

Transferases in Polymer Chemistry

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Abstract Transferases are enzymes that catalyze reactions in which a group is transferred from one compound to another. This makes these enzymes ideal catalysts for polymerization reactions. In nature, transferases are responsible for the synthesis of many important natural macromolecules. In synthetic polymer chemistry, various transferases are used to synthesize polymers in vitro. This chapter reviews some of these approaches, such as the enzymatic polymerization of polyesters, polysaccharides, and polyisoprene.

Keywords Biocatalysis · Enzymatic polymerization · Polyester · Polyisoprene · Polysaccharide · Transferase

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Abbreviations

APP	Allylic diphosphate
Asp	Aspartic acid
ATRP	Atom transfer radical polymerization
CoA	Coenzyme A
CPT	<i>cis</i> -Prenyltransferase
Cys	Cysteine
DMAPP	Dimethylallyl diphosphate
DP	Degree of polymerization
EC	Enzyme Commission
FPP	Farnesyl diphosphate
GAG	Glycosaminoglycan
GGPP	Geranylgeranyl diphosphate
Glc	Glucose
GPP	Geranyl diphosphate
GT	Glycosyltransferase
HA	Hyaluronan
HAS	Hyaluronan synthase
His	Histidine
HMG-CoA	3-Hydroxy-methyl-glutaryl-CoA
IDI	Isopentenyl diphosphate isomerase
IDS	<i>trans</i> -Isoprenyl diphosphate synthase
IPI	IPP isomerase
IPP	Isopentenyl diphosphate
MPP-D	Mevalonate diphosphate decarboxylase
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced form)
NMR	Nuclear magnetic resonance
PHA	Polyhydroxyalkanoate
P3HB	Poly(3-hydroxybutyric acid)
TEMPO	2,2,6,6-Tetramethylpiperidine-1-oxyl
THF	Tetrahydrofuran
TPT	<i>trans</i> -Prenyltransferase
UDP	Uridine diphosphate

1 Introduction

Enzymatic polymerizations are an emerging research area with not only enormous scientific and technological promise, but also a tremendous impact on environmental issues. Biocatalytic synthetic pathways are very attractive as they have many advantages, such as mild reaction conditions, high enantio-, regio- and chemoselectivity, and the use of nontoxic natural catalysts.

Transferases (enzyme classification, class no. 2) are enzymes that catalyze reactions in which a group is transferred from one compound to another. Groups that are transferred are Cl, aldehydic or ketonic residues, acyl, glycosyl, alkyl, nitrogenous, and phosphorus- and sulfur-containing groups [1]. Of the three classes of enzymes used in polymer science so far, transferases are the least applied class of biocatalyst. Despite their potential for synthesizing interesting polymeric materials many transferases are very sensitive biocatalysts, which prevents their isolation on a larger scale and/or their use for synthesizing polymers on a reasonable scale.

The enzyme class of transferases is subdivided into nine subclasses:

EC 2 Transferases

EC 2.1 Transferring one-carbon groups

EC 2.2 Transferring aldehyde or ketonic groups

EC 2.3 Acyltransferases

EC 2.4 Glycosyltransferases

EC 2.5 Transferring alkyl or aryl groups, other than methyl groups

EC 2.6 Transferring nitrogenous groups

EC 2.7 Transferring phosphorus-containing groups

EC 2.8 Transferring sulfur-containing groups

EC 2.9 Transferring selenium-containing groups

This review focuses on acyl- and glycosyltransferases and transferases that transfer alkyl or aryl groups, other than methyl groups (EC 2.3, EC 2.4, and EC 2.5) as in these classes can be found interesting examples for the polymer scientist.

Acyltransferases are, for instance, able to synthesize biological polyesters with properties comparable or sometimes even exceeding polymers based on petrochemical-derived monomers. Acyltransferases are also frequently used to modify macromolecules in food and non-food applications.

For the synthesis of highly defined polysaccharides, glucosylsaccharides are the only option available. Specialized oligo- and polysaccharides for food and medical applications can be synthesized, and also hybrid structures with non-natural macromolecules or surfaces.

Prenyltransferases are responsible for the synthesis of *cis*-polyisoprene in natural rubber particles but can also be used to synthesize polyisoprenes in vitro.

2 Acyltransferases (EC 2.3)

2.1 Polyester Synthase

Polyhydroxyalkanoates (PHAs) are biological polyesters that are produced by a wide variety of bacteria as osmotically inert carbon- and energy-storage compounds that accumulate in the form of granules (see Fig. 1).

PHAs are generated in almost all bacteria under nutrient-limited growth conditions when a carbon source is readily available. PHA production occurs in exponential, late exponential, or in stationary growth phases, depending on the organism. Accumulation of PHA can reach as much as 85% of the dry cell weight. When the environment becomes more hospitable, the PHAs are degraded to the corresponding monomers, which are used as a source of energy for biosynthesis (supplying NADH) and as biosynthetic building blocks. As with all the other polymers discussed above, there are also conditions of growth in which PHA is generated transiently.

Polyhydroxyalkanoate synthase (systematic name: acyl-CoA:3-hydroxybutyrate O-acyltransferase; EC 2.3.1. class) is responsible for the polymerizations of PHAs *in vivo* because it catalyzes the stereoselective conversion of (*R*)-3-hydroxyacyl-CoA substrates to PHAs with the concomitant release of CoA (see Fig. 2) [3].

The residues Cys-319, Asp-480 and His-508 of the class I polyester synthase from *Cupriavidus necator* are conserved in all PHA synthases and were shown to be essential for covalent catalysis [2, 4, 5]. Cys-319 is the proposed catalytic nucleophile that is activated by the general base catalyst His-508.

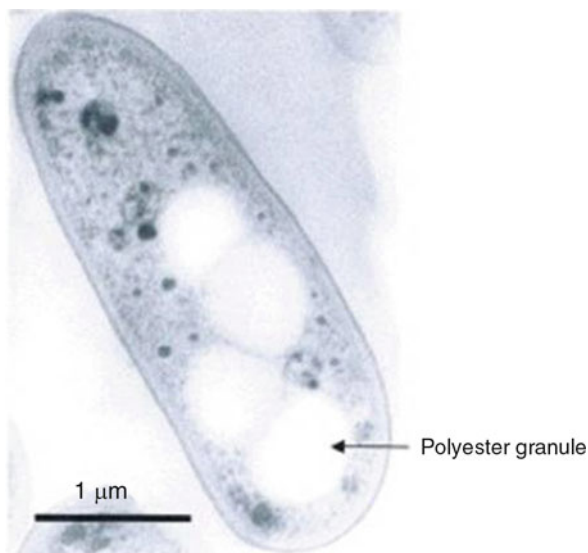


Fig. 1 Electron microscopy image of *Pseudomonas aeruginosa* harboring polyester granules [2] – Reproduced by permission of Portland Press Ltd.

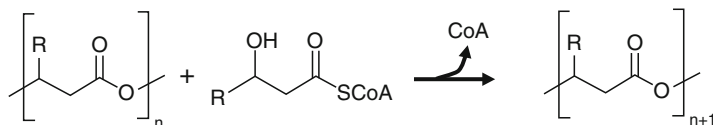


Fig. 2 Reaction catalyzed by polyester synthase

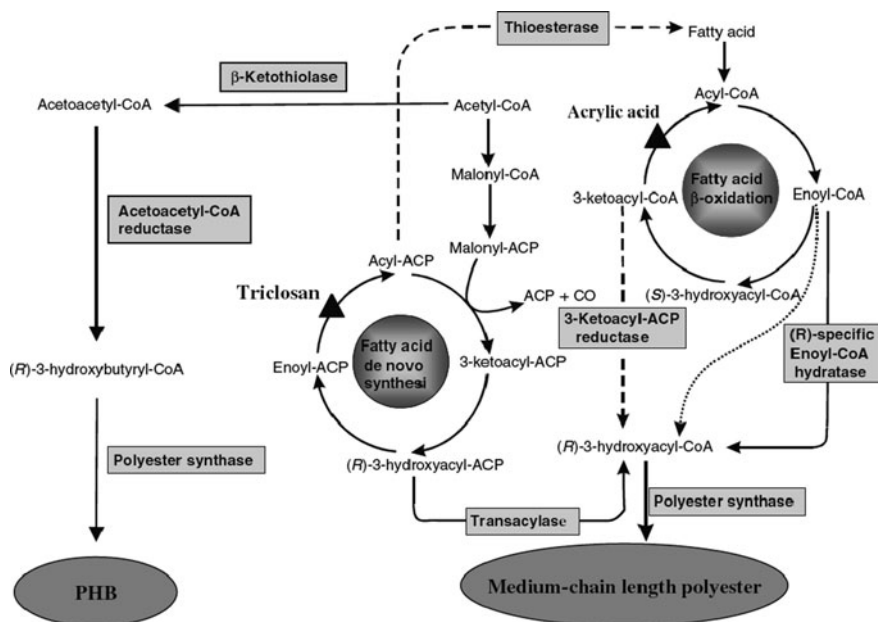


Fig. 3 Metabolic routes towards biopolyester synthesis. *Dashed lines* represent engineered biosynthesis routes. *Triangles* depict targets for inhibitors enabling biopolyester synthesis. Enzymes indicated on *shaded boxes on solid lines* are biopolyester biosynthesis enzymes. With kind permission from Springer Science+Business Media [7]

Reported efforts on engineering the PHA synthase towards better performance and selectivity *in vivo* and *in vitro* were recently assessed in an excellent mini-review by Nomura and Taguchi [6].

