Review

New developments of polysaccharide synthesis
via enzymatic polymerization

By Shiro Kobayashi[*1,†]

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Abstract: This review focuses on the *in vitro* synthesis of polysaccharides, the method of which is "enzymatic polymerization" mainly developed by our group. Polysaccharides are formed by repeated glycosylation reactions between a glycosyl donor and a glycosyl acceptor. A hydrolysis enzyme was found very efficient as catalyst, where the monomer is designed based on the new concept of a "transition-state analogue substrate" (TSAS); sugar fluoride monomers for polycondensation and sugar oxazoline monomers for ring-opening polyaddition. Enzymatic polymerization enabled the first *in vitro* synthesis of natural polysaccharides such as cellulose, xylan, chitin, hyaluronan and chondroitin, and also of unnatural polysaccharides such as a cellulose–chitin hybrid, a hyaluronan–chondroitin hybrid, and others. Supercatalysis of hyaluronidase was disclosed as unusual enzymatic multi-catalyst functions. Mutant enzymes were very useful for synthetic and mechanistic studies. *In situ* observations of enzymatic polymerization by SEM, TEM, and combined SAS methods revealed mechanisms of the polymerization and of the self-assembling of high-order molecular structure formed by elongating polysaccharide molecules.

Keywords: polysaccharide synthesis, enzymatic polymerization, cellulose, chitin, glycosaminoglycan, hybrid polysaccharide

Introduction

Polysaccharides are found in all living cells and play critical biological roles in nature. They belong to one of three major classes of natural biomacromolecules besides nucleic acids and proteins (Fig. 1).[1)–3)] Nucleic acids (DNA and RNA) and proteins are polyphosphates and poly(α-peptide)s, respectively. These two classes have relatively simple structures and their *in vitro* chemical synthesis already became easily accessible; a computer-controlled synthesizer based on the Merrifield method[4)] together with remarkable progresses in genetic engineering[5)] enabled to produce these biomacromolecules automatically. In contrast, polysaccharides are biomacromolecules, in which sugar molecule units are linked via a glycosidic

bond with a complicated structure (Fig. 2).[6)] Although the modification reactions of polysaccharides to prepare a new material like cellulose acetate from cellulose have long been known, chemical synthesis of polysaccharides via the glycosidic bond formation had not been successful, despite many attempts. It is because the control of the *in vitro* glycosidic bond formation is very difficult, and hence, there had been no good method for the production of polysaccharides. Therefore, the development of an efficient method of polysaccharide synthesis had been an unsolved, challenging problem in polymer science for half a century, until our group found a possible solution. The present review describes the first successful *in vitro* synthesis of various polysaccharides mainly achieved by us recently using a new method of polymer synthesis, i.e., *enzymatic polymerization*. Its definition is a "chemical polymer synthesis *in vitro* (in test tubes) via nonbiosynthetic (nonmetabolic) pathways catalyzed by an isolated enzyme".[1)–3),6)–13)]


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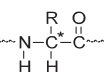
Background

In vivo, polysaccharides are biosynthesized by the catalysis of glycosyltransferases, which catalyze exclusively the formation of a glycosidic bond
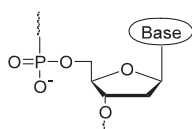
Fig. 1.   Three major classes of natural biomacromolecules.

with the corresponding sugar nucleotides as substrates.[14] Scheme 1 shows typical examples, biosynthesis of natural cellulose and natural chitin, respectively, where UDP-glucose (Glc) and UDP-*N*-acetyl-D-glucosamine (GlcNAc) substrate monomers are polymerized by the catalysis of cellulose synthase and chitin synthase, respectively, both glycosyltransferases.

Polysaccharides are produced by the repeated glycosylations of a glycosyl donor with a glycosyl acceptor to form a glycosidic bond. Scheme 2 shows a typical example of glycosylation reaction to give a glucose dimer, where the anomer carbon (C1) of the glycosyl donor is activated by introducing X group, whereas other hydroxy groups having similar reactivity in both the donor and acceptor are not protected. In forming the product, α- and β-isomers arise with respect to stereochemistry of the anomer carbon and four isomers are conceivable with respect to regioselectivity due to the four reactive hydroxyl groups (excluding 1-OH) in the acceptor. Thus, the reaction involves a possibility to produce
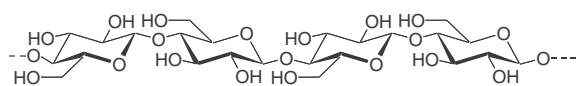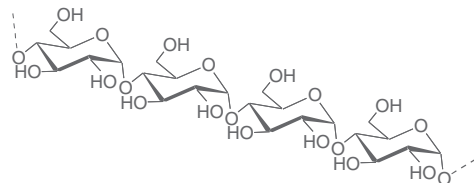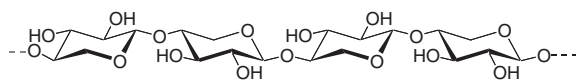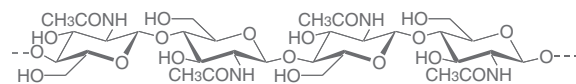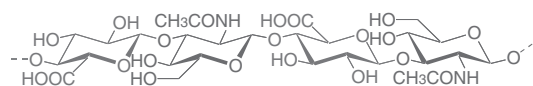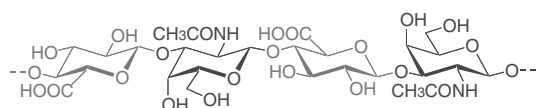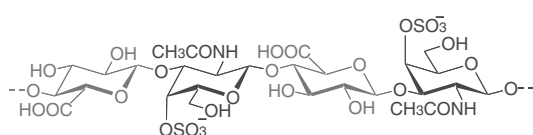
Natural Polysaccharides

Fig. 2.   Typical examples of natural polysaccharides; four homo-polysaccharides consisted of a single repeating unit, and three hetero-polysaccharides consisted of different repeating units.
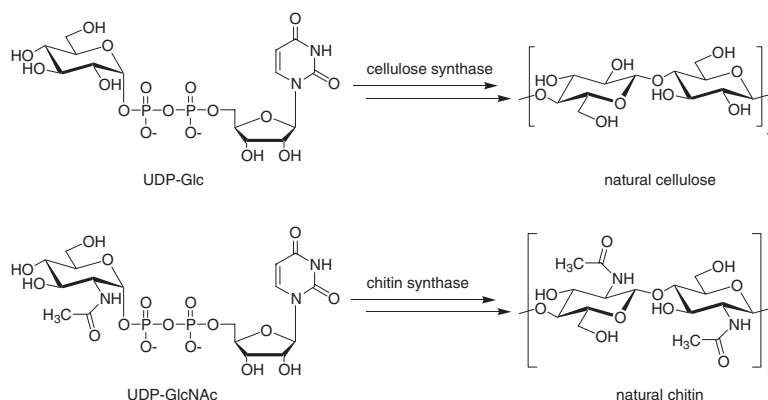
Scheme 1.   *In vivo* biosynthesis of natural cellulose and natural chitin by the corresponding glycosynthase catalysis employing the sugar nucleotide as a specific substrate monomer.
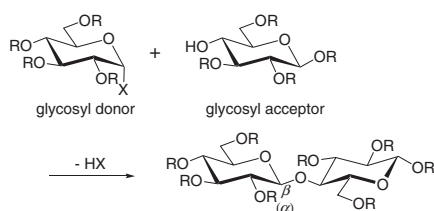
Scheme 2.   Glycosylation is a reaction between a donor and an acceptor (R = H).

at least eight isomers of the glucose dimer, even by this simplest calculation. For reference, following number of isomers was theoretically calculated; cello-trimer is composed of three D-glucose molecules connecting through $\beta(1{\to}4)$ glycosidic linkage, whereas 120 kinds of trisaccharide isomers can be formed from three D-glucose molecules, 1424 isomers from four D-glucose molecules including cello-tetramer, and 17,872 isomers from five D-glucose molecules including cello-pentamer.[15] On the other hand, four L-alanine molecules give only one tetrapeptide isomer, L-alanyl-L-alanyl-L-alanyl-L-alanine. A tetramer from four nucleoside molecules also gives only one isomer. This simple example of comparison between oligo-saccharides and an oligo-peptide as well as an oligo-nucleic acid clearly shows the complexity in carbohydrate chemistry.

The way of activation of the C1 together with a catalyst, therefore, is the key for controlling the glycosylation. Over the century the reaction control has been a central subject in the carbohydrate chemistry. So far, several well-known methods have

been developed, which are chronologically cited: (i) the Koenigs-Knorr method (1901) using bromine or chlorine at the C1 carbon of glycosyl donor with AgClO$_4$ or Hg(CN)$_2$ as catalyst,[15] (ii) the Helferich method (1933) and the Ogawa-modified version (1981), (iii) the Kochetkov orthoester method (1971), (iv) the Sinaÿ imidate method (1978), (v) the Schmidt-modified imidate method (1980) using trichloroacetyl imidate with (CH$_3$)$_3$SiOSO$_2$CF$_3$ as catalyst,[16] and (vi) the Mukaiyama glycosyl fluoride method (1981) using AgClO$_4$-SnCl$_2$ catalyst.[17]

However, all these glycosylation reactions need the protection-deprotection procedures and we considered them not sufficient enough to control the reaction in terms of stereo- and regio-selectivities. The control should be perfect, because the polysaccharide synthesis requires the repetition of the glycosylation many times. Therefore, we decided to develop a superior reaction by ourselves for the synthesis of polysaccharides and reached, at last after in-depth considerations, to conceive using enzymes as catalyst for the *in vitro* synthesis.

Characteristic features of enzymatic reactions

All the bio-substances including biomacromolecules are produced *in vivo* with enzymatic catalysis via biosynthetic pathways. Enzymatic catalysis has following advantageous characteristics in general: (i) a high catalytic activity (high turnover number), (ii) reactions under mild conditions with respect to temperature, pressure, solvent, pH of medium, etc., bringing about energetic efficiency, and (iii) high reaction selectivities of regio-, enantio-, chemo-, and stereo-regulations, giving rise to
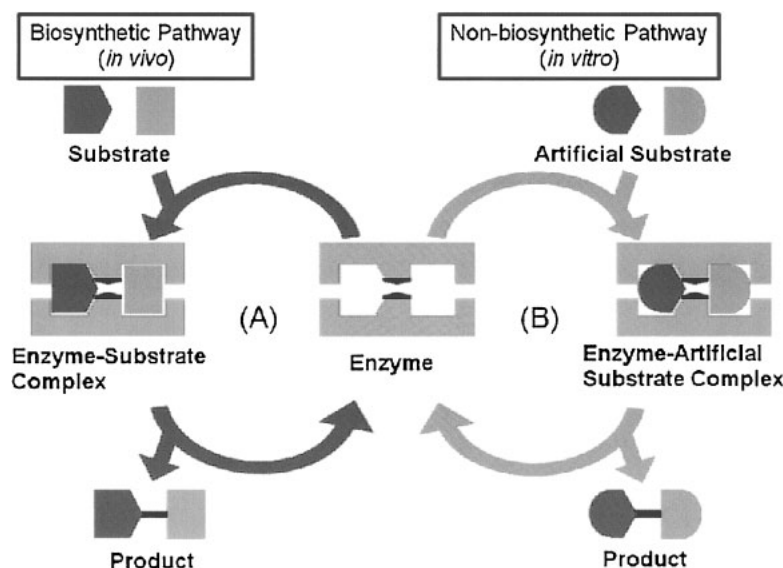
Fig. 3.   A "key and lock" theory showing the enzyme-substrate relationships for (A) an *in vivo* biosynthetic pathway, and (B) an *in vitro* non-biosynthetic pathway.

perfectly structure-controlled products. If these *in vivo* characteristics can be realized for *in vitro* polymer synthesis, we may expect the following outcomes: (i) perfect control of polymer structures, (ii) creation of polymers with a new structure, (iii) a clean process without forming by-products, (iv) a low loading process with saving energy, and (v) biodegradable properties of product polymers in many cases.

The following two aspects are to be emphasized as fundamental and important characteristics in enzymatic reactions. First, a "key and lock" theory proposed by E. Fischer in 1894 pointed out the relationship between substrate and enzyme where a reaction takes place.[18] The theory implies that via *in vivo* biosynthetic pathways a substrate and an enzyme correspond very strictly in a 1:1 fashion like a key and lock relationship. This phenomenon is nowadays understood in the following way; they recognize each other and form an enzyme-substrate complex, shown as the left hand cycle (A) in Fig. 3. The complex formation is due to the supramolecular interactions mainly of hydrogen-bonding. The formation of the complex activates the substrate to induce the reaction and lead to a product with perfect structure control.

Second, L. Pauling demonstrated in 1946 the reason why enzymatic reactions proceed under such mild reaction conditions,[19),20)] i.e., the forma-

tion of the enzyme-substrate complex stabilizes the *transition-state* and much lowers the activation energy compared with the no-enzyme case, which is now well accepted (Fig. 4). In fact, owing to such stabilization of the transition-state the enzyme-catalyzed rate-enhancement reaches normally $10^3 \sim 10^6$, often 10^{12}-fold, and in some cases even 10^{20}-fold![21)]

When the "key and lock" relationship observed *in vivo* reactions is strictly valid also for the enzyme-substrate relationship *in vitro*, there is no possibility to play with *in vitro* reactions. However, enzyme is often dynamic and able to interact with not only a natural substrate but also an unnatural substrate. In the *in vitro* enzymatic polymerization via non-biosynthetic pathways, a monomer is an unnatural (artificial) substrate for the catalyst enzyme. Yet, the substrate is to be recognized and to form an enzyme-artificial substrate complex so that the desired reaction may take place. For this reason, it has been proposed that the monomer is to be designed according to a new concept of a "transition-state analogue substrate" (TSAS), whose structure should be close to that of the transition-state of the *in vivo* enzymatic reaction.[3),6)-12),22)-24)] This is because the enzyme stabilizes the transition-state via complexation with the substrate.[19),20)] Then, the artificial monomer thus appropriately designed forms readily an enzyme-
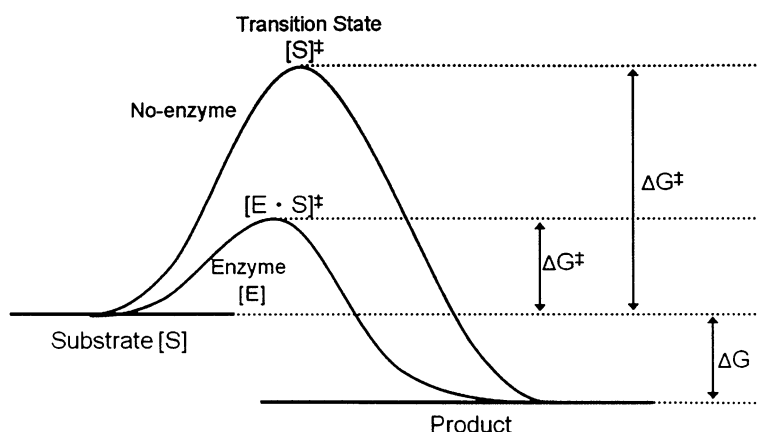
Fig. 4.   Energy profile of an enzyme-catalyzed reaction, lowering an activation energy by stabilizing the transition-state, in contrast to a no-enzyme reaction requiring a high activation energy without stabilization of the transition-state.
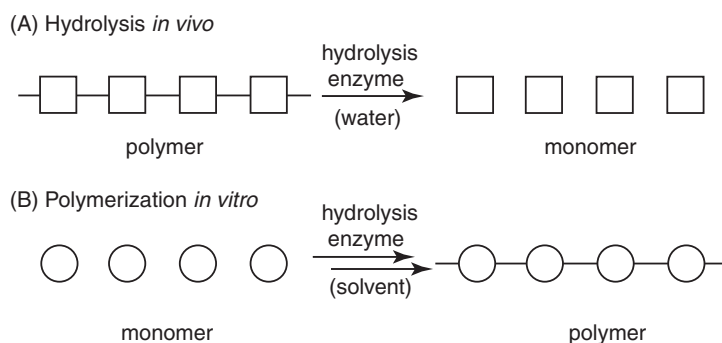

Fig. 5.   Model illustration using a hydrolysis enzyme for polymer synthesis: (A) *in vivo* the enzyme catalyzes the bond-cleavage of a polymer molecule to monomers with hydrolysis, and (B) *in vitro* the same enzyme catalyzes the bond-formation of monomers to produce a polymer molecule.

substrate complex and the reaction is induced to give the product with liberating the enzyme again as shown in the right side cycle (B) in Fig. 3. It is very important that a structurally close transition-state is involved commonly in both cycles (A) and (B).

The above features led us to think the following. Since an enzymatic reaction is reversible and when an enzyme-substrate complex is formed, a "hydrolysis enzyme" catalyzing a bond-cleavage *in vivo* is able to catalyze a bond-formation of monomers *in vitro* (formally a reverse reaction of the hydrolysis) to produce a polymer (Fig. 5). This view was based on a hypothesis that structure of a transition-state is very close in both *in vivo* and *in vitro* reactions, if the *in vitro* reaction would be induced.

Enzymatic polymerization to synthesize natural polysaccharides

A number of scientists had been challenging to find a facile method to *in vitro* synthesize natural polysaccharides; however, the first successful example was actually achieved by our cellulose synthesis. For developing a superior reaction as discussed above, we introduced a hydrolysis enzyme, a glycoside hydrolase, as polymerization catalyst. The hydrolases are extracellular enzymes that are more stable, easier to obtain, and much cheaper compared with glycosyltransferases. Glycoside hydrolases have two types, one is an *endo*-type and the other an *exo*-type enzyme.[25] For the enzymatic polymerization, *endo*-type enzymes have been found efficient, whose shape at the catalytic domain

looks like cleft.[26] Here, we describe the development of the polysaccharide synthesis via enzymatic polymerization, including the situation of the early stage of research.

