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Alginates: Biology and Applications

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Preface

Alginates are well established as food additives and as encapsulation agents in biotechnology. Commercial production from harvested brown seaweeds commenced in the early twentieth century. Alginates belong to exopolysaccharides and are non-repeating copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G) which are linked by 1–4 glycosidic bonds. These comonomers can be arranged in blocks of continuous M-residues (M-blocks), G-residues (G-blocks) or alternating residues (MG-blocks). The comonomer composition and arrangement strongly impact on the alginate material properties, which range in nature from slimy and viscous solutions to pseudoplastic materials. Brown seaweeds and only the two Gram-negative bacterial genera *Azotobacter* and *Pseudomonas* are capable of alginate production. The bacterial alginates are characterized by acetylation of M-residues to a variable extent at positions O-2 and/or O-3. The degree of acetylation was also found to affect the material properties of the alginate. In *Pseudomonas aeruginosa*, for example, alginate is mainly composed of M-residues mediating viscous solution properties required for its function as a biofilm matrix polymer. However, in *Azotobacter vinelandii* a pseudoplastic alginate with a high G-residue content is produced when this bacterium forms a desiccation-resistant cyst under adverse environmental conditions. The mature cysts are surrounded by two capsulelike layers containing a high proportion of alginate to maintain structural integrity and resistance to desiccation. In brown algae alginate is produced as an intercellular gel matrix contributing to the mechanical stability of the plants.

Current knowledge of the genetics, gene regulation and biosynthesis of alginate is mainly based on extensive studies implementing the two bacterial species *A. vinelandii* and *P. aeruginosa*. Seminal work by Govan et al. and Chakrabarty et al. in the early 1980s kick-started research directed towards understanding the molecular mechanisms underlying alginate biosynthesis (Darzins and Chakrabarty 1984; Fyfe and Govan 1980; Ohman and Chakrabarty 1981). Interestingly, although the biosynthesis gene cluster was identified almost 30 years ago, the function of some genes essential for alginate biosynthesis has still not been assigned. The biosynthesis pathway leading to the formation of the activated alginate precursor GDP-mannuronic acid is well understood; however, the polymerization of GDP-mannuronic acid and the export of the resulting alginate are poorly understood. The enzymes (epimerase, transacetylase, lyase) which catalyse modification of the nascent

polymannuronate and hence strongly impact on alginate material properties have been studied in more detail. In particular, the epimerization process, i.e. the introduction of G-residues at the polymer level, is of great interest from an applied point of view. Alginate is currently used for a variety of industrial purposes, and the production was hitherto exclusively based on brown seaweeds. However, the possibility to engineer bacterial production strains capable of producing tailor-made alginates for medical applications especially has become increasingly attractive. This monograph provides an overview of the state of the art of alginate material properties, genetics and biosynthesis as well as applications of alginates and tailor-made alginates in medicine, food and biotechnology.

Palmerston North and Münster — Bernd H.A. Rehm
November 2008 — Alexander Steinbüchel

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Contents

Material Properties of Alginates

Ivan Donati and Sergio Paoletti

Contents

Abstract The present chapter deals with the description of the main characteristics of alginate as a material. Sources and the chemical structure of the polysaccharide are discussed. Particular attention is devoted to the definition of the comonomer sequence and its determination together with chemical and biochemical modifications of alginate. The stability of alginate molecules is discussed from the point of view of the effect on the polymer molecular weight. The characteristics of the polysaccharide in solution are tackled looking both at the equilibrium properties (thermodynamic

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properties) and at the nonequilibrium ones (viscosity in dilute and semidilute solution). The second part of this contribution is focused on the analyses of the ion-binding properties of alginate towards divalent ions. A general description of the "egg-box" model is provided together with a brief overview of recent findings on this topic. The mechanism of hydrogel formation and the description of the mechanical properties of alginate gels in small and large deformation ranges are addressed.

1 Sources and Applications

"Alginate" is a collective term for a family of polysaccharides produced by brown algae (Painter 1983) and bacteria (Gorin and Spencer 1966; Govan et al. 1981). Alginic acid was first discovered, extracted, and patented by Stanford (1881, 1883a, b). This polysaccharide was recognized as a structural component of marine brown algae (*Phaeophyceae*), where it constitutes up to 40% of the dry matter and occurs mainly in the intercellular mucilage and algal cell wall as an insoluble mixture of calcium, magnesium, potassium, and sodium salts (Haug and Smidsrød 1967). The presence of alginate provides the mechanical strength and flexibility of the seaweed and, additionally, acts as water reservoir preventing dehydration once part of the seaweed has been exposed to air. Alginate can thus be regarded as having the same morphophysiological properties in brown algae as those of cellulose and pectins in terrestrial plants.

Several bacteria, such as *Azotobacter vinelandii* and numerous species of *Pseudomonas*, produce an exocellular polymeric material which resembles alginate (Gorin and Spencer 1966; Linker and Jones 1966; Govan et al. 1981). In fact, alginate is an essential part of the protective cyst of dormant *Azotobacter* species and is the main constituent of biofilms of both *Azotobacter* and *Pseudomonas* (Campos et al. 1996), where it protects the bacterium from the host immune system. *Pseudomonas aeruginosa*, an opportunistic pathogen which colonizes the lungs of patients affected by cystic fibrosis, is able to secrete a viscous slime containing notable amounts of alginate (Pedersen et al. 1990). Secretion of alginate by *Pseudomonas syringae* has been reported to be associated with plant infection and colonization (Yu et al. 1999; Keith et al. 2003).

To date, all commercially available alginates have been extracted from brown algae, mainly from *Laminaria hyperborea*, *Macrocystis pyrifera*, *Laminaria digitata*, *Ascophyllum nodosum*, *Laminaria japonica*, *Ecklonia maxima*, *Lessonia nigrescens*, and *Durvillaea antarctica*. The composition of alginate in different seaweeds varies according to seasonal and growth conditions, as well as within different parts of the plant (Andresen et al. 1977; Indergaard and Skjåk-Bræk 1987). This variability in composition stems from the different requirements, in terms of mechanical properties, of the plants. For example, in *L. hyperborea*, which grows in very exposed coastal areas, very high mechanical rigidity is required in the stipe and in the holdfast, whereas high flexibility is needed in the leaves that float on streaming water.

The worldwide annual industrial production of alginate is estimated to be 30,000 metric tons, which is probably less than 10% of the biosynthesized material in crops of macroalgae. These figures allow us to consider such a polysaccharide as

an unlimited and renewable resource even for a steadily growing industry (Draget et al. 2005). In addition, alginate production by fermentation is also technically possible, although it does not meet the requirement of economic feasibility.

The main industrial applications of alginate as a natural polymeric material are linked to its stabilizing, viscosifying, and gelling properties and its ability to retain water. Alginate is largely used as a viscosifier in textile printing because of its shear-thinning characteristics. Its use has also been reported to increase color yield and brightness. In addition, various applications of alginate in the food industry are currently being exploited. The ability of this polysaccharide to stabilize aqueous mixtures, dispersions, and emulsions together with its gel-forming and viscosifying properties represent key features for the use of alginate in food applications. Several alginate-based restructured products (pet food, reformed meat, onion rings, crab-sticks, to name a few) are available on the market for large-scale distribution (Cottrell and Kovacs 1980; Littlecott 1982; Sime 1990). In addition, alginate is widely used as an additive for the production of low-sugar jam, jellies, and fruit fillings (Toft et al. 1986). In these applications, synergistic interactions with proteins and/or other polysaccharides are exploited.

Alginates well meet all the requirements for their use in pharmaceutical and medical applications. They have been largely used in wound dressings, dental impression, and formulations for preventing gastric reflux. However, the most advanced biotechnological and biomedical application of alginate resides in its use as a hydrogel for cell immobilization for applications ranging from production of ethanol from yeast cells and of antibiotics or steroids (Smidsrød and Skjåk-Bræk 1990) to transplantation and cell therapy (Lim and Sun 1980; Winn et al. 1991; Soon-Shiong et al. 1994; Hasse et al. 2000; Joki et al. 2001; Rokstad et al. 2002). In the latter case, alginate gel is used as a selective immune barrier to protect the transplanted cells from the host immune system.

There has been a notable increase in the number of alginate applications in recent years and the possibility of using such a polysaccharide for advanced biomedical therapies requires very detailed knowledge of its molecular characteristics. In fact, a clear understanding of the structure–function relationships is crucial for successful preparation of refined alginates for those applications where specific biochemical and physicochemical features have to be met. In addition, engineering of alginate molecules, by tailor-making their composition and properties or by introducing cell-specific signals, represents an important step forward for future novel applications in the biotechnology field.

2 Chemical Structure and Sequence

Alginate can be regarded as a paradigm of how strongly the macroscopic properties of the polymer depend on the subtle details of its chemical composition and on the ensuing physicochemical properties in well-defined environments. For this reason we decided to provide a critical overview of such structural aspects as a foundation for the behavior of alginate as a material.

2.1 Chemical Composition

Although the discovery of alginate can be traced back to the end of the nineteenth century, the complete elucidation of its chemical structure required several years. The presence of nitrogen in the polymeric structure of the polysaccharide, proposed by Stanford himself, was definitely ruled out by additional investigations, and the presence of uronic acids, specifically mannuronic acid, as a fundamental structural feature of the alginate backbone was found in 1926 (Atsuki and Tomoda 1926; Schimdt and Vocke 1926). The glycosidic bonds connecting C-1 of one mannuronic unit to C-4 of the following one were found to display a β configuration; hence, alginate was believed to be the brown seaweed counterpart of cellulose. However, it was later elucidated (Fisher and Dörfel 1955) that another uronic saccharide, i.e., α-L-guluronic acid (G), was constitutively present in the polysaccharide. This finding revealed the intrinsic complexity of the polysaccharide and boosted intense research on analytical methods for the characterization of the alginate chain from the point of view of the chemical composition. In fact, it soon became very clear that alginate displayed a marked chemical inhomogeneity and that the content of the two different uronic groups varied over a wide range depending on the algal source and the part of the plant considered. Moreover, Haug and coworkers, by means of partial acid hydrolysis and fractionation experiments, succeeded in revealing the block-copolymer nature of alginate. In particular, three fractions were isolated: two of these contained almost exclusively α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues, respectively, while the third one was composed of both uronic acids in almost equal proportion (Haug and Smidsrød 1965; Haug et al. 1966, 1967c).

As such, alginates do not possess a regular repeating unit. They can be described as linear binary copolymers of 1–4-linked M and G residues arranged in a blockwise pattern with homopolymeric regions of G residues (G-blocks) and a homopolymeric region of M sequences (M-blocks) interspersed by regions in which the two groups coexist in a strictly alternating sequence (MG-blocks) (Fig. 1) (Haug et al. 1966).

The saccharide units composing the alginate chain adopt different conformations, i.e., $^4C_1$ and $^1C_4$ in the case of mannuronic acid and guluronic acid, respectively. It follows that diequatorial linkages connect mannuronic acid residues in M-blocks which assume a flat ribbonlike chain conformation. In contrast, diaxially linked guluronic acid residues lead to a buckled and more rigid structure for the G-blocks. MG-blocks are characterized by alternating axial–equatorial and equatorial–axial glycosidic bonds connecting the residues; such variation in the "tacticity" of the bonds accounts for the greater flexibility of the MG-block with respect to the other sequences. As a matter of fact, the rigidity of the chain blocks was reported to decrease along the series GG>MM>MG (Smidsrød and Whittington 1969; Stokke et al. 1993). These results have been revisited recently (Vold et al. 2006) and the block components of natural alginates, when analyzed separately, seem to show essentially the same chain stiffness, irrespective of their chemical composition.

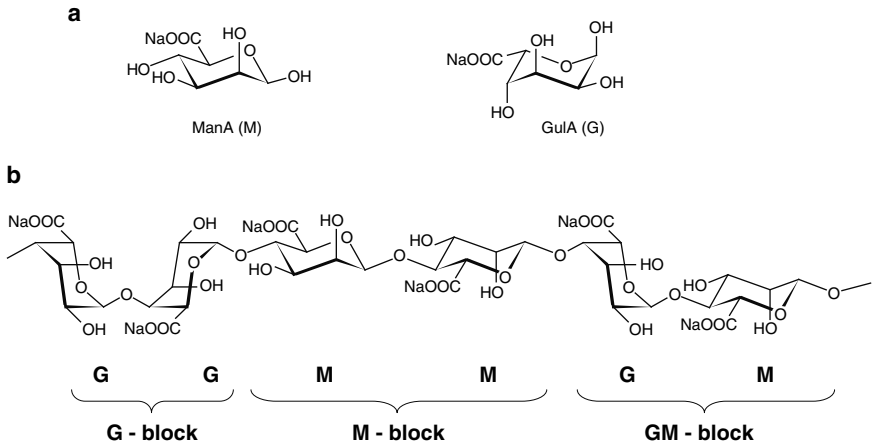

Fig. 1 Alginate chemical structure. **a** The $^{4}C_{1}$ conformation of β-D-mannuronic acid (M) sodium salt and the $^{1}C_{4}$ conformation of α-L-guluronic acid (G) sodium salt. **b** The block composition of alginate with G-blocks, M-blocks, and MG-blocks

2.2 Comonomer Sequence

2.2.1 Sequence Determination and Biological Distribution

The determination of the composition and sequential structure should be regarded as a mandatory task when dealing with alginate. This is because these two features are often key functional attributes which strongly affect the properties of the polysaccharide as well as its final performances both in biotechnological and food applications.

It is certainly tempting to tackle the problem of the (co)monomer distribution in alginate by use of the typical statistical approach developed for synthetic (co)polymers. However, it has been ascertained that the monomer distribution along the alginate chain cannot be described by using Bernoullian statistics (Painter et al. 1968; Smidsrød and Whittington 1969). Moreover, a second-order Markov model can only provide an approximate description of the monomer sequence in the polysaccharide (Larsen et al. 1970).

It is nowadays fully understood that the monomer distribution in alginates is the result of complex biochemical events, the correct identification and quantification of which require that comparatively long monomer sequences be known with extremely high accuracy. Certainly, the knowledge of the bare monomeric composition of alginate does not suffice to determine the sequential structure of alginate (Larsen et al. 1970).

A complete characterization of the polysaccharide, from the point of view of the composition, can be efficiently achieved by high-resolution proton and

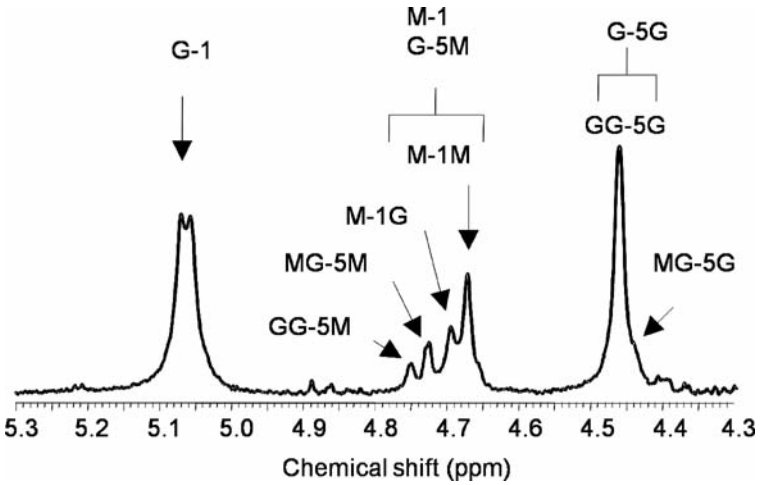

Fig. 2 ^{1}H-NMR spectrum (anomeric region) of an alginate isolated from *Laminaria hyperborea* ($F_G \sim 0.63$). *M-1M* and *M-1G* represent the anomeric proton of an M residue neighboring another M residue or a G residue, respectively. *MG-5M*, *GG-5M*, and *MG-5G* refer to the H-5 proton of the central G residue in an MGM, GGM, or MGG triad, respectively. *G-1* refers to the anomeric proton of G residues and *GG-5G* refers to the anomeric proton of G residues in G-blocks

carbon nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) (Grasdalen et al. 1977, 1979, 1981; Grasdalen 1983). An example of a typical ^{1}H-NMR spectrum of alginate is reported in Fig. 2. Through this technique, the monomeric (F_G, F_M), diadic (nearest neighbors F_{GG}, F_{GM}, F_{MG}, F_{MM}), and G-centered triadic (next-nearest neighbors F_{GGG}, F_{GGM}, F_{MGG}, F_{MGM}) fractions can be calculated. In particular, for long chains in which the end effects can be neglected, the following relations hold:

$$F_G + F_M = 1,$$

$$F_G = F_{GG} + F_{GM} = F_{GG} + F_{MG},$$

$$F_M = F_{MM} + F_{GM} = F_{MM} + F_{MG},$$

$$F_{MG} = F_{GM},$$

$$F_{GG} + F_{MM} + F_{GM} + F_{MG} = 1,$$

$$F_G = F_{GGG} + F_{GGM} + F_{MGG} + F_{MGM},$$

$$F_{GGM} = F_{MGG},$$

$$F_{MG} = F_{GM} = F_{GGM} + F_{MGM}.$$

From the relations above, the average length of G-blocks ($N_{G>1}$) can be evaluated as

$$\overline{N}_{G>1} = \frac{F_G - F_{MGM}}{F_{GGM}}.$$

It should be noted, however, that the ^{1}H-NMR analysis provides an evaluation of the block chain length averaged over the whole G-block population. It follows that this technique, although very powerful, cannot provide information on the distribution of the G-block lengths within the polysaccharide structure. Very recently, a new approach based on the use of specific alginate-degrading enzymes (alginate lyases) coupled with a range of analytical techniques (electrospray ionization mass spectrometry, high-performance anion-exchange chromatography with pulsed amperometric detection, and matrix-assisted laser desorption/ionization time of flight mass spectrometry) allowed these limitations to be overcome by assessing the real length of the block structures in alginates (Holtan et al. 2006).

The determination of the M-centered triads represents a more demanding issue since the ^{13}C-NMR spectrum of the polysaccharides is required. The lack of this information hampers the exact determination of the average length of the MG-blocks in alginates. However, an estimation can be accomplished by considering the ratio F_{MGM}/F_{GGM} for the different polysaccharide samples (Grasdalen et al. 1981).

A parallel and alternative characterization method exploiting circular dichroism (CD) has also been developed. It is based on the determination of the CD spectrum arising from each of the different sequential structures composing alginate, i.e., $[\theta]_{GG}(\lambda)$ for the G-blocks, $[\theta]_{MM}(\lambda)$ for the M-blocks, and $[\theta]_{MG+GM}(\lambda)$ for the alternating sequences. Once these have been determined by using (nearly) pure M-blocks, G-blocks, and MG-blocks, the CD spectrum of an alginate sample, $[\theta]_{alg}(\lambda)$, is considered as a linear combination of the three contributions above weighted for their fraction (Eq. 1) (Morris et al. 1980; Donati et al. 2003b). In such a way the diadic composition of alginate can be calculated using a nondestructive and very accessible technique, although it is less informative than NMR (Donati et al. 2003b).

$$[\theta]_{alg}(\lambda) = F_{GG}[\theta]_{GG}(\lambda) + F_{MM}[\theta]_{MM}((\lambda) + F_{GM+MG}[\theta]_{GM+MG}(\lambda)). \quad (1)$$

The composition and sequential structure of alginates from algal sources can vary over the range ($0.2 < F_G < 0.75$). In general, high contents of guluronic acid are found for alginates from *L. hyperborea* plants, while alginates isolated from *M. pyrifera*, *A. nodosum*, and *L. digitata* are characterized by a lower content of G-blocks (Smidsrød and Skjåk-Bræk 1990; Draget et al. 2005).

Alginates isolated from bacterial strains such as *Azotobacter* and *Pseudomonas* show more extreme and variable composition. While alginate obtained from the former bacterium is a real block copolymer, thus resembling the seaweed product,

alginates isolated from *Pseudomonas* species are characterized by the total lack of contiguous G residues. Even in alginates isolated from *P. aeruginosa* composed of almost 50% guluronic acid, the only G-containing sequence detected is the regular alternating structure (Sherbrock-Cox et al. 1984). This feature is also common to *P. fluorescens* and *P. putida*. An additional complication found in bacterial alginates is represented by the presence of *O*-acetyl groups, which invariably affect functional and material properties (ion binding and swelling) of the alginate itself (Skjåk-Bræk et al. 1986a; Ertesvåg et al. 1996).

Finally, an alginate composed exclusively of mannuronic acid (mannuronan) can be isolated from an epimerase-negative mutant (AlgG$^-$) of *P. fluorescens* (Gimmestad et al. 2003).

An overview of the composition of the seaweed and bacterial alginates is reported in Table 1.

2.2.2 Modification of Composition and Sequence

Alginates, both in seaweed and bacteria, are produced by a postpolymerization reaction of mannuronan which involves an inversion of the configuration at the C-5 position of the uronic acid without breaking of the glycosidic bond. It is not within the scope of this chapter to describe in detail those enzymes. It suffices to stress that seven epimerases were found to be encoded in the genome of the alginate-producing bacterium *A. vinelandii*. These enzymes, named "AlgE1" to "AlgE7," were sequenced, cloned, and overexpressed in *Escherichia coli* strains (Ertesvåg et al. 1994, 1996; Svanem et al. 1999). In such a way, after purification from the cells by sonication, centrifugation, filtration, and ion-exchange chromatography, large amounts of seven different epimerases were produced, allowing for compositional modifications of natural alginates. Each of these recombinant mannuronan C-5 epimerases generates specific nonrandom epimerization patterns, thus allowing the introduction of G residues into the alginate chains in different block structures. For example, the C-5 epimerase AlgE4 is able to convert homopolymeric M-blocks into strictly alternating copolymeric sequences, while the AlgE6 epimerase is a G-block-forming enzyme (Høidal et al. 1999; Hartmann et al. 2002a, b; Holtan et al. 2006). AlgE1 is characterized by the ability to introducing both MG-blocks and G-blocks into a mannuronan sample (Ertesvåg et al. 1998). The seven epimerases mentioned above also showed differences as to the substrate specificity. For example, AlgE5 has been shown to prefer MG-blocks as a substrate, while AlgE1 and AlgE6 prefer mannuronan or substrates containing G-blocks (Holtan et al. 2006). The *in vitro* use of such enzymes makes it possible to produce perfectly tailored alginate sequences not found in nature and opens up the possibility of controlling the length and distribution of blocks within the alginate chain, thus improving polysaccharide performances for several applications. In fact, it has been reported that mannuronan C-5 epimerases can lead to the

Table 1 Chemical composition of some algal and bacterial alginates determined by high-resolution ^{1}H-NMR spectroscopy

Alginate source	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,MG}$	F_{GGG}	F_{MGM}	$F_{GGM,MGG}$	$N_{G>1}$
Algal									
Laminaria hyperborea (stipe)	0.63	0.37	0.52	0.26	0.11	0.48	0.07	0.05	15
Laminaria hyperborea (leaf)	0.49	0.51	0.31	0.32	0.19	0.25	0.13	0.05	8
Macrocystis pyrifera	0.42	0.58	0.20	0.37	0.21	0.16	0.17	0.04	6
Laminaria digitata	0.41	0.59	0.25	0.43	0.16	0.20	0.11	0.05	6
Lessonia nigrescens	0.41	0.59	0.22	0.40	0.19	0.17	0.14	0.05	6
Ascophyllum nodosum	0.41	0.59	0.22	0.38	0.21	0.13	0.14	0.07	5
Laminaria japonica	0.35	0.65	0.18	0.48	0.17				
Durvillaea antarctica	0.32	0.68	0.16	0.51	0.17	0.11	0.12	0.05	4
Bacterial									
Azotobacter vinelandii	0.25–0.75	0.75–0.25	0.07–0.65						
Pseudomonas aeruginosa	0	1	0	1	0	0	0	0	
Pseudomonas fluorescens	0.40	0.60	0	0.2	0.4	0	0.4	0	

F_G denotes the fraction of alginate consisting of guluronic acid; F_{GG} and F_{GGG} indicate the fraction of alginate consisting of guluronic acid in blocks of dimers and trimers, respectively, whereas F_{MM} indicates the fraction of alginate consisting of mannuronic diads; $F_{GGM,MGG}$ indicates the fraction of alginate which starts or ends with a block of guluronic acid; $F_{GM,MG}$ indicates the fraction of alginate consisting of mixed sequences of guluronic and mannuronic acid, with F_{MGM} denoting the fraction of alginate consisting of two mannuronic acids interspaced with guluronic acid; $N_{G>1}$ denotes the average length of guluronic acid blocks as $N_{G>1} = (F_G - F_{MGM})/F_{GGM}$

production of alginates with enhanced gelling properties and stability (Skjåk-Bræk et al. 1986b; Draget et al. 2000).

This enzymatic approach to composition tailoring was found to be extremely valuable in understanding the role of the different sequences in determining the overall alginate properties. In fact, by use of the epimerase technology, alginate samples composed of (almost) exclusively one block sequence were produced (Fig.3, Table 2) (Donati et al. 2005b).

Recently, the combination of different epimerases allowed the design of engineered alginates where the block length and composition are finely tuned (Fig. 4, Table 2) (Mørch et al. 2007). In particular, knowledge of the mode of action and the specificity of the epimerases represents a fundamental tool for creating specific alginates with potentially any desired block composition. This novel and revolutionary approach can open up a completely new market for such a polysaccharide with outcomes covering advanced applications in the biomedical field.

2.3 Natural Substituents

O-Acetyl groups are invariably encountered in bacterial alginates and are the most characteristic feature distinguishing them from the algal ones. In alginates isolated from *A. vinelandii* and *P. aeruginosa* the acetyl groups are associated with the M residues and the ratio of the distribution between C-2 and C-3 is approximately 3:2, although some di-O-acetylated residues have been found (Table 3) (Skjåk-Bræk et al. 1986a). The role of *O*-acetyl groups in bacterial alginate is not completely understood. The presence of *O*-acetyl moieties has an inhibitory effect on mannuronan C-5 epimerization in *A. vinelandii* (Skjåk-Bræk et al. 1985) and on alginate lyase in some strains of *P. aeruginosa* (Linker and Evans 1984). It has been reported that acetyl substituents are necessary for the alginate isolated from *P. aeruginosa* to mediate cell aggregation into microcolonies in the early stages of biofilm development. In fact, an acetylation-defective mutant of such a bacterial strain was unable to form cell clusters and produced a thinner and more homogeneous biofilm (Tielen et al. 2005).

The presence of *O*-acetyl groups in bacterial alginates brings about some notable differences in the solution properties of the polysaccharide and it is believed to perturb the stereoregular sequences, inducing a conformational change (Delben et al. 1982; Skjåk-Bræk et al. 1989b). Theoretical consideration yielded an estimated dimension increase of 20% after acetylation (Stokke et al. 1993) owing to a restriction of the conformational freedom of the polymer chains probably due to a hindered rotation of the monomers. From viscosity investigations, chain expansions were detected for degrees of acetylation of up to 11% (Skjåk-Bræk et al. 1989b).

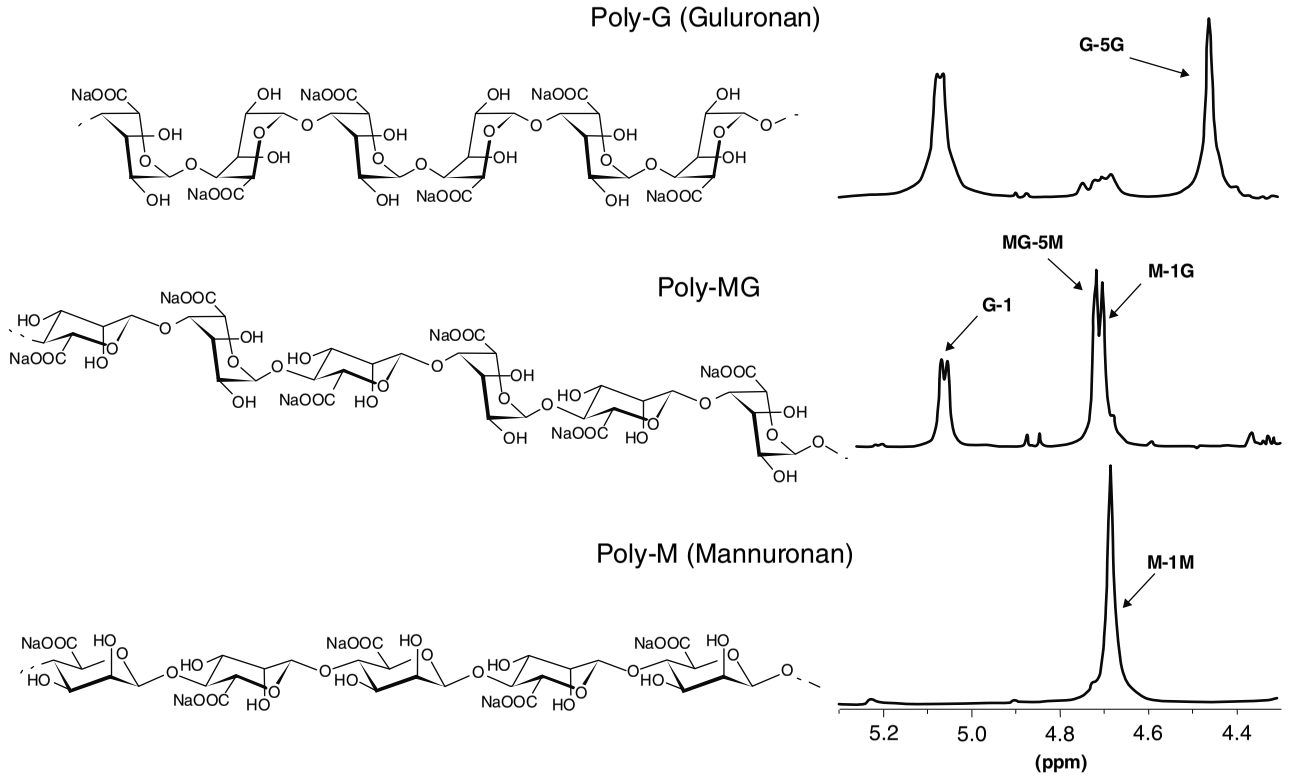

Fig. 3 Structure and ^{1}H-NMR (anomeric region) of polyG (guluronan), polyMG and polyM (mannuronan) (Table 2). See the legend to Fig. 2. (Adapted from Donati et al. 2005b)

Table 2 Chemical compositions of mannuronan and alginates obtained by means of AlgE epimerases

Sample	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,MG}$	F_{GGG}	F_{MGM}	$F_{GGM,MGG}$
PolyM (mannuronan)[a]	0	1	0	1	0	0	0	0
PolyG (guluronan)[a]	0.88	0.12	0.82	0.06	0.06	0.78	0.03	0.04
PolyMG (polyalternating)[a]	0.46	0.54	0	0.08	0.46	0	0.46	0
PolyMG + AlgE1[b]	0.48	0.52	0.05	0.10	0.43	0.04	0.41	0.01
PolyMG + AlgE1[b]	0.77	0.23	0.59	0.05	0.18	0.56	0.16	0.03
PolyMG + AlgE6[b]	0.55	0.45	0.18	0.08	0.37	0.15	0.35	0.03
PolyMG + AlgE6[b]	0.72	0.28	0.50	0.05	0.22	0.47	0.21	0.03

[a]Taken from Donati et al. (2005b)
[b]Taken from Mørch et al. (2007)

2.4 Alginate as a Polyelectrolyte

Apart from the compositional issues characterizing alginate samples, a common feature of mannuronic acid and guluronic acid residues is that they both bear a carboxylic moiety that, at neutral pH, renders alginate a polyanion. The presence of fixed charges on the polysaccharide results in a notable influence on its overall dimension in solution, which is strongly influenced by the presence of added supporting salt. It is well known the ionic strength (I) of the solution is correlated to the Debye length (k^{-1}), i.e., the distance at which the electrostatic interactions are screened out by the supporting salt, according to Eq. 2:

$$\kappa^{-1} \propto \frac{1}{\sqrt{I}}. \tag{2}$$

The vast majority of the physicochemical analyses on alginates have been performed in the presence of 0.1 M sodium chloride. However, properties such as viscosity, the radius of gyration, and persistence length depend on the ionic strength of the medium used. In particular, the following linear relationship was proposed between the intrinsic viscosity of alginate, as polyanion, and the ionic strength (Fig. 5) (Fixman 1964; Smidsrød and Haug 1971):

$$[\eta] = S\frac{1}{\sqrt{I}}, \tag{3}$$

with the slope of the curve, S, expressed as Eq. 4 (Fig. 5, inset) (Smidsrød and Haug 1971):

$$S = B\left([\eta]_{0.1}\right)^{\upsilon}, \tag{4}$$

where $[\eta]_{0.1}$ is the intrinsic viscosity of the polyelectrolyte at 0.1 M sodium chloride, while B is a parameter inversely dependent on the rigidity of the polymer chain. υ is

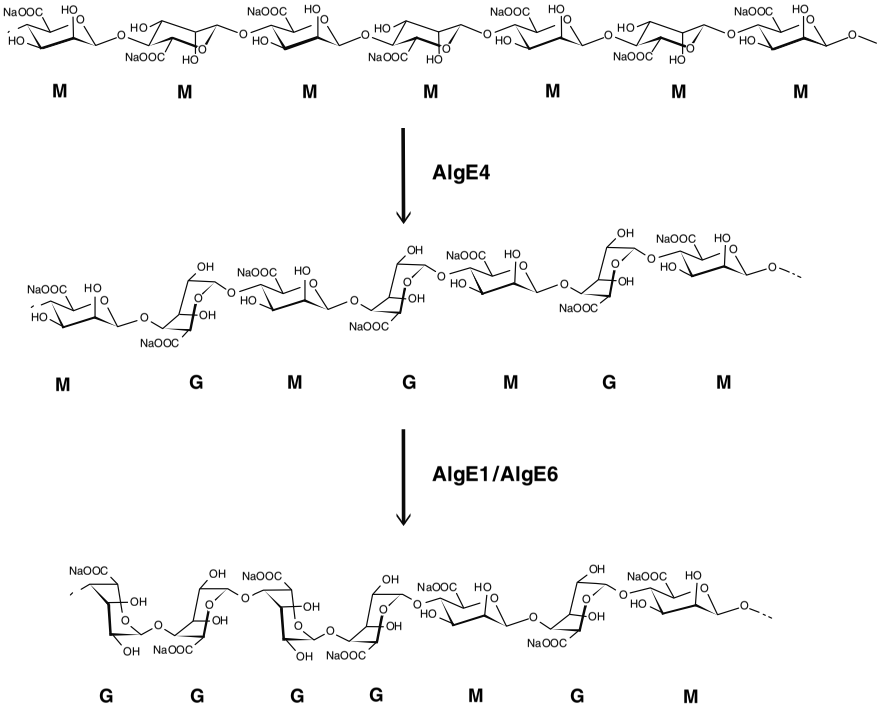

Fig. 4 An enzymatic process to tailor-make alginates. Mannuronan is first converted into polyalternating alginate by the epimerase AlgE4. The use of AlgE1 or AlgE6 in a second reaction step leads to the introduction of G-blocks along the polysaccharide chain. (Adapted from Mørch et al. 2007)

Table 3 Composition and acetylation pattern of some bacterial alginates

Source	F_G	F_M	F_{GG}	$F_{GM,MG}$	F_{MM}	Total (%)	C-2 (%)	C-3 (%)	C-2 and C-3 (%)
A. vinelandii	0.67	0.33	0.54	0.13	0.2	21	13	8	3
P. aeruginosa	0	1	0	0	1	37	23	14	5
P. aeruginosa[a]	0	1	0	0	1	57	34	23	11

Data taken from Skjåk-Bræk et al. (1986)
[a]After acetone reprecipitation

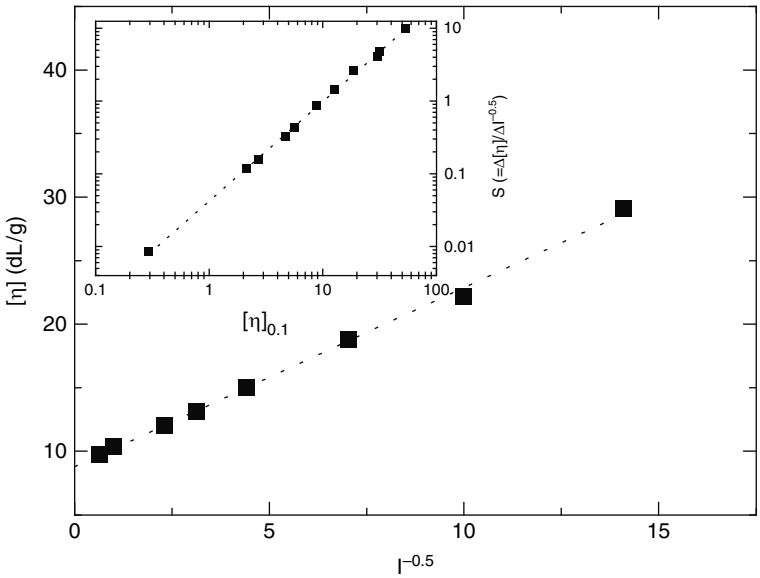

Fig. 5 Dependence of the intrinsic viscosity $[\eta]$ on the inverse of the square root of the ionic strength I for alginate. The *dotted line* represents the linear best fit of the experimental data. *Inset*: Dependence of the slope of the $[\eta]$-$I^{-0.5}$ plot on the intrinsic viscosity measured in the presence of 0.1 M NaCl, $[\eta]_{0.1}$. The *dotted line* represents the linear best fit of the experimental data. (Adapted from Smidsrød 1970; *inset* adapted from Smidsrød and Haug 1971)

usually assumed to be 1.3. Typically, B ranges from 0.04 to 0.05 in the case of alginates with high M content (Smidsrød and Haug 1971; Donati et al. 2004).

The rigidity of the alginate chain is given by its total persistence length, q_T, which is affected by the ionic strength used in the experimental determination. In the model of Odijk (Odijk and Houwaart 1978) q_T of wormlike polyelectrolyte chains is the sum of two contributions:

$$q_T = q_0 + q_E. \tag{5}$$

In this equation, q_0 is the intrinsic persistence length referring to the unperturbed dimensions of alginate chains. They are defined as the conditions in which

excluded-volume effects and nonideal short-range interactions are counterbalanced. The second term, q_E, is the contribution to the total persistence length due to the electrostatic interactions, which depend on the ionic strength; they are supposed to fade out at infinite ionic strength, neglecting any nonideal behavior of the supporting electrolyte. Typically, the intrinsic persistence length of alginate chains is around 12 nm, while the effect of the electrostatic interactions contributes, at an ionic strength of 0.17 M, an additional approximately 3 nm (Vold et al. 2006).

The interactions of counterions with the polyanion (modeled as a homogeneous linear distribution of charges) are theoretically well described by the classical counterion condensation theory (Manning 1969a, b, 1977, 1978). It predicts that some of the monovalent cations will condense into a volume tightly surrounding the chain if the average distance between the charges falls below 7.135 Å (in water at 298 K): it corresponds to a value of the dimensionless linear charge density of 1 (for details, see Sect. 4.1).

Looking at the molecular structure of alginate and considering lengths of 4.35 and 5.17 Å for guluronic acid and mannuronic acid repeating units, respectively, one can conclude that in both cases condensation of monovalent counterions is to be expected. This process conforms well to the theoretical prediction for an ideal system in which purely physical interactions hold.

In contrast, when divalent ions are considered, an additional "nonbonding" chemical affinity of the condensed doubly charged ion for the polyelectrolyte (corresponding to a free energy contribution $G^{0,\mathrm{aff}}$) was shown to be present; they bring in species-specific effects, albeit without violating the general assumption of the condensation theory. In particular, theoretical calculations based on calorimetric measurement revealed that the affinity of nongelling divalent ions, such as Mg^{2+}, decreased along the series G-block > MG-block > M-block, in parallel with the corresponding decreasing values of the linear charge density (Fig. 6) (Donati et al. 2006b).

To some extent of analogy with the theory of "real" solutions with respect to "ideal" solutions, it is possible to address to such a "real" polyelectrolyte model as one in which chemical identity is taken into account in addition to the fundamental long-range nonspecific electrostatic interactions. Still, in the "real" polyelectrolyte the fundamental assumption of the counterion condensation theory is not violated: namely, that the fixed charge on the polyelectrolyte is preserved even in the presence of a fraction of condensed counterions.

2.5 Conformational Properties of the Alginate Chain

The influence of alginate composition on the flexibility of the polysaccharide chain has been discussed and analyzed quite extensively over the years. It was first reported by Smidsrød (1973) that the extension of the polysaccharide chain was dependent on its composition, with the intrinsic flexibility of the blocks decreasing in the order MG > MM > GG. This experimental result, based on light-scattering determinations, was also supported by different computer-modeling studies performed using a Monte Carlo method (Smidsrød and Whittington 1969; Stokke et al. 1993). Overall,

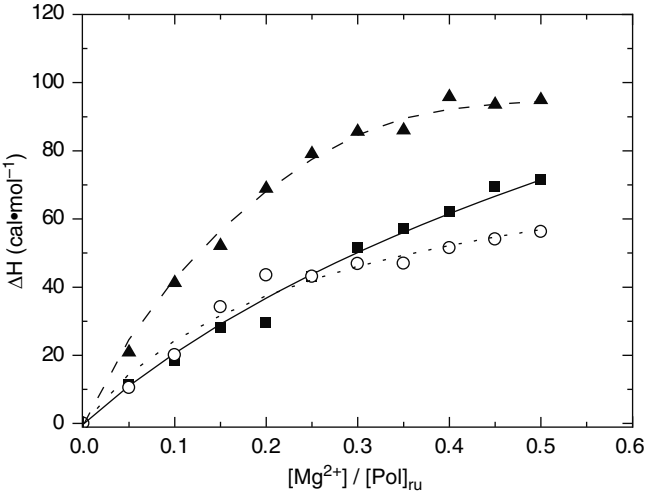

Fig. 6 Dependence on the ratio $[Mg^{2+}]/[Pol]_{ru}$ of the experimental (*symbols*) and theoretical (*lines*) values of the enthalpy of mixing (ΔH_{mix}) for polyG (guluronan, *triangles* and *discontinuous lines*), polyM (mannuronan, *squares* and *continuous line*) and polyMG (*circles* and *continuous dots*). The best fit between the experimental and theoretical curves was achieved by using an intrinsic free energy of affinity, $G^{0,aff}$, of −3.55 kcal·mol^{-1} for polyG, −1.18 kcal·mol^{-1} for polyMG, and −0.30 kcal·mol^{-1} for polyM. (Adapted from Donati et al. 2006b)

such analyses, despite some variability and numerical difference in the predicted chain extension, all pointed to a flexibility of the block components of alginate chain that decreases in the order reported above. These results were supported by the experimental determination of the empirical *B* value for alginates with different compositions. In fact, the stiffness parameter *B* of alginate from *M. pyrifera*, which contains relevant amounts of MG-blocks and M-blocks, was higher than that isolated from *L. hyperborea* stipe, characterized by a higher frequency of G-blocks. In addition, both values were lower than that measured for a MG-block-rich alginate (Smidsrød et al. 1973). Since a higher value of the *B* parameter is associated with higher flexibility, the conclusion drawn was that G-blocks were intrinsically stiffer than the other sequences composing alginate (Smidsrød et al. 1973). Additional evidence on the effect of alginate composition on polymer chain extension came from the evaluation of the persistence length determined by light-scattering methods. In fact, alginate with different compositions displayed a decreasing persistence length upon increasing the M-block and MG-block fractions. In particular, average persistence lengths of 15 and 13 nm were found for alginate from *L. hyperborea* and *A. nodosum*, respectively, while for an MG-block-rich alginate, obtained from the acid-soluble fraction, a value as low as 9.5 nm was observed under the same conditions (Smidsrød et al. 1973). Gamini et al. (1992) compared two alginates isolated from *M. pyrifera* and *L. hyperborea* and found a value of the persistence length of 15 and 20 nm, respectively. Recently, molecular modeling investigations confirmed the differences in stiffness among the blocks present in alginate (Braccini et al. 1999).

The overall scenario presented above has recently been revisited (Vold et al. 2006). The availability of pure alginate samples composed (almost) exclusively of one type of block sequence allowed reinspection of the intrinsic flexibility of the limiting components of the alginate chains. In particular, the empirical *B* parameter of an alginate composed exclusively of MG sequences was found to be markedly lower ($B = 0.038$) (Dentini et al. 2005) than that measured in the 1970s from acid-soluble alginate dominated by MG-blocks ($B = 0.065$). In parallel, the composition of engineered alginates, prepared by means of mannuronan C-5 epimerases, was shown to have a minimal effect on the *B* parameter, pointing to a limited effect of block sequences on the flexibility of the polysaccharide. This is also reflected in the evaluation of the persistence length of the different alginate samples containing largely one single block structure that was found to be around 15 nm independent of the composition (Vold et al. 2006).