PHA polymers can be divided into three main classes with properties that are dependent on their monomer composition. Short-chain-length PHAs have monomers consisting of three to five carbons, are thermoplastic in nature, and generally lack toughness. Medium-chain-length PHAs have monomers consisting of 6–14 carbons and these polymers are elastomeric in nature. The third main class of PHAs includes copolymers made up of short-chain-length and medium-chain-length HA monomers consisting of 3–14 carbons. These PHAs have properties in between those of the first two classes, depending on the mole ratio of monomers, and therefore have a wide range of physical and thermal properties.

Figure 3 shows parts of the *in vivo* metabolic route towards PHAs. The biosynthesis of poly(3-hydroxybutyric acid) (P3HB) requires the condensation of two

acetyl-CoA molecules catalyzed by the β -ketothiolase (PhaA), leading to the formation of acetoacetyl-CoA, which is reduced to (*R*)-3-hydroxybutyryl-CoA by the (*R*)-specific acetoacetyl-CoA reductase (PhaB). (*R*)-3-Hydroxybutyryl-CoA is substrate for the polyester synthase (PhaC) and the direct precursor of P3HB biosynthesis [2, 7]. In contrast to this, medium-chain-length PHAs are produced in vivo from intermediates of fatty acid metabolism (see Fig. 3).

PHAs can consist of a diverse set of repeating unit structures and have been studied intensely because the physical properties of these biopolyesters can be similar to petrochemical-derived plastics such as polypropylene (see Table 1). These biologically produced polyesters have already found application as bulk commodity plastics, fishing lines, and for medical use. PHAs have also attracted much attention as biodegradable polymers that can be produced from biorenewable resources. Many excellent reviews on the in vivo or in vitro synthesis of PHAs and their properties and applications exist, underlining the importance of this class of polymers [2, 6, 7, 12, 26–32].

In the large-scale production of PHAs, the extraction and purification of PHA from biomass is a crucial factor for determining the practical importance of these polymers. It is important that PHAs can be extracted efficiently and easily, much like the extraction of endogenous compounds such as starch, sucrose, and oil.

Table 1 Comparison of the physical properties of the poly(3-/4-hydroxyalkanoate)s with polypropylene. With kind permission from Springer Science+Business Media [8]

Polyester	T_g (°C)	T_m (°C)	Crystallinity	Elongation at break (%)	References
P3HB	15	175	50–80	5	[9]
P3HV	−15, 0	110, 112, 118	56		[10, 11]
P3HB-co-20mol%3HV	−1	145		50	[12]
P3HB-co-10mol%3HHx	−1	127			[13]
P3HB-co-17mol%3HHx	−2	120		850	[13]
P3HB-co-47mol% 3HV-co-16mol% 4HV- co-15mol%3HHx-co- 2mol%3HO	−15	118		1,000	[11]
P3MB	8	100			[14]
P4HB	−40	53		1,000	[10]
P(3HB-co-16%4HB)			43	444	[15]
P3HPE	−11	63			[16, 17]
PHO	−36	59, 61			[18–20]
PHN	−39, −29	48, 54, 58			
P3H6MN	Not determined	65			[19, 20]
PH-p5TV	17	95			[21]
PH6PHx	4	Not determined			[22]
PH5PoxV	14	Not determined			[23]
PH- <i>p</i> -nitroPV	28	Not determined			[24]
PH8-pMPoxO	14	97			[25]
Polypropylene	−15	176	50	400	[12]

T_g glass transition temperature, T_m melting temperature

There are two common protocols used for PHA extraction from bacteria. The conventional one is based on the solubility of PHA in chloroform and insolubility in methanol [33]. After harvest, lipids and other lipophilic components in the bacterial cells are removed by reflux in hot methanol, followed by solubilization of PHA in warm chloroform. PHA from chloroform solvent can be recovered by solvent evaporation or precipitation by addition of methanol. Although highly purified PHA is obtained by this method, a large amount of hazardous solvent is needed to repeat the same process. Thus, this method is not environmentally friendly and unsuitable for mass production of bioplastic. The second protocol is designed to avoid the use of organic solvents. Bacterial cells are treated with a cocktail of enzymes (including proteases, nucleases, and lysozymes) and detergents to remove proteins, nucleic acids, and cell walls, leaving the PHA intact [34].

The first demonstration of *in vitro* P3HB synthesis in aqueous solution was achieved by Gerngross and Martin [35]. The polymer obtained had significantly higher molecular weight than that synthesized *in vivo*. This is probably due to a lack of a chain termination step of the PHA polymerization under *in vitro* conditions, similar to a living polymerization. Many other reports followed [27, 36, 38–44] and were extensively reviewed [2, 6, 7, 12, 26–32].

2.2 Transglutaminase

Transglutaminases (systematic name: protein-glutamine:amine gamma-glutamyltransferase; EC 2.3.2.13) belong to a class of enzymes known as aminoacyltransferases that catalyze calcium-dependent acyl transfer reactions between peptide-bound glutamine residues as acyl donors and peptide-bound lysine residues as acyl acceptors, resulting in the formation of intermolecular ϵ -(γ -glutamyl)lysine crosslinks.

The transamidation mechanism starts with the nucleophilic attack by the thiol group of an active-site cysteine residue (the catalytic triad is composed of Cys-276, His-334, and Asp-358) on the donor substrate γ -carboxamide group, leading to loss of an equivalent of ammonia and formation of a covalent thiolester intermediate. The acyl group of the transient thiolester is transferred to the acceptor amine substrate in the second step [45].

Transglutaminases form a family of related enzymes found in plasma, tissues, and extracellular media in all vertebrates. One of the best-known transglutaminases is the well-characterized human plasma coagulation enzyme Factor XIIIa, activated by thrombin from its tetrameric zymogen during the coagulation process. At a physiological level, tissue transglutaminases are involved in normal cellular processes, including cell adhesion [46], formation of the extracellular matrix [47], and apoptosis [48]. Diminished regulation of transglutaminase activity is implicated in a number of diseases.

Transglutaminases can recognize a broad range of primary amine acyl-acceptor substrates *in vitro*. However, recognition of the acyl-donor substrate *in vivo* is restricted to the γ -carboxyamide of glutamine within an apparently relatively small subset of sequence contexts

In vitro, the enzyme is able to catalyze crosslinking of whey proteins, soy proteins, wheat proteins, beef myosin, casein, and crude actomyosin (which is refined from mechanically deboned meat), improving functional properties such as the texture of food products [49–53]. Bonds formed by transglutaminase exhibit a high resistance to proteolytic degradation [54].

Although the main applications of microbial transglutaminases remain in the food sector, novel potential applications have emerged during the last decade. These applications cover the areas of biomedical engineering, material science, textiles and leather processing. For a recent excellent review by Zhu and Tramper see [55].

Transglutaminases can be used for improving the properties of protein-based fabrics such as wool, leading to a higher tensile strength after chemical or protease pretreatment [56, 57]. Besides crosslinking, transglutaminases were employed for grafting/coating of wool fabrics with silk sericin or keratin, leading to increased bursting strength and softness, and reduced felting shrinkage [58, 59].

In biomedical applications, transglutaminases have been used for tissue engineering materials such as enzymatically crosslinked collagen [60–63] or gelatin scaffolds [64–69]. Even melt-extruded guides based on enzymatically crosslinked macromolecules for peripheral nerve repair have been reported [70].

Transglutaminases have just become recently available in larger quantities and high purity due to microbiological production of the enzyme instead of extraction from animal tissue. It can be expected that this enzyme will be used more frequently in the future for food and non-food applications.

3 Glycosyltransferases (EC 2.4)

Glycosyltransferases (GTs) are important biological catalysts in cellular systems, generating complex cell surface glycans involved in adhesion and signaling processes. Recent advances in glycoscience have increased the demand to access significant amounts of glycans representing the glycome.

GTs catalyze the transfer of a sugar moiety from an activated donor sugar onto saccharide and nonsaccharide acceptors. GTs can be divided into the Leloir and non-Leloir types according to the type of glycosyl donors they use [71]. Non-Leloir glycosyltransferases typically use glycosyl phosphates as donors, whereas Leloir glycosyltransferases utilize sugar nucleotides as donors and transfer the monosaccharide with either retention (retaining enzymes) or inversion (inverting enzymes) of the configuration of the anomeric center. Most of the GTs responsible for the biosynthesis of mammalian glycoproteins and glycolipids are Leloir GTs.

GTs now play a key role for *in vitro* synthesis of oligosaccharides, and the bacterial genomes are increasingly utilized for cloning and overexpression of active

transferases in glycosylation reactions [72–77]. In a recent excellent review by Homann and Seibel, possible ways to tailor-make biocatalysts by enzyme engineering and substrate engineering are summarized [78].