Cellulose synthesis: Finding a new method of enzymatic polymerization. Cellulose is the most abundant organic molecule in the plant world and of course on the Earth; some hundreds billion tons of cellulose are annually photo-synthesized. It is a structural polysaccharide with a linear $\beta(1{\to}4)$ glycosidic bond structure of a dehydrated D-glucose repeating unit. It is a representative of polysaccharides, which had been an important symbolic molecule for Staudinger, Mark, and other prominent polymer scientists as to its structure, molecular weight, and derivatization reactions since the dawn period of polymer science in the 1920s.[27] Therefore, we selected cellulose as the first target molecule for the *in vitro* synthesis of polysaccharides, with taking also the situation below into account.[28]

Chemical synthesis of cellulose had been a challenging problem in polymer chemistry, since the first attempt in 1941.[29] Despite big efforts of the synthesis by many groups, all attempts resulted in no-success for half a century as shown by the following examples. Polycondensation of 2,3,6-tri-*O*-phenylcarbamoyl-D-glucose in CHCl$_3$/DMSO in the presence of P$_2$O$_5$ as a dehydrating agent was carried out to yield a polysaccharide; however, regio- and stereo-regulations of the reaction were not accomplished.[30] Synthesis of polysaccharides with well-defined structure by polycondensation of derivatives of monosaccharides or disaccharides containing a glycosidic hydroxy group, halogen or acetate group failed to form the desired products.[31] Cationic ring-opening polymerization of a bicyclic acetal monomer was a possible way to cellulose synthesis. A polysaccharide of degree of polymerization (DP) ∼20 was obtained; however, regioselective ring-opening did not take place. The major component was a tetrahydrofuran-ring unit and not a six-membered glucopyranose unit.[32],[33] With a carbohydrate chemistry approach, glycosylation to form a $\beta(1{\to}4)$ bond can be repeated in stepwise, leading to oligo- or polysaccharides. Starting from tribenzylated glucoside, four kinds of protecting group with different reactivity were employed and the glycosylation was achieved by using the imidate method. Seven times of the glycosylation gave cello-
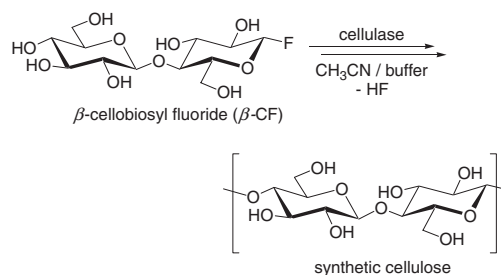
Scheme 3. Cellulase-catalyzed polycondensation of the fluoride monomer (β-CF) to synthetic cellulose.

octamer derivatives and further deprotections were needed to cello-octamer.[34] This stepwise synthesis looked not a practical method for polysaccharide synthesis.

For the superior reaction, we employed cellulase, a hydrolysis enzyme of cellulose, for catalyst. In order for the reaction to proceed, design of a substrate was the key. After the consideration, a substrate monomer of β-cellobiosyl fluoride (β-CF) was designed for catalysis by cellulase, with expecting a new catalytic function of cellulase, i.e., catalysis to form a glycosidic bond repeatedly which is a reverse catalysis to cleave the bond hydrolytically as seen in Fig. 5. It was then found that the reaction proceeded smoothly to produce synthetic cellulose via a single step polycondensation liberating HF molecule (Scheme 3).[28]

A typical polymerization example is given. A mixture of monomer β-CF and cellulase (*Trichoderma viride*, 5 wt % for β-CF) in a mixed solvent of acetonitrile/0.05 M acetate buffer (pH 5) (5:1, v/v) was stirred at 30 °C. When the reaction progressed the initially homogeneous solution gradually became heterogeneous with a white precipitation of the product. After 12 h, the resulting suspension was heated at 100 °C to inactivate the enzyme and poured into an excess amount of methanol/water (5:1) to separate the insoluble part by filtration. The water-insoluble, white powdery material was isolated in 54% yields. The insoluble part was acetylated and the molecular weight of the product was determined by size-exclusion chromatography (SEC) analysis to afford a DP value around 22 and hence a polysaccharide of "cellulose".[35] Further, water soluble cellooligomers were found in the filtrates.

The water-insoluble part was established as the first clear-cut example of "synthetic cellulose". Its
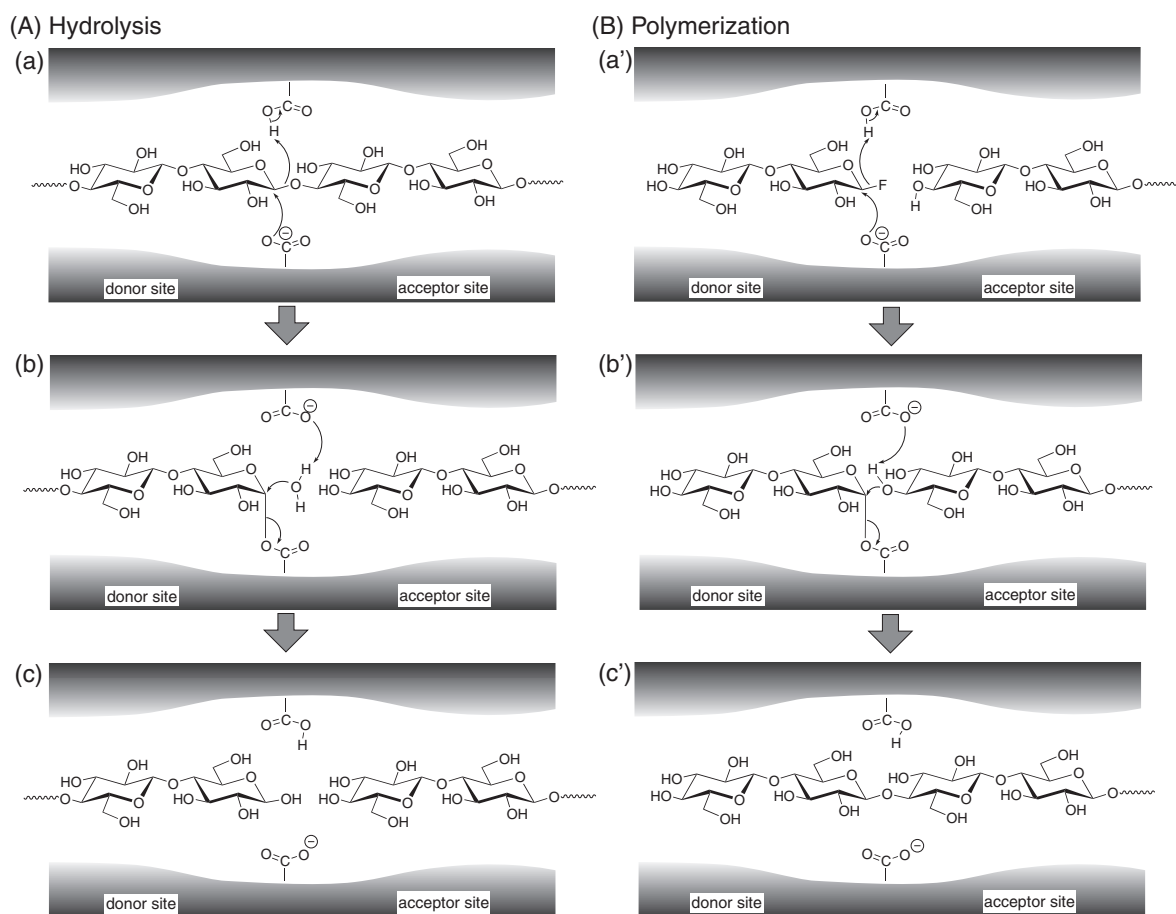
(A) Hydrolysis

(B) Polymerization

Fig. 6. Postulated reaction mechanisms of cellulase catalysis: (A) hydrolysis of cellulose, and (B) polycondensation of β–CF monomer to synthetic cellulose.

CP/MAS ^{13}C NMR and IR spectra confirmed the exclusive $\beta(1{\to}4)$ glycosidic linkage structure. Synthetic cellulose was, of course, readily hydrolyzed by cellulase to give glucose and cellobiose. In addition, thermal behavior of synthetic cellulose was completely similar to that of natural sample; it decomposed around at 260 °C without showing melting point.

It was necessary to use an organic solvent/ buffer mixture as solvent to prevent the hydrolysis of the synthetic cellulose with the cellulase catalysis. Among the solvents examined, acetonitrile was the best; DMF or DMSO was too polar and deactivated the cellulase catalysis. It was also important to use appropriate pH of the buffer.

The discovery of the reaction (Scheme 3) much attracted chemists from many research fields[3] and actually initiated the series of our studies on "enzymatic polymerization"; the cellulose synthesis

opened a new door not only to synthesis of various polysaccharides but also to extensive synthesis of other polymers. These polymers include variously functionalized polyesters by lipase enzyme catalysis and oxidatively polymerized phenolic and polyphenolic substances by oxidoreductase enzyme catalysis.[1),2),7)–9),11)–13)] In addition, lipase-catalyzed ring-opening polymerization of lactones and a macrolide to functional polyesters[36),37)] and synthesis of "artificial Urushi" by a peroxydase catalyst[38)] were reported.

Fig. 6 illustrates the postulated reaction mechanisms of cellulase catalysis in the hydrolysis of cellulose (A) and in the polymerization of β-CF monomer (B).[3),6)–12)] It is generally accepted that cellulases have two carboxylic acid residues for catalysis located in the cleft like domain.[26)] In the hydrolysis (A), one residue pulls (protonation) the glycosidic oxygen, and the other pushes the C1

carbon in the general acid-base mode to assist the glycosidic bond-cleavage (stage a). Then, a highly reactive intermediate (or transition-state) of a glycosyl–carboxylate structure with α-configuration is formed (stage b). This intermediate may have another possible structure of an oxocarbenium carboxylate. A water molecule attacks the C1 of the intermediate from β-side to complete the hydrolysis with formation of the hydrolysate having β-configuration (from stage b to c).

In the polymerization (B), the monomer β-CF is readily recognized and activated at the donor site via a general acid-base mode to cleave the C–F bond to form a highly reactive glycosyl–carboxylate intermediate having α-configuration (from stage a' to b'). The 4-hydroxy group of another monomer molecule or the growing chain end attacks the C1 of the intermediate from β-side to form a β-glycoside (from stage b' to c'). Thus, from β-CF to the product, the inversion of configuration is involved twice ("double displacement mechanism"). The product moves to the right for the next monomer coming in the donor site. The repetition of stages a'–c' is a polycondensation with liberating HF molecule, leading to the production of synthetic cellulose.

In the polymerization, β-CF monomer acted as both a donor and an acceptor. β-CF is considered as a TSAS monomer, because of a common transition-state (or intermediate) structure involved from stage a to b and stage a' to b' in both reactions A and B.[3),6)–12)] In other words, the transition-state comes early, whose structure is probably close to the starting substrate of β-CF. From polymer chemistry, this polymerization proceeds via an "activated monomer mechanism", in contrast to an "active chain-end mechanism" as normally observed in many vinyl polymerizations.

There are four important reasons for the monomer design. First, the monomer is to be recognized and activated by cellulase for the reaction to take place. For this purpose, cellobiose (disaccharide) structure of β-CF was chosen because the smallest unit to be recognized by cellulase was speculated as a cellobiose repeating unit judging from the cellulose structure in Fig. 2. Second, fluorine has an atomic size (covalent radius, 0.64 Å) close to that of oxygen (0.66 Å) constituting the glycosidic bond of cellulose, which gives a desired opportunity for β-CF to be readily recognized by
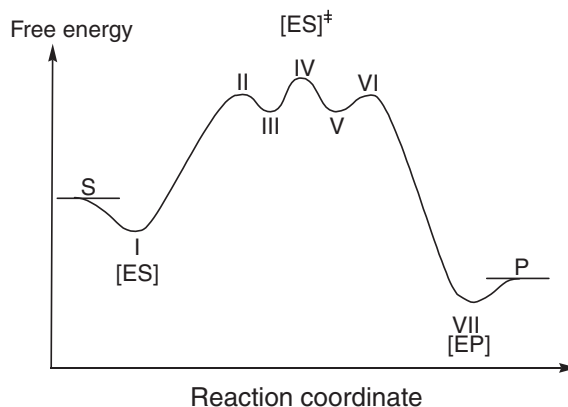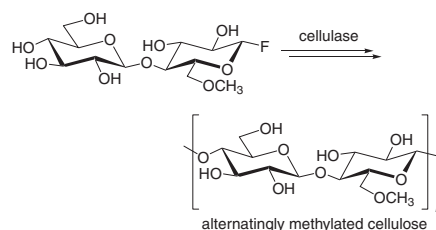

Fig. 7. Reaction pathway of an enzymatic reaction in a detailed expression.

Scheme 4. Synthesis of alternatingly 6-methylated cellulose.

cellulase. Third, fluoride anion is one of the best leaving groups. Fourth, among the glycosyl halides only glycosyl fluorides are stable as the unprotected form, which is required for most enzymatic reactions carried out in the presence of water. After all, combination of cellulase and β-CF readily leads to a transition-state, which is close in structure to that involved in hydrolysis of cellulose by cellulase. These views indeed led us to create the above concept of TSAS, which is essential for the monomer design in all cases.

Here, we should comment on the transition-state and the intermediate, because these are sometimes ambiguous and confusing. We express our concept, as a "transition-state analogue substrate" (TSAS) but not as an "intermediate analogue substrate". In Fig. 4, the reaction pathway of an enzymatic reaction is roughly given. In a more detailed expression, for example, intermediates (I, III, V and VII) and transition-states (II, IV and VI) are shown in Fig. 7. It is highly possible that stages a' and b' in the polymerization correspond to intermediate III (or transition-state II) and inter-
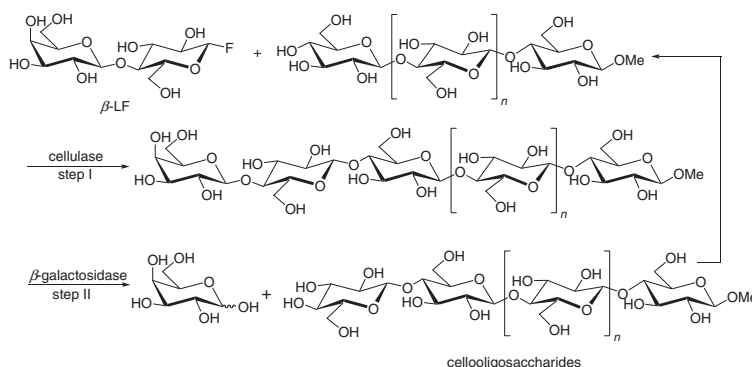
Scheme 5. A stepwise chain elongation to synthesize cellooligosaccharides.

mediate V (or transition-state IV), respectively. However, it is often very difficult to distinguish transition-state and intermediate due to small energy difference and some cases all the stages II–VI are regarded as a transition-state like in Fig. 4. In addition, an efficient enzymatic catalysis is really owing to the stabilization of the transition-state, lowering the activation energy.[19),20)] Therefore, we prefer to use the term TSAS.

It is to be noted that the effectiveness of a disaccharide fluoride structure like β-CF as monomer was argued[39)] and disaccharide fluoride monomers were widely utilized later by other chemists, for examples, for the synthesis of oligo- and polysaccharides.[40),41)] In fact, a fluoride monomer of monosaccharide, β-glucosyl fluoride, was not a good substrate in terms of the polysaccharide yield and molecular weight, giving only a small amount of oligomers. Furthermore, importance of the disaccharide monomer structure was utilized to explain the mechanism of cellulose biosynthesis.[42)]

Strict control of regio- and stereo-selectivities encounters the limitation of substrates adaptable to the enzymatic reaction and becomes an obstacle for the development of this method to the general polysaccharide synthesis. However, we found that glycoside hydrolases show dynamic nature in the substrate specificity and often recognize unnatural substrates. The first such example is the synthesis of 6-O-methylated cellulose as shown in Scheme 4, where 6-methylated β-CF acted as a substrate monomer.[43)]

Cellulose synthesis was extended to an oligosaccharide synthesis, i.e., cellooligosaccharides were prepared using β-lactosyl fluoride (β-LF) as a donor via a step-by-step chain elongation by combined use of cellulase a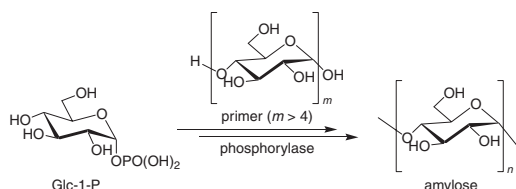nd β-galactosidase (Scheme 5).[44),45)] Cellulase catalyzes the glycosidation of β-LF to the nonreducing 4-hydroxy group of the acceptor but β-LF did not act as an acceptor in the step I. β-Galactosidase cut off a β-galactoside unit from a newly added nonreducing end in the step II.

Mutant enzymes for cellulose synthesis. It is a very strong tool for the mechanistic study to mutate an enzyme and examine the reaction. Very recently, our approach provided important information on the role of the cellulase function. Cellulase (endoglucanase II, EG II) consists of three domains: a cellulose-binding domain (CBD), a linker domain, and a catalytic domain. In the hydrolysis, CBD first binds the crystalline part of cellulose and then the catalytic domain catalyzes the hydrolysis of cellulose molecules, the linker domain linking these two domains. Using biotechnology, two types of protein enzymes were prepared from yeast, the one having all domains (EG II) and the other having only catalytic domain lacking CBD and the linker domain (EG II core) (Fig. 8).[46)] Very interestingly, both enzymes showed high polymerization activity for monomer β-CF, giving synthetic cellulose. With progress of time, the cellulose produced gradually disappeared
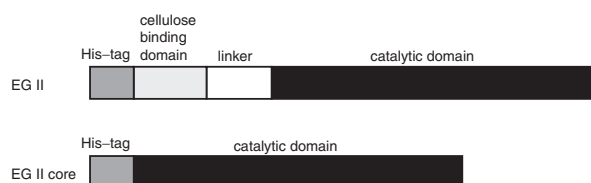
Fig. 8. Schematic representation of EG II expressed by *Saccaromyces serevisiae* and the mutant EG II core.
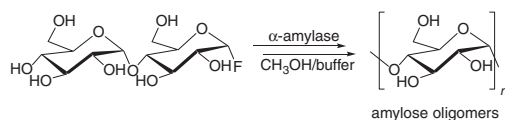
Scheme 6. Enzymatic polymerization of α-maltosyl fluoride monomer.

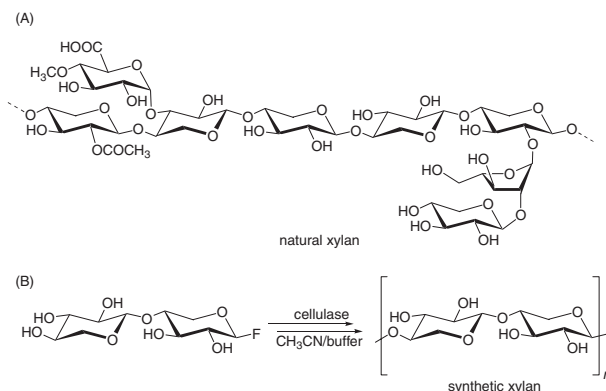Scheme 7. Phosphorylase-catalyzed polymerization of Glc-1-P to amylose.

