbone [198, 199]. Eissa and Khosravi demonstrated the copper wire-catalyzed Click polymerization of di-alkyne-terminated PEG with di-azide-functionalized trehalose prepared by tosylation/acetylation of α,α-D-trehalose followed by azidation reaction (Scheme 12) [199]. The produced alternating linear glycopolymers with triazole linkers were fully characterized and the polymer with a PEG segment of M_n ~600 gmol^{-1} showed a lower critical solution temperature (LCST) at ~39°C, which is known as the fever temperature. This material constituted a new class of temperature-responsive water-soluble glycopolymers.

In the last 5 years, thiol-based Click reactions, that is the reaction of thiols with various functional groups such as alkenes, alkynes, *para*-fluorophenyl, and halides, have also attracted attention due to their versatility for preparing tailor-made macromolecular architectures [200, 201]. Glycopolymers have been synthesized from these reactions, which are simple, compatible with water and oxygen, metal-free, highly efficient, and provide high yields under the employed conditions [100, 101]. Many examples of glycopolymers made from sugars with various functionalities (azide, alkyne, and thiol) incorporated into a polymer either by Clicking onto the polymeric backbone or by polymerizing them as glycomonomers have been reviewed recently [32]. For instance, glucothiose was grafted onto alkyne functional scaffolds via thiol–ene Click reaction. The polymeric backbones were first prepared by RAFT polymerization of (ethylene glycol)methacrylate and



Scheme 11 Synthesis of different glycodendrimers via Click chemistry. Reprinted from [197] with permission. Copyright 2006, American Chemical Society



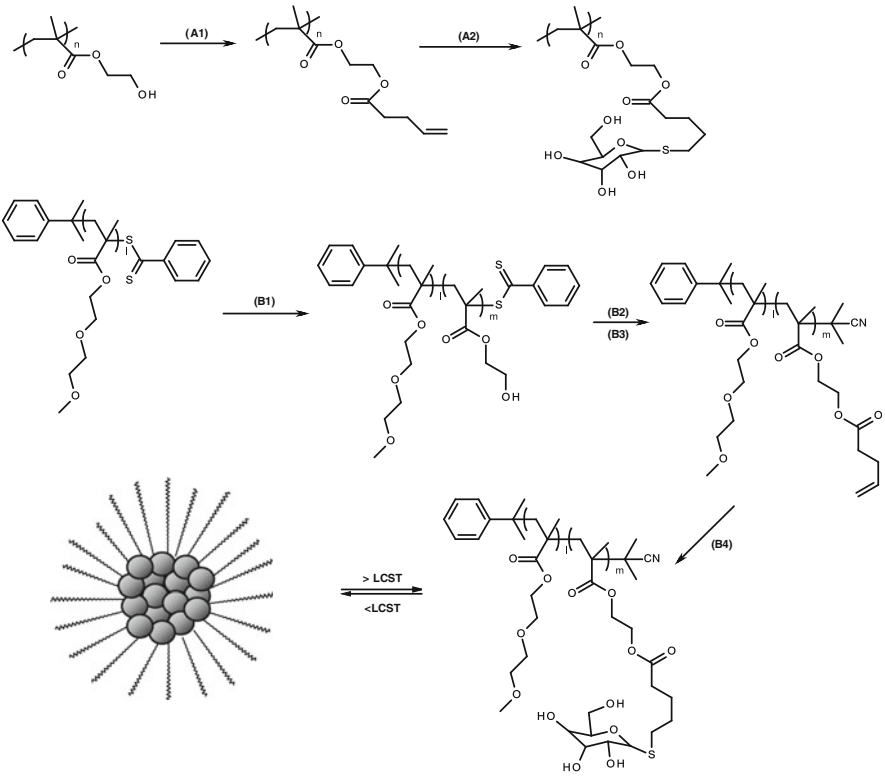
Scheme 12 Synthesis of temperature-responsive glycopolymer [199]

HEMA followed by reaction with 4-pentenoic anhydride resulting in polymers bearing alkene side chains (Scheme 13). The thiol–ene Click reaction was reported to be complete in less than 2 h. The produced glycomicelles showed bioactivity with Con-A [102].

3.2.2 Post-polymerization Functionalization Using Non-Click Reactions

Liu and coworkers [103] reported that bromo-alkane functionalized polymers can be glycosylated in near quantitative yield (98%) simply by the reaction of the bromo groups with a thio-sugar. This thioether formation reaction was utilized to covalently attach protected or unprotected carbohydrate residues to fluorescent conjugated polymers.

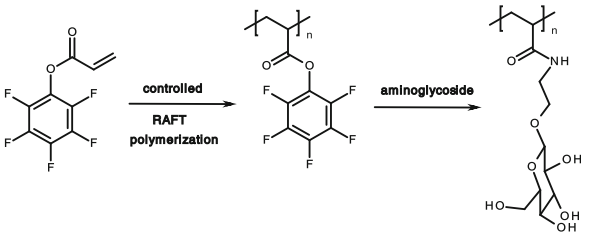
Another attractive method for attaching sugar moieties onto preformed polymers involves the reaction between pendant active esters and amino-sugars. These groups react with amines under very mild reaction conditions, yielding amides quantitatively. Moreover, the high nucleophilicity of amines compared to alcohols retains the selectivity without the need for protecting groups in sugar



Scheme 13 Synthetic strategies for the preparation of glucose-functionalized (*co*)polymers. (*A1*) 4-Pentenoic anhydride, DMAP, pyridine, DMF; (*A2*) UV, glucothiose, DMPA, DMF; (*B1*) HEMA, AIBN, DMAc, 70°C; (*B2*) AIBN, toluene, 80°C; (*B3*) 4-pentenoic anhydride, DMAP, pyridine, DMF; (*B4*) UV, glucothiose, DMPA, DMF. Redrawn from [102]

molecules. Although this method often requires an excess of substrate as well as some purification [202], polymeric activated esters have found broad applications in life science, especially peptide chemistry. Polymers bearing active carbonyl compounds such as *N*-hydroxysuccinimide (NHS) ester, anhydrides, and acid chlorides [19] have been reacted with amino-sugars, including *N*-acetyl lactosamine [203], 2-deoxy-D-glucosamine [19, 204], and galactosamine [19].

Takasu et al. [205, 206] reported the direct introduction of sugar residues into poly(vinyl alcohol) (PVA), although this method is limited by the low reactivity of the hydroxyl functionality. The method was based on the stereoselective glycosylation reaction of a hydroxyl group in PVA and a sugar oxazoline in the presence of an acid as a promoter, producing exclusively the β-*O*-glycoside linkage. Alternatively, PVA was converted into a reactive ester using *p*-nitrophenyl chloroformate followed by reaction with different amino-saccharides. The produced glycopolymers showed specific interaction with Con-A and RCA [124, 207].



Scheme 14 RAFT polymerization of pentafluorophenyl acrylate followed by post-functionalization with an aminoglycoside

Living ROMP has been exploited to polymerize norbornenes functionalized with either pentafluorophenyl or NHS esters to produce a reactive backbone. Subsequent treatment with an amine-functionalized mannose residue in the presence of a carbodiimide coupling agent (required to achieve a high degree of substitution) resulted in glycopolymers with high activity for Con-A, as proven by hemagglutination assays [104]. Efforts have also been made to polymerize active ester monomers by CRP techniques [208]. Müller and coworkers [209] demonstrated the ATRP of activated ester monomers using Cu(I)/bipy in DMSO, which was later optimized by Haddleton et al. [210–212]. Well-defined poly(*N*-acryloxysuccinimide) was substituted quantitatively by gluco- and galactosamines [105]. Müller et al. reported examples of linear and hyperbranched glycopolymers grown successfully from surfaces of multiwalled carbon nanotubes by the "grafting from" strategy with good controllability and high reproducibility using ATRP [213].

Lately, Theato [214] and others [215] have demonstrated the synthesis of well-defined polymeric activated esters using RAFT polymerization. To the best of our knowledge, there is only one communication, reported by Boyer and Davis [101], on the investigation of this methodology to produce glycopolymers (Scheme 14).

Alternating copolymers of maleic anhydride with alkene [216] or *N*-vinyl-pyrrolidone [106] have been employed for the attachment of sugar-amines. The surface of carbon nanotubes has also been functionalized with highly reactive maleic anhydride groups, which were further reacted with long polymer chains or small functional molecules with hydroxyl or amino group [217].

Other methods for modifying polymers with carbohydrates via the post-polymerization technique include the photo-grafting of poly(ethylene terephthalate) (PET) fibers with glycosyl azides [218] and the use of carbohydrates bearing isocyanate [219] or aldehyde [220] groups, which react efficiently with polymers bearing pendant amino residues.

4 Self-assembly of Linear Glycopolymer Conjugates

Linear amphiphilic glycopolymer conjugates that can self-assemble into well-defined nano- to micro-sized structures such as micelles, vesicles (polymersomes), α-helices, and worm-like aggregates are of increasing interest as means for drug

transport and biosensing [34, 221]. These complex supramolecular polymeric structures display bioactivity, which originates from the multivalent effect of the multiple copies of carbohydrate groups presented on the surface of the nanostructure. Moreover, they exhibit three-dimensional structures, which provide greater surface area that may enhance affinity towards lectins. Therefore, although polymers end-functionalized with a single carbohydrate unit are not included in the earlier provided definition of glycopolymers, selected examples of their synthesis and self-assembly are presented and summarized in Table 3.

A number of block copolymers containing carbohydrates that produce self-assembled nanostructures have been developed for receptor-mediated targeting to specific tissues or cells for the delivery of therapeutics [60, 110, 222]. Also, glycopolymers capable of self-assembling into capsule-like structures with sugar functionality presented into the solution have recently found potential as a first step towards cell biomimicry [27, 109, 223]. Compared to natural liposomes, synthetic glycopolymer vesicles (glycopolymersomes) offer all the advantageous features of synthetic polymers (broadly adjustable properties including solubility, stability, elasticity, fluidity, dynamics, and permeability) as well as those of biological polymers (including functionality, multivalency, and biocompatibility) [26, 224]. Nonetheless, designing supramolecular glycomimics that can interact with biological entities through the language of the glycocode remains a central challenge. In this section, successful examples of linear glycopolymer conjugates that are capable of self-assembling into various architectures in solution are highlighted and their fundamental importance and potential applicability in materials science and biomedicine are discussed. The self-assembly behavior of glycoconjugates onto surfaces is not the focus of this article.

Chaikof et al. demonstrated the self-assembly of a glycopolymer-polypeptide A–B–A triblock copolymer, PLG-poly(2-acryloyloxyethyllactoside)-PLG, in water into large spherical micelles. Fourier transform infrared (FTIR) spectroscopy of the triblock copolymers revealed that the α-helix/β-sheet ratio increased with the Pγ-BLG block length. The morphology of the self-assembled structures changed from spheres to lamellae, then to worm-like micelles, by altering the initial copolymer concentration. The multivalent interaction of RCA_{120} with these lactose-installed polymeric aggregates was investigated by UV–vis spectroscopy. It was also claimed that this kind of aggregate may be useful as artificial polyvalent ligands in the investigation of carbohydrate–protein recognition and for the design of site-specific drug delivery systems [80, 81].

A biodegradable material based on PCL-β-PGAMA was developed. It was found that the self-assembled glucose-containing aggregates changed in shape from spherical micelles to worm-like aggregates, then to vesicles with decreasing weight fraction of the hydrophilic PGAMA block as revealed by TEM and fluorescence spectroscopy. The work was extended to prepare a polypseudorotaxane-centered triblock copolymer by inclusion of α-cyclodextrin into the PCL block. Their binding with Con-A was demonstrated by UV–vis spectroscopy and dynamic light scattering (DLS) [76, 77].

In two separate reports, Stenzel et al. exploited two different types of Click chemistry, thiol–ene and CuAAC, to produce thermoresponsive glycopolymer micelles as potential candidates for targeted drug delivery vehicles. A block copolymer containing di(ethylene glycol) methyl ether methacrylate (DEGMA) and HEMA was first prepared by RAFT polymerization; subsequent modification with glucothiose yielded the glycosylated block copolymer [102]. In another study, 2′-(4-vinyl-[1,2,3]-triazol-1-yl)ethyl-*O*-α-D-mannopyranoside monomer was polymerized in the presence of a RAFT agent (3-benzylsulfanylthiocarbonylsulfanyl propionic acid) to yield well-defined polymers with molecular weights up to 51,500 g mol^{-1} and a PDI of ~1.16. The 1,2,3-triazole linkage between mannose and the polymer backbone ensured the formation of highly stable glycopolymers, which did not undergo hydrolysis. The resulting polymer was employed as a macroRAFT agent in the polymerization of NIPAAm in order to generate thermoresponsive block copolymers, which undergo reversible micelle formation at elevated temperatures. The micellar glycopolymer structure showed high affinity to Con-A [98].

A simple procedure to pattern proteins onto surfaces was developed. The carbohydrate-based amphiphilic diblock copolymer, poly{styrene-*block*-[2-(β-D-galactosyloxy)ethyl methacrylate-*co*-styrene]} [PS-*b*-P(GalEMA-*co*-S)], was used for micellization and the production of honeycomb-structured porous films. Lectin binding assays using UV–vis spectroscopy and DLS showed that fluorescent PNA was successfully conjugated with the sugar moieties on the micelles and inside the pores of the porous films (Fig. 4) [62].

A rod–coil amphiphile, tetra-(*p*-phenylene)-*block*-PEO-α-D-mannopyranoside, was shown to form small vesicles that interacted specifically with the pili of a particular *Escherichia coli* bacterial strain [111]. A similar architecture was designed that involved a β-cyclodextrin as a head group that was coupled to polystyrene. The hydrophobic interior of the formed vesicle was used to attach hydrophobic fluorescent dyes and the adamantine-coupled enzyme horseradish peroxidase to the surface [112].

Alexander and Pasparakis [27] elegantly designed double hydrophilic block copolymers, based on PGEMA as one block and the more sparingly water-soluble poly(diethyleneglycol methacrylate) (PDEGMA), that assembled into vesicles displaying glucose functionality on the surface. The size of these vesicles was controlled by comonomer content, block ratio, molar mass, and LCST. These vesicles were considered as artificial cell mimics with potential applications in cell sensing, therapeutics, and synthetic biology.

Otsuka et al. [113] recently reported the self-assembly properties of thermoresponsive hybrid oligosaccharide block copolymers, maltoheptaose-*block*-PNIPAM. Those copolymers were synthesized via CuAAC Click chemistry between an alkynyl-functionalized maltoheptaose and PNIPAM having a terminal azido group prepared by ATRP. Well-defined vesicular morphologies with an approximate diameter of 300 nm and cloud point (T_{cp}) that ranged from ~36 to ~52°C depending on the degree of polymerization were obtained, as confirmed by static and DLS as well as TEM (Fig. 5).

A series of peptide–carbohydrate hybrid block copolymers, such as dextran-β-PBLG [114], dextran-*b*-PS, and hyaluronan-*b*-PBLG [115], have been utilized

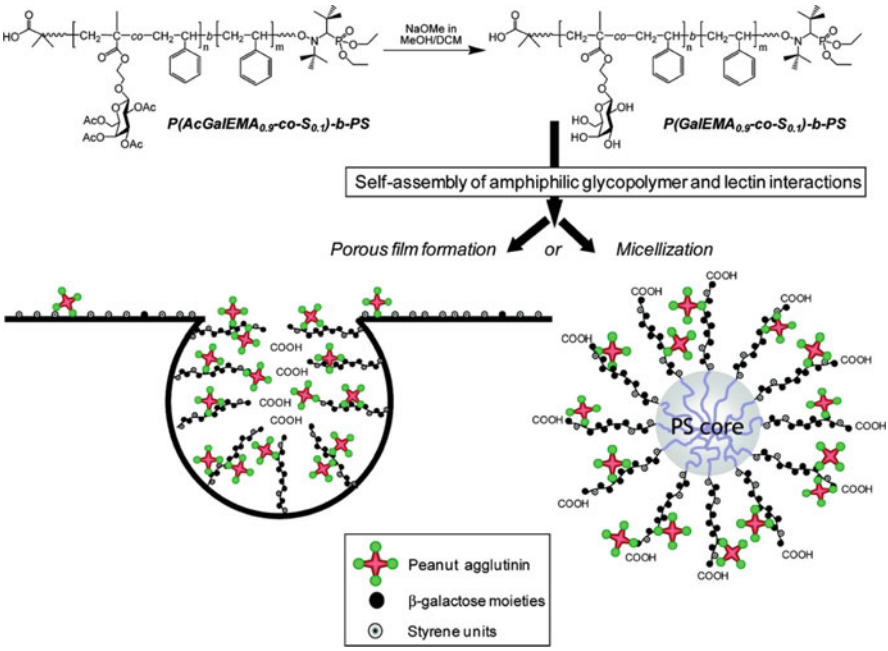


Fig. 4 Synthesis of micelles with glycopolymer shell based on β-galactose moieties (*right*) and honeycomb structured porous films with glycopolymer enriched inside the pore (*left*). These are materials in solution and in solid state, respectively, that can selectively recognize PNA (*Arachis hypogaea*). Reprinted from [62] with permission. Copyright 1998, American Chemical Society

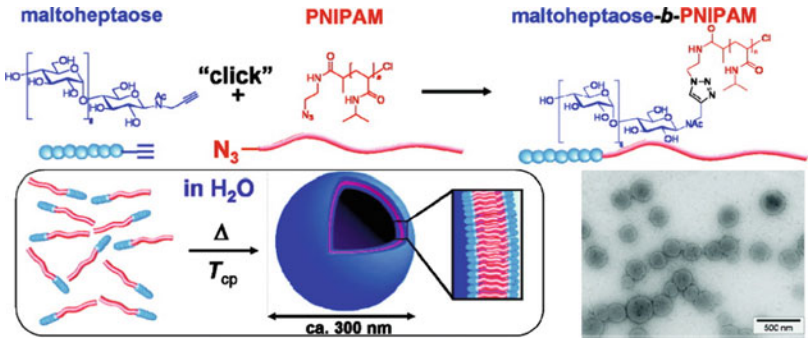


Fig. 5 Thermoresponsive hybrid oligosaccharide block copolymers made via CuAAC Click chemistry and characterized by TEM. Reprinted from [113] with permission. Copyright 2010, American Chemical Society

for polymersome formation. Lecommandoux et al. [115, 125, 126] demonstrated the usefulness of these biohybrid vesicles in drug delivery and tumor-targeting applications, as revealed by both in vitro and in vivo studies.

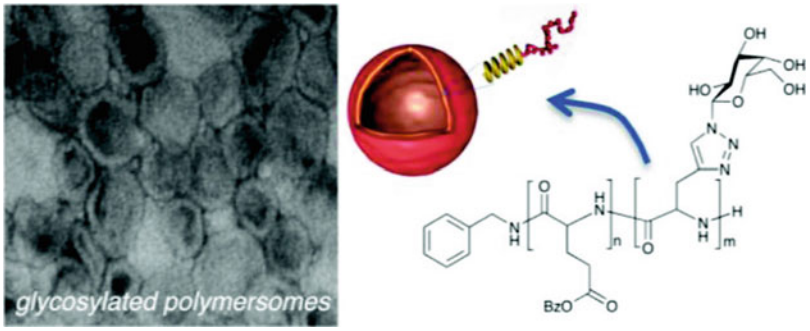


Fig. 6 Glycosylated peptide block copolymers. Reprinted from [107] with permission. Copyright 2011, American Chemical Society

Recently, Lecommandoux and Heise [107] utilized a combination of controlled NCA ROP and Click glycosylation reactions to afford well-defined amphiphilic galactose-containing block copolymers. The method was based on sequential ROP of BLG and propargylglycine (PG) NCAs followed by glycosylation of the poly (PG) block by CuAAC Click reaction using azide-functionalized galactose. The produced copolymers were self-assembled using the nanoprecipitation method to obtain spherical and worm-like micelles as well as polymersomes, depending on the block copolymer composition and the nanoprecipitation conditions (Fig. 6). The self-assembled structures were characterized by DLS, TEM, and AFM. These structures also displayed bioactive galactose units in the polymersome shell, as demonstrated by selective lectin binding experiments with RCA_{120}, not Con-A.

5 Conclusions

The precise and highly sophisticated recognition properties of natural glycoconjugates originate from their controlled structures, including chain length, composition, and topology. In order to mimic these naturally occurring glycoconjugates, a breakthrough was needed in the development of methodologies for synthesizing glycopolymer conjugates with not only a controlled molecular structure but also an organized molecular assembly. This review provides an insight into recent advances in glycopolymer synthesis. Biological functions and self-assembling properties of some glycopolymers are discussed in relation to their structures. Interactions between glycopolymers and lectins are greatly influenced by many factors including the rigidity of the polymer, the density of sugar molecules, and the architecture and molecular weight of the glycopolymer.

In summary, refinements in robust and well-developed techniques in the field of carbohydrate and polymer synthesis are now enabling the production of glycopolymers possessing controlled structures and tuneable interactions with lectins. Indeed, it is anticipated that many glycomimics of commercial significance will be

generated in the near future, exhibiting a biological function surpassing that of the corresponding natural glycoconjugate.

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DNA–Polymer Conjugates: From Synthesis, Through Complex Formation and Self-assembly to Applications

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Abstract With this review, we aim at achieving a comprehensive and up to date look at the broad topic of DNA conjugates as well as summarizing the important trends in the particular field of materials based on nucleic acids.

Keywords Conjugates · DNA · Micelles · Oligonucleotide · Self-assembly · Vesicles

Contents

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Abbreviations

CD	Circular dichroism
cDNA	Complementary DNA
CEPA	2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidite
CPG	Controlled pore glass
DBC	Diblock copolymer
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DIC	*N*,*N*′-diisopropylcarbodiimide
DMT	*N*,*N*-dimethyltryptamine
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
MPEG	Monomethoxy polyethylene glycol
NHS	*N*-hydroxysuccinimide
ODN	Oligodeoxynucleotide
PAGE	Polyacrylamide gel electrophoresis
PB	Poly(butadiene)
PCl	Poly(caprolactone)
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PIB	Poly(isobutylene)
PLGA	Poly(D,L-lactic-*co*-glycolic acid)
PNIPAM	Poly(*N*-isopropylacrylamide)
PPO	Poly(propylene oxide)
QCM	Quartz crystal microbalance
RNA	Ribonucleic acid
siRNA	Small interfering RNA
SPS	Solid-phase synthesis
ssDNA	Single-stranded DNA
TBC	Triblock copolymer
TEM	Transmission electron microscopy
TTP	Thymidine triphosphate

1 Introduction

Owing to their inherent structural and biological properties, nucleic acids are fascinating macromolecules. Indeed, deoxyribonucleic acid (DNA) exhibits an incomparable capability for molecular recognition and plays a crucial role in heredity mechanisms as well as protein synthesis. However, a current focus of

research is the synthesis of DNA–polymer conjugates resulting from the coupling of a nucleotide sequence to a synthetic polymer segment. The development of efficient therapeutic agents based on nucleotide sequence delivery has been largely hampered by their reduced bioavailability, mainly because of their characteristic polyanionic nature. Poor intracellular delivery, limited access to the specific target, and low resistance to nucleases are the major obstacles to the in vivo efficacy of synthetic, non-modified oligodeoxyribonucleotides (ODNs), which therefore limits their use as gene expression regulators in antisense, antigene, or aptamer therapeutic strategies [1, 2]. The low biostability in cells has emerged as the most relevant limit to in vivo applications of synthetic oligonucleotides containing only 3′- and 5′-phosphodiester linkages. Therefore, many research efforts have been devoted, in the past decade, to overcome these drawbacks by the synthesis of synthetic DNA or RNA analogues [3], which is beyond the scope of this review, or by their conjugation to polymers as described herein.

As reviewed here, both water-soluble and self-assembling DNA–polymer conjugates have been synthesized. Of particular interest are the resulting amphiphilic macromolecules, which self-assemble into structures of various shapes. The fine balance between several intermolecular interactions between the self-assembled DNA–polymer conjugates, such as hydrophobic or electrostatic interactions, direct the self-assembly into a given morphology such as spherical core–shell micelles or vesicular structures. Nucleotide sequences are indeed charged polyelectrolytes [4, 5], and colloidal forces govern the stability of the suspension of the resulting like-charged self-assembled structures [6, 7]. However, nucleotide sequences interact through several binding modes other than electrostatic interactions [8, 9]. Therefore, the properties of the self-assembly will affect the function of the DNA fragment involved in the self-assembly. For instance, the higher molecular crowding in vesicular structures than in spherical core–shell micelles is expected to hamper the ability of the self-assembled nucleotide sequences to undergo optimal hybridization. The conjugation of polymers to DNA therefore paves the way for the development of potential applications of high interest as well as for the achievement of a comprehensive understanding of the structure formation and modes of interaction of the self-assembled DNA–polymer conjugates.

As described herein, various chemical routes for the synthesis of DNA–polymer conjugates have been established (Sect. 2), enabling the design of both water-soluble (Sect. 3) and self-assembling macromolecules (Sect. 4) composed of either synthetic or natural polymer segments. The toolbox, which is being constituted with potential pharmaceutical and biomedical applications in mind, is an invaluable device for achieving a comprehensive understanding and eventually establishing a mechanism of the structure formation inherent to the modes of interaction of these novel DNA–polymer conjugates.

2 Synthesis

Existing chemistry routes allow the versatile synthesis of DNA–polymer conjugates through a large choice of the composition, length, and architecture of both ODN sequences and polymer segments. Conjugation of ODN can be conducted at the 2′-position of the ribose sugar, nucleobases and internucleotidic phosphodiester bonds. However, the most common reactions take place at the 5′- or 3′-terminus, due to high accessibility of these positions. It has been reported that this strategy has also another advantage, namely, conjugation through the 3′-terminus enhances the exonuclease resistance of the nucleotide sequence [1]. Conjugation of oligonucleotide is considered difficult because even slight changes in its structure may affect its biological properties (e.g., exonuclease resistance), which is crucial regarding the potential future uses of the resulting DNA–polymer conjugates. Therefore, the specific sites for conjugation should be cautiously chosen. Moreover, conjugation sites may have limited access along the ODN fragment, which also needs to be taken into consideration. An additional issue is that the two polymer and nucleotide sequence entities may show different stabilities to the reaction conditions and may not be congruous [10].