Reference to the conformational aspects of alginate in the specific case of its interaction with gelling divalent ions is given in Sect. 5.

3 Molecular Weight and Stability

3.1 Molecular Weight and Molecular Weight Distribution of Native Alginates

Natural, bacterial, and enzymatically tailored alginate samples are polydisperse with respect to the molecular weight. This can be traced back to two main reasons: (1) the production of polysaccharides is not gene-encoded but it is under enzymatic control; (2) the extraction causes a substantial depolymerization. As a consequence, polydispersity in the degree of polymerization of these samples is more the rule than an exception. For polydisperse systems, the molecular weight is expressed by different averages over the whole distribution of the molecular weights for the sample. The number-average molecular weight ($\overline{M}_{\mathrm{N}}$) and the weight-average molecular weight ($\overline{M}_{\mathrm{W}}$) represent the most common averages used to address this point:

$$\overline{M}_{\mathrm{N}} = \frac{\sum_i N_i M_i}{\sum_i N_i}, \tag{6}$$

$$\overline{M}_{\mathrm{W}} = \frac{\sum_i w_i M_i}{\sum_i w_i} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i}, \tag{7}$$

where N_i is the number of molecules and w_i is the weight of molecules having the given molecular weight M_i.

Typically, commercial alginates have a weight-average molecular weight ($\overline{M}_W$) of approximately 200,000, but alginates with values as high as 400,000–500,000 are available. The molecular weight of native alginate is not known since degradation occurs during the extraction.

The polydispersity index is given by the ratio $\overline{M}_W/\overline{M}_N$ and provides an evaluation of the polydispersity of the sample. The polydispersity index is 1 in the case of a perfectly monodisperse polymer (proteins), while for polydisperse samples the relation $\overline{M}_W > \overline{M}_N$ holds. Typically, alginate samples have a polydispersity index ranging from 1.5 to 3, although values as high as 6 have also been reported (Draget et al. 2005).

Methods based on laser light scattering have proved their reliability in determining the molecular weight of alginate samples over a wide range of molecular sizes (Martinsen et al. 1991). Multiangle laser light scattering coupled with size-exclusion chromatography using a refractive index detector was found to be an efficient and reliable method for the determination of both the molecular weight averages and their distribution (Vold et al. 2006). In addition, the evaluation of the viscosity-average molecular weight ($\overline{M}_V$) can be performed by means of intrinsic viscosity determinations once the Mark–Houvink–Sakurada (MHS) parameters (K, a) are known (see Eq. 12). For the latter average, it generally holds that $\overline{M}_N < \overline{M}_V < \overline{M}_W$, with $\overline{M}_V = \overline{M}_W$ when $a = 1$.

3.2 Chain Degradation

The stability of an alginate molecule is strongly dependent on the conditions to which it is subjected, i.e., temperature, pH, and presence of contaminants. The glycosidic linkages between the sugar monomers of the polysaccharide are susceptible to cleavage both in acidic and in alkaline media. The first condition, which brings about a notable molecular weight reduction at pH values lower than 5, induces an acid hydrolysis of the glycosidic bond. In contrast, alkaline conditions cause chain scission by a β-elimination reaction at the glycosidic bond (Haug et al. 1963, 1967b). The rate of acid hydrolysis for alginate at pH around 3 or 4 was found to be much higher than that of neutral polysaccharides owing to intramolecular catalysis from the nondissociated carboxyl group which can form a favorable six-membered ring (Smidsrød et al. 1966). As to the alkaline conditions, it has been found that, in addition to hydroxyl ions, carbonate and phosphate ions function as catalysts in the β-elimination reaction owing to the general base-catalyzed nature of this reaction (Haug et al. 1967b).

In both acid and alkaline conditions, the decrease in the degree of the polymerization follows first-order kinetics (valid for degrees of polymerization exceeding 10) (Haug et al. 1963):

$$\frac{1}{\mathrm{DP}_t} - \frac{1}{\mathrm{DP}_0} = k\,t, \tag{8}$$

where DP_t and DP_0 are the degree of polymerization at time t and time 0, respectively.

A very efficient way to characterize the degradation of alginate is to evaluate the time-dependent variation of the reciprocal of the intrinsic viscosity (see Sect. 4.2), i.e., $1/[\eta]$, which is proportional to the number of broken bonds in the polysaccharide chain. Such plots, which were found to be linear for $[\eta] > 3$, allow the overall stability of alginate in different pH conditions to be summarized (Fig. 7) (Haug et al. 1963).

Alginate chains are very sensitive to the presence of free radicals that can be generated by the contaminants in the commercial samples (polyphenols) (Smidsrød et al. 1963a, b, 1967). Such radicals are known to depolymerize alginate by an oxidative–reductive depolymerization. The mechanism of degradation involves the auto-oxidation of reducing compounds with subsequent formation of peroxides (ROOH). These will then form hydroxyl radical species that could lower the degree of polymerization of the alginate chain at a very high rate (Smidsrød et al. 1963a, 1967). In addition, notable molecular weight reduction is encountered when alginate is treated with oxidants such as sodium periodate (Larsen and Painter 1969; Smidsrød and Painter 1973; Vold et al. 2006).

In view of these considerations, safe handling conditions for alginate comprise a neutral pH (Haug and Larsen 1963) and limited heating as it is known that all the depolymerization reactions are boosted by temperature. It follows that sterilization using an autoclave should be avoided. Since the use of γ-radiation could also be deleterious for the polysaccharide, filtration over 0.22-μm filters is the recommended sterilization procedure.

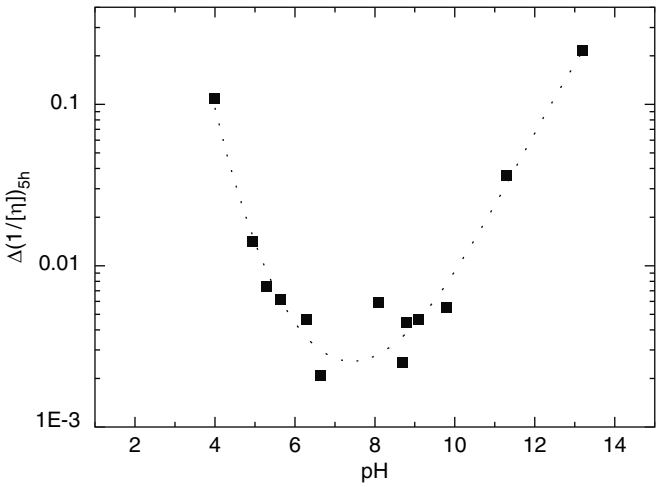

Fig. 7 Degradation of alginate isolated from *Laminaria digitata* measured as the variation of the ratio $1/[\eta]$ after 5 h at different pH and at 68°C. The *line* is drawn to guide the eye. (Adapted from Haug et al. 1963)

In vivo biodegradation of alginates is performed by endolyases which catalyze the splitting of the 1–4 glycosidic bond via a β-elimination reaction. Modifications on the alginate chain, such as acetylation, lead to a net reduction of the enzyme activity. Endolyases are widely distributed in nature in prokaryotic and eukaryotic microorganisms and in bacteriophages that use alginate as a source of carbon. Lyases are found in the bacterial species producing alginate, such as *A. vinelandii* and *P. aeruginosa*, but not in the human gastrointestinal tract. Alginate-degrading enzymes have also been isolated from marine algae, including *L. digitata*. Like epimerases, endolyases exhibit sequence specificity in cleavage of either M or G residues in alginate chains (Gacesa 1992; Wong et al. 2000).

3.3 Chemical Modifications of Alginate

Several attempts to chemically modify alginate to vary its physicochemical properties have been reported. Among all these, the propylene glycol derivative of alginate (PGA) deserves special mention: it is an ester derivative obtained by reaction of propylene oxide with some uronic moieties of alginate. PGA is largely used as a thickener and stabilizer in several food applications mainly because of its solubility in acidic conditions and low sensitivity to the presence of divalent ions (typically Ca^{2+}) (Yilmazer et al. 1991). In addition, the hydrophobic character introduced by the propylene glycol moiety allows this modified polysaccharide to function as a surfactant and emulsifier (Yilmazer and Kokini 1991). Recently, applications of PGA in tissue engineering have been proposed.

Alginate is highly biocompatible and has very interesting features as to mass transport aspects; however, its biological inertness limits the applicability in the field of tissue engineering. In fact, the lack of bioadehesivity (Smetana 1993; Lee and Mooney 2001), i.e., the total absence of biological signals mimicking the extracellular matrix milieu and favoring the cell embedding, hampers the use of alginate hydrogels for the design of so-called third-generation biomaterials. The latter are able to directly intervene in cell recognition processes and therefore could bring about a notable benefit in tissue engineering. Several attempts have been made to overcome such a critical limitation. In particular, the chemical introduction of cell-specific signals, such as peptides and oligosaccharides, has been exploited (Rowley et al. 1999; Yang et al. 2002; Rowley and Mooney 2002; Donati et al. 2003a) and led to an enhancement of (favorable) interactions between the modified alginate and different cell types. However, the chemical modification of alginate brings about the drawback of reduced mechanical stability of its hydrogels. This can be safely traced back to the introduction of side-chain groups also (or even predominantly) on G residues (Donati et al. 2003a), which are the ones involved in gel formation. Recently, this issue was addressed and the use of a chemoenzymatic strategy allowed an engineered alginate modified exclusively on the non-gel-forming M residues to be obtained, and it hence had suitable mechanical properties for tissue engineering applications (Donati et al. 2005a).

4 Alginate Solutions

4.1 Thermodynamic Properties

Sodium alginate, like basically all the monovalent salts of alginate, is highly soluble in water owing to the favorable entropic contribution from the free (noncondensed) counterions. This consideration leads immediately to identification of the presence of a high amount of supporting salt as a limiting condition for the solubilization of the polyanion. In fact, the increase of the ionic strength of the alginate-containing solution causes the entropic gain arising from the polysaccharide counterions to level off. The addition of 1:1 salts, such as potassium chloride, will progressively disfavor the solubility of the alginate chain, leading eventually to phase separation. This salting-out effect can be efficiently used for the fractionation of alginates (Haug 1959a, b). In addition, the presence of ions could have a strong (negative) effect on the kinetics of alginate dissolution, since they act by reducing the water chemical potential difference between the alginate particle and the surrounding liquid. Concentrations of supporting salt even lower that 0.1 M slow down the kinetics of dissolution of alginate particles and limit their solubility.

Besides this unspecific role of added salts contributing to the total ionic strength of the medium, the presence of specific divalent or multivalent ions can dramatically affect the solubility of alginate. This point will be discussed in more detail in the following section; here it suffices to say that divalent ions could induce a large-scale chain aggregation mediating physical contacts between the polymer chains. This results in an extended network formation that leads to phase separation and, possibly, to hydrogel formation.

The solubility of alginate may also be limited owing to the modification of the environmental conditions which do not imply variation in the ionic strength. In fact, the pH of the solution is a critical parameter controlling the solubility of the polysaccharide. It is known that the carboxyl groups of both mannuronic acid (pK_a = 3.38) and guluronic acid (pK_a = 3.65) can be protonated by addition of inorganic acids. Therefore, if the pH of the alginate-containing solution is lowered below the pK_a of the constituting acids, phase separation or hydrogel formation occurs. Several investigations have focused on the pH dependence of alginic acid precipitation. It was found that molecular weight and composition play an important role in determining the pH of the precipitation of the polysaccharide (Haug and Larsen 1963; Haug et al. 1967a). In particular, MG-block rich alginates show a reduced tendency to phase-separate at acidic pH values when compared with G- and M-rich alginates, likely because of their higher degree of “conformational disorder” of the glycosidic bonds (Hartmann et al. 2006).

The determination of the pK_a values of uronic acids composing alginate can be traced back to the work of Haug, who also noticed that the pK_a of alginate chains, $(pK_a)^{pol}$, does not differ significantly from that of its monomeric units. However, considering alginates isolated from different sources, their $(pK_a)^{pol}$ value shows a

slight dependence on the polysaccharide composition. In addition, the $(pK_a)^{pol}$ of alginate was found to depend slightly on the polymer concentration and on the ionic strength. In fact, the $(pK_a)^{pol}$ for *L. digitata* alginate drops from 3.92 to 3.42 and that of *L. hyperborea* alginate drops from 4.41 to 3.74 on passing from a salt-free solution to aqueous 0.1 M sodium chloride (Haug 1964).

Since alginate is an acidic polyelectrolyte, it is important to note that the $(pK_a)^{pol}$ value, as well as the enthalpy of dissociation, ΔH_{diss}, depends on the degree of dissociation, α (Fig. 8): $(pK_a)^{pol}(\alpha)$ and $\Delta H_{diss}(\alpha)$. Focusing on Fig. 8b, it is interesting to note that algal and bacterial (acetylated) alginates behave differently as to $\Delta H_{diss}(\alpha)$ at low and high degrees of ionization. In particular, the ionized state of algal alginates is more favored by enthalpy than the one isolated from bacteria (Delben et al. 1982).

Several calorimetric studies performed on both algal and bacterial alginates have focused on thermodynamic properties such as enthalpy of dilution, which provides a measure of the energy changes in the interaction of the solvated solute species upon dilution (Fig. 9). Owing to the polyelectrolytic nature of alginate, the enthalpy of dilution is a function of its linear charge density, ξ [$\xi = e^2/(Dk_BTb)$, where e is the value of the unitary charge and k_B is Boltzmann's constant, D is the bulk dielectric constant of the medium, T is the absolute temperature, and b is the (average) distance between fixed charges projected along the polyelectrolyte axis]. In particular, the (excess) electrostatic enthalpy change upon mixing the polymeric salt with pure solvent to change its concentration (C_e) from $C_{e,i}$ to $C_{e,f}$, per mole of uronic acid group, is given by Eq. 9 (Paoletti et al. 1985):

$$\Delta H_{dil} = -\frac{1}{2}RT\left(1+\frac{d\ln D}{d\ln T}\right)(1-r)\xi\left(2\ln\frac{1-e^{-\kappa_f b}}{1-e^{-\kappa_i b}}+\frac{\kappa_f b}{e^{\kappa_f b}-1}+\frac{\kappa_i b}{e^{\kappa_i b}-1}\right), \qquad (9)$$

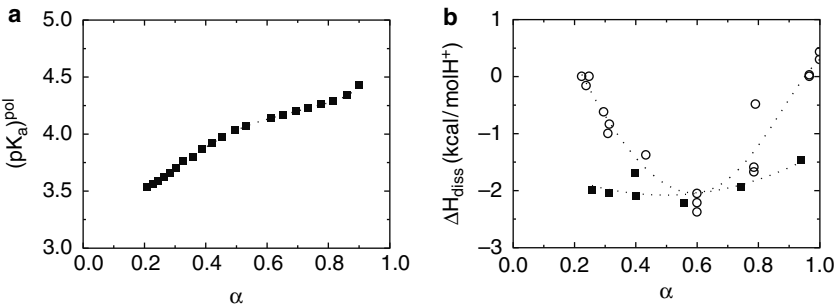

Fig. 8 **a** Apparent dependence of $(pK_a)^{pol}$ on the degree of ionization, α, for (*squares*) algal alginic acid titolated with NaOH in water at 25°C. **b** Enthalpy changes on proton dissociation (ΔH_{diss}) for acetylated (*circles*, degree of acetylation approximately 8%) and deacetylated (*squares*) alginic acid as a function of the degree of ionization, α, in water at 25°C. The *lines* are drawn to guide the eye. (Adapted from Delben et al. 1982)

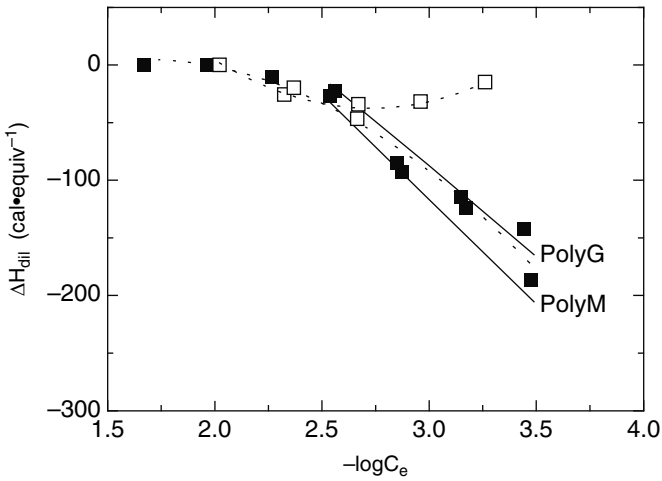

Fig. 9 Dependence of the heats of dilution, ΔH_{dil}, on the negative logarithm of the final polymer concentration, C_e, for acetylated (*open squares*) and deacetylated (*filled squares*) sodium alginates ($\alpha \sim 1$) in water at 25°C. The *dotted lines* are drawn to guide the eye. The heat of dilution, calculated according to Eq. 9 derived from the counterion condensation theory, for polyG (guluronan) and polyM (mannuronan) is also reported. (Adapted and modified from Paoletti et al. 1985)

where κ_i and κ_f are the reciprocal of the Debye length at initial and final ionic strength, respectively, and r is the fraction of monovalent ion condensed on the polyelectrolyte ($r = 1 - \xi^{-1}$).

Equation 9, derived from the counterion condensation theory, was found to hold in the case of algal alginate and to parallel with a very high accuracy its exothermic experimental enthalpy of dilution (Benegas et al. 1992). In particular, the slope of the ΔH_{dil} versus $-\log C_e$ experimental data points out that the algal copolymer falls in-between the theoretical predictions for the two limiting components of homopolymeric sodium mannuronate and guluronate (Fig. 9) (Paoletti et al. 1985). In contrast, when a bacterial (acetylated) alginate is considered, significant differences are detected. In particular, in the latter case the ΔH_{dil} versus $-\log C_e$ curve departs from the expected behavior in a way which is suggestive of a concomitant conformational change of the polymer.

Additionally, for algal alginate samples, a theoretical approach based on the counterion condensation theory was found to correlate very well with the experimental enthalpy of mixing of alginate with monovalent ions, namely, Na^+ (Donati et al. 2006b).

4.2 Nonequilibrium Properties

The viscosity of an alginate solution is a measure of the hydrodynamic volume of the polymer chains. As such, it is strongly affected by the average length of the chains in the ensemble (i.e., it depends on some average of the molecular weight)

and the conditions used (solvent, temperature, ionic strength), which collectively influence both extension (expansion) and flexibility of the polysaccharide sample. An increase of alginate molecular weight will obviously increase the viscosity of the alginate solution, providing some beneficial effects for several applications, in particular in the food industry and textile printing. However, the use of a high molecular weight alginate results in a solution which is difficult to handle (sterile filtration could become a major issue for such systems) and that could reduce cell viability (Kong et al. 2003), owing to the high shear forces required to mix it with cells for cell therapy.

The intrinsic viscosity $[\eta]$ of alginate is experimentally determined in dilute polymer solutions by means of the Huggins (Eq. 10) and Kraemer (Eq. 11) relations:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c, \tag{10}$$

$$\frac{\ln \eta_{rel}}{c} = [\eta] - k''[\eta]^2 c, \tag{11}$$

where η_{sp}, η_{rel}, and c are the specific viscosity, the relativity viscosity, and the alginate concentration, respectively, while k' and k'' are the Huggins and Kraemer constants, respectively.

The value $[\eta]$ is used to evaluate the molecular weight of the alginate sample through the MHS equation:

$$[\eta] = KM_w^a. \tag{12}$$

Generally alginate is considered a semiflexible wormlike chain for which the a parameter of the MHS equation exceeds the theoretical value of a random-coil chain in θ conditions (i.e., 0.5).

Several authors have challenged the determination of the semiempirical K and a parameters for alginate (mainly for samples isolated from *L. hyperborea*) over a wide range of molecular weights. The MHS parameters were first determined as a function of the ionic strength by Smidsrød (1970) (Table 4). It is important to note that the a parameter, describing the polymer chain extension and conformation, exceeds the value expected for Gaussian coils in the θ condition even for the extrapolation at infinite supporting salt concentration ($I \rightarrow \infty$). This effect has been considered as a consequence of the intrinsic rigidity of the polysaccharide chain. Values of a above 0.5 are usually attributed to excluded-volume effects, resulting in an expansion factor over the unperturbed dimensions which increases with molecular weight. The data obtained by Smidsrød were basically confirmed by Mackie et al. (1980), who proposed a set of different MHS semiempirical parameters depending on the ionic strength used in the analysis and on the composition of the alginate samples.

Additional investigations reported a value for a in the MHS equation ranging from 0.92 for a M-rich alginate to 1.13 for a G-rich alginate (Martinsen et al. 1991). In the cases reported, viscosity measurements were performed in combination with

Table 4 Semiempirical parameter *a* obtained for alginate

	Smidsrød (1970)	Mackie et al. (1980)		Berth (1992)	Martinsen et al. (1991)		Vold et al. (2006)
Ionic strength	*L. digitata*	*L. hyperborea*	*A. vinelandii*		*L. hyperborea*	*M. pyrifera*	*L. hyperborea* (stipe or leaf)
0.01	1.15	1.31	1.08				
0.1	1	1.11	0.91	0.90	1.13	0.92	1.01
0.2		1.06	0.86				
0.5		1.00	0.82				
1	0.87						
∞	0.84	0.80	0.73				

wide-angle laser light scattering and low-angle laser light scattering analyses to determine the molecular weight of the samples. Table 4 summarizes the different values of the semiempirical parameter *a* reported for different alginate samples.

Recently, Vold et al. (2006) reported the following relation for alginate samples to be valid for practical reasons for all the alginate samples regardless of the source:

$$[\eta] = 0.00504\, M_w^{1.01} (0.1\text{M NaCl}, T = 20^{\circ}\text{C}). \tag{13}$$

The use of a size-exclusion chromatography with online multiangle laser light scattering and viscometry also suggested that, in the case of *L. hyperborea* alginate, two separate regions in the linear log[η]-logM_w relationship coexist depending on the alginate molecular weight range (Eqs. 14, 15) (Vold et al. 2007).

$$20{,}000 < M_w < 100{,}000: \; [\eta] = 0.0051 M_w^{1.00} \tag{14}$$

$$100{,}000 < M_w < 300{,}000: \; [\eta] = 0.0349\, M_w^{0.83}. \tag{15}$$

The use of light-scattering techniques allowed the determination of the relationship between the radius of gyration of alginate and its molecular weight as

$$\left\langle R_g^2 \right\rangle^{1/2} = 0.095 M_w^{0.54} (M_w > 65{,}000) \text{ (Smidsrød 1970)} \tag{16}$$

or as

$$\left\langle R_g^2 \right\rangle^{1/2} = 0.0352 M_w^{0.60}. \text{ (Vold et al. 2006)} \tag{17}$$

The linearity of the relationship between the reduced specific viscosity and alginate concentration (Eq. 10) is generally fulfilled until a critical concentration, C^*, is reached, which corresponds to a coil overlap factor, $\phi = [\eta]\, C_{Alg}$, of 1. Once the semidilute regime has been reached, the relative viscosity scales strongly with the polymer concentration, namely, according to $\eta_{sp} \alpha C_{Alg}^{3.3-3.1}$ (Morris et al. 1981;

Donati et al. 2007). Alginate solutions in the semidilute regime show, additionally, a marked shear-thinning effect which leads to a reduction of the viscosity upon increase of the shear rate applied. As a consequence, the (viscosity) relaxation time of alginate solution scales as $\tau \mathrm{relax} \propto C_{\mathrm{Alg}}^{1.8}$, thus substantially in good agreement with the value expected for a semidilute entangled system, i.e., $\tau_{\mathrm{relax}} \propto C^{3/2}{}_{\mathrm{Alg}}$.

5 Ion-Binding Properties

The most relevant feature of alginate, from both the industrial and the biotechnological point of view, is connected with its ability to efficiently bind divalent cations, such as Ca^{2+}, Sr^{2+}, and Ba^{2+} to name a few, leading eventually to hydrogel formation. This polysaccharide has shown a highly selective binding ability and the affinity towards different alkaline-earth divalent ions was found to increase in the order $Mg^{2+}<<Mn^{2+}<Ca^{2+}<Sr^{2+}<Ba^{2+}<Cu^{2+}<Pb^{2+}$ (Haug and Smidsrød 1967, 1970). The affinity was found to be strongly dependent on alginate composition and in particular it was reported to increase with increasing amount of G-blocks present in the polymer (Smidsrød 1974).

The dependence of the selective ion-binding ability from the alginate composition led to the proposal of an ion chelation mechanism involving the G sequences in the polysaccharide chain as the main structural feature for the hydrogel formation. In other words, the ion-binding properties of alginate were originally allocated to a specific and strong interchain interaction between stretches of G-blocks and the divalent ion which ends up in the formation of a junction.

Morris and Rees, and Smidsrød proposed the so-called egg-box model to account for the interchain ion binding (Grant et al. 1973; Smidsrød 1974; Morris et al. 1978). According to it, two facing helical stretches of G sequences bind the divalent ion in a chelate type of binding. The selectivity of the process stems from the stringent geometrical requirements of a cavity formed by the diaxially linked G residues that is able to accommodate the divalent ions. In contrast, the different configuration of the glycosidic bonds present in the other block structures (diequatorial or equatorial–axial) would not allow such a tight ion entrapment. It is interesting to point out that a diaxial configuration is also shown by galacturonate residues in pectate. The latter is a polyuronate of vegetal origin strictly correlated with alginate in having very similar affinity for divalent ions and in being α-D-galacturonic acid the "near mirror image" of α-L-guluronic acid (G).

Smidsrød (1974) reported the following relations for the strength of binding of divalent ions to the three alginate fragments (Fig. 10):

GG-blocks: Ba > Sr > Ca >> Mg

MM-blocks: Ba > Sr ≈ Ca ≈ Mg

MG-blocks: Ba ≈ Sr ≈ Ca ≈ Mg

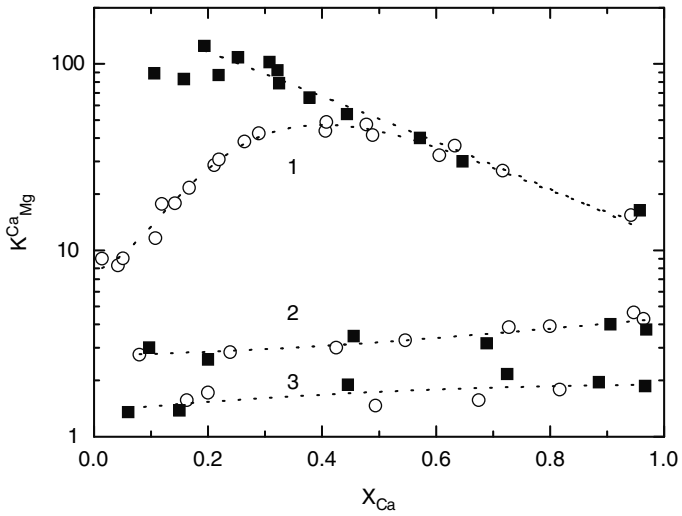

Fig. 10 Selectivity coefficients, K_{Mg}^{Ca}, as a function of ionic composition (X_{Ca}) for different alginate fragments. *Open circles* dialysis of the fragments in their Na^+ form. *Filled circles* dialysis first against 0.2 M $CaCl_2$, then against mixtures of $CaCl_2$ and $MgCl_2$. *Curve 1* fragments with 90% guluronate residues. *Curve 2* alternating fragment with 38% guluronate residues. *Curve 3* fragment with 90% mannuronate residues. The *lines* are drawn to guide the eye. (Adapted from Smidsrød 1974)

Overall, each cross-linking ion (i.e., Ca^{2+}) interacts with two adjacent G residues as well as with two G residues in the opposing chain, thus inducing the formation of junction zones. The latter represent the physical interactions connecting alginate chains together and leading to the formation of the hydrogel. Therefore, the junction zones in the gel can be envisaged as "nanocrystallites" flooded by the "sticky sea" of elastically active chains. On the basis of dialysis experiments, which showed a 4:1 ratio between G residues and calcium ions, a schematic sketch of the possible junction formed in the G-rich alginate has been proposed (Fig. 11).

The formation of the junction, and eventually of the hydrogel, upon addition of the divalent ion can be described as a cooperative process with an unfavorable binding of the first ion and a more favorable binding of the following ones (zipper mechanism). It has been reported that in the case of Ca^{2+}, a minimum of eight to 20 adjacent G units are required for the formation of a stable junction. Also in this case a strict parallel with pectate holds (Kohn and Luknar 1977; Donati et al. 2006a). However, the minimum length of the G-block required for the binding of a cross-linking ion decreases upon increasing the affinity of the latter.

Direct verification of the structural features of the egg-box model is strongly hampered by the lack of crystalline and well-resolved X-ray structures of alginate in the presence of divalent ions such as Ca^{2+} or Sr^{2+}. Most of the crystallographic data

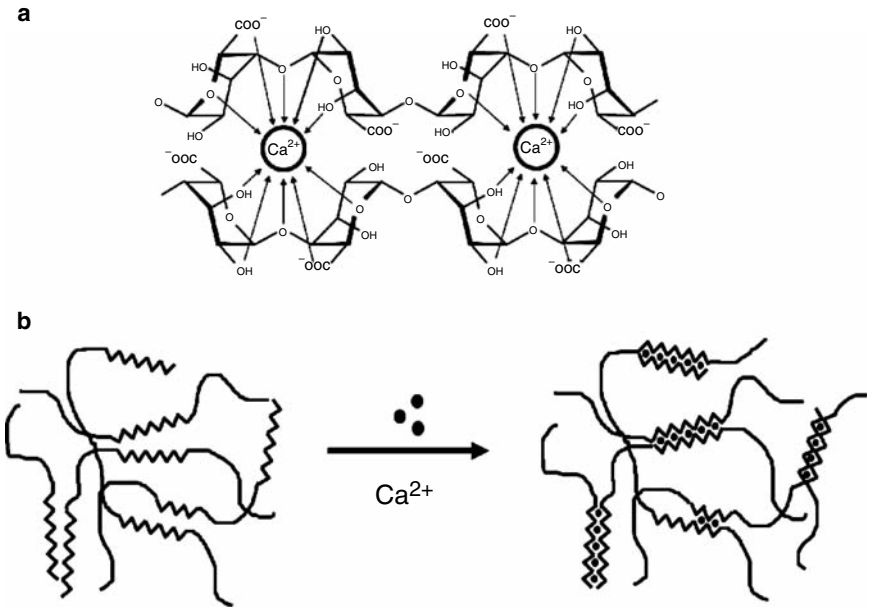

Fig. 11 Binding of divalent cations by alginate – the "egg-box" model. **a** Chelation of divalent cations. **b** Interchain junction formation

available arise from the acidic form of the polysaccharide in the fiber form: the derived structural features are used to predict the crystal structure and ion coordination geometry of Ca^{2+}–alginate junctions in the physical gels (Atkins et al. 1973a, b). Over the years, refinements of the egg-box model have been attempted by means of a reinspection of the original X-ray diffraction data and a slightly modified crystal structure for the calcium-induced junction was proposed on the basis of the "formal" replacement of one water molecule with the divalent ion found in the unit cell of the free acid form of alginate (Arnott et al. 2000). A good-resolution X-ray data for Ca^{2+}–alginate junctions has, however, been achieved recently. These novel experimental data recognized the 2/1 helical structure of the G-blocks in the junctions and helped determine both the stoichiometry and the structural features of the complex between G-blocks and calcium (Sikorski et al. 2007). Additionally, the description of the calcium-induced interchain association of the G-blocks in the hydrogel junctions has been attempted by means of molecular modeling. In this case, the positioning and the coordination of the ion differed, to some extent, from the those in original egg-box (Braccini and Pérez 2001) model and these conclusions were supported by NMR spectroscopy (Steginsky et al. 1992).

From the inspection of the data reported above, it should be concluded that the basic features of the ion-induced alginate junctions were well represented in the original formulation of the egg-box model provided by Morris, Rees and Smidsrød.

Therefore, the description of alginate junctions as composed of dimers or of few laterally associated polymer strands still holds after 30 years.

This overall picture has been reconsidered and rediscussed in view of small-angle X-ray scattering experiments that suggested a lateral association of calcium-containing junctions, far beyond a pure dimerization, takes place once an excess of ion has been provided (Stokke et al. 2000). However, recent X-ray analyses have indicated that alginate junctions are largely composed of polysaccharide chain dimers held together by Ca^{2+} coordination in close resemblance to the egg-box model, with some limited lateral association occurring through unspecific electrostatic interactions (Sikorski et al. 2007).

The divalent ion binding by alginate chains has also been screened by calorimetric and dilatometric methods. In particular, the enthalpy of mixing of alginate with divalent cations of high affinity, such as Ca^{2+} and Pb^{2+}, was exothermic and characterized by a sigmoid shape (Fig. 12) (Cesàro et al. 1988b). This result revealed a contribution of conformational origin (disorder-to-order transition) deriving from the G-rich component of the polysaccharide chain, as corroborated by CD data (Morris et al. 1975; Cesàro et al. 1988a). In addition, such results pointed to the cooperativity of the ion-induced junction formation. Moreover, dilatometric studies revealed that alginate-ion interactions are associated with a positive volume change. It stems from a release of a large number of water molecules (desolvation) from the

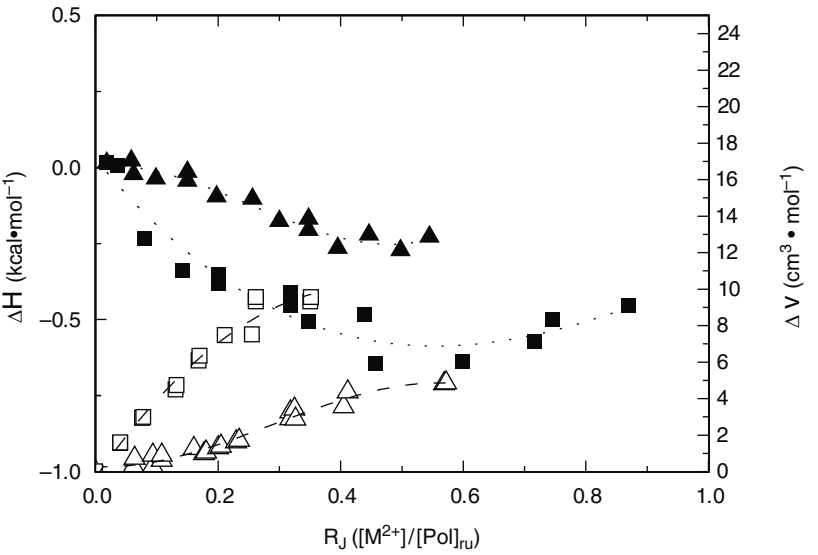

Fig. 12 Enthalpy (*left y*-scale, *filled symbols*) and volume (*right y*-scale, *open symbols*) changes upon mixing of alginate with Pb^{2+} (*squares*) and Ca^{2+} (*triangles*) in aqueous $NaClO_4$ (0.05 M) as a function of R_j (the ratio of the number of moles of ion per monomole of polymer). The *lines* are drawn to guide the eye. (Adapted from Cesàro et al. 1988b)

solvation spheres of both the divalent ion and the chelating polymer chain, bringing about a favorable increase in entropy (Fig. 12). The combination of the quoted results clearly shows that the extremely high affinity of alginate for ions such as Ca^{2+} or Pb^{2+} must be traced back to both negative enthalpy changes and positive entropy changes. A thermodynamic explanation is then given for the only slight temperature dependence of the stability of lyotropic calcium alginate gels, at variance with, for example, the thermolyotropic gels of the sulfated polysaccharides κ-carrageenan and ι-carrageenan with K^+.

The availability of alginate epimerases, which are able to produce alginate polymers composed (almost) exclusively of one block structure represents a very powerful tool to underline the role of the different sequences with respect to their ion-binding properties. In particular, the epimerization of mannuronan with C-5 epimerase AlgE4 allows the production of a pure polyalternating alginate, thus composed exclusively of MG-blocks, which, in spite of the complete lack of G-blocks, was able to form stable hydrogels upon addition of calcium. Moreover, the elongation of alternating sequences in natural alginates, obtained by means of the AlgE4 epimerase, was found to induce a marked increase in the mechanical properties and syneresis of the calcium hydrogels. These novel findings allowed the proposal of the existence, in alginate gels, of three possible junctions. More specifically, the direct involvement of repeating MG units in the formation of both mixed MG/GG and pure MG/MG junctions has been suggested (Fig. 13) (Donati et al. 2005b).

Thanks to the availability of alginates with tailor-made block compositions, the selectivity of binding by the different block structures has been revised, revealing the binding ability of M-blocks towards Ba^{2+} ions and of MG-blocks towards Ca^{2+} and Sr^{2+} ions (Mørch et al. 2006). The overall picture resulting from these analyses helps shed some light on the different effects of the same ion on natural alginates characterized by a different composition (Mørch et al. 2006).

These studies have a profound impact in the alginate field as it is now clear that detailed knowledge of the composition and the ion-binding properties is fundamental for the selection of the alginate in order to have the desired material properties.

6 Alginate Gels

6.1 Gel Formation Properties

6.1.1 Acid Gels

Alginate solutions treated at a pH below the pK_a of uronic acid can, under certain conditions, lead to the formation of acid gels. The latter, in contrast to ionic gels, are equilibrium gels, as proved by swelling studies, and are stabilized by hydrogen bonding (Draget et al. 1994; Smidsrød and Draget 1996). It has been shown that the gel strength of acid gels becomes independent at a pH below of 2.5. However, acid hydrogels

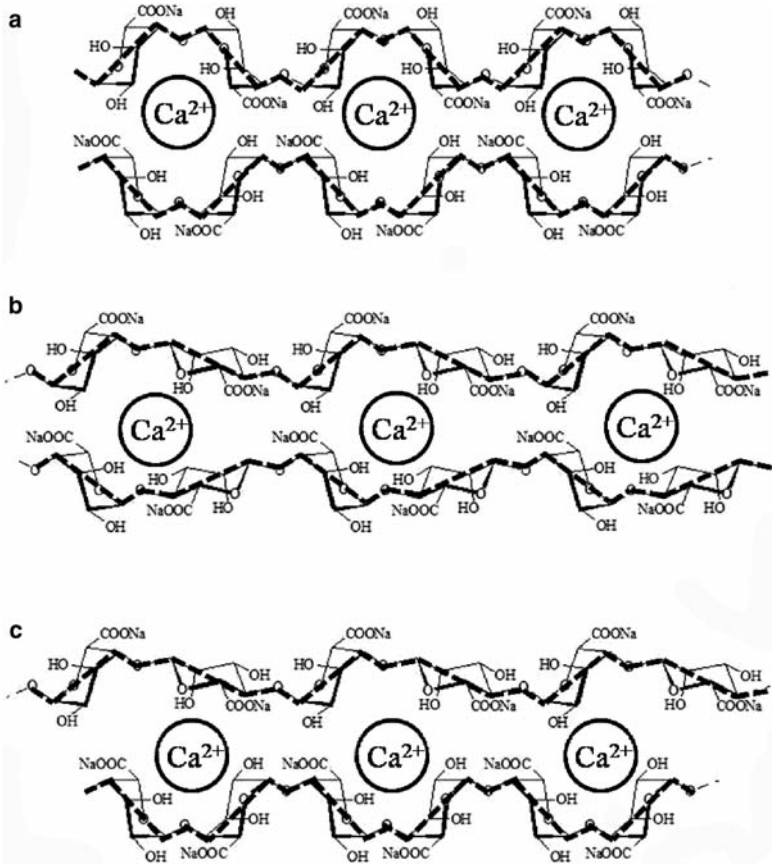

Fig. 13 The three possible junctions in alginate hydrogels: **a** GG/GG junction, **b** MG/MG junction, and **c** GG/MG junction

resemble ionic ones in the sense that the gel strength seems to be correlated with the content of G-blocks in the alginate chain, pointing to a possible cooperativity in the gel formation process. With the exception of some pharmaceutical uses, the number of applications for alginic gels is so far limited (Draget et al. 2005).

6.1.2 Ionic Gels

The treatment of an alginate semidilute solution (1–2%) with a proper divalent cation, i.e., Ca^{2+}, results in the formation of a ionotropic (lyotropic) gel. The ion-binding ability of alginate is the basic feature controlling hydrogel formation. Overall, an alginate gel can be depicted as a continuous network swollen with water where

physical cross-links, represented by the ion-induced junction zones, hold together different polysaccharide chains.

The formation of the hydrogel is favored by the fact that Ca^{2+}–alginate gels have a markedly higher affinity than Na^{+}–alginate solution towards Ca^{2+} ions. This has been explained theoretically as a near-neighbor autocooperative process which predicts that affinity towards a specific ion increases with increasing content of the same ion in the medium (Smidsrød 1974).

The gelling kinetics is strongly dependent on the method used for the introduction of the cross-linking ion and hence homogeneous or inhomogeneous hydrogel can be obtained (Fig. 14).

6.1.2.1 Homogeneous Alginate Hydrogels

To achieve a homogeneous distribution of alginate within the hydrogel, a controlled introduction or release of the cross-linking ion has to be accomplished. Over the years, the internal gelation method has been established as a reliable procedure for achieving a release of calcium ions in a controlled fashion (Draget et al. 1991). This is based on the use of an inactivated form of calcium ($CaCO_3$, Ca-EDTA, calcium citrate) that is mixed with the alginate solution. The controlled release of the cross-linking ion is usually obtained by means of a decrease of pH caused by the addition of a slowly hydrolyzing molecule such as δ-gluconolactone (GDL). The use of calcium carbonate and GDL, once a molar ratio of GDL to $CaCO_3$ of 2 is maintained, guarantees only slightly acidic pH in the final hydrogel, thus with a negligible contribution from acidic alginate gels. In contrast, when Ca-EDTA is used, the

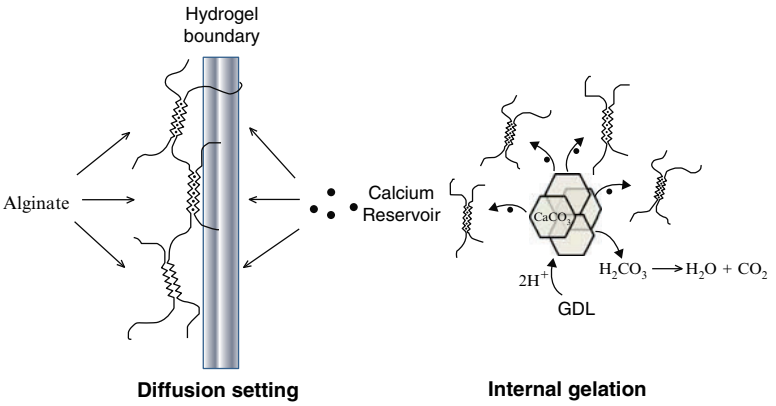

Fig. 14 The diffusion setting and internal gelation methods

presence of some acidic gel cannot be ruled out. Generally, the internally set gels are prepared in calcium-limited conditions to favor handling and the preparation of homogeneous gels. It follows that their mechanical performances will be lower when compared with those of saturated calcium alginate gels.

The kinetics of the gelation process has been studied extensively and it has been found to be influenced by alginate concentration, composition, and particle size of the calcium salt (i.e., $CaCO_3$) (Draget et al. 1991). The influence of alternating sequences in the gelling kinetics on the homogeneous alginate networks has also been explored. In particular, it has been reported that the longer the MG-blocks are in the alginate chains, the faster is the gel setting (Draget et al. 2000).