Scheme 8. (A) typical structure of natural xylan having branching, and (B) enzymatic polymerization to synthetic xylan having a linear structure.

with EG II due to the hydrolysis; however, the product cellulose hardly changed with EG II core. These results suggest that the CBD plays an important role for the hydrolysis of the product, but not for the polymerization of β-CF. The polymerization needs only the function of the catalytic domain.

Amylose synthesis. Amylose is a water-soluble α(1→4)-glucose polymer produced *in vivo* as a substance for energy storage. We extended the cellulase-catalyzed synthesis of cellulose to the synthesis of amylose and designed α-maltosyl fluoride as monomer for the catalysis of α-amylase, a hydrolysis enzyme of amylose. The monomer derived from maltose, a disaccharide component of amylose, readily disappeared by the addition of the enzyme within 1 h to give a mixture of maltooligosaccharides (amylose oligomers) (Scheme 6).[47] The product contained was a mixture of oligomers consisting not only of even- but odd-numbered glucose units, like maltotriose, maltopentaose and matoheptaose. These results indicate that hydrolysis and/or rearrangement of the amylose oligomers occurred by the catalysis of α-amylase. The formation of an α(1→4)-glycosidic linkage looked harder compared with that of β(1→4)-linkage.

For reference, another *in vitro* enzymatic synthesis of amylose is mentioned. Amylose can be synthesized via *in vitro* polymerization of D-glucosyl phosphate (Glc-1-P) catalyzed by potato phosphorylase (Scheme 7)[48] Phosphorylases are known as enzymes in the carbohydrate metabolism *in vivo* to catalyze the phosphorolysis of glycosidic bonds in

oligo- and polysaccharide substrates. Therefore, the reaction belongs to a biosynthetic pathway, and hence, it is not within the context of the enzymatic polymerization defined by us. This approach is the equilibrium-controlled synthesis, and therefore, the reaction needs a large excess amount of Glc-1-P. In addition, the reaction requires a primer of malto-oligomer with minimum length of tetramer and then proceeded in living-like polymerization to form amylose.

In contrast to the reaction like Scheme 7, all the enzymatic polymerizations described here belong to a kinetic approach, where an activated glycosyl donor such as a glycosyl fluoride, designed as a TSAS, forms rapidly the glycosyl-enzyme complex to lead to the product poly- or oligosaccharide (kinetic-controlled synthesis).[10]

Xylan synthesis. Naturally occurring xylan existing mostly in the hemicellulose is an important biomacromolecule composed of a β(1→4)-linked D-xylose main chain, which bears side chains such as arabinose and 4-*O*-methyl-glucuronic acid (Scheme 8A). As in the case of cellulose synthesis, β-xylobiosyl fluoride monomer was designed on the basis of the TSAS concept for the catalysis of cellulase containing xylanase. The enzyme-catalyzed polycondensation of the monomer provided synthetic xylan in good yields having average DP of 23 without side chains under perfect control of regioselectivity and stereochemistry (Scheme 8B).[49]

Chitin synthesis. Chitin is the most abundant organic substance in the animal world, as

(A) Hydrolysis

(a)

(B) Polymerization

(b)

(b')

(c)

(c')
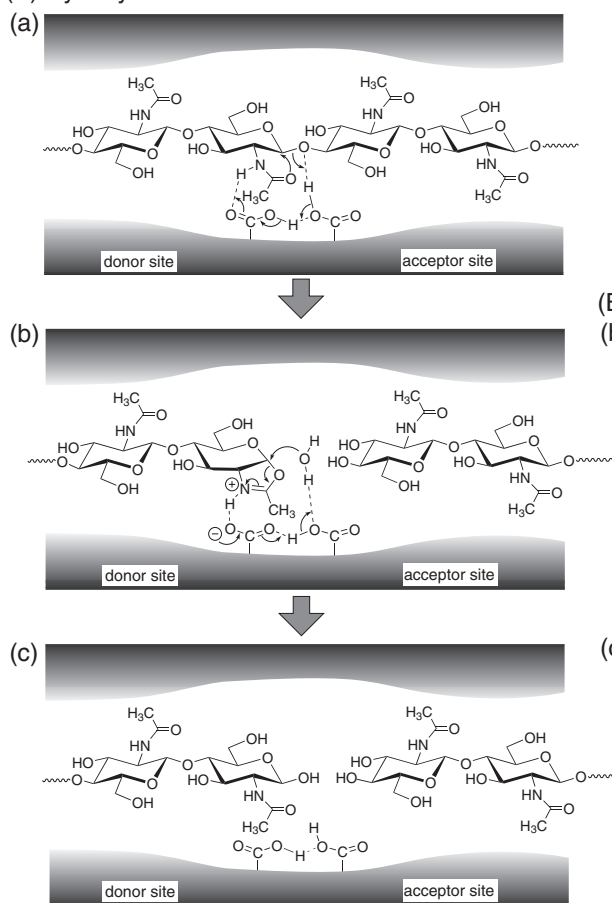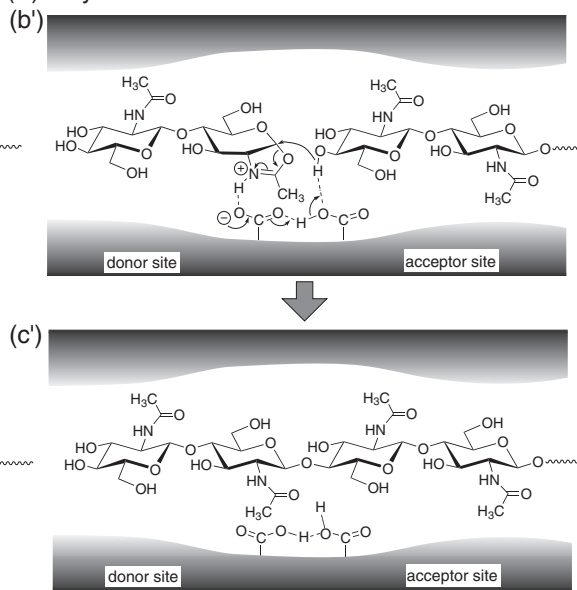
Fig. 9. Postulated reaction mechanisms of chitinase catalysis: (A) hydrolysis of chitin, and (B) ring-opening polyaddition of ChiNAc-oxa monomer to synthetic chitin.

cellulose in the plant world. It is found as a structural polysaccharide widely in animals like invertebrates and its total quantity is estimated as approximately 1% for that of cellulose. Chitin has a linear structure of $\beta(1\rightarrow4)$-linked N-acetyl-D-glucosamine (GlcNAc) as a repeating unit. It attracts much interest in a number of scientific and application purposes as a multifunctional substance. Chitin and its derivatives are frequently utilized for biocompatible, biodegradable and bioactive materials such as immuno-adjuvant substances, inhibitor of metastases of tumor cells, wound-healing materials, additives for cosmetics, and drug carriers.[50]

In vitro synthesis of such an important biomacromolecule of chitin was first reported in 1996, utilizing chitinase from *Bacillus* sp. (family 18) as catalyst.[22),51)] Based on the TSAS concept, an N,N'-diacetylchitobiose [GlcNAc$\beta(1\rightarrow4)$GlcNAc] oxazo-
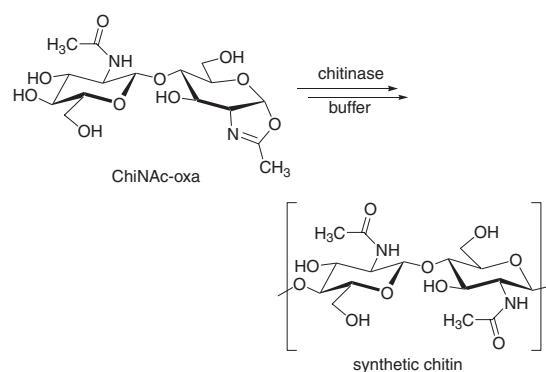
Scheme 9. Chitinase-catalyzed ring-opening polyaddition of the oxazoline monomer (ChiNAc-oxa) to synthetic chitin.

line derivative (ChiNAc-oxa) was designed by speculation and synthesized as monomer. The monomer was recognized and catalyzed by the

chitinase, giving rise to synthetic chitin in quantitative yields via ring-opening polyaddition under perfect control of regioselectivity and stereochemistry (Scheme 9).[22] The DP value was evaluated as 10–20 depending on the reaction conditions employed.

Fig. 9 illustrates the postulated reaction mechanisms of the chitinase catalysis in the hydrolysis of chitin (A) and in the polymerization to synthetic chitin (B). In the hydrolysis (A), the glycosidic oxygen is protonated by one of the carboxylic acid groups immediately after the recognition of chitin in the stage a. The acetamido-oxygen of the chitin unit at the donor site attacks the neighboring C1 carbon to form the corresponding oxazolinium ion stabilized by the other carboxylate, involving the scission of the glycosidic linkage (stage b), which is thus called a "substrate-assisted mechanism".[52] Nucleophilic attack by a water molecule from β-side opens the oxazolinium ring to accomplish the hydrolysis reaction, giving rise to the hydrolysate having β-configuration (stage c).

In the polymerization (B), the monomer having already an oxazoline moiety is readily recognized at the donor site as a TSAS monomer and immediately protonated by the carboxylic acid to form the corresponding oxazolinium ion. The protonation step onto the monomer nitrogen must be much easier, compared with the protonation on the glycosidic oxygen atom. Therefore, a step corresponding to the stage a of the hydrolysis to cleave the glycosidic C–O bond is not necessary. The hydroxy group at the C4 of another monomer or the growing chain end attacks the C1 of the oxazolinium from β-side (stage b'), resulting in the formation of a $\beta(1\rightarrow4)$-glycosidic linkage (stage c'). Repetition of these reactions is a ring-opening polyaddition. The monomer ChiNAc-oxa acted as both donor and acceptor. The key-point is the structure similarity of the transition-state (or the intermediate) involved in both stages b and b'.[3),6)–12),22)–24)] It is to be noted that our speculated design of an oxazoline monomer based on the TSAS concept[22] preceded the paper reporting that the chitinase enzyme belonging to family 18 involves an oxazolinium intermediate via a "substrate-assisted mechanism" during the hydrolysis of chitin.[52]

Further the concept of TSAS was supported by the following experiments. ChiNAc-oxa monomer was subjected to enzymatic polymerization. Hy-
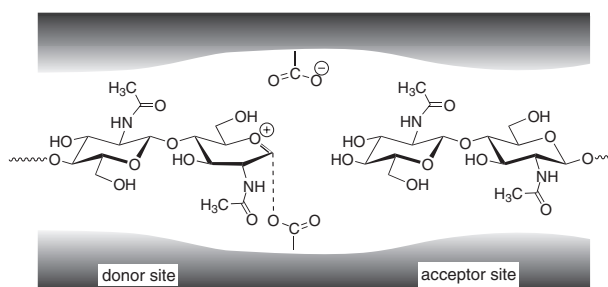

Fig. 10. Lysozyme or chitinase (family 19) catalysis in the hydrolysis of chitin involving an oxocarbenium ion intermediate.

Table 1. Effect of pH on the ring-opening polyaddition of ChiNAc-oxa monomer[a]

pH of phosphate buffer	reaction time[b]/h	yields of synthetic chitin/%
8.0	2.5	38
9.0	20	62
10.6	50	∼100
11.4	150	87
12.9	300	0

[a]Reaction conditions; chitinase 1 wt% at r.t. in a phosphate buffer.
[b]Reaction time for complete monomer consumption.

drolysis enzymes used were chitinase (family 18) involving an oxazolinium intermediate, and lysozyme as well as chitinase (family 19) involving an oxocarbenium ion intermediate (or transition-state) (Fig. 10). With the family 18 enzyme, synthetic chitin was quantitatively obtained after 50 h at pH 10.6, whereas with lysozyme no chitin was produced even after 165 h.[53] This implies that ChiNAc-oxa monomer could not be a substrate for the lysozyme enzyme, since it is not able to act as a TSAS monomer.

The optimal pH for the hydrolysis by chitinase is known as 7.8. Effects of the buffer pH on the ring-opening polyaddition of the monomer showed that the optimal value is around pH 10.6 in terms of the chitin yield (Table 1).[53] At pH 8.0, the reaction time for the complete monomer conversion took 2.5 h; however, the yield of synthetic chitin was 38%. At pH 10.6 it took 50 h and the chitin was obtained quantitatively. This means that the polymerization was very rapid at around pH 8 but the product chitin was also hydrolyzed rapidly; at pH 10.6 the polymerization was exclusively pro-
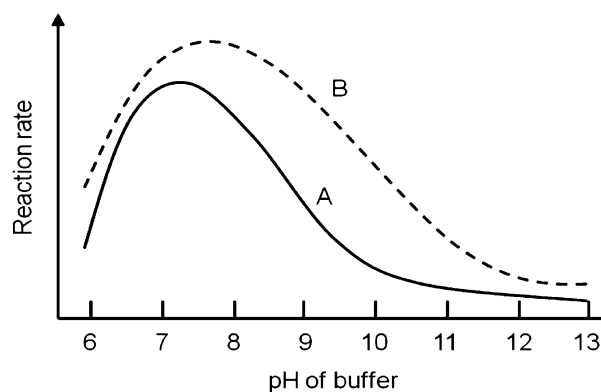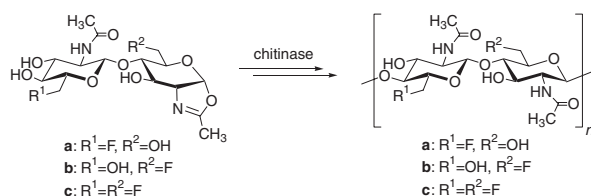
Fig. 11. A rough sketch of pH effects on the rate of chitinase-catalyzed reactions: (A) hydrolysis rate, and (B) polymerization rate.

Scheme 10. Synthesis of fluorinated chitins via chitinase-catalyzed polymerization of fluorinated chitobiose oxazoline monomers, **a**, **b** and **c**.
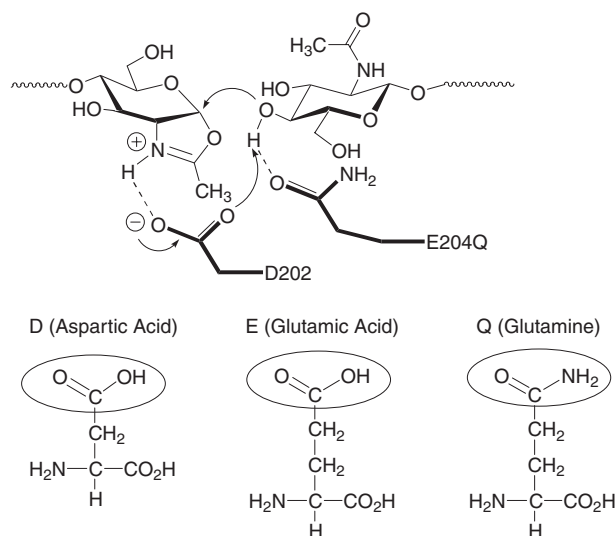

Fig. 12. Polymerization of the oxazoline monomer by the mutant enzyme.

very similar to each other, together with those of natural and synthetic chitins, which are all organized in antiparallel molecular chain alignment, i.e., thermodynamically stable α-chitin. Introduction of fluorine substituent into monomers lowered the polymerizability to some extent.[54] 6-Fluorinated mono-saccharide oxazoline monomer showed a lower reactivity than unsubstituted one with chitinase catalysis and the oxazoline monomer did not induce a glycosylation reaction, showing the importance of the di-saccharide structure.[55]

Furthermore, 6-*O*-carboxymethylated ChiNAc-oxa monomer was selectively dimerized by the chitinase catalysis to produce alternatingly 6-*O*-carboxymethylated chitotetraose.[56]

Mutant enzyme for chitin synthesis. In relevant to the mechanistic aspects, the amino acid residue E204 in wild-type chitinase A1 from *Bacillus circulans* was assumed to be the catalytic acid/base, and D202 serves as a stabilizer for the oxazolinium ion during the hydrolysis. Then, a mutated enzyme E204Q was designed and prepared, where the glutamic acid at the 204th amino acid residue was replaced by glutamine (Fig. 12).[57] The mutant enzyme was employed for the chitin synthesis. As anticipated, it exhibited the catalytic activity for the formation of $(1\rightarrow4)$-β-glycosidic linkage, suppressing completely the hydrolysis of the product, although the total catalytic activity was much reduced. This result shows the mecha-

moted, suppressing the hydrolytic activity. These pH effects are shown roughly in Fig. 11. The polymerization is less sensitive to the pH, probably because it does not require the protonation at the glycosidic oxygen. Therefore, the polymerization is to be carried at a pH where the difference in rate of polymerization and hydrolysis is maximal, so that the polymerization may take place selectively.