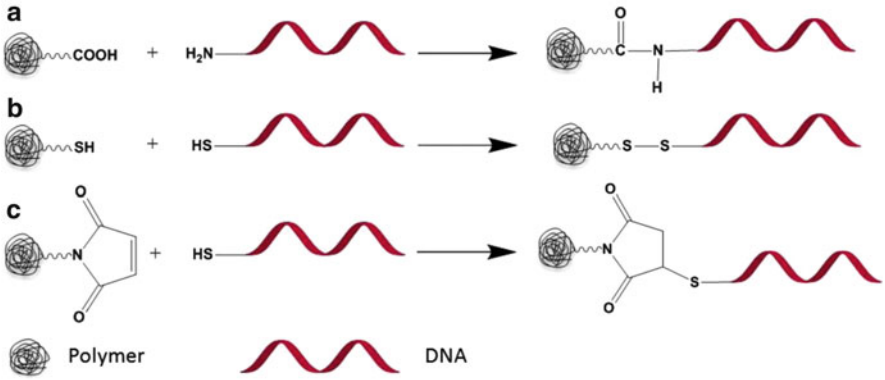
Methods of DNA conjugation to polymers mainly rely on coupling terminal groups (grafting strategy), which is presently the most efficient approach. Basically, there are two principal approaches to the binding of an ODN sequence with a polymer: solid-phase synthesis (SPS) and solution coupling. Solution coupling can be used for achieving both water-soluble and amphiphilic DNA–polymer conjugates. However, carrying out a solution reaction in order to obtain an amphiphilic product usually leads to low yields because of the chemical incompatibility of the two reactants [11]. In the last few years, new methods of DNA conjugation with polymers have been reported, such as enzymatic reactions, as described in the Sect. 2.3.

2.1 Solution Coupling

Solution coupling requires separate syntheses and purification of the DNA sequence and polymer segment, followed by the reaction, which drives the generation of both reversible and irreversible bonds [1]. The polymer reactant is usually used in slight excess.

Three main different methods (see Scheme 1) for coupling DNA and organic polymer segments in water have been reported: amide bond formation [12], disulfide bond formation [13], and Michael addition [13].

In the first approach, the components are coupled by a peptide bond. The amino group of the ODN fragment reacts with the activated carboxyl group of the polymer. Among the most popular activating agents used for this kind of reaction are *N*,*N*′-diisopropylcarbodiimide (DIC), *N*,*N*′-dicyclohexylcarbodiimide

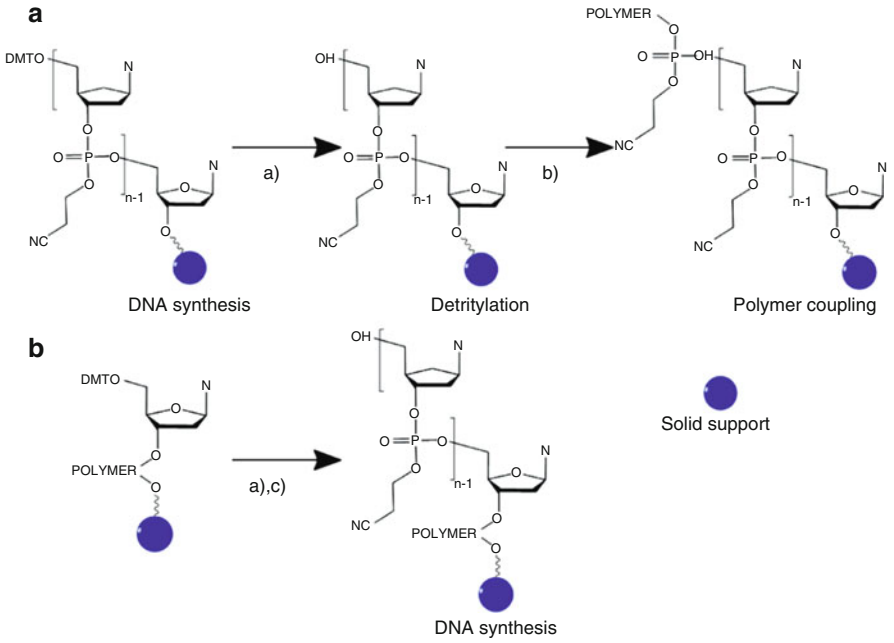


Scheme 1 Solution coupling strategies for DNA conjugation with polymers: (**a**) amide bond formation, (**b**) formation of disulfide bridges between the thiol groups of both components, and (**c**) Michael addition of maleimide to ODN fragments functionalized with a sulfhydryl terminal group [11] (figure adapted with permission of Wiley-VCH, Weinheim)

(DCC), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). In order to bind an ODN with a polymer by a disulfide linkage, both entities have to be modified with sulfhydryl groups, one of these groups being activated. For a Michael addition reaction, a thiol modification of the ODN is also required. Linkage is formed by the nucleophilic addition of thiol to an electron-deficient carbon. Polymer is usually modified with maleimide or acrylic acid. This reaction should be carried out at neutral pH. All the types of reactions mentioned above are very efficient and convenient. High yields of DNA–polymer conjugates achieved by solution coupling have been reported with segments such as poly(*N*-isopropylacrylamide) (PNIPAM) [14], polyethylene glycol (PEG) [15], and several polysaccharides [2]. Synthesis of water-soluble DNA derivatives can be prepared in standard laboratory conditions in a very straightforward way, without an expensive automatic DNA synthesizer. Moreover, DNA sequences with various modifications are commercially available.

2.2 Solid-Phase Synthesis

It was previously mentioned that the preparation of amphiphilic DNA–polymer conjugates by solution coupling is rather challenging, especially in achieving satisfying yields. Therefore, SPS is mainly used. However, SPS could be also used in order to achieve water-soluble compounds [16, 17]. Choosing this strategy avoids performing tedious chemistry and purification steps [18]. Moreover, over the course of the SPS reaction, potentially reactive groups along the ODN fragment are protected and thus the number of side products is limited. There are two classes of SPS strategies: programmed, fully automated reaction in a DNA synthesizer and

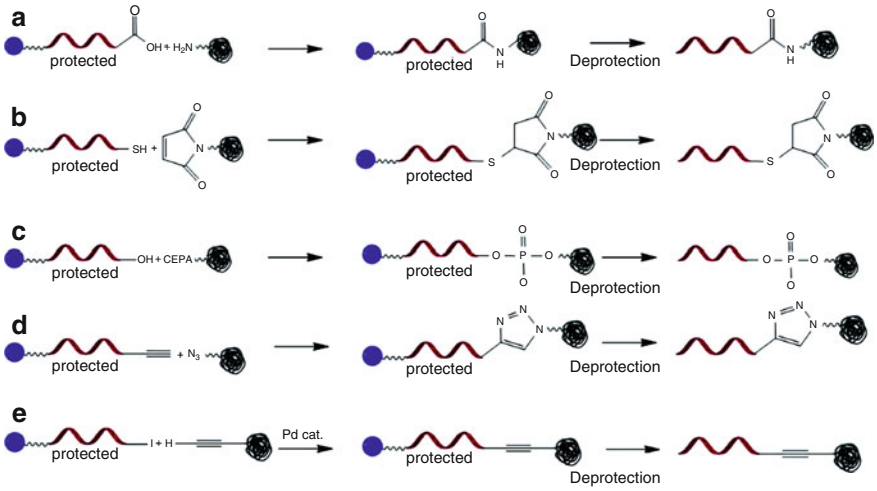


Scheme 2 Synthetic scheme of the coupling of DNA with polymers at the (**a**) 5′-end and (**b**) 3′-end of the DNA: (*a*) deblocking of DMT, (*b*) coupling of activated CEPA to the 5′-end, and (*c*) standard synthesis with nucleoside phosphoramidite. Subsequently, in order to obtain the conjugate, cleavage from the solid support is conducted prior to the washing out of the 2-cyanoethyl groups [19] (figure adapted with permission of the Royal Society of Chemistry)

modified methods that take advantage of simple tools like syringe filters. The advantages of automated synthesis are precise control over the reaction, large scale and fine reproducibility but the cost of using this approach is quite high [19]. On the other hand, with modified methods, a product that perfectly matches special needs can be produced [19].

The most fundamental method in automated DNA–polymer conjugate synthesis is coupling to the 5′-end of oligonucleotides using 2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite (CEPA) reactive groups (Scheme 2, top). CEPA-modified polymers react with the detritylated 5′-end of ODNs bound to a solid support, usually a controlled pore glass (CPG) resin. To couple the polymer segment at the 3′-end of the DNA (Scheme 2, bottom), it is necessary to begin with the solid-supported polymer or to carry out reverse synthesis, in which the desired compound is attached to the 3′-end of the sequence elongated in the 5′- to 3′-end direction. After cleavage from the resin and deprotection with a concentrated ammonia solution, ODN-based copolymers could be achieved [19].

Disadvantages of the synthesis of DNA copolymers without a synthesizer are less convenience and more time consumption but this type of method is especially helpful in the case of solvent or catalyst incompatibilities. The conditions of post-DNA synthesis change depending on factors such as chemo-stability, catalyst and



Scheme 3 SPS strategies that can be used to achieve DNA block copolymers: (**a**) amide bond formation, (**b**) Michael addition, (**c**) phosphodiester bonding, (**d**) Huisgen reaction, and (**e**) Sonogashira–Hagihara reaction [17] (figure adapted with permission of Wiley-VCH, Weinheim)

chemical structures. However, ex situ approaches allow the preparation of linear and brush-type DNA–polymer conjugates [11]. We briefly present the different strategies for ex situ synthesis on a solid support. The mechanisms are the same as in the case of reactions conducted in aqueous solution [18, 19]. A Huisgen reaction occurs between an azide-modified oligonucleotide and an alkyl-terminated component to form a 1,2,3-triazole (Scheme 3d). This reaction is almost quantitative and can be conducted in aqueous solution. The efficiency of a Huisgen reaction is greatly increased when it is catalyzed by Cu^{I} [20, 21]. Sonogashira–Hagihara coupling is a reaction between terminal alkynes and an aryl halide, catalyzed by palladium complexes. The reaction is conducted in the presence of catalytic amounts of Cu^{I} and an amine base (Scheme 3e) [17, 22].

2.3 Enzymatic Reactions

The greatest disadvantage of the SPS approach is restriction to short lengths of the nucleic acid sequence and polymer segments due to the limited diffusion of the reactants through the pores of the solid support, which limits the use of porous solid beads, for example, to low molecular weight polymer segments to be coupled to the ODN [23].

In order to overcome this obstacle, elegant molecular biology methods have been adapted to the synthesis of DNA–polymer conjugates. DNA polymerase chain reactions (PCR) hold great promises because they allow the generation of

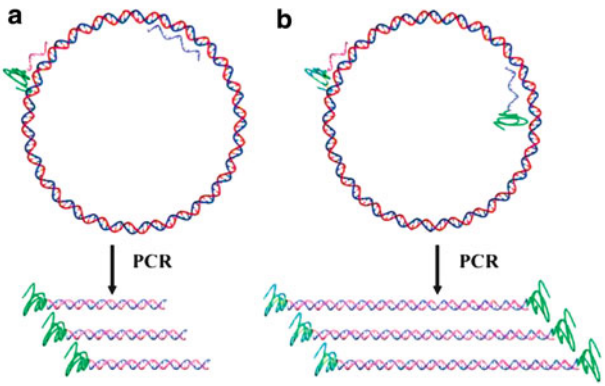


Fig. 1 Building-up of (**a**) DNA diblock and (**b**) DNA triblock copolymers using PCR (Safak et al. [23]) (reprinted with permission of Wiley-VCH, Weinheim)

DNA–polymer conjugates with longer DNA fragments. Herrmann and coworkers applied this strategy to the synthesis of DNA–polymers conjugates. The DNA fragment coupled to the polymer segment acts as a primer to generate double-stranded (ds) diblock copolymers (DBC) with DNA fragments of extended length up to 1,578 bp. A total average molecular weight greater than 1,000 kDa and monodisperse nucleic acid chains could be achieved.

In brief, prior to PCR, ssDNA copolymers, which act as primers, were prepared by solution coupling (PEG, PNIPAM) or by SPS (PPO). The plasmid pBR322 was used as a template for the PCR reaction and the four deoxyribonucleotide triphosphates served as monomers; the *Taq* DNA polymerase was chosen as the enzyme. The length of the DNA fragment was tuned by choosing different annealing sites of the primer on the template (Fig. 1) [24].

Starting from a ssDNA diblock copolymer-like primer results in a triblock dsDNA architecture. Symmetric as well as asymmetric triblock chain configurations could be achieved, i.e., A–DNA–A or A–DNA–B (where A and B are different polymers) [23].

Herrmann and coworkers demonstrated in 2009 that the use of PCR enables the generation of even more complex block copolymer architectures, like DNA–A–DNA and A–DNA–A–DNA–A conjugates. In these cases, the DNA conjugates and DNA nanoparticles act as primers. To produce triblock copolymers composed of nucleic acid sequences of length up to 500 bp, the authors used a triblock copolymer composed of two identical 22-mer ssODNs attached to PEG (ssTB1). The pBR322 plasmid acts as the template for PCR. In order to obtain pentablock structures, ssTB1 and several ssDNA diblock copolymers were implemented in PCR [24].

The same group also showed how to control the size of amphiphilic DNA block copolymer nanoparticles by enzymatic reaction. In brief, particles obtained from block copolymers synthesized in a DNA synthesizer were incubated with terminal deoxynucleotidyl transferase (TdT) at 37°C in the presence of Co^{2+} and

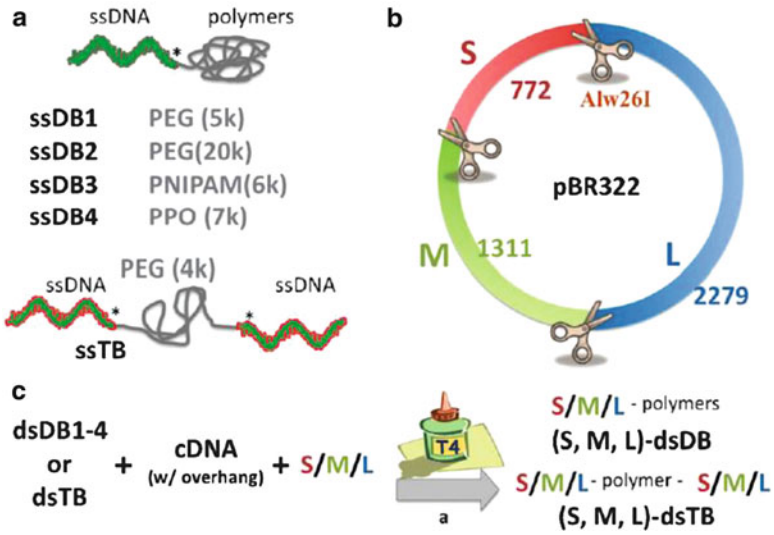


Fig. 2 Synthesis of DBCs with extended nucleic acid segments. (**a**) Four ssDNA DBCs and a ssTBC were synthesized; (**b**) enzymatic digestion of pBR322 by Alw26I gives three dsDNA fragments (*S*, *L*, and *M*); (**c**) mixed components in the presence of T4 DNA ligase gives extended dsDBCs or dsTBCs [26] (figure reproduced with permission from Royal Society of Chemistry)

deoxythiamine triphosphate (dTTP). Subsequent to reaction, the growth of the nanoparticles was analyzed by various techniques like scanning force microscopy (SFM), fluorescence correlation spectroscopy (FCS) and polyacrylamide gel electrophoresis (PAGE) [25].

More recently, Herrmann and coworkers proposed a new synthetic strategy for the preparation of polymer conjugates with extremely long DNA blocks, inspired by the field of molecular biology and based on enzymatic restriction and ligation. This method can be applied to the generation of both hydrophilic DNA–polymer conjugates as well as amphiphilic DBCs, with DNA lengths varying between 800 and 2,300 bp and M_w in the range of 3000 kDa. These studies also clearly show that thermoresponsive, hydrophobic and hydrophilic polymers are in accordance with the activity of the respective enzymes used to achieve the synthesis. The same components as for PCR can be used starting with the synthesis of single-stranded di- and triblock copolymers. DNA fragments of three different lengths (small, middle, long; S, M, L), used to eventually achieve copolymers composed of large DNA fragments, were obtained by a process of circular plasmid DNA digestion by a DNA restriction enzyme (Alw26I) and subsequent separation using gel electrophoresis. In a next step, the ssDBC or ssTBC is reacted with the appropriate complementary DNA (cDNA), i.e., a nucleotide sequence composed of overhangs, which allows sticking the ssDNA and dsDNA fragments (S/M/L). Simple stirring and incubation yield the elongated double-stranded diblock or triblock architectures after enzymatic ligation (Fig. 2) [26].

In 2010, for the first time, Baccaro and Marx reported the enzymatic synthesis of DNA copolymers, composed of a DNA backbone and an organic polymer as a side chain synthesized by PCR. First, analogues of a thymidine-bearing polymer moiety (PEG) were prepared and subsequently converted into triphosphates. In order to incorporate polymer-functionalized building blocks (TTP), primer and nucleotide templates (304 and 1,062 bp, respectively) containing one adenine (A) residue were used. Subsequently, the original TTP moieties were substituted by modified triphosphates under the activity of the thermophilic enzyme $9°N_m$ DNA polymerase [27].

3 Water-Soluble DNA–Polymer Conjugates

We will next describe the most recently reported DNA–polymer conjugates, resulting from the covalent binding between a DNA fragment and a water-soluble polymer segment such as a carbohydrate, a poly(amine) or a PEG polymer segment. The resulting water-soluble macromolecules reveal a high potential for applications in pharmacology, gene therapy and chemotherapy, as summarized and further discussed in this section.

A large variety of reports describe the conjugation of polymers to achieve DNA–polymer conjugates. An overview of the three major classes and their potential uses in therapeutics, diagnostics and nanotechnology is given in the sections on (1) DNA–carbohydrate conjugates, (2) DNA–polyamine conjugates, and (3) DNA–synthetic polymer conjugates.

3.1 DNA–Carbohydrate Conjugates

DNA–carbohydrate conjugates are mainly considered for their ability to improve the poor cell- or tissue-specific delivery of nucleotide sequences through cell-receptor-mediated endocytosis. In Nature, glycolipids and glycoproteins play key roles in the recognition processes that occur at the cell surface, whereas glycoconjugates take part in various vital processes such as extracellular matrix–cell interactions, cell–cell adhesion, and viral invasion. Carbohydrate–protein interactions play a central role in cellular recognition since multi-antennary carbohydrates are anchored to the proteins or lipids that constitute the cell membranes. The preparation of carbohydrate–oligonucleotide conjugates by SPS or solution phase coupling has been comprehensively reviewed elsewhere [2]. Intensive investigations of carbohydrate–protein interactions have been reported and evidence their enhanced cellular uptake. Herein, potential future applications of the resulting DNA–carbohydrate conjugates are highlighted.

Investigations on the interactions of glycosylated nucleotide sequences with proteins and the effective cell-specific delivery of DNA–carbohydrate conjugates

have been reported [2, 28–30]. Glycosylation of the bases increases the resistance of nucleotide sequences to degradation by nucleases and, as such, a modified DNA could be enrolled in the regulation of transcription. DNA sequences composed of glycosylated bases could be located in T7 phages, bacteriophages SP-15 and RL38JI, protozoa *Trypanosoma brucei*, other kinetoplastids, and in the related eukaryote *Euglena gracilis*. Similarly, RNA sequences bearing glycosylated bases were found in rabbit and rat liver. It is thus straightforward to assume that DNA–carbohydrate conjugates can provide advantages other than enhanced cellular uptake (such as in vivo organ localization) and enhanced bioefficiency (like low toxicity of the components of the conjugates, even at elevated levels). In addition to the structural homogeneity of both the starting material and product, the components of the conjugates are in defined relative proportions to each other. These advantages of DNA–carbohydrate conjugates have been assessed in antisense biotechnology through the in vitro study of the efficiency of glycosylated DNA. To this end, mono- and disaccharide phosphoramidites, solid phase supported carbohydrates, glycosylated nucleoside phosphoramidites, and DNA–carbohydrate conjugates resulting from the post-synthetic conjugation of reactive sugar derivatives with nucleotide sequences, have all been systematically studied [21, 31–33]. The major outcome from these studies was the possibility to modulate the fundamental role conferred by the polymer segment to cell-to-cell communication (e.g., recognition properties by specific cell-surface membrane receptors such as lectins) by modifying polymer properties such as composition, length and stereochemistry.

Hyaluronic acid, a linear polysaccharide composed of a repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine, is the main ligand of a transmembrane glycoprotein CD44 that is overexpressed by many cancer cells [34]. Besides, hyaluronic acid conjugates of cancer drugs have been shown to exhibit increased uptake by cancer cells [35]. Galactosylated PEG-oligonucleotide phosphorothioate conjugates have therefore been synthesized and showed enhanced cellular uptake via the galactose-specific asialoglycoprotein-receptor-mediated endocytosis, similarly to antigenic peptides targeted to dendritic cells subsequent to an appropriate glycoconjugation [36].

There are therefore two main areas of potential application for DNA–carbohydrate conjugates. First, these conjugates can be used effectively for the investigation of biological processes in vitro. For instance, a detailed study of DNA sequences conjugated to 2-deoxy-5-[(β-D-glucopyranosyl)oxymethyl] uridine (nucleoside J) has been carried out in vivo [37, 38]. This hypermodified nucleoside has been found in *T. brucei* and in telomeres [37, 39], and in chromosome internal repeats of some primitive eukaryotes [40]. Interestingly, J-containing DNA was found to be resistant to degradation by nucleases [38].

The second main area of application results from the high selectivity of DNA–carbohydrate conjugates for cell recognition and increased specific cellular uptake due to stability against nucleases. 5-Neoglycoconjugates have revealed excellent cell-type specificity and cellular uptake in vitro and in vivo [41–45]. For instance, Hangeland et al. [41] demonstrated that the neoglycoconjugate

DNA–methyl-phosphonate is delivered to human hepatocellular carcinoma in a ligand-specific manner, reaching a peak value of 26 pmol per 106 cells (a 43-fold enhancement over the pristine oligonucleotide) [41]. As the uptake of the DNA–neoglycopeptide conjugates is significantly enhanced as compared to the free neoglycopeptide or when the carbohydrate moieties are enzymatically removed, one can confirm cell-specific oligonucleotide uptake.

3.2 DNA–Polyamine Conjugates

Being positively charged at physiological pH, polyamines form complexes with negatively charged nucleotide sequences. The resulting complexes, known as polyplexes, might interact with the negatively charged phospholipids of the cell membrane and, hence, enhance the cellular uptake of nucleotide sequences [46–53]. Polyamines and complexes thereof with DNA or RNA sequences, which are held by non-covalent electrostatic interactions, are frequently used as non-viral vectors for gene delivery applications [47, 48]. Dendrimeric polyamines are also extensively used as transfection agents. In the cellular environment, polyamines take over the function of the phosphate counterions. In addition to their role in regulation of cell growth and maintenance of cell viability, natural polyamines such as putrescine, spermine and spermidine are involved in the regulation of gene function [47–50]. Besides, stabilization of DNA and RNA duplexes has been evidenced in vitro [52, 53], especially that of duplexes assembled by imperfect base pairing [54]. It is also worth noting that some oligoamines, such as spermine and spermidine, are naturally occurring metabolites known to complex in cells with RNA [46].

Covalent DNA–polyamine conjugates have thus received much attention as potential enhancers of the delivery and biodistribution of therapeutic nucleotide sequences. Several possibilities for polyamine conjugation to nucleotide sequences have been reported [55–60]. Most of these reports describe polyamine tethering to a base. Oligoamines, either linear or branched, are usually attached to either the 5′-terminal hydroxyl function of the nucleotide sequence or to the C_5 site of a pyrimidine base. Alkyl-modified ω-bis(4-hydroxybutyl)spermine molecules have been incorporated as trifluoroacetyl-protected phosphoramidite reagents at the 5′-terminus of nucleotide sequences, giving a long linear spermine-phosphate tail. The coupling efficiency of the resulting conjugates has been reported to be as high as 90–96%. Enhanced stability of DNA and RNA duplexes in vivo has been assessed subsequent to conjugation [31, 61].

Using an easy and versatile procedure for oligonucleotide–polyamine conjugation [46], polyamines were conjugated to distinct terminal and internal positions along nucleotide sequences. Upon polyamine attachment to terminal nucleosides, the hybridization efficiency with the complementary nucleotide sequence increased with the number of cationic amines. However, tethering to an internal nucleoside resulted in a considerable decrease in duplex stability. However, in phosphorothioates

(a variant in which one of the non-bridging oxygen atoms is replaced by sulfur) no significant effect on the stability of the duplex has been monitored. Furthermore, the conjugates exhibited higher target downregulation ability with increasing polyamine chain length in a human melanoma cell culture assay. The main outcome of these investigations was the finding that the duplex secondary structure is not dependent on the length of the attached polyamine. As assessed by melting point determinations, only the stability is affected and increases with increasing polyamine length when conjugation occurs at a terminal position. Correlation between duplex stability and in vitro antisense efficiency was also observed [46].

A phosphate-modified spermine has also been conjugated to nucleotide sequences, increasing the counter-strand affinity as evidenced by monitoring the corresponding melting temperatures [62]. Biophysical and in vitro data supported the potentially favorable properties expected for DNA–polyamine conjugates. However, modification of 2′-deoxy-2′-succinylamido-uridine led to substantial decrease in counter-strand affinity when incorporated internally in DNA [62].

Coupling through the amino group of polyamines such as spermine or spermidine of carboxyl-modified nucleotide sequences has been reported [46, 63, 64]. For instance, oligonucleotides were synthesized with (*N*,*N*-dimethyltryptamine) on a reverse-phase purification cartridge using standard procedures. The full-length modified nucleotide sequence, still fully protected and bound to the solid support, was reacted with the polyamine. After selective cleavage of the benzyl ester in a phase-transfer reaction catalyzed by palladium nanoparticles, the respective polyamines were coupled to the carboxyl-modified nucleotide using diisopropylcarbodiimide/1-hydroxybenzotriazole activation of the carboxylic group, resulting in an amide bond formation between the nucleotide sequence and the polyamine [46]. The biochemical, biophysical, and in vivo data support the idea that modified oligonucleotides exhibit very high nuclease resistance due to the presence of the positively charged polymer segment. In addition, because of their charge, the modified oligonucleotides show enhanced cellular permeability through cell membranes and high affinity for binding to the target RNA [64].