An established industrial process is the fermentative production of oligo- and polysaccharides (α -glucans) by lactic acid bacteria, e.g., dextran by *Leuconostoc mesenteroides*. Such polymers are used in the food industry, as additives for dyes, and in health care [79, 80]. Differences in the type of glycosidic linkage, the degree and type of branching, and the molecular mass of glucans available at present show promising variations in structural and functional properties, which need to be elucidated further. Known are dextrans with different structural specificities [α -1,6-bound glucose (Glc) backbone, with α -1,2 and α -1,3 side chains] [81, 82], mutan (α -1,3-bound Glc units) [83], alternan (α -1,6- and α -1,3-bound Glc units) [84], and amylose/reuteran (α -1,4- bound Glc units) [85]. In addition to defined polymers, oligosaccharides with specific structures are urgently needed. Oligosaccharides currently produced for commercial markets, including isomaltooligosaccharides, leucrose, and palatinose, are of interest in the fields of food, pharmacy, and cosmetics because of their ability to prevent and treat diseases from various biological origins [86, 87].

In this section, enzymes in the EC 2.4. class are presented that catalyze valuable and interesting reactions in the field of polymer chemistry. The Enzyme Commission (EC) classification scheme organizes enzymes according to their biochemical function in living systems. Enzymes can, however, also catalyze the reverse reaction, which is very often used in biocatalytic synthesis. Therefore, newer classification systems were developed based on the three-dimensional structure and function of the enzyme, the property of the enzyme, the biotransformation the enzyme catalyzes etc. [88–93]. The Carbohydrate-Active enZYmes Database (CAZy), which is currently the best database/classification system for carbohydrate-active enzymes uses an amino-acid-sequence-based classification and would classify some of the enzymes presented in the following as hydrolases rather than transferases (e.g. branching enzyme, sucrases, and amylomaltase) [91]. Nevertheless, we present these enzymes here because they are transferases according to the EC classification.

3.1 Phosphorylase

In the field of polymer science, the most extensively used transferase is phosphorylase (systematic name: (1 \rightarrow 4)- α -D-glucan:phosphate α -D-glucosyltransferase; EC 2.4.1.1). Although this enzyme is responsible for the depolymerization of linear α -(1 \rightarrow 4) glycosidic chains in vivo it can also be used to synthesize linear α -(1 \rightarrow 4) glycosidic chains (amylose) in vitro.

In vivo linear α -1,4-glucans are synthesized from ADP-glucose by the enzyme glycogen synthase [94–97]. The enzyme, as well as the monomer, are quite sensitive and therefore most researchers (at least in the field of polymer science) prefer to use phosphorylase for the synthesis of amylose.

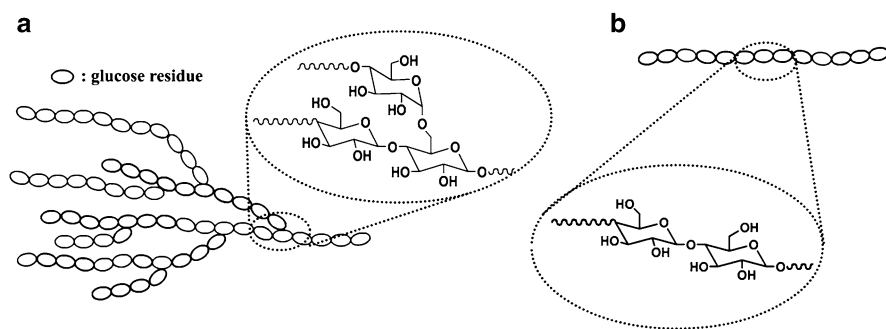


Fig. 4 Structure of (a) amylopectin and (b) amylose

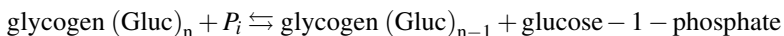
Amylose is one component of starch, which is the most abundant carbohydrate storage reserve in plants. Carbohydrates such as starch function as a reservoir of energy for later metabolic use. It is found in many different plant organs, including seeds, fruits, tubers, and roots, where it is used as a source of energy during periods of dormancy and regrowth.

Starch granules are composed of two types of α -glucan, amylose and amylopectin, which represent approximately 98–99% of the dry weight. The ratio of the two polysaccharides varies according to the botanical origin of the starch.

Amylose is a linear molecule in which the glucose units are joined via α -(1 \rightarrow 4) glucosyl linkages. Amylopectin is a branched molecule in which about 5% of the glucose units are joined by α -(1 \rightarrow 6) glucosyl linkages (see Fig. 4).

In animals, a constant supply of glucose is essential for tissues such as the brain and red blood cells, which depend almost entirely on glucose as an energy source. The mobilization of glucose from carbohydrate storage provides a constant supply of glucose to all tissues. For this, glucose units are mobilized by their sequential removal from the non-reducing ends of starch. For this process three enzymes are required *in vivo*:

1. Glycogen phosphorylase catalyzes glycogen phosphorolysis (bond cleavage of the α -(1 \rightarrow 4) bonds by the substitution of a phosphate group) to yield glucose-1-phosphate:



Phosphorylase is only able to release glucose if the unit is at least five units away from a branching point.

2. Glycogen debranching enzyme removes α -(1 \rightarrow 6) glycogen branches, thereby making additional glucose residues accessible to glycogen phosphorylase.
3. Phosphoglucomutase converts glucose-1-phosphate into glucose-6-phosphate, which has several metabolic fates.

The glycogen phosphorolysis of phosphorylase can be reverted, which makes it possible to enzymatically polymerize amylose as well as hybrid structures with amylose as outlined in the following section.

3.1.1 Enzymatic Polymerization of Amylose with Glycogen Phosphorylase

The existence of a phosphorylating enzyme in a higher plant was first reported by Iwanoff, who observed that an enzyme he found in the germinating vetches *Vicia sativa* liberates inorganic phosphate from organic phosphorous compounds [98]. Shortly after, the same enzyme was found in other vetches and wheat [99, 100], rice and coleseed [101], barley and malt etc. Bodnár was the first to report a progressive disappearance of inorganic phosphate (thus the reverse reaction) while incubating suspended flour from ground peas in a phosphate buffer [102]. Cori and Cori demonstrated that animal tissues contain an enzyme that acts upon glycogen as well [103–106]. Cori et al. suggested that the product of this reaction is α -glucopyranose-1-phosphoric acid (also called Cori-ester), which was confirmed later by Kiessling [107] and Wolfrom and Pletcher [108].

Glycogen phosphorylases belong to the group of vitamin B6 enzymes bearing a catalytic mechanism that involves the participation of the phosphate group of pyridoxal-5'-phosphate (PLP). The proposed mechanism is a concerted one with a front-side attack, as can be seen in Fig. 5 [109]. In the forward direction, e.g.,

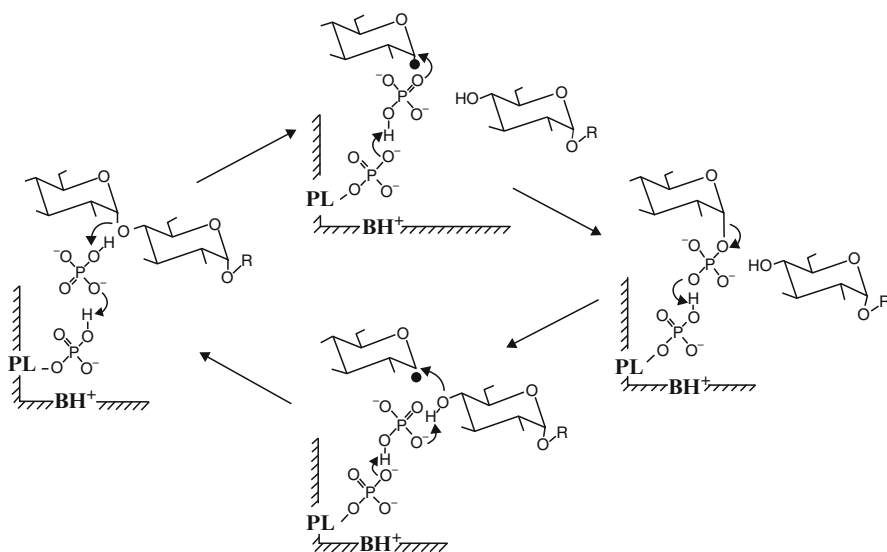


Fig. 5 Catalytic mechanism of glycogen phosphorylases. The reaction scheme accounts for the reversibility of phosphorolysis of oligosaccharides (*R*) in the presence of orthophosphate (*upper half*) and primer-dependent synthesis in the presence of glucose-1-phosphate (*lower half*). PL enzyme-bound pyridoxal; BH⁺ a general base contributed by the enzyme protein. Reprinted with permission from [109]. Copyright 1990 American Chemical Society

phosphorolysis of α -1,4-glycosidic bonds in oligo- or polysaccharides, the reaction is started by protonation of the glycosidic oxygen by orthophosphate, followed by stabilization of the incipient oxocarbenium ion by the phosphate anion, and subsequent covalent binding of the phosphate to form glucose-1-phosphate. The product, glucose-1-phosphate, dissociates and is replaced by a new incoming phosphate.

In the reverse direction, protonation of the phosphate of glucose-1-phosphate destabilizes the glycosidic bond and promotes formation of a glucosyl oxocarbenium ion–phosphate anion pair. In the subsequent step, the phosphate anion becomes essential for promotion of the nucleophilic attack of a terminal glucosyl residue on the carbonium ion. This sequence of reactions brings about α -1,4-glycosidic bond formation and primer elongation.