6.1.2.2 Inhomogeneous Alginate Hydrogels

Hydrogels characterized by an inhomogeneous distribution of the polysaccharide can be obtained by means of the diffusion setting. This is performed by allowing a cross-linking ion (e.g., Ca^{2+}) to diffuse from a larger outer reservoir into an alginate solution. In this case, very rapid gelling kinetics occurs at the front of the diffusing ion, resulting in an instantaneous formation of the hydrogel. This approach has been largely used in the preparation of alginate beads for the entrapment of biological material and for cell immobilization (Strand et al. 2002). In the case of the setting of gel beads obtained from diffusion, highly inhomogeneous alginate distributions have been reported, with a fivefold increase in alginate concentration at the surface and a very low concentration (virtually zero) in the center of the gel. Hence, the polymer is mainly located on the external part of the bead, while a very soft and liquid core is found in its central part. Inhomogeneity of alginate beads has been studied by several methods, including synchrotron radiation induced X-ray emission, magnetic resonance imaging, confocal microscopy, and Raman spectroscopy (Thu et al. 2000; Strand et al. 2003; Heinemann et al. 2005). The polymer gradient within the gel can be controlled by careful selection of alginate molecular weight, concentration, and composition, as well as of the cross-linking ion used and of its concentration (Thu et al. 2000; Strand et al. 2003; Mørch et al. 2006). In general, low molecular weight alginate and (relatively) low concentrations of the gelling ion will give the highest polysaccharide gradient throughout the hydrogel. In contrast, a high molecular weight alginate and a high concentration of gelling ions will increase the homogeneity of the hydrogel (Skjåk-Bræk et al. 1989a). In general, a high inhomogeneity of the hydrogel may be preferred in the preparation of microcapsules characterized by lower porosity (Martinsen et al. 1992) and higher resistance against swelling (Thu et al. 1996b).

From the theoretical point of view, the strong and virtually irreversible site binding of the cross-linking ion by two facing G-blocks in the alginate chain has been regarded as the main cause of the uneven distribution of alginate within the gel. The cross-linking ions rapidly diffuse towards the center of the droplet owing to the difference in the concentration between the reservoir and the alginate bead. The diffusion of gelling ions will create a sharp gelling zone that moves from the surface towards

the center of the gel. The activity of alginate (and of the gelling ion) will be zero in this zone, and alginate molecules will diffuse from the internal, nongelled part of the gelling body towards the zero-activity region. However, the diffusion of alginate (a polyanion) is enhanced, with respect to self-diffusion on a noncharged macromolecule, by the coupled diffusion of the counterion (Na^+). The latter, owing to the unbalanced concentration between the alginate bead and the reservoir, will rapidly diffuse towards the gelling front, dragging the alginate molecule for electroneutrality reasons.

The addition of a nongelling ion, such as sodium chloride, to the reservoir (gelling bath) containing the cross-linking ion reduces the diffusion of the counterion of alginate towards the external part of the bead. As a consequence, the coupled diffusion between the counterions and the macromolecule is hampered and the resulting gel is homogeneous with a uniform distribution of the polysaccharide throughout the bead (Skjåk-Bræk et al. 1989a; Strand et al. 2003).

6.1.3 Syneresis

"Syneresis" is the term used to describe the slow shrinking of alginate upon increasing the divalent cross-linking ion concentration. Macroscopically, syneresis is detected as a release of water from the gel with a consequent decrease in its dimensions and increase in polymer concentration (Fig. 15). This partial collapse of the network increases the stability of the hydrogel and reduces its porosity: both features are highly advantageous in the case of the use of alginate for microencapsulation for cell transplantation (Martinsen et al. 1992; Thu et al. 1996b).

The alginate gel shrinkage has been reported to depend on the composition of the alginate sample. In fact, alginates containing long G-blocks will shrink less than alginates with shorter G-blocks owing to the formation of strong irreversible junctions, thus hindering reorganization of the network structure (Martinsen et al. 1989). In addition, syneresis increases with increasing calcium concentration, gelling time (Martinsen et al. 1989), and alginate molecular weight (Draget et al. 2001).

Although macroscopically very well characterized, the molecular details of syneresis are still debated. Undoubtedly, MG-blocks in alginate chain do play an important role in determining gel shrinkage. In fact, a correlation between the frequency of alternating sequences in the polysaccharide and the loss of water by the hydrogel has been reported. This was traced back to the higher flexibility of the MG-blocks, with respect to the other blocks present in alginate, thus allowing a denser packing of the gel network (Draget et al. 2001). As a consequence, lateral association of calcium-junction zones, beyond dimerization, has been suggested to account for the syneresis of alginate hydrogels (Stokke et al. 2000).

More recently, it has been underlined that the length of the MG-blocks, rather than their overall amount, determines the extent of the collapse of the hydrogel. Therefore, syneresis was thought to be a result of a "zipping" of long alternating sequences in secondary MG/MG junctions upon increasing the Ca^{2+} concentration (Donati et al. 2005b).

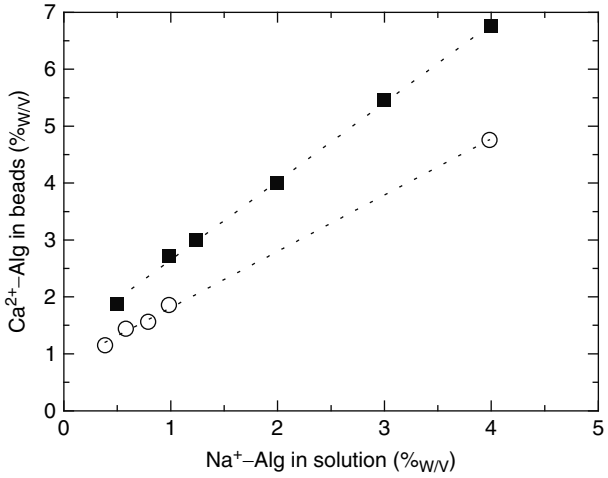

Fig. 15 Calium alginate concentration in the gel bead versus sodium alginate concentration in the solution for samples isolated from *Macrocystis pyrifera* (*filled squares*) and *L. hyperborea* stipe (*open squares*). (Adapted from Martinsen et al. 1989)

It is likely that both mechanisms contribute, to a different extent, to the final shrinkage of the alginate hydrogel.

6.2 Hydrogel Stability

Alginate hydrogels are easily obtained by using a proper cross-linking ion, which, being chelated by the G sequences in the alginate chain, induces the formation of the three-dimensional network. As such, the presence of a semisolid state is guaranteed by the physical interactions between the ion and some of the uronic moieties composing the polysaccharide chain. These interactions are by far less resistant than chemical linkages and their depletion markedly affects the hydrogel stability. It follows that the major limitation to the use of alginate gel is represented by the presence in the medium of compounds able to chelate divalent ions as well as by a high concentration of competing ions. Treatment of alginate gels with one of these two systems causes gel swelling, variation of its porosity, and eventually dissolution.

It is well known that the volume of a polyelectrolyte hydrogel in a solvent is determined by the equilibrium between the osmotic pressure (Π_1^{osm}), which tends to increase the overall dimensions of the hydrogel, and the elastic retractive force (Π_1^{el}), which, in contrast, acts to impede volume expansions:

$$\Pi_1^{osm} + \Pi_1^{el} = 0. \tag{18}$$

From the theoretical point of view, the same concept can be rephrased by stating that, under equilibrium conditions, the chemical potential of the solvent inside the gel ($\mu_{solv,In}$) equals that outside the gel ($\mu_{solv,Out}$), i.e., in the surrounding solution. For a polyelectrolyte gel this reads

$$\mu_{solv,In} = \mu_{solv,Out} \rightarrow \Delta\mu_{solv} = \Delta\mu_{mix} + \Delta\mu_{ion} + \Delta\mu_{el}. \quad (19)$$

The polymer–solvent mixing term, $\Delta\mu_{mix}$, basically depends on the interactions between the polymer and the solvent (hydration of the polymer) (Flory 1953; Hoffman 2002). This term has been reported to be negligible in the case of alginate gels (Moe et al. 1993).

An osmotic contribution facilitating gel swelling arises from the difference in chemical potential of the water owing to uneven distribution of ions between the inside and outside of the gel, $\Delta\mu_{ion}$. In this case, the osmotic swelling force results from the presence of the counterions of the fixed charges on alginate. These mobile counterions cannot leave the hydrogel, because the electroneutrality condition must always be fulfilled, and therefore their concentration will always be greater in the gel than outside. Hence, an osmotic pressure is exerted on the semipermeable membrane of the hydrogel, causing an increase of its volume. The swelling of the hydrogel is opposed by the physical cross-links (network junctions) which cause an elastic network retraction force, $\Delta\mu_{el}$ (Moe et al. 1993; Hoffman 2002).

The overall equilibrium volume of the polyelectrolyte hydrogel is determined by the balance of the contributions reported. In view of these considerations, it can be easily understood that the presence of competing ions, as well as of chelating agents, acts in two ways, both contributing to hydrogel network swelling. First, calcium ions are removed from the junctions, hence decreasing the number of physical cross-links and reducing the elastic network retraction force. Second, the replacement of the divalent cross-linking ions with monovalent ones increases the number of dissociable counterions (one calcium ion is replaced by two sodium ions) and thus the osmotic pressure.

From what was reported above, it is clear that phosphate, citrate, lactate, and EDTA should be avoided when dealing with calcium alginate hydrogels since the presence of such chelating agents will remove the cross-linking ion from the network junctions. Similarly, high concentrations of competing ions, such as Na^+ in physiological saline solution (i.e., 0.15 M NaCl), will hamper the stability of the network.

Several strategies have been devised to overcome the limited stability of alginate gels in physiological conditions. As a first choice, the replacement of calcium ions with stronger binding ones, such as Ba^{2+} or Sr^{2+}, coupled with the use of alginate chains containing a high fraction of G residues has been reported (Smidsrød et al. 1972; Mørch et al. 2006). A notable increase in the stability of the alginate hydrogel was noticed and, although the toxicity of these ions used for biomedical applications still represents a concern, no Ba^{2+} leakage was detected from hydrogels prepared from high-G alginate after extensive rinsing (Thu et al. 1996a). In addition to this approach, the use of polycations, such as poly(L-lysine), poly(L-ornithine),

chitosan, and polyethyleneimine, for the preparation of a polyanion-polycation complex membrane has been explored (Lim and Sun 1980; Tanaka et al. 1984a; Thu et al. 1996a; Gåserød et al. 1998; Taqieddin and Amiji 2004; de Vos et al. 2006; Blasi et al. 2006; Lawrie et al. 2007). The strong electrostatic binding taking place between the two oppositely charged polyelectrolytes account for an increase of the physical interactions and for a partial discharging of the polymer. Very stable beads can be prepared depending of the alginate hydrogel dimensions, the concentration of the polycation, and the exposure time. However, the presence of a polycation on the surface of the hydrogel, when implanted as a bioreactor in the body, might evoke a strong immunitary reaction with the formation of a fibrotic capsule around the bead which impedes the correct exchange of nutrients and oxygen to the biological material entrapped within the network. Although this adverse effect can be reduced by an additional coating with a polyanion, a complete suppression of the inflammatory response by the polycation is still an open issue.

Another approach for stabilizing alginate gels comprises the introduction of covalent cross-links in addition to the physical (ion-induced) ones. Various chemical techniques have been attempted, including covalent grafting of alginate with synthetic polymers, a combination of covalent and ionic cross-links on the polysaccharide, as well as the direct reticulation of poly(L-lysine) on alginate (Hertzberg et al. 1995; Dusseault et al. 2005, 2008). A drawback in the methods based on the direct chemical modification of alginate is the lack of selectivity. In fact, grafting of side chain groups occurs mainly on the G residues of the polysaccharide, thus hampering gel formation by the modified alginate. A new approach has recently been shown to be able to overcome this limit by using a combination of chemical grafting of methacrylate moieties onto mannuronan, composed exclusively of M-blocks, and of different mannuronan C-5 epimerases (Rokstad et al. 2006).

Finally, the composition of the alginate chain was found to have a profound impact on dimensional stability. In particular, the presence of long alternating sequences in the polymer, introduced by an enzymatic approach, leads to very stable alginate gel beads that do not swell upon treatment with saline solution (Fig. 16) (Mørch et al. 2007).

6.3 Mechanical Properties

Alginate gels and microbeads are largely used for food and biomedical applications and consequently they should have suitable mechanical properties to withstand the stresses (compression and shear) exerted. Therefore, it is highly desirable to clearly understand the molecular features controlling the stiffness and elasticity of the network to be able to select the proper alginate sample for different applications.

From the theoretical point of view, the elasticity of a network is a measure of its deformation under stress and it is often described by the rubber elasticity theory. The classical rubber elasticity theory was derived for chemical networks in which the energy-at-break of the cross-links is so large that only the (entropy-

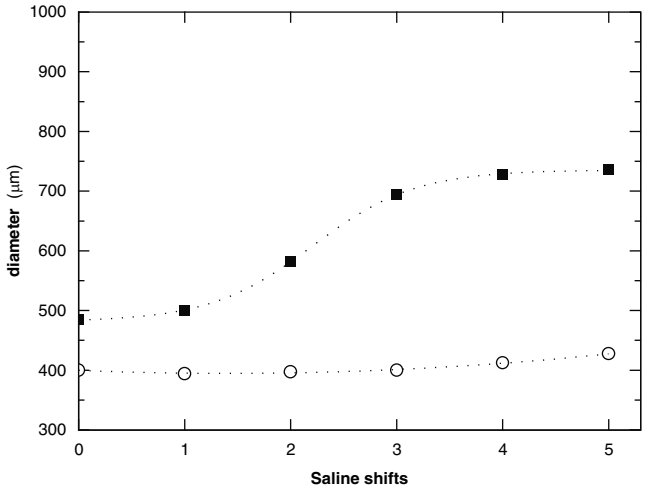

Fig. 16 Dimensional stability of alginate beads obtained from *L. hyperborea* alginate ($F_G = 0.66$) (*squares*) and from an epimerized alginate ($F_G = 0.64$) (*circles*). The *lines* are drawn to guide the eye. (Adapted from Mørch et al. 2007)

dominated) behavior of the polymer stretches connecting them needs to be considered to explain and describe elasticity. In addition, this theory describes the cross-links connecting the elastically active networks as stable and pointlike. Upon gel deformation, the free-energy increase of the network is caused by a decrease of entropy. According to the rubber elasticity approach, the shear modulus (G) can be related to the number of elastically active chains per unit volume (n),

$$G = nkT = \frac{cRT}{M_c}, \tag{20}$$

or, if loose polymer ends that do not contribute to the network elasticity are taken into consideration,

$$G = \frac{c\,R\,T}{M_c}\left(1 - \frac{2\,M_c}{\overline{M}_w}\right), \tag{21}$$

where k is Boltzmann's constant, T the absolute temperature, c the weight concentration of the polymer, R the gas constant, M_c the average molecular weight of an elastic chain, and $\overline{M}_w$ the weight-average molecular weight of the uncrosslinked polymer.

The applicability of the rubber elasticity theory in the case of physical gels (including biopolymer gels) has been repeatedly questioned. In fact, even in the

presence of cooperative sequences of interchain contacts held together by weak forces, the overall energy-at-break of the gel junctions may become comparable to the elastic (free) energy of the rubberlike component. Alginate gels certainly do not strictly meet the conditions required by the rubber elasticity theory. In fact, cross-links are reversible junctions rather than permanent pointlike contacts and the chains between the junctions are restricted in their movements (although topological restrictions do not, per se, exclude the feasibility of a purely entropic description of the network; Gardel et al. 2004). Therefore, a purely entropic approach certainly does not capture all the physics of these systems: rather, both entropic and enthalpic terms contribute to the elasticity of alginate gels. The enthalpic contribution is negligible at small deformations (Doi and Kuzuu 1980; Higgs and Ball 1989) but it becomes important when large strains are exerted on the hydrogel. In contrast, in the small deformation range, where the linearity between σ and γ is fulfilled, the entropic contribution is expected to prevail (Higgs and Ball 1989; Linke et al. 1998).

In general, the rheological characterization of alginate gels is performed to quantify the apparent modulus of rigidity based on small deformations in the longitudinal direction (compression or elongation) or on small-shear deformation. The first method provides Young's modulus, *E*, while the latter gives the shear modulus, *G*, where

$$G = \frac{E}{2(1+\rho)}, \tag{22}$$

with ρ *the* Poisson ratio (0.5 for incompressible materials).
The viscoelastic nature of the alginate-based networks, like the other polysaccharide-based systems, can be explored by dynamic measurements which allow determination of the elastic (G') and loss (G'') moduli.

Alginate hydrogels are cold-setting physical networks and their setting is independent of temperature (see Section 5). However, since alginate networks are considered to be nonequilibrium gels, the kinetics of gel setting, as well as the properties of the final system, will depend on the thermal history.

The sol–gel transition in the case of alginate gels is accomplished by addition of divalent ions such as calcium. Several models have been proposed to describe the cross-linking of polymers at their critical gel point and the one formulated by Winter and Chambron (Winter and Chambon 1986; Chambon and Winter 1987) was found to be applicable in the case of alginate gels (Liu et al. 2003; Lu et al. 2005, 2006). In particular, a power-law dependence of the storage and loss moduli on the angular frequency was predicted to hold at the critical gel point:

$$G'(\omega) \approx G''(\omega) \approx \omega^n, \tag{23}$$

where *n* is the critical gel exponent. In the case of alginate, *n* was found to be basically independent of the molecular weight for low molecular weight alginate samples, having an almost constant value of 0.71, which is in good agreement with the percolation theory predictions (de Gennes 1979; Stauffer 1985; Martin et al. 1988).

In contrast, for high molecular weight alginate chains n decreases upon increasing the polymer concentration (Lu et al. 2005). At the same time, the fractal dimension at the critical gelation point for the high molecular weight alginate shows a concentration dependence, while it is basically independent of concentration for the low molecular weight alginate, pointing to a more densely filled network in the former case.

6.3.1 Small Deformation Range

The small deformation range refers to the part of the mechanical deformation curve for alginate in which a linear relationship holds between the strain (ε) on the network and the stress exerted (σ) (Hooke's law). The elastic modulus is frequently used to measure the gel strength of alginate networks. In particular, Young's modulus (E) is easily calculated from the slope of the linear ε–σ curve recorded in uniaxial compression:

$$\frac{F}{A} = E\frac{\Delta l}{l} \Rightarrow \sigma = E\varepsilon. \tag{24}$$

From the technical point of view, the mechanical properties of alginate depend on the deformation rate, the extent of deformation, and eventual rupture of the gel (Mitchell and Blanshard 1976).

In general, the elastic modulus is affected by the amount of cross-linking ion used, the concentration and composition of the alginate, and, to certain extent, its molecular weight. The dependence of the mechanical properties on the concentration of the cross-linking ion can be easily understood, as a higher number of junctions (and hence a higher number of elastically active chains per unit volume) will be formed when a higher number of divalent ions are provided to the system. For this specific reason, calcium-saturated alginate hydrogels are better performing than the calcium-limited ones. By the same means (i.e., number of elastically active chains per unit volume), the polysaccharide concentration will have a strong impact on the elastic modulus of the alginate gel. In particular, it has been reported that Young's modulus of alginate gels scales with the polysaccharide concentration according to the power law $E \propto c^2$ (Smidsrød and Haug 1972). This correlation is particularly useful when syneresis occurs and the variation of the concentration of the polysaccharide in the gel has to be accounted for (Martinsen et al. 1989).

Alginate gels made by calcium diffusion (saturated gels) showed a molecular-weight dependence of the elastic modulus up to a molecular weight of the polysaccharide of approximately 100,000. In contrast, internally set gels show more pronounced molecular-weight dependence, with the elastic modulus being affected by the dimension of the chain up to a molecular weight of 300,000 (Draget et al. 1993). This has been correlated with the limited amount of calcium generally used for the preparation of the latter hydrogels, which would induce a higher presence of loose ends (nonelastically active chains) in the network (Draget et al. 2005).

The type of divalent cation used for the gelation also has a notable impact on the mechanical properties of the alginate hydrogels (Smidsrød 1974; Mørch et al. 2006). It has been reported that the minimum length of G-blocks required for junction formation decreases upon increasing the affinity of the ions towards the alginate chains (Stokke et al. 1991). As a consequence, cations that bind strongly to the uronic moieties present in the polysaccharide form a higher number of junctions (and hence elastically active chains) per unit volume. This effect, combined with a higher strength of the junction itself, leads to an increase of the mechanical properties (Young's modulus) of the hydrogel (Stokke et al. 1991). From a general point of view, the rigidity of alginate gels decreases in the order Pb>Cu,Ba>Sr>Cd>Ca>Ni>Zn>Co>Mn.

The composition of the polysaccharide plays an important role in the mechanical properties of the hydrogels. In particular, the gel strength was found to correlate with the length of the G-blocks ($N_{G>1}$) rather than with the overall content of the guluronic acid residues (fraction of guluronic acid units, F_G). A profound effect on Young's modulus was detected for $N_{G>1}$ ranging from approximately 4 to 15 (Fig. 17).

Additional evidence for the importance of the composition for the mechanical properties of alginate gel was recently reported. In fact, it was noted that the enzymatic elongation of alternating sequences (MG-blocks) in the polysaccharide chain leads to an increase of Young's modulus, E. This effect was first attributed to the higher intrinsic flexibility of the MG-blocks with respect to the other blocks composing alginate (Draget et al. 2000). However, the possible involvement of alternating sequences in calcium-induced junctions, whether heteropolymeric MG/GG or homopolymeric MG/MG junctions, provides an additional explanation for their effect on E (Donati et al. 2005b). In addition, it has been reported that the different block structures could have different binding ability with respect to the divalent ions.

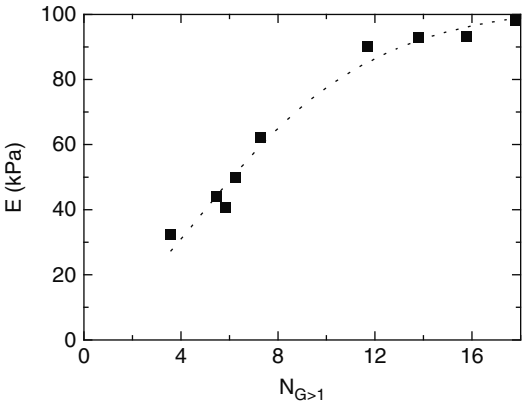

Fig. 17 Young's modulus of alginate gels as a function of the average G-block length ($N_{G>1}$). (Adapted and modified from Skjåk-Bræk et al. 1986b)

6.3.2 Large Deformation Range

The study of large deformation properties for alginate gels is undoubtedly highly correlated with their application in food engineering, where typical processes such as cutting/slicing and eating have to be considered. However, a clear understanding of the network deformation behavior certainly also has a deep impact in the development of alginate-based materials to be used as bioreactors for regenerative medicine, since the forces exerted onto the gel implants likely exceed the linear stress–strain response.

It is commonly known that the stress-strain curve of alginate gels displays a linear region that extends approximately up to a deformation of 10–12% of the specimen. However, for most saturated alginate gels the stress–strain linearity holds up to a maximum deformation of approximately 6–8%. The linear region is followed by a nonlinear trend described as "strain-hardening," which is a rather general phenomenon for biopolymer gels and consists of an upturn of the stress-strain curve. In addition, when calcium-saturated alginate gels are considered, a region of plastic behavior, where notable deformations are caused by a small increase in the stress applied, can be found (Fig. 18). It has been shown that the presence and extension of the "plastic region" is related to the presence of long alternating sequences within the alginate chain (Mørch et al. 2008).

Several interpretations for the mechanism of the strain hardening of hydrogels have been proposed. Flory argued that the upturn of the stress–strain curve is mainly attributed to strain-induced crystallization. In contrast, Treloar (1949) proposed a

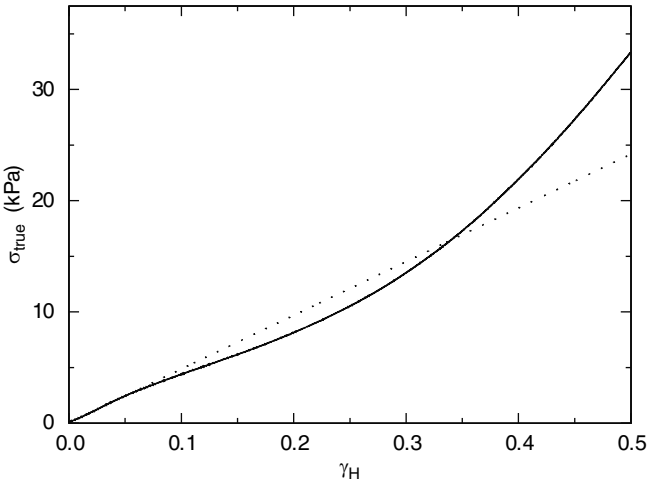

Fig. 18 Dependence of the true stress [$\sigma_{true} = \sigma(1-\varepsilon)$] on the Hencky strain [$\gamma_H = -\ln(1-\varepsilon)$] for saturated hydrogels from *L. hyperborea*. The *dotted line* represents the continuation of the linear stress-strain relationship. (Donati I and Paoletti S, unpublished data)

non-Gaussian network elasticity approach, thus based on the finite extensibility of the polymer chains, that adequately explained the nonlinear behavior.

Different investigations have focused on the large deformation properties of alginate gels (Mitchell and Blanshard 1976; Mitchell 1980; Mancini et al. 1999a; Zhang et al. 2005, 2007). In all cases, the presence of ion-induced junctions – which are by far larger that the pointlike cross-links of the natural rubber networks – is considered the main factor responsible of the upturn of the stress-strain curve and in general for the large deformation properties of alginate networks. Zhang et al. (2007) have recently proposed the possible direct involvement of junctions in the strain hardening of alginate gels. According to their model, the overall stress, σ, can be described as being composed of two contributions:

$$\sigma = \sigma_C + \sigma_J, \tag{25}$$

where σ_C is the stress arising from the (entropic) deformation of elastically active chains and σ_J is the stress produced by the (energetic) deformation of the junctions.

In practice, at small strains the physical (ion-induced) junctions will not be deformed and a purely entropic description of the system holds. In contrast, when a large strain is reached, the stress will be transmitted to the network junctions deforming them and, eventually, causing their breakage (Zhang et al. 2007). As such, the rod-like junction zones contribute to a deformation-dependent stress which is the main cause of the strain-hardening of the hydrogel. This latter contribution has been described by the mathematical model of the finite deformation elasticity of gels formed from stiff, rodlike polymer chains (Doi and Kuzuu 1980), which predicts a nonlinear stress-strain curve dominated by an energetic (enthalpic) contribution.

In the case of an alginate network, the fracture gel stress, i.e., the stress required to cause network breakage, can be safely correlated with the strength and number of junctions per unit volume. However, there is no direct correlation between the rupture strength and the elastic modulus (Mitchell and Blanshard 1976; Mitchell 1980). In fact, it has been reported that hydrogels from M-rich alginates, despite their lower elastic modulus, typically show a higher rupture strength than those obtained from G-rich alginates. Therefore, M-rich alginates are recognized to be more elastic, while G-rich alginates form more brittle gels. The length of the junction is also correlated with the rupture strength, as it is easily expected that upon application of the stress the shorter the junction is, the lower is the stress required to break it. After the breakage of the junction, the stress will be transferred to the neighboring junctions, eventually accelerating gel failure.

It is interesting to notice that for alginate gels the fracture stress depends upon both the divalent cation used and the polysaccharide concentration, while the fracture strain is not apparently affected by these two parameters. Moreover, alginate hydrogels being viscoelastic materials, the fracture characteristics are affected by the deformation rate (Zhang et al. 2005). However, the fracture stress, in contrast to the elastic modulus of the alginate gels, does not become constant above a certain molecular weight (Mitchell 1979).

An empirical characterization of the behavior of alginate gels at large deformations has been proposed recently (Mancini et al. 1999b). In particular, the stress (σ)–strain (ε) curves have been shown to be accurately reproduced by a power equation (Peleg and Campanella 1989):

$$\sigma = \varphi \varepsilon^{p}, \tag{26}$$

where φ is the rigidity constant that represents a measure of stiffness, while p is the degree of concavity. When $p = 1$, Eq. 26 reduces to Hooke's law (and φ coincides with the modulus of elasticity), while for $p > 1$ or $p < 1$, an upward or downward concavity, respectively, is detected. In the case of alginate, n was found to be approximately 2.17 independent of the alginate composition and concentration (Mancini et al. 1999b). Equation 26 was also found to hold at the gel fracture point, while the work for rupture was found to be very well represented by the following relation (Mancini et al. 1999b):

$$L_{\mathrm{R}} = \int_0^{\sigma_{\mathrm{R}}} \sigma \, \mathrm{d}\varepsilon = k \frac{\varepsilon_{\mathrm{R}}^{n+1}}{n+1}, \tag{27}$$

where σ_{R} and ε_{R} are the stress and strain at failure, respectively.

Mitchell and Blanshard (1976) discussed the viscoelastic behavior of the alginate gels by means of creep compliance measurements. The experimental curves were satisfactorily modeled using a combination of one Maxwell and two Voigt elements. From such measurements, the authors concluded that the cross-links in the hydrogel were not permanent but, upon the application of sufficiently high stresses, displayed the tendency to move and, eventually, to break. These reorientations did not seem to be present when small deformations were applied, but rather they could be regarded as a characteristic of the large deformation range.

Recently, creep compliance measurements (Fig. 19) were used to point to a possible role of long MG-block sequences in alginate gels. The experimental curves were modeled accurately by means of one Maxwell and one Voigt model and the Newtonian viscosity, η_{N}, was obtained for alginate gels prepared under different experimental conditions (Eq. 28).

$$J(t) = J_0 + J_1 \left(1 - e^{-t/\tau}\right) + \frac{t}{\eta_{\mathrm{N}}}, \tag{28}$$

where $J(t)$ is the measured compliance, J_0 and J_1 are the compliance values of the Maxwell and Voigt springs, respectively, and τ is the retardation time associated with the Voigt element.

It is to be noted that upon treatment of natural alginate samples with the AlgE4 epimerase, which causes an elongation of the MG-blocks, an increase of η_{N} was detected. This effect was traced back to the presence of extended secondary MG/MG junctions which, by bridging alternating sequences in the gel network via an ion-mediated mechanism, enhance the frictional forces between polymer chains, reducing their slipping (Donati et al. 2005b).

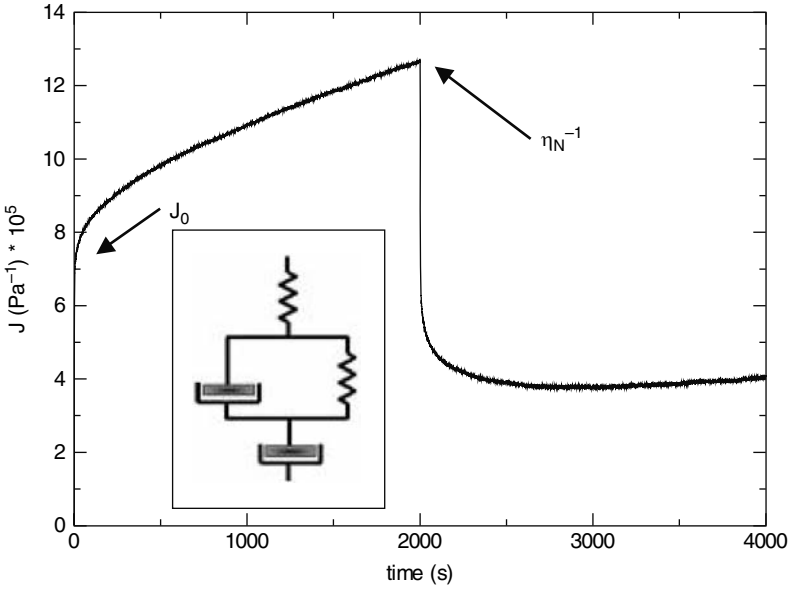

Fig. 19 Example of viscoelastic behavior (*creep compliance curve*) for *L. hyperborea* alginate. *Inset*: The combination of one Maxwell and one Voigt model used to fit the experimental data. (Adapted from Donati et al. 2005b)

6.4 Diffusion Properties

Diffusion properties of alginate hydrogels are determined by the pore size and the pore size distribution within the network. It has been reported that calcium-treated alginate forms networks characterized by a pore size between 50 and 1,500Å (Andresen et al. 1977).

Self-diffusion of small molecules is very little affected by the alginate hydrogel. In fact, the rates of self-diffusion of glucose and ethanol have been reported to be as high as about 90% of their corresponding values in water (Tanaka et al. 1984b). The diffusion of larger molecules, such as proteins, is somehow restricted, although proteins with molecular weight as high as 300,000 are able to diffuse out of the hydrogel with a rate that depends on their molecular size (Tanaka et al. 1984b; Martinsen et al. 1989).

The diffusion of molecules through alginate hydrogels depends upon both the cross-linking ion concentration and the polymer concentration and composition. The use of a higher concentration of divalent ions, such as calcium, will induce the formation of a more compact gel with a possible collapse of some junctions. This leads to a pore size increase and thus greater diffusion of the protein (Gåserød et al. 1998). At variance, the replacement of Ca^{2+} ions by Ba^{2+} ions does not seem to lead to modifications in the permeability of the alginate hydrogels (Mørch et al. 2006).

An increase of the polysaccharide concentration induces a reduction in the pore size, while the increase in the content of the G residues in the polymer chain has the opposite effect. The latter effect has been correlated with the condensation of junctions upon the addition of the divalent ion which is favored by the presence of long G-blocks (Martinsen et al. 1989; Gåserød et al. 1998).

The coating of alginate with a polycation, such as poly(L-lysine), chitosan and polyethyleneimine (Tanaka et al. 1984a; Kulseng et al. 1997; Gåserød et al. 1999), enhances the polymer density on the surface of the bead, thus limiting the diffusion of the proteins. In particular, a proper polycation coating of alginate bead leads to a selective capsule permeability, thus allowing the free diffusion of proteins as small as insulin, but preventing diffusion of antibodies through the capsule membrane. These results represent the basis for proposing alginate gel beads for the encapsulation of Langerhans islets for the treatment of type I diabetes.

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Alginate Production: Precursor Biosynthesis, Polymerization and Secretion

Bernd H.A. Rehm

Contents

Abstract The activated intracellular precursor of alginate biosynthesis is GDP-mannuronic acid. Carbon sources are oxidized to acetyl-CoA, which enters the citric acid cycle, providing the intermediate oxaloacetate. Oxaloacetate is converted via gluconeogenesis into fructose 6-phosphate. The central metabolite fructose 6-phosphate derived from gluconeogenesis is then converted to the activated alginate precursor GDP-mannuronic acid employing alginate-specific biosynthesis enzymes. This conversion requires four enzymatic steps catalysed by bifunctional phosphomannose isomerase:GDP-mannose pyrophosphorylase, phosphomannomutase and GDP-mannose dehydrogenase. GDP-mannuronic acid is polymerized to alginate by a membrane-anchored glycosyltransferase which is presumably represented by Alg8, which has been suggested to be a subunit of a multiprotein complex spanning the cytoplasmic membrane (Alg44), the periplasm (AlgX, AlgK, AlgG, AlgL) and the outer membrane (AlgE). These periplasmic proteins have been proposed to form

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a scaffold guiding the nascent alginate chain through the periplasm to the alginate-specific channel protein AlgE in the outer membrane. AlgE has been suggested to facilitate export of alginate through the outer membrane. The extended periplasmic C terminus of membrane-anchored Alg44 shows similarities to membrane fusion proteins and might colocalize the alginate polymerase (Alg8) with the export protein AlgE. The cytoplasmic N-terminal loop of Alg44 comprises a PilZ domain required for binding of the secondary messenger bis-(3′–5′)-cyclic dimeric guanosine monophosphate, the binding of which was found to be required for alginate production

1 Introduction

Alginates represent a family of non-repeating unbranched exopolysaccharides composed of various amounts of (1–4)-linked β-D-mannuronic acid and its C5-epimer α-L-guluronic acid. These sugar acids are distributed in blocks of continuous β-D-mannuronic acid residues (M-blocks), α-L-guluronic acid residues (G-blocks), or as alternating residues (MG-blocks) (Rehm and Valla 1997; Rehm 2005b). Alginates are synthesized by brown seaweeds and by bacteria belonging to the genera *Pseudomonas* and *Azotobacter* (Rehm 2002). The distribution of comonomer residues and in particular the presence of G-blocks were found to be similar in algal alginates and in alginates derived from *Azotobacter vinelandii*, whereas alginates from pseudomonads are different in that they lack G-blocks (Skjak-Braek et al. 1986). These structural differences lead to different material properties reflecting the different biological functions of the alginates (see "Material properties of alginates" by Donati and Paoletti, this volume). In brown algae and *Azotobacter* cysts (a dormant stage) alginate serves as a cell wall constituent, whereas in *Pseudomonas* it contributes to the biofilm matrix. In the opportunistic human pathogen *Pseudomonas aeruginosa* alginate is considered a virulence factor contributing to the formation of persistent biofilms after infection of the lungs of cystic fibrosis (CF) patients (Kobayashi 2005). Knowledge of alginate biosynthesis is most advanced in *P. aeruginosa* and hence in this chapter I will focus on this bacterium.

The cytosolic biosynthesis steps leading to the formation of the activated precursor GDP-mannuronic acid have been extensively investigated and are well understood; however, the actual polymerization and secretion of alginate is poorly understood. Recent studies suggested the formation of a multiprotein complex spanning the cytoplasmic membrane, the periplasm as well as the outer membrane (Jain and Ohman 2005; Oglesby et al. 2008; Remminghorst and Rehm 2006b, c).

Future research dedicated to unravelling these final steps of alginate production will be motivated by medical interest with respect to the design of alginate biosynthesis inhibitors as well as by biotechnological applications implementing the engineering of improved bacterial production organisms. Bacterial fermentation using production organisms obtained by metabolic engineering approaches should enable the biotechnological production of a wide range of defined alginates suitable for high-value applications as biomaterials in medicine (Rehm 2005; Rehm 2009). The unique material properties of alginates have already led to a variety of industrial applications, such

as stabilizing, thickening and gelling agents in food production and immobilization of cells in pharmaceutical and biotechnology industries (Paul et al. 1986). Commercial alginates are currently exclusively produced from brown seaweeds.

2 Alginate Precursor Biosynthesis

The alginate precursor GDP-mannuronic acid is synthesized in the cytosol by stepwise conversion of the central metabolite fructose 6-phosphate (Fig. 1). Three alginate-specific enzymes catalyse the four biosynthesis steps and these enzymes

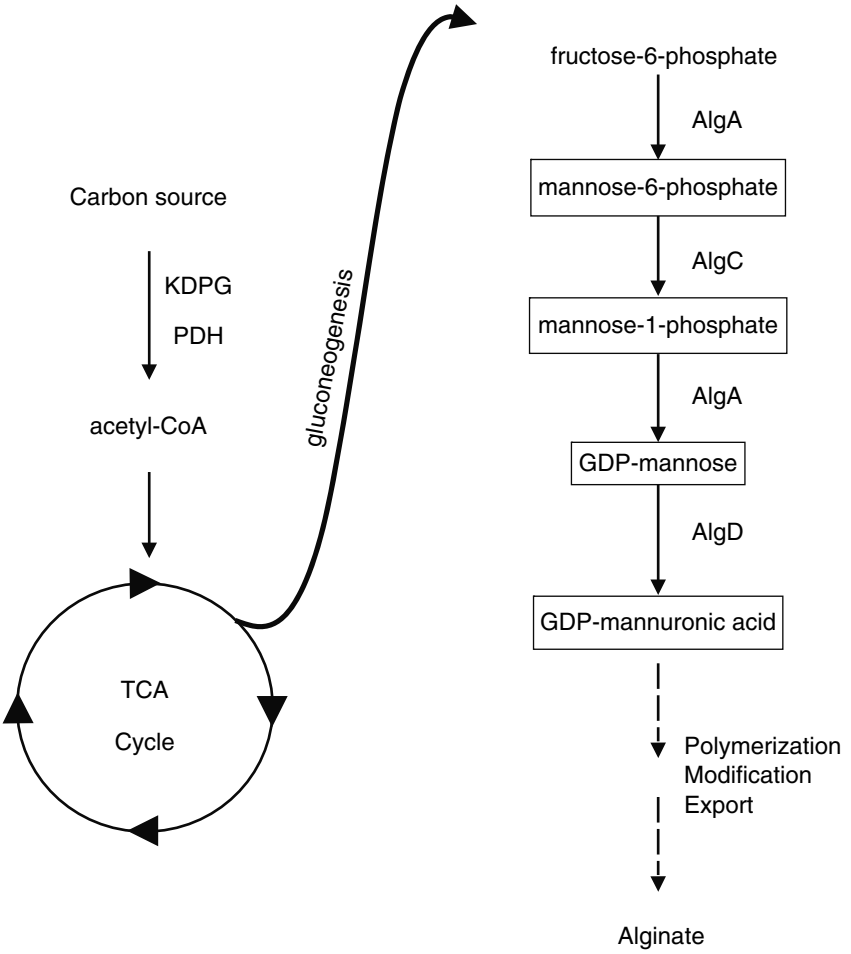

Fig. 1 Biosynthesis route of alginate in bacteria. *KDPG* ketodeoxyphosphogluconate pathway (Entner–Doudoroff pathway), *PDH* pyruvate dehydrogenase, *AlgA* phosphomannose isomerase–GDP-mannose pyrophosphorylase, *AlgC* phosphor-mannomutase, *AlgD* GDP-mannose dehydrogenase. *Boxed* intermediates are precursors of alginate. *Dashed arrows* indicate unknown biosynthesis steps

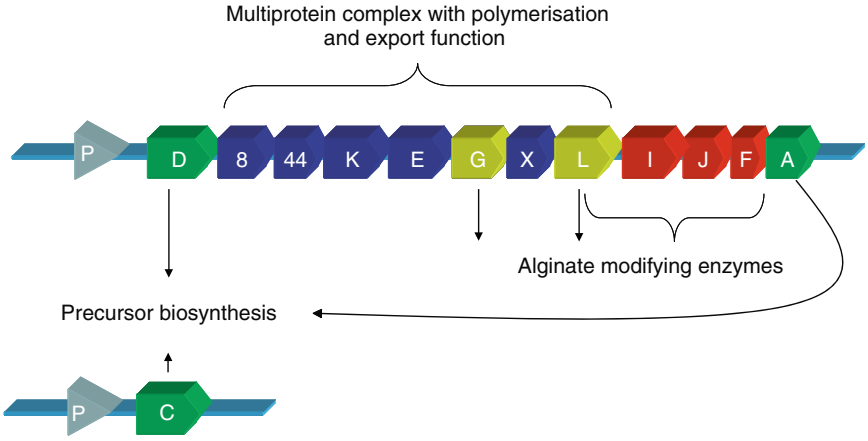

Fig. 2 The alginate biosynthesis operon and functional assignment of the genes

have been extensively characterized. Two of the genes (*algD*, *algA*) encoding these enzymes are localized in the alginate biosynthesis gene cluster and one gene (*algC*) is not colocalized (Fig. 2).

2.1 Alginate Biosynthesis Genes

The first alginate biosynthesis genes were discovered in *P. aeruginosa* motivated by the medical significance of this bacterium as an opportunistic human pathogen which often establishes chronic infections in the lung of CF patients (May et al. 1991). Alginate as a virulence factor contributes to the establishment of persistent biofilms after infection (Gacesa 1998; Russell and Gacesa 1988).

The alginate biosynthesis genes and their function have been compiled in a recent review (Rehm 2002). Seminal research by Darzins and Chakrabarty (1984) created the foundation for the identification and analysis of alginate biosynthesis genes in *P. aeruginosa*. The whole genome sequence of *P. aeruginosa* is now available and has already enabled functional genomic studies with respect to alginate biosynthesis (Lizewski et al. 2004; Stover et al. 2000). At least 24 genes were found to be directly involved in alginate biosynthesis in *P. aeruginosa*, with a few genes encoding proteins not exclusively involved in alginate biosynthesis (Table 1) (Gaona et al. 2004; Goldberg et al. 1993; Ledgham et al. 2003; Lizewski et al. 2004; Olvera et al. 1999; Wu et al. 2004; Ye et al. 1994). Twelve biosynthesis genes are colocalized in the alginate biosynthesis gene cluster, the transcription of which is regulated by one promoter upstream of the *algD* gene (Fig. 2) (Chitnis and Ohman 1993). How alginate biosynthesis gene transcription is regulated is discussed in "Alginate gene regulation" by Ohman in this volume.