Chitinase showed a dynamic nature for substrate recognition; it catalyzed the ring-opening polyaddition of fluorinated sugar-oxazoline monomers to produce fluorinated chitin derivatives (Scheme 10).[54],[55] The polymerization took place effectively at pH 8.0–9.0 and 30–40 °C, giving rise to alternatingly 6-fluorinated chitin derivatives from monomers **a** and **b**, and fully 6-fluorinated chitin derivative from monomer **c** under perfect control of regioselectivity and stereochemistry. The molecular weight (M_n) of a water-insoluble part was up to 1,400–1,600 from **a** and **b** (yield ~40%) and up to 1,620 from **c** (yield ~23%); the other parts were water-soluble with lower molecular weight. Crystalline structures of polymers **a** and **b** were
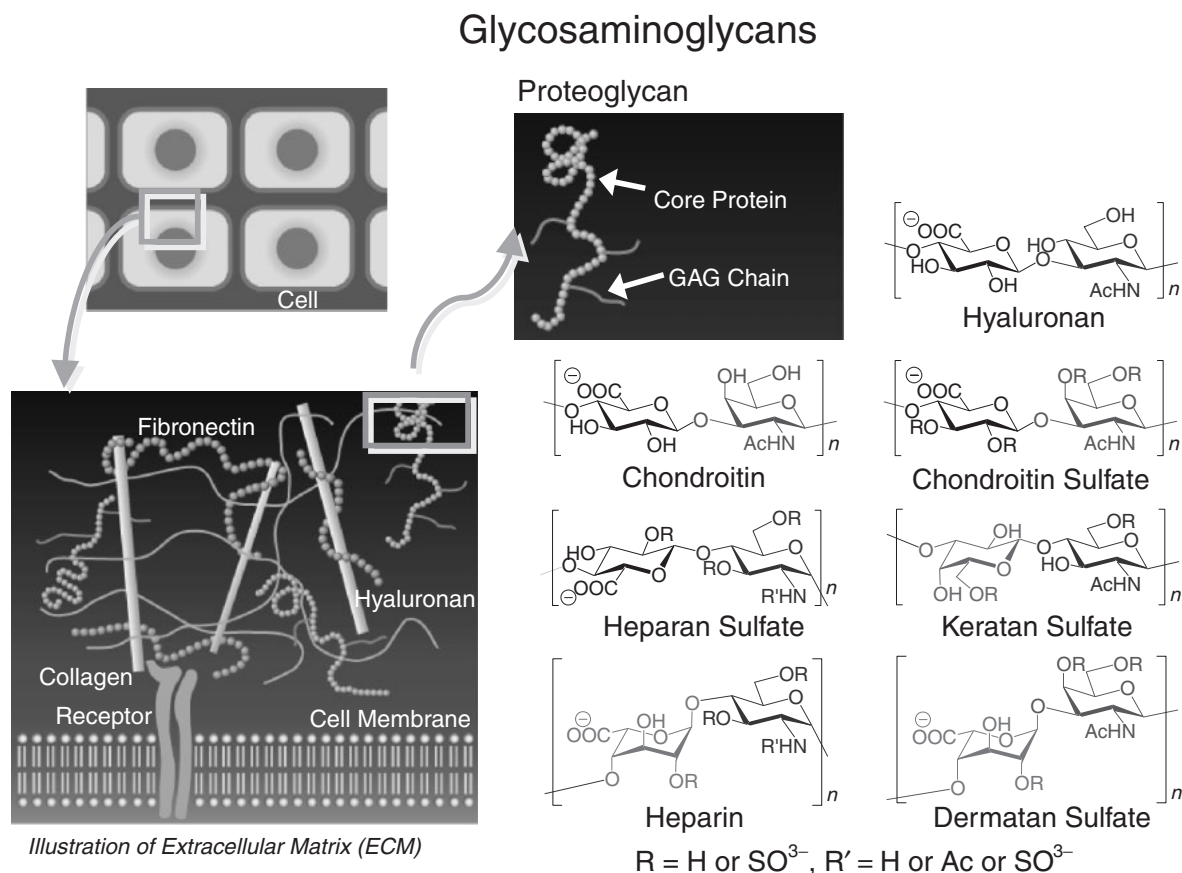
Glycosaminoglycans

Fig. 13. Glycosaminoglycan (GAG) chains are linked to core proteins, which form proteoglycans. Structures of seven typical glycosmainoglycans are given.

nistic difference between hydrolysis and polymerization. The former needs both carboxy groups, whereas the latter inherently does not need the second carboxyl group. This observation accords with the sketch in Fig. 11; the polymerization proceeded at a higher pH.

Glycosaminoglycans. Extracellular matrices (ECMs) are substances found between living cells (Fig. 13).[58] ECMs are constituted from fibronectins, collagens, proteoglycans, etc. Proteoglycans are formed from core proteins to which many (normally over 100) polysaccharide chains are linked. These are all linear hetero-polysaccharides called glycosaminoglycans (GAGs). GAGs are one of the classes of naturally occurring bioactive hetero-polysaccharides which are consisted of alternatingly connecting a hexosamine [N-acetyl-D-glucosamine (GlcNAc) or N-acetyl-D-galactosamine (GalNAc)] and an uronic acid [D-glucuronic acid

(GlcUAc) or L-iduronic acid (IdoA)].[59] In Fig. 13 the structures of typical seven GAG molecules are given. GAGs construct ECMs through association with core proteins and also collagens, fibronectins, etc., providing scaffolds for cellular proliferation and differentiation in morphogenesis and regeneration of tissues.[58] Thus, GAGs play critical roles in living organisms, and are frequently used as therapeutic materials and food supplements. However, investigation of GAG functions at a molecular level is currently under challenging. Therefore, GAG samples with well-defined structure are strongly demanded.

Hyaluronidase (HAase) is one of the endo-β-N-acetylglucosaminidase responsible for metabolism of hyaluronan (hyaluronic acid, HA), chondroitin (Ch) and chondroitin sulfate (ChS).[59] The enzyme catalyzes hydrolysis of such GAGs via a substrate-assisted mechanism similarly to a chitinase involv-

(B) Fluoride-type monomer

hyaluronoglucuronidase

Natural HA chain

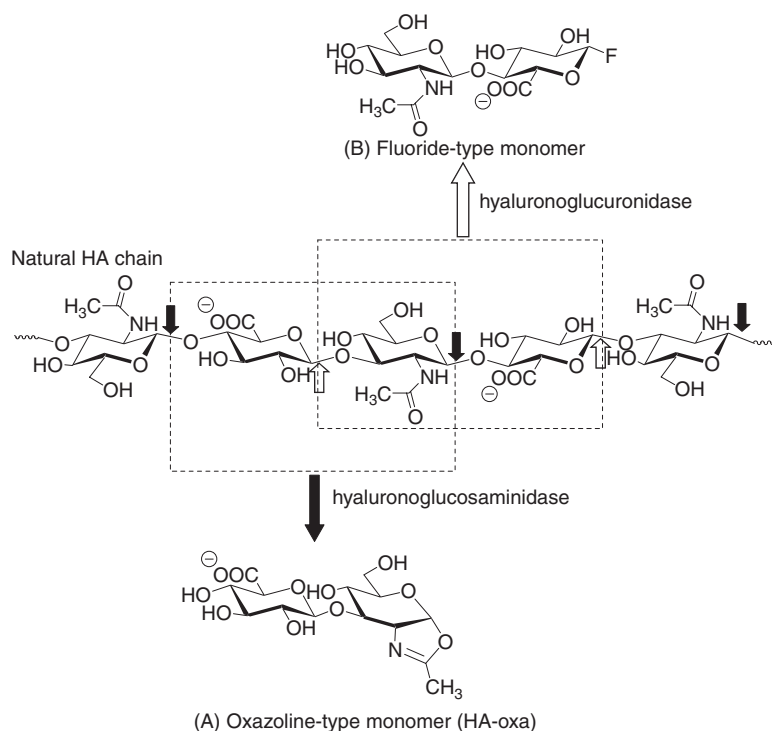hyaluronoglucosaminidase

(A) Oxazoline-type monomer (HA-oxa)

Fig. 14.   Monomer design for bond-cleaving points on HA by HAase (black arrows) and hyaluronoglucronidase (white arrows) and possible monomer structures for the enzymes: (A) Oxazoline-type monomer for HAase catalysis, and (B) Fluoride-type monomer for the catalysis of hyaluronoglucronidase.

ing an oxazolinium ion intermediate.[60] On the basis of the TSAS concept for their synthesis, a molecular design of monomer structure bearing an oxazoline moiety allowed the production of HA, Ch and ChS via ring-opening polyaddition by HAase catalysis.[61],[62]

Hyaluronan synthesis. Hyaluronan (HA) was found in 1934, which is a high molecular weight biopolysaccharide contained in the vitreous humor of cattle eyes.[6] HA is widely found, in particular, in the skin, the rooster comb, the synovial joint fluid, the vitreous humor of eyes, etc. Biological functions of HA are concerned with the formation of a strong network of viscoelastic ECMs, the activation of intracellular signaling, the matrices formation around cumulus cells during ovulation and fertilization, and others. Such multi-functions of HA have been utilized in medical and pharmaceutical fields.

In vivo synthesis of HA is achieved in the plasma membrane by the catalysis of HA synthase through alternating addition of GlcNAc and GlcUAc via $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ fashion, respec-

tively, employing the corresponding uridine-5′-diphospho(UDP)-sugar substrates. A number of chemists have challenged to *in vitro* synthesize such an indispensable molecule of HA; however, an octasaccharide derivative was the largest molecule prepared by organic chemistry to date.[63]

The first successful *in vitro* synthesis of HA was reported via enzymatic polymerization.[23] As illustrated in Fig. 14, HA is composed of a hetero-disaccharide repeat, i.e., $\beta(1\rightarrow4)$-linked *N*-acetyl-hyalobiuronate [GlcUAc$\beta(1\rightarrow3)$GlcNAc]. For the monomer design, there are two possibilities of monomer structure derived from the hetero-disaccharide structure on the basis of the TSAS concept: (A) an oxazoline-type monomer, and (B) a fluoride-type monomer.

Due to the availability of the enzyme, we employed the oxazoline monomer (HA-oxa(R)) for HA synthesis. The monomer was readily recognized and catalyzed by HAase under perfect structure control, providing for the first time the *in vitro* synthetic HA (R=CH₃) in good yields (Scheme 11).[23] The M_n and M_w of synthetic HA

Table 2. Polymerization of HA-oxa(R) monomers to synthetic HA and HA derivatives

monomer R	enzyme[b]	pH	time/h	yield/%	$M_n{}^c$	$M_w{}^c$
	polymerization[a]				synthetic HA or HA derivative	
methyl	OTH	7.0	48	72	4,400	9,800
methyl	OTH	7.5	52	78	5,500	13,800
				53^d	13,300	22,000
methyl	OTH	8.0	60	73	5,700	14,900
methyl	OTH	9.0	72	65	4,600	11,300
methyl	BTH	7.5	60	53	7,800	17,600
				34^d	17,700	25,000
methyl	bee venom	7.5	72	trace	—	—
methyl	H–OTH	7.5	6	69	6,800	19,500
ethyl	OTH	7.5	48	65^d	6,900	17,500
n-propyl	OTH	7.5	60	47^d	4,500	13,500
vinyl	OTH	7.5	48	50^d	5,900	16,800

[a]In a carbonate buffer (50 mM) at 30 °C; monomer conc., 0.1 M; amount of enzyme, 10 wt % for monomer. [b]OTH, ovine testicular HAase (560 units/mg); BTH, bovine testicular HAase (500 units/mg). [c]Determined by SEC calibrated with hyaluronan standards. [d]Isolated yields after purification.
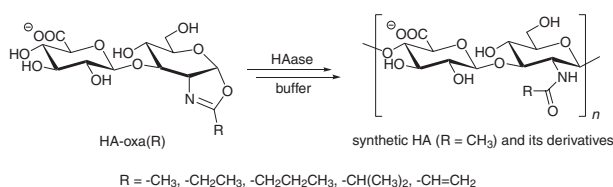

Scheme 11. HAase-catalyzed ring-opening polyaddition of HA-oxa(R) monomers to synthetic HA and HA derivatives.

reached high molecular weight values, 17,700 (corresponding to ∼90 saccharide units) and 25,000, respectively. Further, 2-substituted oxazolines of GlcUAcβ(1→3)GlcNAc like 2-ethyl-, 2-n-propyl-, 2-vinyl derivatives (HA-oxa(R)) were also polymerized effectively by HAase catalysis, giving rise to unnatural HA derivatives with the corresponding N-acyl group. Particularly, an N-vinyl (acrylamido) HA derivative has potentials for utilization as macromonomer, telechelics and so forth, leading to the application for medical materials. Some of polymerization results are given in Table 2.[64]

These results motivated us to carry out HAase-catalyzed copolymerization between various 2-substituted HA oxazoline monomers, giving rise to some unnatural HA derivatives (Scheme 12).[65] The copolymerization took place at 30 °C in a carbonate buffer to give copolymers, various unnatural HA derivatives, with M_n exceeding 10,000 in a good yield within 48 h. The results clearly show that the
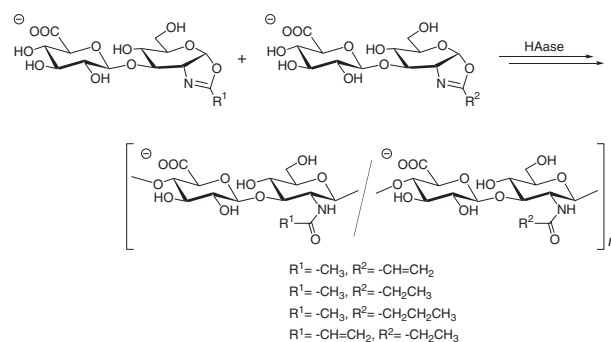
Scheme 12. HA-catalyzed copolymerization of HA-oxa(R) to produce four unnatural HA derivatives.

cross propagation of these monomers is feasible by using HAase as catalysis; therefore, this enzymatic copolymerization will serve as one of important technologies for constructing various GAGs.

Chondroitin synthesis. Chondroitin (Ch) exists predominantly as a carbohydrate part of proteoglycans or in the higher organisms as a precursor of Ch sulfate (ChS), mainly found in cartilage, cornea, and brain matrices. Many papers reported the biological functions of Ch and ChS, for examples, maintaining cartilage elasticity, regulation of tissue morphogenesis, and promotion of neurite outgrowth and neuronal migration, which frequently associate with the sulfation patterns.[66] Such important biomacromolecules of Ch and ChS
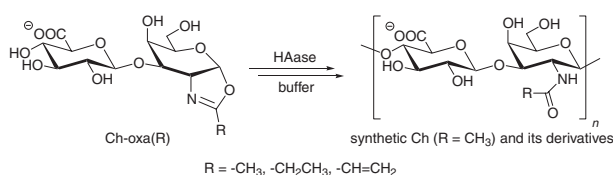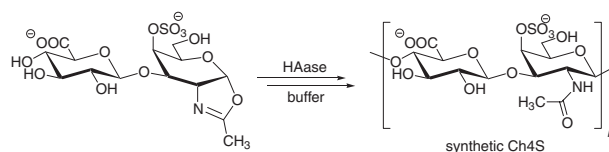
Scheme 13. HAase-catalyzed ring-opening polyaddition of Ch-oxa(R) monomers to synthetic Ch and Ch derivatives.

Scheme 14. HAase-catalyzed ring-opening polyaddition to synthetic Ch4S.

have been synthesized for the first time via non-biosynthetic pathways by ring-opening polyaddition catalyzed by HAase.[24),67)]

Ch is composed of the hetero-disaccharide repeating unit, GlcUAcβ(1→3)GalNAc (*N*-acetyl-chondrosine), which is connected through a β(1→4)-glycosidic linkage (Fig. 13). Therefore, on the basis of TSAS concept an oxazoline monomer (Ch-oxa(R)) was designed again for the synthesis of Ch via HAase-catalyzed ring-opening polyaddition (Scheme 13).[24)] In addition to the 2-methyl-oxazo-line monomer, polymerization of various oxazoline monomers with 2-ethyl, 2-*n*-propyl, 2-isopropyl, 2-vinyl, and 2-phenyl substituents was investigated. Monomers with methyl, ethyl and vinyl substituents were efficiently catalyzed by HAase at pH 7.5, providing synthetic Ch and its derivatives bearing the corresponding *N*-acyl groups under perfect structure control. Molecular weight (M_n) of these products reached ∼5000, which correspond to that of naturally occurring Ch. Monomers with 2-iso-propyl, and 2-phenyl substituents were not cata-lyzed by the enzyme probably due to steric bulki-ness of these substituents.

Chondroitin 4-sulfate synthesis. In living cells biosynthesis of ChS is performed by catalysis of the specific glycosyltransferases with uridine 5′-diphospho(UDP)-GlcUAc and UDP-GalNAc as substrates, and the sulfotransferases with 3′-phosphoadenosine 5′-phosphosulfate; however, synthesis mechanism of ChS is not fully understood. Biological activity of ChS in living systems is closely associated with the sulfation patterns, but the sulfation reaction is not well controlled and hence the naturally occurring ChS has a variety of structure. The ChS is classified into five groups according to the major component in the molecule, i.e., ChS–A mainly containing GlcUAcβ(1→3)GalNAc4S unit (sulfated at C4 in GalNAc; A unit), and other four groups; ChS–C, ChS–D, ChS–E, and ChS–K. These natural ChS

variants contain other units in various proportions, for example, ChS–A from whale cartilage incorpo-rates C unit in about 20% as a minor unit, and a shark cartilage ChS–C has D unit in about 10%. Thus, incorporation of minor units causes substan-tial structural diversity of ChS molecules, which makes the results on ChS functions indistinct. Synthesis of ChS with a uniform structure has been challenged by many scientists; up to now hexasac-charides consisting of structural units A, C and D have been synthesized as the longest ChS variants via conventional chemical methods. The investigation of the biological activities of naturally occurring ChS at a molecular level demands a structurally well-defined ChS sample. Therefore, we challenged the synthesis of ChS–A with a uniform structure via enzymetic polymerization.[67)]

HAase-catalyzed polymerization of a Ch-oxa(CH$_3$) monomer with a sulfate group at the C4 of GalNAc successfully progressed at pH 7.5, giving rise to synthetic Ch4S having the sulfate group exclusively at the C4 of GalNAc unit in high yields within a couple of hours (Scheme 14).[67)] Surpris-ingly, the enzymatic polymerization proceeded at the temperatures ranging widely from 0 to 50 °C. All the spectroscopic data supported the Ch4S structure. The highest M_n and M_w values of synthetic Ch4S were 18,400 and 36,500, respective-ly. These values are to be compared with those of natural Ch (∼4,600 and ∼6,800, respectively), and natural ChS–A (28,800 and 34,800, respectively, from whale cartilage). M_n of synthetic Ch4S corre-sponds to 72–74 saccharide units. The synthetic Ch4S provides with a desired ChS–A sample with-out contaminated units at a molecular level.