This mechanism accounts for retention of configuration in both directions without requiring sequential double inversion of configuration. It also provides for a plausible explanation of the essential role of PLP in glycogen phosphorylase catalysis: the phosphate of the cofactor PLP and the substrate phosphates approach each other within a hydrogen-bond distance, allowing proton transfer and making the phosphate of PLP into a proton shuttle that recharges the substrate phosphate anion.

The fact that glycogen phosphorylase can be used to polymerize amylose was first demonstrated by Schäffner and Specht [110] in 1938 using yeast phosphorylase. Shortly after, the same behavior was also observed for other phosphorylases from yeast by Kiessling [111, 112], muscles by Cori et al. [113], pea seeds [114] and potatoes by Hanes [115], and preparations from liver by Ostern and Holmes [116], Cori et al. [117] and Ostern et al. [118]. These results opened up the field of enzymatic polymerizations of amylose using glucose-1-phosphate as monomer, and can be considered the first experiments ever to synthesize biological macromolecules *in vitro*.

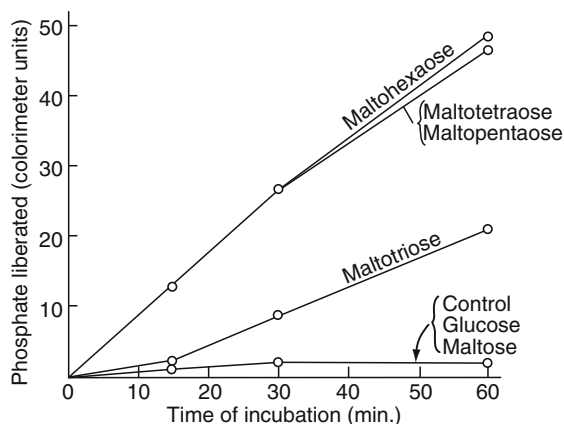
Recently Kuriki and coworkers succeeded in producing glucose-1-phosphate *in situ* during the enzymatic polymerization of amylose. By using sucrose phosphorylase or cellobiose phosphorylase, the monomer was produced during the polymerization from inorganic phosphate and sucrose or cellobiose, respectively [119–121].

One of the remarkable properties of phosphorylase is that it is unable to synthesize amylose unless a primer is added (poly- or oligomaltosaccharide):



The kinetic behavior of the polymerization of amylose using potato phosphorylase with various saccharides as primers was first studied by Hanes [115]. Green and Stumpf [122] failed to detect priming action with maltose but were able to confirm all other results by Hanes. Weibull and Tiselius [123] found that the maltooligosaccharide of lowest molecular weight to exhibit priming activity was maltotriose, which was confirmed by Whelan and Bailey [124], who also showed that maltotriose is the lowest member of the series of oligosaccharides to exhibit priming activity (see Fig. 6).

Fig. 6 Priming activity of glucose and maltooligosaccharides in the enzymatic polymerization using potato phosphorylase and glucose-1-phosphate as monomer [124] – Reproduced by permission of Portland Press Ltd.



Whelan and Bailey were also able to clarify the polymerization mechanism of the enzymatic polymerization with phosphorylase [124]. Their results showed that the polymerization follows a “multichain” scheme in contrast to a “single-chain” scheme that was also proposed by some authors. In the multichain polymerization scheme, the enzyme–substrate complex dissociates after every addition step, whereas in the single-chain scheme each enzyme continuously increases the length of a single primer chain without dissociation.

By studying the polydispersities of amyloses obtained by enzymatic polymerization with potato phosphorylase from maltooligosaccharides of various lengths, Pfannemüller and Burchard were able to show that the reaction mechanism of the polymerization with maltotriose as primer varies from its higher homologs [125]. The amyloses built by polymerization from maltotetraose or higher show a Poisson distribution [126], which can be expected from a polymerization following a multichain scheme (random synthesis occurs and all the primer chains grow at approximately equal rates). However, a bimodal broad distribution was observed when maltotriose was used as primer. The authors found that in the case of maltotriose as a primer the reaction can be divided into a start reaction and the following propagation, the rate of the first reaction being 400 times slower than the rate of the propagation. Due to this start reaction not all chains start to grow at the same time, which results in a broader distribution. The propagation again follows a multichain reaction scheme. Suganuma et al. [127] were able to determine the exact kinetic parameters of the synthetic as well as the phosphorolytic reaction using maltotriose and higher maltooligosaccharides as primer and were able to confirm the results of Whelan and Bailey [124] and Pfannemüller and Burchard [125].

3.1.2 Hybrid Structures with Amylose Blocks

The strict primer dependence of the glycogen phosphorylases makes them ideal candidates for the synthesis of hybrid structures of amylose with non-natural materials

(e.g., inorganic particles and surfaces, synthetic polymers). For this, a primer functionality (maltooligosaccharide) can be coupled to a synthetic structure and subsequently elongated by enzymatic polymerization, resulting in amylose blocks. Various examples of these types of hybrid materials are outlined in the following section.

Amylose Hybrids with Short Alkyl Chains

Pfannemüller et al. showed that it is possible to obtain carbohydrate-containing amphiphiles with various alkyl chains via amide bond formation. For this, maltooligosaccharides were oxidized to the corresponding aldonic acid lactones, which could subsequently be coupled to alkylamines [128–136]. Such sugar-based surfactants are important industrial products with applications in cosmetics, medical applications etc. [137–139]. The authors were also able to extend the attached maltooligosaccharides by enzymatic polymerization using potato phosphorylase, which resulted in products with very interesting solution properties [140, 141].

Amylose Brushes on Inorganic Surfaces

Amylose brushes (a layer consisting of polymer chains dangling in a solvent with one end attached to a surface is frequently referred to as a polymer brush) on spherical and planar surfaces can have several advantageous uses, such as detoxification of surfaces etc. The modification of surfaces with thin polymer films is widely used to tailor surface properties such as wettability, biocompatibility, corrosion resistance, and friction [142–144]. The advantage of polymer brushes over other surface modification methods like self-assembled monolayers is their mechanical and chemical robustness, coupled with a high degree of synthetic flexibility towards the introduction of a variety of functional groups.

Commonly, brushes are prepared by grafting polymers to surfaces by, e.g., chemical bonding of reactive groups on the surface and reactive end groups of the attached polymers. This “grafting to” approach has several disadvantages as it is very difficult to achieve high grafting densities and/or thicker films due to steric crowding of reactive surface sites by already adsorbed polymers.

The “grafting from” approach (polymers are grown from initiators bound to surfaces) is a superior alternative because the functionality, density, and thickness of the polymer brushes can be controlled with almost molecular precision.

The first surface-initiated enzymatic polymerization reported was the synthesis of amylose brushes on planar and spherical surfaces [145]. For this, silica or silicone surfaces were modified with self-assembled monolayers of (3-aminopropyl)trimethoxysilane or chlorodimethylsilane, respectively. To these functionalities, oligosaccharides were added via (a) reductive amidation of the oligosaccharides to surface-bound amines, (b) conversion of the oligosaccharide to the according aldonic acid lactone and reaction with surface bound amines, and (c) incorporation

of a double bond to the oligosaccharide and subsequent hydrosilylation to surface-bound Si-H functions. The surface-bound oligosaccharides could be enzymatically elongated, using potato phosphorylase and glucose-1-phosphate as monomer, to amylose chains of any desired length. The degree of polymerization could be determined by spectrometric measurement of the liberated amount of inorganic phosphate [146], which was confirmed by cleavage of the amylose brushes (either enzymatically or by prior incorporation of light-sensitive spacers) and subsequent characterization of the free amylose chains. The obtained amylose-modified surfaces showed good chiral discrimination when employed as column materials in chiral affinity chromatography. Modification of the OH groups of the amylose brushes even enhanced the separation strength of the developed column materials (Loos, unpublished results). The results were recently confirmed by Breiting, who attached maltooligosaccharides to surfaces via acid-labile hydrazide linkers, and enzymatically extended the chains using potato phosphorylase [147].

Copolymers with Amylose

The combination of oligo- or polysaccharides with non-natural polymeric structures opens up a novel class of materials. By varying the chain topology of the individual blocks as well as of the whole copolymer, the type of blocks, the composition etc., a complete set with tailor-made properties can be designed.

Amylose is a rod-like helical polymer consisting of α -(1 \rightarrow 4) glycosidic units. A measurement of the stiffness of a polymer is afforded by the so-called persistence length, which gives an estimate of the length scale over which the tangent vectors along the contour of the chains backbone are correlated. Typical values for persistence lengths in synthetic and biological systems can be several orders of magnitude larger than for flexible, coil-like polymers. Rod-like polymers have been found to exhibit lyotropic liquid crystalline ordered phases, such as nematic and/or layered smectic structures with the molecules arranged with their long axes nearly parallel to each other. Supramolecular assemblies of rod-like molecules are also capable of forming liquid crystalline phases. The main factor governing the geometry of supramolecular structures in the liquid crystalline phase is the anisotropic aggregation of the molecules.

Copolymeric systems with amylose are therefore systems in which at least one component is based on a conformationally rigid segment, and are generally referred to as rod-coil systems [148–151]. By combining rod-like and coil-like polymers, a novel class of self-assembling materials can be produced since the molecules share certain general characteristics typical of diblock molecules and thermotropic calamitic molecules. The difference in chain rigidity of rod-like and coil-like blocks is expected to greatly affect the details of molecular packing in the condensed phases and thus the nature of thermodynamically stable morphologies in these materials. The thermodynamically stable morphology probably originates as the result of the interdependence of microsegregation and liquid crystallinity. From this point of view, it is very fascinating to compare the microstructures originating in solution and in the bulk for such materials.