Table 1 The alginate biosynthesis genes from *Pseudomonas aeruginosa*

Gene	Gene product/function or putative function[a]
algD	GDP-mannose dehydrogenase
Alg8	Glycosyltransferase/polymerase-export function?
Alg44	c-di-GMP binding-activation/membrane fusion protein?
algK	Periplasmic protein/multiprotein complex assembly
algE	Outer-membrane porin/alginate export?
algG	Mannuronan C-5-epimerase/biosynthesis?
algX	Periplasmic protein with high sequence similarity to *algJ*/scaffold protein sequestering MucD
algL	Alginate lyase/biosynthesis?
PA1167	Alginate lyase (polyguluronate lyase)/biosynthesis?
algI	O-Acetylation
algJ	O-Acetylation
algF	O-Acetylation
algA	Phosphomannose isomerase/GDP-mannose pyrophosphorylase
algB	Member of *ntrC* subclass of two-component transcriptional regulators (cognate sensor kinase is KinB)
algC	Phosphomannomutase
algH	Unknown function
algR	Regulatory component of two-component signal transduction system (cognate sensor kinase is FimS)
algQ	Histone-like transcriptional regulator binds to *algD* promoter
algP	Histone-like transcriptional regulator binds to *algD* promoter
algZ	AlgR cognate sensor (alginate and motility regulator)
algU	Homologous to *Escherichia coli* σ^E global stress response factor
mucA	Anti σ factor
mucB	Anti σ factor?
mucC	Regulator?
mucD	Homologous to *E. coli* serine protease DegP
algW	Homologous to *E. coli* serine protease DegS
mucP	Homologous to *E. coli* RseP protease involved in activation of AlgU via regulated intramembrane proteolysis cascade
mucE	Periplasmic or outer-membrane protein involved in activation of AlgU via regulated intramembrane proteolysis cascade
mucR	Alginate specific c-di-GMP synthesizing enzyme

Modified according to Rehm (2002)
c-di-GMP bis-(3′–5′)-cyclic dimeric guanosine monophosphate
[a]Putative function indicated by ?

The alginate biosynthesis genes encoding enzymes catalysing the synthesis of the precursor GDP-mannuronic acid from fructose 6-phosphate have all been functionally assigned and characterized (Table 1, Fig. 1) (Rehm and Valla 1997).

2.2 GDP-Mannuronic Acid Biosynthesis

Alginate biosynthesis enzyme activities were initially assessed in the brown alga *Fucus gardneri* by Lin and Hassid (1966) and 10 years later in *A. vinelandii* (Pindar and Bucke 1975). The central metabolite fructose 6-phosphate derived

from gluconeogenesis was found to be a precursor for the synthesis of GDP-mannuronic acid. The alginate-specific and bifunctional enzyme phosphomannose isomerase (PMI)/GDP-mannose pyrophosphorylase (GMP), encoded by the *algA* gene, catalyses the initial and third steps of GDP-mannuronic acid synthesis starting from fructose 6-phosphate (Fig. 1) (May et al. 1994). The PMI-catalysed reaction channels the fructose 6-phosphate towards alginate biosynthesis via the formation of mannose 6-phosphate (Shinabarger et al. 1991). Mannose 6-phosphate is then converted to mannose 1-phosphate by the phosphomannomutase (AlgC) (Zielinski et al. 1991). The AlgC enzyme has been found to show additionally phosphoglucomutase activity and its role in rhamnolipid and lipopolysaccharide biosynthesis was demonstrated experimentally (Goldberg et al. 1993; Olvera et al. 1999; Ye et al. 1994). The GMP activity of AlgA then converts the mannose 1-phosphate to GDP-mannose, with concomitant hydrolysis of GTP (Shinabarger et al. 1991). AlgA has been studied in more detail and amino acids required for the GMP enzyme activity were identified through site-directed mutagenesis of the *algA* gene (May et al. 1994). Replacement of Lys-175 by arginine, glutamine or glutamate resulted in a 470–3,200-fold increased K_m for mannose 1-phosphate when compared with the wild-type enzyme. These results suggested a role of Lys-175 in the binding of the substrate mannose 1-phosphate. Replacement of Arg-19 by glutamine, histidine or leucine resulted in a fourfold to sevenfold increased K_m for GTP when compared with the wild-type enzyme. Hence, Arg-19 might be involved in the binding of GTP. Limited proteolysis analysis showed that the C terminus is essential for PMI activity but not for GMP activity, suggesting that the bifunctional PMI/GMP protein is composed of two independent enzymatic domains (May et al. 1994).

GDP-mannose dehydrogenase (GMD), encoded by the *algD* gene, is a key enzyme in the biosynthesis of alginate and catalyses the almost irreversible oxidation of GDP-mannose to GDP-mannuronic acid. This is the committed step in alginate biosynthesis and it was shown to represent the metabolic bottleneck in alginate-overproducing mucoid strains of *P. aeruginosa* (Tatnell et al. 1993, 1994). Thus, AlgD has been conceived as a potential target to inhibit alginate production and hence to combat *P. aeruginosa* infections. GMD inhibitors have been identified and GMD inhibition has been shown to increase the susceptibility of a mucoid strain of *P. aeruginosa* to tobramycin, which is widely used for the treatment of CF lung infections (Snook et al. 2003). Thus, GMD inhibitors can be conceived as potential drugs which impair alginate production and the formation of characteristic biofilms and thereby strongly aid the efficiency of antibiotics. The absence of enzymes corresponding to AlgD in humans makes these inhibitors very specific by presumably avoiding severe side effects. GMD belongs to a small family of NAD^+-dependent four-electron-transfer dehydrogenases, which include UDP-glucose dehydrogenase (UGD), histidinol dehydrogenase and 3-hydroxy-3-methylglutaryl-CoA reductase. UGD and GMD have been proposed to share a similar reaction mechanism by using a single active site to catalyse the two-step conversion of an alcohol to the respective acid via a thiohemiacetal intermediate. The structure of UGD from *Streptococcus pyogenes* has been resolved and showed that the enzyme forms a dimer (Campbell et al. 2000). Biochemical characterization of the *P. aeruginosa* GMD revealed

an allosteric and cooperative behaviour, which suggested that the enzyme at least forms an oligomer composed of six subunits (Naught et al. 2002; Roychoudhury et al. 1989). In a more recent study, the crystal structure of the *P. aeruginosa* GMD in complex with its cofactor NAD(H) and reaction product, GDP-mannuronic acid, was determined at a resolution of 1.55 Å (Snook et al. 2003). The crystal structure was used to shed light on the multistep reaction catalysed by GMD. The reaction comprises four steps: (1) oxidation of a hydroxyl to an aldehyde; (2) nucleophilic attack by a thiol to form the thiohemiacetal intermediate; (3) oxidation of the intermediate to a thioester; (4) release of the product by hydrolysis. On the basis of amino acid sequence alignments and a structural comparison with UGD, the cysteine at position 268 has been proposed as the active-site nucleophilic thiol (Campbell et al. 2000; Roychoudhury et al. 1992). The significance of this residue was confirmed by the sensitivity of GMD to thioreactive agents (Shankar et al. 1995). Accordingly, the crystal structure of GMD showed that the Cys-268 thiol group and the oxygens of the carboxylate group of the mannuronic acid could be within 2.4 Å. Thus, the thiol group as a potent nucleophile presumably serves as the active site required for formation of the proposed thiohemiacetal intermediate.

3 Alginate Polymerization and Secretion

The polymerization of exopolysaccharides usually requires the activity of membrane-bound glycosyltransferases which catalyse the transfer of an activated sugar moiety onto a receptor molecule while forming a glycosidic bond. The Wz-dependent capsular polysaccharide polymerization/secretion pathways in *Escherichia coli* have been extensively studied and serve as a model for the biosynthesis of various bacterial exopolysacharides (Whitfield 2006). Repeating sugar units are synthesized attached to a lipid carrier by glycosyltransferases at the cytoplasmic membrane. The lipid carrier linked repeating unit is then transferred across the cytoplasmic membrane and polymerization occurs at the periplasmic side of the membrane. The polymerized repeating units are subsequently secreted through specific pores in the outer membrane (Whitfield 2006). However, alginate represents a non-repeating exopolysaccharide and no alginate-related lipid carrier intermediate could be identified so far. Hence, alginate polymerization and transfer across the membrane might be based on a different molecular mechanism. The polymerization and secretion of alginate are barely understood and extensive research will be required to shed light on these molecular processes (Rehm and Valla 1997; Rehm 2005).

3.1 Alginate Polymerization

The polymerization of mannuronic acid residues to alginate could resemble the lipid carrier independent cellulose synthesis in bacteria such as *Gluconacetobacter xylinus* (Ross et al. 1991). The cellulose synthase comprises a multiprotein complex of 420

kDa which resides in the cytoplasmic membrane and catalyses the processive polymerization of glucose by using UDP-glucose as a substrate. New findings related to bacterial cellulose biosynthesis were recently summarized by Valla et al. (2009).

The protein Alg8, presumably encoding a glycosyltransferase, and Alg44 were predicted to be transmembrane proteins as well as subunits of the alginate polymerase (Oglesby et al. 2008; Remminghorst and Rehm 2006b, c). Recently, Alg8 was identified as a key membrane protein for the production of alginate and multiple copies of Alg8 resulted in significant overproduction of alginate (Remminghorst and Rehm 2006c). The same study showed for the first time the in vitro synthesis of alginate by using an envelope fraction of *P. aeruginosa*. Interestingly, separation of cytoplasmic membrane and outer membrane abolished alginate polymerization activity, which strongly suggested a coordinated polymerization and secretion as well as the requirement of a multiprotein complex spanning the cytoplasmic membrane, the periplasm and the outer membrane (Fig. 3) (Remminghorst and Rehm 2006c). Additionally, the isogenic *alg8* deletion mutant did not secrete uronic acids, which were proposed to be derived from alginate lyase (AlgL) mediated degradation of misguided and unprotected alginate when found to be produced by isogenic *algK*, *algX* and *algG* (epimerase gene) deletion mutants, respectively. Interestingly, an AlgL gene deletion mutant of *P. aeruginosa* showed a swollen periplasm shortly after induction of alginate production, which suggested that AlgL does not only degrade misguided alginate but also contributes to the proposed protein scaffold (Fig. 3) (Jain and Ohman 2005). In contrast to these proposed scaffold-forming proteins (AlgK, AlgX, AlgG, AlgL), which were proposed to play a role in guidance

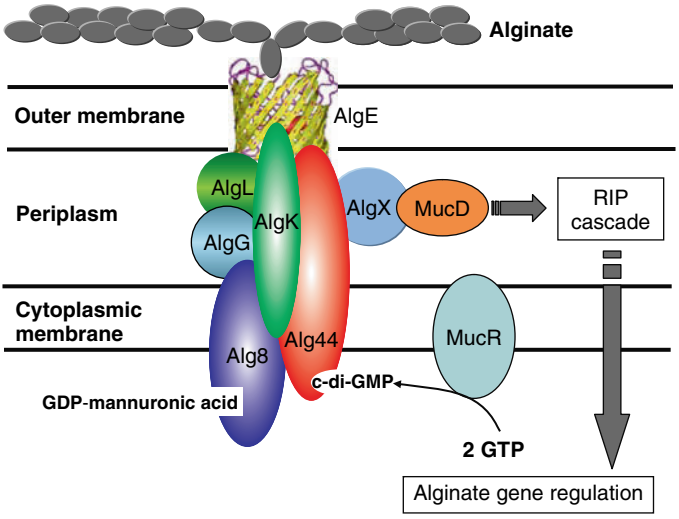

Fig. 3 Model of the proposed multiprotein complex involved in alginate polymerization/export. *c-di-GMP* bis-(3′–5′)-cyclic dimeric guanosine monophosphate, *RIP* regulated intramembrane proteolysis, *Alg* alginate gene (for a description see Table 1)

and protection of the nascent alginate chain, Alg8 seems to be directly involved in alginate polymerization. Hydrophobic cluster analysis of Alg8 indicated similarities to processive β-glycosyltransferases (e.g. cellulose or chitin synthase) (Saxena et al. 1995), which are transmembrane proteins that catalyse the formation of polysaccharide or oligosaccharide chains by transferring the sugar residue from an activated donor substrate to a growing acceptor molecule. These β-glycosyltransferases are related on the basis of their sequence pattern and especially with respect to the presence of conserved motifs and catalytic residues (Saxena et al. 2001). The catalysis is believed to involve a general base, which assists in the deprotonation process of the nucleophilic hydroxyl of the acceptor, and an oxocarbenium ion like transition state similar to that proposed for glycosidases (Ünligil et al. 2000). A threading model of Alg8 was developed on the basis of the crystal structure of SpsA, a glycosyltransferase involved in spore coat polysaccharide formation of *Bacillus subtilis* (Charnock and Davies 1999; Remminghorst and Rehm 2006c). In a recent study, a refined model for the membrane topology of Alg8 was developed using PhoA (alkaline phosphatase) and LacZ (β-glactosidase) reporter enzyme fusions, respectively (Oglesby et al. 2008; Remminghorst et al. 2009). Evidence for a large cytoplasmic loop containing the active domains and five transmembrane domains as well as for a short periplasmic loop was obtained. The presence of a cytoplasmic loop was further confirmed by successful production of only the cytoplasmic domain as soluble protein (Remminghorst et al. 2009). The C-terminal transmembrane domain of Alg8 was found to be essential for the in vivo polymerization reaction. Conserved amino acid residues of Alg8 were subjected to site-specific mutagenesis. The predicted active-site residues in the D133, D188-x-D190, L336xxR339W340 motif as well as in D295/D296 and K297 were found to be required for in vivo polymerization activity (Oglesby et al. 2008; Remminghorst et al. 2009).

Alg44 was recently demonstrated to be a membrane protein with a periplasmic C terminus and was found to be essential for alginate polymerization (Merighi et al. 2007; Remminghorst and Rehm 2006b). A refined membrane topology model of Alg44 was obtained by constructing further PhoA fusions which showed a central transmembrane domain (amino acid residues 159–177) preceded by an N-terminal transmembrane domain (amino acid residues 1–30) presumably serving as a membrane anchor (Oglesby et al. 2008). The N-terminal cytoplasmic loop comprises the PilZ domain, which is required for binding of the secondary messenger bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (Fig. 3). The PilZ domain in Alg44 is essential for alginate biosynthesis, which was recently verified experimentally by assessing the capability of modified and truncated Alg44 to restore alginate biosynthesis in an *alg44*-negative mutant of *P. aeruginosa* (Merighi et al. 2007). Thus, similar to bacterial cellulose synthesis, c-di-GMP has been verified as an important activator of bacterial alginate biosynthesis which adds an additional posttranslational regulatory layer to the already complex regulatory network which controls alginate gene expression. Interestingly, more than 33 enzymes (diguanylate cyclases, phosphodiesterases) have been found to be involved in the control of intracellular levels of c-di-GMP in *P. aeruginosa* (Kulasakara et al. 2006). It has been proposed that these enzymes contrib-

ute to the formation of localized c-di-GMP pools mediating differential regulation of various responses, such as flagella biogenesis and exopolysaccharide biosynthesis. To identify the diguanylate cyclase which provides a c-di-GMP pool for Alg44 and hence for activation of alginate, a putatively membrane anchored diguanylate cyclase PA1727 (designated MucR) which also comprises a sensor domain was analysed in more detail. An isogenic knockout of this gene caused a 38-fold decrease in alginate production in *P. aeruginosa*, which suggested that this diguanylate cyclase catalyses c-di-GMP formation required for activation of alginate biosynthesis (Hay et al., 2009). The extended periplasmic C terminus of Alg44 shows similarities to membrane fusion proteins such as MexA, a membrane-bridging protein involved in the multidrug efflux system of *P. aeruginosa* (Akama et al. 2004). Hence, Alg44 could function as a subunit of a periplasmic protein scaffold and/or link Alg8 in the cytoplasmic membrane with the outer membrane protein AlgE (Remminghorst and Rehm 2006b). Experimental evidence for a function of the C terminus of Alg44 in colocalization and stabilization of a protein complex comprising Alg8 in the cytoplasmic membrane and AlgE in the outer membrane was obtained by detecting reduced levels of AlgE in an *alg44*-negative mutant (Oglesby et al. 2008). A C-terminal deletion of only 24 amino acid residues of Alg44 did not enable restoration of alginate production, further suggesting that Alg44 might act as a copolymerase essential for alginate polymerization.

AlgK and AlgX are periplasmic proteins and the respective deletion mutants showed secretion of free uronic acids presumably owing to AlgL-catalysed degradation of misguided periplasmic alginate (Gutsche et al. 2006; Jain and Ohman, 1998; Jain and Ohman 2005).

A pull-down experiment with Strep-tag-labelled AlgX produced in an isogenic *algX*-negative mutant of *P. aeruginosa* resulted in copurification of MucD, which suggested that AlgX specifically interacts with the serine protease MucD (Gutsche et al. 2006). However, no evidence for interaction with the other proposed subunits of the alginate polymerase/secretion complex could be obtained (Gutsche et al. 2006). Since knockout mutants of the *mucD* gene resulted in a mucoid phenotype, MucD was proposed as a negative regulator of alginate biosynthesis (Boucher et al. 1996; Wood and Ohman 2006; Yorgey et al. 2001). The negative regulator function was recently shown to require the serine protease activity (Wood and Ohman 2006). AlgX as a subunit of the alginate polymerase/secretion complex might sequester MucD in the periplasm and thereby interfere with its negative regulatory function. In a recent study, a regulated intramembrane proteolysis (RIP) cascade in the envelope of *P. aeruginosa* was proposed that ultimately leads to degradation of membrane-anchored MucA. Degradation of MucA causes the release of sequestered alginate-specific sigma-factor AlgU, which then enables transcription of the alginate biosynthesis genes (Qiu et al. 2007) (see "Alginate gene regulation" by Ohman, this volume, for further details). In the absence of AlgX, MucD could interfere with RIP activation of alginate gene transcription by degrading proteins such as AlgW, MucP, MucE and MucB (Fig. 3). These proteins have been proposed to be involved in the RIP cascade (Qiu et al. 2007).

The AlgK sequence showed an apparent signal peptide characteristic of a lipoprotein. AlgK-PhoA as well as AlgK-β-lactamase fusion proteins were found to show reporter enzyme activity, which suggested a periplasmic subcellular localization while they were probably anchored in the cytoplasmic membrane (Aarons et al. 1997; Jain and Ohman 1998). Overall the amino acid sequence of AlgK does not indicate a specific function of the protein in alginate polymerization/secretion. However, the AlgK sequence shows four Sel1-like repeats (SLR) which are characteristic of proteins forming modular architectures and which all seem to serve as adaptor proteins for the assembly of macromolecular complexes (Mittl and Schneider-Brachert 2007). Thus, AlgK might play a key role in the assembly of the alginate polymerization/secretion multiprotein complex. Recently, AlgK was subjected to a structural analysis in an attempt to verify the presence of the putative SLR motifs and provide insight into the function of AlgK. AlgK could be crystallized and preliminary X-ray data were obtained (Keiski et al. 2007). On the basis of density calculations, the authors estimated that four molecules of the protein are present in the asymmetric unit (Keiski et al. 2007). Size-exclusion chromatography results using purified AlgK provided evidence that AlgK forms a dimer in solution, suggesting that two dimers constitute the asymmetric unit in crystallized AlgK.

AlgX, AlgK, Alg44, AlgG together with AlgL were proposed to form a protein scaffold in the periplasm surrounding the nascent alginate chain and guiding the polymer to AlgE in the outer membrane (Fig. 3) (Gutsche et al. 2006; Jain and Ohman 1998, 2005; Jain et al. 2003; Remminghorst and Rehm 2006a, b), while AlgL might also clear the periplasm from misguided alginate and AlgG introduces guluronic acid residues (Bakkevig et al. 2005). Interestingly, except for the AlgX-MucD interaction, none of the proposed protein-protein interactions in the alginate polymerization/secretion multiprotein complex were confirmed experimentally.

3.2 Alginate Export

An early comparative analysis of outer-membrane protein profiles from various mucoid and non-mucoid *P. aeruginosa* strains indicated the presence of a 54-kDa protein exclusively found in mucoid strains (Grabert et al. 1990); hence, it was proposed that this outer-membrane protein could play a role in alginate biosynthesis. Shortly after the detection of the mucoid-specific 54-kDa outer-membrane protein, the *algE* gene in *P. aeruginosa* was identified as an essential gene for alginate production and which is also part of the alginate biosynthesis gene cluster (Chu et al. 1991). In the same study the *algE* gene product was identified as an insoluble presumably membrane bound protein with an apparent molecular mass of 54 kDa. Purification of the 54-kDa protein after solubilization with octylglucoside enabled N-terminal sequencing which confirmed that the 54-kDa protein corresponded to AlgE (Grabert et al. 1991).

The fact that AlgE is strictly associated with the alginate-overproducing mucoid phenotype of *P. aeruginosa* triggered a study investigating the immunogenicity of

AlgE as well as the use AlgE as an antigen for antibody capture in diagnostic applications (Rehm et al. 1994b). Purified and native AlgE was injected into rabbits and a strong antibody response 9 weeks after injection suggested a strong immunogenicity of AlgE (Rehm et al. 1994b). Hence, it was conceived that AlgE could be used for antibody detection in sera from CF patients. An antibody-capture ELISA with AlgE as an antigen was established and the analysis of 41 sera from CF patient showed a strong correlation with the infection status of the CF patients (Rehm et al. 1994b). None of the 23 control sera from healthy humans showed significant levels of anti-AlgE antibodies. Interestingly, CF patients who became infected only a few weeks before the blood samples were taken already showed a significant anti-AlgE antibody titre, suggesting the conversion to the mucoid form occurs early after infection with non-mucoid forms (Rehm et al. 1994b). Since CF patients are initially infected by non-mucoid *P. aeruginosa* and the mucoid form, which correlates with the establishment of a chronic infection, emerges in the CF lung during infection, the AlgE-based ELISA might represent an important differential diagnostic tool to assess the infection status. The infection status significantly informs the antibacterial treatment regimen, because mucoid *P. aeruginosa* shows a different and mainly increased antibiotic resistance, when compared with the non-mucoid variant. The strong AlgE immunogenicity also suggested an application of this mucoid-specific outer-membrane protein as part of a multicomponent experimental vaccine which could mediate protection against colonization with mucoid forms of *P. aeruginosa* (Rehm et al. 1994b).

AlgE is only represented as a minor protein with a low copy number in the outer membrane. To produce significant amounts of AlgE for functional analysis, *algE* was overexpressed in *E. coli* using a T7-promoter-based overexpression system. Recombinant and mature AlgE was localized to the outer membrane of *E. coli* by using immunoblotting analysis. This analysis suggested that the signal peptide was properly cleaved off during the secretion process, while traces of unprocessed AlgE were still detectable in the cytoplasmic membrane (Rehm et al. 1994a). Recombinant AlgE was solubilized from the *E. coli* outer membrane and purified by immobilized metal ion affinity chromatography followed by anion-exchange chromatography. Outer-membrane proteins have been described to function as general or specific porins, which form a β-barrel and facilitate the transport of various or specific compounds through the outer membrane (Buchanan 1999; Delcour 2002; Schulz 1996). Thus, to assess whether AlgE functions as such a channel protein it was subjected to electrophysiological analysis using planar lipid bilayers. These experiments demonstrated that AlgE can spontaneously incorporate into planar lipid bilayers while causing single-channel current fluctuations with a low mean conductance of 0.76 nS and a very short mean open-state lifetime of 0.7 ms (Rehm et al. 1994a). The electrophysiological analysis suggested that AlgE forms a channel protein in the outer membrane with unusual properties hitherto not described for other outer-membrane channel proteins (Delcour 2002). The AlgE channel was characterized in more detail by using differently sized cations and anions, respectively. Only the size of the anion had an impact on the mean single-channel conductance and increasing anion size lowered the mean single-channel conductance. These findings suggested that AlgE

forms an anion-specific channel which could facilitate the export of the anionic alginate through the outer membrane. This proposed function of AlgE was further supported by the finding that GDP-mannuronic acid can partially block the AlgE channel (Rehm et al. 1994a). Topological models of AlgE and its homologue AlgJ from *A. vinelandii* have been developed on the basis of secondary structure predictions, hydrophilicity analysis and known outer membrane protein structures, resulting in the proposal of a β-barrel consisting of 18 β-strands (Fig. 3) (Rehm et al. 1994a; Rehm 1996). Recently, homology modelling was used to generate a three-dimensional model of AlgE suggesting a pore diameter suitable for alginate export (Rehm 2002). These data supported the hypothesis that AlgE forms an alginate-specific pore which enables export of the nascent alginate chain through the outer membrane. The AlgE protein is the only outer-membrane protein which has been found to be required for alginate biosynthesis and which might be colocalized with the alginate polymerase by the membrane fusion protein function of Alg44.

4 Conclusion and Future Perspectives

Although the alginate biosynthesis gene cluster was identified in the late 1980s, the functional assignment of various genes presumably contributing to alginate polymerization/secretion is still at an early stage. Except for the AlgX–MucD interaction, none of the proposed protein-protein interactions required to form an envelope-spanning multiprotein complex have been confirmed experimentally. Further extensive research will be required to shed light on the molecular mechanisms underlying alginate polymerization and secretion as well as the c-di-GMP-mediated activation. The understanding of the final steps in alginate production might inform the design of specific inhibitors when considering the role of alginate as a virulence factor and/or it might help to overcome the production bottleneck when considering alginate as an important biopolymer for medical and biotechnological applications. Although commercial production of alginate relies almost entirely on cheap algal sources, the ability to engineer bacterial alginates will make continuous fermentative production using bacteria increasingly attractive. Knowledge of how alginate composition and molecular weight contribute to material properties and knowledge of what material properties are required to meet medical specifications will provide an enormous opportunity for the use of engineered bacteria for the production of tailor-made alginates.

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Bacterial System for Alginate Uptake and Degradation

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Contents

Abstract Most alginate-assimilating bacteria secrete degrading enzymes, i.e., alginate lyases, into the extracellular fraction or periplasm, and incorporate the resultant alginate oligosaccharides through their cytoplasmic membrane. The Gram-negative bacterium *Sphingomonas* sp. A1 can directly incorporate the polysaccharide into the cytoplasm, without degradation, through a mouthlike pit on the cell surface, periplasmic binding proteins, and an ATP-binding cassette importer in the cytoplasmic

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membrane. This uptake system (superchannel) is distinct from channels or transporters responsible for importing low molecular weight substrates. The constituent proteins are inducibly expressed and organized at the superchannel when flagellin homologues, as cell surface receptors, recognize the external alginate. Cytoplasmic alginate lyases with different substrate specificities and action modes help degrade the alginate into monosaccharides. The strain A1 superchannel can be transplanted to other sphingomonads through membrane engineering. This chapter reviews the bacterial system for alginate uptake and degradation by considering the structure and function of each molecule in the superchannel.

1 Introduction

Alginate is a linear polysaccharide produced by brown seaweed and certain bacteria. Two monosaccharides, β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G), constitute the polysaccharide molecules, and are arranged in three regions: polyM, polyG, and heteropolymeric random sequences (polyMG) (Gacesa 1988). Brown seaweed alginate is widely used in the food, chemical, and pharmaceutical industries as a stabilizing, thickening, or emulsifying reagent because the polymer chelates metal ions and forms a highly viscous solution (Jensen 1993). Oligosaccharides derived from brown seaweed alginate function physiologically as growth factors for microbial, plant, and animal cells, such as bifidobacteria, brassica, and human keratinocyte (Akiyama et al. 1992; Kawada et al. 1997, 1999; Iwamoto et al. 2003, 2005). Cytokine is also inducibly produced from mouse macrophage cells in the presence of the oligosaccharides (Kurachi et al. 2005).

Two Gram-negative bacterial species, *Azotobacter vinelandii* and *Pseudomonas aeruginosa*, produce and secrete alginate extracellularly. Distinct from the seaweed polymer, bacterial alginate molecules are often acetylated at the mannuronate residue (Remminghorst and Rehm 2006). During the conversion of *A. vinelandii* vegetative cells to desiccation-resistant cysts, the extracellular alginate becomes a major component of the cyst exine (Moreno et al. 1998). An opportunistic pathogen, *P. aeruginosa*, produces extracellular biofilms, including alginate, as important virulence factors during lung infections in cystic fibrosis patients (May and Chakrabarty 1994). These biofilms often protect *P. aeruginosa* cells from phagocytic cells and/or antibiotics, making biofilm-dependent diseases difficult to treat (Schweizer and Boring 1973).

Alginate-depolymerizing enzymes thus hold promise as biochemicals for processing edible seaweed alginate and removing bacterial biofilm alginate. A large number of alginate-assimilating microbes have been isolated from the soil, seawater, or wastewater, and some marine algae and mollusks have been found to produce alginate-degrading enzymes (Wong et al. 2000). To the best of our knowledge, all of the alginate-degrading enzymes that have been characterized to date strictly catalyze a β-elimination reaction, not a hydrolytic reaction, thus indicating that the enzymes are exclusively categorized as lyases. Although both polysaccharide lyases and hydrolases cleave the glycosidic bond in substrate molecules, the products released

through the lyase reactions have unsaturated saccharides at the nonreducing terminus, with a double bond between C-4 and C-5. There are various types of substrate specificity, i.e., a preference for polyM, polyG, and/or polyMG. There are also two modes of action among the alginate lyases, *endo* or *exo*.

When microbes utilize macromolecules for their growth, such as polysaccharides and proteins, the microbial cells commonly excrete degrading enzymes across the cytoplasmic membrane and then incorporate the degraded low molecular weight products into the cytoplasm. This is true of most alginate-assimilating microbes, and no microbial direct uptake system for alginate without degradation has ever been demonstrated, except for *Sphingomonas* sp. A1 (strain A1). Strain A1 has an unexplored specific and elegant system for alginate assimilation (Momma et al. 2000). Strain A1 also produces several alginate lyases with different substrate specificities and modes of action in the cytoplasm. The structure and function relationships of alginate lyases have been clarified through X-ray crystallography (Yoon et al. 1999; Yamasaki et al. 2005).

This chapter primarily deals with the structural and functional bases for the strain A1 system for the direct uptake and degradation of alginate. It also discusses applications of the bacterial system to bioremediation, sugar technology, and biofuel generation.

2 Alginate-Assimilating Bacterium

Strain A1 belongs to the genus *Sphingomonas*, reclassified as a new member of the *Proteobacteria* α-4 subclass from *Pseudomonas* in 1990. Sphingomonads, bacteria in the genus *Sphingomonas*, including strain A1, are Gram-negative rods, aerobic, nonfermentative, polymixin B-resistant, and yellow-pigmented (White et al. 1996). Strain A1 is the only sphingomonad that assimilates alginate. Recently, phylogenetic, biochemical, and taxonomical analyses have been used to divide sphingomonads into four different genera, i.e., *Novosphingobium*, *Sphingobium*, *Sphingomonas sensu stricto*, and *Sphingopyxis* (Takeuchi et al. 2001).

2.1 Pit Formation on the Cell Surface

Sphingomonads are peculiar among Gram-negative bacteria in that their outer membrane includes glycosphingolipids (GSL) with long-chain base dihydrosphingosin in place of lipopolysaccharides (Kawasaki et al. 1994). In fact, some sphingomonads have been specifically shown to have many large pleatlike substances on their cell surface (Hashimoto et al. 2005c). Possibly owing to the presence of GSL-dependent pleatlike substances, specific cell surface properties for sphingomonads have been reported (White et al. 1996; Kinjo et al. 2005), e.g., hydrophobicity and antigenicity. In addition to the presence of GSL in the outer membrane, strain A1 has two

characteristic features, pit formation on the cell surface and no secretion of alginate lyases from the cytoplasm.

Electron microscopy indicates that strain A1 also has many large pleats on the cell surface (Fig. 1a) (Hisano et al. 1996). Distinct from those of other sphingomonads, strain A1 cells grown on alginate form a mouthlike pit on the cell surface, with a size

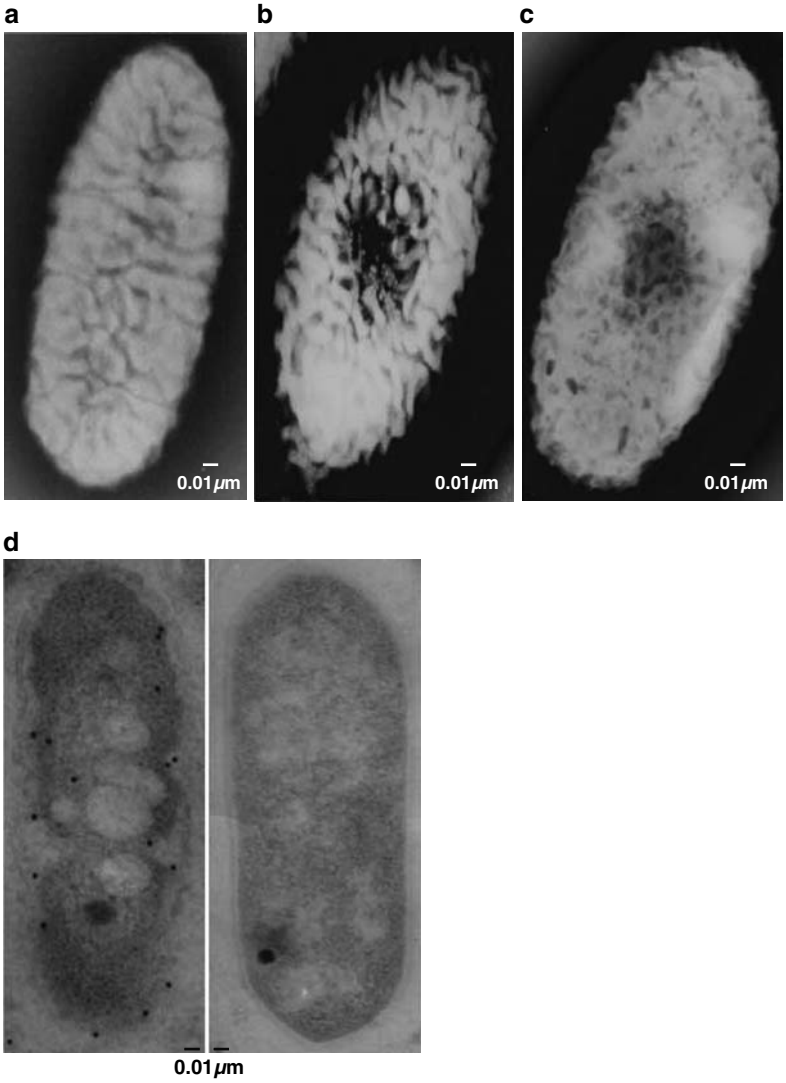

Fig. 1 Electron micrographs of *Sphingomonas* sp. A1. **a** Strain A1 cell grown in the absence of alginate; **b** strain A1 cell grown on alginate; **c** *p6*-disruptant cell grown on alginate; **d** localization of flagellin homologue p5 in strain A1 cells by immunogold electron microscopy (*left* strain A1 cell grown on alginate; *right* strain A1 cell grown in the absence of alginate)

of around 0.1 μm (Fig. 1b). This cell surface pit is formed through the reorganization and/or fluidity of the pleats. As far as we know, this is the first finding of a pit-forming bacterium in the history of microbiology. The pit formation is dependent on the presence of alginate in the external milieu, i.e., the pit forms in the presence of alginate, but disappears in the absence of the polymer. That is, the pit formation is reversible. Electron microscopy with alginate-staining dye has shown alginate concentrated in the pit, indicating that the pit functions as a funnel or concentrator for alginate. Since strain A1 secretes no alginate lyases, these bacterial cells must be equipped with a direct uptake system for alginate without degradation.

2.2 Genome Sequence

To clarify the direct uptake system for alginate in strain A1, the bacterial genome sequence was completely determined (Hashimoto et al. 2005c). This is the first sphingomonad to have its genome sequence determined. Strain A1 also has a circular double-stranded DNA plasmid (pA1) other than a chromosome (Harada et al. 2006).

The chromosome of strain A1 comprises a circular double-stranded DNA consisting of 4,622,788 base pairs encoding about 4,800 genes. The GC content is relatively high (62%) among Gram-negative bacteria. The significant homology observed in 30% of whole genes between strain A1 and *P. aeruginosa* (Stover et al. 2000) is probably due to the reclassification of sphingomonads from pseudemonads, suggesting that strain A1 has evolved from pseudomonads. A large number of genes coding for alginate and pectin-degrading enzymes are found in the chromosome of strain A1. Similar to alginate, pectin is also a polyuronate. Strain A1 has also been experimentally demonstrated to assimilate pectin by cytoplasmic pectin-degrading enzymes, suggesting that strain A1 has become somewhat specialized to incorporate polysaccharides in its growth and has developed a direct uptake system. In fact, the genes responsible for alginate uptake and degradation are assembled into a cluster, as described later (Fig. 2)

The plasmid pA1 comprises 46,557 base pairs coding for 49 genes with a GC content of 65% (Harada et al. 2006). The genes encoded in pA1 show a significant sequence similarity to those in the self-transmissible promiscuous incompatibility (Inc) group P-1β plasmid. The genetic organization of pA1 is also comparable to that of the IncP-1β plasmid. However, unlike any reported IncP-1 plasmids, pA1 contains no inserted mobile genetic elements.

3 Alginate Uptake

The cell surface pit is responsible for the direct uptake of alginate in strain A1. The formation and disappearance of the pit on the bacterial cell surface are, respectively, dependent on the presence and absence of alginate in the external milieu. This indicates

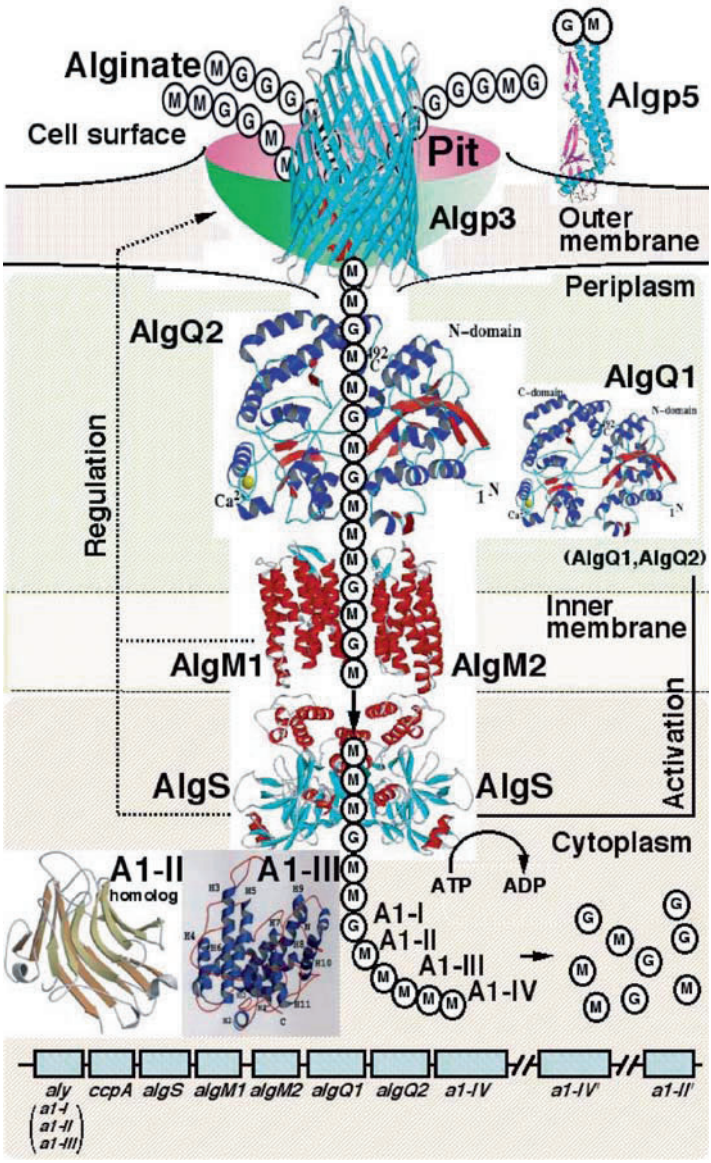

Fig. 2 Superchannel system for alginate uptake and degradation in strain A1. *G* L-guluronate, *M* D-mannuronate, *aly* gene for alginate lyases (A1-I, A1-II, and A1-III), *ccpA* catabolite-control protein gene, *algS*, *algM1*, and *algM2* ABC importer genes for alginate import, *algQ1* and *algQ2* genes for alginate-binding proteins, *a1-IV* alginate lyase A1-IV gene, *a1-II′* alginate lyase A1-II′ gene, *a1-IV′* alginate lyase A1-IV′ gene, *Algp3* TonB-dependent transporter, *Algp5* alginate receptor

that comparative analyses, using transcriptomics and proteomics, of both bacterial cells grown in the presence of alginate and bacterial cells grown in its absence would contribute to a clarification of the direct uptake system for alginate.

3.1 Cell Surface Receptors

Two flagellin homologues, p5 (40 kDa) and p6 (31 kDa), have been found to be inducibly expressed in the outer membrane by two-dimensional polyacrylamide gel electrophoresis dependent proteome analysis (Hashimoto et al. 2005a). Although flagellin is well known to be a component of the helical filament of flagella, strain A1 shows neither cell motility nor flagella formation. A p6 gene disruptant shows a significant growth retardation in a medium using alginate as the sole carbon source, although the disruption of the p5 gene has no effect on the growth of strain A1, even in the presence of alginate. The double disruption of the p5 and p6 genes results in growth failure, indicating that these flagellin homologues might be essential for cell viability. There are significant cell surface structural differences between the p6 gene disruptant and the wild type of strain A1. The gene disruptant showed incomplete pit formation and a network structure on the cell surface rather than a pleated one (Fig. 1c), suggesting that flagellin homologues regulate cell surface structures in strain A1. Thus, strain A1 flagellin homologues were overexpressed in *Escherichia coli* cells, purified, and characterized (Hashimoto et al. 2005a).

Immunoelectron microscopy using anti-p5 antibody indicated that p5 was inducibly expressed in alginate-grown strain A1 cells and exclusively localized in the cell envelope (Fig. 1d, left). Strain A1 cells grown in the absence of alginate produced only small amounts of flagellin homologues (Fig. 1d, right). Since p5 was expressed as a cell surface protein in the presence of alginate, the interaction between p5 and alginate was analyzed using a surface plasmon resonance biosensor. Both proteins, p5 and p6, specifically bound to alginate with high affinity and two alginate-binding sites were located in the flagellin homologues. The dissociation constants (K_d) in the two sites for p5 and alginate were determined to be 1.3×10^{-7} and 2.6×10^{-9} M. The high affinity of p5 for alginate may suggest that the protein functions as a cell surface receptor for alginate as is seen in a CD44-like receptor, a mammalian transmembrane receptor for hyaluronan (Turley et al. 2002).

To clarify the structure and function relationship of the cell surface receptor for alginate, the crystal structure of truncated p5 ($p5\Delta N_{53}C_{45}$) was determined at 2.0 Å resolution (Maruyama et al. 2008) (Fig. 2). The truncated p5 ($p5\Delta N_{53}C_{45}$) had mutations with a lack of N-terminal 53 residues and C-terminal 45 residues. Two regions (flagellin_N and flagellin_C motifs) at the N- and C-termini were conserved in bacterial flagellins, while the central domains (flagellin_IN motif) varied to some extent. The flagellins were, therefore, divided into two groups, on the basis of the presence or absence of the flagellin_IN motif. $p5\Delta N_{53}C_{45}$ is the first structure of a flagellin_IN-motif-containing flagellin to be determined, although the crystal structure of the *Salmonella typhimurium* flagellin, which lacks the flagellin_IN motif, was previously determined (Yonekura et al. 2003). $p5\Delta N_{53}C_{45}$ consists of an α-domain rich in α-helices and a β-domain rich in β-strands. The α-domain is composed of N- and C-terminal regions, while the β-domain has a central region. The α-domain is structurally similar to the D1 domain of the *S. typhimurium* flagellin. The β-domain has a structure that resembles the finger domain of the

bacteriophage T4 baseplate protein (Leiman et al. 2004). The deletion mutant analysis suggested that residues 20–40 and 353–363 are involved in alginate binding. Since $p5\Delta N_{53}C_{45}$ includes no alginate-binding regions, a structural analysis of the cell surface receptor with a whole sequence is now in progress.

In addition to p5, the *E. coli* flagellin (FliC) also interacted with alginate, suggesting that alginate binding is clearly common to the bacterial flagellins (Hashimoto et al. 2005a). Bacterial flagellins are generally the flagella components responsible for cell motility (Aizawa 1996), and some pathogenic bacteria interact with hosts through flagellins (Hayashi et al. 2001). To the best of our knowledge, the behavior of the strain A1 flagellin homologues p5 and p6 is the first finding showing that a flagellin has physiological roles, especially alginate binding, in addition to its function as a propeller. This yields valuable hints to the origin, evolution, and function of flagella. One possibility is that a flagellin may be created first as a cell surface protein able to recognize nutrients present in the external milieu and/or to regulate cell surface structures, before changing into flagellar proteins, or vice versa. We can at least state that microbes with flagella can access materials containing alginate. But why alginate? Alginate is a polyanion and as such is able to interact with cations (Llanes et al. 2000). If, as we understand, alginate was the first polysaccharide produced in the sea early in biological history, and ferrous ions were abundant in the prebiotic ocean before oxygen was produced by cyanobacteria (Walker 1987), our results suggest the hypothesis that flagella were first created by ancient microbes to obtain alginate as a carbon and energy source for growth and/or as a carrier for ferrous and other metal ions.