The Ch-oxa(CH$_3$) monomers with a sulfate group at the C6, and at both the C4 and C6 were not polymerized at all. These results suggest that HAase distinguished a sulfate group at the different position on the GAG chains. Synthetic Ch4S is expected to be a good medical material due to the defined uniform structure.
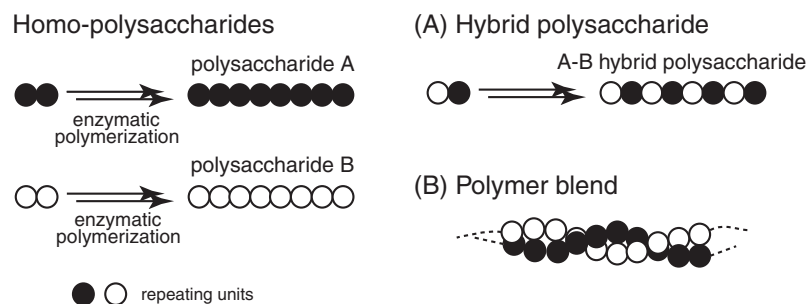
Fig. 15.   Model illustration of (A) a hybrid polysaccharide, and (B) a polymer blend from two homo-polysaccharides.
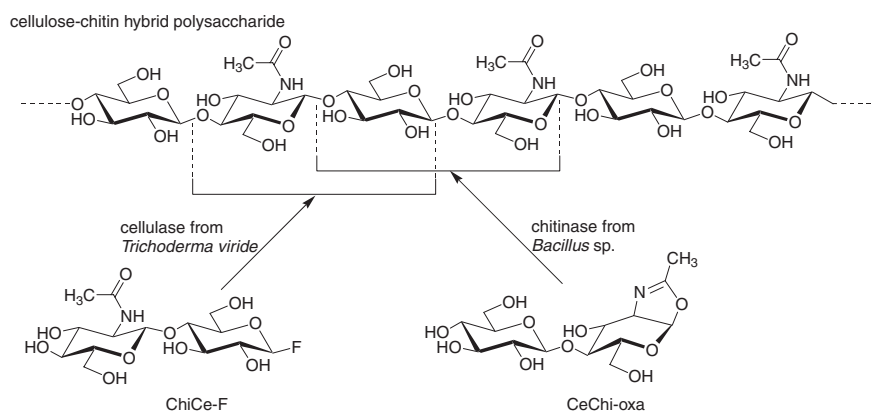

Fig. 16.   Two kinds of monomer designed for the synthesis of a cellulose–chitin hybrid polysaccharide.

Enzymatic polymerization to synthesize unnatural hybrid polysaccharides

All the above enzymatic polymerizations for the synthesis of natural and unnatural polysaccharides were achieved by a single step reaction. By utilizing this advantageous characteristics, new hybrid polysaccharides have been developed and attracted much attention as a novel class of high performance polysaccharides.[68] Hybrid polysaccharides are defined as polymers consisting of different kinds of repeating saccharide units in a single polymer chain; they are unnatural polysaccharides, completely distinguished from a polymer blend of two homo-polysaccharides (Fig. 15). Hybrid polysaccharides have potentials of displaying novel functions derived from the characteristic structures of intramolecular hybridization, in addition to those of natural homo-polysaccharides.

Cellulose–chitin hybrid polysaccharide.
This polysaccharide is a hybrid of cellulose, the most abundant in the plant world, and chitin, the
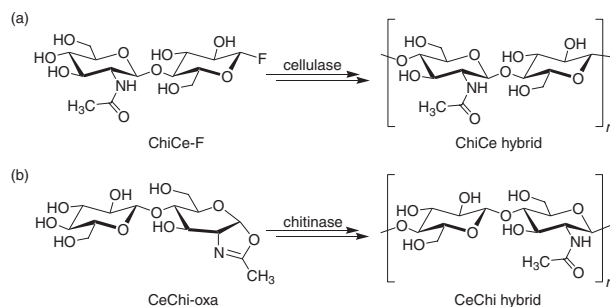
Scheme 15.   Enzymatic polymerizations to a cellulose–chitin hybrid polysaccharide.

most abundant in the animal world. On the basis of the TSAS concept, two kinds of candidate monomers were designed from the viewpoint of repeating structure of a cellulose–chitin hybrid polysaccharide, that is, a β-fluoride monomer of GlcNAcβ(1→4)Glc (ChiCe-F) and an oxazoline monomer of Glcβ(1→4)GlcNAc (CeChi-oxa) (Fig. 16). ChiCe-F is suitable as a TSAS monomer
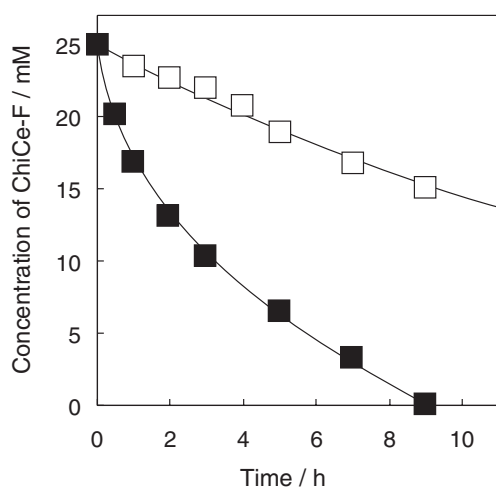
Fig. 17.　Reaction time-courses of monomer ChiCe-F with cellulase (■) and without enzyme (□). Reaction conditions: In acetonitrile–acetate buffer (50 mM, pH 5.0) mixture (5:1, v/v); amount of enzyme, 5 wt % for ChiCe-F; reaction temperature, 30 °C; initial concentration of ChiCe-F, 25 mM.
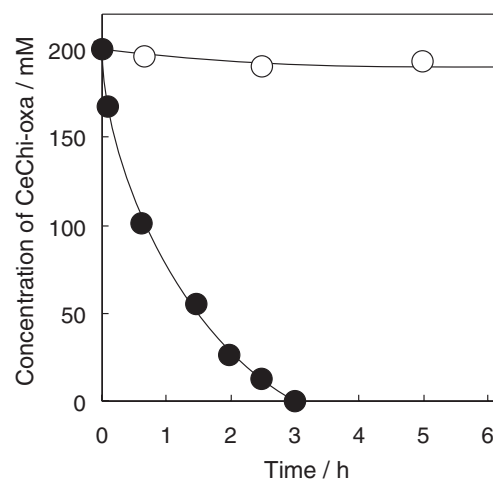
Fig. 18.　Reaction time-courses of CeChi-oxa with chitinase (●) and without enzyme (○). Reaction conditions: In a carbonate buffer (10 mM, pH 10.5); amount of enzyme, 5 wt % for CeChi-oxa; reaction temperature, 30 °C; initial concentration of CeChi-oxa, 200 mM.

Table 3.　Typical results of enzymatic polymerization of ChiCe-F and CeChi-oxa

	polymerization[a]				hybrid polymer		
enzyme	monomer	pH	initial conc./mM	time[b]/h	yield[c]/%	$M_n{}^d$	$M_w{}^d$
cellulase	ChiCe-F	5.0	25	9	55	2,820	3,070
cellulase	ChiCe-F	5.0	50	8	63	2,840	3,220
chitinase	CeChi-oxa	10.5	200	3	34	3,750	4,170
chitinase	CeChi-oxa	11.0	200	10	46	4,030	4,660

[a]In a CH$_3$CN-acetate buffer (50 mM) mixture (5:1, v/v) for cellulase-catalyzed polymerization, or in a carbonate buffer (10 mM) for chitinase-catalyzed polymerization; amount of enzyme, 5.0 wt % for the monomer; reaction at 30 °C. [b]Indicating the time for disappearance of corresponding monomer. [c]Isolated yields. [d]Determined by SEC using pullulan, cellooligosaccharides and chitooligosaccharides standards.

for polycondensation catalyzed by cellulase from *Trichoderma viride* to form β(1→4)-glucosidic linkage, and CeChi-oxa is acceptable as a TSAS monomer for ring-opening polyaddition induced by chitinase from *Bacillus* sp. (Scheme 15).[69]

　　The enzymatic polycondensation of monomer ChiCe-F took place successfully under similar reaction conditions to those in cellulose synthesis (Fig. 17). With enzymatic catalysis, ChiCe-F disappeared within 9 h, providing a cellulose–chitin hybrid polysaccharide (ChiCe hybrid) as a white precipitate. Without enzyme, ChiCe-F gradually decomposed without formation of any precipitate, which is a hydrolyzed product of ChiCe-F. Notably, the C2′ acetamido group in ChiCe-F is sterically

bulkier than the hydroxy group in β-CF, suggesting that the recognition of monomer by the enzyme is loose for the C2′ substituent and the catalysis is less interfered by that substituent. Molecular weight (M_n) of the ChiCe hybrid polysaccharide reached 2,800, which corresponds to 16 saccharide units. Interestingly, the enzyme produced the hybrid polysaccharide under various pH conditions ranging from 3.0 to 7.0 with M_n 2,600–2,800, corresponding to 15–16 saccharide units, in good yields (Table 3).[69] A mechanism for the polycondensation by cellulase catalysis is similarly considered as that of the cellulose synthesis; however, it is strange that monomer ChiCe-F with a bulky *N*-acetyl group at C2 was still recognized, activated, and glycosy-
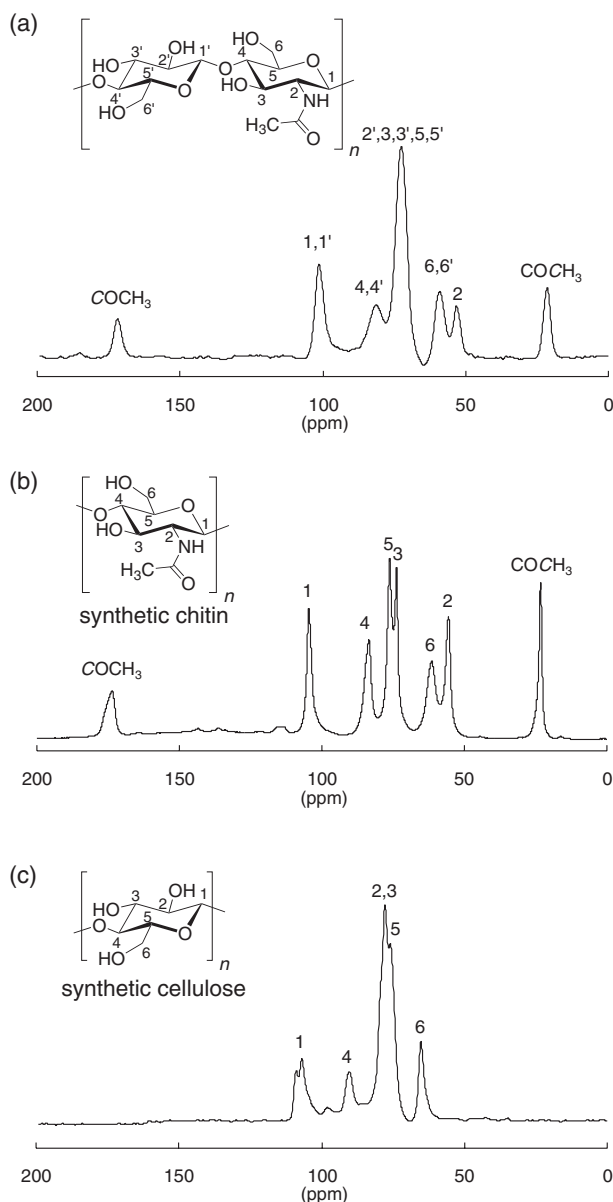
(a)

(b)

synthetic chitin

(c)

synthetic cellulose

Fig. 19. CP/MAS ^{13}C NMR spectra of (a) cellulose–chitin hybrid, (b) synthetic chitin, and (c) synthetic cellulose.

(a)

(b)

(c)

Fig. 20. X-ray diffractograms of (a) cellulose–chitin hybrid, (b) synthetic chitin, and (c) synthetic cellulose.

lated with liberating HF molecule, repeatedly, by the cellulase catalysis. A similar phenomenon was also observed for a C2 substituent.[45]

Monomer CeChi-oxa was smoothly polymerized via ring-opening polyaddition catalyzed by chitinase (Fig. 18). With the enzyme catalysis, CeChi-oxa disappeared completely within 3 h, and a white precipitate of CeChi hybrid polysaccharide was formed during the reaction. Without enzyme, the monomer gradually decomposed, and it re-

mained in 95% after 3 h without formation of a precipitate. M_n of the CeChi hybrid reached about 4,000, which corresponds to 22 saccharide units. This enzyme also catalyzed the polymerization under wide range of pH conditions from 6.0 to 11.0 with average M_n around 3,800 in good yields (Table 3).[69] The ring-opening polycondensation mechanism was explained analogously to that of the chitin synthesis.

Chemical structure of the cellulose–chitin hybrid polysaccharide was confirmed by CP/MAS ^{13}C

Fig. 21. Possible two monomers XyCe-F and CeXy-F for synthesis of a cellulose–xylan hybrid polysaccharide by a xylanase-catalyzed polymerization.

NMR spectrometry (Fig. 19a). Assignment of the signals was performed by comparing with those of synthetic chitin (Fig. 19b)[22] and synthetic cellulose (Fig. 19c).[28] Two significant signals derived from acetamido group of GlcNAc unit were found at $\delta 20$ (methyl) and $\delta 175$ (carbonyl) in Fig. 19a as well as in Fig. 19b. Furthermore, the C2 carbon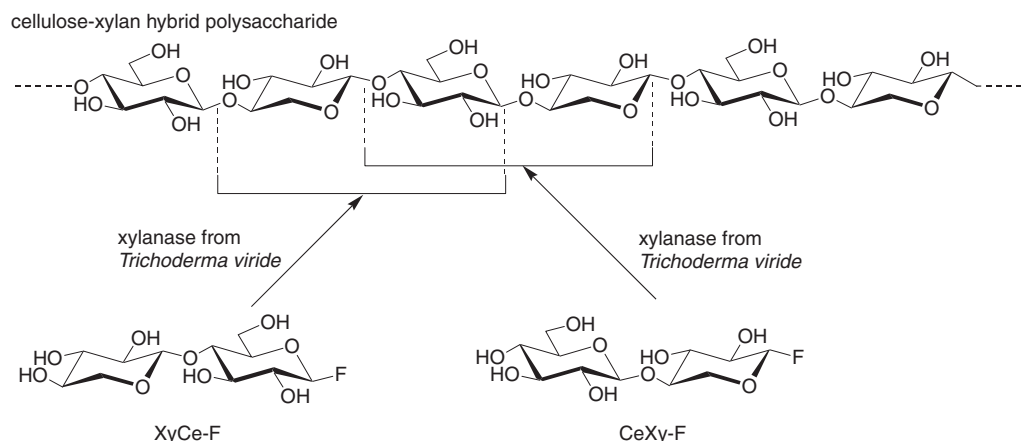 of GlcNAc was observed at $\delta 55$. Signals derived from Glc unit in Fig. 19a were also detected at similar δ values from synthetic cellulose depicted in Fig. 19c. Signals from the C4 and C4′ carbons were observed at around $\delta 81$, exhibiting that the product has the $\beta(1{\rightarrow}4)$-linked glycosidic bond exclusively.

MALDI-TOF mass spectrum of a low molecular weight part of the CeChi hybrid showed the peaks at every m/z of 365, which corresponds to the molecular mass of the repeating disaccharide unit. Thus, the structure of the hybrid was confirmed to be an alternatingly $\beta(1{\rightarrow}4)$-connected Glc and GlcNAc, a cellulose–chitin hybrid polysaccharide.

It is interesting that no significant peaks were observed by the X-Ray diffraction (XRD) of the CeChi hybrid polysaccharide (Fig. 20a). This is in contrast to that of synthetic chitin showing characteristic peaks of α-chitin ($2\theta = 9.3, 19.2, 23.4$ and 26.1), which forms antiparallel molecular organization (Fig. 20b), and to that of synthetic cellulose assembling in antiparallel molecular chain packing (cellulose II), which provides an XRD spectrum exhibiting sharp peaks at $2\theta = 12.2, 19.9,$ and 22.0 (Fig. 20c). These results clearly indicate that no crystals were formed from the hybrid polysaccharide. Both α-chitin and cellulose II have antiparallel

molecular chain organizations. However, the crystal structures of them have different dimensions of cell units, therefore, the hybrid polysaccharide is probably difficult to form either a crystalline structure or a higher-ordered assembly.[69]

Cellulose–xylan hybrid polysaccharide. Xylan has an interesting character of forming supramolecular interaction with cellulose, for example, adsorption onto cellulose fibrils during the pulping process. Therefore, a cellulose–xylan hybrid polysaccharide comprised of alternating $\beta(1{\rightarrow}4)$-glucose (Glc) and $\beta(1{\rightarrow}4)$-xylose (Xyl) is attractive as a biomacromolecule bearing cell wall functions within a single chain.[70]

For the synthesis of a cellulose–xylan hybrid polysaccharide, there are two kinds of glycosidic linkages; $\beta(1{\rightarrow}4)$-glucosidic linkage and $\beta(1{\rightarrow}4)$-xylosidic linkage (Fig. 21). Therefore, two monomers, Xyl$\beta(1{\rightarrow}4)$Glc β-fluoride (XyCe-F) and Glc$\beta(1{\rightarrow}4)$Xyl β-fluoride (CeXy-F), are designed for potential enzymes such as cellulase and xylanase.

Polycondensation of monomer XeCe-F was carried out in a 5:1 (v/v) mixture of acetonitrile–acetate buffer (pH 5.0) using a variety of enzymes as catalyst.[70] Interestingly, cellulase from *Trichoderma viride* exhibited little activity for the polycondensation, and the partially purified cellulase did not catalyze at all. These results indicate that the cellulase active for the synthesis of cellulose was difficult to catalyze polymerization of XyCe-F, suggesting that the enzyme required the methylol group (-CH$_2$OH) at the nonreducing end for dis-

playing the polymerization activity. In contrast, xylanase from *Trichoderma viride*, a hydrolysis enzyme of xylan, was effective for the polymerization, giving rise to a XyCe hybrid polysaccharide in a 58% yield as a white precipitate. MALDI-TOF mass spectrum of the peracetylated hybrid showed the peaks with equal distance of m/z 504, which corresponds to the molecular mass of the peracetylated disaccharide unit, the DP reaching 24. ^{13}C NMR analysis confirmed the structure of the XyCe hybrid having alternatingly connected $\beta(1{\to}4)$-Glc and $\beta(1{\to}4)$-Xyl units. Monomer CeXy-F was also polymerized by xylanase, affording the CeXy hybrid polysaccharide in good yields. The DP reached 18 by MALDI-TOF/MS analysis.