Practical applications in which copolymers are characterized by some degree of structural asymmetry have been suggested. For instance, a flexible block may be chosen because it donates a flexural compliance, whereas the more rigid portion offers tensile strength. In addition to the mechanical properties, the orientational order and the electrical conductance of certain rigid blocks could be exploited in optical and electrical devices.

Comb-type and linear block copolymer systems with enzymatically synthesized amylose have been reported and are outlined in the following section.

Comb-Type Copolymers with Amylose

The first comb-like structures synthesized by enzymatic “grafting from” polymerization from a polymeric backbone were reported by Husemann and Reinhardt [152, 153]. Acetobromo oligosaccharides were covalently bound to 6-trityl-2,3-dicarbanilyl-amylose chains and subsequently elongated by enzymatic polymerization using potato phosphorylase, the result being amylopectin-like structures with various degrees of branching. Pfannemüller and coworkers extended this work by grafting amylose chains onto starch molecules. The modified starches were studied by the uptake of iodine and by light scattering measurements of carbanilate derivatives [154] and appeared to be star-like in electron microscopy studies [155].

A full series of star-, network- and comb-like hybrid structures with oligosaccharides were synthesized by Pfannemüller and coworkers (see Fig. 7) and it was shown that the attached oligosaccharides can be extended via enzymatic polymerization using potato phosphorylase [128, 129, 136, 156, 157].

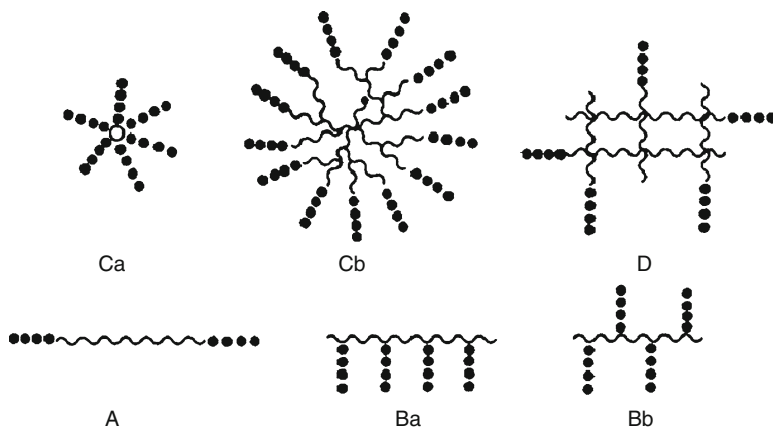


Fig. 7 Maltotetraose hybrids with various carriers resulting in different chain architectures: *A* poly(ethylene oxide); *Ba* and *Bb* poly(acrylic acid), amylose, cellulose, and other polysaccharides; *Ca* cyclodextrin and multifunctional acids; *Cb* amylopectin; *D* crosslinked poly(acryl amide) [156] – Reproduced by permission of Wiley

Another type of comb-like amylose hybrid synthesized via enzymatic grafting using phosphorylase is based on polysiloxane backbones. To achieve these structures, double bonds were incorporated to the reducing end of oligosaccharides, which were then attached to poly(dimethylsiloxane-*co*-methylsiloxane) copolymers via hydrosilylation [158, 159] or to silane monomers, which were subsequently polymerized to polysiloxanes [160]. Various mono-, di-, tri- and oligosaccharides were attached to the siloxane backbones and their solution properties studied with viscosimetry and static and dynamic light scattering [161]. The pendant oligosaccharide moieties could be extended by enzymatic “grafting from” polymerization [162, 163].

Kobayashi and colleagues succeeded in attaching maltopentaose to the *para* position of styrene and performed free-radical polymerizations towards the homopolymers [164, 165] as well as towards copolymers with acrylamide [164]. Kobayashi and coworkers also reported the successful attachment of maltopentaose to poly(L-glutamic acid) [166]. Kakuchi and coworkers showed that the saccharide-modified styrene monomers could also be polymerized by TEMPO-mediated controlled radical polymerization [167]. Amylose-grafted polyacetylenes were recently reported by Kadokawa et al. [168]. Maltooligosaccharide-grafted polyacetylene was synthesized by Rh-catalyzed polymerization of *N*-propargylamide monomers having a maltooligosaccharide chain [168] and by attaching maltoheptaonolactone to amine-functionalized polyacetylene [169]. Kadokawa and coworkers also succeeded in covalently attaching maltooligosaccharides to natural biopolymers such as cellulose, chitin, and chitosan [170–172].

In all cases, the authors could successfully elongate the attached oligosaccharide structures using enzymatic polymerization, the product being comb-type block copolymers with amylose.

Linear Block Copolymers with Amylose

Various linear block copolymers of the AB, ABA, and ABC type containing enzymatically polymerized amylose blocks were reported. Ziegast and Pfannemüller converted the hydroxyl end groups of poly(ethylene oxide) into amino groups via tosylation and further reaction with 2-aminoalkylthiolate [173]. To the resulting mono- and diamino-functionalized poly(ethylene oxide), maltooligosaccharide lactones were attached and subsequently elongated to amylose via enzymatic polymerization [174]. Pfannemüller et al. performed a very detailed study on the solution properties of the synthesized ABA triblock copolymers because they can be considered model substances for “once-broken rod” chains [175]. With static and dynamic light scattering, the authors found that the flexible joint between the two rigid amylose blocks has no detectable effect on the common static and dynamic properties of the chain. Using dielectric measurements however, it became obvious that the directional properties of the electric dipoles of the broken rigid chains showed a different behavior to the non-broken rods (pure amylose). Akyoshi et al. also synthesized amylose-*b*-poly(ethylene glycol) block copolymers via enzymatic

grafting from oligosaccharide-terminated poly(ethylene oxide) and studied the solution properties of these amphiphilic block copolymers by static and dynamic light scattering [176, 177].

It was also shown that the enzymatic polymerization of amylose could be started from oligosaccharide-modified polymers that are not soluble in the medium of polymerization (aqueous buffers). Amylose-*b*-polystyrene block copolymers could be synthesized by attaching maltooligosaccharides to anionically synthesized amino-terminated polystyrene, and subsequent enzymatic elongation to amylose [178, 179]. Block copolymers with a wide range of molecular weights and copolymer compositions were synthesized via this synthetic route. The solution properties of star-type as well as crew-cut micelles of these block copolymers were studied in water and THF, and the according scaling laws established [180]. In THF, up to four different micellar species were detectable, some of them in the size range of vesicular structures, whereas the crew-cut micelles in water were much more defined. Bosker et al. studied the interfacial behavior of amylose-*b*-polystyrene block copolymers at the air–water interface using the Langmuir–Blodgett technique [181].

Recently, two groups reported controlled radical polymerizations starting from maltooligosaccharides (ATRP [182] and TEMPO-mediated radical polymerization [183]), which will certainly lead to new synthetic routes towards amylose-containing block copolymers.

Even though the products are not block copolymer structures, the work of Kadokawa and colleagues should be mentioned here. In a process that the authors named “vine-twining polymerization” (after the way that a vine plant grow helically around a support rod), the enzymatic polymerization of amylose is performed in the presence of synthetic polymers in solution, and the authors showed that the grown amylose chains incorporate the polymers into its helical cavity while polymerizing [184–191].

3.2 Branching Enzymes

The formation of the α -(1 \rightarrow 6) glucosyl branches of amylopectin and glycogen is synthesized by branching enzymes (systematic name: (1 \rightarrow 4)- α -D-glucan:(1 \rightarrow 4)- α -D-glucan 6- α -D-[(1 \rightarrow 4)- α -D-glucano]-transferase; EC 2.4.1.18).

This enzyme catalyzes the formation of α -(1 \rightarrow 6) branching points by cleaving an α -(1 \rightarrow 4) glycosidic linkage in the donor substrate and transferring the nonreducing end-terminal fragment of the chain to the C-6 hydroxyl position of an internal glucose residue, which acts as the acceptor substrate [192]. Depending on its source, branching enzymes have a preference for transferring different lengths of glucan chains [193–196].

Recently, the in vitro synthesis of amylopectin- or glycogen-like structures via a tandem reaction of phosphorylase and branching enzyme was reported [197–201].