3.2 Outer-Membrane Transporters

Two proteins, p7 (28 kDa) and p8 (20 kDa), were overproduced in the outer membrane of alginate-grown strain A1 cells (He et al. 2004, 2008). Although p7 is similar to bacterial lipoproteins in its primary structure, it was found to have no lipid moiety by N-terminal sequence analysis and mass spectrometry. p7 was found to bind to alginate most efficiently at neutral pH with a K_d of 3.6×10^{-8} M. p8 is primarily similar to the polyhydroxyalkanoate granule associated protein of *Ralstonia eutropha* (Hanley et al. 1999). The disruptant of the p8 gene showed a significant growth retardation in an alginate medium. p8 was found to bind to alginate most efficiently at pH 4.0 with a K_d of 1.3×10^{-7} M. The p7 or p8 binding affinity for alginate was not much higher than that of the cell surface alginate receptor, suggesting that both proteins can associate and release the polymer. In addition to alginate molecules, p8 also exhibited an affinity for granules consisting of alginate and metal ions. The above-mentioned results indicated that p7 and p8 are outer-membrane proteins binding to alginate and facilitate the concentration of alginate in the pit on the cell surface of strain A1.

Four proteins with molecular masses of around 75 kDa, i.e., p1 (78 kDa), p2 (71 kDa), p3 (74 kDa), and p4 (72 kDa), were inducibly overexpressed in the

outer membrane of alginate-grown strain A1 cells (Hashimoto et al. 2005a). The outer-membrane proteins showed a significant sequence identity with TonB-dependent outer-membrane transporters. A mutant with a disruption of the p1, p2, p3, or p4 gene showed a significant growth retardation in the alginate medium, suggesting that each protein was involved in alginate assimilation. The typical outer-membrane transporters so far analyzed incorporate iron-bound siderophores, such as ferrichrome, enterobactin, enterochelin, and citrate (Coulton et al. 1986; Pressler et al. 1988; Lundrigan and Kadner 1986; Koedding et al. 2004) into the periplasm, with a proton motive force generated from an inner-membrane complex (TonB-ExbB-ExbD) (Larsen et al. 1999). Alginate potentially chelates iron (Sreeram et al. 2004), suggesting that p1, p2, p3, and/or p4 may incorporate iron into the periplasm using alginate as a siderophore. Homology modeling indicated that p3 constitutes a tunnel-like β-barrel structure spanning the outer membrane (Fig. 2). After the alginate-metal ion complex had been concentrated by p8 in the pit, the iron-bound alginate was transported across the outer membrane through the action of the TonB-dependent transporters, p1, p2, p3, and/or p4.

3.3 Periplasmic Binding Proteins

In the presence of alginate, strain A1 cells inducibly express two proteins, AlgQ1 (59 kDa) and AlgQ2 (59 kDa), in the periplasm. Both mediate the transfer of alginate from the outer membrane (pit) to the inner membrane (Fig. 2). AlgQ1 and AlgQ2 are mutually similar in primary structure and were found to specifically bind to alginate with a K_d of 2.3×10^{-7} and 1.5×10^{-7} M, respectively (Momma et al. 2005). Neighboring genes code for AlgQ1 and AlgQ2 in the genome of strain A1 (Fig. 2, bottom).

X-ray crystallographic studies indicate that the overall structures of AlgQ1 and AlgQ2 are almost identical. Both alginate-binding proteins consist of two N- and C-domains with an α/β structure (Momma et al. 2002, 2005) (Fig. 2). The N- and C-domains are connected by three linker loops, and the cleft formed between the two domains accommodates alginate molecules. Conformational change occurs in alginate-binding proteins through alginate binding and release (Mishima et al. 2003; Momma et al. 2005). In contrast to a ligand-free form whose N- and C-domains are wide open, ligand-bound proteins adopt a closed form (Fig. 3a). Between the open and closed forms, the root-mean-square deviation of the equivalent Cα atoms that were within a distance of 2.0 Å of one another was 0.67 Å (295 Cα atoms). These values were 0.42 Å for the N-domain (224 Cα atoms) and 0.47 Å for the C-domain (264 Cα atoms). The N-domains of the open and closed forms were superimposed, and the rotation and translation required to superimpose the C-domains were determined to be 30° and 0.5 Å, respectively. During the conversion of the open cleft to the closed one, there was a concerted shift of the Glu396 side chain, which moved up into the cleft owing to alginate binding. This ligand-induced movement of Glu396 might be the trigger for the motion that enabled the other

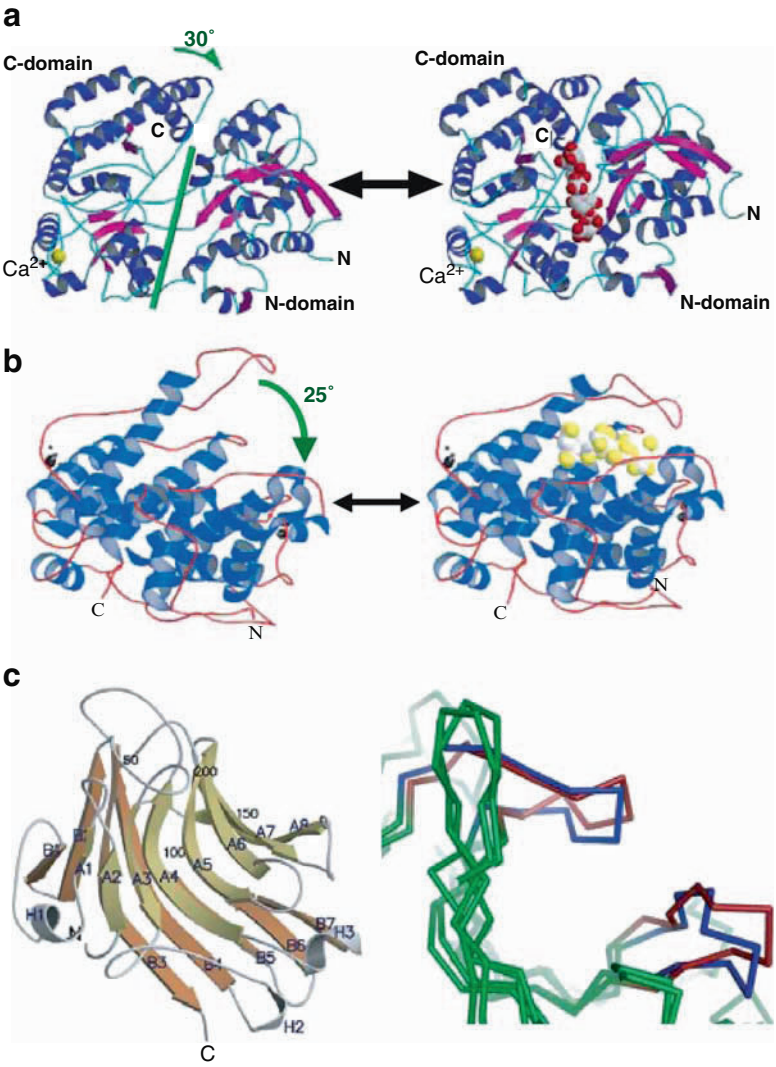

Fig. 3 Protein dynamics. **a** Alginate-binding protein AlgQ2 (*left* open form, *right* alginate-bound closed form); **b** alginate lyase A1-III (*left* open form, *right* alginate-bound closed form); **c** alginate lyase A1-II′ (*left* overall structure, *right* two flexible loops colored *blue* or *red*)

domain to participate in ligand binding and ultimately to engulf the bound alginate. The Glu396 movement accompanied the exclusion of a water molecule from the binding site. The major driving force for the hinge closing in alginate-binding proteins is, therefore, the exclusion of a water molecule from the binding site. The shift in the equilibrium on alginate binding might also be enhanced by the

interaction of alginate molecules with Glu396, which caused the resulting perturbation of the hinge favoring the closed form.

The clefts in AlgQ1 and AlgQ2 were larger than those observed in other substrate-binding proteins, such as maltose-binding proteins (Momma et al. 2005, Quiocho et al. 1997). Computational simulation indicated that at least a linear hexasaccharide could be accommodated in the active cleft of alginate-binding proteins, thus demonstrating that this larger space in the cleft enables AlgQ1 and AlgQ2 to bind to macromolecules. Distinct from sugar-binding proteins with aromatic and polar residues located in the cleft (Quiocho et al. 1997), many positively charged residues, as well as aromatic ones, are arranged in the cleft of alginate-binding proteins (Mishima et al. 2003; Momma et al. 2005). Positively charged residues in the active cleft of alginate-binding proteins contribute to the specific binding to acidic polysaccharides, such as alginate. Van der Waals contacts and hydrogen bonds are formed between alginate and binding proteins. The nonreducing terminal residues of alginate molecules are tightly bound to alginate-binding proteins. Thus, AlgQ1 and AlgQ2 specifically recognize and bind to the nonreducing saccharide of alginate, and deliver the polymer to the ATP-binding cassette (ABC) importer in the inner membrane.

3.4 Inner-Membrane ABC Importer

Bacterial ABC importers typically consist of four subunits (two ATP-binding proteins and two transmembrane subunits) (Davidson and Chen 2004). In most Gram-negative bacteria, such as *E. coli*, ABC importers accompany a periplasmic binding protein mediating the transport of the substrate from the outer membrane to an ABC importer in the inner membrane, and the genes coding for the binding protein and ABC importer are assembled into a cluster (Boos and Shuman 1998). A system consisting of ATP-binding proteins, transmembrane domains, and an associated periplasmic binding protein is thus crucial for substrate import. This is also true in the ABC importer system for alginate in strain A1, i.e., the strain A1 genetic cluster for the alginate import system contains five genes for the ATP-binding proteins (AlgS/AlgS), transmembrane domains (AlgM1 and AlgM2), and two alginate-binding proteins (AlgQ1 and AlgQ2), in that order (Momma et al. 2000) (Fig. 2, bottom). The strain A1 alginate ABC importer has a general architecture composed of two molecules of AlgS (40 kDa) and a heterodimer of AlgM1 (37 kDa) and AlgM2 (33 kDa) (Fig. 2). AlgM1 and AlgM2 are homologous to the permease domain of a bacterial ABC importer. P-loop/Walker and ABC-signature motifs are also conserved in AlgS, which exhibits ATPase activity in the dimeric form. In the alginate ABC importer, AlgM1 and AlgM2 function as permeases for the transport of alginate from the periplasm to the cytoplasm using the energy derived from ATP hydrolysis, catalyzed by the AlgS homodimer.

The three-dimensional structure of the alginate ABC importer has been modeled through homology modeling and docking simulation (Fig. 2). AlgM1 and AlgM2 each

contain six transmembrane α-helices. A pore for the passage of alginate is formed at the interface between AlgM1 and AlgM2. A crystallographic analysis of AlgS indicates that the protein includes an α/β structure (Mishima et al. 2001). The interface between the transmembrane domains (AlgM1-AlgM2) and ATP-binding proteins (AlgS-AlgS) is stabilized by the interaction of a short α-helix of AlgM1 and AlgM2 extruding to the cytoplasm with an ENI motif and a Q loop of AlgS.

Although a dimeric form of AlgS is active as ATPase in vitro, even after separation from the alginate ABC importer, the ATPase activity is substantially increased in the presence of both transmembrane domains (AlgM1 and AlgM2) and periplasmic binding proteins (AlgQ1 and AlgQ2), complexed with alginate (Momma et al. 2005). The activation of AlgS in the dimer form requires the participation of the transmembrane domains (AlgM1 and AlgM2) and alginate-bound proteins (AlgQ1 and AlgQ2), thus suggesting that information on the structural change induced in the binding proteins through alginate binding is transferred to a dimer form of AlgS via mediation by the transmembrane domains. As is seen in the *E. coli* maltose transport system (Oldham et al. 2007), alginate-bound AlgQ1 or AlgQ2 initiates the transport cycle. Once AlgS has bound to ATP, domains in the alginate-bound AlgQ1/AlgQ2 open wide (release of alginate), and the periplasmic entrance is inducibly produced by the transmembrane domains (AlgM1 and AlgM2). Subsequently, alginate is transported to the cytoplasm by the ABC importer using the energy generated by the ATP hydrolysis.

The expression of the alginate ABC importer affects the pit formation on the cell surface (Momma et al. 2000). Mutants with disruption of the ABC importer genes form no apparent pit on the cell surface and fail to assimilate alginate. In conclusion, the clever system used by strain A1 for alginate uptake is composed of a pit on the cell surface, periplasmic binding proteins, and an ABC importer, which function cooperatively.

4 Alginate Degradation

Alginate molecules contain three blocks, polyM, polyG, and polyMG. In strain A1, alginate incorporated into the cytoplasm through the alginate uptake system is degraded to the constituent monosaccharides by endotype and exotype alginate lyases with different substrate (block) specificities.

4.1 Endotype Alginate Lyases

Strain A1 cells produce three endotype alginate lyases in their cytoplasm, A1-I (65 kDa), A1-II (25 kDa), and A1-III (40 kDa) (Yoon et al. 2000) (Fig. 2). These three endotype alginate lyases originate from a single gene (Murata et al. 1993). In the genome of strain A1, this gene (*aly*) is located in the vicinity of the operon for

the alginate uptake system (Fig. 2, bottom). Po (71 kDa) is first synthesized in an inactive form of alginate lyase as a precursor of the gene product. A1-I is converted from Po as an active form after the cleavage of the N-terminal peptide (6 kDa). A1-I is subsequently processed into N-terminal A1-III and C-terminal A1-II in an autocatalytic manner. A1-III and A1-II are specific for polyM and polyG, respectively, indicating that A1-I is a combined form of A1-III and A1-II, and has the characteristics of both enzymes.

On the basis of their primary structures, polysaccharide lyases are classified into 18 families (PL-1–PL-18) in the carbohydrate-active enzyme (CAZY) database (B. Henrissat, P. Coutinho, and E. Deleury, http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Alginate lyases are categorized as PL-5, PL-7, PL-14, PL-15, PL-17, and PL-18. Most bacterial alginate lyases are classified into two families, PL-5 and PL-7, and the alginate lyases in these families are generally specific for polyM and polyG, respectively. Endotypes A1-III and A1-II belong to PL-5 and PL-7, respectively, and thus A1-I becomes a member of a new family PL-5 + 7. Some pseudomonads, such as *P. aeruginosa* PAO1 (Stover et al. 2000) and *Pseudomonas syringae* pv. *tomato* DC3000 (Buell et al. 2003) have both PL-5 and PL-7 alginate lyases. Distinct from strain A1, including the PL-5 + 7 family's enzyme gene, the PL-5 and PL-7 enzyme genes of pseudomonads are located separately in their bacterial genomes. It is postulated that A1-I is an original alginate lyase, and that the A1-III and A1-II genes derived from the A1-I gene, independently evolving into various genes belonging to the PL-5 and PL-7 families through duplication, modification, and translocation. The genome sequence of strain A1 provides hints to the molecular diversity and evolution of alginate lyases as follows (Miyake et al. 2004).

In addition to the genes for A1-I, A1-II, and A1-III, the genome of strain A1 contains a novel alginate lyase (A-II′, 31 kDa) gene, although no A1-II′ is expressed in strain A1 cells even in the presence of alginate, and the location of the A1-II′ gene in the genome of strain A1 is far from the gene cluster for alginate uptake and degradation. A1-II′ is a member of PL-7, on the basis of the primary structure. Distinct from A1-II, A1-II′ shows a broad substrate specificity (polyM, polyG, and polyMG). A phylogenetic analysis of the alginate lyases categorized into PL-5 and PL-7 indicated that A1-I has a central position in the phylogenetic tree, being an intermediate between PL-5 and PL-7. This suggested that the A1-II′ gene was generated from the A1-II gene through separation from the A1-I gene, subsequent duplication, modification, and translocation.

Since alginate lyases have potential applications as biochemicals for processing edible seaweed alginate and removing bacterial biofilm alginate, the structure and function relationship of these alginate lyases has been analyzed. An X-ray crystallographic analysis demonstrated that PL-5 A1-III consists of 12 α-helices constituting an α_6/α_5-barrel structure as a basic frame (Yoon et al. 1999) (Fig. 3b). The enzyme has a tunnel-like cleft covered with a lid loop consisting of residues 57–90. A comparative structural analysis of substrate-free and bound A1-III indicated that the loop is situated about 16.8 Å above the glycosidic linkage to be cleaved in the ligand-free form (open conformation), while this distance is 8.6 Å in the closed formation (Yoon et al. 2001; Mikami et al. 2002). The movement of the lid loop is essential

for enzyme catalysis, activation of the catalytic center Tyr246 through the formation of the Tyr68–Tyr246 pair, the binding of substrates, and the release of products. The catalytic reaction of polysaccharide lyases has a three-step mechanism (Linker et al. 1956): (1) the removal or neutralization of the negative charge on the C6 carboxylate anion by residue I; (2) the abstraction of the C-5 proton at subsite + 1 by a general base (residue II); and (3) proton donation to the O4 atom at subsite + 1 by a general acid (residue III). The alginate tetrasaccharide-bound structure of A1-III indicates that His192 functions as residue I, and Tyr246 as both residues II and III. The catalysis by A1-III with a single Tyr as an active center can probably be regarded as a novel β-elimination reaction.

X-ray crystallographic studies revealed that PL-7 A1-II′, which had a broad substrate specificity, consists of two β-sheets constituting a β-sandwich structure. A1-II′ has an active site in the cleft covered with two flexible loops (residues 133–145 and 193–203) (Yamasaki et al. 2005). The flexibility of these loops is essential for substrate binding, i.e., the accommodation of substrates in the active site (Ogura et al. 2008) (Fig. 3c). Both G and M in alginate molecules are bound to the active site as follows: the enzyme interacts appropriately with the substrate's hydroxyl groups, although the substrate's carboxyl groups are recognized by specific residues. These structural characteristics provide A1-II′ with its broad substrate specificity. In the A1-II′ reaction, Gln189 (residue I) neutralizes the negative charge of the carboxyl group, His191 (residue II) abstracts the C-5 proton, and Tyr284 (residue III) donates the proton to the glycoside bond to be cleaved.

4.2 Exotype Alginate Lyases

Alginate is depolymerized to unsaturated disaccharides, trisaccharides, and tetrasaccharides with different M to G ratios as final products through the reaction of the endotype alginate lyases A1-I, A1-II, and A1-III (Yoon et al. 2000). Alginate oligosaccharides are further exolytically degraded to the constituent monosaccharides by alginate lyase A1-IV (86 kDa) (Hashimoto et al. 2000). The resultant monosaccharides are nonenzymatically converted to an α-keto acid (4-deoxy-L-erythro-5-hexoseulose uronic acid). The genes coding for A1-I, A1-II, A1-III, and A1-IV are included in the genetic cluster for the system for alginate uptake and degradation (Murata et al. 1993; Momma et al. 2000; Hashimoto et al. 2000) (Fig. 2, bottom), and this genetic cluster was found to be inducibly expressed in the presence of alginate through transcriptomics by DNA microarray. Therefore, the alginate uptake system is linked with the degradation system, and the expression is coordinately regulated.

A1-IV exolytically acts on alginate polysaccharides and oligosaccharides in saturated form, as well as on unsaturated oligosaccharides, indicating that A1-IV can degrade saturated and unsaturated alginate polysaccharides and oligosaccharides with various M and G compositions (Miyake et al. 2003). A1-IV is, therefore, involved in the complete degradation of alginate in strain A1. Exotype A1-IV, with its broad substrate specificity, is peculiar among alginate lyases in that the enzymes so far

analyzed prefer either polyM or polyG, and act on alginate endolytically, although there have been a few reports on alginate lyases that depolymerize both polyM and polyG, and show exolytic activity (Wong et al. 2000). To the best of our knowledge, no alginate lyase has previously been reported that exolytically acts on unsaturated and saturated alginate molecules of various lengths and M to G ratios, except for A1-IV. This is why A1-IV shows no significant identity with any other function-known proteins.

Accompanying the bacterial genome sequence analysis, novel genes encoding hypothetical proteins homologous with A1-IV were found in the genomes of some bacteria, including strain A1. One such protein, A1-IV′ (90 kDa) of strain A1, catalyzes the cleavage of glycosidic bonds in alginate molecules through the β-elimination reaction, and releases unsaturated disaccharides and trisaccharides as the main products, indicating that the enzyme is an endotype alginate lyase (Hashimoto et al. 2005b). On the other hand, *Agrobacterium tumefaciens* Atu3025 (88 kDa), which is similar to A1-IV, was identified as an exotype alginate lyase (Ochiai et al. 2006). On the basis of their primary structures, A1-IV and other A1-IV homologues contribute to the establishment of a novel polysaccharide lyase family, PL-15, suggesting the evolutionary route of alginate lyases in PL-15 (Hashimoto et al. 2005b). As was seen in the case of A1-II′, A1-IV′ was not expressed in strain A1 cells grown on alginate. It is thought that the A1-IV′ gene was generated from the A1-IV gene and that A1-IV was the original protein of PL-15.

In conclusion, strain A1 produces diverse alginate lyases classified into the families PL-5, PL-7, PL-5 + 7, and PL-15, which contain almost all bacterial alginate lyases, suggesting the possibility that strain A1 can be regarded as the original producer of alginate lyases.

5 Concluding Remarks and Future Perspectives

A large number of genome sequencing projects are now being carried out, and the complete gene sequences of over 700 organisms, ranging from viruses to human, have been determined. Several “omics” technologies, such as transcriptomics, proteomics, and glycomics, have facilitated the interpretation of the enormous amount of information obtained in genome sequencing. Comprehensive analyses of genes, proteins, and saccharides using these technologies readily and simultaneously contribute to our understanding of interesting strain A1 cellular events, especially from the pattern of global gene expression.

5.1 Superchannel as Alginate Uptake System

When microbes utilize macromolecules for their growth, they usually produce extracellular macromolecule-degrading enzymes, and incorporate the degraded low molecular weight products through specific transporters, channels, and/or porins. On the other

hand, strain A1 employs an alternative elegant means for the degradation of macromolecules (Fig. 2) (Murata et al. 2008). Once cell surface receptors (p5 and p6) recognize the external alginate, the resultant signals are transmitted to the DNA. On the basis of signal transduction, genes responsible for alginate uptake and degradation are expressed. Alginate is concentrated in the cell surface pit where alginate-binding proteins (p7 and p8) are inducibly expressed in the presence of alginate, and is then incorporated by TonB-dependent transporters (p1–p4). Periplasmic alginate-binding proteins (AlgQ1 and AlgQ2) deliver the polysaccharide from the outer membrane to the inner membrane. The inner-membrane ABC importer (AlgM1–AlgM2/AlgS–AlgS) directly incorporates alginate into the cytoplasm with the energy generated by AlgS. This is a novel macromolecule uptake system independent of extracellular degrading enzymes, which we call a "superchannel." This assimilation method (swallow type) for macromolecules is energetically and substantially more efficient and economical than the extracellular-degrading-enzyme-dependent assimilation method (chewing type), since it minimizes the degraded product loss from diffusion that is observed in the chewing-type method. The significance of the novel pit-dependent ABC importer system for macromolecule uptake is that cell surface molecules are in a fluidized state and are rearranged to create a molecular apparatus. Similar to the strain A1 superchannel, other pit or pore-dependent transport systems are found in bacterial and human cells (Sugawara et al. 1999; Andrews et al. 1990; Noronha et al. 2000; Shimizu 1999). This pit formation may be considered to be the original model for the endocytosis and/or phagocytosis of eukaryotic cells, and this notion can be applied to the uptake of other macromolecules across the cell membrane, such as DNA and proteins.

5.2 Molecular Transplantation of Superchannel

An understanding of the direct uptake system, i.e., superchannel, present in strain A1 is not only beneficial in clarifying the macromolecule transport mechanism in microbial cells, but also has a potential application for improving in the permeability of various compounds across cell membranes.

The widespread use of chemicals, such as solvents, herbicides, insecticides, and many industrial chemicals, has led to the contamination of soils, waste streams, and various other sites, and has directly or indirectly had hazardous effects on many organisms through food chains. Among these chemicals, dibenzofuran, a dioxin analogue, is a highly toxic compound generated in combustion processes and certain chemical syntheses of haloaromatics. To eliminate it, as well as other hazardous compounds, several physicochemical processes have been considered, such as high-temperature incineration, but these methods are not always economically feasible for the treatment of pollutants, which are present at low concentrations and in soluble/insoluble forms. More efficient and convenient biological methods, using microorganisms, are now sought to assist in their removal from contaminated sites.

At present, most bioremediation studies are restricted to the isolation of microbes capable of degrading environmental pollutants and analyses of their genes/enzymes

involved in the degradation pathway. Among the pollutant-degrading microbes so far isolated and analyzed, sphingomonads are the most promising microbes for potential bioremediation applications. Sphingomonads are widely distributed in nature and are frequently found in various soils and aqueous environments.

Sphingomonas wittichii RW1 (strain RW1) shows significant growth using dibenzofuran as a source of carbon and energy (Wittich et al. 1992). To enhance the permeability of the molecules, the molecular transplantation of the strain A1 superchannel to strain RW1 was conducted through the introduction of the genetic cluster for the alginate uptake system (Aso et al. 2006). The strain RW1 transformant with the genetic cluster formed a pit on the cell surface, while no pit was observed in the wild type of strain RW1 (Fig. 4). The wild type of strain RW1 showed a lag time during the first few days, and a longer cultivation time (4 days) was required for the complete removal of dibenzofuran. In comparison with the wild type of strain RW1, the transformant degraded the dibenzofuran rapidly, within 2 days.

After being given the strain A1 genetic cluster, the poly(propylene glycol) degrading *Sphingomonas subarctica* and polydextrose-degrading *Sphingomonas sanguis* also formed the pit constitutively on their cell surface and showed significant macromolecule-degrading abilities (Aso et al. 2006).

On the basis of the results described above, the molecular transplantation of the superchannel has been established as a novel practical biotechnology for the molecular breeding of microbial degraders suitable for the bioremediation of various environmental pollutants with low to high molecular weights.

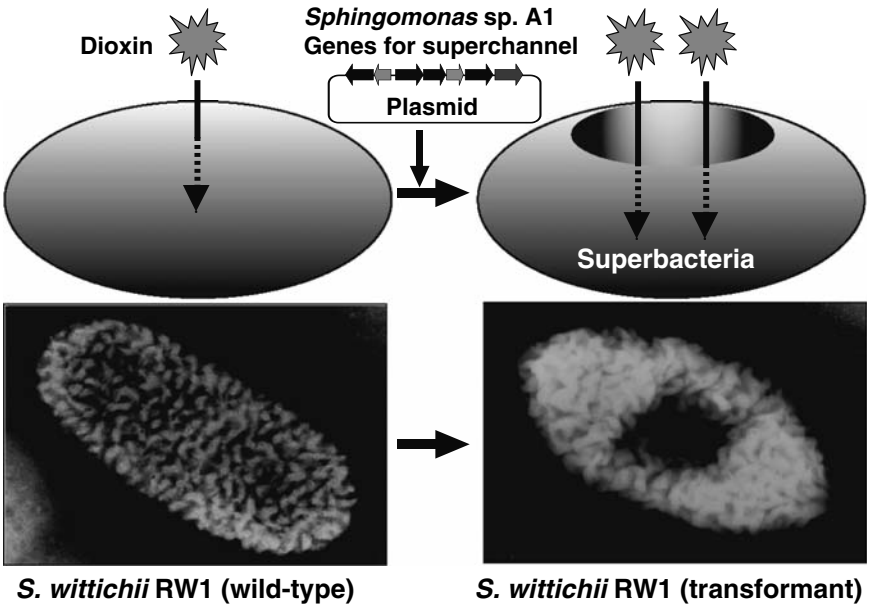

Fig. 4 Molecular transplantation of the superchannel

5.3 Application of the Alginate Degradation System

Alginate degradation products are expected to have applications in various food, chemical, and medical formulations because of the safety record of these polymers. A wide range of industries have long been interested in alginate-modifying enzymes. The use of alginate lyases will enable the preparation of physiologically and technologically novel and available materials in the agricultural, food, and pharmaceutical fields. The results presented in this chapter, therefore, provide a possible and promising way, when taken together, of obtaining a more complete picture of the utilization of alginate, as well as of the renewable oligosaccharides that are obtainable in large quantities at low cost.

In the genome of strain A1, several genes coding for alginate lyases with different substrate specificities and modes of action were found. These enzymes are promising for removing and modifying alginate. Pathogenic bacteria, such as *P. aeruginosa*, are known to produce exopolysaccharides as a capsulelike biofilm that facilitates interaction with and complete adherence to target cells (Costerton et al. 1999). This biofilm often forms a firm barrier and inhibits the penetration of antimicrobial agents, making it difficult to treat biofilm-dependent bacterial infections. Degradation of the bacterial exopolysaccharides might lead to the establishment of a novel therapy for biofilm-dependent bacterial infectious diseases. The application of A1-III, with its high activity for *Pseudomonas* alginate, might be feasible for the treatment of such diseases (Sakakibara et al. 2002).

The physiological function of oligosaccharides is of importance. Alginate oligosaccharides depolymerized with A1-I enhance the proliferation and/or differentiation of bifidobacteria (Akiyama et al. 1992) and some plant cells (Murata et al. 1993), making it possible to use alginate oligosaccharides as growth factors for bifidobacteria in the food industry or as biochemical fertilizers in agriculture. The alginate oligosaccharides obtained with A1-I also promote the differentiation and proliferation of human epithelium cells (Hashimoto et al. 2002), indicating that these oligosaccharides might be applicable as epidermal growth factors in place of bovine pituitary extract, the utilization of which is thought to be hazardous owing to the occurrence of bovine spongiform encephalopathy (Kawada et al. 1997).

Biofuel is expected to become a new energy source in place of fossil fuels, such as petroleum and coal (Gray et al. 2006). In particular, technologies for the production of bioethanol are sought and the industrial production of ethanol from sugarcane and corn starch has been developed (Gray et al. 2006). The use of foodstuffs to produce ethanol, however, causes serious social problems, such as insufficient supply and a sudden rise in prices for the crops utilized. Lignocellulose is thought to be the next target for bioethanol production, but some technical problems regarding how to remove the lignin and hydrolyze the cellulose still need to be overcome. Recently, marine biomass has been focused on as a possible material for bioethanol production. Alginate, a major part of this marine biomass, makes up 30–60% of dried seaweed. The strain A1 exotype alginate lyase A1-IV, with its broad substrate specificity, degrades alginate to the constituent monosaccharides, indicating that it is feasible to use this enzyme for the saccharification of alginate. The α-keto acid

derived from the resultant monosaccharides is further converted to pyruvate and glyceraldehyde 3-phosphate through successive reactions of cytoplasmic reductase A1-R (28 kDa), kinase A1-K (35 kDa), and aldolase A1-A (24 kDa) (Ochiai et al. 2008). This metabolic pathway has also been observed in *P. aeruginosa* (Preiss and Ashwell 1962). The tricarbon chemicals pyruvate and glyceraldehyde 3-phosphate can be converted into ethanol. In fact, strain A1 cells grown on alginate under a limited oxygen level synthesize ethanol, suggesting that strain A1 may become a potent ethanologen through assimilating alginate, although the production level of ethanol should be enhanced through metabolic engineering.

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Enzymatic Alginate Modification

Helga Ertesvåg, Svein Valla, and Gudmund Skjåk-Bræk

Contents

Abstract Alginate is a linear 1-4-linked copolymer of β-D-mannuronic acid and its C-5-epimer α-L-guluronic acid. The polymer is produced by some algae and bacteria, and is used for numerous purposes in industry. Alginate is initially synthesized as mannuronan, which is then modified at the polymer level by mannuronan C-5-epimerases, alginate lyases, and *O*-acetylases. This generates a variety of heteropolymers where properties such as viscosity, chain stiffness, gel formation, water-binding potential, and immunogenicity are dependent on the action of the

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modifying enzymes. Both alginate lyases and C-5-epimerases can be used in vitro to tailor alginates for specific purposes. The lyases may also be used as tools to better define the sugar monomer sequences of an alginate sample.

1 Introduction

Alginates are structurally heterogenous polysaccharides produced by bacteria belonging to the genera *Pseudomonas* and *Azotobacter*, and by brown seaweeds and some calcareous red algae (Stanford 1883; Gorin and Spencer 1966; Linker and Jones 1966; Govan et al. 1981; Okazaki et al. 1982). In bacteria these polymers probably serve many different functions, as illustrated, for example, by *P. aeruginosa*, where they provide the producing organism with a selective advantage during serious infections of patients suffering from the disease cystic fibrosis (Ramsey and Wozniak 2005). In *A. vinelandii* they play a critical role in the ability of the organism to enter a desiccation-resistant state designated "cyst" (Sadoff 1975). Brown seaweed alginates serve a role in maintaining the integrity of the cell wall, analogous to cellulose and pectins in green land plants, and provide the stiffness or elasticity needed depending on the environment (Haug et al. 1974). The function in red algae is not known although it has been suggested that alginate is involved in the calcification process (Bilan and Usov 2001).

All alginates isolated from natural sources are linear 1-4-linked copolymers of the two sugar monomers β-D-mannuronic acid (M) and its C-5-epimer α-L-guluronic acid (G) (Fig. 1). However, alginate structures can otherwise be quite heterogeneous, even when they are isolated from different parts of the same organism (e.g., seaweed blades and stipes) or from bacteria exposed to different environmental conditions. The structural differences relate to the following: (1) the ratio between M and G residues may vary over a wide range (Ertesvåg et al. 1996); (2) the distribution pattern of the monomers along the polymer chains may vary drastically, even if the fractional composition is similar (Skjåk-Bræk et al. 1986a); (3) bacterial alginates are O-acetylated to varying degrees in the 2 and/or 3 positions, while this is not seen in seaweed alginates (Skjåk-Bræk et al. 1986a); (4) alginate chain lengths vary and are heterogenous even when alginate is extracted from a single source. All these four structural differences have very important consequences for the physicochemical properties of the polymer, and the producing organisms have taken advantage of the structural diversity to serve their functional needs. The diversity also forms the basis for a widespread and varied use of the polymer in industry.

Generally, the presence of G residues in alginates opens the potential for formation of polymer gels in the presence of divalent cations such as Ca^{2+}, but this form of gel formation can only take place if the G residues are found as consecutive stretches (designated "G-blocks") (Fig. 1). The lengths and numbers of these blocks affect several important physical properties of the gels, such as stiffness, swelling, and porosity. The G residues may also be present as alternating sequences, (MG)*n*, designated "MG-blocks" (Fig. 1). Finally, alginates with a very low content of G

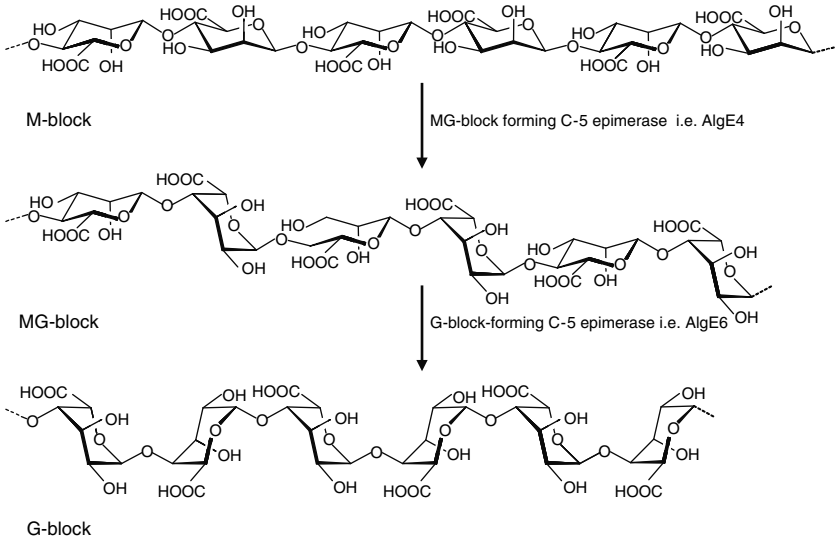


Fig. 1 Alginate structure. Hexamers of the three different structural elements are shown. The mannuronan C-5-epimerases will convert consecutive stretches of β-D-mannuronic acid (M-blocks) to blocks containing single α-L-guluronic acid residues or consecutive stretches of α-L-guluronic acid residues (G-blocks)

residues are referred to as being rich in M-blocks (Fig. 1). Such alginates cannot form cationic gels, but can form acidic gels (Draget et al. 1994). For further details on the relation between monomer composition and physicochemical properties, see Smidsrød and Draget (1996). An additional interesting observation is that some types of alginates exhibit immune-stimulating properties. These activities which include stimulation of cytokine synthesis through the Toll-like receptor system, is correlated with a high content of M interspaced with single G residues, although the sequential structure of the epitope remains to be identified (Flo et al. 2000).

The biotechnological uses of alginates cover a wide range of applications owing to the consequences of the structural diversity (Smidsrød and Draget 1996). They are, for example, used to affect food texture owing to the ability to form viscous solutions (related to polymer chain lengths), for immobilization of cells owing to the ability to form cationic gels (Draget et al. 2000), and as a feed additive in fish farming owing to their immunogenic properties (http://www.biomar.no).

Since all commercial alginates are currently harvested from brown seaweeds, different structural types of alginates must be selected from different types of alga or from specific algal tissues, i.e., alginate enriched in G for making mechanically strong gels is found in the stipes and holdfast of *Laminaria hyperborea*, a plant growing in very exposed coastal areas. Alginates with a high M content (for softer and more elastic gels) can be extracted from the leaves of the same plant or from other species such as *Laminaria digitata*, *Ascophyllum* sp, and *Lessonia* sp. Alginate can also to some extent be fractionated, but the efficiency is low (Haug et al. 1967).

Another advantage of using seaweed alginates compared with using those from bacteria is that they can generally be produced more cheaply from seaweeds than from bacteria. However, bacterial alginates not only present a wider structural variety, but they also offer the potential of designing new alginate structures to meet demands for alginates with improved or new functional properties.

For many types of application, such as for pharmaceuticals, price per unit weight is not necessarily the most critical parameter, but rather the product quality in terms of homogeneity and structural reproducibility and availability. For such purposes there is certainly a potential for using alginates produced by bacteria, as one could envision both stable production and unique quality. To achieve this one must be able to tailor-make the alginate structures in vitro or in vivo, and in this chapter the methods available for doing this are discussed.

2 Mannuronan C-5-Epimerases

Homopolysaccharides with only one type of bond are synthesized using a nucleotide sugar and one glycosyltransferase. This concept may be extended to linear heteropolysaccharides by suggesting that several glycosyltransferases cooperate, and that the polysaccharide structure will be a product of their relative reaction rates, substrate requirements, and precursor availability. This was also suggested for the biosynthesis for alginate, as Lin and Hassid (1966b) claimed to be able to isolate the potential precursors GDP-D-mannuronic acid and GDP-L-guluronic acid from the brown algae *Fucus gardnerii*. The same authors also showed the presence of enzymes necessary to incorporate mannose 1-phosphate into alginate using ^{14}C-labeled mannose derivatives (Lin and Hassid 1966a). However, Haug and Larsen (1969) were able to show that the bacterium *A. vinelandii* secretes an enzyme that epimerizes M-residues to G-residues on the polymer chain, and this was the first example of an epimerase working on a polymer. This suggested that alginate is synthesized as a homopolymer, as was later confirmed by Chitnis and Ohman (1990). A mannuronan C-5-epimerase was also identified in the algae *Pelvetia canaliculata* (Magdwick et al. 1973). Mammalian cells were found to synthesize heparin in a similar way, where a glucuronan C-5-epimerase converts some of the D-glucuronic acid residues to L-iduronic acid residues (Lindahl et al. 1972).

2.1 Early Studies on the Secreted Mannuronan C-5-Epimerase from *A. vinelandii*

Mannuronan C-5-epimerase could be partly purified from the *A. vinelandii* culture broth using ammonium sulfate precipitation (Larsen and Haug 1971). Further purification was later obtained by using ion-exchange and affinity chromatography (Skjåk-Bræk and Larsen 1982b; Skjåk-Bræk et al. 1985). When the partially

purified enzyme was incubated with alginate in the presence of tritiated water most of the radioactivity was found in the G residues, but a smaller amount was also found in M residues (Larsen and Haug 1971). The authors suggested a model in which the first step in the catalytic reaction is the abstraction of H-5 followed by addition of hydrogen from water to the other side of the uronic acid residue.

The carbazole method can also be used to follow the epimerization reaction (Knutson and Jeanes 1968; Haug and Larsen 1971), since G gives rise to a higher color intensity than M. Infrared light may also give an estimate of the G content (Mackie 1971), and it is possible to measure the increase in G content using G-specific lyases as was first described by Currie and Turvey (1982). However, a more accurate method involves the use ^{3}H-5-labeled mannuronan as a substrate. During epimerization, this hydrogen atom is exchanged with water and the reaction can be measured by quantifying the radioactivity remaining in the soluble phase after the alginate has been precipitated by ethanol (Skjåk-Bræk and Larsen 1982a).

These methods can quantify the increase in G content as the epimerization reaction proceeds, but cannot provide information regarding the distribution pattern of the G residues (G-blocks or MG-blocks). This can to some extent be achieved by partial acid hydrolysis of the polymer followed by acid precipitation, since the MG-blocks are more soluble in acid than G-blocks and M-blocks (Haug et al. 1967). However, ^{1}H-NMR and ^{13}C-NMR spectroscopy (Grasdalen et al. 1979, 1981; Grasdalen 1983) is now the preferred method since it can be used to obtain accurate data on the frequencies of the different dimer and trimer distributions in the alginate polymer.

Even in the first papers describing the secreted mannuronan C-5-epimerases it was mentioned that the concentration of divalent calcium ions affected activity (Haug and Larsen 1969, 1971). However, if the reaction was allowed to proceed to the end point, an intermediary concentration of Ca^{2+} (about 1 mM) gave rise to the highest number of G residues. Ca^{2+} also increased the stability of the enzyme. Later, with use of purified epimerase, it was confirmed that at low concentrations of Ca^{2+} G-blocks were preferably made, while at higher concentrations MG-blocks dominated (Larsen et al. 1986; Skjåk-Bræk et al. 1986b). One hypothesis that could explain this observation is that the increase in Ca^{2+} changed the action pattern of the enzyme from a predominantly single-chain mechanism to a multiple-attack mechanism with increasing number of calcium ions (Larsen et al. 1986). However, in a report by Ofstad and Larsen (1981), it was stated that different batches of ammonium sulfate precipitated epimerase varied both regarding their optimal calcium ion concentration for activity and the monomer sequence distribution in the polymer reaction product. These authors suggested that the lack of reproducibility could be explained by assuming the presence of two enzymes with different calcium optimums and preference with respect to the epimerization patterns introduced. The relative amounts of these two enzymes could then vary among different batch cultivations of the bacterium. As discussed later, this prediction later turned out to be correct, except that the complexity is even greater than they anticipated.

2.2 Cloning and Characterization of the Mannuronan C-5-Epimerases from *A. vinelandii*

To resolve the issues described in the previous section and also to obtain larger quantities of the epimerase, the gene or genes encoding them had to be cloned. The purified proteins were separated by sodium dodecyl sulfate polyacrylamide gel elecrophoresis, and the N-terminal sequence of the protein corresponding to the most intense band (122 kDa) was determined. A degenerate nucleotide probe was constructed on the basis of this information and was used to screen a genomic bacteriophage λ library of *A. vinelandii* (Ertesvåg et al. 1994). Positive clones were identified, and parts of one of these were subcloned into a plasmid vector. Surprisingly, several of the plasmids expressed active mannuronan C-5-epimerases in *Escherichia coli* (Ertesvåg et al. 1994, 1995). The insert in the phage was then sequenced and it was found to contain a gene encoding a protein with the expected N-terminal sequence, but also genes encoding parts of two other homologous proteins. Further analyses of other positive phage clones finally resulted in the identification of five different homologous genes (Ertesvåg et al. 1995). Furthermore, Southern blotting revealed that the *A. vinelandii* chromosome contains even more homologous sequences. The last of these genes were finally cloned by a second screen of the genomic library (Svanem et al. 1999), and it is now clear that the total number of homologous genes is eight, but only seven of them (*algE1–algE7*) encode proteins displaying mannuronan C-5-epimerase activity after expression in *E. coli*. The product of the last gene, designated "*algY*," has no known function (Svanem et al. 1999).