3.3 DNA–Synthetic Polymer Conjugates

PEG is an uncharged, water-soluble, non-toxic, non-immunogenic polymer and is therefore an ideal material for protecting active biomolecules. PEG is amphipathic and expected to improve the transport and cellular association properties of nucleotide sequences. PEG is known to play an important role in the pharmacokinetic behavior of therapeutic proteins. In addition to serving as ligands themselves, they can also serve as linkers for conjugating other ligands [15]. Besides, several reports evidenced that the covalent attachment of PEG to therapeutic proteins (a process commonly referred to as PEGylation) leads to improved aqueous stability, reduced immunogenenicity, and reduced toxicity as well as increased in vivo circulation times [65–69]. PEG has been extensively utilized in drug delivery systems as a

shielding material for active substances [64, 65]. When injected in vivo, therapeutic biomolecules such as peptides or proteins lead in most cases to a fast immune response and therefore exhibit rather short circulation lifetimes and low therapeutic efficiencies [67, 68]. PEG bioconjugation allows a significant improvement in the in vivo response to such therapeutic agents. Hence, the field of protein PEGylation has grown tremendously within the last 20 years. In most of these approaches, PEG was used as a biocompatible protecting material. PEG has also been coupled to a wide variety of biomolecules, for example lipids (PEGylated liposomes), polysaccharides, enzymes, and antibodies [66]. A description of this mature area of pharmaceutical science is beyond the scope of this review and is available in several excellent publications [67, 68].

The effect of the different high molecular weight PEG chains on the biological properties of conjugated antisense oligonucleotides has thus been demonstrated. For instance, of the two different conjugates of an anti-HIV 12-mer oligonucleotide tested for antisense activity in MT-4 cells [70], only the oligonucleotide conjugated to the linear monomethoxy PEG (MPEG) showed anti-HIV activity. The 12-mer, when conjugated to a branched $(MPEG)_2$, was inactive, as was the unmodified oligonucleotide. Also, an oligonucleotide that targets human ICAM-1 has been conjugated to a series of PEG esters of 550, 2,000, and 5,000 average molecular weight (corresponding to 11, 44, and 110 ethylene glycol residues) [70]. This study indicated that PEG interferes with the cellular permeation in vitro with or without cationic lipids present. Therefore, it is not surprising that PEG is by far the most used synthetic polymer for bioconjugation [71]. PEG was also extensively studied for shielding plasmid DNA for non-viral gene delivery [71–73]. However, in some particular cases, PEG was not directly bound to the genetic material but was coupled to polycationic segments, which form electrostatic complexes with DNA oligonucleotides (supramolecular bioconjugation).

4 Self-assembling DNA–Polymer Conjugates

As already mentioned, the main obstacles to using nucleotide sequences as such are their limited plasma half-life as well as limited cellular penetrability and uptake. There is therefore an increasing interest in designing nucleic acid-decorated nanostructures that have improved cell penetrability and can potentially be used as carriers. With this aim, nucleotide sequences have been grafted to hydrophobic polymers to induce the formation of self-assembled nanostructures [11, 19, 74]. The resulting structures possess unique recognition properties because of their interaction with the DNA sequence complementary to that involved in the self-assembly. These thus appear as alternatives to self-assembled structures derived from poly (peptides) [75] or synthetic polymers [76]. Due to the inherent property of self-recognition of oligonucleotide sequences, the self-assembled DNA–polymer conjugate structures are suitable candidates for targeted delivery vehicles. Drugs could be targeted to specific cells through hybridization between the self-assembled

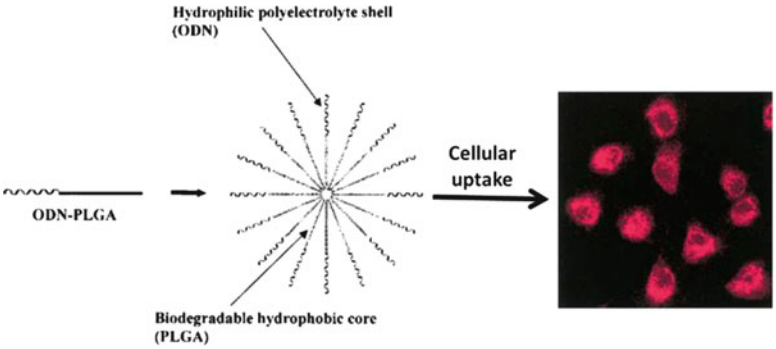


Fig. 3 Micelle formation by the amphiphilic DNA–polymer conjugate resulting from the coupling of nucleotide sequences and poly(D,L-lactic-*co*-glycolic acid) in aqueous medium. Confocal image shows cellular uptake of rhodamine-labeled micelles transported within NIH3T3 mouse fibroblast cells [77] (figure adapted with permission of American Chemical Society)

DNA–polymer conjugate and the cDNA labeled with a ligand of a cell surface receptor. There is thus an enhanced interest in designing self-assembled structures composed of oligonucleotides for their potential application in drug delivery and biosensing to achieve advances in the biomedicine, industrial, and technological fields [11, 19, 74, 76].

4.1 Self-assembly of Micellar Structures

Self-assembly of amphiphilic DNA–polymer conjugates into micelles of various shapes in dilute aqueous solution has recently drawn much attention because of their potential applications in biomedicine and nanoscience. When a hydrophobic polymer segment is coupled to a nucleotide sequence it may self-assemble in aqueous solution to give rise to core–shell micelles composed of a hydrophobic core surrounded by a hydrophilic corona of the DNA fragment, which may act as excellent vehicle for targeted delivery. Naked nucleotide sequences reveal a limited plasma half-life and poor cell penetrability. Upon conjugation to a ligand to scaffold their self-assembly, blood stream circulation time through avoidance of renal exclusion and cellular uptake is enhanced owing to their typical size in the submicrometer range.

Park and coworkers therefore designed DNA–polymer conjugates that self-assemble into micelles for the targeting of antisense oligonucleotide to cells. The c-myc antisense nucleotide sequence was conjugated to biodegradable poly (D,L-lactic-*co*-glycolic acid) (PLGA). The hydrophobic PLGA comprises the core of the resulting micelles whereas the hydrophilic DNA fragments comprise the corona. These micelles were readily taken up by fibroblast cells (Fig. 3). Because PLGA is a biodegradable polymer, a random hydrolytic scission of the PLGA

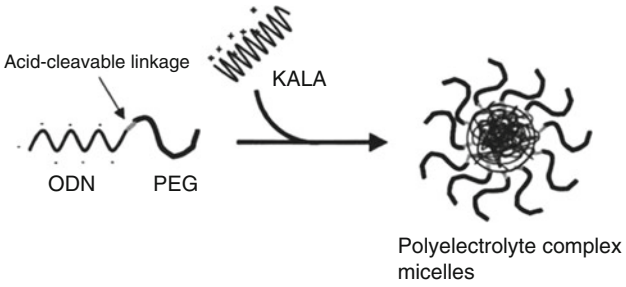


Fig. 4 Formation of polyelectrolyte complex micelles self-assembled from ODN–PEG conjugate and the peptide KALA [78] (figure reproduced with permission of American Chemical Society)

backbone yields water-soluble oligo(lactic-*co*-glycolic acid), which results in the slow degradation of the PLGA core and subsequent release of the antisense oligonucleotide from the corona [77].

In a subsequent study, the same research group used a DNA–PEG micellar system for antisense oligonucleotide delivery. The antisense oligonucleotide was conjugated to PEG through an acid cleavable phosphoramidite linkage. When the fusogenic peptide KALA was complexed to this conjugate through electrostatic interaction with the negatively charged ODN, a complex was assembled with the positively charged KALA. The inner polyelectrolyte complex core is surrounded by the PEG chains constituting the corona, which protects the ODN from enzymatic degradation and binding to serum proteins. In addition to steric hindrance inferred by the neutral flexible PEG chains, the solubility of these nanoparticles in aqueous media is improved (Fig. 4). Because ODN are conjugated to PEG through an acid-labile linkage, the ODN could be released from these micellar structures in the endosomal compartment where the conditions are acidic. It was demonstrated that these polyelectrolyte micelles are transported into cells far more efficiently than the pristine ODN itself and also exhibited higher anti-proliferative activity against smooth muscle cells [78].

Mirkin and coworkers reported another class of amphiphilic DNA–polymer conjugates generated by SPS that assemble into micellar structures in aqueous solution and used their molecular recognition property to hybridize them with gold nanoparticles, resulting in the construction of stimuli-responsive structures of higher order [79]. Alcohol-terminated polystyrene was activated with 2-cyanoethyl *N*,*N*′-diisopropylchlorophosphoramidite prior to polymer reaction with the 5′-hydroxyl group of the oligonucleotide strand (5′-A_5-ATCCTTATCAATATT-3′) bound to the CPG solid support (Fig. 5, top). The resulting DNA–polystyrene conjugates self-assemble in aqueous solution to form micelles having sizes in the range of 13–18 nm (Fig. 5, bottom left). The core of the micelles is composed of the polystyrene whereas the shell is made of the DNA fragments. When the DNA sequence complementary to that involved in the self-assembly is conjugated to gold nanoparticles, in the presence of these micelles, molecular recognition is induced by hybridization, which leads to higher ordered structures. These structures

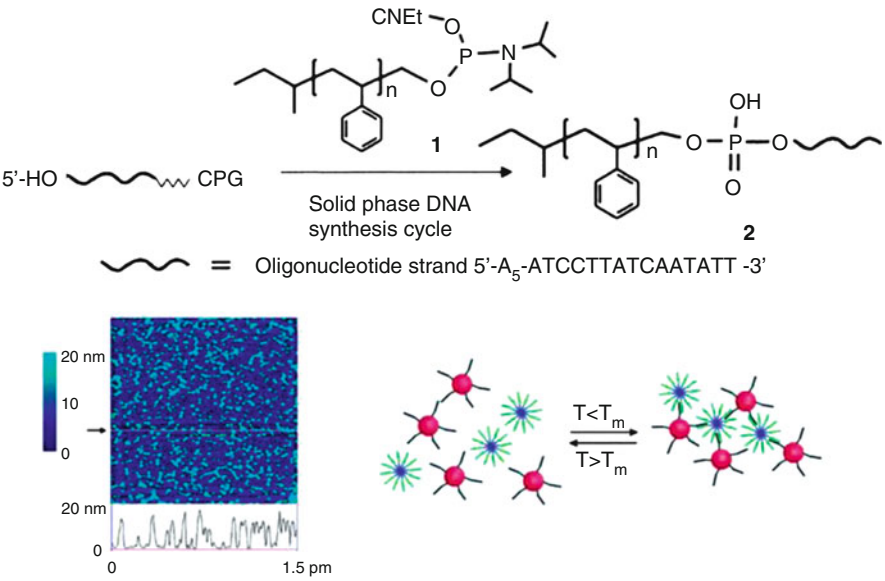


Fig. 5 *Top*: synthesis of DNA–polystyrene conjugates (*2*) using 2-cyanoethyl *N*,*N*′-diisopropyl-chlorophosphoramidite (*1*) as activator. *Bottom left*: image shows self-assembly of conjugates into micellar structures (13–18 nm). *Bottom right*: assembly of higher ordered structures formed by addition of gold nanoparticles modified with the complementary sequence of the DNA involved in the self-assembly that undergoes hybridization. Reversible disassembly at high temperature (above the "melting temperature", T_m) by breaking of the duplex [79] (figure adapted with permission of American Chemical Society)

are reversibly disassembled by heating them above the "melting temperature" (T_m) of 57.8°C (Fig. 5, bottom right).

Herrmann and coworkers resorted to the strategy developed by Mirkin and coworkers to construct a novel class of amphiphilic DNA–polymer conjugates through SPS. Here, DNA was coupled to a poly(propylene oxide) (PPO) polymer segment of low glass transition temperature and of higher biocompatibility than the previously reported polymers used for the synthesis of DNA–polymer conjugates. Hydroxy-group-terminated PPOs were activated by 2-cyanoethyl phosphoramidite chloride prior to coupling to the 5′-hydroxyl end of a 22-mer oligonucleotide sequence bound to a CPG solid support. After removal of the solid support and protecting groups by ammonia, the resulting hybrid was purified by PAGE to yield DNA-*b*-PPO copolymers that self-assemble in aqueous solution to give micelles with a hydrophobic PPO core and a shell composed of the nucleotide sequences. These micelles were used as nanoreactors to carry out organic reactions [80]. Various chemical reactions like Michael addition, amide bond formation, and isoindole formation were carried out at the surface of the micelle by chemical modification of the 5′-end of the cDNA by sulfhydryl, amino, carboxylic, or maleimide groups. Owing to the hybridization property of cDNA strands, the reactants are thus in close proximity and the reaction occurs more efficiently,

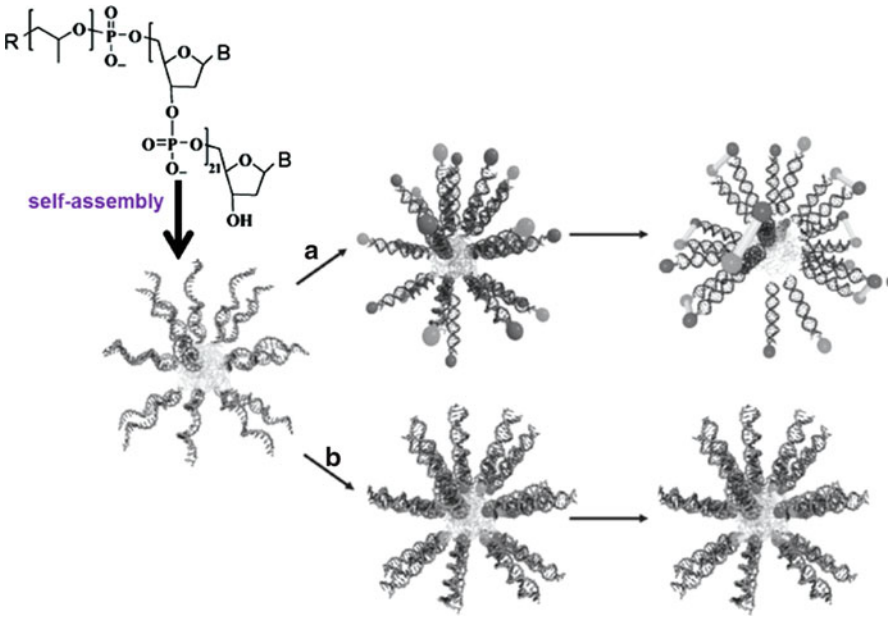


Fig. 6 DNA-*b*-PPO self-assembles into micelles, which are used as templates for carrying organic reactions (*a*) on the surface by equipping the reactants at the 5′-end of the cDNA or (*b*) in the core by equipping the reactants at the 3′-end of the cDNA [80] (figure adapted with permission of Wiley-VCH)

leading to a higher yield than other templates. It was also possible to spatially define the location of product formation by these reactions, i.e., to control whether the reaction takes place at the surface of the micelle or in the inner core by attaching the reactants at either the 5′- or 3′-end (Fig. 6).

In a subsequent study, they also demonstrated the potential for application of these micelles in biomedicine by loading the anticancer drug doxorubicin inside the hydrophobic cores of the self-assembled structures [10]. For this, the 5′-end of the cDNA was modified with folic acid units to target the micelles to the receptors present on the cell surface of human Caco-2 cancerous cells. It was demonstrated that the cellular uptake of these micelles was dramatically increased by folic acid conjugation and that the viability of the cancerous cells was drastically reduced, thus showing that the self-assembled DNA–polymer conjugates could act as novel delivery vehicles for targeting of drugs to cancerous cell lines presenting folic acid receptors (Fig. 7).

In another study, the same research group demonstrated that these micelles can undergo a morphological transition from spherical to rod-like structures upon hybridization with long DNA sequences, which were selected in such a way that they encode several times the complementary sequence of the DNA composing the self-assembling conjugate [81]. Although hybridization with small single-stranded complementary sequences did not affect the micellar morphology, hybridization

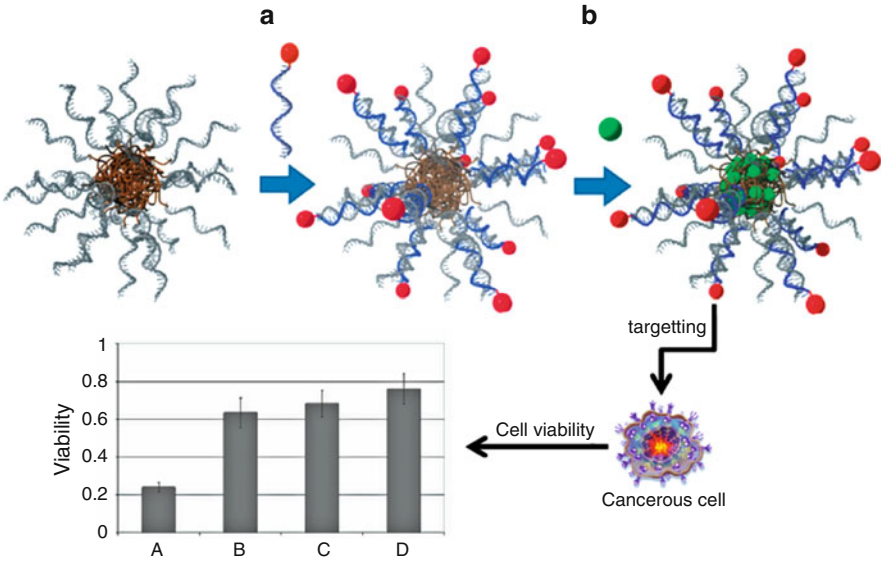


Fig. 7 Drug delivery system based on self-assembling DNA–polymer conjugates. (*a*) Folic acid (FA, *red dots*) is covalently attached to the cDNA sequence and hybridized with self-assembled DNA–polymer conjugates to reveal FA units at the periphery of the self-assembly. (*b*) Anticancer drug doxorubicin (Dox, *green dots*) is loaded into the hydrophobic core of the micelles. Due to hydrophobic interactions of Dox with PPO, the drug accumulates in the interior of the block copolymer self-assembly. These micelles can be targeted to human Caco-2 cancerous cells. *Bar graph* shows the viability of cells after incubation with Dox-loaded micelles covalently linked to FA, (*A*) Dox-loaded micelles equipped with targeting units, (*B*) Dox-loaded micelles in the presence of non conjugated FA, (*C*) Dox-loaded micelles in the absence of any targeting unit, (*D*) Folic-acid-conjugated micelles in the absence of Dox [10] (figure adapted with permission of Wiley-VCH)

with long cDNA sequences triggered a transition from spherical to rod-like structures (Fig. 8a). They further demonstrated that the rod-like morphology is much more efficient as a delivery vehicle to cancerous cells than the micellar structures [82] since their cellular uptake was 12 times more effective than their spherical counterparts (Fig. 8b).

Another study of induction of a morphological transition by conjugating a DNA sequence to a polymer has been recently reported by Gianneschi and coworkers [83]. They synthesized a brush-type amphiphilic oligonucleotide by grafting DNA fragments to a hydrophobic block copolymer. Self-assembly of this amphiphilic DNA-brush copolymer into micelles of various shapes takes place depending on the hydrophobic-to-hydrophilic balance, as represented by cones in Fig. 9. The hydrophilicity is inferred by the DNA fragment made up of 19-mer nucleotide sequences. When the hydrophilic-to-hydrophobic balance is disturbed due to digestion of the oligonucleotides by a DNAzyme, the hydrophobic weight increases, which results in a morphological transition from micellar to cylindrical structures.

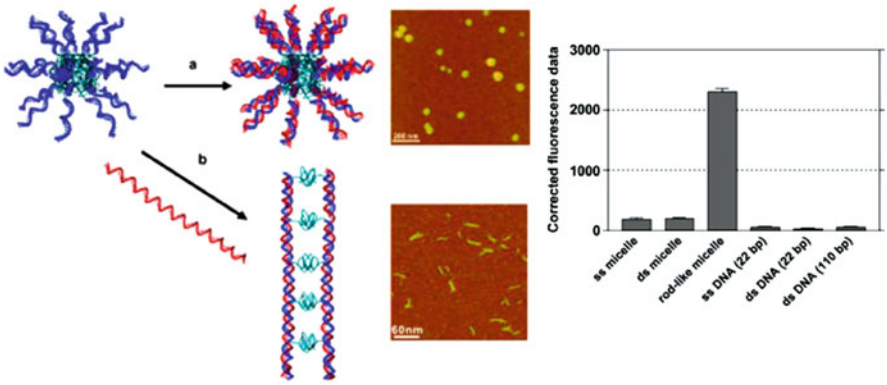


Fig. 8 (*a*) Base pairing of the DNA involved in the self-assembly of the DNA–PPO conjugate with a short complementary sequence yields micelles with a double-stranded corona. The overall spherical shape of the self-assembly is maintained. (*b*) Hybridization with long DNA sequences results in rod-like micelles consisting of two double helices aligned in parallel. Images show internalization of the nanoparticles investigated by fluorescence spectroscopy after cell lysis. *Bar graph* shows the results obtained for spherical and rod-like micelles compared with those obtained for the pristine DNA (*bars* represent the mean of three experiments) [82] (figure adapted with permission of Wiley-VCH)

Alternatively, when the cDNA sequence is not fully complementary and only 9 complementary bases of the 19 of the sequence composing the self-assembling DNA–copolymer conjugate (In_1) are added, such that it hybridizes within the shell, transition back to spheres is observed because it is the most stable configuration for accommodating the bulky duplex strands. When the exactly complementary 19-base sequence (In_2) is added, In_1–In_2 duplex strands are formed due to the higher stability of the longer 19-base duplex as compared to the 9-base duplex. As a result, the spherical micelles are broken and re-assemble into cylindrical structures (Fig. 9).

In another recent study, using the quartz crystal microbalance (QCM), Gauffre and coworkers demonstrated the DNA-driven attachment of block copolymers to surfaces [84]. DNA–copolymer conjugates were synthesized by conjugation of a 22-mer ssDNA sequence to the readily self-assembling poly(ethylene oxide)-*block*-poly (caprolactone) diblock copolymer (PEO-*b*-PCl). In aqueous solution, the resulting hybrid self-assembles into micellar structures composed of a PCl hydrophobic core surrounded by a hydrophilic corona composed of PEO and DNA. Gold-coated quartz crystal sensors were functionalized with an "anchor" nucleotide sequence using standard gold–thiol surface chemistry. The DNA "arm" of the micelles, self-assembled by the DNA–copolymer conjugated to PEO-PCl, was hybridized with a "linker" oligonucleotide, composed of a sequence allowing binding to the anchor. When a solution of these micelles flowed over the quartz crystal surface functionalized with complementary sequences of the linker, binding of the micelles occurred through hybridization. Alternatively, when the nucleotide antisense sequence of the arm (as-arm) flowed into the QCM chamber, reversible detachment from the surface by competitive displacement was evidenced by QCM (Fig. 10).