Chitin–xylan hybrid polysaccharide. Cellulose–chitin and cellulose–xylan hybrid polysaccharides were successfully prepared via enzymatic polymerization; however, these were insoluble in aqueous media, providing the products as precipitates. This is probably due to rigidity of a molecular chain resulting from similarity in component monosaccharide units leading to intramolecular hydrogen bonding, that is, Glc is different from GlcNAc only in the C2 substituent (hydroxy and acetamide, respectively) and from xylan in the presence or absence of the C6 methylol group. A chitin–xylan hybrid polysaccharide consisting of alternatingly connected $\beta(1{\to}4)$-GlcNAc and $\beta(1{\to}4)$-Xyl was anticipated to show good solubility in aqueous media; the monosaccharide units are structurally quite different in the C2 substituent (acetamide in GlcNAc and hydroxy in Xyl) and in the presence and absence of the C6 methylol group.[71]

Two kinds of monomer were desiged from the structural viewpoint of a chitin–xylan hybrid polysaccharide. Monomer XyChi-oxa bearing an oxazoline moiety is suitable for the catalysis of chitinase as a TSAS monomer to form $\beta(1{\to}4)$-N-acetylglucosaminide linkage (Scheme 16). Another monomer of a fluoride-type was for xylanase catalysis to produce $\beta(1{\to}4)$-xyloside linkage. However, highly toxic reagents such as organotin compounds are required to synthesize the fluoride-type monomer; therefore, monomer XyChi-oxa was employed for the synthesis of the XyChi hybrid. The monomer was successfully consumed and polymerized via ring-opening polyaddition, giving rise to the hybrid polysaccharide in good yields. The polymerization proceeded under broad range of pH conditions from 7.0 to 10.0; at pH 9.7 was produced the product hybrid in the highest yield of 76% within 4 h. M_n of the hybrid reached 1500 ($M_w = 2700$), which corresponds to 8–10 saccharide units. The chitin–xylan hybrid polysaccharide was a water-soluble $\beta(1{\to}4)$-polysaccharide.
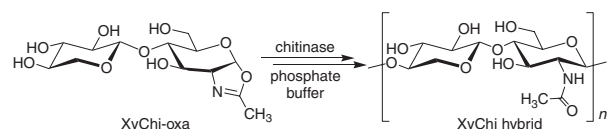
^{13}C NMR spectrum in D_2O confirmed the structure of the XyChi hybrid composed of $\beta(1{\to}4)$-GlcNAc and $\beta(1{\to}4)$-Xyl. MALDI-TOF/MS further confirmed the alternating structure, which showed the peaks with the equal distance of m/z 335 corresponding to the molecular mass of the repeating disaccharide unit of the XyChi hybrid (Fig. 22).[71] It is very interesting that higher molecular mass peaks were observed on the spectrum, indicating that the hybrid with such higher molecular weight (up to 10,000) was produced. This looked rather strange, because polysaccharides with such a higher molecular weight are normally very hard to be detected by MALDI-TOF method; it is probably due to the soluble nature of the hybrid polysaccharide, even with such a molecular weight.[71]

Chitin–chitosan hybrid polysaccharide. Chitosan is an N-deacetylated derivative of chitin, i.e., a polymer of $\beta(1{\to}4)$-linked D-glucosamine (GlcN) produced practically by alkaline hydrolysis of chitin; some fungi produce chitosan as a structural support. Since the biological activities of chitosan heavily depend on the extent of N-deacetylation, developing new synthetic methods of chitosan with well-defined structure is important to elucidate the relationship between a carbohydrate structure and its activities. Chitosan is soluble in acidic media such as acetic acid, in contrast to the insolubility of chitin in almost all solvents. Furthermore, 50% N-deacetylated chitin exhibits outstanding solubility in water. Therefore, a chitin–chitosan hybrid polysaccharide is anticipated soluble in water as a novel unnatural polysaccharide having both nature of chitin and chitosan.

Scheme 16.   Chitinase-catalyzed synthesis of a chitin–xylan hybrid polysaccharide.
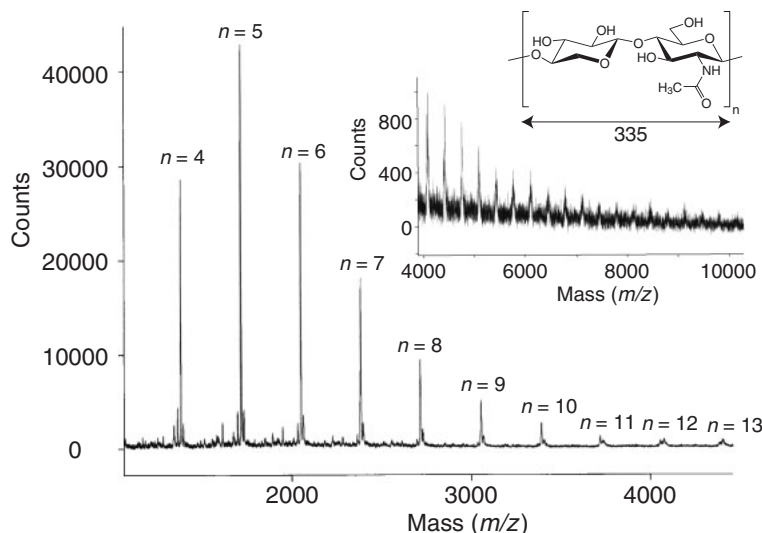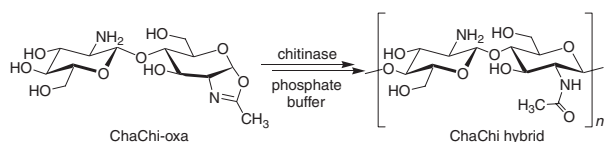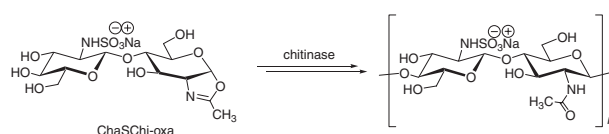
Fig. 22.   MALDI-TOF/MS mass spectrum of a XyChi hybrid obtained under positive ion mode. The inset shows the magnified spectrum in the higher molecular mass area.

Scheme 17.   Chitinase-catalyzed synthesis of a chitin–chitosan hybrid polysaccharide.

Scheme 18.   Chitinase-catalyzed synthesis of a chitin-*N*-sulfonated chitosan hybrid polysaccharide.

To achieve the synthesis, an oxazoline-type monomer (ChaChi-oxa) was designed as a TSAS monomer by chitinase (*Bacillus* sp.) catalysis. The monomer was polymerized via ring-opening poly-condensation manner to produce the desired hybrid polysaccharide having the structure of $\beta(1\rightarrow4)$-*N*-acetylglucosaminide linkage (Scheme 17).[72] The best result was obtained at pH 9.0 and 30 °C with 5 wt % chitinase from *Serratia marcescens* in terms of both the yield (75%) and molecular weights of the chitin-chitosan hybrid ($M_n = 2{,}020$, $M_w = 4{,}280$); the M_n value corresponds to 10–12 saccharide units.

Chitin-*N*-sulfonated chitosan hybrid poly-saccharide. An unnatural chitin-*N*-sulfonated chitin hybrid polysaccharide, i.e., an alternatingly *N*-sulfonated chitin derivative, was synthesized via ring-opening polyaddition of an *N*-sulfonated chitobiose oxazoline derivative (ChaSChi-oxa) catalyzed by chitinases from *Bacillus* sp. and *Serratia marcescens* (both family 18) (Scheme 18).[73] The product polymer is the first example of a hybrid of natural (chitin) and unnatural (*N*-sulfonated chitosan) polysaccharides. The polymerization proceeded in a phosphate buffer at 30 °C in a homogeneous system, giving rise to a water-soluble polysaccharide in good yields (up to 62%) under total control of regioselectivity and stereochemistry. The chemical structure of the hybrid polysaccharide has a repeating unit of GlcNS$\beta(1\rightarrow4)$GlcNAc connecting through $\beta(1\rightarrow4)$ glycosidic linkage. The M_n value reached 4,180, which correspond to 18–20 saccharide units.

From a viewpoint of monomer structure, the C-2′ substituent sulfonamido group in monomer ChaSChi-oxa is sterically much bulkier and strongly anionic, compared to that in other monomers employed for the chitinase-catalyzed polymerizations. Therefore, the results obtained here strongly imply that the C-2′ position in the monomer is not deeply involved in the catalysis of family 18 chitinases, indicating a potential to create chitin derivatives alternatingly having various C-2′ sub-

stituents. Furthermore, the product polymer was expected to become an important material for investigation of biological activities of *N*-sulfonate group in a chitin molecule at a molecular level.[73]

## Supercatalysis of hyaluronidase for enzymatic polymerization

During the course of our studies on enzymatic polymerization, we found a supercatalytic nature of HAase for the glycosaminoglycan (GAG) synthesis. HAase catalyzes a polymerization of a wide variety of monomers to give not only natural GAGs but many unnatural ones.[23),24),61),62),64),65),67),68),74)] These behaviors are the new findings of enzymatic catalysis, which is really beyond a "key and lock" theory.

Glycosaminoglycan hybrid synthesis. A typical example of supercatalysis is shown. Hybrid GAGs have been prepared for the first time via a single step copolymerization catalyzed by HAase. The reaction provided the first example of enzymatic copolymerization between monomers produc-

ing HA, Ch, and Ch4S homopolymers that have a different main-chain structure (Scheme 19).[75]

Two monomers HA-oxa and Ch-oxa were copolymerized in a regio- and stereoselective manner, giving rise to HA–Ch hybrid in good yields, where two comonomers showed a similar copolymerizability and the copolymerization proceeded homogeneously. The SEC profile of the reaction mixture showed a single molecular weight distribution, indicating that the copolymerization proceeded successfully to give a copolymer HA–Ch hybrid with M_n around several thousands without formation of homopolymers. ^{13}C NMR analysis showed that the hybrid has both repeating unit structures of HA and Ch.

HAase-catalyzed copolymerization of HA-oxa with Ch4S-oxa proceeded also successfully to produce an HA–Ch4S hybrid with M_n over 14,000 in good yields. Copolymer compositions of these hybrids were controllable by varying the comonomer feed ratio (Table 4).[75] Copolymerization proceeded homogeneously, where two monomers were consumed in a close consumption rate. The SEC profile of the reaction mixture showed a single molecular weight distribution, indicating that the copolymerization proceeded to give a copolymer of HA–Ch4S hybrid without formation of homopolymers. ^{13}C NMR analysis showed that the hybrid has both repeating unit structures of HA and Ch4S. Furthermore, these hybrids were effectively digested by the catalysis of *Streptomyces hyalurolyticus* hyaluronidase to verify the copolymer hybrid structures. Thus, these reactions demonstrated a novel and facile method for the production of various hybrid GAGs. This method will permit to easy access to intramolecular polysaccharides hybrid-
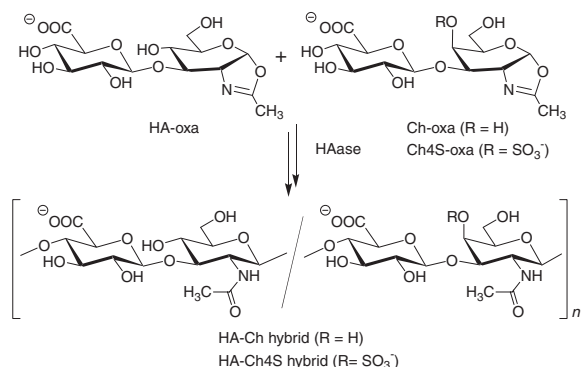
Scheme 19. HAase-catalyzed copolymerization to HA–Ch and HA–Ch4S hybrids.

Table 4. Enzymatic copolymerization of HA-oxa with Ch4S-oxa

entry	copolymerization[a]			copolymer (HA-Ch4S hybrid)				
	comonomer, feed ratio		time[b]/h	composition[c]		yield[d]/%	$M_n{}^e$	$M_w{}^e$
	HA-oxa : Ch4S-oxa			HA-oxa : Ch4S-oxa				
1	1	0	3.5	1	0	55	15,000	25,000
2	0.75	0.25	7.0	0.74	0.26	56	14,000	24,000
3	0.50	0.50	7.0	0.50	0.50	60	14,000	25,000
4	0.25	0.75	8.0	0.25	0.75	63	15,000	26,000
5	0	1	1.5	0	1	68	16,000	27,000

[a]In 50 mM phosphate buffer at pH 7.5; total monomer concentration, 0.10 M; enzyme, HAase (OTH, 3,720 units/mg), 10 wt% for total monomers; reaction at 30 °C. [b]Indicating the time for complete consumption of both monomers. [c]Determined by ^{1}H NMR analysis. [d]Isolated yields after purification. [e]Determined by SEC calibrated with hyaluronan standards.
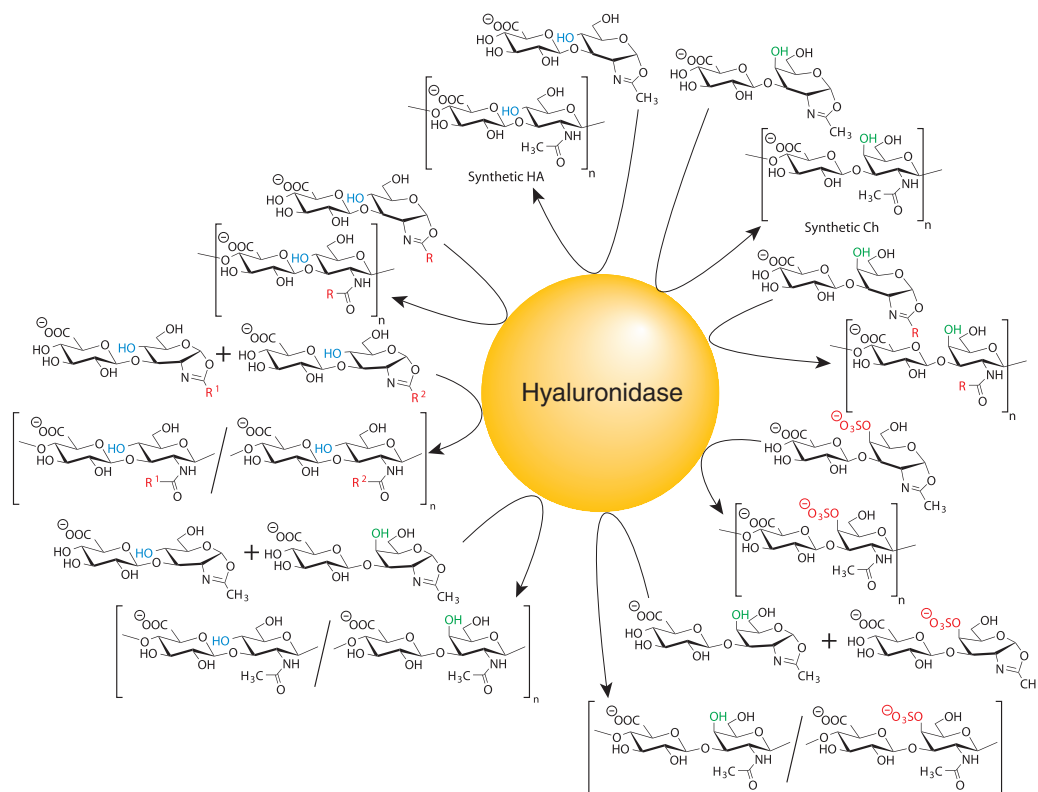
A Supercatalyst of Hyaluronidase

Fig. 23.   Illustration of hyaluronidase supercatalysis, showing the multi-catalyst functions for the enzymatic polymerization of various substrate monomers to lead to many natural and unnatural glycosaminoglycans.

ization. Such hybrid GAGs may serve as a novel class of tools for investigation of their functions in various scientific fields such as polymer chemistry, biochemistry, organic chemistry, medicine, pharmaceutics, and enzymology.

A supercatalyst of hyaluronidase. As observed above, a hyaluronidase (HAase) showed a wide spectrum of catalysis for enzymatic polymerization of a variety of substrate monomers with controlling stereochemistry and regioselectivity perfectly. This is not usual in view of enzymatic catalysis specificity where the key and lock relationship is normally observed. These behaviors of the multi-catalyst functions are far beyond the "key and lock" theory, therefore, the HAase is a *super-catalyst* for the enzymatic polymerization. This situation can be illustrated in Fig. 23.[74]

High-ordered molecular structure formation

One of the most advantageous characteristics of the enzymatic polymerization is that a product polysaccharide can be obtained directly by a single step reaction. It is due to the fact that a TSAS monomer is not protected, and hence, the product needs no de-protection step after the reaction as normally the cases for carbohydrate chemistry. This situation allowed to examine an *in-situ* molecular-level self-assembling of the product polysaccharide during the polymerization, by the direct observation of the on-going reaction system.

Self-assembling of synthetic cellulose. Cellulose forms typically two allomorphs of high-ordered molecular structure; cellulose I is of parallel molecular chain packing structure and thermodynamically metastable, and cellulose II is of antiparallel molecular chain packing structure and thermodynamically more stable. Surprisingly, nature normally forms cellulose I in spite of thermodynamically less favored allomorph, and hence, cellulose I was believed for a long time as an allomorph that only nature is able to form. Once cellulose I is converted to cellulose II, it never comes
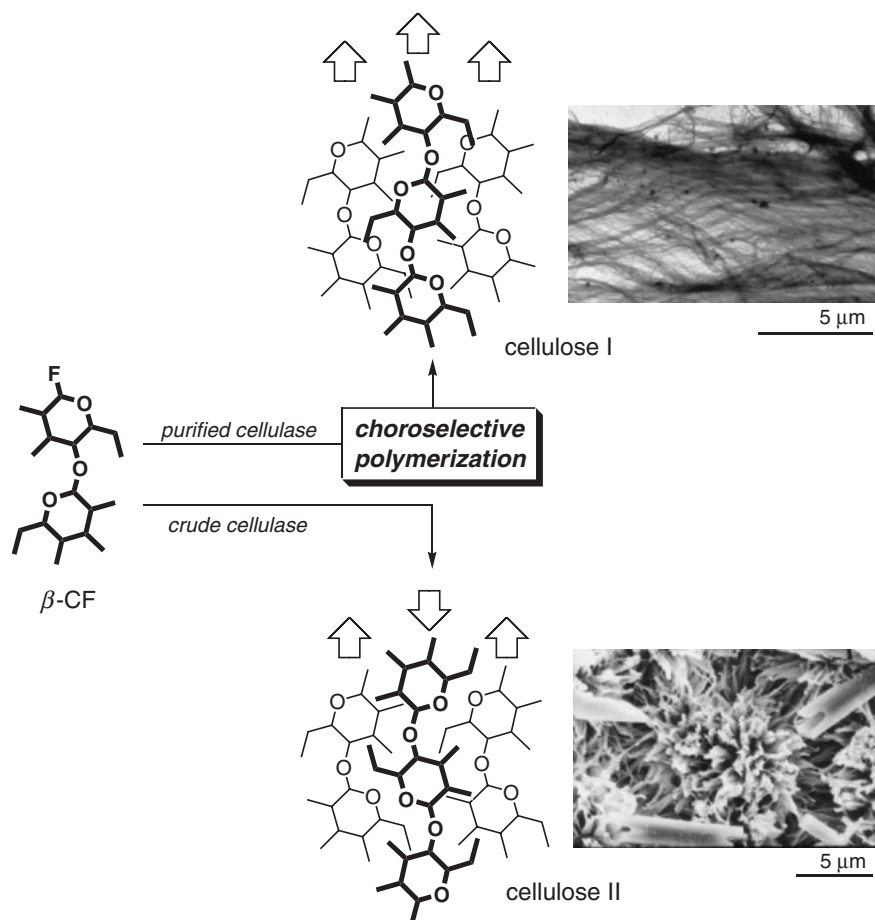
Fig. 24.   Typical two allomorph structures of cellulose I microfibril (parallel) via choroselective polymerization by TEM observation
and cellulose II spherulite (antiparallel) by SEM observation.

back to cellulose I. The *in vitro* formation of synthetic cellulose I allomorph, therefore, had been a dream for polymer scientists and biologists. Such a dream was at last realized during the course of collaboration with botanists by purification of the enzyme and also by varying the solvent composition. The polycondensation of β-cellobiosyl fluoride (β-CF) using a partially purified cellulase provided a microfibril of synthetic cellulose I, which was verified by the direct observation of the reaction by transmission electron microscopy (TEM) measurement.[76] On the other hand, the polycondensation using a crude cellulase mixture led to the formation of a synthetic cellulose II spherulite observed by scanning electron microscopy (SEM) (Fig. 24).[77]

These behaviors were explained as follows. A mixture of acetonitrile/buffer solvent and β-CF forms micelles, since β-CF is one of surfactants.