The secreted epimerases are all modular enzymes containing one or two A-modules and one to seven R-modules (Fig. 2). Within each module type the range of homology is between 50 and 100%. The two A-modules from AlgE1 were expressed separately in *E. coli* and both were shown to be active epimerases (Ertesvåg and Valla 1999), proving that this module contained the catalytic site of the enzyme. The presence of the R-modules increased the activity of the enzyme about tenfold. Interestingly, this increase was found when the R-modules were located both N-terminally and C-terminally with respect to the A-module (Ertesvåg and Valla 1999). The R-modules contain four to seven imperfect direct repeats of a nine amino acid long motif found in many proteins exported by ATP binding cassette transporters (Delepelaire 2004). Such type I secreted proteins do not contain an N-terminal secretion signal, but some feature of their C-terminal sequence is necessary for secretion. The genes (*eexDEF*) needed for secretion of the epimerases have been identified (Gimmestad et al. 2006).

The nonameric repeat has been shown to bind calcium (Baumann et al. 1993), and 3D-structure studies of the R-module of AlgE4 support this conclusion (Aachmann et al. 2006). Furthermore, binding and 3D-structure studies of the AlgE4 A-module have demonstrated that this catalytic unit also binds Ca^{2+} (Ertesvåg and Valla 1999; Rozeboom et al. 2008).

The different epimerases produce alginates with distinct epimerization patterns; AlgE2 and AlgE5 seem to predominantly epimerize M residues located next to a

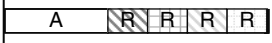
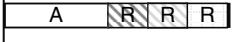
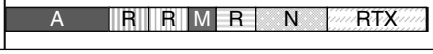
Enzyme	Modular structure	End products	Other activities
AlgE1		G-and MG-blocks	
AlgE2		G-blocks (short)	Weak lyase activity
AlgE3		G-and MG-blocks	
AlgE4		MG-blocks	
AlgE5		G-blocks (medium)	Weak lyase activity
AlgE6		G-blocks (long)	
AlgE7		G-and MG-blocks	Lyase activity
PsmE		G-blocks (long)	Deacetylase

Fig. 2 Modular structure and end products of the secreted mannuronan C-5-epimerases. The relationships within the group of A-modules and R-modules (Svanem et al. 1999) are shown by similarities and differences in *cross-hatchings* and *gray tones*. The *Pseudomonas syringae* enzyme also contains two additional calcium-binding modules (*M* and *RTX*) and a module displaying alginate deacetylase activity (*N*). The weak lyase activity of AlgE2 and AlgE5 (0.1–0.3% of the reactions) is probably a result of failed epimerase reactions

preexisting G residue, generating G-blocks (Ertesvåg et al. 1999; Ramstad et al. 1999), while AlgE4 does not do this, and therefore forms alternating sequences, (MG)*n*, in the reaction product (Høidal et al. 1999). As can be seen from Fig. 2, the A-modules of AlgE2 and AlgE5 belong to one homology group of A-modules, while the A-module of AlgE4 belongs to another group. AlgE1 and AlgE3 both contain two A-modules, one from each of these two groups. And when they were expressed separately, the A1-modules make G-blocks, while the A2-modules introduce alternating structures (Ertesvåg et al. 1998b, 1999). However, the A-module of AlgE6 groups with that of AlgE4, but still this enzyme introduces G-blocks into the alginate (Svanem et al. 1999). A study using hybrid A-modules between AlgE2A and AlgE4A might reconcile these findings, since it was shown that only certain parts of the A-module are important for the epimerization pattern (Bjerkan et al. 2004).

The A-module of AlgE7 is similar to that of AlgE6. However, the end product after incubation of mannuronan with AlgE7 is relatively small oligomers with G residues at the reducing end and an unsaturated residue at the nonreducing end, identifying AlgE7 as being both a mannuronan C-5-epimerase and an alginate lyase (Svanem et al. 2001). The same residues are important for both the epimerase and lyase activities, emphasizing the hypothesis proposed by Gacesa (1987) that alginate epimerases and lyases share the first steps of the catalysis.

The grouping of the epimerases into those generating G-blocks and those preferably introducing alternating structures is fairly crude; there are differences both in the relative numbers of G-blocks and alternating structures made (Fig. 2), the acceptance of alternating structures as a substrate for further epimerization, and the requirements for Ca^{2+} (Ertesvåg et al. 1999; Campa et al. 2004; Holtan et al. 2006).

In the more biological context it has been shown that different epimerases are expressed at different times in the life cycle of *A. vinelandii*, probably reflecting a need for different alginates in the vegetative capsule and in the cyst (Høidal et al. 2000). The crucial role of these enzymes in cyst formation was recently confirmed by studies of the phenotypes of mutants in which all the *algE* genes had been deleted (Steigedal et al. 2008).

A gene encoding a secreted mannuronan C-5-epimerase designated PsmE has also been identified in *P. syringae* (Bjerkan et al. 2004). This enzyme contains one A-module and three R-modules (Fig. 2). A second module putatively binding calcium is interspersed between the last two R-modules. These modules are followed by a module encoding an alginate deacetylase and lastly by a third type of a calcium-binding module. PsmE was produced recombinantly in *E. coli* and was shown to introduce G-blocks into alginate. Recombinantly produced PsmE is able to remove acetyl groups on acetylated alginate and thus uses acetylated alginate as a substrate for epimerization, in contrast to the AlgE epimerases (Bjerkan et al. 2004). G-block-containing alginates have not been reported from *Pseudomonas* sp. However, *psmE* was identified as a gene expressed at low (18°C) temperature (Ullrich et al. 2000), and it is possible that alginate has not yet been prepared from the relevant strains growing at the conditions required for production of such an alginate. A-modules are only found in the genomes of *A. vinelandii* and *P. syringae* among all the genomes presently deposited in GenBank.

2.3 Cloning and Characterization of the Bacterial Periplasmic Mannuronan C-5-Epimerases

Chitnis and Ohman (1990) were able to isolate two mutants of *P. aeruginosa* that produced alginate with no G residues, and cloned a DNA fragment that complemented this defect. The DNA fragment was later expressed in *E. coli* and was shown to encode a mannuronan C-5-epimerase that was localized in the periplasm (Franklin et al. 1994). The enzyme was inhibited by acetyl groups on the alginate. Later, similar enzymes were cloned and characterized from *A. vinelandii* (Rehm et al. 1996) and *P. fluorescens* (Morea et al. 2001; Gimmestad et al. 2003). All three characterized AlgG epimerases introduce only single G residues (no G-blocks) into the alginate. However, in a later study, Jerga et al. (2006) claimed that AlgG from *P. aeruginosa* can produce G-blocks in vitro. AlgG does not need calcium for activity (Rehm et al. 1996; Jerga et al. 2006).

AlgG is not only needed for epimerization of the nascent polymer, it also has a structural function in transporting the polymer out of the cell, presumably by being

part of a periplasmic protein complex (Gimmestad et al. 2003; Jain et al. 2003). The *A. vinelandii* AlgG epimerase has fairly low activity in vivo and in vitro (Rehm et al. 1996; Steigedal et al. 2008), indicating that the AlgE epimerases determine the G distribution in this bacterium.

2.4 Mannuronan C-5-Epimerases from Algae

As already mentioned, mannuronan C-5-epimerase activity has been described from several brown algae. However, genes encoding putative algal epimerases seem to be difficult to express recombinantly. Nyvall et al. (2003) were able to show that *L. digitata* expresses several genes sharing significant sequence similarities with the bacterial epimerases, especially the AlgG group. The expression pattern of these genes coincided with the level of mannuronan C-5-epimerase, strengthening the hypothesis that these genes encode such enzymes. The data indicated that *L. digitata* encodes a family of at least 21 mannuronan C-5-epimerases. It seems probable that the high number of different epimerases enables the algae to tailor alginates according to the needs of the season and tissue concerned, an explanation analogous to that for the existence of the AlgE epimerase family in *A. vinelandii*. Alginate-related genes from red algae have not yet been reported.

2.5 Structure of the Mannuronan C-5-Epimerases

The 3D structures of the A- and R-modules of AlgE4 were recently published (Aachmann et al. 2006; Rozeboom et al. 2008), and were both found to be right-handed parallel β-rolls (Fig. 3a). The A-module consists of one β-helix with positively charged residues facing a shallow groove. One calcium ion was identified in the structure (Fig. 3b) (Rozeboom et al. 2008). When the crystal was soaked in a solution containing a tetramer of M residues, it was found that the oligomer bound with the reducing end closest to the N-terminal end of the A-module. The R-module forms a narrower β-roll that also binds an oligomer of M residues. On the basis of this, it was proposed that the whole enzyme probably forms one elongated protein with the positively charged alginate binding site spanning both modules (Aachmann et al. 2006).

The catalytic site of AlgE4 seems to consist of Asp152, His154, and Tyr149 (Fig. 3b). This assumption is consistent with the structure data, and even conservative substitutions of one of these amino acids decreased the enzyme activity to less than 1% (Rozeboom et al. 2008). The studies also showed that this catalytic site is very similar to the corresponding site reported for five alginate lyases. The similarity between the lyases was first observed by Osawa et al. (2005). AlgG from *P. aeruginosa* is predicted to be structurally similar to that of the A-module (Douthit et al. 2005), and also shares some sequence similarity to AlgE4 around the catalytic site.

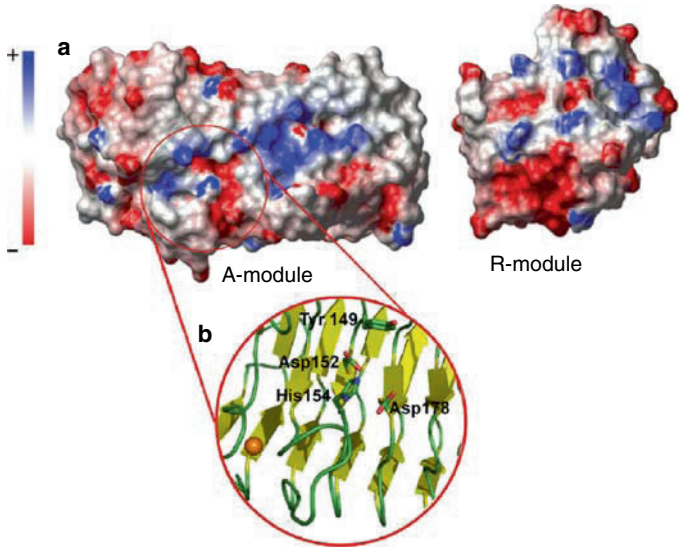


Fig. 3a Electrostatic surface model of the A- and R-modules of AlgE4. **b** The catalytic site of AlgE4 is enlarged and shown in stick representation. The bound Ca^{2+} ion is shown as an *orange sphere*. The models were constructed using MOLMOL (Koradi et al. 1996) and PyMOL (DeLano 2002). (**a** Reproduced with permission from Achmann et al. 2006)

Rozeboom et al. (2008) also proposed a catalytic mechanism for the epimerases based on the structure and activities of mutant proteins (Fig. 4). In this model Asp152 (or possibly Asp178) protonates the +1 M residue. Tyr149 abstracts the C-5 proton from the protonated sugar, while His154 subsequently donates a proton to C-5 from the other side of the sugar.

2.6 Mode of Action of the Secreted Mannuronan C-5-Epimerases

Each of the AlgE1–AlgE7 epimerases generates specific nonrandom epimerization patterns when acting upon mannuronan or any other alginate as a substrate. Such nonrandom block structures are typical in all alginates and have traditionally been attributed to the processive mode of action of polymer-modifying enzymes. "Processivity" refers to the average number of times a reaction is repeated between association and dissociation of the enzyme-substrate complex. High processivity is common for enzymes taking part in the replication and modification of RNA and DNA (Kornberg and Baker 1992). However, any enzyme that has more than one substrate-binding subsite and performs multiple modifications on a substrate may display processivity (Hartmann et al. 2002). Several examples of this type of action have been confirmed for polysaccharides, mainly for exolytic enzymes (Ernst et al. 1998;

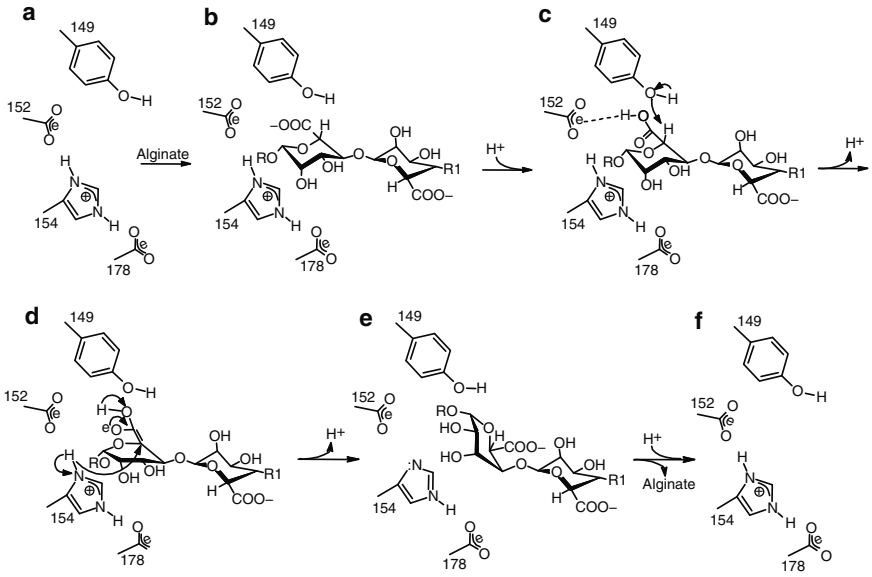


Fig. 4 Proposed catalytic mechanism of AlgE4. The alginate enters the catalytic site (**a**, **b**). The carboxylate group is protonated, allowing it to form a hydrogen bond with Asp152 (or Asp178) (**c**). Tyr149 is deprotonated (via Arg195), and the alkoxide group extracts H-5 from the mannuronic acid residue in the catalytic site (**c**). A double bond is formed, resulting in a nearly planar hex-4-enepyranosyluronate residue (**d**). The protonated His145 performs a nucleophilic attack on the C-5 atom of this sugar from the *si* face, flipping the sugar ring into the $^1C_4$ chair conformation of guluronic acid (**d**, **e**). The carboxylic acid moiety is deprotonated and leaves the active site (**e**, **f**). His154 is protonated again and the epimerase is ready to perform a new reaction (**f**). (Modified with permission from Rozeboom et al. 2008)

Breyer and Matthews 2001). In alginates, enzyme processivity influences whether G units are introduced at random or successively as blocks. The formation of long blocks could thus be explained by a high degree of processivity. However, these nonrandom patterns can also arise from a preferred attack mechanism by which the affinity for the substrates depends on preexisting G residues. To distinguish between these two mode of action experimentally is, however, difficult.

Results from kinetic experiments using time resolved NMR spectroscopy and modeling based on Monte Carlo simulation suggest that AlgE4 acts processively (Høidal et al. 1999; Hartmann et al. 2002). Evidence for processivity also comes from single molecular pair unbinding studies of mannuronan and AlgE4 using atomic force microscopy (Sletmoen et al. 2004). The degree of processivity was estimated, using specific degrading enzymes, NMR, electrospray ionization mass spectrometry, and capillary electrophoresis. On average, AlgE4 epimerizes ten M residues before the enzyme-substrate complex dissociates, implying that the enzyme, converting every second residue, propagates a distance corresponding to 20 M residues (Campa et al. 2004).

AlgE1 and AlgE6 introduce G-blocks in a processive manner when acting on polyMG as a substrate. (Holtan et al. 2006). The remaining epimerases act according to either a preferred attack mechanism (AlgE2 and AlgE5) or a combination of the two mechanisms. A polymer-modifying enzyme, independent of whether it acts processively or in a preferred mode, would have to interact with more than one residue in the polymer substrate. AlgE4 requires a hexameric mannuronan oligomer as the minimum size of the substrate to support epimerase activity, while seven to eight residues and eight to ten residues appear to be the minimum size for AlgE6 and AlgE1, respectively (Holtan et al. 2006).

The recently elucidated crystal structure (Rozeboom et al. 2008) of the A-module reveals that AlgE4A has the structural basis for processivity, since the substrate is partially enclosed in a groove with a relatively large interaction surface, and with a clamp at each side of the active site that would enclose the polymer and help it to slide rather than dissociate.

2.7 Enzyme Specificity; Epimerization of Nonnatural Uronans and Poly(guluronic acid)

The AlgE epimerases are specific for polymeric β-1•4-linked mannuronic acid found naturally in alginates or in C-6-oxidized mannose-containing polysaccharides. AlgE1, AlgE4, and AlgE6 are able to epimerize M-residues in various nonnatural uronans obtained by a selective 2,2,6,6-tetramethylpiperidine-1-oxyl-mediated hypohalite oxidation of mannose containing polysaccharides, leading to new polyelectrolytes with enhanced gelling properties in aqueous systems containing Ca^{2+} ions (Crescenzi et al. 2000, 2002).

The epimerization reaction of D-ManA to L-GulA is analogous to the conversion of D-GlcA into L-IdoA (Lindahl et al. 1972; Hagner-McWhirter et al. 2000) in the biosynthesis of heparin, heparan sulfate, and dermatan sulfate, and glucuronan C-5-epimerase is the only other known polymer-level C-5-epimerase. It has therefore been of considerable interest to establish if mannuronan C-5-epimerases also could epimerize polymer-linked GlcA. In 2000 an astonishing paper by Chang et al. (2000) was published in which they claimed that epimerase isolated from *A. vinelandii* was able to epimerize polymer-linked uronic acid in a range of C-6-oxidized polymers (cellulose, amylose, pullulan, and chitosan) and in native pectin. Over the years, we and other groups have tried to reproduce their results but without success (Holtan 2006) (A. Hotchkiss, USDA ARS, USA, and V. Crescenzi and M. Dentini, University La Sapienza, Rome, Italy, personal communications). Both wild-type and recombinant epimerases have been tested alone or in combinations on the following substrates: C-6-oxidized cellulose and amylose, microbially produced glucuronan from *Rhizobium meliloti*, pectate, and heparin precursors such as *E. coli* K5 capsular polysaccharide and sulfaminoheparosan. No epimerization was detected.

There is, however, indicative evidence that the epimerase binds to GlcA-containing polymers. In a study on the action of the mannuronan C-5-epimerases on C-6-oxidized

konjac glucomannan (Crescenzi et al. 2002) a GlcA-α-L-(1→4)GulA-β-D-(1→4) ManA sequence could be identified after incubation with AlgE4 and AlgE6. This indicates that the AlgE4 subsite (−1) is able to bind to β-D-glucuronic acid. Increasing the AlgE4 and AlgE6 incubation time led to depolymerization of the polymer by a β-elimination mechanism. Similar observations have been reported for C-6-oxidized cellulose (β-(1→4)-D-glucuronic acid) after incubation with AlgE4 (Crescenzi et al. 2000; Crescenzi et al. 2002). This indicates that GlcA, which is a C-2-epimer of ManA, can to some extent be accepted in the active site of AlgE4 mannuronan C-5-epimerase such that the proton on C-5 is abstracted although epimerization to L-iduronic acid is not taking place. Because GlcA is able to recognize β-(1,4)-D-glucuronic acid and act as a lyase, only subtle conformational modifications of AlgE4 or another epimerase might turn it into an active glucuronan C-5-epimerase.

2.8 In Vitro Epimerization with AlgE Epimerases

Alginate gels represent very challenging semisolid structures with applications in biotechnology and industrial fields. The ability to control and tune the mechanical properties represents a key feature for improving the applications of such biomaterial. The availability of AlgE epimerases provides a powerful tool to design the sequential structures and allows monitoring of the effect of compositional modifications on the functional properties of alginate gels. The AlgE enzymes, which in their natural state act in the exocellular environment, are soluble, stable, and require no cofactor except calcium ions. They have been proven to be very effective in modifying all types of alginate in vitro (Mørch et al. 2007). In particular AlgE1, AlgE4, and AlgE6 are technically useful owing to their lack of lyase activity. AlgE4 is highly effective in converting M-blocks into polyMG sequences in any block-structured alginate. When acting on pure mannuronan, this enzyme is able to convert the entire polymer into polyMG (Fig. 1) without breaking the polymer backbone (Hartmann et al. 2002). As described earlier, AlgE1 and AlgE6 generate long G-blocks either by elongating preexisting G-blocks or by condensing them by epimerizing single M residues flanked by Gs (Fig. 1). PolyG is still difficult to make since the calcium ions, required by the enzyme, are gradually depleted owing to their cooperative binding to the G-blocks generated. Moreover, binding of calcium ions could lead to gel formation, rendering the molecules less accessible as a substrate for the enzyme. With use of suboptimal concentrations of calcium ions in the presence of high concentrations of antigelling ions such as sodium, high molecular weight alginates with 97% G were obtained (Holtan et al. 2006). Since three to four M residues at the reducing and nonreducing ends cannot be attacked by the enzyme, this probably represents the end point of the epimerization.

The availability of pure alginates resembling separately the three extreme block sequences present in the natural polysaccharide, namely, G-blocks, M-blocks, and MG-blocks, was found to be a fundamental tool to point out their role in the final hydrogel (Donati et al. 2005)

3 Alginate Lyases

The viscosity of alginates is mostly dependent on molecular weight, although the G distribution affects the stiffness of the chain and thereby also the viscosity. Alginate lyases split the polymer by β-elimination, leaving an unchanged saturated uronate on the reducing end and an unsaturated (4-deoxy-L-*erythro*-hex-4-enepyranosyluronate) residue, symbolized by Δ, at the nonreducing end. Alginate lyases are widely distributed in nature, in organisms growing on alginate as a carbon source, in alginate-producing organisms, and in some bacteriophages (Hashimoto et al. 2009). In this review we concentrate on lyases produced by alginate-producing bacteria and those used to characterize alginate structure.

3.1 Alginate Lyases as Tools To Determine Alginate Structure

Specific degrading enzymes are invaluable tools for structural analysis of macromolecules. This is also the case with alginate-specific degrading enzymes, of which all known can be classified as lyases. The specificity of these enzymes is commonly directed towards one of the residues in the glycosidic linkage, i.e., the lyase from *Klebsiella aerogenes* cleaves G-G and G-M specifically for the guluronate in the glycon position, leaving a reducing guluronate end and an unsaturated end originating from either M or G (Boyd and Turvey 1978). The lyase from *A vinelandii* is specific for M in the glycon position, cleaving M-M and M-G bonds (Ertesvåg et al. 1998a). Other enzymes are specific for the sugars on both sides of the bond, such as the M-M-specific lyase from *P. alginovora* (Chavagnat et al. 1996). Although the chemical identity of the aglycon is lost by the lyase-catalyzed reaction, the unsaturated end functions as a chromophore (UV 230 nm) and is also easily identified by NMR. Combined with mass spectrometry and NMR, specific lyases have been used successfully in analyses of subsite specificity for the epimerases (Campa et al. 2004) as well as in analysis of block length and block length distribution (Holtan et al. 2006). We are currently using enzyme engineering in our search for more specific lyases to be used in alginate sequencing.

3.2 An Alginate Lyase Is Needed To Remove Aberrantly Localized Alginate

In all alginate biosynthetic gene clusters sequenced so far a gene, *algL*, encoding an alginate lyase has been found. Initially this was surprising since degradation of a newly synthesized polymer seems a futile reaction. It was suggested that AlgL could be involved in determination of the polymer length, making primers for alginate synthesis or releasing the cells from alginate (Boyd et al. 1993). Later it

was shown that AlgG, AlgK, and probably AlgX form a protein complex guiding the nascent alginate chain from the polymerase in the inner membrane and through the secretion pore AlgE (AlgJ in *A. vinelandii*) in the outer membrane (Rehm et al. 1994; Rehm 1996; Jain and Ohman 1998; Gimmestad et al. 2003; Jain et al. 2003; Robles-Price et al. 2004). If any of these three proteins are missing, the product is unsaturated oligomers, suggesting that the protein complex protects the alginate against AlgL. It was then shown that alginate production in the absence of AlgL is lethal to the cells (Bakkevig et al. 2005; Jain and Ohman 2005). The explanation seems to be that the polyanion alginate in the periplasm will attract cations and thus create an osmotic stress that leads to cell lysis. If one of the alginate-producing complexes does not function properly, this will happen, and AlgL is present in the periplasm as a repair system to remove these lethal molecules.

P. aeruginosa has been found to encode a second alginate lyase (Yamasaki et al. 2004) that prefers MG-rich alginate. The biological function of this lyase is not known.

4 Alginate Acetylation

Bacterial, but not algal, alginates are acetylated at O2, O3, or both (Skjåk-Bræk et al. 1986a). Acetylation increases the water-binding capacity of alginate and its viscosity (Skjåk-Bræk et al. 1989). It has also been shown that acetylation increases intermolecular association of alginate molecules (Windhues and Borchard 2003).

The genes, *algI, algJ* (*algV* in *A. vinelandii*), and *algF* encoding the proteins needed for acetylation are encoded in the alginate biosynthetic gene clusters (Franklin and Ohman 1996; Vazquez et al. 1999). However, the gene products are not necessary for alginate production (Shinabarger 1993). Still, acetylation takes place in the periplasm (Franklin and Ohman 2002), indicating that although the nascent alginate is not accessible to AlgL it is accessible to AlgF.

For *P. aeruginosa*, acetylation is necessary for the formation of microcolonies during the early stages of biofilm development (Tielen et al. 2005). This suggests that acetylation of alginate is important for cell–cell attachment. Acetylation will also protect the alginate against degradation by many alginate lyases. For *A. vinelandii*, the G distribution of the alginate is determined by the secreted mannuronan C-5-epimerases. Since these enzymes will not epimerize acetylated residues, acetylation may be a way for the bacterium to determine the final degree of epimerization.

5 Use of Alginate-Modifying Enzymes for Biotechnological Purposes

The studies on alginate biosynthesis have established that alginate is synthesized as the homopolymer mannuronan, and that the diversity of structural and functional properties found in different alginates results from the action of C-5-epimerases,

lyases, and acetylases. So far the secreted mannuronan C-5-epimerases have the greatest potential from a biotechnological point of view. Pure mannuronan can now be produced by mutants lacking epimerases (Gimmestad et al. 2003). When this polymer is used as a substrate for the AlgE epimerases, alone or in combination, it is thus for the first time feasible to produce alginates with predetermined structures. In addition to compositionally homogeneous alginates (mannuronan, guluronan, and polymers with strictly alternating M–G) (see Fig. 1), this could also include other alginates with extreme composition and sequential structure not found in nature. Of particular interest is a type of alginate comprising only G-blocks interspaced with polyMG sequences. This material, which was made by a two-step conversion of polyM with AlgE4 and AlgE1, forms very strong, elastic, nonswelling gels, particularly suitable for making immune-protective microcapsules for cell transplantations (Mørch et al. 2007, 2008).

Specific alginate lyases have also been used for preparation of pure uronate blocks (Heyraud et al. 1998). Studies on oligomeric G-blocks have shown that they can affect many properties of an alginate gelling system (Jørgensen et al. 2007). Alginate lyases have also been suggested as mucolytic agents in the treatment of cystic fibrosis (Mrsny et al. 1994)

A further challenge would be to produce tailored alginates in vivo. This might be done by first making an epimerase-negative strain (including an epimerization-defective mutation in *algG*) of *A. vinelandii* and then introducing specific epimerase genes controlled by a heterologous promoter. *A. vinelandii* has a high alginate lyase activity which needs to be controlled to obtain alginate of a sufficiently high molecular weight, but it has the ability to secrete the AlgE epimerases and has been shown to make alginates with a high fractional content of G residues (Skjåk-Bræk et al. 1986a).

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Alginate Gene Regulation

Dennis E. Ohman

Contents

Abstract Alginate is an important virulence factor of *Pseudomonas aeruginosa*, and so our understanding of alginate gene regulation is best understood in this species. Expression of the *algD* operon for alginate biosynthesis is only highly expressed in mucoid clinical isolates that usually have pathoadaptive *mucA* mutations. The three major regulators of the *algD* promoter (P*algD*) are AlgR, AlgB, and AmrZ. Each binds to DNA sites relatively far upstream from the start of *algD* transcription. AlgR and AlgB are two-component regulators. AmrZ is an Arc-like positive regulator of P*algD*, but can also be a negative regulator. A global role for these regulators is also emerging. Expression of P*algD* and the regulators are under the control of σ^{22}, an extracytoplasmic function alternative sigma factor. σ^{22} activity is under posttranscriptional control by membrane-bound MucAB. Release of σ^{22} sequestration can occur as a result of cell wall stress, which activates proteases, including AlgW (DegS-like) protease, to degrade MucA.

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1 Introduction

Pseudomonas aeruginosa is a common opportunistic pathogen, and persons with the autosomal recessive disease cystic fibrosis (CF) are especially susceptible. A striking feature of strains infecting the CF pulmonary tract is the frequent conversion in vivo to a highly mucoid colony morphology, which is due to alginate overproduction (Linker and Jones 1966; Evans and Linker 1973). Alginate is a capsule-like exopolysaccharide that loosely adheres to the cells, and so most of it is found in the culture supernatant. Mucoid *P. aeruginosa* tends to persist in the lungs of CF patients, causing chronic bronchopulmonary infection (Govan and Harris 1986), and cannot be eliminated from the lungs despite aggressive antibiotic therapy (Frederiksen et al. 1997). The appearance of mucoid strains correlates with the formation of a bacterial biofilm containing microcolonies, the development of anti *P. aeruginosa* antibodies, inflammation, and a generally poor prognosis for the patient (Hoiby and Olling 1977; Lam et al. 1980; Pedersen 1992). The CF patient is eventually overwhelmed by the chronic infection as the number of *P. aeruginosa* in the sputum becomes as high as 10^8 cfu/ml (Seale et al. 1979). In addition, adults with chronic obstructive pulmonary disease have also been observed to have mucoid *P. aeruginosa* in their sputum samples (Brauer et al. 2008). There is evidence that the mucoid phenotype confers several selective advantages to the bacterial invader, which include increased resistance to phagocytosis (Schwarzmann and Boring III 1971; Baltimore and Mitchell 1982; Pedersen et al. 1992; Pier et al. 2001). Although alginate is synthesized by many species of *Pseudomonas* and by *Azotobacter*, the regulation of alginate in *P. aeruginosa* has been most intensively studied owing to the role of this capsule-like exopolysaccharide in pathogenesis. Thus, this review of alginate gene regulation will emphasize that which is known in *P. aeruginosa*.

2 Hierarchical Regulation of the Alginate Operon

All but one of the genes (i.e., *algC*) for alginate biosynthesis in *P. aeruginosa* are in a large operon (Chitnis and Ohman 1993), which is often called the "*algD* operon." This 12-gene operon begins with *algD* and is located at 3.96-Mb on the 6.26-Mb physical genomic map of strain PAO1 (Stover et al. 2000). In typical *P. aeruginosa* strains, the *algD* operon is nearly silent, but it shows high activity in clinical mucoid strains (Deretic et al. 1987). The *algD* promoter is large and complex in that four major transcription factors are required for its expression in mucoid *P. aeruginosa*. There is a hierarchy of regulation in that an alternative sigma factor, σ^{22} encoded by *algT*/*algU*, controls the *algD* promoter (P*algD*) directly and also controls the expression of the other major regulators required for expression of the *algD* operon, i.e., AlgR, AlgB, and AlgZ (Wozniak and Ohman 1994). Each of these regulators binds to P*algD* and is described in the following sections. Conversion to the mucoid phenotype and alginate overproduction as observed in clinical isolates is typically

due to mutations in *mucA*, which encodes a posttranscriptional negative regulator of σ^{22} (Martin et al. 1993b; Boucher et al. 1997; Mathee et al. 1997).

3 AlgR, a Two-Component Regulator

The *algR* gene (PA5261), located at 5.92-Mb on the PAO1 chromosome map, encodes a 27.6-kDa protein of the two-component regulator family. AlgR (also called "AlgR1") footprints to two segments far upstream of the *algD* promoter (–479 to –457 and –400 to –380 upstream of the transcription start site) and to a third binding site (–50 to –30) near the *algD* messenger RNA start site that is in an inverted orientation (Fig. 1); all binding sites share a consensus core sequence (ACCGTTCGTC) (Kato and Chakrabarty 1991; Mohr et al. 1991, 1992).

AlgR contains a conserved N-terminal domain typical of response regulators (Fig. 2a) of two-component signal transduction systems (Deretic et al. 1989). The C-terminal domain has a LytTR domain (Fig. 2a) similar to LytR in *Staphylococcus aureus* (Nikolskaya and Galperin 2002), which is found in a variety of bacterial transcriptional regulators that bind to a specific DNA sequence pattern. Also under AlgR control is *algC*, which is not located in the *algD* operon. The *algC* gene encodes a phosphomannose/glucose mutase that is required for both alginate and lipopolysaccharide biosynthesis. AlgR binds to three regions upstream and downstream of the *algC* transcription start site (Fujiwara et al. 1993).

Upstream of *algR* is *algZ* (PA5262, also called "*fimS*"), which encodes the 40-kDa sensory component that interacts with AlgR (Whitchurch et al. 1996; Yu et al. 1997). AlgZ has a LytS histidine kinase signal transduction domain related to that of LytS, a sensor in *S. aureus* (Fig. 2a). However, AlgZ lacks other recognizable nucleotide binding motifs that are typical of histidine protein kinases (Yu et al. 1997). The *algZ–algR* genes appear to form an operon, and it is under σ^{22} positive control (Wozniak and Ohman 1994; Whitchurch et al. 1996). The start of *algZ* transcription is −73 bp from the start of translation (Wozniak and Ohman 1994). However, AlgZ is not required for alginate production, suggesting that AlgR has

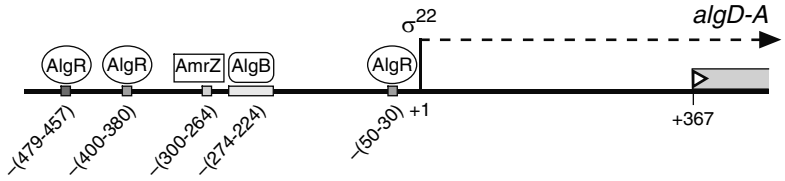

Fig. 1 Map of the *algD* promoter (P*algD*), which controls expression of the 12-gene operon (*algD, 8, 44, K, E, G, X, L, I, J, F, A*) for alginate biosynthesis in *Pseudomonas aeruginosa*. The *closed arrowhead* at +1 base pairs indicates the start of transcription, which is under σ^{22} control. The relative locations of the upstream binding sites for major regulators AlgR, AlgZ, and AlgB are indicated. The start of *algD* translation is at +367 base pairs from the start of transcription

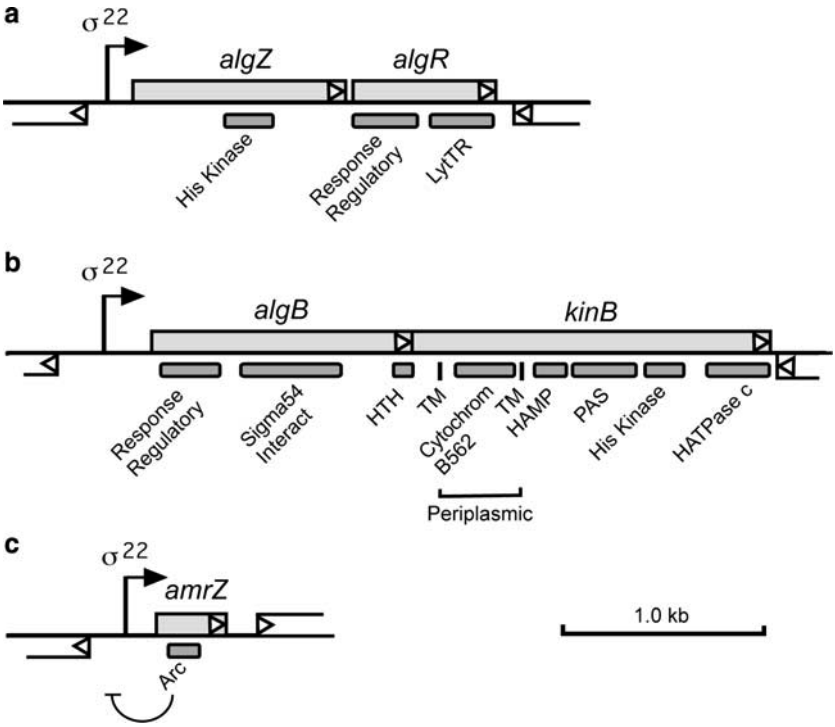

Fig. 2 Gene maps of the major P*algD* regulators: **a** AlgZ-AlgR, **b** AlgB-KinB, and **c** AmrZ. All are under the transcriptional control of σ^{22}. The names and locations of functional domains, obtained from the *Kyoto Encyclopedia of Genes and Genomes* (http://www.genome.jp/), are indicated on each open reading frame and are described in the text

both phosphorylation-dependent and phosphorylation-independent mechanisms of gene activation (Whitchurch et al. 1996, 2002; Yu et al. 1997; Ma et al. 1998). Even when the predicted phosphorylated residue of AlgR (Asp-54) was mutated to asparagine, alginate production was still activated (Ma et al. 1998).

Although AlgZ is not required for alginate production in mucoid (*mucA*$^-$) *P. aeruginosa*, AlgZ and AlgR are both required for type IV pilin mediated motility (i.e., twitching motility) on a solid surface (Whitchurch et al. 1996, 2002). The phosphorylation of AlgR at Asp-54 is essential for direct activation of the *fimU-pilVWXY1Y2E* operon, which is required for the assembly and export of a functional type IV pilus (Belete et al. 2008). AlgR also controls hydrogen cyanide production (Carterson et al. 2004) and activates expression of approximately 58 genes as shown by microarray analysis (Lizewski et al. 2004). In addition, AlgR represses the Rhl quorum-sensing system in nonmucoid biofilms but not in planktonic culture, suggesting a contact-dependent regulatory mechanism (Morici et al. 2007). In all, this suggests a more global role for AlgR in *P. aeruginosa* than strictly alginate gene regulation.

4 AlgB, an NtrC-Family Two-Component Regulator

A mutation in the *algB* gene blocks expression of P*algD* in mucoid (i.e., *mucA*$^-$) *P. aeruginosa* (Goldberg and Ohman 1984, 1987). The *algB* gene (PA5483) maps at 6.17-Mb on the chromosome and like *algR* encodes a two-component regulator (Wozniak and Ohman 1991; Goldberg and Dahnke 1992). Early attempts to demonstrate a direct interaction between AlgB and P*algD* were unsuccessful, suggesting that AlgB may control P*algD* indirectly (Woolwine and Wozniak 1999). However, recent studies have shown that AlgB binds within a region located between –274 and –224 (Fig. 1) relative to the start of P*algD* transcription (Leech et al. 2008). Thus, AlgB belongs to a subclass of NtrC family proteins that can activate promoters that utilize a sigma factor other than σ^{54} in that the P*algD* utilizes σ^{22} in mucoid *P. aeruginosa*. A footprint analysis has not yet been reported to determine the binding site of AlgB.

The 49-kDa AlgB has an N-terminal response regulator domain (Fig. 2b) with the conserved Asp-16, Asp-59, and Lys-109 residues that are typically involved in phosphorylation. The central portion of AlgB has a σ^{54} interaction domain and two Walker boxes (probably representing an AAA+ ATPase domain), which puts AlgB in the NtrC class of transcription enhancer-binding proteins (Wozniak and Ohman 1991). Although NtrC class regulators typically utilize σ^{54} (σ^{N}) promoters, a mutation in *rpoN* (encoding σ^{54}) in mucoid (i.e., *mucA*-defective) *P. aeruginosa* has no effect on *algD* transcription or the mucoid phenotype (Mohr et al. 1990; Totten et al. 1990). The C-terminus of AlgB contains a helix-turn-helix for DNA binding that belongs to the Fis family of regulatory proteins (Fig. 2b).

Immediately downstream of *algB* is *kinB* (PA5484), which encodes a 66-kDa histidine protein kinase that is the cognate environmental sensor of AlgB (Ma et al. 1997). The *algB–kinB* genes form an operon that is under σ^{22} control, although the *algB* transcriptional start site has not yet been determined. The N-terminus of AlgB has two transmembrane domains that form a relatively large periplasmically localized loop as shown by PhoA fusion analysis (Ma et al. 1997). This loop is often the "sensory" domain of histidine protein kinases, and AlgB contains a cytochrome *b*-562 domain within it (Fig. 2b). In the cytochrome, this domain binds a single heme prosthetic group. Centrally, there is a HAMP domain, which is a cytoplasmic helical linker domain common to many receptor histidine kinase and methyl-accepting signaling proteins. Next is a PAS fold, which has been shown in other proteins to act as a sensor for oxygen and redox. There is a histidine kinase A domain, which is similar to that found in the signal-transducing histidine kinase, CheA. This domain contains the conserved "H box" where autophosphorylation at His-385 is predicted to occur. The C-terminus contains an HATPase c domain that is structurally related to ATPase domains of histidine kinases, and this contains the conserved N, D/F, and G boxes.

To show experimentally that KinB has histidine protein kinase activity, a purified KinB derivative containing the C-terminal cytoplasmic region was demonstrated in vitro to undergo progressive autophosphoryation in the presence of [γ-^{32}P]ATP, and it was shown that the phosphoryl label could be rapidly transferred

to purified AlgB (Ma et al. 1997). This reaction is blocked by substitutions of the residues conserved in histidine protein kinases. This provides evidence that autophosphorylation occurs at His-385 with transfer of the phosphate to Asp-59 in the response regulatory domain of AlgB (Ma et al. 1997).

The presence of this active two-component regulatory system suggests that AlgB regulates alginate gene expression in response to environmental or physiological changes. However, KinB is not required for the mucoid phenotype in a *mucA*-defective background (Ma et al. 1998). Even *algB* alleles expressing proteins blocked for phosphorylation, with a D59N substitution or even with the N-terminal response regulatory domain (i.e., 145 residues) deleted, could still restore the mucoid phenotype to an *algB* null mutant of *P. aeruginosa*. Nevertheless, there is evidence that KinB plays a regulatory role in the cell by phosphorylating AlgB. A microarray analysis has shown that as many as 89 genes require both AlgB and KinB for maximal expression, and thus AlgB has a global role beyond alginate gene regulation (Leech et al. 2008).

5 AmrZ, an Arc-Like DNA-Binding Protein

The *amrZ* gene (PA3385, located at 3.79-Mb on the PAO1 map) was originally called "*algZ*" as an alginate regulator (Baynham and Wozniak 1996; Baynham et al. 1999), but was later renamed "*amrZ*" for "alginate and motility regulator" (Baynham et al. 2006). When *amrZ* is mutated in a mucoid strain, there is no alginate production or detectable *algD* transcription (Baynham et al. 1999). A footprint analysis shows that AmrZ binds to a 36-bp sequence that contains an 8-bp direct repeat (GCCATTAC) and is centered at −282 bp relative to the *algD* start of transcription (Baynham and Wozniak 1996) (Fig. 1).

The *amrZ* gene is under σ^{22} positive control and encodes a 12-kDa protein that is a DNA-binding protein of the ribbon–helix–helix family showing homology to the Mnt and Arc repressors of *Salmonella* bacteriophage 22 (Baynham and Wozniak 1996; Baynham et al. 1999). These proteins have an N-terminal β-sheet involved in recognizing and binding DNA. AmrZ contains an Arc-like DNA binding domain (Fig. 2c), and mutations in AmrZ within this β-sheet result in loss of DNA binding (Ramsey et al. 2005; Baynham et al. 2006).

AmrZ can be either a gene activator or a gene repressor. Like AlgR, AmrZ has a role in type IV pilus biosynthesis and thus twitching motility. A mutation in *amrZ* results in no type IV pilin expression, and the amount of PilA (the major pilin subunit) on the surface of the cells is much reduced (Baynham et al. 2006). In contrast, AmrZ binds two sites at its own promoter, where it functions as an autorepressor (Ramsey et al. 2005). AmrZ is also a negative regulator of flagellum biosynthesis. In mucoid *P. aeruginosa*, where *amrZ* shows high expression (due to deregulated σ^{22} activity), AmrZ blocks flagellum biosynthesis by binding to the promoter of *fleQ*, which encodes the master regulator of the flagella regulon (Tart et al. 2006). Thus, AmrZ is a multifunctional regulator of alginate and motility.