Phosphorylase catalyzes the polymerization of glucose-1-phosphate in order to obtain linear polysaccharide chains with α -(1 \rightarrow 4) glycosidic linkages; the glycogen

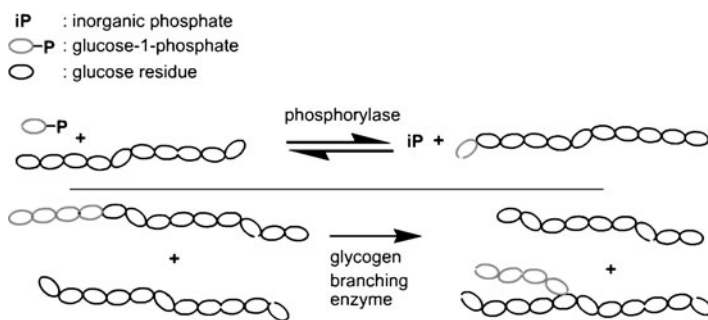


Fig. 8 Reactions catalyzed by glycogen phosphorylase (*above*) and glycogen branching enzyme (*below*)

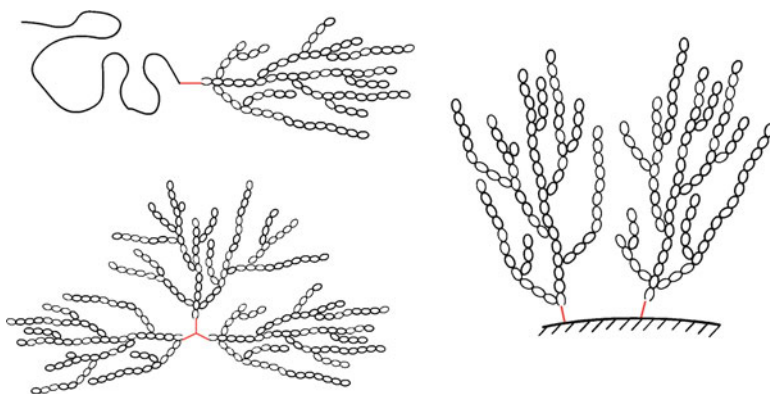


Fig. 9 Hybrid structures with amylopectin

branching enzyme is able to transfer short, α -(1 \rightarrow 4) linked, oligosaccharides from the non-reducing end of starch to an α -(1 \rightarrow 6) position (see Fig. 8). By combining the branching enzyme with phosphorylase, it becomes possible to synthesize branched structures via a one-pot synthesis because phosphorylase will polymerize linear amylose and the glycogen branching enzyme will introduce the branching points, which are again extended by phosphorylase.

As shown above, hybrid structures bearing amylose blocks can be synthesized by covalent attachment of primer recognition units for phosphorylase and subsequent enzymatic “grafting from” polymerization. Following the same route, we are currently synthesizing hybrid materials bearing (hyper)branched polysaccharide structures (as shown in Fig. 9) with the described tandem reaction of two enzymes. The branched structure, high amount of functional groups, and biocompatibility of these structures make these architectures suitable for applications in the biomedical field and in the food industry.

3.3 Glucansucrases

Another very valuable glycosyltransferase for the construction of well-defined polysaccharides is glucansucrase (dextransucrase; systematic name: sucrose:(1 \rightarrow 6)- α -D-glucan 6- α -D-glucosyltransferase; EC 2.4.1.5).

Recently Dijkhuizen and coworkers reported on a family of glucansucrases found in *Lactobacillus reuteri*, which convert sucrose into large, heavily branched α -glucans [85]. One of these glucansucrases (GTF180) [202] produces an α -glucan with (α 1 \rightarrow 3) and (α 1 \rightarrow 6) glycosidic linkages. Kamerling and coworkers could show that the (1 \rightarrow 3, 1 \rightarrow 6)- α -D-glucan of *L. reuteri* strain 180 has a heterogeneous structure with no repeating units present [203]. It contains only α -D-Glcp-(1 \rightarrow 6)-units in terminal position. All α -D-Glcp-(1 \rightarrow 3) units were shown to be 6-substituted, and the polysaccharide is built-up from different lengths of isomalto-oligosaccharides, interconnected by single (α 1 \rightarrow 3) bridges. The unique polysaccharide structure produced was suggested to be prebiotic [204].

The GTF180 enzyme shows large similarity with other glucansucrase enzymes, but has a relatively large N-terminal variable region. Truncation of the enzyme, by deletion of the variable region, had no effect on the linkage distribution of the α -glucan produced [85].

Seibel and coworkers succeeded in constructing various new complex glycoconjugates containing thioglycosidic linkages to different glycopyranosides (galactose, glucose, neuraminic acid) (producing branched thiooligosaccharides) by changing the chemoselectivity of the various glucansucrases from α -1,6- to α -1,2-, α -1,3- or α -1,4-linked glucose [205].

Seibel and coworkers also showed that the mutagenesis is an effective tool for altering the regioselectivity and acceptor-substrate specificity of glucansucrase GTFR of *Streptococcus oralis*, a dextran-producing enzyme. By random mutagenesis, they were able to switch the regioselectivity and acceptor specificity of GTFR of *S. oralis* towards synthesis of (a) various short chain oligosaccharides or (b) novel (mutan) polymers with completely altered linkages, without compromising its high transglycosylation activity [206].

3.4 Levansucrase

Dijkhuizen and coworkers identified and characterized a *Lactobacillus levansucrase* (systematic name: sucrose:[6]- β -D-fructofuranosyl-(2 \rightarrow) n α -D-glucopyranoside 6- β -D-fructosyltransferase; EC 2.4.1.10) from *L. reuteri* strain 121, which could produce a high molecular weight levan polysaccharide from fructose [207].

3.5 Amylomaltase

Glycosyltransferases are also used extensively to modify natural polysaccharides. Thermoreversible gels that retrograded reversibly – comparable to gelatin

gels – from enzymatically modified starch using amylomaltase were reported recently [208–213].

Amylomaltase (4- α -glucanotransferase; systematic name: (1 \rightarrow 4)- α -D-glucan: (1 \rightarrow 4)- α -D-glucan 4- α -D-glycosyltransferase; EC 2.4.1.25) catalyzes the glucan-chain transfer from one α -1,4-glucan to another α -1,4-glucan (or to the 4-hydroxyl group of glucose), or within a single linear glucan molecule to produce a cyclic- α -1,4-glucan [213–215]. The enzyme was first found in *Escherichia coli*, but seems to be distributed in various bacterial species with different physiological functions. In *E. coli*, amylomaltase is expressed with glucan phosphorylase from the same operon. Amylomaltase is a member of the maltooligosaccharide transport and utilization system and plays a role in converting short maltooligosaccharides into longer chains, upon which glucan phosphorylase can act [216]. The name amylo-maltase is used for the microbial 4- α -glucanotransferases, whereas the plant counterparts are usually called disproportionating enzymes (D-enzymes) [212, 214, 215].

Typically, enzymatic modification of starch employs hydrolyzing enzymes such as α -amylase, pullulanase, and glucoamylase. These hydrolyze the α -1,4- or α -1,6-glycosidic bonds in amylose and amylopectin by first breaking the glycosidic linkage and subsequently using a water molecule as acceptor substrate. Amylo-maltases also initially break the glycosidic linkage but, instead of water, they use another oligosaccharide as an acceptor substrate and form a new glycosidic linkage. Amylo-maltases can use high molecular weight starch as both donor and acceptor molecule and can catalyze the transfer of long α -1,4-glucan chains [217], or even highly branched cluster units of amylopectin.

3.6 Hyaluronan Synthase

Hyaluronan (HA) is a nonsulfated non-epimerized linear glycosaminoglycan (GAG) existing in vivo as a polyanion of hyaluronic acid and composed of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine [GlcA β (1 \rightarrow 3)GlcNAc β (1 \rightarrow 4)] [218–220]. It is a major constituent of the extracellular matrix (ECM) of the skin, joints, eye, and many other tissues and organs. Despite the simple structure of this macromolecule, the complexity of its physico-chemical properties and biological functions is tremendous. HA has extraordinary hydrophilic, rheological, and signaling properties and is viscoelastic. This naturally occurring biopolymer is dynamically involved in many biological processes, such as embryogenesis, inflammation, metastasis, tissue turnover, and wound healing.

The isolation, purification, and identification of nearly pure HA have been the center of scientific interest for many decades. The bacterial production of HA by *Streptococcus equi* [221] and *Streptococcus zooepidemicus* [222] enabled it to be produced in larger quantities than could be achieved by extraction methods alone. HA produced by *S. equi* has a lower molecular weight than does HA produced by *S. zooepidemicus*, which has a MW of about $1.8\text{--}2 \times 10^6$ Da with a yield of

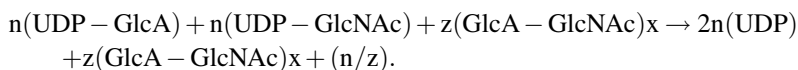
around 4 g HA per liter of the cultivated solution. At present, HA from various sources, with different degrees of purity and molecular weights, is available for medical applications.

HA is synthesized by hyaluronan synthase (systematic name: alternating UDP- α -*N*-acetyl-D-glucosamine: β -D-glucuronosyl-(1 \rightarrow 3)-[nascent hyaluronan] 4-*N*-acetyl- β -D-glucosaminyltransferase and UDP- α -D-glucuronate:*N*-acetyl- β -D-glucosaminyl-(1 \rightarrow 4)-[nascent hyaluronan] 3- β -D-glucuronosyltransferase; EC 2.4.1.212) [223–226]. Hyaluronan synthase (HAS) is a single protein glycosyltransferase that is able to transfer two different monosaccharides, whereas most glycosyltransferases catalyze one glycosidic transfer reaction exclusively.

Markovitz et al. successfully characterized the HAS activity from *Streptococcus pyogenes* and discovered the enzyme's membrane localization and its requirements for sugar nucleotide precursors and Mg^{2+} [227]. DeAngelis et al. were the first to succeed in the molecular cloning and characterization of the Group A Streptococcal gene encoding the protein HasA, known to be in an operon required for bacterial HA synthesis [228, 229]. Following this, sequences of the genes encoding other HAS proteins were identified using molecular biological techniques [220, 224, 225, 230–243]. However, still little is known about the structure and mechanism of HAS.