Cellulase enzymes locate on the micelle surface. From an active enzyme a cellulose molecule elongates and then macromolecular chains start to assemble to form higher-ordered structure. When cellulase is purified, a distance between active enzymes is close to each other to readily facilitate a parallel packing structure of elongating cellulose molecules, leading to cellulose I (Fig. 25A). For this type of high-order structure control, a novel concept "choroselective polymerization" (meaning; space-selective polymerization) was proposed (Fig. 24).[78] In contrast, with crude cellulase a distance between active enzymes is rather far because the content of the active enzyme was believed to be much less than 1% in the crude enzyme,[76] and then elongating molecules form a thermodynamically controlled stable cellulose II with an antiparallel packing structure (Fig. 25B).
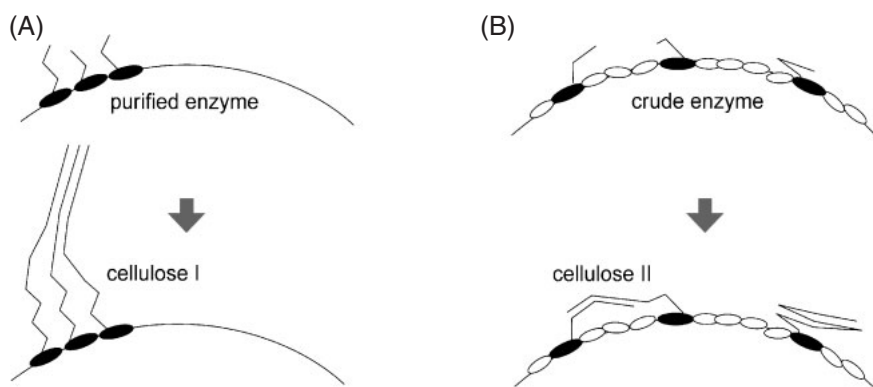
Fig. 25.   Postulated models for the formation of (A) synthetic cellulose I by a purified cellulase, and (B) synthetic cellulose II by a crude cellulase.

Recently, a detailed mechanism of self-assembling process of synthetic cellulose during enzymatic polymerization of β-CF has been revealed by collaboration with polymer physicists.[79]–[81] The investigation was made as one of general problems of chemical reactions at specific sites and reaction-induced self-assembling process of reaction products in the context of non-equilibrium phenomenon and pattern formation. The polymerization and self-assembling processes were explored at *real time* and *in-situ* by a combined small-angle scattering (SAS) method [combining small-angle neutron scattering (SANS), small-angle X-ray scattering (SAXS), ultra-SANS and ultra-SAXS], together with wide-angle X-ray scattering (WAXS) and field-emission scanning electron microscopy (FE-SEM).[81]

Typical SANS results are given; the SANS profiles with the progress of the polymerization reaction of β-CF catalyzed by cellulase exhibited the change in the scattered intensity expressed by characteristic power law behavior of $q^{-\alpha}$, which was estimated as q^{-4} before the polymerization and $q^{-3.7}$ after the polymerization (Fig. 26). These results suggest a smooth surface of the enzyme association before the polymerization and eventually a rough surface with a fractal structure after the polymerization.

According to the SANS results and the known information, the following pictures were proposed for the whole polymerization reaction as illustrated in Fig. 27.[81] Over the length scale ($r < 200$ nm) covered by the time-resolved SANS, the enzyme association has a flat, smooth surface with a sharp
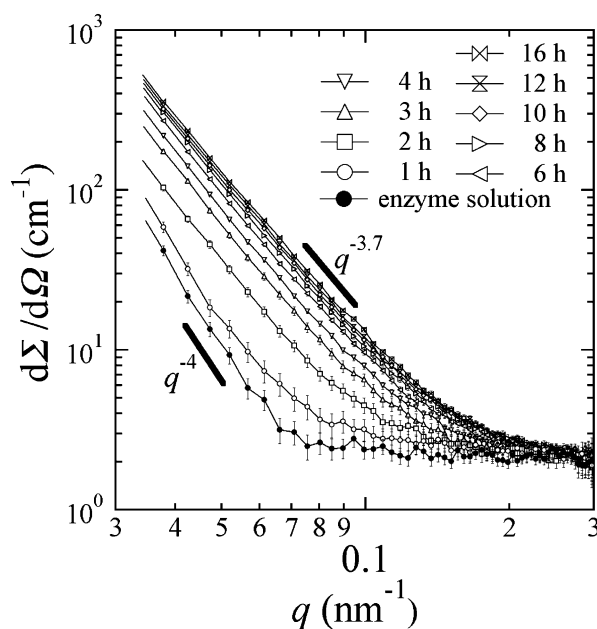

Fig. 26.   Time evolution of the SANS profiles during the enzymatic polymerization process for cellulose synthesis. The SANS profile from the enzyme solution is also included as a reference.

interface boundary against reaction medium as schematically illustrated in part (a). One can see an active enzyme molecule of $\sim$5 nm in diameter in (b). The active enzyme contains a special part of the so-called cleft[26] having $\sim$3 nm in length and $\sim$0.5 nm along the cross-sectional directions. The cleft is composed of two subsites, donor and acceptor sites, bordered at the active center as shown in part (c) where a substrate monomer is recognized and activated at the donor site for glycosylation
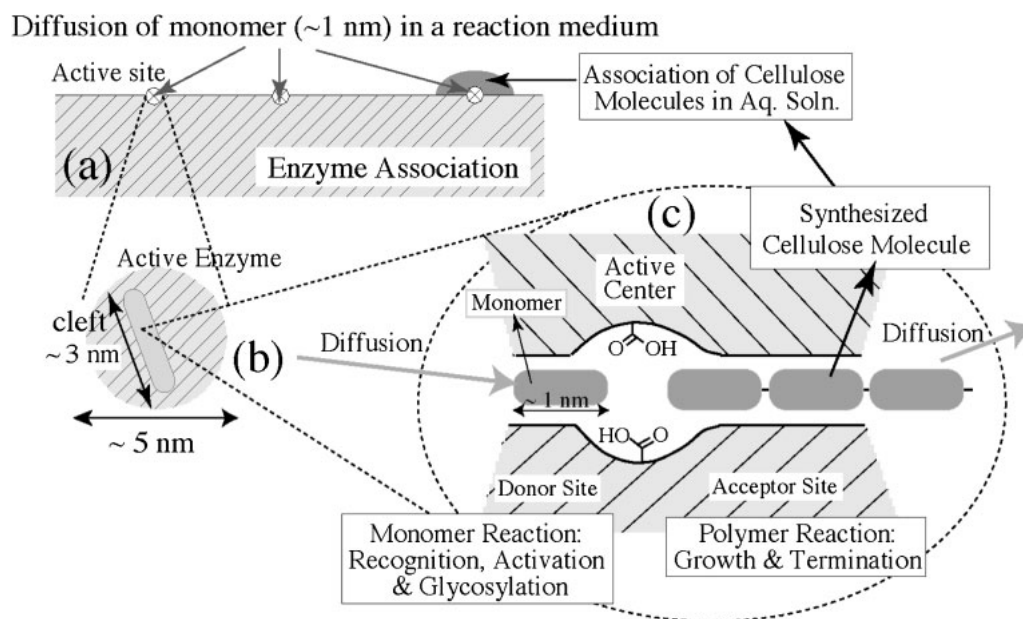
Fig. 27. Schematic illustrations of cellulose synthesis at a specific reaction field of enzyme associations to be observed by the combined SAS method.
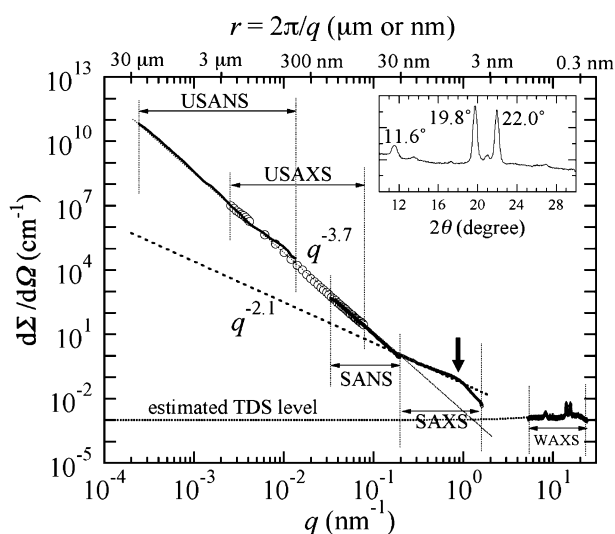

Fig. 28. Combined SAS profiles (USANS, USAXS, SANS, and SAXS) and WAXS profile obtained for the *in vitro* enzymatic polymerization system, which was reaction-terminated at 24 h after onset of the polymerization. Inset shows enlarged WAXS profile, where 2θ is Bragg angle.

Fig. 29. FE-SEM images for the self-assembled synthetic cellulose molecules obtained with three different magnifications; (a) × 1,000, (b) × 10,000, and (c) × 200,000.

reaction (via liberation of HF) and growing polymer chain is located at the acceptor site.

Fig. 28 shows that a combined SAS method together with WAXS method covers the wide q range as wide as 5 orders of magnitude. It was surprising that self-similarity of the surface roughness is extending from 30 nm (though the value being apparent one) to ~30 μm. Strikingly, there have been no reports that successfully observed the self-similar surface roughness extending over such a remarkably wide length scale. In the figure, the thermal diffuse scattering (TDS) was evaluated not to be contributing to the background scattering.

In order to confirm further the surface structures, FE-SEM observations were performed to show typical FE-SEM images for the synthetic cellulose, taken at three different magnifications (Fig. 29). Surprisingly, the self-similar rough sur-

face structure could also be clearly observed in the figures. The size of the largest and smallest surface structural elements are estimated to be about 30 μm and 30 nm from parts (a) and (c), respectively. The self-similar surface structure of the synthetic cellulose was thus successfully captured, that is reflected by a fractal structure.

The results strongly suggest the followings: β-CF is recognized and activated at the active site of the enzyme located on the micelle with a smooth interface. Then, a large number of cellulose molecules are produced on the enzyme association from each active site by the enzyme-catalyzed polycondensation, leading to the formation of the aggregated (self-assembled) structure with a rough surface (fractal dimension $D_s = 2.3$). The interior of the aggregates is suggested to have a branched (dendritic) structure from mass fractal dimension $D_m = 2.1$ (Fig. 30).[81]

All the results obtained are summarized as follows: (i) Even in the aqueous reaction medium free from monomers, enzymes (cellulase) as a catalyst aggregate themselves into associations with characteristic length more than 200 nm. (ii) Cellulose molecules created at each active site of enzymes associate via self-assembling around the enzyme associations and the cellulose aggregates thus formed are characterized by an object having surface fractal dimensions D_s, increasing from 2 (smooth surface) to 2.3 (rough surface with fractal structure) with reaction time. (iii) At the end of the reaction, the fractal structure extends over a surprisingly wide length scale ranging from ~30 nm to ~30 μm (3 orders of magnitude). This unique self-assembly of the reaction products is proposed to be caused by the following factors: (a) an extremely large number of polymers are repeatedly created at an active center of the catalyst in the enzyme association; (b) the polymers formed keep springing out from the narrow space of the active center in the catalyst toward the reaction medium, where (c) the polymers associate themselves into aggregates, because they are insoluble in the medium. In relevant to the above point (a), a total number of cellulose molecules N_{total} created per an active enzyme (defined by "total turnover number") during the whole reaction process was roughly estimated to be 2.8×10^5. This value well reflects the characteristic feature of high turnover number of the enzyme.[81] It is to be noted that this
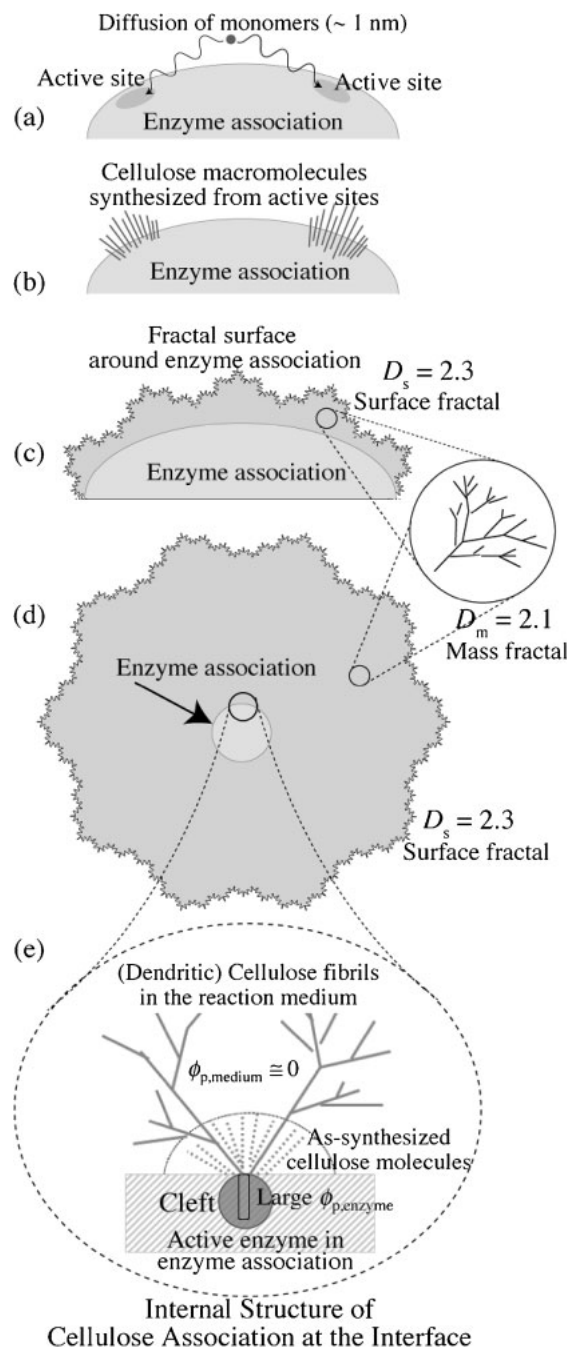
Fig. 30. Schematic models of self-assembling process of synthetic cellulose on the surface of enzyme associations. The weight ratios of the cellulose created per an active enzyme and per a crude enzyme in the final stage of polymerization are roughly estimated as 1.4×10^4 and 14, respectively. The cellulose aggregate surrounding the enzyme association has enough free space for diffusion of monomers from the reaction medium into the active sites and of terminated polymers from the active sites into the reaction medium.

Fig. 31. Electron micrographs of synthetic chitin spherulites: (A) a bundlelike assembly of α-chitin ribbons by TEM observation after 3 h incubation, and (B) a dendritic assembly of α-chitin ribbons by SEM observation after 25 h incubation.
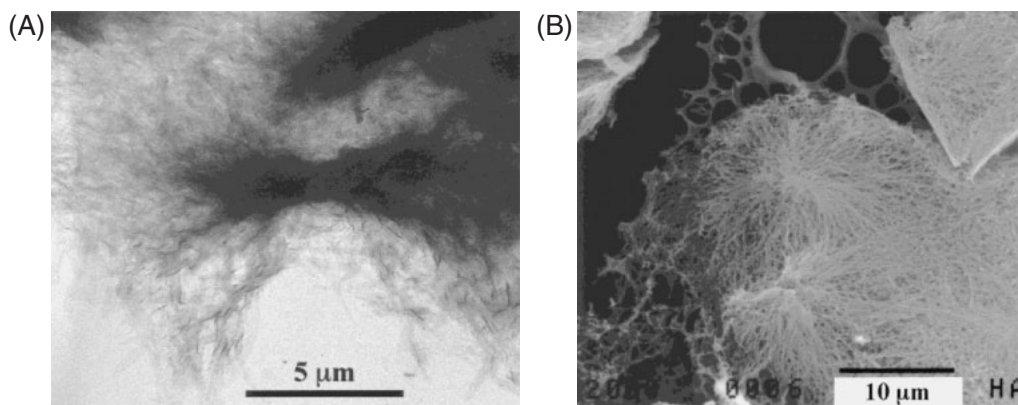
type of approach to investigate the system under-going *in vitro* enzymatic synthesis of cellulose as a model system by using the combined SAS method together with real-space analyses will be applicable to many open nonequilibrium systems in nature, particularly in biological systems.

Self-assembling of synthetic chitin. A polymerization system of the ring-opening polyaddition of oxazoline monomer (ChiNAc-oxa) to produce synthetic chitin was directly observed by using optical microscopy in combination with TEM and SEM techniques.[82] Platelike single crystals of α-chitin were first formed, which have the stable antiparallel chain packing structure. The crystals were gradually shaped into ribbons by the rapid growth along with the α axis with the crystalline thickness around 10 nm. The α-chitin ribbons then aggregated to form bundlelike assemblies (A) or dendritic assemblies (B) as the ribbon concentration in solution increased as shown in Fig. 31. They further grew into spherulites by spraying and branching, which displayed birefringence with a Maltese cross by polarization microscopic observation. This synthetic chitin spherulite, in which a number of α-chitin ribbons radiated from a common center, is different from the helicoidal textures composed of α-chitin microfibrils which were already known as a typical three dimensional organization of chitin.