6 Ancillary Regulators of the *algD* Operon

Other regulators of *algD* expression have also been described. AlgQ (AlgR2) is a positive regulator of nucleoside diphosphokinase, which is necessary for the formation of GDP-mannose, a precursor in alginate biosynthesis (Kim et al. 1998). AlgP (AlgR3) is a highly basic histone-like protein required for normal *algD* expression (Deretic and Konyecsni 1989, 1990; Kato et al. 1990), but it does not appear to bind P*algD*. Integration host factor is a global regulator that has been shown to play a role in *algD* expression (Wozniak and Ohman 1993; Delic-Attree et al. 1996, 1997). RpoS, the stationary-phase sigma factor, is also required for high-level alginate production (Suh et al. 1999). CysB, required for cysteine biosynthesis, can act as an activator of *algD* expression (Delic-Attree et al. 1997). Unlike *mucA* mutants, a mucoid strain with an undefined *muc-23* mutation depends on σ^{54} (RpoN) for expression of P*algD* instead of σ^{22} (AlgT/AlgU)(Boucher et al. 2000).

The intracellular level of the bacterial second messenger bis-(3′–5′)-cyclic-GMP (c-di-GMP) also regulates alginate production. Alg44, encoded by the *algD* operon, has a PilZ domain that functions as a c-di-GMP receptor, and substitutions in the PilZ conserved residues results in loss of c-di-GMP binding and the ability to produce alginate in *P. aeruginosa* (Merighi et al. 2007). Alg44 is an inner-membrane protein required for alginate polymerization, and its PilZ domain is located in a cytoplasmically localized domain (Oglesby et al. 2008). Growth conditions may affect alginate production. The environment of the CF lung has a high osmolarity, and dehydration has been reported to contribute to the activation of the *algD* promoter (Berry et al. 1989; DeVault et al. 1991). The addition of calcium (1.0 and 10 mM $CaCl_2$) to the culture results in tenfold thicker biofilms and eightfold increase in the expression of *algD* in the mucoid CF strain, FRD1 (Sarkisova et al. 2005), but the regulatory mechanisms are not yet understood. Anaerobic growth conditions have also been shown to stimulate alginate production in normally nonmucoid *P. aeruginosa* (Hassett 1996).

7 Alternative Sigma Factor, σ^{22}

The *algT* gene (PA0762, also called "*algU*") is located at 0.83 Mb on the PAO1 genome map and encodes a 22-kDa alternative sigma factor called "σ^{22}" (also called "AlgT," "AlgU," "σ^{E}") (Deretic et al. 1994; DeVries and Ohman 1994). Early studies showed that *algT* is essential for alginate production in mucoid strains and that it is genetically linked to the "switch to mucoid" gene (originally called "*algS*" and now called "*mucA*") (Flynn and Ohman 1988a, b). Overexpressed from a multicopy plasmid, *algT* can activate the mucoid phenotype in typical nonmucoid strains of *P. aeruginosa* like PAO1 and in several other *Pseudomonas* species (Goldberg et al. 1993). σ^{22} shows sigma factor activity in in vitro transcription assays (Hershberger et al. 1995). *Escherichia coli* σ^{E} is sufficiently similar to *P. aeruginosa* σ^{22} that the *E. coli rpoE* gene can complement an *algT(algU)* mutation in *P. aeruginosa*, restoring the mucoid phenotype (Yu et al. 1995). σ^{22} promotes transcriptional activity at promoters with the

consensus sequence GAACTT (−35) … TCtga (−10) (DeVries and Ohman 1994; Govan and Deretic 1996). P*algD* in *P. aeruginosa* matches this consensus. The *algT* product positively regulates its own transcription at two starts of transcription that also have the σ^{22} consensus sequence (DeVries and Ohman 1994; Schurr et al. 1995)

The *algT* gene is in an operon with four other downstream genes called "*mucA–mucB–mucC–mucD*" (Fig. 3), which are named "*muc*" for the mucoid phenotype caused by mutations in this region (Martin et al. 1993b). MucA (21 kDa) is an inner-membrane protein with one transmembrane domain, and it functions to posttranslationally control σ^{22} activity (Mathee et al. 1997). MucA acts as an anti-sigma factor that binds σ^{22} and can inhibit its ability to transcribe from P*algD* in in vitro transcription assays (Schurr et al. 1996; Xie et al. 1996). As mentioned already, mucoid conversion observed in CF clinical isolates of *P. aeruginosa* is typically due to mutations in *mucA* that occur in vivo (Martin et al. 1993b; Boucher et al. 1997). A common mutation, called the "*mucA22* allele," has a single base pair deletion in a string of five Gs that results in a prematurely truncated MucA.

The nature of the selection pressure for mucoid conversion in the CF lung has received much speculation. Treatment of a biofilm culture of nonmucoid *P. aeruginosa* PAO1 with activated polymorphonuclear leukocytes can result in mucoid conversion (Mathee et al. 1999). When CF isolates are cultured in the laboratory, the mucoid phenotype is typically unstable, especially under nonaerated conditions (Ohman and Chakrabarty 1981). This is usually due to mutations in the *algT* gene instead of by reversion to restore the wild-type *mucA* allele (DeVries and Ohman 1994; Schurr et al. 1994).

MucB (PA0764, also called "AlgN") is a 34.6-kDa negative regulator of σ^{22} activity (Goldberg et al. 1993; Martin et al. 1993a). MucB is a periplasmic protein (Mathee et al. 1997) that binds the periplasmic domain of MucA (L.F. Wood and D.E. Ohman, unpublished data). Mutation in *mucB* leads to a partially mucoid phenotype, probably owing to the lack of MucA-MucB interactions (Martin et al. 1993a). Thus, MucA–MucB apparently forms a signal transduction complex that normally keeps σ^{22} transcriptional activity low until it is affected by an appropriate environmental stress signal. However, in the CF pulmonary environment, there is a strong selection for *mucA* defects to bypass this control circuit and deregulate the negative control over σ^{22} transcriptional activity. The 16-kDa MucC open reading frame appears to be translationally coupled to MucB but its role is unclear (Fig. 3).

MucD (PA0766) has high similarity to *E. coli* HtrA (DegP) (Fig. 3), a periplasmic serine protease involved in the proteolysis of abnormal proteins and that is required

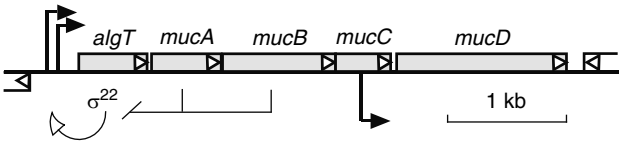

Fig. 3 Map of the *algT*(*algU*) operon of *P. aeruginosa*. The *two closed arrowheads* upstream indicate starts of transcription under autoregulatory control by the *algT* product, σ^{22}. MucA-MucB posttranscriptionally control the activity of σ^{22}. The *closed arrowhead within mucC* represents a strong secondary promoter (not under σ^{22} control) for expression of *mucD*, which encodes a periplasmic HtrA-like protease

for resistance to oxidative and heat stress (Ohman et al. 1996; Boucher et al. 1997). Mutation in *mucD* results in a semimucoid phenotype, especially on *Pseudomonas* isolation agar (Boucher et al. 1997; Wood and Ohman 2006). The *mucD* gene has a secondary promoter, independent of σ^{22}, that is curiously located in the middle of *mucC* (Wood and Ohman 2006). Thus, the phenotype of a *mucC* mutation is complicated by its effects on the *mucD* promoter and *mucD* expression. A mutant expressing a single-copy *mucD* allele encoding MucD altered in its serine protease motif (S217A) is defective in temperature resistance and alginate gene regulation, and so it is a lack of MucD proteolytic activity that activates the mucoid phenotype (Wood and Ohman 2006).

8 Role of σ^{22}-MucAB and Regulated Proteolysis in a Cell Wall Stress Response

P. aeruginosa σ^{22} belongs to the extracytoplasmic function subfamily of sigma factors, which are distantly related to σ^{70} and generally play roles in stress responses (Lonetto et al. 1994; Missiakas and Raina 1998; Helmann 2002). *E. coli* σ^{E}, a well-characterized extracytoplasmic function sigma factor, is sequestered by RseAB proteins (which resemble MucAB) and together they form an envelope-stress response system (Alba and Gross 2004). The σ^{E}–RseAB complex sequesters the sigma factor until envelope stress (e.g., misfolded envelope proteins) signals DegS protease and YaeL/RseP protease to sequentially degrade the anti-sigma factor RseA in a process called "regulated intramembrane proteolysis," which derepresses sigma factor activity (Alba and Gross 2004).

Given this striking similarity to the σ^{E}-RseAB complex, a broader role for σ^{22} in *P. aeruginosa* is emerging as an extracytoplasmic cell wall stress response system. *P. aeruginosa* σ^{22} controls several genes other than those for alginate production, including *rpoH*, a gene encoding σ^{32}, a homologue of the major heat-shock sigma factor (Schurr and Deretic 1997). A proteomic study, using a 2D-gel analysis of all cellular proteins, showed that unregulated σ^{22} in a mucoid PAO1 *mucA22* strain causes elevated expression of *dsbA*, encoding disulfide bond isomerase (Malhotra et al. 2000). A genomic analysis using DNA microarrays showed that the expression of numerous genes having a wide array of functions is coinduced with alginate genes in a mucoid PAO1 *mucA* strain compared with the wild type (Firoved and Deretic 2003). Thus, like σ^{E}–RseAB in *E. coli*, the *P. aeruginosa* σ^{22}–MucAB membrane complex could be a signal transduction mechanism that responds to cell stress to activate the expression of many genes.

To identify stress stimuli that cause transcriptional induction of P*algD*, a plate bioassay has been employed where a plasmid-borne *algD* promoter is fused to a chloramphenicol acetyltransferase cartridge (P*algD-cat*) to provide chloramphenicol resistance as a marker for P*algD* activation (Fig. 4a). This revealed that antibiotics affecting peptidoglycan synthesis and other agents disruptive to the cell wall have a strong effect on P*algD* induction in wild-type *P. aeruginosa* PAO1 (Wood et al. 2006). For instance, treatment of wild-type *P. aeruginosa* PAO1 carrying the P*algD-cat* plasmid leads to a ring of growth around D-cycloserine, a peptidoglycan synthesis

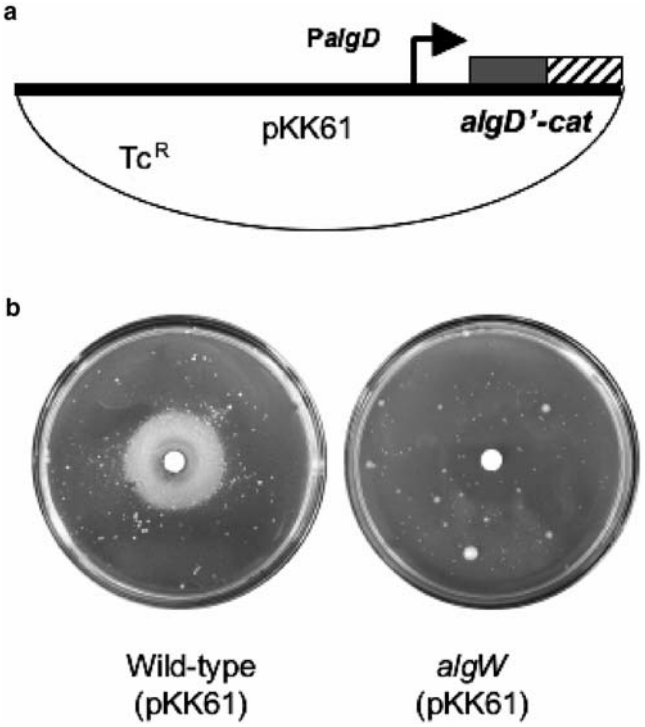

Fig. 4 Bioassay for environmental factors that activate P*algD* expression. **a** The broad host range plasmid pKK61 contains the complete P*algD* region fused to a *cat* (chloramphenicol acetyltransferase) reporter gene, which confers chloramphenicol resistance when the plasmid-encoded P*algD* is activated. **b** Agar plates containing chloramphenicol are spread with *P. aeruginosa* strains carrying pKK61. Wild-type PAO1 (*left*) shows no growth except at the center where D-cycloserine was deposited, which activates P*algD* owing to its induction of a cell wall stress response. An *algW* mutant of PAO1 (*right*), defective in expression of AlgW (DegS-like) protease, fails to show P*algD-cat* expression in the presence of D-cycloserine, indicating a role for this protease in the cell wall stress response that derepresses σ^{22} activity

inhibitor (Fig. 4b). This induction is blocked by mutations in the genes for σ^{22}, AlgB, and AlgR. A transcriptome analysis using microarrays verified that sublethal levels of D-cycloserine activate expression of genes in the *algD* operon. D-Cycloserine also activated many of the same genes, seen in earlier microarray assays (Firoved and Deretic 2003), that are associated with σ^{22} derepression (Wood et al. 2006). Thus, in wild-type nonmucoid *P. aeruginosa*, the σ^{22} regulon responds to cell wall stress.

There is also evidence for regulated intramembrane proteolysis (RIP) control of σ^{22} activity as seen with σ^{E} in *E. coli*. A gene called "*algW*" (PA4446 at 4.9 Mb on the PAO1 map), originally described for its mutant phenotype affecting alginate production (Boucher et al. 1996), is the functional homologue of DegS protease in *P. aeruginosa*. In the bioassay described above, loss of AlgW protease blocks P*algD* induction by D-cycloserine (Fig. 4b), and overexpression of *algW* in PAO1 results in a mucoid phenotype even in the absence of cell wall stress (Wood et al.

2006). These data suggest that AlgW protease plays a major role in σ^{22} activation. Other evidence for RIP in alginate gene regulation came about through a chance discovery following random transposon mutagenesis that overexpression of PA4033 (called "*mucE*") causes mucoid conversion in *P. aeruginosa* (Qiu et al. 2007). PA4033 is a 9.5-kDa envelope lipoprotein, and its overproduction and accumulation derepresses σ^{22} in a manner that may be similar to that seen in *E. coli* where overexpression of porins causes σ^{E} derepression (Alba and Gross 2004). The effect of MucE overexpression on alginate production also requires the AlgW protease and another protease (termed "MucP"), which is a homologue of RseP/YaeL protease in *E. coli*; evidence for MucA degradation is also observed (Qiu et al. 2007).

A model for P*algD* activation is shown in Fig. 5. MucAB sequesters σ^{22} in the inner membrane, thus preventing its interaction with promoters and RNA polymerase, which keeps P*algD* nearly silent. In the CF pulmonary environment there is a selection for *mucA* mutations that make MucA defective, thus releasing σ^{22} to cause deregulation of the system, and allowing maximal expression of P*algD*. In wild-type (MucA^{+}) *P. aeruginosa*, cell wall stress (e.g., peptidoglycan inhibitors, misfolded porins) activate a RIP cascade in which the AlgW and YaeL/MucP proteases degrade MucA to release σ^{22}, thus allowing P*algD* expression (Fig. 5).

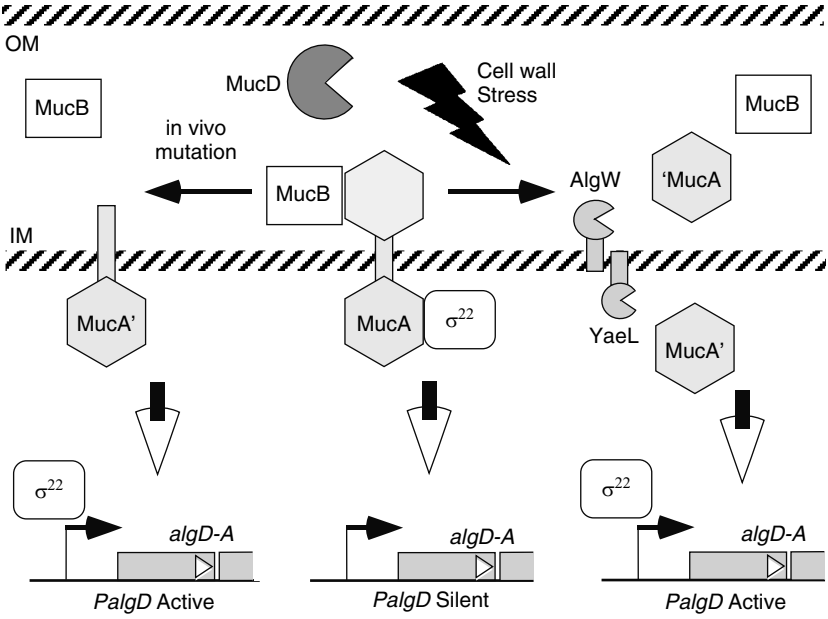

Fig. 5 Model for P*algD* activation in *P. aeruginosa*. In the *center* is shown MucAB sequestering σ^{22} at the inner membrane to prevent interaction with promoters and RNA polymerase, which keeps P*algD* nearly silent. On the *left* is shown the effect of a *mucA* mutation that truncates MucA prematurely (a common event in the cystic fibrosis pulmonary environment), and thus release of σ^{22} causing maximal expression of P*algD*. On the *right* is shown the effect of cell wall stress (e.g., peptidoglycan inhibitors, misfolded porins) on wild-type *P. aeruginosa*, which activates the AlgW and YaeL/MucP proteases to degrade MucA and thus release σ^{22} for P*algD* expression

Other proteases are also being examined for effects on alginate gene regulation. A mutation in the Prc-like protease (PA3257) of *P. aeruginosa*, originally reported to affect alginate production (Reiling et al. 2005), causes poor induction of P*algD-cat* by D-cycloserine, suggesting that it also can play a role in the response to cell wall stress (Wood et al. 2006). MucD, the periplasmic HtrA-like protease is encoded by the *algT* operon, and its function may be to degrade misfolded proteins in that compartment. When MucD is missing by mutation, misfolded proteins in the periplasm signal the RIP cascade, activation of the AlgW protease cascade, and expression of P*algD* (Fig. 5). There is new evidence that three other proteases, ClpP (PA1801), ClpX (PA1802), and ClpP2 (PA3326), may have a role in the degradation of MucA fragments in the cytoplasm (Qiu et al. 2008).

9 Conclusions

Activation of the *algD* promoter and thus alginate biosynthesis is remarkably complex and is linked to a system for stress response. Two regulators (AlgR and AlgB) are proteins of the two-component regulator family, and yet their cognate sensors are not required for P*algD* expression in mucoid *P. aeruginosa*. Two alginate regulators (AlgR and AmrZ) are also involved in motility. The fact that the P*algD* region is about a kilobase in size and requires multiple transcriptional activators suggests the formation of a high-order loop structure allowing for multivalent contacts between the multiple transcription factors. The requirement for three transcriptional activators (AlgR, AlgB, AmrZ) to activate P*algD* suggests that RNA polymerase may be contacted simultaneously by all three. It is unusual for a bacterial promoter to be controlled by the binding of three activators, especially when it is positioned so far upstream from the transcription start site. The binding of integration host factor, which has strong DNA-binding properties, may help to promote the interactions of RNA polymerase with the three regulatory proteins. In addition, it is intriguing that P*algD* has such a long (367-nucleotide) 5′ untranslated sequence (Fig. 1), but its role in regulating P*algD* expression has as yet not been explored.

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Role of Alginate in Bacterial Biofilms

Larry J. Halverson

Contents

Abstract The prevalence of obtaining alginate overproducing stains from lungs of patients with cystic fibrosis and since alginate is an epiphytic fitness and plant pathogenic virulence trait has promoted inquires into the biological function of alginate. Clues into the role of alginate have been revealed by exploring biofilm matrix composition and alginate biosynthesis regulation at the transcriptional and the posttranslational level. Thus, we are refining our appreciation of the types of environmental stressor that activate alginate production and how surface growth may be an important attribute necessary for alginate production. Alginate production likely occurs under conditions in which cues of environmental stresses and biofilm development processes are integrated into regulatory networks controlling alginate production in a fashion that promotes survival of biofilm residents.

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1 Introduction

Direct observation of a variety of habitats (soil, water, animals, plants) has established that most microbes reside on surfaces to create a structured biofilm ecosystem rather than as planktonic individuals. By definition, biofilms are assemblages of microbial populations encased in an extracellular matrix adhered to each other and/or to surfaces or interfaces (Costerton et al. 1995). Matrix components can include lipids, proteins, nucleic acids, and/or polysaccharides, such as alginate. Interest in the contribution of alginate to cell adherence to biotic and abiotic surfaces and as the scaffolding material of biofilms led to the development of *Pseudomonas aeruginosa* as a model for biofilm studies. This focus was due, in part, to the isolation of alginate-overproducing strains from chronic *P. aeruginosa* infections in the cystic fibrosis (CF) lung (Costerton et al. 1995; Ramsey and Wozniak 2005). Remarkable discoveries have occurred in biofilm research this past decade, particularly on the transition from a motile to a sessile lifestyle, and the production of matrix polysaccharides (Hickman et al. 2005; Lee et al. 2007; Merighi et al. 2007). Moreover, we are gaining new insight into the natural conditions under which bacteria produce alginate and the benefits it confers onto biofilm residents.

It is clear from genomic and genetic data that the ability of bacteria to make alginate is widespread amongst members of the ribosomal RNA homology group I of pseudomonads and several *Azotobacter* species (Cote and Krull 1988; Fett et al. 1992; Gacesa 1998). Alginate plays a key role as a virulence factor of plant-pathogenic pseudomonads, in plant epiphytic fitness, and the encystment process of *Azotobacter* species in addition to *P. aerguinosa* infections of CF lungs. Despite the nearly universal ability of *Pseudomonas* species to produce alginate, we have been unable to determine the exact benefits of alginate production. The role of alginate production in natural environments is not well understood. This is a consequence of difficulties in finding laboratory conditions favoring alginate production and the rapid loss of alginate production by overproducers isolated from CF lungs when they are cultivated in the laboratory. Yet, recent findings are providing new insights into biofilm conditions, the environmental factors, and novel regulatory mechanisms controlling alginate production, in particular in the opportunistic human pathogen *P. aeruginosa*. From these studies a picture is emerging revealing the central role of alginate in biofilm lifestyle strategies and stress tolerance, in an environmental context- and species-dependent manner that could lead to increased ecological success of its producers.

2 Bacterial Exopolysaccharides

The fact that bacteria produce extracellular polysaccharides is well recognized, largely because of their involvement in both beneficial and detrimental bacteria–host interactions, and environmental stress tolerance. Extracellular polysaccharides have been classified as capsular or exocellular polysaccharides, although the distinction between the two is operational (methodological). In many cases this distinction is based

on how tightly the polymers are associated with the cell. Secreted polysaccharides represent the most extensively studied component of the biofilm matrix and are often assumed to be the most abundant extracellular component. Alginate is usually isolated as an exopolysaccharide, and the extent to which it is more tightly associated with the cell is unclear. Depending upon the species and environmental conditions, the amount and types of exopolysaccharide produced can vary extensively. For a long time it was assumed that alginate was the primary exopolysaccharide matrix component of *P. aeruginosa* biofilms (Wozniak et al. 2003). It is increasingly clear that alginate is one of many exopolysaccharides that can be made by various *Pseudomonas* species. Although fluorescent pseudomonads can make other exopolysaccharides, alginate biosynthesis capabilities are the most conserved exopolysaccharide biosynthetic capability on the basis of bioinformatic analysis of sequenced genomes. Hence, alginate production likely plays a fundamentally important role in the ecological success of biofilm residents.

3 Alginate Structure and Properties

Alginate consists of a linear polymer of 1,4-β-linked mannuronic acid and its epimer, guluronic acid. These monomers may be arranged in homopolymeric or heteropolymeric block structures. The mannuronate and guluronate confer anionic characteristics to the exopolysaccharide. *P. aerguinosa* alginates are normally highly O-acetyated on the second and/or third position of the D-mannuronic acid residue, yielding highly diverse block structures with various degrees of acetylation (Gacesa 1998). The extent of O-acetylation and the block structure define the physiochemical properties of alginate. The high molecular mass of bacterial alginates and its negative charge ensure that the polymer is highly hydrated. Extensive O-acetylation of alginate increases the water-binding capacity of the polymer. See "Material properties of alginates" by Donati and Paoletti in this volume for a more detailed description of the physicochemical properties of alginates.

In certain cases, cations such as calcium or magnesium can facilitate gelation by acting as an ionic bridge between polymer strands. Temperature has also been shown to influence gelation, with gels being stabilized at lower temperatures. Therefore, the gelling properties are influenced by the chemistry and structure of the alginate, as well as the physicochemical environment. Alginates which contain polyguluronate form rigid gels in the presence of Ca^{2+}, which may contribute a structural role to the outer cyst cell wall of *Azotobacter vinelandii* (Gacesa 1998). In contrast, the absence of polyguluronate blocks, such as in *P. aeruginosa*, produces relatively flexible gels in the presence of Ca^{2+}.

The physicochemical properties of alginate depend predominantly on the structure and composition of the alginate. Thus, the water-binding, gel-forming, virulence, and immunogenic properties of alginate will be dependent on controlling the level of acetylation, epimerization, and molecular weight of alginate. At present little is known on how modulation of these chemical properties influences the beneficial attributes of alginate production. For example, it is conceivable that alginates

produced under water-limiting conditions will be more acetylated than those produced under water-replete conditions since acetylation is directly associated with the water-holding properties of the polymer.

4 Regulation of Alginate Biosynthesis

Alginate biosynthesis and gene regulation have been under scrutiny for a number of years and are the focus of several chapters in this monograph (See Alginate production: precursor biosynthesis, polymerization and secretion" by Rehm and "Alginate gene regulation" by Ohman in this volume for more information on alginate biosynthesis and gene regulation). Briefly, expression of the alginate biosynthetic operon is under the control of the *algD* promoter, with the exception of *algC*, which is also involved in lipopolysaccharide and rhamnolipid synthesis (Zielinski et al. 1991). A key regulatory element controlling transcription from the *algD* promoter is the alternative sigma factor AlgT, also known as AlgU or σ^{22} (Deretic et al. 1994). Besides controlling alginate production, AlgT is the *P. aeruginosa* equivalent of the extreme heat-shock sigma factor σ^{E} of *Escherichia coli* (Yu et al. 1995). The *E. coli* σ^{E} signal transduction pathway responds to cell envelope stress and is activated by an intramembrane proteolysis cascade that liberates sequestered cytosolic σ^{E}. Congruent with the functions played by the σ^{E} of *E. coli*, AlgT is involved in the regulation of a number of other systems in *P. aeruginosa*, such as protection from heat shock, osmotic stress, and exogenous oxidants (Firoved and Deretic 2003; Martin et al. 1994; Yu et al. 1995). AlgT activity is repressed by the gene products of the *mucABCD* locus. One of these genes (*mucA*) encodes an anti-sigma factor functioning at the protein level by binding to AlgT (Schurr et al. 1996; Xie et al. 1996). The diversity and the nature of the regulatory elements affecting *algD* expression reflect the integration of various environmental signals influencing release of sigma AlgT from the negative regulator *mucA* (Fig. 1). Numerous studies have implicated extreme heat, high osmolarity, desiccation, slow growth rates, oxygen availability, oxidative stress, and cell wall disrupting antibiotics as environmental insults that could disrupt *mucA* sequestration of cytosolic AlgT. The universality of the signals influencing alginate production in pseudomonads has not been demonstrated, although both *P. aerguinosa* and *P. syringae* share some conserved signals, including high concentrations of reactive oxygen species and high osmolarity, for activating transcription from alginate promoters (Keith and Bender 1999). By analogy with other sigma/anti-sigma factor systems, MucA (and MucB) likely controls AlgT activity in response to environmental stimuli, permitting physiological and transient release of AlgT, even in the absence of *muc* mutations. Future work will need to focus on environmental signals or other regulatory proteins that increase AlgT activity in nonmucoid strains: much of our understanding of alginate gene regulation has been explored in *mucA* mutant strains.

Historically, studies concerning the regulation of alginate biosynthesis have focused on transcriptional control of the *algD* promoter and *algT* expression in *P. aeruginosa*.

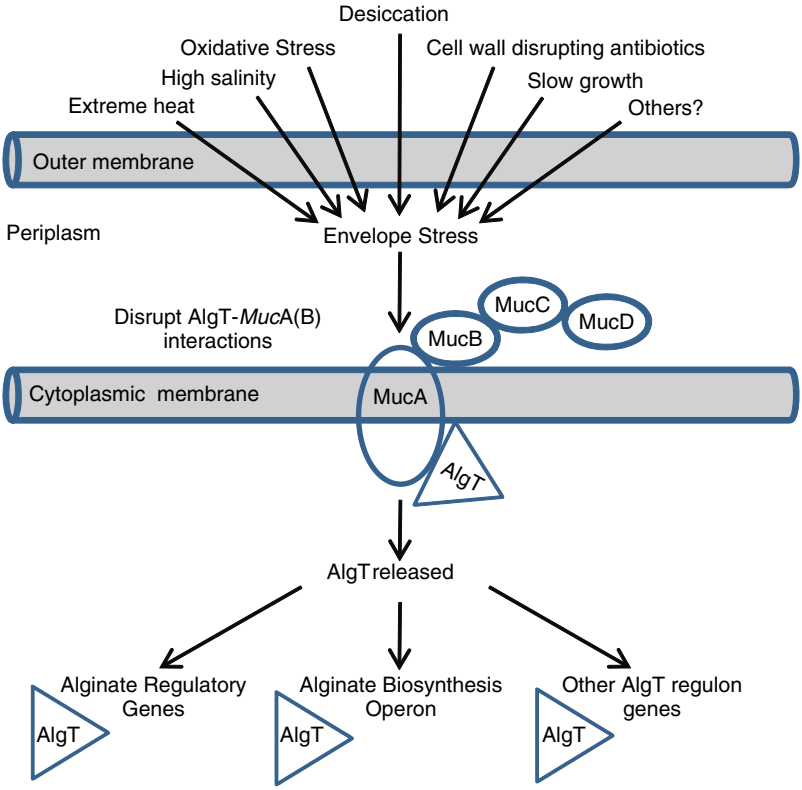

Fig. 1 Model of environmental stress regulation of alginate biosynthesis. The illustration is a summary of the regulation of alginate gene expression derived from various sources (Deretic et al. 1994; Schnider-Keel et al. 2001; Suh et al. 1999; Wood et al. 2006; Wood and Ohman 2006; Yu et al. 1995)

Recently, it was discovered that one of the alginate biosynthesis proteins, Alg44, contained a PilZ domain, which function as receptors of the secondary signaling molecule cyclic diguanylate (c-di-GMP) (Merighi et al. 2007). These secondary signaling molecules control a number of cellular processes in bacteria by functioning as cofactors for enzymes and regulatory proteins (Galperin 2004). Intracellular concentrations of c-di-GMP are regulated by the opposing activities of diguanylate cyclases that synthesize and phosphodiesterases that degrade the signaling molecule. In general, high c-di-GMP levels promote biofilm development, including formation of multilayer bacterial mats at air–liquid interfaces called "pellicles" (Lee et al. 2007) and exopolysaccharide production (Hickman et al. 2005). Thus, c-di-GMP functions coordinately with transcriptional mechanisms in influencing *P. aeruginosa* exopolysaccharide production, including alginate (Merighi et al. 2007). Alg44 presumably is a transmembrane protein that functions in facilitating the extrusion of polymerized alginate through the outer membrane pore AlgE, and was shown to bind c-di-GMP. Overexpression of a phosphodiesterase led to a significant reduction

in alginate production, while, in contrast, elevated levels of c-di-GMP significantly enhanced alginate production (Merighi et al. 2007). These results demonstrated an important role for c-di-GMP in alginate biosynthesis/export. The question remains whether surface growth is a requirement for c-di-GMP activation of alginate production, much like surface growth is a requirement for activation of the Wsp chemosensory system for *P. aeruginosa* biofilm formation and Pel exopolysaccharide production (Guvener and Harwood 2007; Lee et al. 2007). Posttranslational control of alginate production may explain previous inconsistencies between *algD* gene expression at substratum interfaces (Davies et al. 1993) and the apparent lack of alginate in biofilms formed on glass in flow-cell systems.

5 Role of Alginate in Biofilm Formation

Various exopolysaccharides have been shown to be required for the initial attachment of a bacterium to a surface or for the structural development of mature biofilms. The paradigm organism for studying bacterial biofilms is *P. aeruginosa*, and it was presumed that it produced alginate as the primary exopolysaccharide in biofilms. The first line of evidence supporting an involvement of alginate in adherence to inert surfaces was based on the demonstration that both *algC* and *algD* genes are upregulated following adherence of mucoid strains to glass surfaces (Davies et al. 1993; Davies and Geesey 1995). Subsequently, it was demonstrated that alginate overproduction, caused by a *mucA* mutation, altered biofilm developmental patterns, resulting in biofilms containing more biomass than those formed by the wild type (Hentzer et al. 2001). Biofilms made by alginate overproducers are significantly different from those made by nonalginate overproducing strains, with attached cells of alginate overproducers growing more exclusively as discrete microcolonies, resulting in lower substratum coverage and high structural heterogeneity (Hentzer et al. 2001; Nivens et al. 2001). Absence of alginate O-acetylation substantially decreases surface adherence, thereby delaying biofilm initiation (Nivens et al. 2001). Alginate overproduction by *mucA* mutants can, in some cases, interfere with biofilm growth on glass surfaces in flow-cell systems (Nivens et al. 2001). For mucoid strain *P. aeruginosa* FRD1, calcium addition stimulates biofilm thickness by at least tenfold compared with the strain without added calcium. Scanning confocal laser microscopy showed increased spacing between cells for the thick biofilms, and Fourier transform infrared spectroscopy revealed that the material between cells is primarily alginate (Sarkisova et al. 2005).

One concern with these finding is that high-level AlgT activity in the *mucA* mutant could result in expression of genes other than those involved in alginate production and it is those genes that contribute to the altered biofilm phenotype of the alginate overproducer. However, this phenotype was not observed in biofilms formed by an *algDmucA* double mutant, indicating alginate overproduction is causal to the altered biofilm phenotype. Alginate overproduction leads to a dramatic increase in resistance to the antibiotic tobromycin, which could explain why *P. aeruginosa* biofilms are so recalcitrant to antibiotic therapy (Wozniak et al. 2003).

The role of alginate in biofilm formation and development in flow-cell systems was challenged by composition analyses of the exopolysaccharide matrix and confocal microscopy in both *P. aerguinosa* and *P. syringae* biofilms, which revealed that alginate was not a significant component of the matrix (Laue et al. 2006; Stapper et al. 2004; Wozniak et al. 2003). Not only was alginate not detected in the biofilms, transcriptional reporter gene data indicated that alginate biosynthetic gene expression was not induced on initiation of biofilm formation in a microtiter plate assay. Additionally, Wozniak and colleagues (2003) showed that the structural and antibiotic resistance profiles of wild-type and *algD* mutant biofilms were indistinguishable. This led to speculation that other polymers, such as DNA or lipopolysaccharide, were the primary scaffolding component. Recent evidence suggests that *P. aeruginosa* is capable of producing several other exopolysaccharide constituents, a hydrophobic glucose-rich polymer called "Pel" (Friedman and Kolter 2004a, b) and a galactose–mannose-rich polymer called "Psl" (Jackson et al. 2004; Ma et al. 2006, 2007; Matsukawa and Greenberg 2004). Much less is known about these polymers than is known about alginate, but it appears that they contribute to biofilm formation and maintenance in flow-cell systems. However, the role of alginate is unquestionably important in CF pathogenesis. This implies that the onset of biofilm formation in the CF lung during initial colonization likely precludes the switch to a mucoid phenotype (Wozniak et al. 2003). The transition from a nonmucoid to a mucoid phenotype may involve a switch from an unidentified exopolysaccharide, possibly Pel or Psl, which may be the primary exopolysaccharide in non-CF-lung habitats. In light of recent data (Chang et al. 2007), one environmental signal stimulating alginate production in the CF lung could be water starvation.

6 Alginate Creates a Hydrated Biofilm Microenvironment

Bacteria exist in a variety of habitats that are routinely or periodically not saturated with water and residents must integrate cues on water abundance into lifestyle strategies. As in fully hydrated systems, biofilms in unsaturated habitats are encapsulated in an exopolysaccharide layer (Chang and Halverson 2003). Since many exopolysaccharides are hygroscopic, their presence presumably creates a more hydrated microenvironment in the immediate vicinity of the cell, thereby contributing to desiccation tolerance. Despite the long-standing assumption that exopolysaccharide production contributes to desiccation tolerance, there has been relatively little evidence to support the notion that exopolysaccharides, or a particular exopolysaccharide component such as alginate, ameliorate the stresses bacterial cells actually experience under desiccating conditions. Recently, Chang et al. (2007) provided direct evidence that alginate functions to maintain cellular hydration and biofilm formation under desiccating conditions. Moreover, alginate is integral to biofilm architecture under water-limiting conditions but not under water-replete conditions (Chang et al. 2007), which is consistent with reports that alginate does not contribute to biofilm formation in fully hydrated flow-through systems (Wozniak et al. 2003).

Chang et al. (2007) assessed the extent to which alginate production by wild-type and *algD* mutants of *P. putida*, *P. aeruginosa*, and *P. syringae* occurs in response to water limitation. Alginate production increased dramatically under water-limiting but not under high osmolarity conditions, and was regulated at the transcriptional level on the basis of an *algD* promoter transcriptional fusion assay. Figure 2 shows *P. aeruginosa* and *P. syringae* colony biofilms cultivated under water-starvation conditions encapsulated in an alginate-containing matrix. The inability to make alginate decreased exopolysaccharide volume (Fig. 2) and resulted in decreased desiccation tolerance of all three *algD* mutants compared with their respective wild-type species (Chang et al. 2007). To further understand the role of alginate, they used two complementary approaches to assess whether alginate creates a more hydrated microenvironment that protect cells by slowing the rate of cellular drying. Using *P. putida* as a model, they demonstrated that cells encapsulated with alginate perceive less water stress than those without alginate following a desiccation stress, as measured by an intracellular water potential biosensor (Axtell and Beattie 2002). Second, alginate decreased the extent of membrane dehydration, given that the wild type exhibited fewer membrane fatty acid changes reflective of desiccation-mediated membrane stress compared with the *algD* mutant. Collectively, the results indicate that alginate retains water in the cell microenvironment either directly, owing to its hygroscopic properties, or through its ability to influence biofilm architecture that

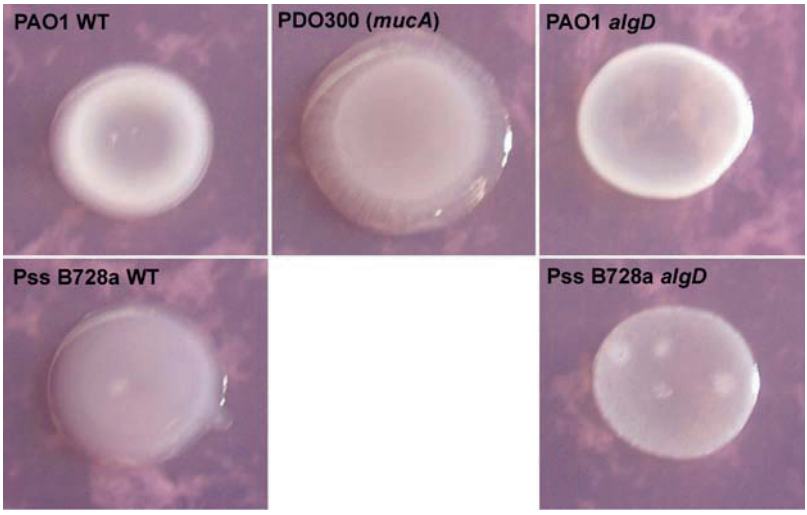

Fig. 2 Morphology of 2-day-old *Pseudomonas aeruginosa* PAO1 and *P. syringae* pv. *syringae (Pss)* B728a colony biofilms under water-limiting conditions. Water starvation stimulates a mucoid PAO1 wild type and PssB728a wild type, a hypermucoid PDO300 *mucA*, and a nonmucoid PAO1 *algD* and PssB728a *algD* colony morphology. Water starvation was imposed by poly(ethylene glycol) amendments to tryptone yeast extract (TYE) medium to lower the water potential by 1.5 MPa as described previously (Chang and Halverson 2003). All strains have a nonmucoid colony morphology on TYE without poly(ethylene glycol) (not shown)

reduces evaporative water loss, or both. Significantly, their findings suggest that the prevalence of alginate biosynthesis capabilities among pseudomonads is because alginate production capabilities are an important fitness trait in soil, the rhizosphere, aerial leaf surface, or the CF lung that can, at times, be water-limited.

7 Alginate Production in the CF Lung

P. aeruginosa infections of lungs of CF patients initially occur as nonmucoid variants. As the *P. aeruginosa* population expands, it diversifies, yielding strains with traits uncharacteristic of environmental isolates. The same traits are consistently acquired during chronic infection of different patients, suggesting that there is a conserved pattern of evolution by which this opportunistic pathogen adapts to the CF airway (Deretic et al. 1994). The phenotypes acquired by *P. aeruginosa* over the course of CF infections reflect alterations in diverse aspects of their biology. One commonly isolated variant is for those that exhibit a mucoid colony morphology, due to the overproduction of alginate. Thus, alginate is presumed to protect *P. aeruginosa* in the CF lung. Once the infecting bacteria start overproducing alginate, the infection is very difficult to eradicate and is associated with a poor prognosis (Burns et al. 2001; Ciofu et al. 2008; Singh et al. 2000). An important visual observation of CF sputum samples suggested that *P. aerguinosa* in the CF lung forms biofilms (microcolonies) consisting of exopolysaccharide-embedded cells (Deretic et al. 1994; Singh et al. 2000). Studies on CF-derived mucoid *P. aeruginosa* isolates in early biofilm studies led to the assumption that alginate is the primary exopolysaccharide in biofilms formed by mucoid and nonmucoid isolates, although this perspective is changing. Inhibition of adherence of nonmucoid but not mucoid strains to eukaryotic cells grown in vitro by alginate antibodies was also considered evidence for the role of alginate in biofilm formation. Consequently, the role of alginate in the formation of *P. aeruginosa* biofilms has been the focus of numerous studies, as described earlier in this chapter, leading to the conclusion that alginate is not a component of the biofilm matrix in flow-cell systems (Wozniak et al. 2003). The question arises as to the identity of the environmental parameters that exert such a strong selective pressure favoring alginate-overproducing variants in the CF lung.

Mucoidy arises owing to mutations in one of several different negative regulators (usually *mucA*) of the alternative sigma factor AlgT, leading to uncontrolled AlgT activation of the alginate biosynthesis genes, as described earlier. Consequently, these variants overproduce alginate, as illustrated in Fig. 2, where the normally mucoid *mucA* mutant strain PDO300 becomes hypermucoid compared with its wild-type parent under water-limiting conditions. Water starvation stimulates the AlgT-mediated envelope stress response in pseudomonads (van de Mortel and Halverson 2004). Besides controlling alginate production, AlgT also controls a large repertoire of other genes involved in stress tolerance (Yu et al. 1995). Since the presence of uncontrolled AlgT activity in mucoid isolates interferes with normal cellular physiological function, the selective advantage of mucoid forms must be

critical for the ability of the organisms to persistently infect CF patients. Alternatively, it is the altered physiological function (Firoved and Deretic 2003) and not alginate production per se that provides a competitive advantage in the CF lung. The metabolic costs associated with alginate production must be great since mucoid strains cultivated in the laboratory rapidly lose alginate-production capabilities and many CF isolates have mutations in *mucA* and in *algT*, resulting in nonmucoid variants.

There is an increasing body of evidence indicating that the CF lung environment is water-limited. The periciliary liquid layer depletion model suggests that CF airway epithelia exhibit abnormally high rates of airway surface liquid absorption (Mall et al. 2004; Matsui et al. 1998, 2005). Presumably, loss of water volume from the CF epithelia results in thickening of mucus, which restricts its transport and provides a favorable environment for microbial colonization (Mall et al. 2004; Matsui et al. 2006). Mucus production may counter water loss if the mucus water sorption properties are sufficient to counter the high rate of liquid adsorption. Biophysical measurements suggest mucus is sufficiently dehydrated to restrict bacterial motility and small-molecule diffusion (Matsui et al. 2005, 2006). In light of recent reports indicating that water deprivation stimulates alginate production (Chang et al. 2007), selective pressure for variants with high AlgT activity, such as *mucA* variants, may be particularly strong in the CF lung environment.