The in vitro synthesis of HA oligomers and polymers using HAS and UDP-sugars was reported by the group of Paul DeAngelis. The monosaccharide units from UDP-GlcNAc and UDP-GlcA are transferred sequentially in an alternating fashion to produce the disaccharide repeats of the heteropolysaccharide. Recombinant derivatives of one HAS, PmHAS from the gram-negative bacterium *Pasteurella multocida* type A [233], have proved to be very useful for chemoenzymatic syntheses of both oligosaccharides [244] and polysaccharides [245, 246].

In 2004, the PmHAS was employed in synchronized, stoichiometrically controlled polymerization reactions in vitro to produce monodisperse HA polysaccharide preparations [246]. Reaction synchronization is achieved by providing the HAS with an oligosaccharide acceptor to bypass the slow polymer initiation step in vitro. All HA chains are elongated in parallel and thus reach the same length, yielding a population of narrow size distribution. The synthase adds all available UDP-sugar precursors to the nonreducing termini of acceptors in a non-processive fashion, as in the following equation:



Therefore, size control is possible. For example, if there are many termini (i.e., z is large), then a limited amount of UDP-sugars will be distributed among many molecules and thus result in many short polymer chain extensions. Conversely, if there are few termini (i.e., z is small), then the limited amount of UDP-sugars will be distributed among few molecules and thus result in long polymer chain extensions. With this, it became possible to synthesize highly defined HA polymer standards that can be used for the characterization of polysaccharides by, for instance, size exclusion chromatography equipped with a multi-angle light scattering detector [247].

4 Transferases that Transfer Alkyl or Aryl Groups, Other than Methyl Groups (EC 2.5.)

Rubber is synthesized by plants via a side branch of the isoprenoid pathway by the enzyme rubber transferase (*cis*-prenyl transferase; systematic name: poly-*cis*-polyprenyl-diphosphate:isopentenyl-diphosphate polyprenylcistransferase; EC 2.5.1.20). Surprisingly, although this process has been studied for decades, due to the labile nature of the rubber transferase and the fact that it is a membrane-associated enzyme present in relatively low abundance, the identification of its protein subunits remain elusive. For some recent reviews on rubber biosynthesis, please refer to [248–251].

Natural rubber, *cis*-1,4-polyisoprene, is a strategically important plant-derived commodity required for the manufacture of numerous industrial, medical, and household items. Although more than 2500 plant species are known to produce rubber, *Hevea brasiliensis* (Brazilian rubber tree) is currently the sole source of natural rubber. Most countries depend on imports of *H. brasiliensis* rubber to sustain demand. Despite the increasing demand for natural rubber, the acreage for rubber trees has diminished in recent years. Furthermore, decades of inbreeding have rendered commercial *H. brasiliensis* varieties susceptible to abiotic stress and pathogen attack. Due to this, an increasing interest in the development of additional sources of natural rubber can be observed. Research on rubber-producing transgene plants, and on identification of the genes involved in its biosynthesis and regulation has intensified in recent years. To achieve a sustainable production of rubber from natural sources, our understanding of the molecular mechanism of rubber biosynthesis needs to be improved.

Prenyltransferases are a class of enzymes that transfer allylic prenyl groups to acceptor molecules. Prenyl transferases commonly refer to prenyl diphosphate synthases (even though the class of prenyl transferases also includes enzymes that catalyze the transfer of prenyl groups to acceptors that include not only isopentenyl diphosphate (IPP) but also aromatic compounds and proteins etc.).

The reactions catalyzed by prenyltransferases are unique and interesting from a mechanistic viewpoint. The reaction starts with elimination of the diphosphate ion from an allylic diphosphate (APP) to form an allylic cation, which is attacked by the IPP molecule, with stereospecific removal of a proton to form a new C–C bond and a new double bond in the product. By repeating this type of condensation between IPP and the allylic prenyl diphosphate product, prenyltransferase can synthesize a prenyl diphosphate with a certain length and stereochemistry fixed by its specificity (see Fig. 10) [252].

Rubber is synthesized and sequestered on cytosolic vesicles known as rubber particles. Rubber transferase is localized to the surface of the rubber particles, and biosynthesis is initiated through the binding of an allylic pyrophosphate (APP, a pyrophosphate, produced by soluble *trans*-prenyl transferases) primer. Progressive additions of IPP molecules ultimately result in the formation of high molecular weight *cis*-1,4-polyisoprene. The rubber transferase also requires a divalent cation, such as Mg^{2+} or Mn^{2+} , as cofactor.

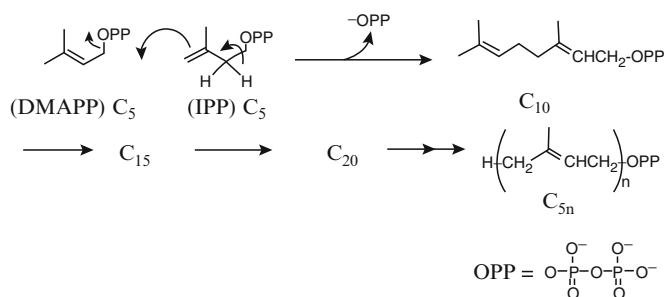


Fig. 10 Reactions catalyzed by prenyltransferases

The prenyl chain elongation catalyzed by prenyltransferases is quite unique because the reaction proceeds consecutively and terminates precisely at discrete chain lengths according to the specificities of the enzymes. The chain length of products varies so widely that it ranges from geraniol (C₁₀) to natural rubber (C > 5000).

The identification and characterization of the genes and enzymes involved in rubber biosynthesis have been slow compared with those involved in the synthesis of other biopolymers. In fact, most of the studies thus far reported begin with rubber particles.

Enzymatically active, partially purified (washed) rubber particles can be isolated such that, when provided with an appropriate APP primer, magnesium ion cofactor, and IPP monomer, rubber is produced *in vitro* [253–255]. Fresh latex can be separated by centrifugation into three phases. The bottom fraction (20% of the latex) contains membrane-bound organelles. The middle fraction is called the C-serum. The top fraction phase contains the rubber particles. Biochemical studies have established that latex in this fractionated form is unstable. These studies also suggest that the bottom fraction is required for initiation of polymer synthesis.

The essential precursor (APP) and monomer (IPP) are synthesized *in vivo* via the isoprenoid pathway, with cytosolic acetyl-CoA being the primary building block for the synthesis of rubber. Acetyl-CoA is converted to IPP through a pathway involving the intermediate 3-hydroxy-methyl-glutaryl-CoA [256–258]. IPP is transformed into dimethylallyl diphosphate (DMAPP) by isomerization. DMAPP primes the sequential head-to-tail condensations of IPP molecules by *trans*-prenyltransferases to form geranyl diphosphate (GPP, C₁₀, monoterpenoids), farnesyl diphosphate (FPP, C₁₅, sesquiterpenoids) and geranylgeranyl diphosphate (GGPP, C₂₀, diterpenoids) (Fig. 11).

Rubber molecules are synthesized from one APP molecule, which initiates the reaction, and the rubber polymer (*cis*-1,4-polyisoprene) is then polymerized by sequential condensations of the non-allylic IPP (magnesium cations are a required cofactor) with release of a diphosphate at each condensation. After initiation and elongation, a termination event occurs in which the rubber molecule is released from the enzyme. Despite the similar process, remarkable differences exist between plant species with respect to enzymatic reaction mechanisms and product molecular weight.

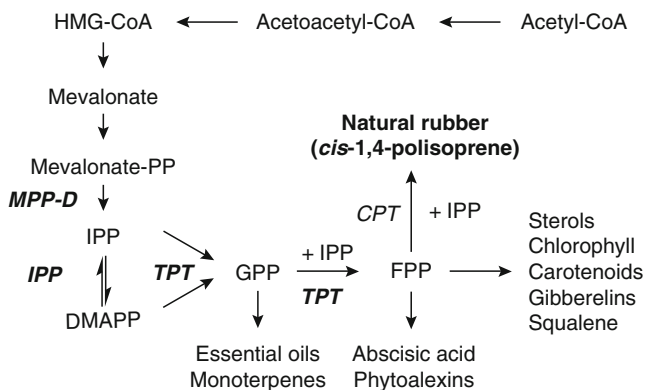


Fig. 11 Natural rubber is produced from a side branch of the ubiquitous isoprenoid pathway, with 3-hydroxy-methyl-glutaryl-CoA (HMG-CoA) as the key intermediate derived from acetyl-CoA by the general mevalonic-acid pathway. Mevalonate diphosphate decarboxylase (MPP-D) produces IPP, which is isomerized to DMAPP by IPP isomerase (IPI). IPP is then condensed in several steps with DMAPP to produce GPP, FPP and GGPP by the action of a *trans*-prenyltransferase (TPT). The *cis*-1,4-polymerization that yields natural rubber is catalyzed by *cis*-prenyltransferase (CPT), which uses the non-allylic IPP as substrate. Reprinted from [248], with permission from Elsevier

The exact mechanism of polymer initiation is unknown. Initiation of rubber synthesis has been studied in several plants and a common finding is that the end groups found in low molecular weight rubber (such as rubber from goldenrod and *H. brasiliensis* leaves) are not made up of *cis*-isoprene units, unlike the bulk of the rubber [259, 260]. Structural studies [261, 262] have led to the suggestion that the C15 FPP may be the most common initiator *in vivo*, at least in *H. brasiliensis*.