It should be noted that the above TEM, SEM and a combined SAS studies on the direct observation of the *in situ* polymerization reaction are a completely new approach in polymer science. These studies are outcomes of the typical cooperative

works between scientists of chemistry, physics and biology fields, which allows the elucidation of not only a chemical reaction mechanism at a nano-sized level but also a mechanism of high-ordered structure formation from a nano-sized to micron-sized level.

Conclusion

This review has focused on the polysaccharide synthesis via enzymatic polymerization, which we mainly developed within these two decades. The polymerization using a hydrolysis enzyme as catalyst and a designed monomer based on the new concept of a transition-state analogue substrate (TSAS) enabled the first chemical synthesis of several important natural polysaccharides like cellulose and chitin, and also new unnatural polysaccharides. All these polysaccharides had been difficult to synthesize by the conventional methods. Stereochemistry and regioselectivity were perfectly controlled during the polymerization to give synthetic polysaccharides with a well-defined structure. Polysaccharides synthesized such as hyaluronan (HA), chondroitin (Ch), and chondroitin 4-sulfate (Ch4S) belong to polymers having one of the most complicated structure ever prepared via *in vitro* test tube reactions. Some of synthetic polysaccharides were functionalized by introducing a reactive group, e.g., acryloylated HA and Ch were readily obtained. Various unnatural hybrid polysaccharides, typically a cellulose-chitin hybrid and an HA/Ch hybrid, have been created by finding a new catalyst function for the designed monomers. Supercatalysis of hyaluronidase (HAase) was disclosed as showing an unusually wide spectrum of

enzymatic catalysis. The enzymatic polymerizations to synthetic cellulose and synthetic chitin provided with fundamental information on biosynthesis mechanism, which much contributed to a basic science. The *in situ* direct observations of the enzymatic polymerization by electron microscopy and small-angle scattering methods revealed mechanisms concerning the polymerization reaction as well as the high-order molecular structure formation by the elongating polymer molecules. Interestingly, the surface of synthetic cellulose aggregates showed a fractal structure over a wide length scale in 3 orders of magnitude.

The above synthesis techniques and related information can be further applied for developing and designing new materials like telechelics, macromonomers, end-functionalized polymers, polymer gels, bio-active materials and polymeric drugs derived from these poly- and oligosaccharides. In addition, mutation of enzyme is a new technique to modify the catalyst function and the mutant was very useful for mechanistic studies, which is an important future direction. Furthermore, a recent paper showed that enzymatic catalysis bridges the study on the *in vivo* biosynthesis of natural biomacromolecules and that on the *in vitro* polymerization for synthesis of such polymers,[83] suggesting also a future direction of research.

References

1) Kobayashi, S., Shoda, S. and Uyama, H. (1995) Enzymatic polymerization and oligomerization. Adv. Polym. Sci. **121**, 1–30.

2) Kobayashi, S., Shoda, S. and Uyama, H. (1997) Enzymatic catalysis. *In* Catalysis in Precision Polymerization (ed. Kobayashi, S.). John Wiley & Sons, Chichester, England, Chap. 8.

3) Kobayashi, S. (2005) Highlight: Challenge of synthetic cellulose. J. Polym. Sci. Part A: Polym. Chem. **43**, 693–710.

4) Atherton, E. and Sheppard, R.C. (eds.) (1989) Solid-Phase Peptide Synthesis: A Practical Approach. Oxford Univ. Press, Oxford.

5) Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (eds.) (2002) Manipulating Proteins, DNA, and RNA. *In* Molecular Biology of the Cell. 4th edition, Garland Science, New York.

6) Kobayashi, S. and Ohmae, M. (2006) Enzymatic polymerization to polysaccharides. Adv. Polym. Sci. **194**, 159–210.

7) Kobayashi, S. (1999) Enzymatic polymerization: A new method of polymer synthesis. J. Polym. Sci. Part A: Polym. Chem. **37**, 3041–3056.

8) Kobayashi, S., Uyama, H. and Ohmae, M. (2001) Accounts: Enzymatic polymerization for precision polymer synthesis. Bull. Chem. Soc. Jpn. **74**, 613–635.

9) Kobayashi, S., Uyama, H. and Kimura, S. (2001) Enzymatic polymerization. Chem. Rev. **101**, 3793–3818.

10) Kobayashi, S., Sakamoto, J. and Kimura, S. (2001) *In vitro* synthesis of cellulose and related polysaccharides. Prog. Polym. Sci. **26**, 1525–1560.

11) Kobayashi, S. and Uyama, H. (2002) Enzymatic polymerization. *In* Encyclopedia of Polymer Science and Technology. 3rd ed. (ed. Kroschwitz, J.I.). Wiley, New York (website).

12) Kobayashi, S. and Ohmae, M. (2007) Polymer synthesis and modification by enzymatic catalysis. *In* Macromolecular Engineering: Precise Synthesis, Materials Properties, Applications (eds. Matyjaszewski, M., Gnanou, Y. and Leibler, L.). Wiley-VCH, Weinheim, pp. 400–477.

13) Kobayashi, S. and Uyama, H. (2003) Biomacromolecules and bio-related macromolecules. Macromol. Chem. Phys. **204**, 235–256.

14) Taniguchi, N., Honke, K. and Fukuda, M. (eds.) (2002) Handbook of Glycosyltransferases and Related Genes. Springer, Tokyo.

15) Schmidt, R.R. (1986) New methods for the synthesis of glycosides and oligosaccharides — Are there alternatives to Koenigs-Knorr method? Angew. Chem. Int. Ed. Engl. **25**, 212–235.

16) Schmidt, R.R. and Michel, J. (1980) Angew. Chem. Int. Ed. Engl. **19**, 731–732.

17) Mukaiyama, T., Murai, Y. and Shoda, S. (1981) Chem. Lett., 431–432.

18) Fischer, E. (1894) Ber. **27**, 2985–2993.

19) Pauling, L. (1946) Chem. Eng. News **24**, 1375.

20) Kollman, P.A., Kuhn, B., Donini, O., Perakyla, M., Santon, R. and Bakowies, D. (2001) Acc. Chem. Res. **34**, 72–79.

21) Borman, S. (2004) Chem. Eng. News **23** (Febru-

ary), 35.

22) Kobayashi, S., Kiyosada, T. and Shoda, S. (1996) J. Am. Chem. Soc. **118**, 13113–13114.

23) Kobayashi, S., Morii, H., Itoh, R., Kimura, S. and Ohmae, M. (2001) J. Am. Chem. Soc. **123**, 11825–11826.

24) Kobayashi, S., Fujikawa, S. and Ohmae, M. (2003) J. Am. Chem. Soc. **125**, 14357–14369.

25) Crout, D.H.G. and Vic, G. (1998) Curr. Opin. Chem. Biol. **2**, 98–111.

26) Davies, G. and Henrissat, B. (1995) Structure **3**, 853–859.

27) Mark, H. (1980) Cellul. Chem. Technol. **14**, 569–581.

28) Kobayashi, S., Kashiwa, K., Kawasaki, T. and Shoda, S. (1991) J. Am. Chem. Soc. **113**, 3079–3084.

29) Schlubach, H.H. and Luehrs, L. (1941) Liebigs Ann. Chem. **547**, 73–85.

30) Husemann, E. and Mueller, G.J.M. (1966) Makromol. Chem. **91**, 212–230.

31) Kochtkov, N.K. (1987) Synthesis of polysaccharides with a regular structure. Tetrahedron **43**, 2389–2436.

32) Micheel, F., Brode, O.-E. and Reinking, K. (1974) Liebigs Ann. Chem. 124–136.

33) Uryu, T., Yamaguchi, C., Morikawa, K., Terui, K., Kanai, T. and Matsuzaki, K. (1985) Macromolecules **18**, 599–605.

34) Nakatsubo, F., Takano, T., Kawada, T. and Marakami, K. (1989) *In* CELLULOSE, Structural and Functional Aspects (eds. Kennedy, J.F., Phillips, G.O. and Williams, P.A.). Ellis Horwood, Sussex, pp. 201–206.

35) In the carbohydrate chemistry, a "polysaccharide" refers to a molecule with more than ten monosaccharide units: Kennedy, J.F. and White, C.A. (1979) Polysaccharides. *In* Comprehensive Organic Chemistry (eds. Barton, D., Ollis, W.D. and Haslam, E.). Pergamon Press, Oxford, vol. 5, pp. 755–815.

36) Uyama, H., Takeya, K. and Kobayashi, S. (1993) Proc. Jpn. Acad., Ser. B **69**, 203–207.

37) Namekawa, S., Uyama, H. and Kobayashi, S. (1998) Proc. Jpn. Acad., Ser. B **74**, 65–68.

38) Ikeda, R., Tsujimoto, T., Tanaka, H., Oyabu, H., Uyama, H. and Kobayashi, S. (2000) Proc. Jpn. Acad., Ser. B **76**, 155–160.

39) Samain, E., Lancelon-Pin, C., Ferigo, F., Moreau, V., Chanzy, H., Heyraud, A. and Driguez, H. (1995) Carbohydr. Res. **271**, 217–226.

40) Fort, S., Boyer, V., Greffe, L., Davies, G.J., Morez, O., Christiansen, L., Schuelein, M., Cottaz, S. and Bringuez, H. (2000) J. Am. Chem. Soc. **122**, 5429–5437.

41) Faijes, M., Imai, T., Bulone, V. and Planas, A. (2004) Biochem. J. **380**, 635–641.

42) Saxena, I.M., Brown, Jr. R.M., Fevre, M., Geremia, R.A. and Henrissat, B. (1995) J. Bacteriol. 1419–1424.

43) Okamoto, E., Kiyosada, T., Shoda, S. and Kobayashi, S. (1997) Cellulose **4**, 161–172.

44) Kobayashi, S., Kawasaki, T., Obata, K. and Shoda, S. (1993) Chem. Lett., 685–686.

45) Karthaus, O., Shoda, S., Takano, H., Obata, K. and Kobayashi, S. (1994) J. Chem. Soc. Perkin Trans. 1, 1851–1857.

46) Nakamura, I., Yoneda, H., Maeda, T., Makino, A., Ohmae, M., Sugiyama, J., Ueda, M., Kobayashi, S. and Kimura, S. (2005) Macromol. Biosci. **5**, 623–628.

47) Kobayashi, S., Shimada, J., Kashiwa, K. and Shoda, S. (1992) Macromolecules **25**, 3237–3241.

48) Ziegast, G. and Pfannemueller, B. (1987) Carbohydr. Res. **160**, 185–204.

49) Kobayashi, S., Wen, X. and Shoda, S. (1996) Macromolecules **29**, 2698–2700.

50) Mi, F.L., Shyu, S.S., Lin, Y.M., Wu, Y.B., Peng, C.K. and Tsai, Y.H. (2003) Biomaterials **24**, 5023–5036.

51) Sato, H., Mizutani, S., Tsuge, S., Ohtani, H., Aoi, K., Takasu, A., Okada, M., Kobayashi, S., Kiyosada, T. and Shoda, S. (1998) Anal. Chem. **70**, 7–12.

52) Tews, I., van Scheltinga, A.C.T., Perrakis, A., Wilson, K.S. and Dijkstra, B.W. (1997) J. Am. Chem. Soc. **119**, 7954–7959.

53) Kiyosada, T., (1998) PhD Thesis, Tohoku University.

54) Makino, A., Ohmae, M. and Kobayashi, S. (2006) Macromol. Biosci. **6**, 862–872.

55) Makino, A., Sakamoto, J., Ohmae, M. and Kobayashi, S. (2006) Chem. Lett. **35**, 160–161.

56) Ochiai, H., Ohmae, M. and Kobayashi, S. (2004) Chem. Lett. **33**, 694–695.

57) Sakamoto, J., Watanabe, T., Ariga, Y. and Kobayashi, S. (2001) Chem. Lett., 1180–1181.

58) Bernfield, M., Götte, M., Park, W.P., Reizes, O., Fitzgerald, M.L., Lincecum, J. and Zako, M. (1999) Functions of cell surface heparan sulfate proteoglycans. Ann. Rev. Biochem. **68**, 729–777.

59) Stern, R. and Jedrzejas, M.J. (2006) Hyaluronidases: their genomics, structures and mechanisms of action. Chem. Rev. **106**, 818–839.

60) Marković-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P.J., Müller, U. and Schirmer, T. (2000) Structure **8**, 1025–1035.

61) Kobayashi, S., Itoh, R., Morii, H., Fujikawa, S., Kimura, S. and Ohmae, M. (2003) J. Polym. Sci. Part A: Polym. Chem. **41**, 3541–3548.

62) Kobayashi, S., Ohmae, M., Fujikawa, S. and Ochiai, H. (2005) Macromol. Symp. **226**, 147–156.

63) Blatter, G. and Jaquinet, J.C. (1996) Carbohydr. Res. **288**, 109–125.

64) Ochiai, H., Mori, T., Ohmae, M. and Kobayashi, S. (2005) Biomacromolecules **6**, 1068–1084.

65) Ochiai, H., Ohmae, M., Mori, T. and Kobayashi, S. (2007) Biomacromolecules **8**, 1327–1332.

66) Maeda, N. and Noda, M. (1998) J. Cell. Biol. **142**, 203–216.

67) Fujikawa, S., Ohmae, M. and Kobayashi, S. (2005) Biomacromolecules **6**, 2935–2942.

68) Ohmae, M., Fujikawa, S., Ochiai, H. and

Kobayashi, S. (2006) J. Polym. Sci. Part A: Polym. Chem. **44**, 5014–5027.

69) Kobayashi, S., Makino, A., Matsumoto, H., Kunii, S., Ohmae, M., Kiyosada T., Makiguchi, K., Matsumoto, A., Horie, M. and Shoda, S. (2006) Biomacromolecules **7**, 1644–1656.

70) Fujita, M., Shoda, S. and Kobayashi, S. (1998) J. Am. Chem. Soc. **120**, 6411–6412.

71) Kobayashi, S., Makino, A., Tachibana, N. and Ohmae, M. (2006) Macromol. Rapid Commun. **27**, 781–786.

72) Makino, A., Kurosaki, K., Ohmae, M. and Kobayashi, S. (2006) Biomacromolecules **7**, 950–957.

73) Makino, A., Nagashima, H., Ohmae, M. and Kobayashi, S. (2007) Biomacromolecules **8**, 188–195.

74) Kobayashi, S., Ohmae, M., Ochiai, H. and Fujikawa, S. (2006) Chem. Eur. J. **12**, 5962–5971.

75) Ochiai, H., Fujikawa, S., Ohmae, M. and Kobayashi, S. (2007) Biomacromolecules **8**, 1802–1806.

76) Lee, J.H., Brown, R.M. Jr., Kuga, S., Shoda, S. and Kobayashi, S. (1994) Proc. Natl. Acad. Sci. USA **91**, 7425–7429.

77) Kobayashi, S., Hobson, L.J., Sakamoto, J., Kimura, S., Sugiyama, J., Imai, T. and Itoh, T. (2000) Biomacromolecules **1**, 168–173, 509.

78) Kobayashi, S., Okamoto, E., Wen, X. and Shoda, S. (1996) J. Macromol. Sci. Pure Appl. Chem. **A33**, 1375–1384.

79) Tanaka, H., Koizumi, S., Hashimoto, T., Kurosaki, K., Ohmae, M. and Kobayashi, S. (2006) Physica B **385**–**386**, 814–817.

80) Hashimoto, T., Tanaka, H., Koizumi, S., Kurosaki, K., Ohmae, M. and Kobayashi, S. (2006) Biomacromolecules **7**, 2479–2482.

81) Tanaka, H., Koizumi, S., Hashimoto, T., Kurosaki, K. and Kobayashi, S. (2007) Macromolecules **40**, 6304–6315.

82) Sakamoto, J., Sugiyama, J., Kimura, S., Imai, T., Itoh, T., Watanabe, T. and Kobayashi, S. (2000) Macromolecules **33**, 4155–4160, 4982.

83) Puskas, J.E., Gautriaud, E., Deffieux, A. and Kennedy, J.P. (2006) Natural rubber biosynthesis — A living carbocationic polymerization? Prog. Polym. Sci. **31**, 533–548.

Profile

Shiro Kobayashi was born in 1941. He graduated from Kyoto University, Faculty of Engineering, Department of Synthetic Chemistry in 1964 and started his research career in the graduate school supervised by Professor Junji Furukawa on polymer synthesis and by Professor Takeo Saegusa on organic reactions. After receiving PhD degree in 1969 from Kyoto University, he studied cationic reaction mechanisms for two years at Case Western Reserve University as a postdoctoral fellow with Professor George A. Olah. In 1972, he joined the same Department, Kyoto University as a research associate and restarted to study polymer synthesis. In the meantime, he stayed at Mainz University as a Humboldt fellow with Professor Helmut Ringsdorf in 1976. Following the lectureship in Kyoto University, he was appointed as a full professor of Tohoku University, Department of Applied Chemistry in 1986 and started studies on enzymatic polymerization, which opened a new field of polymer synthesis chemistry. Enzymatic polymerization is a green process and enabled for the first time the chemical synthesis of various natural and unnatural polysaccharides, functionalized polyesters and polymers from phenolic compounds. He moved to Kyoto University, Department of Materials Chemistry in 1997 and officially retired in 2005. Since then he has been a distinguished professor at Kyoto Institute of Technology. His research interests include polymer synthesis chemistry, organic reactions, and material science. His pioneering work on enzymatic polymerization is now well known worldwide. He was awarded the Award of the Chemical Society of Japan for Young Chemists (1976), the Award of the Society of Polymer Science Japan (1987), the Cellulose Society of Japan Award (1996), the Humboldt Research Award (1999), the Chemical Society of Japan Award (2001), the John Stauffer Distinguished Lecture Award (2002), the SPSJ Award for Outstanding Achievement in Polymer Science and Technology (2004), Medal with Purple Ribbon (2007), and others. He currently serves as a member of (executive) advisory board and editorial (advisory) board for fourteen international journals. Since 1999, he has been a foreign member of the Northrhine Westfalian Academy of Science, Germany.