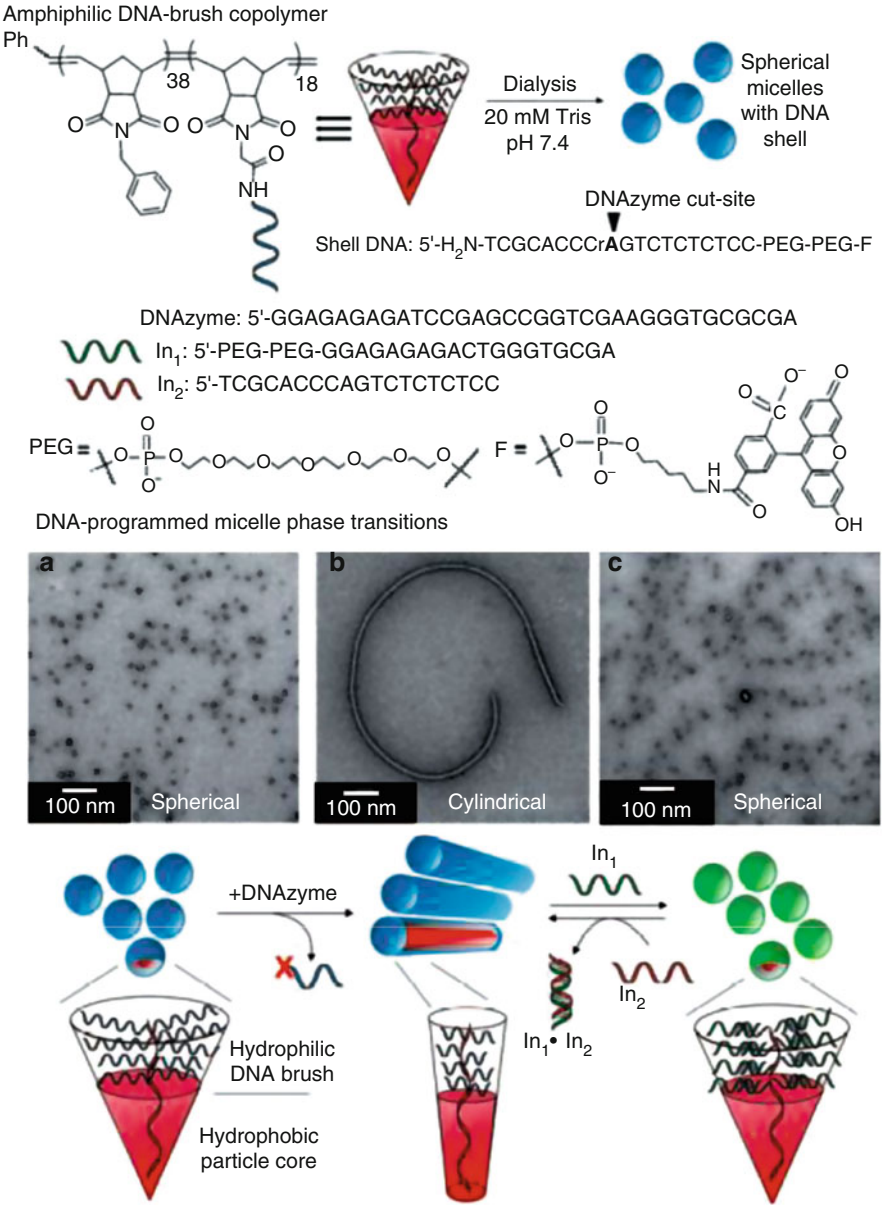


Fig. 9 *Above*: assembly of DNA-brush copolymers into micelles of spherical or cylindrical shape. Amphiphiles are represented as *cones*, with the hydrophobic domain *highlighted*. *Below*: TEM images of (**a**) 25 nm spherical micelles assembled from initial DNA–copolymer conjugates composed of a hydrophobic core made of polymer and a corona of ssDNA; (**b**) cylindrical morphology resulting from the reaction with a DNAzyme; and (**c**) spherical micelles formed after addition of 9-mer oligonucleotide (In_1); these micelles break down on subsequent addition of 19-mer oligonucleotide (In_2) [83] (figure reproduced with permission of Wiley-VCH)

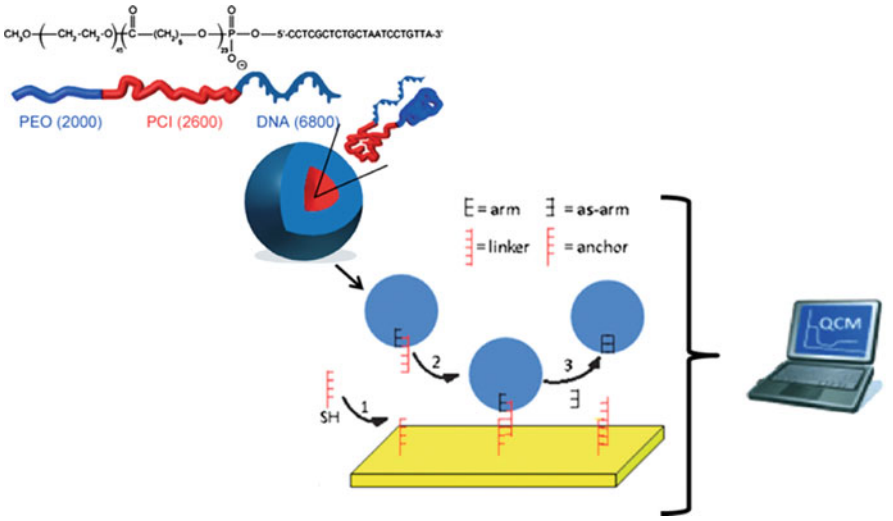


Fig. 10 *Above*: chemical structure of the PEO-PCl-ssDNA conjugate (with the 22-mer oligonucleotide sequence "arm") and representation of a DNA micelle. *Below*: DNA-controlled attachment of block copolymer micelles on surfaces monitored by measuring the time-dependent frequency and dissipation changes induced by mass adsorption on the QCM sensor in the flow mode. *Step 1*: addition of the "anchor"-thiolated oligonucleotide. *Step 2*: addition of the micelles bearing the "linker" oligonucleotide, followed by rinsing. *Step 3*: treatment with the as-arm oligonucleotide (competitive displacement to detach the micelles) [84] (figure adapted with permission of Wiley-VCH)

Park and colleagues recently used micelles self-assembled from DNA–polymer conjugates to encapsulate metal nanoparticles [85]. DNA strands synthesized on beads were directly coupled to a phosphoramidite-terminated polystyrene. The resulting self-assembled amphiphilic conjugates efficiently encapsulated metal nanoparticles inside their core to yield new hybrid micelles with drastically enhanced binding capability to cDNA sequences even at very low salt concentrations, at which isolated DNA strands do not hybridize. The hybrid DNA nanostructure recognizes cDNA with a very high selectivity and can differentiate single base mismatches between complementary strands, as detected with fluorescently labeled DNA probes. Given the high binding efficiency and selectivity, the hybrid material should be extremely effective in duplex DNA detection applications (Fig. 11).

4.2 Self-assembly of Vesicular Structures

Aside from the self-assembly of DNA–polymer conjugates into micelles, the formation of vesicular structures has been recently reported. The vesicles result from the self-assembly of an amphiphilic DNA–polymer conjugate resulting from

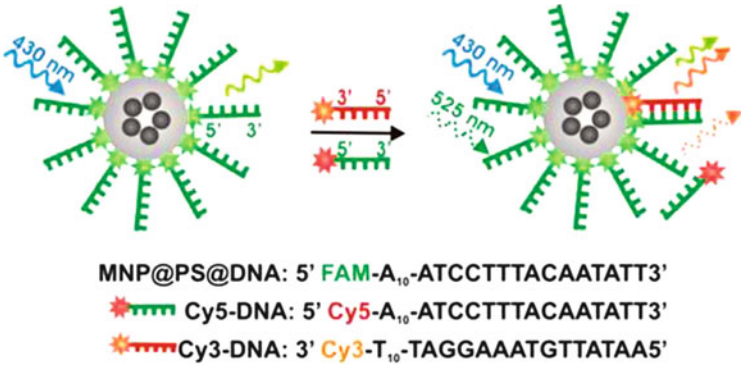


Fig. 11 Preferential binding of target DNA to DNA–polymer conjugate micelles. When both complementary ssDNA sequences of the DNA composing the corona of the micelle and a sequence of the same composition were mixed with the micelles, the cDNA selectively formed a duplex with the DNA involved in the self-assembly rather than with isolated ssDNA. *Wavy arrows* indicate how fluorescence spectra can be used to monitor binding [85] (figure reproduced with permission of Wiley-VCH)

the coupling between a highly hydrophobic polymer segment and a DNA fragment. The self-assembly into vesicular structures is of major interest for potential pharmaceutical and biomedical applications because both water-soluble and hydrophobic substances could be loaded and specifically delivered to cells.

Vebert-Nardin and colleagues reported for the first time the self-assembly of vesicular structures self-assembled from DNA–polymer conjugates through coupling of a suitable polymer segment to a 12 nucleotide sequence [18, 86, 87]. Strongly hydrophobic poly(butadiene) (PB) or poly(isobutylene) (PIB) were coupled to a nucleotide sequence by SPS. The synthetic hydrophobic polymers were selected according to their low glass transition temperature to confer sufficient water solubility to the resulting amphiphilic macromolecule and induce non-kinetically frozen self-assembled structures. The synthesis was performed by coupling of the oligonucleotide functionalized with a carboxylic acid group at the 5′-terminus with the amino-terminated synthetic polymer segment. The grafting between the DNA fragment and the polymer segment is thus conducted prior to the cleavage from the CPG resin.

Circular dichroism (CD) evidenced that neither the coupling of the polymer segment to the nucleotide sequence nor the chemical composition of the synthetic polymer segment affects the chain configuration of the oligonucleotide (see Fig. 12). The CD spectra of the nucleotide prior and subsequent to polymer modification with either PIB or PB are strictly identical. The wavelengths of the ellipticity maxima, as expected, slightly shifted with the composition of the nucleotide sequence. The minimum at 245 nm is typical of a B-form conformation of a DNA fragment, corresponding to a compact cylindrical chain configuration of the nucleotide sequence [88, 89].

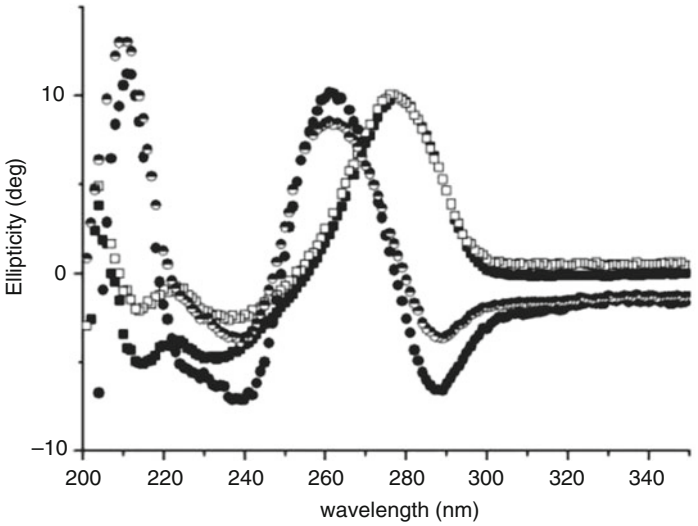


Fig. 12 CD spectra of PIB_{31}–G_7A_5 (*solid circles*), PB_{65}–T_5C_7 (*half solid circles*), PIB_{31}–C_7T_5 (*solid squares*) and free C_7T_5 (*open squares*) [87] (figure adapted with permission of Royal Society of Chemistry)

The amphiphilic DNA–polymer conjugate can thus be regarded as a macromolecule consisting of a 4.08-nm ssDNA fragment covalently linked to a hydrophobic flexible polymer segment, with a Flory radius in a bad solvent of about 4 nm [90]. The full length of the self-assembled macromolecule is thus about 8 nm. A combination of conventional characterization techniques have demonstrated the self-assembly of vesicular structures by both PB– and PIB–DNA conjugates, the sizes ranging between 15 and 150 nm through PIB modification. Conjugation with PB induces the self-assembly of slightly larger structures with sizes ranging between 20 and 150 nm. Figure 13 is a representative transmission electron micrograph (TEM) of a suspension of such vesicular structures [87] self-assembled from PIB_{31}–A_5G_7.

To assess the formation of hollow spherical structures, the authors resorted to co-assembly of the DNA–polymer conjugate in the presence of a pore-forming natural protein. This channel protein regulates the permeability of the vesicular membrane, which ensures exchange between the inner aqueous pool of the vesicular structure and the aqueous surrounding. The vesicular structure can be assessed by the successful encapsulation of the enzyme lactoperoxidase in the inner aqueous pool through monitoring of the enzymatic activity. The enzyme produces di-tyrosine, a fluorescent compound, upon reaction of its tyrosine substrate in the presence of hydrogen peroxide. Diffusion of the products and educts of the enzymatic reaction through the channel protein embedded in the membrane of the vesicular structures could thus be demonstrated by fluorescence spectroscopy (see Fig. 14).

The potential for biological applications of these vesicular structures has been assessed by monitoring a positive bacterial response to surfaces onto which the

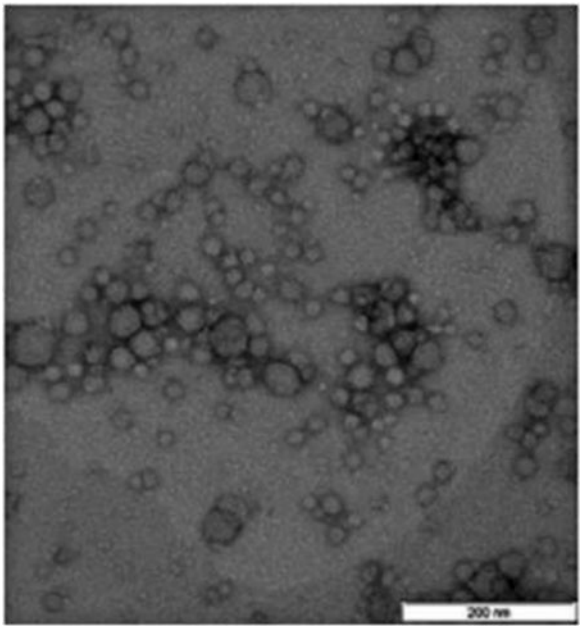


Fig. 13 Self-assembled PIB_{31}–G_7A_5 as observed by TEM; *scale bar*: 200 μm [87] (figure adapted with permission of Royal Society of Chemistry)

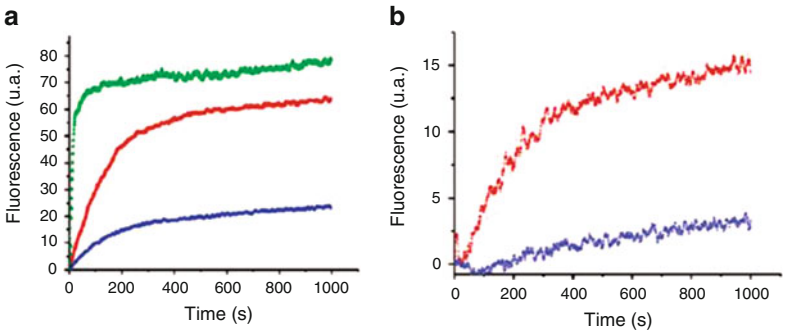


Fig. 14 Fluorescence spectroscopy (emission wavelength at 415 nm) represents (**a**) the enzymatic activity of lactoperoxidase at enzyme concentrations of 5, 1, and 0.5 μM (from *top* to *bottom*) and (**b**) the comparative activity of the vesicles resulting from co-assembly of the DNA–PIB conjugate with channel proteins (*upper curve*) and non-porous self-assemblies (*lower curve*) [87] (figure adapted with permission of Royal Society of Chemistry)

vesicular structures have been immobilized [91] as well as a reduced elasticity of a model phospholipid membrane upon interaction with the DNA–polymer conjugate [92]. This is expected to have greater biological implications than if the DNA–polymer conjugate is solely incorporated into the cell membrane.

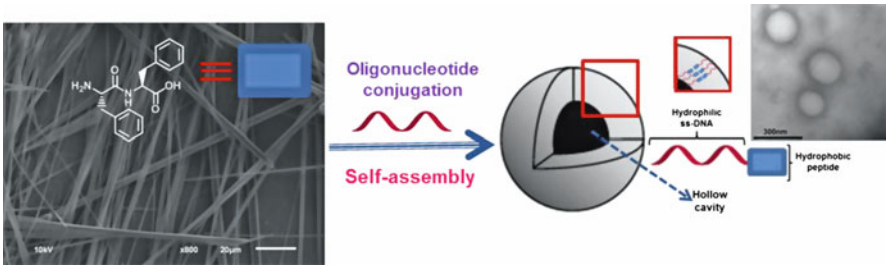


Fig. 15 Morphological transition of diphenylalanine nanotubes to vesicular structures on conjugation of a 12-mer oligonucleotide sequence [93] (figure reproduced with permission of Royal Society of Chemistry)

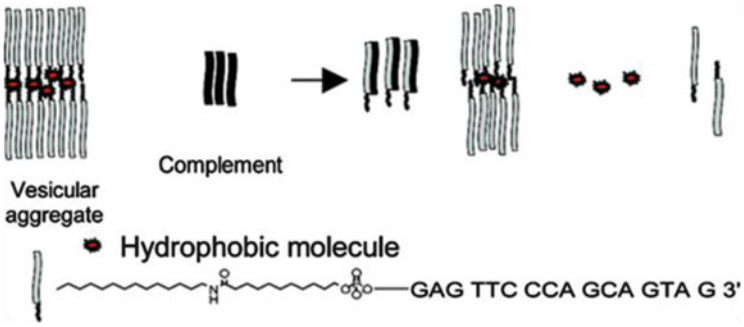


Fig. 16 Entrapment and release of a dye from structures self-assembled by a lipophilic DNA hybrid [94] (figure reproduced with permission of American Chemical Society)

In a recent work, Vebert and coworkers illustrated formation of vesicular structures by grafting oligonucleotides to an amyloid fibril-forming dipeptide [93]. When a 12-mer oligonucleotide was conjugated to diphenylalanine, a common structural motif at the 19 and 20 positions of β-amyloids, there was spontaneous morphological transition from a fibrillar to a vesicular morphology (Fig. 15). Efficient encapsulation of a hydrophilic dye and its pH-triggered release was used to further assess their potential for future use as stimuli-responsive carriers.

Dentinger et al. designed a novel class of amphiphilic compounds that assemble in aqueous solution into vesicular structures. A 16-mer oligonucleotide strand modified by a C_{10} carboxylate linker was coupled to a long alkyl chain that was terminated at one end with an amino group. This conjugate self-assembles in solution to form vesicular structures that efficiently immobilize lipophilic dyes like pyrene and Orange T inside their hydrophobic membrane [94]. These dye molecules could be released by addition of cDNA sequences because their addition leads to hybridization resulting in a destabilization of the vesicular structures (Fig. 16).

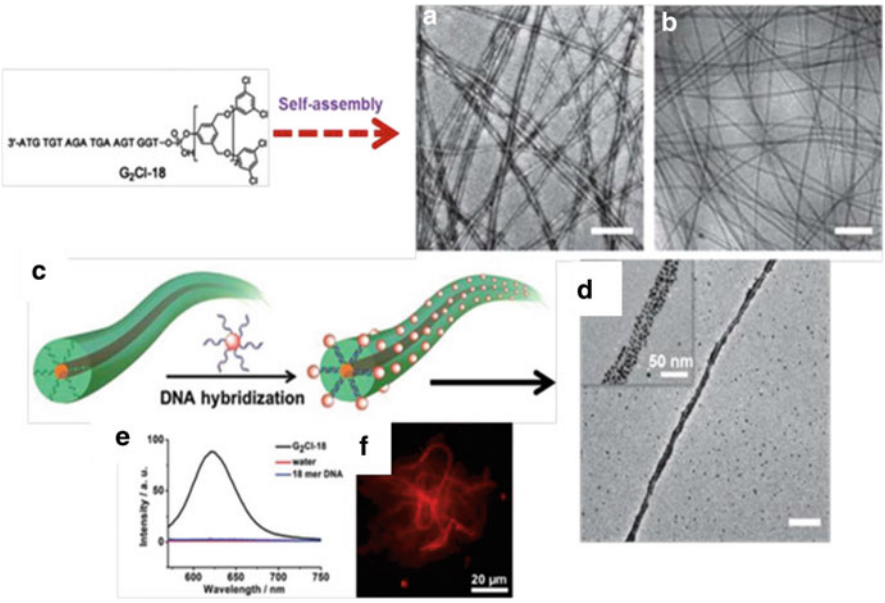


Fig. 17 Images of nanofibers formed by self-assembly in aqueous solution: (**a**) negatively stained TEM and (**b**) cryo-TEM. (**c**) Hybridization of DNA-modified gold nanoparticles with nanofibers. (**d**) TEM images of the hybridization of G2Cl-18 nanofibers with 5 nm gold nanoparticles containing complementary ssDNA. (**e**) Fluorescent emission spectra of Nile Red in aqueous solution in the presence or absence of hybrid. (**f**) Fluorescent image of the nanofibers after Nile Red encapsulation [95] (figure reproduced with permission of Royal Society of Chemistry)

4.3 Self-assembly of Fibrils and Hydrogels

In a recent study, Liu and coworkers reported the synthesis of a novel amphiphilic DNA dendron conjugate by coupling a poly(benzyl ether) dendron functionalized with dichlorobenzene to an 18-mer DNA via a phosphoramidite bond [95]. They demonstrated that this novel hybrid self-assembles into fibrillar structures and that when the cDNA sequence was conjugated to gold nanoparticles addition to the fibrils led to a uniform deposition of gold nanoparticles along the fibers, as evidenced by TEM. They also demonstrated that these conjugates efficiently encapsulate the hydrophobic Nile Red dye, thus illustrating a potential application of this class of nanomaterials as vehicles for delivery of hydrophobic drugs (Fig. 17).

There are also reports that mixing of DNA with polymers leads to formation of hydrogel by crosslinking. When photo-crosslinkable polyvinyl alcohol (azide-unit pendant water-soluble photopolymer; AWP) was mixed with DNA and the resulting mixture irradiated with ultraviolet light, a hydrogel film was formed, due to photo-crosslinking of DNA and AWP, that shows expansion and contraction in response to the media in which it is soaked. It expands in pure water whereas it

contracts in NaCl and CTAB solutions in response to osmotic stress. This phenomenon could be applied for the development of gel-based devices for biosensing applications [96]. In another related study, chitosan and Pluronic polymers were acrylated separately to form photo-crosslinkable polymer. When these acrylated polymers were mixed with plasmid DNA and the temperature increased to 37°C, chemical crosslinking of Pluronic and chitosan hydrogels occurred, the DNA being complexed within these gels. DNA released from these gels showed superior transfection efficiency [97].

In a recent publication, the comparable swelling properties of DNA–polymer conjugate hydrogels were described as highly logical when driven by specific cDNA recognition [98].

4.4 Composite DNA–Polymer Assemblies

Composites are made up of two or more materials with the aim of utilizing the merits of individual constituents to achieve desired synergistic properties and to take advantage of both components. In one such work, Herrmann and coworkers made DNA-functionalized blend micelles by mixing two copolymers. A DNA–polymer conjugate, PPO-*b*-DNA, was mixed with Pluronic F127 (PEO-*b*-PPO-*b*-PEO), which self-assembles into micelles more stable than the PPO-*b*-DNA, which are however of comparable size and can be easily functionalized. When the copolymers are mixed and crosslinked in the core with a crosslinking reagent, a semi-interpenetrating network of PPO from the Pluronic is formed that confers stability to the DNA micelles against disassociation upon dilution or lowering of the temperature (Fig. 18). The core of such micelles can be further used for encapsulation of hydrophobic drugs and the corona can be hybridized with cDNA sequences [99]. The mixed micelles can be foreseen for application as non-immunogenic smart delivery vehicles.

In another study, the DNA micelles were used as scaffolds to assemble viral capsids. Below the critical micelle concentration the micelles are prone to disassociation, therefore amphiphilic DNA–polymer conjugates, which assemble into micelles, were used to induce the assembly of the Cowpea Chlorotic Mottle Virus capsids at neutral pH [100]. The resulting nanocontainers made up of viral capsids were shown to be very stable against dilution and potentially excellent carriers for hydrophobic and hydrophilic drugs in biomedicine (Fig. 18a).

5 Miscellaneous

In a recent report, star polymers having an azide functional motif were conjugated to alkyne-containing DNA using an azide–alkyne cycloaddition click reaction. When a star polymer DNA hybrid was mixed with another star polymer DNA

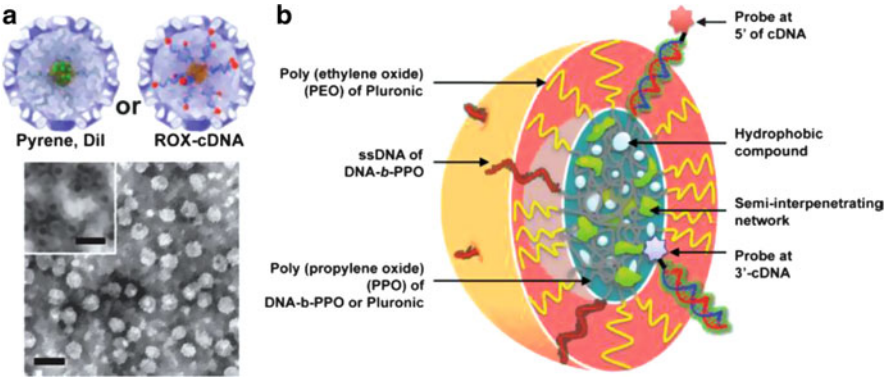


Fig. 18 Composite micelles consisting of antisense oligonucleotides and (**a**) viral capsids or (**b**) synthetic polymers. (**a**) Micelles of DNA amphiphiles loaded with either small hydrophobic compounds (*top left*) or with hydrophilic compounds by hybridization (*top right*) were used to template virus capsid formation at neutral pH. TEM images show micelles incorporated into virus capsids with $T = 1$ or 2 geometry and an empty capsid formed at pH 5.0 as control (*inset*). *Scale bars*: 40 nm. (**b**) Representation of a blend micelle. Diblock DNA copolymer PPO-*b*-DNA was mixed with a triblock copolymer Pluronic (PEO-*b*-PPO-*b*-PEO) composed of the same hydrophobic block, PPO [21] (figure reproduced with permission of Royal Society of Chemistry)

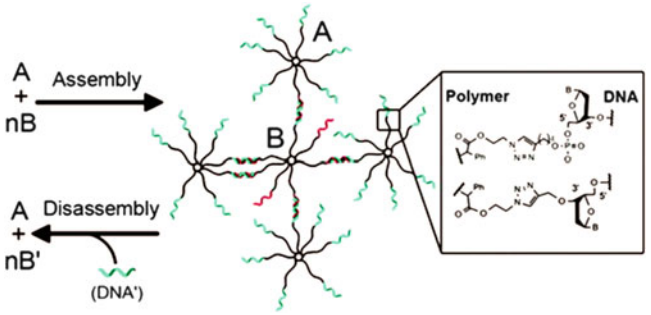


Fig. 19 DNA-directed assembly of star polymers [101] (figure adapted with permission of American Chemical Society)

hybrid composed of the cDNA sequence, self-assembly was induced [101]. Due to hybridization, higher order assembled structures were achieved. The size of the assembled particles could be controlled by varying the ratio of both star polymers. A 1:1 ratio yielded 9 nm particles while a 1:10 ratio yielded 20 nm particles, as measured by dynamic light scattering. In the presence of excess cDNA these assemblies were disrupted, providing an efficient way of designing stimuli-responsive DNA-based nanomaterials (Fig. 19).

Self-assembly of DNA–polymer conjugates has also been used for developing a novel DNA chip for gene diagnosis. A DNA chip is made of thousands of nucleotide sequence attached in a grid pattern and is a very powerful tool in genome

analysis, enabling researchers to identify whether test samples contain a particular DNA or RNA. In this context, Yokoyama et al. have used self-assembled DNA polymer for detection of single nucleotide polymorphism. They synthesized a self-assembling DNA–conjugated polymer based on polyacrylic acid (PAA) for DNA chip fabrication. 3-(2-Pyridyldithio) propionyl hydrazide (PDPH), for promoting adsorption on gold substrate, and a 20-mer ssDNA were both covalently attached to PAA as side chains. This DNA–PAA conjugate spontaneously immobilized on a gold substrate. Single nucleotide polymorphism (SNP) target sequences showed less than 15% of the intensity of that of fully matched target DNA, thus making the DNA chip highly sensitive for SNP detection [102].

In a related study, the authors used self-assembled DNA–polymer conjugates composed of hydrophilic DNA and hydrophobic polymer segments that had been modified with disulfide bridges to facilitate their adsorption onto gold substrates leaving hydrophilic DNA exposed to the solution and thus readily available for hybridization, making these chip useful for biosensing applications [103].