Although many different APPs are effective initiators of rubber biosynthesis, only IPP can be used as the source of isopentenyl monomer for the *cis*-1,4-polymerization of the rubber polymer.

All rubber transferases exhibit similar kinetic constants and pH optima, and are able to accept a similar range of APPs as initiating substrate [263, 264]. *In vitro* studies have shown that several compounds (DMAPP, GPP, FPP, and GGPP) can initiate rubber biosynthesis, with a faster rate of rubber biosynthesis the longer the APP (up to C15 or C20) [254, 265]. Non-natural APPs were also shown to be able to function as a primer for the rubber biosynthesis [266].

The cDNAs of the *cis*-prenyltransferase of *H. brasiliensis* was successfully identified and expressed in *E. coli*. The *in vitro* polymerization of IPP after initiation with FPP using the expressed *cis*-prenyltransferase resulted in low degrees of polymerization [267, 268]. After addition of rubber particles to this polymerization, the molecular weight increased tremendously [269]. It can be concluded that the rubber particles are essential for rubber biosynthesis. Katarina Cornish established a detailed structural model of the *in vivo* synthesis of natural rubber in the rubber particle monolayer membrane and partially explained this behavior (see Fig. 12) [251].

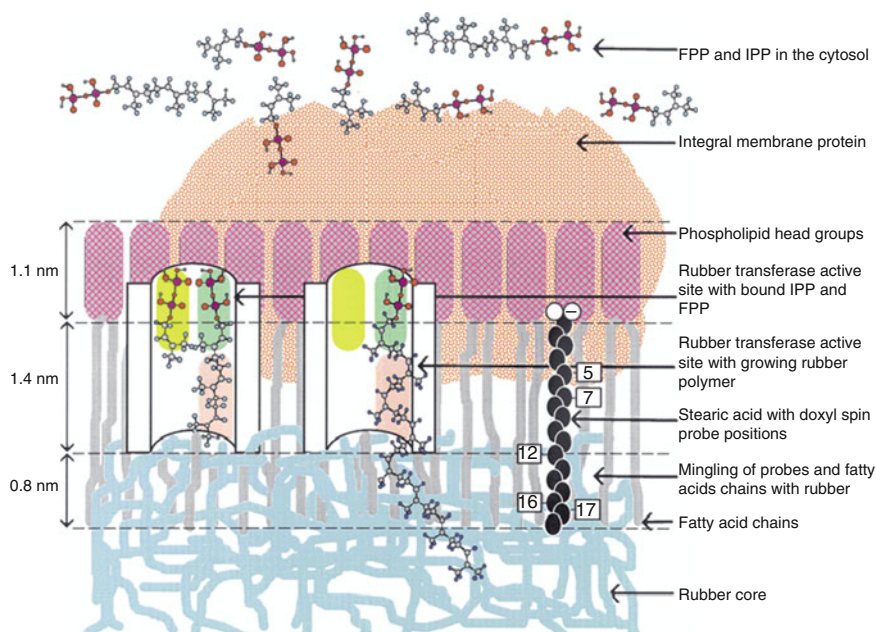


Fig. 12 Scheme depicting, to scale, a section of the surface monolayer biomembrane surrounding a *P. argentatum* rubber particle and the intermingling of the phospholipid fatty acid chains with the pure rubber core. Natural rubber is synthesized from IPP with an APP, most probably FPP, as an essential initiator. Both substrates are hydrophilic and are synthesized in the cytosol. The rubber transferase active site containing IPP (yellow) and allylic-PP (green) binding sites, and a non-specific hydrophobic binding region (pink) is drawn twice. The site on *left* of the schema contains bound IPP and FPP (the magnesium cofactor is not shown). Catalysis then would occur, leading to condensation of the two molecules and the release of pyrophosphate. The active site drawn in the *middle* of the schema is shown containing a short molecule of natural rubber (*cis*-1,4-polyisoprene) originally initiated by FPP. The *trans,trans* tail of the rubber molecule derived from FPP is visible. The active sites are positioned so that the hydrophilic pyrophosphate of FPP is aligned to the polar headgroup region of the membrane. Kinetic studies suggest that FPP traverses the active site as shown. Both models show the same physical position for the membrane–rubber interface [251] – Reproduced by permission of The Royal Society of Chemistry

Rubber appears to be a metabolic dead-end because there have been no findings of enzymes capable of breaking down the rubber in latex. The exact termination reaction of the rubber polymerization is not known. Different end-groups have been detected by NMR in rubber purified from a range of species, indicating that molecule dephosphorylation and release may involve esterification, cyclization, or hydrolysis [262].

In Hevea, the MW of the polymer has a bimodal distribution with some polymers in the range of 106 Da and others 105 Da. Both the Hevea polymers and the C55–C120 oligomeric isoprenes appear to have well-controlled molecular weights [270]. In vitro studies reveal the size of the polymer is related to the relative concentrations of the putative primer (farnesyl pyrophosphate or geranylgeranyl pyrophosphate)

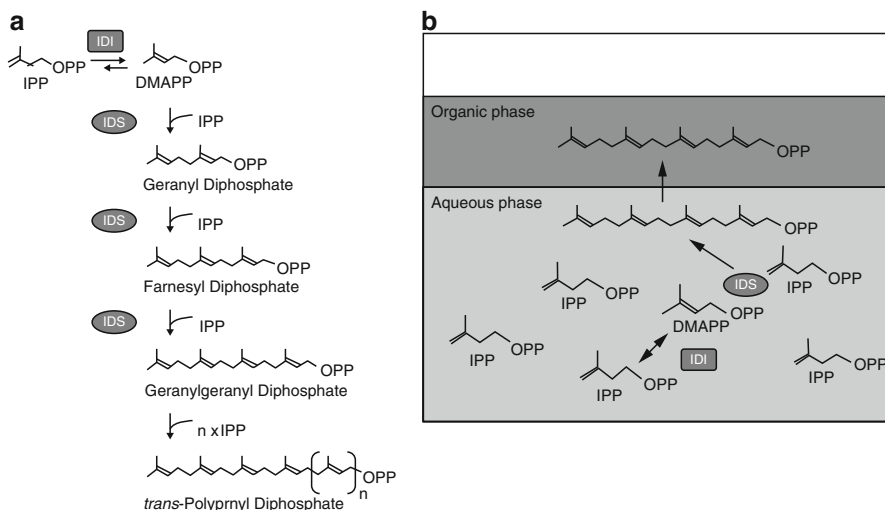


Fig. 13 Synthesis of polyisoprenyl diphosphates. **(a)** Biosynthetic pathway in archaea. **(b)** Concept of the organic–aqueous dual-phase system. The light- and dark-shaded areas indicate the aqueous and organic phases, respectively. Double-headed and single-headed arrows indicate isomerization by IDI and consecutive condensation by IDS, respectively. Reprinted from [271], with permission from Elsevier

and IPP [270]. The higher the ratio of primer to substrate, the shorter the chains. Thus, it is likely that the granule and its associated proteins in conjunction with the elongation protein(s) will play a critical role in chain length control and will be different from in vitro studies.

Recently Fujiwara et al. reported on the in vitro polymerization of *trans*-polyisoprene using the enzymes isopentenyl diphosphate isomerase (IDI) and *trans*-isoprenyl diphosphate synthase (IDS) [271]. IDI catalyzes the interconversion of IPP and DMAPP. IDS can now catalyze the polymerization of IPP from DMAPP as outlined above for the synthesis of natural rubber, and as outlined in Fig. 13a. However, the condensation process is inhibited due to hydrophobic interaction between IDS and hydrocarbon of the longer products. The hydrophobic chain of the elongating product does not readily protrude into the aqueous phase and it tends to interact with the enzyme. To achieve an efficient in vitro synthesis, the authors used an organic–aqueous two-liquid phase system to successfully synthesize (low molecular weight) *trans*-polyisoprene (see Fig. 13b).

5 Conclusions

It is obvious that transferases are powerful catalysts for the enzymatic synthesis of interesting polymer systems such as polyesters, polysaccharides, and polyisoprenes. Considering the range of reactions that transferases can in principle catalyze, it can

be expected that more catalytic systems towards other polymeric materials will be developed in the future.

Despite their potential for synthesizing interesting polymeric materials many transferases are very sensitive biocatalysts, which prevents their isolation on a larger scale and/or their use for synthesizing polymer on a reasonable scale. However, it can be expected that with new biotechnological methods it will become possible to obtain transferases with enhanced stability that can be used more frequently in polymer synthesis. Driven by the discovery of many novel enzymes, synthesis routes in which one or all of the steps are biocatalytic have advanced dramatically in recent years through advances in recombinant DNA technology (which allows both more efficient production and targeted or combinatorial alterations of individual enzymes), and through developments towards higher stability and volumetric productivity. Design rules for improving biocatalysts are increasingly precise and easy to use.

Considering the macromolecules discussed in this chapter (polyester, polysaccharides, and rubber) there can be no doubt that new approaches to synthesize them via enzymatic polymerization will have a huge impact.

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