6 Conclusion

This review aimed at achieving an up-to-date report on the development of DNA–polymer conjugates. This area of research focuses on combining the fascinating properties of DNA with the continuous progress achieved in polymer science, in particular synthesis, development of characterization methods, and theory. Of particular interest are the mechanisms of copolymer self-assembly and the hybridization between cDNA strands to assemble the famous Watson–Crick double helix. Both water-soluble and amphiphilic DNA–polymer conjugates have been synthesized to date. The coupling between a DNA fragment with a polymer segment results in a macromolecule that is expected to have enhanced intracellular delivery and specific targeting of biochemically active, potentially therapeutic DNA fragments. This fast expending field of polymer science is thus foreseen to enable the observation and manipulation of the biochemical activity of living cells and to ultimately solve vital issues in medical and cell biology.

However, several modes of interactions such as hydrogen bonding, aromatic stacking, and electrostatic forces play crucial roles in the formation of structures resulting either from complex formation or self-assembly of DNA–polymer conjugates. A fine balance between these interactions governs the properties of the resulting structures such as size and morphology and particularly affects the function of the conjugated DNA fragment. This review article clearly demonstrates that we currently have a toolbox of both water-soluble and self-assembling conjugates that vary in their composition and architecture, which will enable researchers, through systematic studies of the function of the conjugated DNA fragment, to establish a mechanism of structure formation and modes of interactions of these newly developed DNA–polymer conjugates and to design structures of optimal biological activity.

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Synthesis of Terpene-Based Polymers

Junpeng Zhao and Helmut Schlaad

Abstract Terpenes or terpenoids are a large class of diverse biological compounds derived from isoprene. Due to their abundance in nature and desirable properties, there has been great interest in producing polymers with terpenes as either functional entities or as the main constituent. Terpene-based polymers have found applications as biomedical or liquid crystalline materials and, more importantly, have greatly contributed to the concept of sustainable polymer chemistry. The design and preparation of terpene-based polymers have involved different chemical strategies and a wide variety of polymerization techniques, making use of the chemical functionalities in terpene molecules, e.g. (conjugated) double bonds, hydroxyl and carboxyl groups. This review describes the synthetic methodologies for terpene-based polymers, classified by the position of terpene entities in the polymer chains, i.e., main chain, terminal or central group, and pendant group.

Keywords Bioconjugate · Biohybrid · Polymerization · Synthesis · Terpene

Contents

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Abbreviations

Ac	Acetyl
ADMET	Acyclic diene metathesis
AIBN	α,α′-Azobisisobutyronitrile
ATRP	Atom transfer radical polymerization
BPO	Benzoyl peroxide
Bu^i	Isobutyl
Bz	Benzyl
CDI	1,1-Carbonyldiimidazole
Chol	Cholesterol or cholesteryl
CholCl	Cholesteryl chloroformate
Cy	Cyclohexyl
DCC	Dicyclohexylcarbodiimide
DMAP	4-(*N*,*N*-Dimethylamino)pyridine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
Et	Ethyl
LC	Liquid crystal/crystalline
*m*CPBA	*m*-Chloroperbenzoic acid
Me	Methyl
Men	Menthol
MMA	Methyl methacrylate
M_n	Number-average molecular weight
MW	Molecular weight
NHS	*N*-Hydroxysuccinimide
Oct	Octoate
PCL	Poly(ε-caprolactone)
PDI	Polydispersity index
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
Ph	Phenyl
PLA	Poly(lactic acid)
PNIPAM	Poly(*N*-isopropylacrylamide)
Pr	Propyl
*p*TSA	*p*-Toluenesulfonic acid
Py	Pyridine
RAFT	Reversible addition-fragmentation chain transfer
ROMP	Ring-opening metathesis polymerization

ROP	Ring-opening polymerization
TBD	1,5,7-Triazabicyclo[4.4.0]dec-5-ene
T_g	Glass transition temperature
THF	Tetrahydrofuran

1 Introduction

Bioconjugate or biohybrid polymers, comprising both synthetic polymer and biological entities, have been one of the most appealing research topics in polymer chemistry and many other related fields over the past decades [1–3]. The combination of the properties of synthetic and biological components has proven to be a powerful strategy for creating advanced functional macromolecular materials with great potential for various (bio-related) applications [4–11]. Bioconjugate polymers with polypeptides, proteins, nucleic acids, and polysaccharides as biological components have been extensively studied in terms of synthetic approaches, characterization methods, properties, and potential applications, as reviewed in the other contributions to this special issue on biosynthetic polymer conjugates.

To our awareness, there are more classes of biological compounds in addition to the above-mentioned ones that have been employed in the field of polymer science. Terpenes, exhibiting a carbon skeleton of isoprene units, are a class of naturally occurring compounds with a large diversity of chemical structures and bioactivities. Terpenes are of great significance in both nature and human life as, e.g., pheromones, flavors, fragrances, and nutrients [12]. Some of them even exhibit important pharmacological functions for the treatment of many diseases, including cancer [13–15]. Ever since the middle of the last century, attempts have been made to involve terpenes in polymer preparation, either as the main constituent of the polymer or as a functional biological entity in polymer conjugates. This is mainly due to the fact that (1) the chemical structure of many terpenes contain such moieties as (conjugated) double bonds, hydroxyl or carboxyl groups, which can be utilized in polymer preparation and/or functionalization; (2) the use of naturally abundant and biorenewable terpenes to make (functional) polymers meets the need for sustainable development and reduces dependency on mineral oil; (3) terpene entities possess certain desirable properties, such as biodegradability, lipophilicity, bioactivity, and liquid crystallinity, making the polymer or polymer conjugates useful for many applications, such as for biotechnological [16–20] or optical materials [21–25]. Despite the considerable quantity of studies on terpene-based polymers, the reports still remain disperse. No topic has been reviewed as an entirety up to now, which is probably because of the widely differing research objectives for these materials. Herein, we review the various methodologies for synthesis of terpene-based polymers, with emphasis on recent advances in this field.

Polymers or polymer conjugates can be made from terpene molecules either directly or after certain chemical modifications. Generally, three types of terpene-based polymers can be found in the literature and are classified herein by the

position of terpene entities in the polymer: main chain, terminal or central group, and pendant group. The first type (polyterpenes, Sect. 2), is related closely to the concept of using terpenes as renewable monomer sources [26], whereas preparation of the other two types (terpene–polymer conjugates, Sects. 3 and 4) has mostly been aimed at the study and application of their structure-related properties, e.g., self-association, bioactivity, and liquid crystallinity. A very limited number of terpenes have been employed so far, especially for the terpene–polymer conjugates. It should be noted that copolymers containing polyisoprene, the synthetic counterpart of natural rubber, are extensively studied and used in many fields and will not be included in this review (for further reading, see [27–31]).

2 Polyterpenes

Despite the very large number of naturally occurring terpenes, only a very few other than isoprene can undergo polymerization or be readily transformed into a monomer undergoing chain polymerization. So far, only a few monoterpenes like myrcene, alloocimene, limonene, or pinene have been submitted to chain-growth polymerizations. Carvone is difficult to polymerize directly but can be oxidized to a lactone and then polymerized via ring-opening polymerization (ROP) to yield a polyester. Bile acids can be transformed into unsaturated macrocycles and submitted to ring-opening metathesis polymerization (ROMP). Chain-growth polymerization reactions of terpenes will be described in Sect. 2.1.

Alternatively, some native terpenes like bile acids, which carry hydroxyl and carboxyl functionalities in the same molecule, can undergo step-growth reactions, usually polycondensations, which will be described in Sect. 2.2.

2.1 Chain-Growth Polymerizations

2.1.1 Terpene Monomers

The acylic monoterpenes myrcene (7-methyl-3-methylene-1,6-octadiene) and ocimenes (α-ocimene: 3,7-dimethyl-1,3,7-octatriene, alloocimene: 2,6-dimethyl-2,4,6-octatriene) (Fig. 1a–c) represent unsaturated hydrocarbons bearing conjugated double bonds, which in principle can be polymerized by radical, anionic, and cationic procedures as well as by coordination polymerization processes.

Myrcene

Myrcene is a highly reactive compound that undergoes spontaneous thermal polymerization even in air. Polymerization is effectively inhibited in the cold

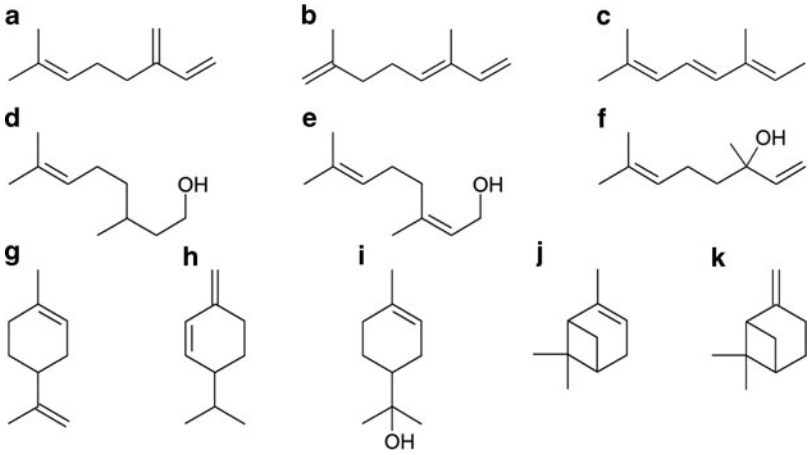


Fig. 1 Chemical structures of (co-)polymerizable monoterpenes: (**a**) myrcene, (**b**) α-ocimene, (**c**) alloocimene, (**d**) citronellol, (**e**) geraniol, (**f**) linalool, (**g**) limonene (dipentene), (**h**) phellandrene, (**i**) α-terpineol, (**j**) α-pinene, and (**k**) β-pinene

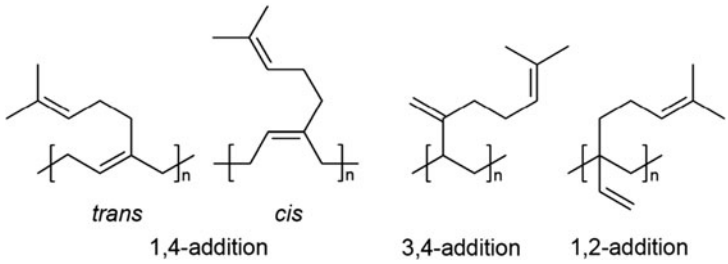


Fig. 2 Repeat units in polymyrcene

(refrigerator) and by 0.1 wt% *p-tert*-butylcatechol, indicating that polymerization follows a free radical mechanism. However, it is thought that the reaction involves more than one mode of polymerization, though not further specified [32].

Hydrogen peroxide-initiated radical polymerization of myrcene in *n*-butanol solution at 100°C yields OH-terminated polymyrcenes (besides a considerable fraction of side products and dimerized species) with M_n in the range of 2–4 kg/mol and fairly low polydispersity index (PDI) = 1.3–1.4. The polymyrcenes consist of predominantly 1,4 structural units (77–85% 1,4 and 15–23% 3,4) (Fig. 2), comparable to polyisoprenes, which is further supported by the low glass transition temperature (T_g) of below −50°C [33]. The polymerization of myrcene with potassium persulfate catalyst in emulsion has also been described, yielding a polymyrcene with predominantly 1,4 structure and relatively low molecular weight (MW; inherent viscosity 1.3) [34]. Fractions of *cis*- and *trans*-1,4 units have not been determined, neither in these nor in the following examples.

Copolymers of myrcene with styrene, methyl methacrylate (MMA), or *p*-fluorostyrene and of myrcene with styrene and 1,3-butadiene, to yield a synthetic rubber, have been synthesized by free radical copolymerization [35] and by emulsion polymerization [36], respectively.

1,3-Butadiene and myrcene have been copolymerized by either batch or continuous processes using finely divided alkali metal (Na or K) as catalyst and ether (preferably diethyl ether or 1,4-dioxane) as solvent at 25–95°C. Conversions of 90% or higher were obtained within 6–24 h. Other terpenes, like α-terpinene, dipentene (racemic limonene), or β-pinene, react little or not at all with 1,3-butadiene, while the copolymerization of alloocimene with 1,3-butadiene gives a low conversion of partially gelled copolymer [37].

The *n*-butyllithium-initiated polymerizations of myrcene proceed in a "living" manner in benzene (5–30°C) as well as in tetrahydrofuran (THF; −30–15°C). Quantitative conversions can be obtained within 2 h (benzene, 30°C) or less than 1 h (THF, 15°C). The polymers have MWs in the range of 5–30 kg/mol, and PDI values are 1.4–1.6 (benzene) and 1.1–1.5 (THF). The polymyrcenes prepared in benzene consist of 85–89% 1,4 units and 11–15% 3,4 units, similar to those obtained by radical polymerization (see above). Increasing either polymerization temperature or initiator concentration causes an increase of the fraction of 3,4 units. On the other hand, polymyrcenes prepared in THF exhibit 40–50% 1,4 units, 39–44% 3,4 units, and 10–18% 1,2 units. Also here, the amount of 1,4 units is found to decrease with increasing polymerization temperature or initiator concentration. The copolymerization of myrcene and styrene results in the formation of block-like or tapered copolymers. The initial copolymers formed in benzene are rich in myrcene, and styrene is preferably incorporated at later stages of the reaction; the situation is reversed when the copolymerization is performed in THF [38].

ABA triblock copolymers have been prepared by sequential anionic polymerization of styrene (A), myrcene (B), and again styrene. Polymerizations were initiated by *sec*-butyllithium at room temperature using benzene as the solvent. The resulting thermoplastic elastomeric copolymers had average MWs in the range of 2–100 kg/mol; the PDI and microstructure of the polymyrcene block were not provided [39].

The cationic polymerization of myrcene with boron trifluoride etherate (BF_3OEt_2) yields a polymer with a different structure, as depicted in Fig. 2, bearing just one double bond per repeat unit. It has been hypothesized that the structure of this polymyrcene is identical to that of the polymer obtained by the cationic polymerization of β-pinene (see below). Further details on the polymerization mechanism were not provided; however, it must involve some intramolecular cyclization of the myrcene [34]. Myrcene might also be polymerized using metal halide catalysts in hydrocarbon or halogenated solvents, though not further specified [40].

Myrcene has also been polymerized using Ziegler-type catalysts. It polymerizes readily by treatment with 3 mol% of $AlBu^i_3$ and $TiCl_4$ ([Al]/[Ti] = 2–2.5), the polymers with the highest MW being produced at 0°C (monomer conversion ~80%). Nevertheless, the 1,4-polymyrcenes are of relatively low MW with intrinsic viscosities of 0.3–1.0. Quantitative monomer conversions and higher MW

1,4-polymyrcenes (intrinsic viscosity 2.0–5.5) can be obtained with a catalyst composed of $AlBu^{i}{}_3$ and VCl_3 ([Al]/[V] = 5–8.5). However, batches usually contain considerable amounts (15–20%, sometimes more) of insoluble, crosslinked product [34].

Ocimene–Alloocimene

α-Ocimene can be polymerized, like myrcene, in a free-radical process in *n*-butanol solution at elevated temperature using hydrogen peroxide as the initiator. However, the monomer conversion is just 30% and only oligomers ($M_n \sim 1$ kg/mol) are produced. It is noteworthy that this procedure is less suited or not suitable for the polymerization of terpenes with hindered vinyl double bonds, i.e., carvone, dipentene, or α-pinene [33].

The anionic polymerization of alloocimene with a catalyst system comprising a metal, e.g., sodium, and an aliphatic ether, e.g., 1,2-dimethoxyethane, yields a polymer with predominantly 2,3- and 6,7-units, and hence pendant dimethylbutadienyl groups (see Fig. 3). Polyalloocimenes with such a microstructure are especially useful as reactive intermediates, for instance readily forming Diels–Alder adducts with activated olefins [41].

The cationic polymerization of alloocimene, also ocimene and myrcene, has been achieved with metal halide catalysts (e.g., stannic chloride, aluminum chloride, or boron trifluoride) in liquid phase (e.g., ethyl chloride, ethylene dichloride, or benzene) at a temperature range of −35°C to about 200°C. MWs and microstructures of the products were not provided [40]. Cationic (or radical) copolymerizations of alloocimene (or myrcene) with for instance styrene yield resinous copolymers [42].

Linear polyalloocimenes can be obtained with $AlBu^{i}{}_3-TiCl_4$ catalyst ([Al]/[Ti] = 3) in heptane solution at preferably −15°C [43]. Other Ziegler-type catalysts ($AlBu^{i}{}_3-VCl_3$) and acid catalysts (BF_3OEt_2 and $TiCl_4$) also affect the cationic polymerization of alloocimene, all producing linear polymers with similar microstructures, i.e., 60–80% 4,7 units (*cis*/*trans* not specified) and 20–40% 6,7 units. The melting range of the prepared polyalloocimene samples was 90–150°C. Little or no cyclopolymerization was observed [44].

However, alloocimene has been found to undergo cationic cyclopolymerization (see Scheme 1) when treated with BF_3OEt_2 in ice-cold ethyl chloride solution (like myrcene, see above). The prepared polyalloocimene was found to be soluble in

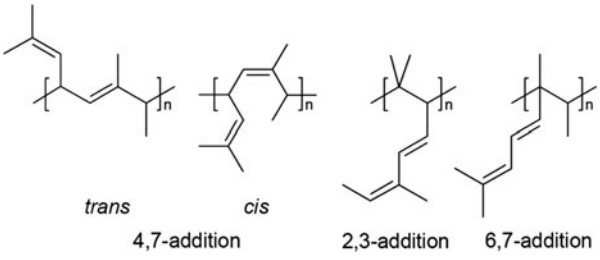
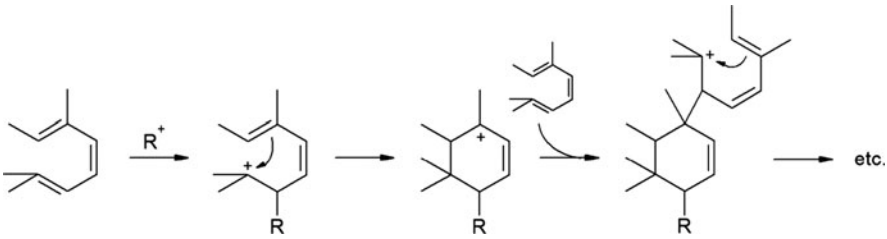


Fig. 3 Repeat units in polyalloocimene



Scheme 1 Cationic cyclopolymerization of alloocimene (R = alkyl) [45]

benzene, dioxane, and halogenated solvents and to form brittle fibers when extruded through a fine orifice (melting point 85–87°C). The polymer takes up bromine readily and can be oxidized to a dark product upon heating in air [45].

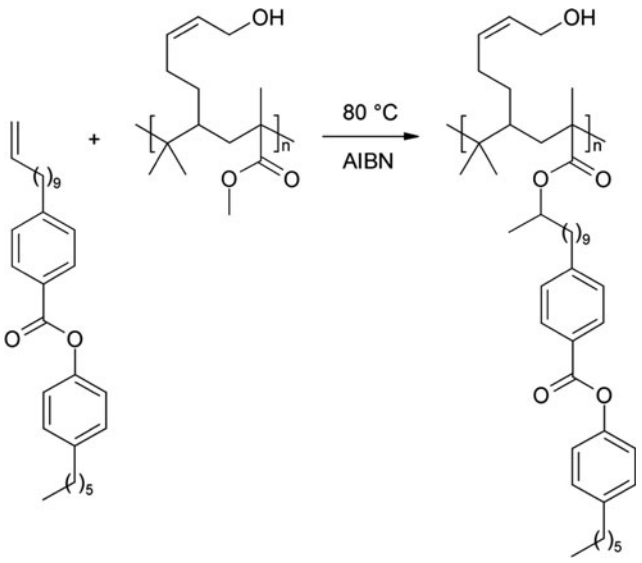
Isobutylene and alloocimene (1.6–2.8 mol%) have been copolymerized with $EtAlCl_2$ as the catalyst in *n*-heptane/methyl chloride 1:1 (v/v) solution at −70°C. The resulting linear copolymers had block-like structures and MWs in the range of 145–260 kg/mol (no PDI given). In contrast to the previous examples, the copolymer contained only traces of alloocimene units with conjugated unsaturation [46].

Citronellol–Geraniol–Linalool–Terpineol

Acyclic monoterpenes carrying alkene and OH groups, like citronellol, geraniol, and linalool (Fig. 1d–f), have been directly copolymerized in radical processes. Alternating copolymers of styrene and citronellol were synthesized by radical polymerization using either α,α′-azobisisobutyronitrile (AIBN) or benzoylperoxide-*p*-acetylbenzylidenetriphenyl arsoniumylide as initiator in xylene solution at 80°C. MWs of the copolymers were not given (only intrinsic viscosities) [47]. Citronellol has also been copolymerized with vinyl acetate (xylene, 60°C, BPO) [48], *n*-butyl methacrylate (xylene, 60°C, BPO) [49], acrylonitrile (xylene, 75°C, BPO) [50], acrylamide (xylene, 80°C, BPO) [51], and styrene/MMA (xylene, 80°C, BPO) [52]. Geraniol has been copolymerized with styrene (xylene, 80°C, BPO) [53], acrylonitrile (DMF, 70°C, BPO) [54], and MMA (xylene, 80°C, BPO) [55]. Poly(geraniol-*co*-MMA), having M_n = 10 kg/mol and broad MW distribution (PDI = 3.5), was further reacted with a vinyl-terminated mesogen to yield a liquid crystal (LC) polymer (Scheme 2) [55].

Linalool has been copolymerized with styrene (xylene, 80°C, BPO-*p*-acetylbenzylidenetriphenyl arsoniumylide) [56], vinyl acetate (xylene, 60°C, BPO) [57], acrylonitrile (xylene, 75°C, BPO) [58], and acrylamide (xylene, 75°C, BPO) [59]. A terpolymer of linalool, styrene, and MMA has also been prepared (xylene, 80°C, BPO) [60].

Alternating copolymers of α-terpineol (Fig. 1i) with *n*-butyl methacrylate (xylene, 80°C, BPO) [61], MMA (xylene, 80°C, AIBN) [62], and *N*-vinyl pyrrolidone (dioxane, 80°C, BPO) [63] have been reported. The free-radical



Scheme 2 Synthesis of an LC polymer from poly(geraniol-*co*-MMA) [55]

copolymerization of α-terpineol and MMA can be initiated in xylene at 30°C using AIBN as photoinitiator [64]. Poly(α-terpineol-*co*-MMA) serves as precursor for the synthesis of LC polymers (cf. Scheme 2) [65].

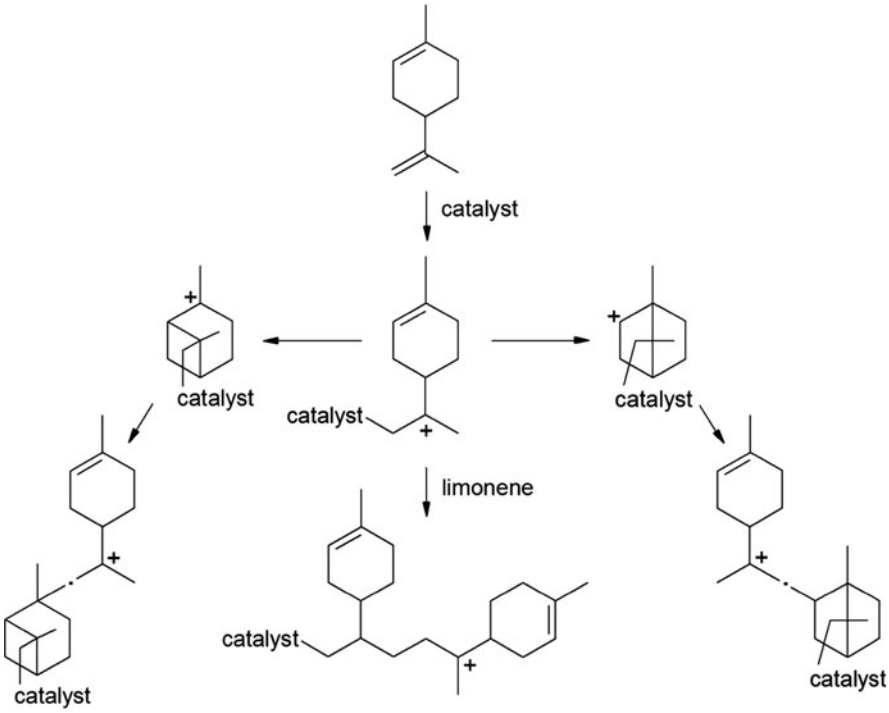
Limonene–Phellandrene

d-Limonene (Fig. 1g) is polymerized with aluminum chloride as (Friedel–Crafts) catalyst in toluene solution at 40–45°C. The polylimonenes exhibit rather low MWs (~1.0–1.2 kg/mol) and softening points in the range of 125–136°C [66].

Polymerization of *d*-limonene with Ziegler-type catalysts (aluminum alkyl-metal halide, 1:1 molar ratio) in *n*-heptane solution at 25–30°C (reaction time 7 days) yields only low MW and almost completely racemized products. The structure of the polymer, which is identical to that obtained by cationic initiation with $TiCl_4$ or BF_3OEt_2, is found to have more than one structural unit. A polymerization mechanism (Scheme 3) is proposed, leading predominantly to bicyclic saturated units of the camphene-type and/or pinane-type. Polymerization via the pendent isopropenyl groups of limonene appears to be less favored [67].

Several reports describe the radical copolymerization of limonene with vinyl monomers, i.e., maleic anhydride, acrylonitrile, MMA, styrene, vinyl acetate, and *N*-vinyl pyrrolidone. The coordination copolymerization of limonene oxide and carbon dioxide has also been reported, see below.

The radical copolymerization of *d*-limonene and maleic anhydride has been performed in THF solution at 40°C using AIBN as the initiator and a reaction

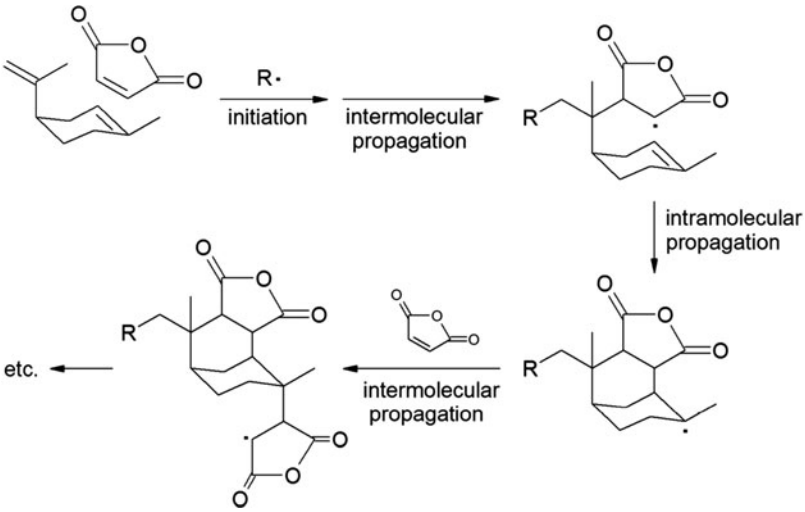


Scheme 3 Proposed mechanism for the cationic polymerization of limonene [67] (reproduced with permission from John Wiley & Sons)

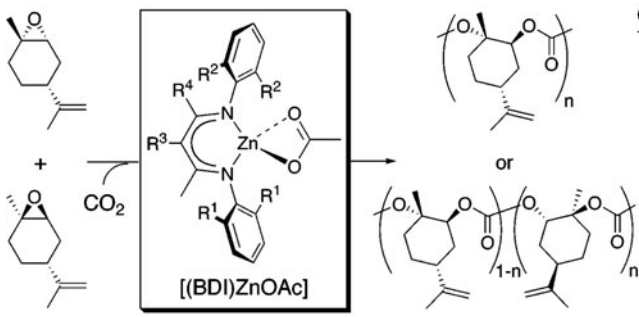
time of 1–2 days. It is suggested that the limonene readily undergoes inter–intramolecular cyclopolymerization with maleic anhydride to yield a 1:2 alternating copolymer (Scheme 4). Copolymers exhibit low MWs (1–1.3 kg/mol) and are optically active [68].

Limonene can also be copolymerized with acrylonitrile (in DMF at 70°C, initiator AIBN) [69], MMA (xylene, 80°C, BPO) [70], styrene (xylene, 80°C, AIBN) [71], *N*-vinylpyrrolidone (dioxane, 80°C, AIBN) [72], and *N*-vinyl acetate (dioxane, 65°C, AIBN) [73], always producing alternating copolymers. Radical addition of limonene occurs via the exocyclic isopropenyl group (in contrast to the cationic system, see above). Also, a terpolymer of limonene, MMA, and styrene has been prepared by free-radical copolymerization (xylene, 80°C, BPO) [74]. Poly (limonene-*co*-MMA) can be converted into a LC polymer (cf. Scheme 2) [75].

The alternating copolymerization of *cis*/*trans*-limonene oxide and carbon dioxide can be achieved with β-diiminate zinc acetate complexes (Scheme 5). The balance between high catalyst activity and selectivity is optimal with catalyst complex 8 (see Scheme 5, right) at 25°C. Catalysts exhibits high selectivity for the *trans* diasteriomer (% *trans* in the copolymer is >98%). The biodegradable polycarbonates have MWs in the range of 4.0–10.8 kg/mol, which can be controlled by the [epoxide]/[Zn] ratio, CO_2 pressure, and reaction time. They also have narrow



Scheme 4 Proposed mechanism for the radical cyclocopolymerization of limonene and maleic anhydride [68]



Complex	R^1	R^2	R^3	R^4
2	Et	Et	H	CH_3
3	^{i}Pr	^{i}Pr	H	CH_3
4	Me	Me	H	CH_3
5	Et	^{i}Pr	H	CH_3
6	Et	Et	H	CF_3
7	^{i}Pr	Et	H	CF_3
8	Et	^{i}Pr	H	CF_3
9	Et	Et	CN	CH_3
10	^{i}Pr	Me	CN	CH_3

Scheme 5 Copolymerization of limonene oxide and CO_2 using β-diiminate zinc acetate complexes [76] (reprinted with permission from American Chemical Society)

MW distributions (PDI ~ 1.15). Catalysts exhibit high selectivity for the *trans* diasteriomer (*% trans* in the copolymer is >98%) [76].

Functional hyperbranched polymers have been obtained by ROMP of dicylopentadiene in monoterpenes, i.e., *d*-limonene and limonene oxide (also β-pinene) using a second generation ruthenium initiator. Chain transfer occurs during polymerization when the terpene alkene reacts with the growing chain, producing a hyperbranched poly(dicyclopentadiene) bearing the terpene at terminal and semi-dendritic units. MWs of the polymers are in the range of 2.1–54.4 kg/mol (PDI = 1.2–2.4) and T_g = 60–160°C, depending on the number of branch points, MW, chain end polarity, and quantity of chain ends [77]. Linear functional ROMP polymer has been prepared in a similar way [78].

Phellandrene (Fig. 1h) is cationically polymerized using various Lewis acids ($EtAlCl_2$, $SnCl_4$, and BF_3OEt_2) in CH_2Cl_2 or methylcyclohexane (or 1:1 mixtures) solution at −78°C. The polymers are obtained in ~80% yield and have M_n = 3.7–6.0 kg/mol (PDI = 1.8–2.0) and T_g ~ 130°C [79].

α-Pinene and β-Pinene

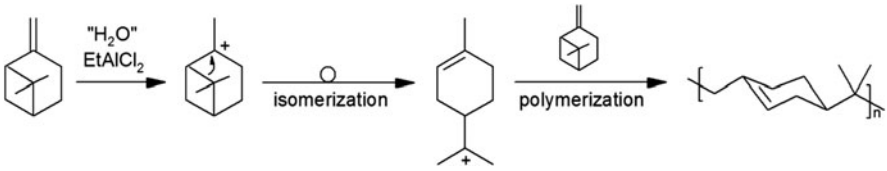
Of the two pinene monomers (Fig. 1j, k), which can be isomerized into each other (cf. Scheme 3), the α-isomer exhibits an endocyclic double bond and is thus the less reactive (and also less frequently used) in polymerization reactions. However, the polymerization of α-pinene was reported as early as 1937, using $AlCl_3$ as catalyst in hydrocarbon (i.e., benzene, toluene, xylene, or hexane) solution at <15°C, yielding ~75%. Polymerization in the presence of aromatics, with $AlCl_3$ as Friedel–Crafts catalyst, takes place without the interaction of aromatic and terpene. However, structures and MWs of the polymers formed were not given [80]. A later comparative study shows that the polymerization of α-pinene produces 35% or less solid polymer with MWs of 0.6–0.7 kg/mol, depending on the catalyst used (β-pinene; yield up to 96%, MW = 0.8–3.1 kg/mol). The molecular structure of the oligo(α-pinene) was, however, not provided [66].

Ziegler-type catalysts affect a polymerization of α-pinene (also limonene, see Scheme 3), producing a polymer whose structure is still unresolved. The structure of the poly(α-pinene) is supposed to be different from that of poly(*d*-limonene) and contain more than one type of bicyclic recurring unit (cf. Scheme 3) [67].

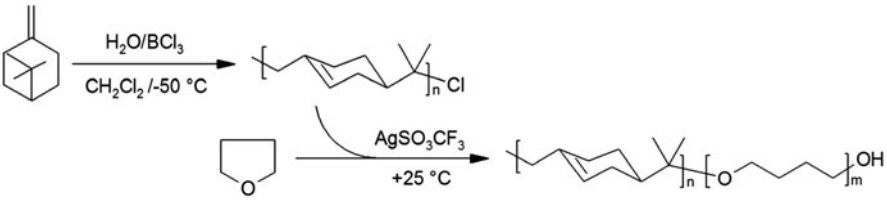
The polymerization of α-pinene with aluminum bromide in xylene (20–25°C, 24 h) and subsequent hardening of the product (250°C, atmospheric pressure) yields a resin with a softening point of 125°C. Replacing aluminum bromide by the chloride produces resins with lower yields and softening points. Also, the grafting of α-pinene (or mixtures of α-pinene with up to 30 mol% of β-pinene) onto polyethylene has been reported [81].

Copolymers of α-pinene and styrene have been obtained by cationic copolymerization using either $AlCl_3$, benzene, 10°C [82], or $SbCl_3/AlCl_3$, toluene, −80°C [83]. In the first case, copolymers with MWs = 2.3–3.1 kg/mol and softening temperatures of 82–85°C were obtained.

Likewise, β-pinene with its exocyclic vinyl group is readily polymerized by cationic techniques; however, the polymers obtained have rather low MWs of≤3.4 kg/mol [80, 84–86]. High molecular weight poly(β-pinene) with MW up to 40 kg/mol (PDI ~ 2.2) can be obtained with the "H_2O"/$EtAlCl_2$ system ("H_2O" indicates adventitious moisture impurities). The polymerizations are carried out in mixtures of methyl chloride/methylcyclohexane (preferred composition 50:50) at −80°C. Quantitative monomer conversions are reached within 20 min or less. The repeat unit of the poly(β-pinene) is found to consist of a cyclohexene unit in the main chain (Scheme 6), which reflects isomerization polymerization [87]. With $AlCl_3$ etherates, e.g., $AlCl_3OPh_2$, the polymerization can be performed even at room temperature and low catalyst concentration (2.5–5.5 mM, $[\beta\text{-pinene}]_0$ = 0.55 M) to yield polymers with M_n = 9–14 kg/mol



Scheme 6 Cationic isomerization polymerization of β-pinene [88]



Scheme 7 Two-step cationic copolymerization of β-pinene and THF [86]

(PDI ~ 2). It is possible to use non-chlorinated solvents like toluene, which is an attractive feature for industrial application of the process [88].

(−)-β-Pinene has been polymerized with various Lewis acids ($EtAlCl_2$, $Et_3Al_2Cl_3$, $ALCl_3$, $TiCl_4$, $SnCl_4$, and BF_3OEt_2) in CH_2Cl_2 and/or methylcyclohexane at −80°C. Stronger Lewis acids, like $AlCl_3$, induce very fast polymerization (complete monomer conversion within 30 s) but produce polymers with lower MWs, due to β-proton elimination. Best results, i.e., complete monomer conversion and highest MW of >20 kg/mol (PDI ~ 2), are obtained with $EtAlCl_2$ and $Et_3Al_2Cl_3$, exhibiting moderate Lewis acidity, in a 1:1 mixed solvent of CH_2Cl_2 and methylcyclohexane. The poly(β-pinene) (M_n = 25.1 kg/mol) is optically active and has T_g ~ 90°C; thermal degradation starts around 300°C. Glass transition and degradation temperatures raise to 130°C and >400°C, respectively, upon saturation of the poly(β-pinene) by hydrogenation (conditions: *p*-toluenesulfonyl hydrazide, xylene, reflux, 5 h) [79].

Partial hydrolysis of organoaluminum compounds, e.g., $AlEt_3$ or $AlBu^i_3$, with water (solvent toluene, 0°C) produces an active catalyst for the polymerization of β-pinene at room temperature (halogen-free conditions). Products are hard poly(β-pinene) resins with a softening point of >100°C [89].

1,4-Bis(2-chloro-2-propyl)-benzene (dicumyl chloride) and 1,3,5-tris(2-chloro-2-propyl)-benzene (tricumyl chloride) in combination with BCl_3 (preferably in CH_2Cl_2/*n*-hexane or CH_3Cl solution at −50°C) have been used to synthesize linear bi-functional (*tert*-chloro) and three-arm star poly(β-pinene)s, respectively. However, low yields (≤20%) and MWs (<2.6 kg/mol) of the polymers indicate the occurrence of rapid termination and/or transfer reactions to monomer. Furthermore, *tert*-chloro-ended poly(β-pinene)/silver triflate can initiate the polymerization of THF at room temperature to produce a poly(β-pinene)-*block*-poly(THF) copolymer (Scheme 7). Unreacted poly(β-pinene) is removed by precipitation of the crude product into *n*-hexane [86, 90].

A few examples of copolymerizations of β-pinene/isobutylene ($EtAlCl_2$, EtCl, −110°C) [84], β-pinene/styrene ($AlCl_3$, CH_2Cl_2, −78°C or 30°C) [85, 91] ($AlCl_3$, xylene, −78°C or 30°C) [85, 92], β-pinene/styrene/α-methylstyrene ($AlCl_3$, xylene, 30°C) [92], and β-pinene/maleic anhydride (AIBN or BPO, bulk, 50–100°C) [93] have been described.

The polymerization of β-pinene by irradiation with ^{60}Co γ-rays *in vacuo* gives a polymeric material that partially precipitates from the liquid monomer. However, polymerization is accompanied by concurrent isomerization of β-pinene to dipentene and other isomers. There is evidence for the participation of ions in the mechanism [94]. γ-Irradiation of α-pinene at 30°C, on the other hand, results in formation of hydrogen, isomers of α-pinene (dipentene and ocimene), and about 5% of polymeric material [95].

2.1.2 Terpene-Derived Monomers

Although the list of polymerizable terpene alkenes in Fig. 1 may, for whatever reason, not be complete, a large number of native terpenes remain that cannot be submitted to any kind of controlled chain-growth polymerization process. Terpenes may eventually be converted into polymerizable monomers, as has for instance been demonstrated for menthone, carvone, and bile acids, i.e., cholic acid and lithocholic acid (see the structures in Fig. 4).

Menthone

Menthone transforms into a polymerizable monomer by Bayer–Villiger oxidation to the seven-membered lactone menthide (oxidant *m*-chloroperbenzoic acid, *m*CPBA). Controlled ROP of menthide can be achieved using a zinc alkoxide catalyst (see Scheme 8) (toluene, room temperature) to yield aliphatic polyesters with predictable MWs up to 91 kg/mol and narrow MW distributions (PDI ~ 1.1) [96].

Renewable ABA triblock copolymers of (−)-menthide (B) and lactide (A) have been prepared by sequential ROPs aiming at the production of thermoplastic elastomers and pressure-sensitive adhesives [97–99].

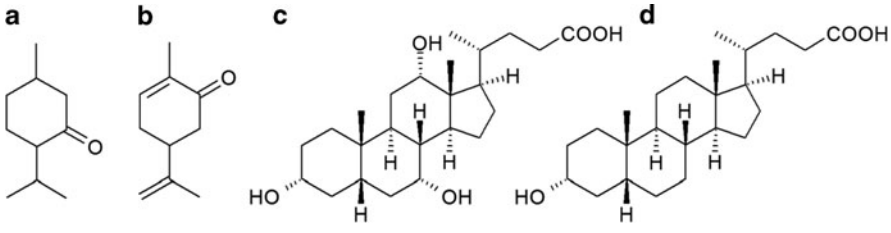
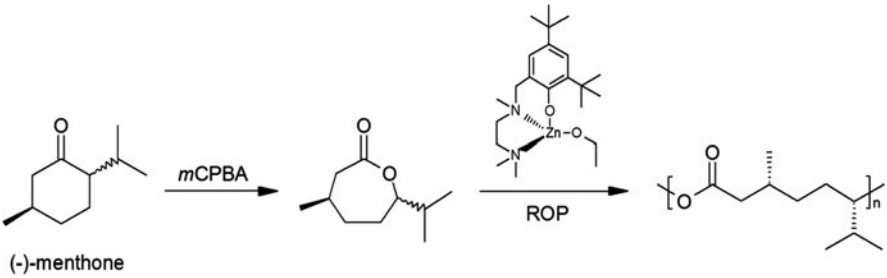
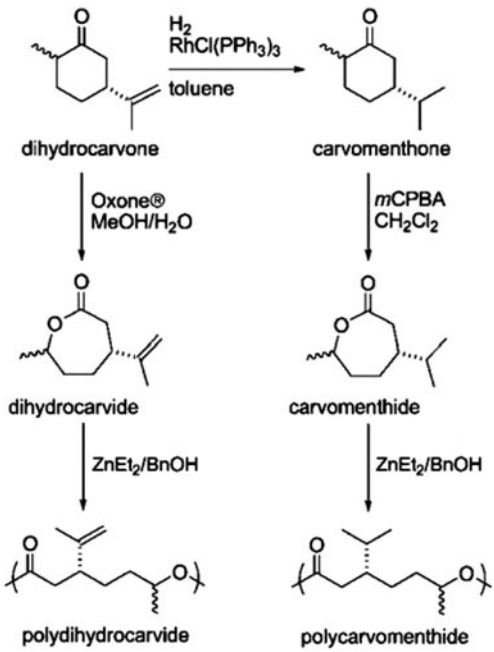


Fig. 4 Chemical structures of (**a**) menthone, (**b**) carvone, (**c**) cholic acid, and (**d**) lithocholic acid



Scheme 8 Transformation of menthone into menthide and subsequent catalytic ring-opening polymerization [96]

Scheme 9 Transformation of carvone into lactone and subsequent ring-opening polymerization [102] (reprinted with permission from Royal Society of Chemistry)



Carvone

Carvone is a monocyclic monoterpene vinyl ketone undergoing dimerization, but no polymerization, upon treatment with sodium amide. Strong acids or bases promote the rearrangement of carvone into 5-isopropyl-2-methylphenol (carvacrol), thus inhibiting polymerization [100]. However, it has been reported that a long-term treatment of carvone with alcoholic base at room temperature results in the formation of a polymer. This polycarvone undergoes depolymerization upon heating, indicating a fairly low ceiling temperature of the polymerization reaction [101].

Alternatively, hydrogenated carvone can be converted into a polymerizable lactone by Bayer–Villiger oxidation (Scheme 9). Hydrogenation of carvone may yield two cyclic ketones, i.e., dihydrocarvone and carvomenthone, which can be oxidized to the

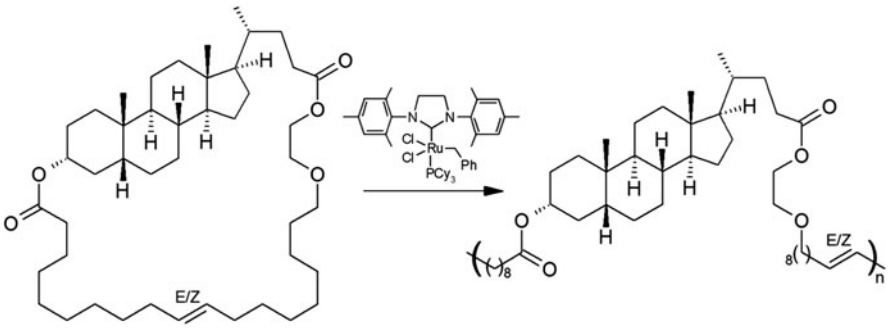
corresponding lactones, i.e., dihydrocarvide and carvomenthide, using either Oxone® (a commercially available stable potassium triple salt, $KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$) or *m*CPBA. The two lactones have been polymerized in bulk at 100°C using benzyl alcohol as the initiator and $ZnEt_2$ as the catalyst. MWs of the resulting polyesters are in the range of 0.8–60 kg/mol (PDI = 1.1–1.4). Random copolymers of the dihydrocarvide and carvomenthide have been prepared with the aim of controlling the olefin functionality along the polymer chain [102].

The dihydrocarvone can also be oxidized to an epoxy lactone, 7-methyl-4-(2-methyloxiran-2-yl)oxepan-2-one, with *m*CPBA and subsequently be used as bifunctional monomer and crosslinker in ROPs. Homopolymerizations using benzyl alcohol as the initiator and either diethyl zinc or tin(II) 2-ethylhexanoate as the catalyst produce only low molecular weight polyesters (<2.5 kg/mol). Copolymerizations with ε-caprolactone give flexible crosslinked materials [103].

Cholic and Lithocholic Acid

Native bile acids can form polymers via polycondensation processes (see Sect. 2.2, step-growth polymerizations) but cannot be directly polymerized via chain-growth mechanisms. However, lithocholic acid can be converted into a diene by esterification of COOH and OH at C3 with ω-alkenyl alcohol and acid chloride. The diene is closed to a macrocycle (using benzylidene-bis(tricyclohexylphosphine) dichlororuthenium, a first generation Grubbs' catalyst, in high dilution), which is then polymerized at high concentration by entropy-driven ROMP using [1,3-bis-(2,4,6-trimethylphenyl)-2-imidazoliudinylidene)dichloro-(phenylmethylene)-(tricyclohexylphosphine)ruthenium], a second generation Grubbs' catalyst (Scheme 10). High molecular weight polyesters (M_n = 59–152 kg/mol, PDI = 1.6–1.9) are produced, besides a small fraction of cyclic oligomers (<10%), in high yields. It is noteworthy that the diene can be polymerized via acyclic diene metathesis (ADMET) polycondensation, but producing much lower molecular weight polyesters than ROMP [104].

The same procedure has been applied to synthesize shape memory polymers based on cholic and lithocholic acid [105]. Also, high molecular weight copolyesters

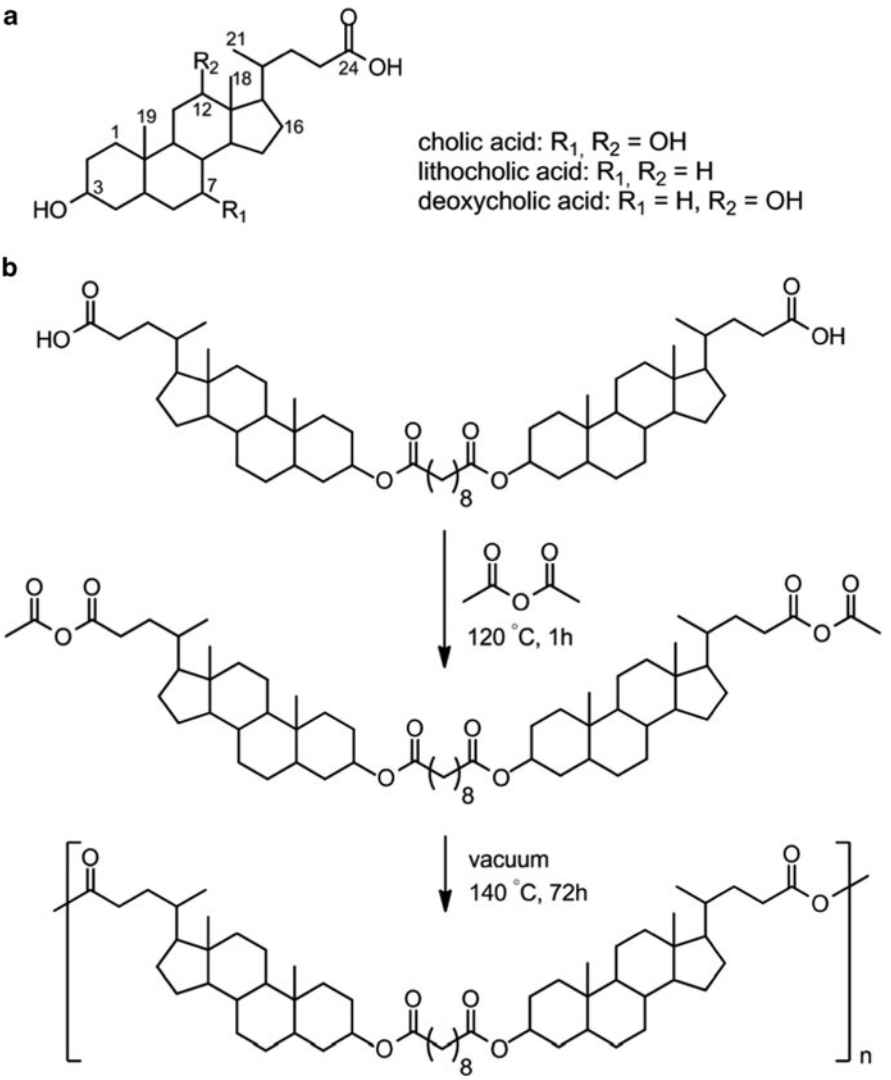


Scheme 10 Entropy-driven ROMP of a lithocholic acid-based macrocycle [104]

(M_n = 70–226 kg/mol, PDI = 2.0–2.5) based on lithocholic and ricinoleic acid have been prepared [106].

2.2 Step-Growth Polymerizations

Bile acids (see the structures in Scheme 11a) have been subject to polycondensation using their COOH and OH functionalities [107–109]. The first attempt was made in



Scheme 11 (**a**) Structures of bile acids and (**b**) synthesis of polyanhydride from dimers of lithocholic acid [110] (reproduced with permission from American Chemical Society)

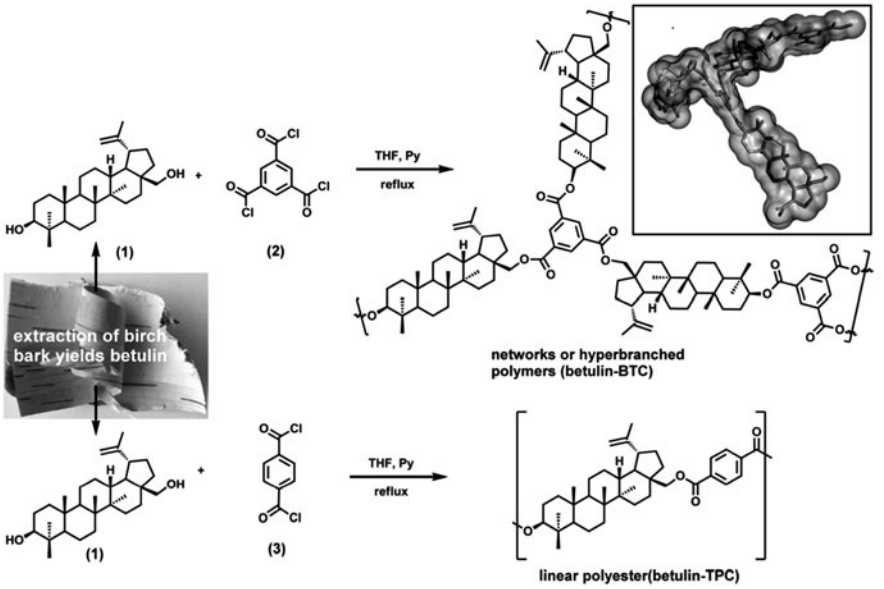
toluene at 90–110°C using *para*-toluenesulfonic acid (*p*TSA) as the catalyst. Polymers with M_n = 4–5 kg/mol were obtained, while crosslinking was observed due to the reaction at C7 and C12 position [107]. The use of a lipase (from *Candida antarctica*) gave regioselective oligocondensation (at 50°C) of cholic acid at C3 position, the M_n of the oligomer being 0.92 kg/mol (PDI = 3.5). The oligocondensation of a mixture of cholic acid and 11-methacroylaminoundecanoic acid results in the formation of a oligo(cholic acid ester) (M_n = 1.1 kg/mol) bearing a radically polymerizable methacryloyl end group, which successively undergoes radical polymerization generating a comb-shaped polymer with oligo(cholic acid ester) side chains (M_n = 33 kg/mol) [108]. Polycondensation of bile acids has been conducted at room temperature using a mixture of diisopropyl carbodiimide and a 1:1 salt of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) and *p*TSA, which allows selective polycondensation at the C3 position and gives a relatively high (apparent) MW of 50–60 kg/mol [109].

A biodegradable polyanhydride has been prepared by polycondensation of a lithocholic acid dimer (Scheme 11b). The homopolymer has a T_g of 85°C and a melting point of >250°C, both of which can be lowered by the incorporation of a comonomer (sebacic acid). The polymers have been subjected to degradation and release studies, using *p*-nitroaniline as the model drug. The degradation and release rates are found to be dependent on the copolymer composition, and no apparent toxicity is observed in vivo [110].

Myrcene–maleic anhydride Diels–Alder adduct and the corresponding diacid have been used as monomers for the polycondensation with diethylene glycol, resulting in unsaturated polyesters. The polyesters exhibited air-drying properties on crosslinking with styrene [111].

Betulin, extracted from birch bark, has been used as a difunctional monomer to undergo polycondensations with acid chlorides, resulting in hyperbranched (networks) or linear polyesters (Scheme 12). The stiff structure of betulin and the geometric positioning of the OH groups prevent close packing of polymer chains to yield a microporous structure, making these polycondensates candidates for gas separation membranes [112].

Thiol-ene addition has been used to functionalize terpenes, including (*R*)-(+)- and (*S*)-(−)-limonene and (−)-β-pinene, with OH and ester moieties. These reactions are regioselective and can be controlled to yield monofunctional, difunctional, or heterodifuctional monomers (Scheme 13a, b). Difunctional and heterodifunctional monomers have been subjected to polycondensation using 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) as a catalyst. Oligomers or low MW products with M_n < 10 kg/mol are obtained from homopolymerization or copolymerization with short-chain diols (Scheme 13c). Copolymerization with long-chain fatty acid-derived diols or diesters gives polyesters with higher MWs (M_n up to 25 kg/mol) (Scheme 13d) [113].



Scheme 12 Synthetic route toward (*top*) microporous betulin-based polyester networks or hyperbranched structures (*inset*: 3D representation of the shown segment) and (*bottom*) linear, soluble polyesters [112] (reprinted with permission from Wiley-VCH Verlag GmbH & Co. KGaA)

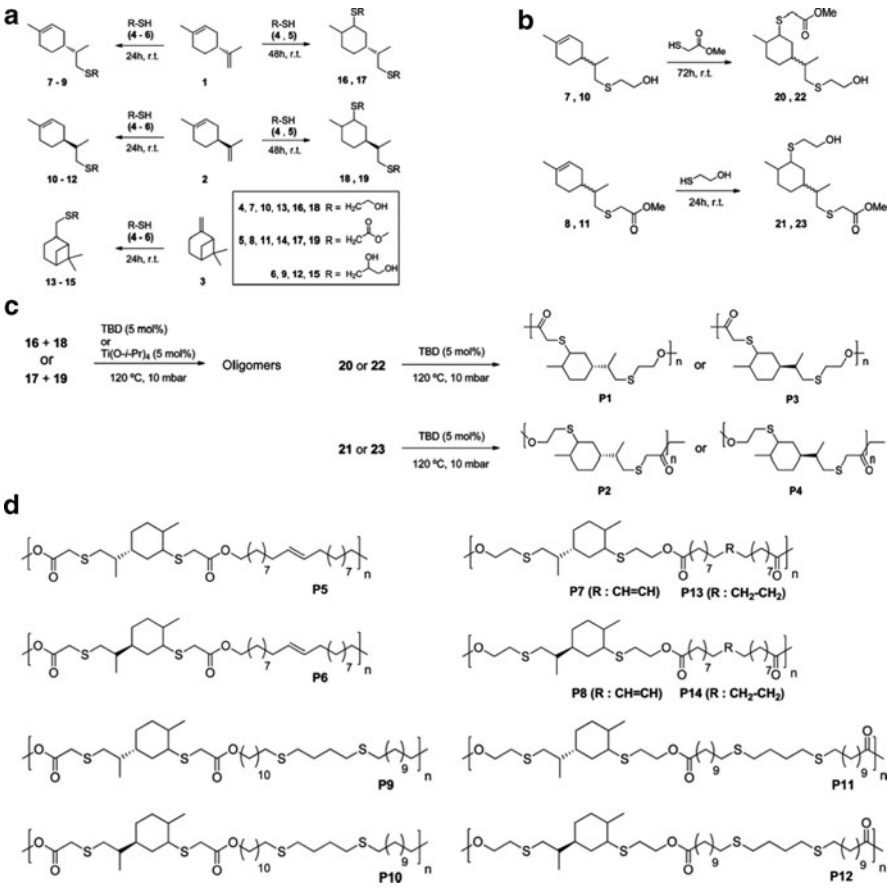
3 Polymers with Terminal or Central Terpene Entities

Many terpenes bear reactive groups such as hydroxyl and carboxyl, which can be used for covalent attachment of terpene entities to end-functionalized polymers (Sect. 3.1) or initiation of polymerization reactions (Sect. 3.2). Sometimes, chemical modification needs to be done to turn OH or COOH into other functional groups for these two purposes. The combined properties of the biological and synthetic components have made terpene–polymer conjugates, with one or two terpene moieties at chain end(s) or in the center, very attractive materials. Nevertheless, only a few terpenes have been used for this purpose, mostly cholesterol and bile acids.

3.1 Attachment of Terpene Entities to Polymer Chain Ends

3.1.1 Cholesterol

The OH functionality on cholesterol (Chol) can be used directly for the esterification with COOH end-functionalized polymers, by the aid of dicyclohexylcarbodiimide (DCC) and DMAP (Scheme 14) [114]. Star-shaped and linear poly (ethylene glycol) (PEG) with terminal Chol entities have been prepared, and used

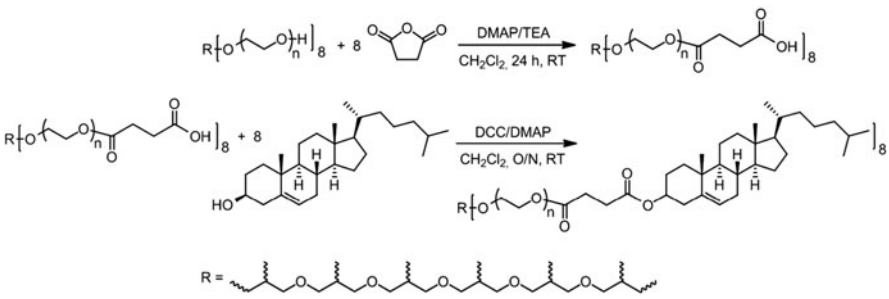


Scheme 13 (**a**) Mono- and diaddition of thiols to terpenes. (**b**) Addition of thiols to monoaddition products generating heterodifunctional terpene monomers. (**c**) Polycondensation of terpene-derived monomers. (**d**) Polycondensation of limonene- and fatty acid-based monomers to polyesters [113] (reprinted with permission from American Chemical Society)

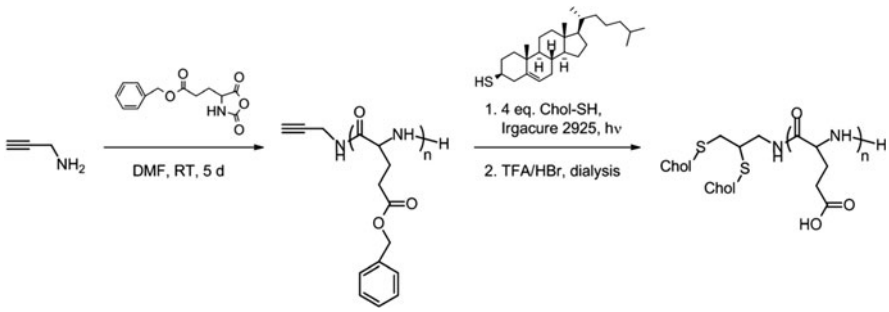
to form hydrogel through the host–guest inclusion with star-shaped PEG end-functionalized with β-cyclodextrin [114–116]. The degree of esterification is >90% for linear PEG and 64–84% for star-shaped PEG.

The esterification has also been implemented in an alternative way, namely, the OH on Chol is firstly turned into COOH (by succinic anhydride) and reacted with OH-ended PEGs. High degree of functionalization (>90%) is achieved for PEGs with MW ranging from 0.4–10 kg/mol. These Chol–PEG conjugates are used to modify non-ionic surfactant vesicles as a potential drug delivery system [117].

Cholesterol has also been coupled to polymer chain ends after other chemical modifications on its OH position. The most-reported method uses cholesteryl chloroformate (CholCl), which is commercially available, to react with OH or NH_2 end-functionalized polymers (i.e., PEG [118, 119] and polyacrylamide derivatives [120, 121]). Quantitative functionalization can be achieved with either 1 equiv. or



Scheme 14 Synthesis of star-shaped PEG with terminal Chol entities [114] (reproduced with permission from American Chemical Society)



Scheme 15 Synthesis of poly(glutamic acid) carrying two Chol entities at the α-chain end by thiol-yne click chemistry [125] (reproduced with permission from Royal Society of Chemistry)

excess of CholCl, with regard to the polymer end groups. 2-Cholesteryl-2-oxo-1,3,2-dioxaphospholane has been synthesized and reacted with polyisoprene with a tertiary amine end group, generating a polymer conjugate with a zwitterionic linkage [122]. Near quantitative functionalization is obtained with tenfold excess of the cholesteryl compound. Recently, thiol-ene/yne click chemistry has been used to attach a Chol moiety to polymer chain ends, facilitated by the commercial availability of thiocholesterol. Thiol-ene chemistry at poly(ε-caprolactone) (PCL) chain end is conducted under UV irradiation (1.5 h) with a very large excess of thiol (10 equiv.) and photoinitiator (2,2-dimethoxy-2-phenylacetophenone, 3.5 equiv.), achieving quantitative addition of Chol at the chain end [123]. Thiocholesterol was added to allyl-terminated poly(2-isopropyl-2-oxazoline) under UV light ($\lambda > 300$ nm) in the absence of photoinitiator [124]. For thiol-yne chemistry at a poly(γ-benzyl-L-glutamate) chain end (Scheme 15), low amounts of Irgacure 2959 (photoinitiator) and 4 equiv. of thiol were used. After 4 h of UV irradiation only the bis-addition product could be detected, though quantitative yield was not claimed [125].

The introduction of lipophilic Chol terminal groups modifies the solution properties of the polymer conjugates, enabling them to self-associate into ordered structures (e.g., lipid structure) [118, 120, 125]. Some of these conjugates are considered to be advantageous for the fabrication of drug delivery systems [119, 121].

3.1.2 Bile Acids and Other Terpenes

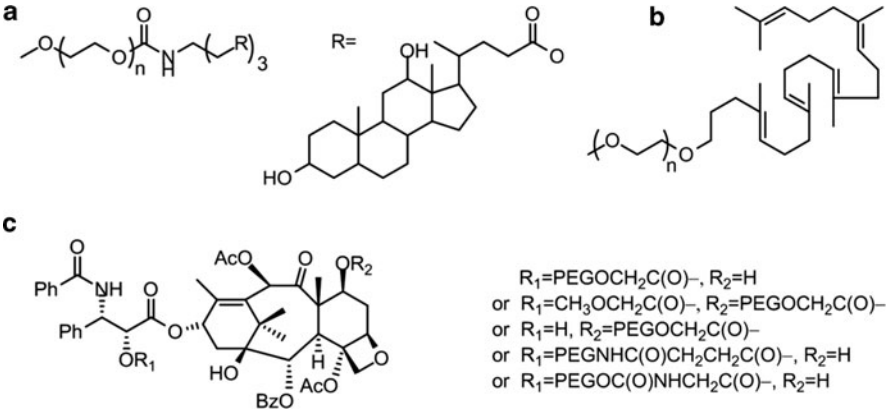
Scheme 16 depicts some of the PEG–terpene conjugates discussed in this section. The polymer conjugates with bile acids (i.e., deoxycholic acid and cholic acid) have also been made by condensation reactions of their COOH functionalities with OH [126] or NH_2 [127–129] end-functionalized polymers. Various coupling reagents have been used, including DCC/DMAP [126], 1,1-carbonyldiimidazole (CDI) [127], and DCC/DMAP/NHS [128, 129]. High degrees of substitution have been achieved regardless of the coupling method and linkage formed (ester or amide). The aqueous self-assembly behavior and potential application as e.g., drug carrier system, of the bile acid conjugates with PEG or poly(*N*-isopropylacrylamide) (PNIPAM) have been studied.

Recently, squalene–PEG conjugates have been made, using end-modified trisnorsqualene and PEG, and have been used to stabilize squalenoyl prodrugs [130]. Conjugates of PEG and taxol derivatives have been prepared and used as water-soluble prodrugs [131, 132]. Attempts are being made to selectively couple PEG at different positions. Release of the drug can be triggered by the hydrolysis of conjugate linkages (ester, amide, or carbamate moieties). The MW of the PEG chain is found to be an important factor for a controlled drug release.

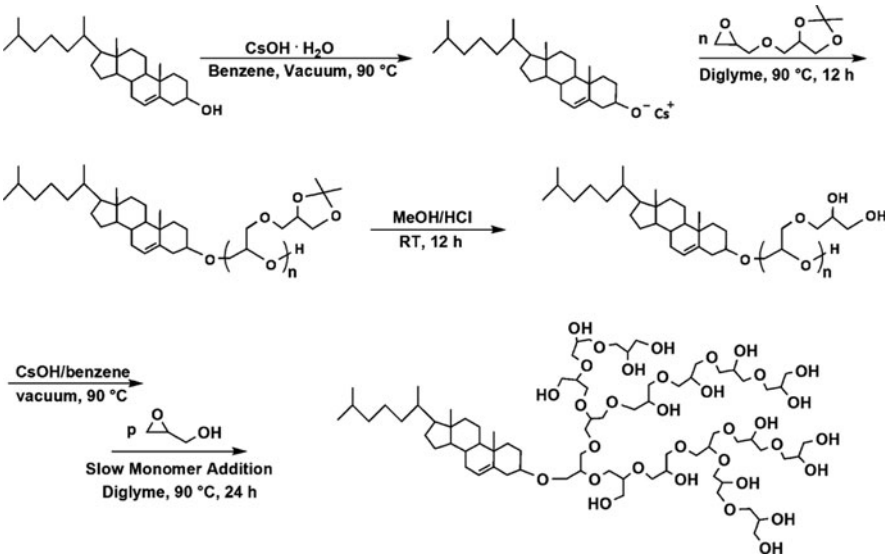
3.2 *Polymerization Initiated from Functional Terpenes*

3.2.1 Cholesterol (and Other Terpenes with 1-2 OH) to Linear (and Hyperbranched) Polymers

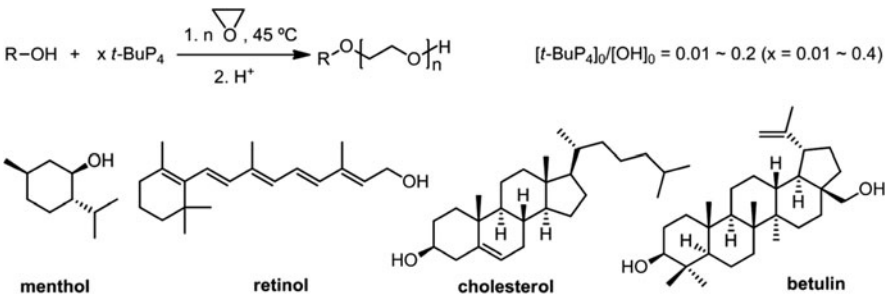
Chol-based amphiphilic polymer conjugates can be prepared by anionic ROP of epoxy monomers from the OH site. Multifunctional polymeric lipids with linear or hyperbranched polyether as the hydrophilic component have been prepared, using



Scheme 16 Molecular structures of PEG conjugates containing (**a**) deoxycholic acid [126], (**b**) squalene [130], and (**c**) taxol derivative [131, 132]



Scheme 17 Synthesis of hyperbranched polyglycerol using cholesterolate cesium [134] (reprinted with permission from American Chemical Society)



Scheme 18 Synthesis of terpene–PEO conjugates by *t*-BuP_4-promoted anionic ROP of ethylene oxide [135]

either potassium naphthalide or cesium hydroxide as the deprotonation agent (Scheme 17). High loading of the catalysts (90% deprotonation) is needed for high initiation efficiency. However, the poor solubility of metal-based catalyst is always an obstacle for this purpose [133, 134].

The anionic ROP of ethylene oxide from terpene alcohols, including Chol, menthol, retinol, and betulin, can be achieved using a metal-free polymerization promoter, i.e., phosphazene base *t*-BuP_4. Nearly quantitative initiation efficiency can be achieved even when *t*-BuP_4 is used in very low amount (0.01–0.2 equiv. of OH groups, Scheme 18), indicating that the proton transfer among active and dormant chain ends during the polymerization is much faster than the both the initiation and propagation.

The different terpene entities are expected to affect the thermal and solution properties of these terpene–poly(ethylene oxide) (PEO) conjugates [135].

A Chol-based radical initiator can be prepared by reacting Chol with 4,4′-azobis (4-cyano-pentanoic acid chloride) and used to polymerize vinyl monomers. Either 2-mercaptoethanol or thiocholesterol is used as chain transfer agent, generating amphiphilic polymer conjugates with Chol entities at one end or both ends, respectively. Higher amount of chain transfer agent is needed to achieve low PDI, with the concomitant limitation to low MWs [136, 137].

Hydroxyl-functionalized terpenes have been used to polymerize cyclic esters or carbonates (lactide, ε-caprolactone, glycolide, and trimethylene carbonate) with the aid of metal-based catalyst such as $AlEt_3$ or $Sn(Oct)_2$, generating biodegradable polymer conjugates [138–146]. Early studies reported the synthesis of poly(lactic acid) (PLA) with $AlEt_3$ and different OH-functionalized terpenes, including vitamins (e.g., α-tocopherol and ergocalciferol), flavors (e.g., menthol and geraniol), and hormones (e.g., testosterone and pregnenolone), as initiating systems (Fig. 5) [138, 139]. Equimolar amounts of $AlEt_3$ and OH were used. The polymerizations were conducted at 60°C without significant side reactions, and the incorporation of every terpene in the polymer products was proven to be successful. The reactivity of the initiating system varies with different terpenes, for example α-tocopherol/$AlEt_3$ proves to be less efficient than others due to the more sterically hindered and less nucleophilic phenol. These biodegradable PLA–terpene conjugates are expected to have great potential as components in controlled-release systems, taking into account the retarded release of the bioactive terpenes.

Chol–PLA conjugates (short PLA) can be synthesized by polymerization either in toluene solution with 1 equiv. of $AlEt_3$ to 3 equiv. of Chol or in bulk with a low amount

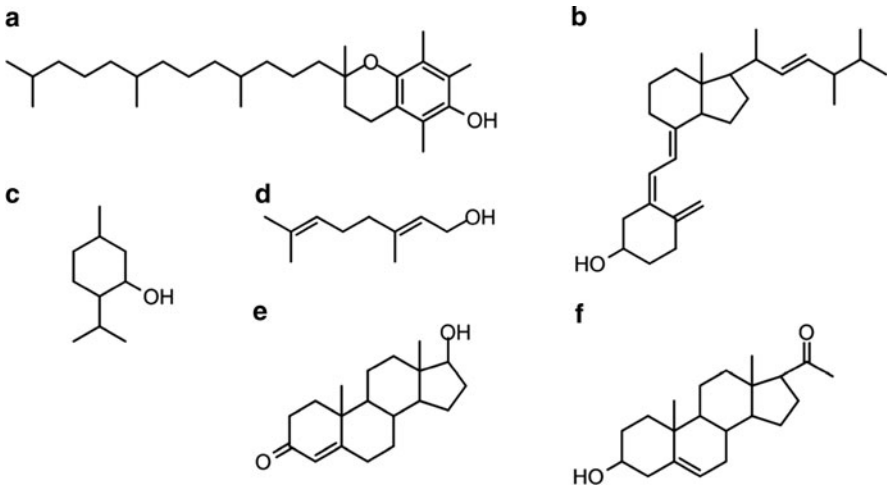


Fig. 5 Structures of OH-functionalized terpenes used as initiators for the ROP of lactide: (**a**) α-tocopherol, (**b**) ergocalciferol, (**c**) menthol, (**d**) geraniol, (**e**) testosterone, and (**f**) pregnenolone

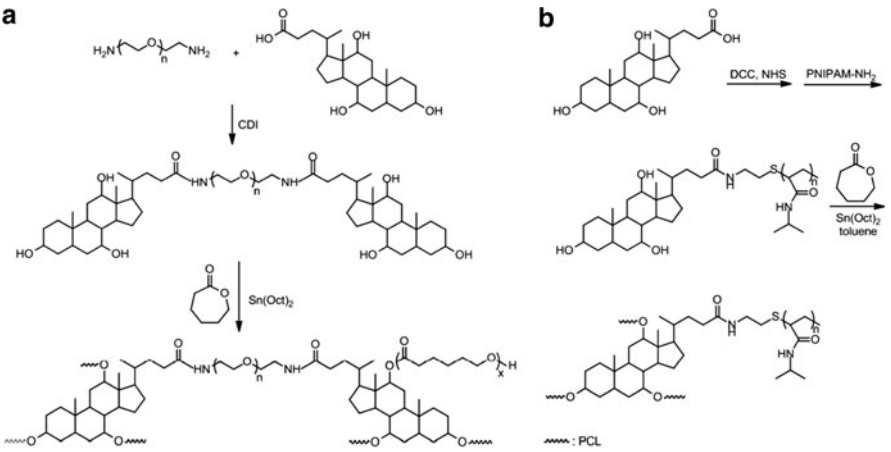
of $Sn(Oct)_2$ (<0.1 equiv.). High isolation yields are not achieved because of the good solubility of short PLA chains in the precipitant (methanol). The polymer conjugates can be further functionalized at the OH terminus with other bioactive components such as indomethacin, rhodamine B, α-amino acids, dendritic poly(L-lysine), etc. [140, 141]. These bifunctional PLA conjugates have exhibited interesting self-ordering behavior, and are expected to be useful biomaterials for cell and tissue engineering, with the Chol moiety improving cell interaction. Chol–polyester, including PCL [142], poly(lactide-*co*-glycolide) (PLGA) [143], and poly(trimethylene carbonate) (PTMC) [144], can be prepared with (for PCL) or without (for PLGA and PTMC) $Sn(Oct)_2$ catalyst. Longer reaction time seems to be needed for the polymerizations without the catalyst, but direct comparison is difficult to make since different monomers are used in both cases. LC structures can be formed when the polyester chains are short. The (enzymatic) degradation of these Chol–polyester conjugates has been studied; a lamella-like porous structure on the Chol–PLGA sample surface is found upon degradation. Drug-release properties were evaluated in vitro, and the incorporation of Chol moiety in the Chol–PCL conjugate was found to improve cell proliferation. Additionally, PCL with a Chol moiety at both ends has been synthesized by coupling two monofunctional Chol–PCL conjugates to one hexamethylene diisocyanate [145]. This bifunctional conjugate has proven to be miscible with high MW PCL and thus is expected to be useful for the modification of PCL-based biomaterials.

3.2.2 Bile acids to Star-Shaped Polymers

The OH and COOH functionalities on bile acids have been used, either directly or after chemical modification, as initiating sites to prepare star-shaped polymer conjugates with a bile acid central core [127, 129, 147–151]. The OH groups on cholic acid are utilized to grow polyester or polycarbonate arms [127, 129, 147]. 2,2-Dimethyltrimethylene carbonate can be polymerized from the OH groups without catalyst (the COOH entity is believed to accelerate polymerization) [147]. The resulting three-arm star-shaped polycarbonate has a higher degradation rate than its linear counterpart.

Amphiphilic star-shaped copolymers with a cholic acid core comprising PCL tethered on the OH sites and linear PEO or PNIPAM tethered on the COOH site have been prepared by combination of ROP and $COOH–NH_2$ coupling reaction (Scheme 19) [127, 129]. The polymerization of ε-caprolactone in these cases was catalyzed by $Sn(Oct)_2$. The star-shaped amphiphilic or thermo-responsive copolymers were studied to assess their micellization behavior and degradation rate, for potential use as "smart" drug-release systems.

Anionic ROP of epoxides have been performed using the OHs on bile acid molecules as initiating sites, with potassium naphthalene as the deprotonation agent [148–150]. Prior to the polymerization, the COOH is turned into OH by reduction or amidation. The molecular characterization verified that all (2–4) OH groups are involved in the initiation with either 1 or 0.25 equiv. of initial deprotonation, indicating fast proton transfer during the polymerization among active and dormant chain ends in DMSO. The aggregation behavior in a water solution of PEG stars with



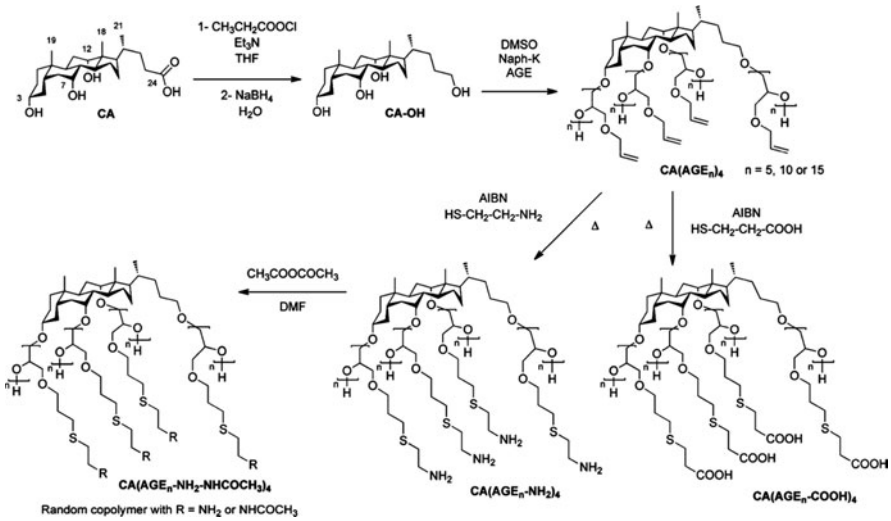
Scheme 19 Preparation of amphiphilic star-shaped copolymers comprising (**a**) cholic acid core, PCL arms, and hydrophilic PEO [127] (reproduced with permission from John Wiley & Sons) or (**b**) PNIPAM chains [129] (reproduced with permission from Elsevier)

bile acid cores shows strong dependence on the PEG chain length as well as the number of arms. Interestingly, the two-armed lithocholate derivative shows the strongest tendency to associate and exhibited two phase transitions during heating from a low (ca. 5°C) to high (ca. 82°C) temperature [149]. Star polymers with cholic acid core and poly(allyl glycidyl ether) arms were prepared and subjected to thiol-ene chemistry to introduce COOH or NH_2 groups in the arms (Scheme 20). The modified polymer conjugates exhibited responsiveness to both pH and temperature [150].

Cholic acid has also been transformed into a multifunctional atom transfer radical polymerization (ATRP) initiator for the polymerization of *t*-butyl acrylate. The ester groups are hydrolyzed to yield poly(acrylic acid) arms. In the low MW range, aggregation of the star-shaped polymer conjugate in water shows greater dependence on the length of the arm than on the degree of ionization. All these star-shaped water-soluble polymer conjugates, also referred to as "molecular umbrellas", are expected to have great potential as for instance drug carriers. Generally, short arms are required to maintain the property brought in by the bile acid core [151].

4 Polymers with Pendent Terpene Entities

Another class of terpene-based polymer bioconjugates includes (co)polymers with pendent terpene moieties. Generally, there are two ways to synthesize these structures: (1) graft terpene molecules onto the polymer backbones, and (2) polymerize terpene molecules decorated with polymerizable moieties. This second method is different from the polymerization reactions described in Sect. 2, since



Scheme 20 Preparation of star-shaped polymer conjugates with cholic acid (*CA*) core and polyether arms functionalized with pendant COOH or NH_2 groups [150] (reprinted with permission from American Chemical Society)

the terpene moieties are incorporated as pendant groups but are not the constituent of the main chain, and therefore the chemical structures as well as the physical and biological properties of the terpenes are mostly maintained. Compared to the mono- or bifunctional terpene–polymer conjugates described in Sect. 3, these multifunctional conjugates preserve and exhibit more profoundly the properties of terpene entities. This is seemingly more favorable for macromolecular design and has aroused extensive studies on the synthetic methodology and on properties and applications. The prepared multifunctional (co)polymer conjugates are mostly subject to studies either on their solution properties, i.e., aqueous self-assembly and aggregation behavior, sometimes further extended to biotechnological applications including drug delivery and gene transfection, or on their LC behavior and application as optical materials. Also, the combination of these two aspects, namely the impact of LC properties on the aqueous self-assembly behavior, has been reported. Other terpenes, which are suitable based on their chemical structures, have scarcely been employed.

4.1 Grafting of Terpenes onto Polymer Chains

CholCl is quite often used to anchor Chol moieties onto backbone originally containing OH or NH_2 groups [121, 152–156]. The backbone copolymers reported include PEG derivatives with pendent NH_2 groups [152], PNIPAM-based

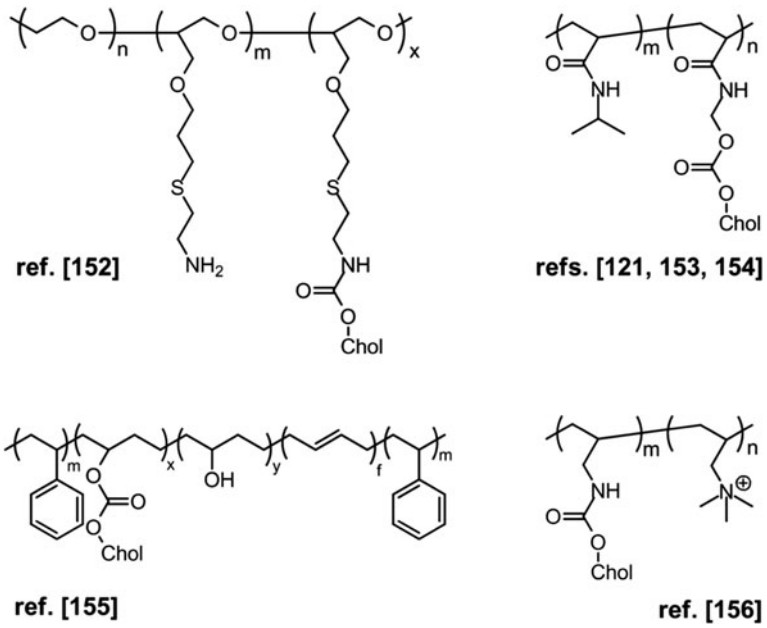
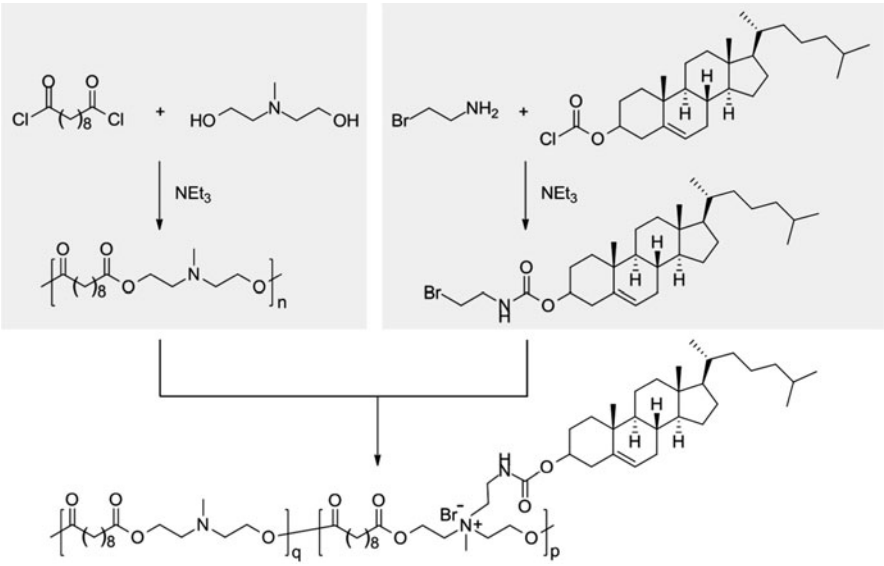


Fig. 6 Copolymers with pendent Chol moeties introduced via CholCl

polyacrylamide derivatives with pendent OH groups [121, 153, 154], polystyrene-*block*-(hydroxylated polybutadiene)-*block*-polystyrene triblock copolymers [155], and poly(allyl amine) [156] (Fig. 6).

Though complete consumption of CholCl may not be achieved in the coupling reaction, the percentage or fraction of Chol moieties in the conjugates can be reasonably controlled by two parameters: (1) the original percentage of backbone repeat units containing NH_2 or OH groups, and (2) the feed ratio between CholCl and NH_2 or OH groups. The self-assembly and/or thermo-responsiveness in water has been investigated for those amphiphilic bioconjugate copolymers. Applications in gene transfection and drug delivery have been estimated. For instance, a cationic PEG derivative with Chol moieties expresses higher transfection efficiency than the copolymer without Chol moieties [152], and a PNIPAM derivative with pendent Chol moieties exhibits higher drug encapsulation efficiency than the Chol-end-functionalized PNIPAM derivative, which also varies with different drugs [121]. Study on the stabilization of single-walled carbon nanotubes in nonpolar solvent with Chol-containing copolymers reveals that more Chol moieties decrease the polarity of the copolymer conjugate and thus inflict poor dispersion [155]. Coupling of Chol to a copolymer backbone has also been realized by COOH–NH_2 amidation with the assistance of either CDI [157] or DCC [158], achieving high but not complete consumption of the reactive sites on the backbone.

Quaternization (the reaction between alkyl bromide and tertiary amine moiety) has been reported for the attachment of Chol moieties onto a (co)polymer backbone



Scheme 21 Preparation of a biodegradable copolymer by polycondensation and attachment of pendent Chol moieties by quaternization [159]

(Scheme 21) [159–161]. CholCl is firstly reacted with 2-bromoethylamine to introduce an alkyl bromide moiety in Chol structure. The Chol-Br is then subjected to quaternization reaction with the polycondensation product of sebacoyl chloride and *N*-methyldiethanolamine, which bear tertiary amine moieties as main-chain constituent, to yield poly[(*N*-methyldiethyleneamine sebacate)-*co*(cholesteryl oxocarbonylamido ethyl methyl bisethylene ammonium bromide) sebacate]. The degree of quaternization can be roughly controlled by the feed ratio of Chol-Br and backbone units; however, no higher than 60% can be achieved, even with 1.5 equiv. of Chol-Br [159] and is even lower when the backbone polymer is linked with a PEG block [160]. The (co)polymer conjugates self-assemble into cationic micelles in aqueous solution at low pH and are used for gene and drug delivery. Enhanced efficiency of gene transfection and intracellular protein delivery has been observed and ascribed to a more stable core–shell structure of the micelles and to an improved cellular uptake through a cellular Chol uptake pathway [159–161].

Hydrosilylation has been used to attach Chol or menthol moieties to polysiloxanes [162–168]. In most cases, double bonds are introduced to the terpenes at the OH position with a spacer (Fig. 7), and then the modified monomers are grafted onto polysiloxanes with Si–H moieties, i.e., polymethylhydrosiloxane. Hexachloroplatinate hydrate has been used as catalyst with usually a little excess of the olefin. The degree of hydrosilylation varies from ca. 50 to 100%, depending on monomers and their combinations. Thermal, optical, and mechanical properties, with emphasis on the LC phase transition, of the terpene-modified polysiloxanes have been studied and modulated with different alkyl spacers, comonomers

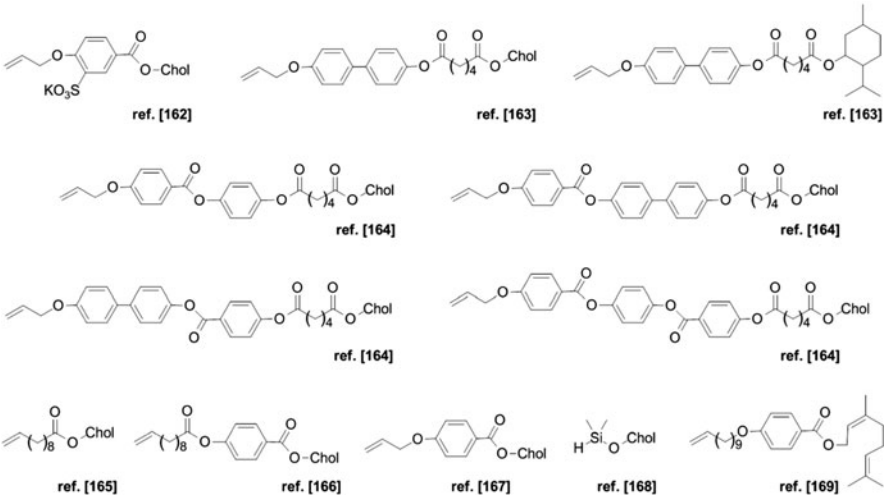


Fig. 7 Structures of terpene-based monomers used for the modification of polysiloxanes via hydrosilylation

[162–164], and difunctional/tetrafunctional crosslinkers [165–167]. The role of the two parts in the hydrosilylation can be reversed, namely, the Si–H group can be introduced to Chol and C=C to polysiloxane (as pendant groups) [168], where the impact of polysiloxanes structure (linear and branched) on the properties of the conjugates is also revealed. Apart from Chol and menthol, polysiloxane with geraniol pendant groups has been made via a similar strategy, and a smectic A phase observed for the polymer conjugate [169].

Hexamethylene diisocyanate has been frequently used as linking agent for attaching Chol moieties onto polymers with pendant NH_2 (NH) groups, such as polyallylamine [170] and poly(L-lysine) [171]. Chol is usually reacted with a large excess of hexamethylene diisocyanate to assure monofunctionalized product, after which the intact isocyanato group is used to attach Chol moieties onto the backbone. The degree of functionalization is tunable on the basis of the feed ratio, which (together with host–guest interaction with β-cyclodextrin) can be used to control the secondary structure formation of the conjugate polymer in water [171]. The same method has been used to hydrophobically modify polysaccharides, which can self-assemble into functional nanoparticles or nanogels [172–176].

Finally, azide–alkyne click chemistry has been used to attach Chol, modified with a propargyl group, to poly(3-azidomethyl-3-methyloxetane), prepared by a cationic ROP [177]. High conversion of the azide groups is achieved with excess of Chol-propargyl. As mentioned, there are a number of methods to attach Chol (and/or menthol), with OH functionalities, to synthetic polymers so as to combine the properties of the two. The same synthetic methods should be applicable to other (functional) terpenes, which would certainly expand the family of terpene-based bioconjugate polymers.

4.2 Polymerization of Terpene-Based Monomers

The polymerization of monomers comprising both a chemically inert terpene entity (mostly menthol, cholesterol, or bile acids) and a readily polymerizable moiety has been another frequently utilized pathway towards (co)polymer conjugates with pendent terpene moieties. A number of polymerization techniques have been employed, including both conventional and living/controlled polymerization methods.

4.2.1 Menthol–Cholesterol

Figure 8 shows the structures of vinyl monomers (Fig. 8a–d) and acetylene monomers (Fig. 8e) derived from Chol and menthol (Men). Chol has, without doubt, been the most appealing terpene for use in this area because of the mesomorphic properties of the monomers and (co)polymers as well as the simplicity and monofunctionality of the Chol structure, which allows quite feasible chemical modification to introduce polymerizable functionalities. In early studies, cholesteryl and cholestanyl (meth)acrylate were made by reacting Chol and dihydrocholesterol with (meth)acryloyl chloride [178–181]. For thermal polymerization, better yields are obtained in the isotropic phase than in the mesomorphic phase because of better mobility of monomer. Solution polymerization with radical initiators gives better yields, higher MWs, and less profound side reactions as compared to bulk thermal polymerization. Later, monomers with different spacers between the (meth) acryloyl group and Chol (or menthol) moieties were prepared [182–193]. Styryl has also been employed as the polymerizable moiety [194]. These monomers are mostly polymerized by free-radical polymerization [184–190, 192–194] with or without comonomers. Laser-initiated polymerization [183] and photopolymerization (UV light, in cholesteric LC phase) [187] have also been reported. Most of these synthetic studies are aimed at creating new LC (co)polymers with spacers and/or comonomers modulating the phase behavior and optical properties [185–188, 190, 192, 193]. Some conjugate copolymers have been prepared to introduce recognition sites (Chol-imprinted polymer) as the receptor for Chol and other steroids [189, 194], or to introduce hydrophobic contents to induce self-association in water [184].

Living/controlled radical polymerizations have been employed recently for Chol-based vinyl monomers, including ATRP [195–197] and reversible addition-fragmentation chain transfer (RAFT) [198, 199], which allowed the preparation of well-defined diblock copolymers (low PDI) with one block being totally synthetic (hydrophilic) and the other block being the Chol-pending conjugate (hydrophobic). What has been of great interest is how the LC nature of the hydrophobic block affects the self-assembly in aqueous solution. Ellipsoidal vesicles are formed by PEG-*block*-poly(cholesteryl acryloyloxyethyl carbonate) and found to exhibit 2D smectic order [196]. Long nanofibers are formed by poly(*N*,*N*-diethylacrylamide)-*block*-poly(cholesteryl methacryloyloxyethyl carbonate), while the block copolymer

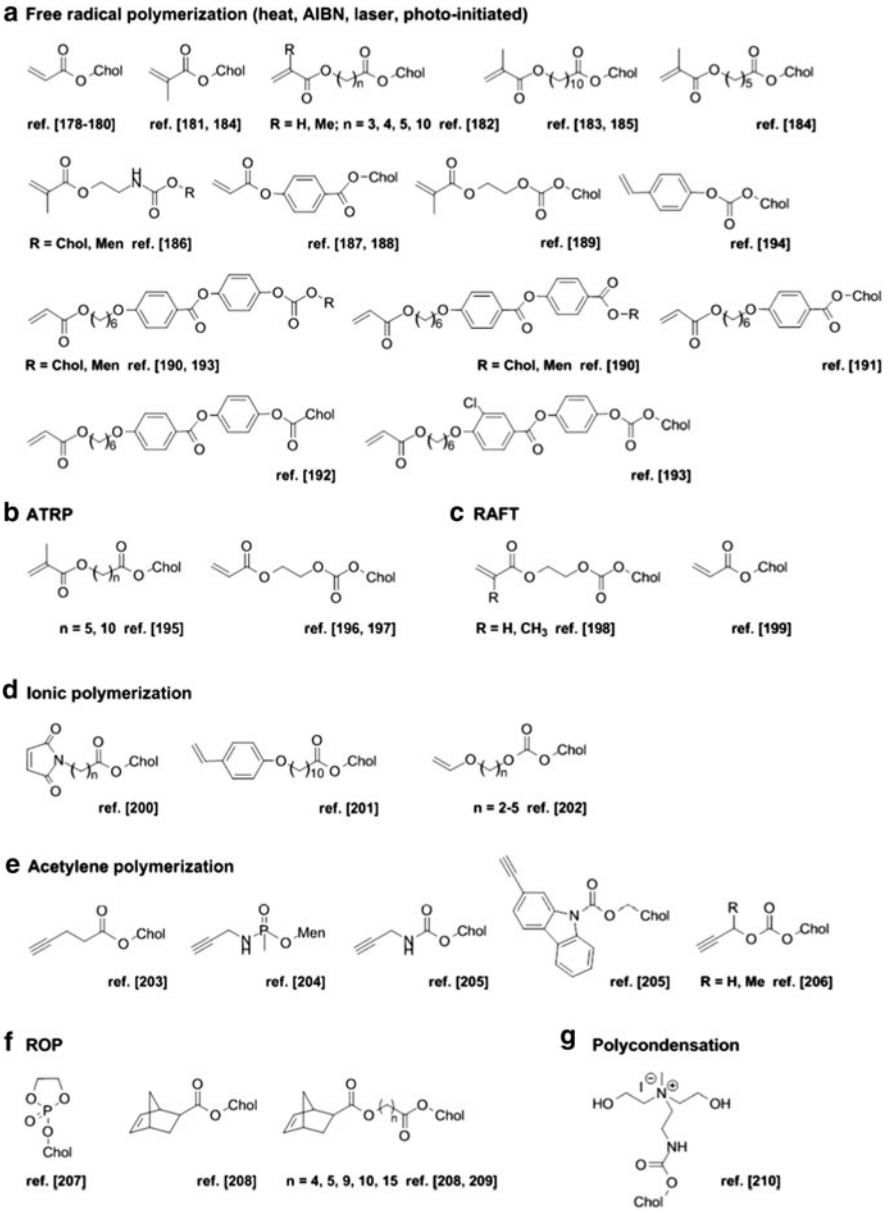


Fig. 8 Structures of monomers derived from cholesterol and menthol

with a polystyrene hydrophobic block and the same hydrophobic content forms vesicles in solution [198].

Attempts to polymerize Chol-based vinyl monomers by ionic procedures have also been made [200, 201]. Living cationic polymerization of Chol-based vinyl ether yields

well-defined di(tri)block copolymers or random copolymers (PDI $\leq$ 1.25) with other bulky vinyl ethers [202]. Apart from vinyl monomers, acetylene monomers containing Chol or menthol moieties have been synthesized and polymerized with transition metal catalysts [203–206]. The chirality and LC properties of the pendant groups, together with the conjugated structure of the backbone, make these polyacetylene derivatives interesting materials for electrical and optical applications.

Heterochain (co)polymers with Chol pendant groups have been prepared by ROP. A cyclic phosphate monomer with Chol moiety (Fig. 8f) has been prepared and copolymerized with two other cyclic phosphate monomers followed by ATRP graft polymerization to introduce hydrophilic side chains [207]. The Chol moieties help this amphiphilic polyphosphate-based graft copolymer to form stable associations in water and to improve the encapsulation of anticancer drugs.

Monomers comprising norbornene and Chol moieties with or without alkyl spacers (Fig. 8f) have been synthesized and polymerized by ROMP using second generation Grubbs' catalysts [208]. A smectic A mesophase is formed only when the spacer is long enough. The Chol-based ROMP monomer can also be copolymerized with two others, i.e., with a short PEG side chain and a crosslinking unit, to create a shape memory polymer network [209], where the smectic A mesophase of Chol moieties plays an important role in the shape memory behavior.

A biodegradable polyphosphoester bearing Chol pendant groups (Fig. 8g) has been produced by polycondensation and used for gene delivery [210].

4.2.2 Bile Acid-Derived Monomers

The chemical structures of monomers derived from cholic acid, the most frequently used bile acid in this respect, are shown in Fig. 9. The methacrylate derivatives of bile acids, containing one (lithocholic acid), two (deoxycholic acid), or three (cholic acid) OH groups (see Scheme 11a), have been prepared. The COOH is protected by an ester group, and the OH at C3 position can be selectively functionalized with methacryloyl and a spacer in between [211, 212]. The (co)polymerization is initiated by AIBN at elevated temperature with or without comonomers (i.e., styrene and MMA). High MWs are acquired at low monomer conversion.

Selective methacrylation of the three OHs in cholic acid have been studied and the reactivity order is found to be C3 > C12 > C7 [213]. Attempts have also been made to improve the hydrophilicity of the bile acid-based (co)polymer conjugates and to explore their properties and potential application in aqueous systems. The OH at C3 position has been turned into NH_2 and the methacrylamide derivatives of bile acids have been compared with methacrylate derivatives. The former are found to undergo more feasible (free radical) polymerization resulting in more hydrophilic polymer [214, 215]. The stereoisomerism of the polymerizable moieties has also been studied. The 3β-epimers are found to polymerize more easily than 3α-epimers; moreover, the polymer of the 3β-epimers presents higher hydrophilicity. Further increased hydrophilicity is achieved by copolymerization with hydrophilic monomers, i.e., methacrylic acid and 2-hydroxyethyl methacrylate, and by selective

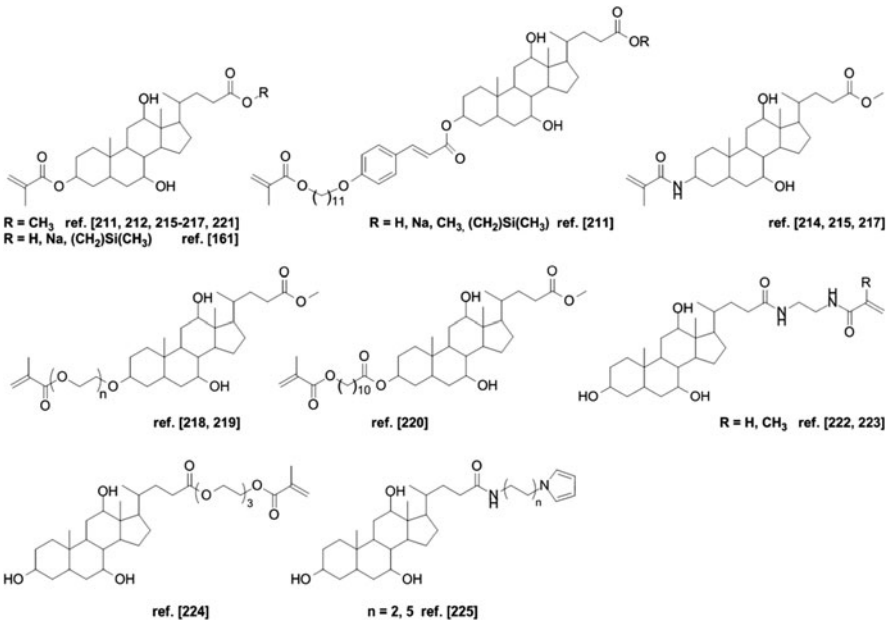


Fig. 9 Chemical structures of monomers derived from cholic acid

hydrolysis to produce an unprotected (or charged) bile acid COOH group [216, 217]. With high content of comonomer, the copolymer is readily soluble in water. Another monomer has been prepared, with PEG oligomer incorporated as a spacer between the methacryloyl and cholic acid moieties. The hydrophilicity of the polymer shows great dependence on the length of the spacer [218]. PEG–cholic acid derived monomer (low content) has been copolymerized with NIPAM (high content) to give a water-soluble copolymer responding to temperature, salt, and pH when COOH groups in cholic acid moieties are liberated [219]. Differing from the polymer formed by Chol-based methacrylates, polymers with bile acid pendant groups do not form any LC structure [220]. Nevertheless, they show great potential as matrix material for mineralization of biominerals [221].

The (meth)acryloyl moiety is sometimes incorporated in cholic acid at the COOH position through a ethylenediamine linker [222, 223]. The conjugate monomer is copolymerized with acrylamide derivatives to produce copolymers with tunable thermo-responsiveness. A monomer of this type with PEG oligomer between methacryloyl and cholic acid moieties has also been prepared and polymerized by ATRP, giving a "comb-shaped" polymer with relatively low MW and low PDI [224].

Pyrrole derivatives bearing bile acid moieties have been synthesized and electrochemically polymerized. The electrochemical properties and stability of the polymer film are found to depend strongly on the length of the alkyl spacer in the monomer [225].

5 Conclusions

Terpenes have been used for decades to make polymers because of their abundance in nature, readily usable chemical functionalities, and the interesting properties they have brought to polymers, like hydrophobicity, bioactivity, and liquid crystallinity. The synthetic methodologies include a large variety of polymerization techniques (to polymerize terpenes or terpene-based monomers) and coupling reactions (to attach terpene entities to synthetic polymers). The use of biosourced terpenes not only meets the urgent need for sustainable chemistry but also exploits the huge potential regarding material properties and biological functions. However, despite all the achievements made in the field of controlled polymerization of terpenes and synthesis of terpene-based polymers, this field is still a challenge to polymer chemists and open for new discoveries and developments.

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