8 Alginate Protects Cells from Antimicrobial Agents

Alginate is clearly produced in the CF lung since antibodies to *P. aerguinosa* alginate have been detected in CF patients, although no mucoid cultures were isolated from sputum samples (Pedersen et al. 1990). With use of alginate-specific antibodies it was also shown that shortly after infection of mice alginate is expressed (Pier et al. 2004). Additionally, alginate gene expression has been observed in nonmucoid *P. aeruginosa* in the lungs of patients with CF and in a mouse model (Bragonzi et al. 2005). Evidence indicates that CF lungs are initially colonized by nonmucoid strains, followed by the appearance of mucoid variants. Alginate does provide protection to *P. aeruginosa* in the CF lung. Infection of the CF lung causes inflammatory cells to be recruited to the site of infection, where they release reactive oxygen species to kill the invading microbes and, unfortunately, cause extensive tissue damage (Ramsey and Wozniak 2005). Alginate can suppress leukocyte functions and nonopsonic phagocytosis and promote adhesion to eukaryotic cell surfaces (Mai et al. 1993a, b; Ramphal and Pier 1985). Complementing this effect is the reported ability of alginate to scavenge reactive oxygen species such as superoxide radicals and hypochlorite, which are likely to play an important role during in vivo killing by macrophages and neutrophils (Learn et al. 1987; Simpson et al. 1989). Furthermore, increased AlgT activity also causes increased expression of antioxidant systems in *P. aeruginosa*, leading to additional protection from reactive oxygen species (Firoved and Deretic 2003; Firoved et al. 2004). Alginate also protects *P. aeruginosa* cells from interferon-γ-mediated macrophage

killing (Leid et al. 2005). Alginate probably provides a physical and chemical barrier, thereby protecting bacteria from phagocytosis.

There has been much speculation that alginate production by biofilm cells increases resistances to antibiotics, although the mode of growth may be more important than alginate production in resisting antibiotics. It is difficult to ascertain the extent to which alginate overproduction contributes to increased antibiotic resistance since increased AlgT activity may induce genes other than those for alginate production that confer increased antibiotic resistance properties. However, it was shown that in nonmucoid *P. aerguinosa* biofilms exposed to subinhibitory levels of the β-lactam antibiotic imipenem, alginate gene transcription was upregulated and coincided with increased alginate production and biofilm volume (Bagge et al. 2004). This observation was subsequently verified to demonstrate that cell-wall inhibitory antibiotics activate alginate biosynthesis and that the AlgW and Prc proteases play a fundamentally important role (Wood et al. 2006). These observations are consistent with cell-wall inhibiting antibiotics in stimulating the envelope stress response, presumably because disruption of the cell wall leads to disruption of the AlgT-MucA signal transduction system. In contrast, the macrolide antibiotic azithromycin blocks quorum-sensing and alginate production, which increases cell sensitivity to complement (Hoffmann et al. 2007). This may explain why azithromycin is clinically effective.

9 Alginate in Phytopathogenic Interactions

The virulence of numerous phytopathogenic bacteria has been correlated with their ability to produce exopolysaccharides. Several studies have shown that the major exopolysaccharide produced by *P. syringae in planta* is alginate in water-soaked leaves (Fett and Dunn 1989). *P. syringae* can also produce the polysaccharide levan, although it is less clear whether it is made *in planta*. The infection of host plants by *P. syringae* involves epiphytic (surface) colonization, entry, establishment of infection sites in the intercellular spaces (apoplast), multiplication within host tissue, and production of disease symptoms. During infection of nonhost plants, the hypersensitive response (HR) functions to restrict pathogen growth and spread. Alginate production has also been associated with increased epiphytic fitness and resistance to toxic molecules (Keith and Bender 1999; Kidambi et al. 1995; Yu et al. 1999). The aerial surface of plants can be an extremely harsh environment, with high levels of solar radiation and fluctuating cycles of wetness. It is likely a suite of adaptations are necessary for epiphytes to survive the harsh leaf environment, and alginate may play a role. Disruption of alginate lyase (*algL*) by transposon mutagenesis revealed that an alginate-deficient *P. syringae* pv. *syringae* mutant was unable to colonize the leaf surface of nonhost plants as well as the wild type (Yu et al. 1999). Evaluation of epiphytic fitness was based on survival on tomato leaves that were kept dry in a low-humidity greenhouse environment. This suggests

that alginate was particularly important for colonizing dry leaf surfaces. However, lesions formed by the alginate-deficient mutant were less severe, possibly because of their lower *in planta* populations (Yu et al. 1999). Collectively, these results suggested that alginate contributes to virulence by facilitating colonization or dissemination of the bacterium *in planta* (Yu et al. 1999). In other words, higher population sizes on leaf surfaces were a consequence of better *in planta* survival.

Although alginate has been isolated from plants infected by *P. syringae*, the signals and timing of alginate gene expression *in planta* have not been fully described. On the basis of various reporter gene studies, the alginate biosynthesis operon is expressed in susceptible plants (Keith et al. 2003). Significantly, *algD* gene expression occurred more quickly and to a greater extent in resistant than in susceptible tomato 'Rio Grande' plants (Keith et al. 2003). Expression of *algD* was evident in individuals colonizing the leaf surface, suggesting biofilm formation is not a prerequisite for alginate production. Within 12h after inoculation, microscopic HRwas visible in resistant tomato cultivars and the incompatible host tobacco inoculated with *P. syringae* pv. *tomato*. Yet, expression of *algD* was visible within 8–12h after inoculation. When the incompatible host tobacco was inoculated with an *hrpC* mutant of *P. syringae*, the HR did not occur and *algD* expression was substantially lower. *hrpC* mutants do not elicit a HR in tobacco (Keith et al. 2003). Collectively the data suggest that signals that proceed the HR may stimulate alginate gene expression in *P. syringae*. One possible signal for *algD* expression is superoxide anion since the oxidative burst, which involves the production of potentially cytotoxic quantities of hydrogen peroxide and superoxide anion, is associated with the HR. The oxidative burst may occur before the HR is visible and reactive oxygen species can induce alginate gene expression in *P. syringae* (Yu et al. 1999).

Another possible signal is water stress associated with the HR. The HR is a complex form of programmed cell death that is generally characterized by the presence of brown, dead plant cells at the infection site. The HR is also associated with cessation of the growth and spread of the bacteria. Interestingly, evidence of alginate gene expression by *P. syringae* pv. *tomato in planta* (Yu et al. 1999) preceding the HR coincides with inhibitory levels of water stress during the HR (Wright and Beattie 2004). Wright and Beattie used a transcriptional fusion between the *E. coli proU* promoter that responds to both the solute and matric components of the total water potential and responds specifically to low water potential and not to ion toxicity or oxidative stress. By measuring the water potential that virulent and avirulent *P. syringae* encounter during their interactions with *Arabidopsis thaliana*, they observed that water potential decreases occurred within 4h after inoculation of the plants and well before visible signs of the HR (Wright and Beattie 2004). The water potential sensed by the bacteria during pathogenesis was sufficient to prevent cell division in the majority of cells in culture. It is conceivable that in addition to or coincident with reactive oxygen species mediated induction of alginate synthesis *in planta*, water starvation occurs during the initial stages of the HR. Alginate may be responsible for the water-soaked appearance of lesions given it is highly hydrophilic and would help maintain moisture in the apoplast to offset HR-mediated desiccation of the infection site.

10 Concluding Remarks and Future Perspectives

Research in the last few years has shed new light onto the potential function of alginate production in bacterial biofilm development and environmental stress tolerance. Much research still has to be done to further define the environmental conditions under which alginate production occurs. Clearly, alginate production is controlled by factors that stimulate the envelope stress response, such as water starvation (van de Mortel and Halverson 2004) and cell-wall inhibiting antibiotics (Wood et al. 2006). Other environmental stressors may stimulate alginate production in a habitat- and species-dependent manner. The recent report that c-di-GMP functions to control alginate production at the posttranslational level provides a foundation on which to further refine our understanding of how this regulatory mechanism influences alginate production. For example, is surface growth a perquisite for c-di-GMP activation of alginate production by *P. aeruginosa* much like it is for production of the hydrophobic Pel polysaccharide? How is alginate production coordinated with the production of other exopolysacchrides that are components of the biofilm matrix since alginate may be only a minor component of the matrix of nonmucoid strains? Of particular interest will be to reveal the spatiotemporal patterns of alginate production within biofilms, particularly since alginate production is energetically demanding under conditions amendable to activating the envelope stress response. Since exopolysaccharides are a shared resource, alginate production by certain members of the microbial community may provide protection to non-alginate-producing residents. If this is the case it could explain, in part, the fact that not all *P. aeruginosa* strains isolated from the CF lung environment overproduce alginate and why some have lost the ability to make alginate. The prevalence of alginate biosynthesis abilities amongst pseudomonads implies that alginate is an important fitness trait under certain environmental conditions or in particular habitats. Understanding the competitive advantages of producing alginate by biofilm residents will provide great insight into the biology of various host-pathogen interactions.

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Microbial Production of Alginates: Physiology and Process Aspects

Wael Sabra and An Ping Zeng

Contents

Abstract Presently, most of the alginate produced commercially is still obtained from algae, although it is subjected to variations in quality and quantity due to changes of climate and sources. Alginate can also be biotechnologically produced by species of two families of heterotrophic bacteria, *Pseudomonas* and *Azotobacter*. Efforts have been made in the past to produce alginate-like polymers from these bacteria. The association of virulence with alginate production in most *Pseudomonas* spp. has made *Azotobacter vinelandii* the most promising candidate for the industrial production of alginate. Nevertheless, for specific and well-defined applications, especially in biomedical and pharmaceutical fields, the production of *Pseudomonas* alginate has attracted increasing attention. Microbial alginate production has been widely investigated in batch, fed-batch, and continuous cultures. This chapter summarizes current knowledge of physiology and process aspects in view of potential industrial production of microbial alginate.

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1 Introduction

As early as 600 B.C., seaweed was used as food for man, but algin, a component of seaweed, was only first discovered in 1880 by the British chemist Stanford. Pure alginic acid was prepared in 1896 by Akrefting. In 1929 the company Kelco began commercial production of alginate and introduced milk-soluble alginate as an ice cream stabilizer. The unique physical properties of alginate enable it to be used as a stabilizer, viscosifier, and gelling agent in the food and beverage, paper and printing, biomaterials, and pharmaceutical industries. However, the high production costs or price of these products, and the environmental impacts associated with seaweed harvesting and processing, have prevented a major breakthrough in various applications. In the middle of the twentieth century, an interesting possible commercial avenue for a microbial polysaccharide was paved by the discovery that strains of *Pseudomonas aeruginosa* (Linker and Jones 1966) and *Azotobacter vinelandii* (Gorin and Spencer 1966) secrete extracellular polyuronides that closely resemble alginic acid recovered from brown algae. In the meantime, the interest in microbial alginate has also been very much medically oriented because of its association with the pathogenicity in strains of *P. aeruginosa*. The fast development of pharmaceutical applications of this polymer as well as the discovery of its unique immunological properties aroused the interest of industrial biochemists in developing a microbially optimized production process of this useful compound. Microbial alginate production has been investigated to date in batch, fed-batch, and continuous cultures. In this chapter we first give a short overview of commercial production of alginate and then summarize several aspects of physiology, such as the biological roles of alginate production in bacteria and bioprocessing, such as optimal conditions for microbial alginate production in bioreactors.

2 Commercial Production of Alginate

The extraction of alginate from algal material is illustrated schematically in Fig. 1. In the first step, acidification of insoluble counterion-alginate (Na^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, etc. via ion-exchange equilibrium with the seawater) is done by extracting the milled algal tissue with 0.1–0.2M mineral acid. Alginic acid is then brought into solution by neutralization with an alkali such as sodium carbonate or sodium hydroxide to from water-soluble sodium alginate. Removal of other algal materials is done by various separation methods, such as sifting, flotation, centrifugation, and filtration. Subsequently, sodium alginate is precipitated by addition of alcohol, calcium chloride, or mineral acid, reconverted to the sodium form if needed, and finally dried and milled. About 25 years ago, Young (1983) suggested a similar route for microbial production of alginate, as shown in Fig. 2. It should be emphasized that to maximize the microbial alginate production on a commercial scale, the biological role of alginate formation should be well understood. In fact there are still many questions remaining to be answered in this respect.

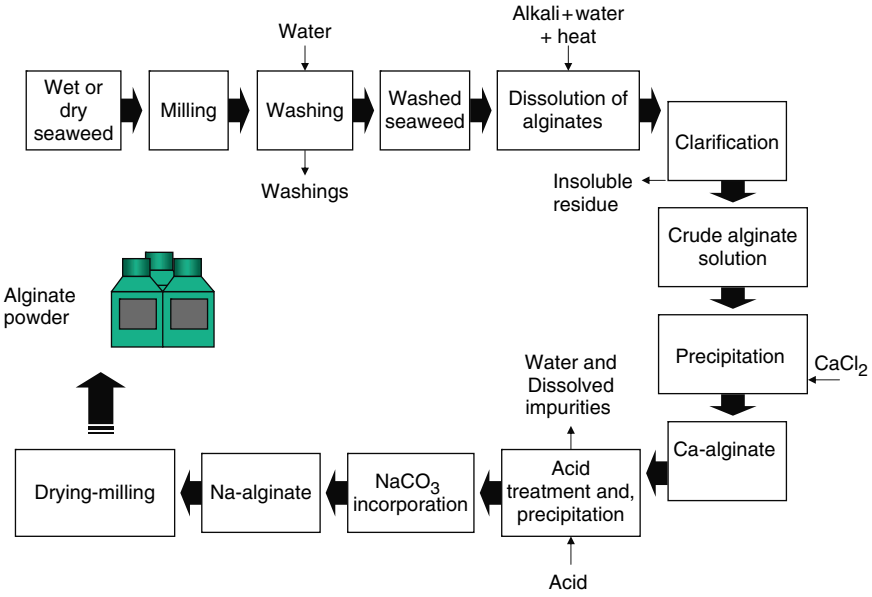

Fig. 1 The manufacture of sodium alginate from seaweed

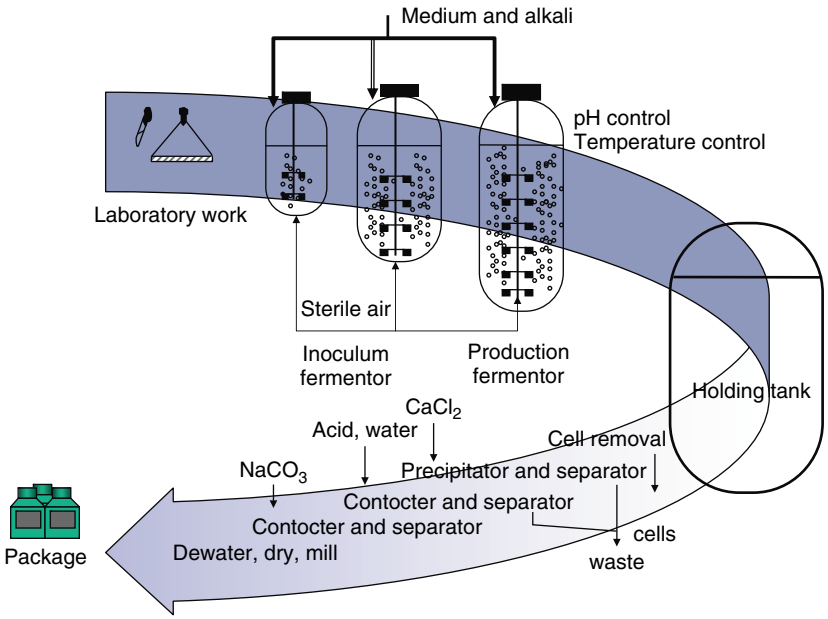

Fig. 2 The production of the microbial alginate (as proposed by Young 1983)

3 Biological Role of Alginate Production in Bacteria

In natural environment in which microorganisms capable of producing exopolysaccharides (EPS) are found, the polysaccharides may be associated with virulence as in the case of plant or animal pathogens, or protect the microbial cell against desiccation and attack by bacteriophages and protozoa or act against macrophages as in the case of human pathogens. The polymers can vary considerably in their chemical structures, and some microorganisms can synthesize more than one type of EPS in accordance with different environmental stimuli.

3.1 Azotobacter vinelandii

3.1.1 Alginate Synthesis Is Essential for Cyst Formation

A. vinelandii is distinguished from the rest of the family *Azotobacteriaceae* by the presence of a characteristic life cycle which includes the formation of a spherically dormant cyst. For the successful formation of the cyst both poly(hydroxybutyric acid) and alginate are essential. Alginate is an essential component of the cyst. This polysaccharide coating protects the cyst from desiccation and unfavourable conditions. The intine (inner coat) and exine (outer coat) layers of the cyst contain different types of alginate. The exine alginate is a polysaccharide with an unusually high content of consecutive guluronic acid residues (G-block), which results in a much higher stiffness. The intine alginate is rich in poly(mannuronic acid) residue. The existence of a large number of alginate-modifying enzymes (epimerases, lyases, and acetylase) in the differentiation process was reported for *A. vinelandii* (Holdal et al. 2000). Furthermore, the correlation between encystment and the release of epimerases indicates that epimerases are, additionally, cell- and cyst-associated. Cysts have been reported to survive in dry soil for several years. Under favourable conditions, the alginate coating will swell and the cyst germinates (Skjak-Braek 1992; Nunez et al. 1999; Holdal et al. 2000; Young and Park 2007; Steigedal et al. 2008).

3.1.2 Alginate Capsule Formation as One of the Mechanisms of Nitrogenase Protection

The association of alginate production with cyst formation and its structural significance do not explain the abundant production of this polymer during vegetative growth of *A. vinelandii*. Indeed, under conditions permitting vegetative growth, alginate production plays different roles depending on the environmental conditions.

A. vinelandii is a gram-negative bacterium, which fixes N_2 non-symbiotically and aerobically. Among bacteria, aerobic nitrogen-fixing microbes are rare. They are mainly found in the family *Azotobacteriaceae*, which comprises the genera

Azotobacter, *Azomonas*, *Beijerinchia*, and *Derxia*. *Pseudomonas methanitrificans*, a methane-oxidizing organism also fixes N_2 aerobically. *Mycobacterium flavum* and possibly one or two related species are also aerobic nitrogen fixers. The remainder of the aerobic bacteria which can fix N_2 are facultative anaerobes which fix N_2 only when they are grown anaerobically (Gottschalk 1988; Lichtl et al. 1997). The majority of nitrogen fixers belong to the group of strictly anaerobic microorganisms (Postgate 1971, 1974).

The nitrogenase enzyme complex, which catalyses the reduction of N_2 to ammonia, is highly sensitive to oxygen. However, members of the diazotrophic *Azotobacter* are able to grow under fully aerated conditions (Linkerhägner and Oelze 1995, 1997; Sabra et al. 1999, 2000). They do not fix nitrogen optimally at high dissolved oxygen concentrations (pO_2) but tend to be microaerophilic in the sense that their N_2 fixation is most effective at low pO_2 though they are obligate aerobes. Obligate aerobes such as *A. vinelandii* are proposed to use two mechanisms for protecting the nitrogenase system against oxygen damage (Fig. 3) (Haddock and Jones 1977; Kuhla and Oelze 1988; Liu et al. 1995; Moshiri et al. 1995; Linkerhägner and Oelze 1997; Duyvis et al. 1998). The hypothesis of respiratory protection postulates that the unusually high activities of cellular oxygen consumption, characteristic for azotobacters, prevent the diffusion of O_2 into the cells and thus to the nitrogenase. Whenever this steady state becomes disturbed by an increase in the pO_2 or phosphate limitation, a conformational protection, i.e. the switch-off of nitrogenase activity,

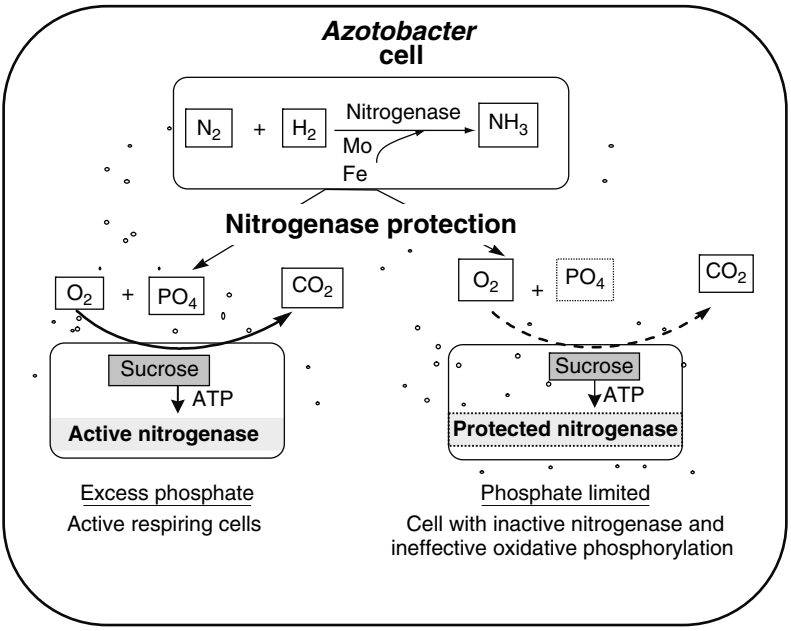

Fig. 3 Proposed mechanisms for the protection of nitrogenase against damage by oxygen in *Azotobacter*

protects nitrogenase proteins. Additional protection of the nitrogenase may also be provided by a decrease in the cellular surface area per cell volume as observed by Post et al. (1982).

Previously, we observed that in a phosphate-limited continuous culture, the specific rate of oxygen consumption (qO_2) of the cells increased as pO_2 was elevated from 1 to 5% air saturation (Sabra et al. 2000). qO_2 remained, however, essentially constant when pO_2 was above 5%. These results cannot be interpreted in terms of the 'respiratory protection' concept. It was observed that both the molecular weight and the guluronic acid content of the alginate formed in these cultures monotonically increased with pO_2. Transmission electron microscopy of negatively stained *A. vinelandii* showed that cells at lower and higher pO_2 formed capsules with significant differences in the thickness and compactness of the polysaccharide (Fig. 4). These results revealed that under nitrogen-fixing conditions the bacterium tends to build alginate capsules with different composition according to the external pO_2, implying that its production may contribute to a protection of nitrogenase against oxygen damage. Indeed, the results obtained in our laboratory (Sabra 1998; Sabra et al. 1999, 2000) and those obtained by Post et al. (1983) and Boiardi (1994) indicate that respiration, at least above a certain oxygen concentration, is not the prevailing mechanism for nitrogenase protection.

The fact that nitrogen-fixing *Azotobacter* cells grown at their maximum specific oxygen consumption rate form compact capsules (Fig. 4), and that the intracellular oxygen level ($C_{O2,\,int}$) in these nitrogen-fixing cells should not exceed the microaerobic- anaerobic range (Linkerhägner and Oelze 1997), indicates that the permeability of the cell wall to oxygen should decrease with increasing pO_2 ($C_{O2,\,ext}$) after reaching the maximum specific respiration rate ($qO_{2\,max}$). Under these conditions, the specific oxygen consumption rate (q_{O2}) can be represented by

$$q_{O2} = qO_{2\,max} = P\,(C_{O2,ext} - C_{O2,int}) = \text{constant},$$

where P is the permeability and is given by

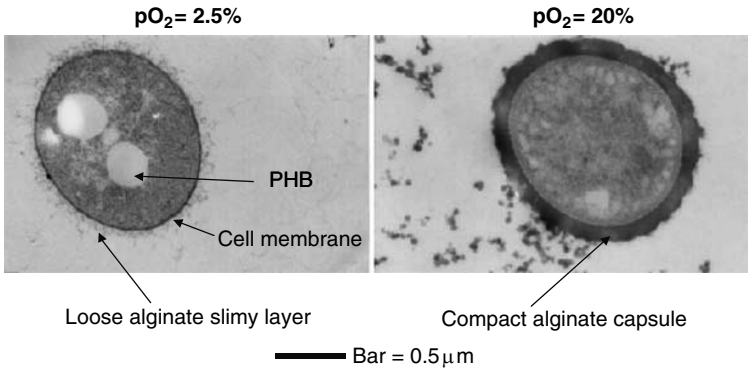

Fig. 4 Thin-section electron micrograph of *Azotobacter vinelandii* cells grown at low (2.5% of air saturation) and high (20%) dissolved oxygen concentrations (pO_2)

$$P = D_{O2}\, A/d.$$

Here, D_{O2} represents the oxygen diffusion coefficient through the cell membrane (cm^2s^{-1}), A the specific cell surface area (cm^2g^{-1}), and d the cell wall thickness (cm).

To achieve a constant qO_2 value and to maintain a low intracellular oxygen concentration at an elevated level of pO_2 in the medium, the organism could respond in three different ways according to the above equations: (1) decrease in the diffusion coefficient of oxygen (D_{O2}) through a dense capsule, (2) decrease in the specific surface area (A); and (3) increase in the capsule thickness (d). All these will result in a decreased oxygen permeability. Post et al. (1982) reported a decrease in the specific surface area as a response to an increased oxygen concentration. The involvement of the first and third possibilities as protection mechanisms for the nitrogenase was suggested by Sabra et al. (2000, 2001). Studies on continuous culture of *A. vinelandii* growing diazotrophically with different oxygen concentrations indicated that alginate capsules are formed even in the presence of a high shear rate in a bioreactor. Moreover, the alginate capsule was more compact and dense at higher pO_2 (Fig. 4). It was therefore postulated that the compact alginate layer formed around the cell acts also as a diffusion barrier for oxygen, which limits its transfer to the extremely oxygen sensitive nitrogenase enzyme. To finally confirm this, measurements of intracellular oxygen concentrations and quantitative knowledge of the sensitivity of nitrogenase towards oxygen are necessary. Figure 5 illustrates differences in the intracellular oxygen concentration as a function of pO_2 for a normal aerobic microorganism with constant permeability and a nitrogen-fixing bacterium with varied permeability of the cell wall.

In general, it is reasonable to state for vegetative cells of *A. vinelandii* that alginate has no single function, but rather provides the cell with a multitude of protective properties under various environmental conditions. Alginate can even act as a protective barrier against heavy metal toxicity, as an ion-exchange system with enhanced selectivity for Ca^{2+}, or provide the bacterium with a hydrophilic, negatively charged coating which creates a barrier against attack and adverse environmental conditions (Fyfe and Govan 1983). Hammad (1998) revealed that alginate-encapsulated *Azotobacter chrococcum* is better protected against the depressive effect of phages than are non-immobilized cells. The presence of eight different types of epimerase together with lyases and acetylase represent excellent tools for the cell to manufacture polymers of different and unique properties to cope with the external environment.

3.2 Pseudomonads

3.2.1 Role of Alginate in Biofilm Formation

Alginate is a key component of biofilm of *P. aeruginosa* and certain other pseudomonads (see "Role of Alginate in Bacterial Biofilms" by Halverson, this volume). Alginate biosynthesis and biofilm formation can be triggered by a number of

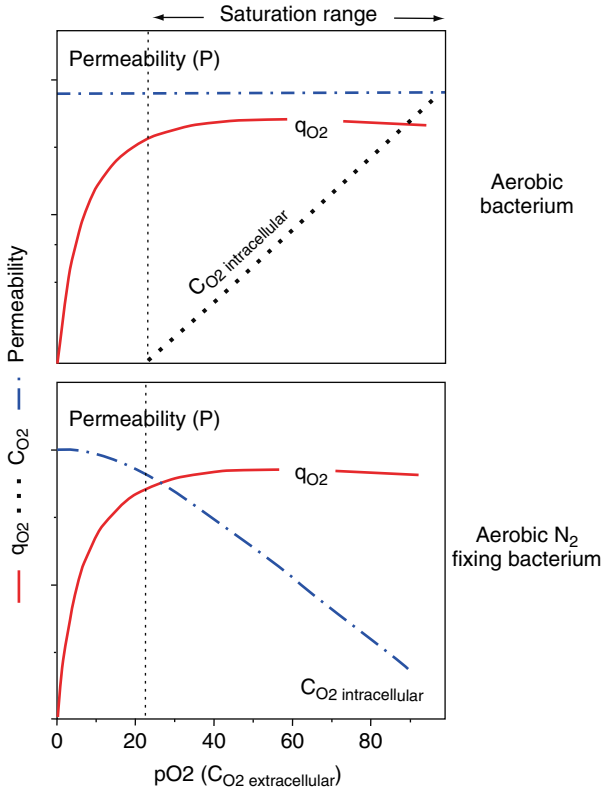

Fig. 5 Differences in the intracellular O_2 concentration as function of pO_2 in culture for a normal aerobic bacterium with constant permeability and a nitrogen-fixing bacterium with varied permeability of the cell wall

environmental factors, including high osmolarity, ethanol, nitrogen, or phosphate limitation (Govan and Deretic 1996; Wagner and Iglewski 2008). It is recognized that oxygen plays an important role in the formation of alginate and mucoidy appearance in this bacterium (Sato et al. 1984; Krieg et al. 1986; Bayer et al. 1990; Leitäo and Sa-Correia 1997; Xu et al. 1998). Oxygen-dependent upregulation of transcription of alginate genes was normally found in highly mucoid strains of *P. aeruginosa* (Leitäo and Sa-Correia 1997). In an experiment with a mixture of mucoid and non-mucoid strains (1:1), Krieg et al. (1986) found that aeration favourably selects the growth of only mucoid phenotypes. Non-mucoid strains were reported to be sensitive to oxygen. In this connection it should also be mentioned that not all cystic fibrosis isolates that appear non-mucoid are truly non-mucoid, but, instead, may belong to the stress-conditio -dependent category of mucoid mutants. Recently, it was shown that in a non-mucoid PAO1 strain of *P. aeruginosa*, alginate formation can be triggered in chemostat culture under oxidative stress conditions (Sabra et al. 2002; Kim et al. 2003). Interestingly, and despite the lack of the oxygen-sensitive nitrogenase in *P. aeruginosa*, this bacterium exhibits better growth in the microaerophilic range as *A. vinelandii*, (Sabra et al. 2000, 2001, 2002).

The preference of *P. aeruginosa* for microaerophilic growth is in accordance with findings from genomic analysis for this strain (Croft et al., 2000). Analysis of the genomic DNA sequences of *P. aeruginosa* PAO1 showed that this strain possesses a 15-kb cluster of genes encoding a microoxic respiration system, similar to that used by nitrogen-fixing bacteria, including genes encoding homologues of nitrogen fixation/microoxic regulatory cascade proteins (fixL, fixJ, and fixK/anr). These genes enable the preferred microaerobic growth of nitrogen-fixing bacteria such as *A. vinelandii*. For *P. aeruginosa*, they might help in its unusual survival under the conditions in the biofilm (Boyd and Chakrabarty 1995). We further investigated the mechanisms by which *P. aeruginosa* protects itself against oxidative stress (Sabra et al. 2002; Kim et al 2003). The *P. aeruginosa* strain PAO1 can create microaerophilic growth conditions by at least two mechanisms: (1) reduction of the transfer rate of oxygen and (2) formation of a polysaccharide capsule on the cell surface (Sabra et al. 2002; Kim et al. 2003; Sabra et al. 2003). It is postulated that the blockage of oxygen transfer may play an important role in the defence of this pathogen against reactive oxygen intermediates. Figure 6 shows the difference between *Pseudomonas* cells grown under nearly anaerobic conditions, in flasks, and in a pO_2-controlled chemostat culture. The difference in capsule morphology of *P. aeruginosa* from that of *A. vinelandii* may reflect the variations in capsule structures, in particular the occurrence of a G-G-block in the latter, and hence the production of a compact alginate layer around the nitrogen-fixing bacterium (Figs. 4, 6)

3.2.2 Alginate and Mucoidy Prevalence in Clinical *P. aeruginosa* Strains

Over the years, a prime target for antimicrobial therapy has been *P. aeruginosa*. However, despite constant improvements in antibiotic research, *P. aeruginosa* remains impossible to eradicate. The association of mucoid strains of *P. aeruginosa* with chronic pulmonary infection in cystic fibrosis patients is well recognized (Pritt et al. 2007). Evidence for their emergence in vivo was first proven by Doggett et al. (1966). However, in contrast to their emergence in vivo, when they are cultured in vitro, mucoid strains of *P. aeruginosa* tend to revert to the non-mucoid form. The particu-

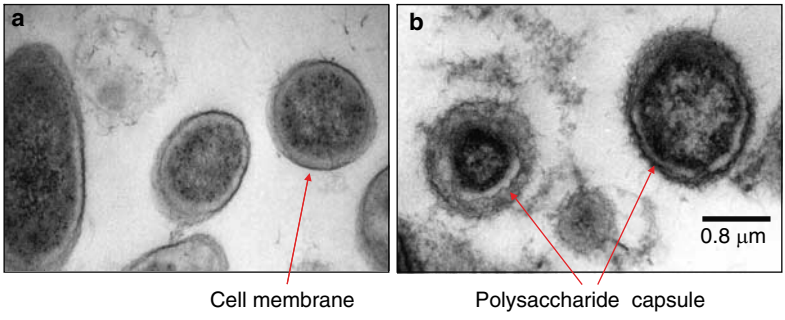

Fig. 6 Transmission electron microscopy of ultrathin sections of negatively stained *Pseudomonas aeruginosa* cells grown under different conditions: **a** cells from shake flask culture (pO_2 = 0); **b** cells grown under controlled oxygen pressure (pO_2 = 10% of air saturation)

lar cause for the conversion to mucoidy is not fully understood in *P. aeruginosa*. In the course of phage typing, Martin (1973) observed mucoid growth of *P. aeruginosa* immediately around areas of phage lysis and obtained mucoid variants after subculturing from such areas. Prolonged antibiotic therapy has been proven as a causative factor in the emergence of mucoidy in patients with cystic fibrosis (Govan and Fyfe 1978; Alkawash et al. 2006; Wood et al. 2006; Prett et al. 2007; Goh et al. 2008). Indeed, isolation of alginate-producing mutants of *P. fluorescens, P. putida*, and *P. mendocina* was done through treatment with antibiotics. Moreover, in vivo, aged biofilms with a high concentration of alginate materials are significantly less susceptible to antibiotic therapy (Anwar et al. 1992; Alkawash et al. 2006). Oxidative burst in neutrophils in contact with *P. aeruginosa* in the lung and the release of oxidative radicals was reported to be another reason for the emergence of mucoidy (Mathee et al. 1999; Sabra et al. 2002, Kim et al. 2003).

3.2.3 Other Pseudomonads

For non-pathogenic alginate-producing pseudomonads no natural ecological niche was reported. This suggests that in the majority of natural habitats, alginate biosynthesis provides no advantage to the organism. Non-mucoid pseudomonads are routinely isolated from natural environments. Since EPS are known to chelate heavy metals, it was also reported that alginate formation was induced, in plant-parasitic pseudomonads, by treatment with a bacteriocidal spray containing copper. Thus, the secretion of alginate may contribute to some plant-bacterial diseases. Although alginate is an important virulence factor in the infection of plants by some phytopathogenic pseudomonads, the precise purpose of this polysaccharide remains unclear. Especially for some pseudomonads that produce more than one polysaccharide. For example, *P. syringae* pv. *glycinea* produces levan and acetylated alginates in vitro when grown on sucrose or glucose as the carbon source, respectively, yet alginate is the only polysaccharide isolated from the infected soybean leaves (Osman et al. 1986; Laue et al. 2006; Schenk et al. 2008). Sodium chloride and ethanol were shown to significantly increase alginate production in a variety of fluorescent pseudomonads, suggesting that osmolarity and dehydration may be general signals for the production of this polysaccharide (Kidambi et al. 1995; Chang et al. 2007).

4 Optimal Conditions for Microbial Alginate Production

4.1 Fermentation Conditions for Optimal Alginate Production in A. vinelandii

Attempts to increase alginate productivity through medium formulation were the focus of many studies (Horan et al. 1981; Okabe et al. 1981; Lebrun et al. 1994; Savalgi and Savalgi 1992; Pena et al. 1997; Clementi et al. 1999; Seanez et al. 2001;

Saude et al. 2002; Saude and Junter 2002: Trujillo-Roldán et al. 2003; Díaz-Barrera et al. 2007; Galindo et al. 2007; Pena et al. 2008). The addition of fixed nitrogenous compounds to the culture medium was reported controversially in the literature. Brivonese and Sutherland (1989) concluded that varying the source of peptone used in the medium could alter alginate yield by up to 30%, suggesting a more specific role for nitrogenous nutrients. Furthermore, growth in the presence of ammonium inhibits alginate formation in *A. vinelandii* (Brivonese and Sutherland 1989; Sabra 1998). To understand the precise role of nitrogenous compounds in alginate production, pO_2 must be accurately controlled. However, most of the work in this respect was done in uncontrolled shake flask cultures. This may explain the contradictory results obtained with A. *vinelandii*.

The crucial effect of controlling pO_2 explains also the controversially reported results with respect to phosphate (Deavin et al. 1977; Jarman et al. 1978; Chen et al. 1985; Sabra et al. 1999). On the one hand, Brivonese and Sutherland (1989) reported that phosphate might simply act to buffer the medium and used a phosphate-rich medium (7.5g K_2HPO_4). On the other hand, observed a drop in alginate yield in a medium with excess phosphate was observed and for their continuous culture studies phosphate limitation gave the maximum alginate production (Sabra et al. 1999). Moreover a correlation between phosphate limitation, respiratory requirement of the cell in term of the respiratory quotient (RQ), and alginate production by diazotrophically grown cells of *A. vinelandii* was demonstrated by Sabra et al. (1998, 1999). They showed that in phosphate-limited batch or continuous culture, the highest specific alginate production was accompanied by a RQ value around 0.8 at an intermediate value of pO_2 (2–5% air saturation). This RQ value was, indeed, near the theoretical optimal RQ value (0.8) calculated on the basis of the stoichiometry of bioreactions leading to alginate formation from sucrose. This optimal RQ appeared to be a useful parameter for the control of alginate production by A. *vinelandii*.

It is generally recognized that the control of oxygen supply is of critical importance for alginate production by *A. vinelandii* especially when it is grown diazotrophically. We showed previously that, under controlled microaerophilic conditions, a very narrow range of pO_2 exists for both optimal alginate and biomass production under diazotrophic conditions (Sabra et al. 1999). This extreme oxygen sensitivity renders the studies on cell physiology with respect to the effects of different nutritional conditions difficult and sometimes controversial (see above). Indeed, it was shown that under phosphate-limited conditions, results obtained under controlled microaerophilic conditions differed significantly from those of flask cultures with the same phosphate concentrations (Sabra et al. 1999). The effect of increasing the viscosity of the culture broth due to the polymer production and cell growth on the effective pO_2, on the one hand, and the decrease in pH due to alginate secretion, on the other hand, make such heterogeneous environments in uncontrolled culture unsuitable for investigating the physiology of such a process. Several studies on *Azotobacter* showed that for optimal alginate formation, pO_2 must be accurately controlled in a microaerophilic range. Oxygen limitation in this bacterium leads to increased accumulation of poly(β-hydroxybutyrate) (Horan et al. 1983; Parente et al. 1998; Sabra et al. 2000; Noguez et al. 2008). The narrow microaerophilic range of

alginate production by a diazotrophically growing culture of *Azotobacter* remains a challenging issue in large-scale production. Recently, it was concluded that alginate production does not increase linearly with increasing pO_2, owing to the fact that the dense alginate capsule around the cell (at high pO_2) exhibits not only a mass-transfer barrier against oxygen but also against other medium constituents and hence lowers the biomass and alginate productivity. In contrast, the soft alginate slimy layer at intermediate pO_2 exhibits lower mass-transfer resistance to other nutrients and thus permits the continuous production of alginate (Sabra et al. 2000).

The effect of other growth limitations on alginate production has also been thoroughly investigated. Iron and/or molybdate limitation gave the highest specific rates for alginate production (Deavin et al. 1977; Annison and Couperwhite 1986b; Ferrala et al. 1986). Ca^{2+} limitation, on the other hand, did not enhance alginate and biomass formation in diazotrophically grown cells (Annison and Couperwhite 1986b; Obika et al. 1993). This may reflect the importance of epimerase activity, which is known to be dependant on the Ca^{2+} level, for the survival of these cells. The chelation of Ca^{+2} through the addition of EDTA to the fermentation medium resulted in the production of alginate with a preponderance of mannuronic acid residues (Couperwhite and McCallum 1974; Annison and Couperwhite 1986a). Surprisingly, under sucrose limitation in a fed-batch culture, conditions where the cell would be expected to make the most efficient use of its available carbon and energy substrate, alginate was produced at similar rates as for other limitations (Sabra 1998; Sabra et al. 2001). This was also observed in carbon-limited chemostat culture by Deavin et al. (1977). The formation of alginate under carbon limitation may indicate how important alginate biosynthesis is for the survival of this diazotrophically grown bacterium in controlled pO_2 culture. This means that the protection of nitrogenase through the formation of a compact capsule around the cell under carbon limitation is the predominating mechanism for respiratory protection, so wasting the limited carbon source is not advantageous for cell survival. In agreement with this assumption, the guluronic acid content and the relative molecular weight of alginate produced after the onset of carbon limitation in a fed-batch culture increased (Sabra 1998). Although the carbon-limited culture produced alginate of high quality (higher molecular weight and higher guluronic acid content), a lower yield was obtained in comparison with that of a normal batch process (3 and $4.9 gl^{-1}$ for fed-batch and batch culture, respectively) (Sabra 1998).

4.2 Alginate Production by P. aeruginosa

The fluorescent pseudomonads have the potential to produce many different types of EPS constituents, including, for example, levan, marginalan, cellulose, and alginate, in addition to several uncharacterized polymers (Fialho et al. 1990; Fett et al. 1995; Kachlany et al. 2001; Jackson et al. 2004; Laue et al. 2006; Priester et al. 2006). Despite the nearly universal ability of *Pseudomonas* species to produce alginate, *P. aeruginosa* was the one used in most studies (Qiu et al. 2008; Stehling et al. 2008), because of its association with virulence in cystic fibrosis patients. Indeed, identifying environmental factors influencing the expression of alginate by the

opportunistic pathogen may help to understand the process of pathogenesis. Moreover, it was recently concluded that mucoid and non-mucoid phenotypes can be expressed according to the host's tissues or environment (Stehling et al. 2008).

It has been well recognized that oxygen plays an important role in the formation of alginate and mucoidy appearance in *P. aeruginosa* (Sato et al. 1984; Krieg et al. 1986; Leitäo and Sä-Correia 1997; Xu et al. 1998; Sabra et al. 2002; Kim et al. 2003). Oxygen-dependent upregulation of transcription of alginate genes was normally found in highly mucoid strains of *P. aeruginosa* (Leitäo and Sä-Correia 1997). Moreover, in an experiment with a mixture of mucoid and nonmucoid strains (1:1), (Krieg et al. 1986) found that aeration favourably selected the growth of mucoid phenotypes. In fact, we reported recently that oxidative stress may cause the conversion to mucoidy (Sabra et al. 2002). Generally, alginate production in vitro is increased upon nutrient (carbon, phosphate, nitrogen, iron) deprivation or exposure to increased sodium chloride, ethanol, desiccation, and oxygen (Ma et al. 1998; Kim et al. 2003; Chang et al. 2007).

4.3 Kinetics of Alginate Production

Alginate production by *A. vinelandii* in phosphate-limited batch cultures was found to parallel cell growth and to cease when growth stopped (Sabra et al. 1999). This was in agreement with the results of Deavin et al. (1977). The first attempt to describe the kinetics of alginate production was made by Klimek and Ollis (1980). They used the experimental data from Deavin et al. (1977) in batch cultures to describe the rate of alginate production by the well-known Luedeking and Piret kinetics (Luedeking and Piret 1959), and a growth-associated form allowed this fermentation processes to be described well. However, in continuous culture, they found that the same strains showed a specific alginate production rate that was relatively independent of growth rate (Deavin et al. 1977; Jarman et al. 1978). These controversial results for batch and continuous cultures were later explained by the fact that alginate synthesis might have been inhibited in the late stage of the batch culture by oxygen limitation that did not occur in continuous culture, owing to pO_2 control (Margaritis and Pace 1985). The contribution of the non-growing-cell metabolism to alginate production was observed by Clementi et al. (1995) using *A. vinelandii* DSMZ 576, and alginate production continued after the organisms entered the stationary phase. They also confirmed that alginate production was only partially associated with growth, with a 75–80% contribution of non-growth-associated production. Recently, we showed that for *A. vinelandii* DSMZ 93–541b at a fixed growth rate the specific alginate production rate (q_{alg}) was dependent on the pO_2 applied (Fig. 7). Furthermore, a general trend was observed, namely the q_{alg} peaked at intermediate pO_2 (2–5% air saturation). It may, therefore, be concluded that the kinetics of alginate production depends not only on the growth rate (Brivonese and Sutherland 1989; Sabra et al. 2000) but also on other culture conditions.

Because of the association of *P. aeruginosa* with pathogenesis, the results of very few kinetic studies have been published on this organism (Krieg et al. 1986). Moreover, most of the work reported in the literature concerning alginate production, if any, was done in petri plates or uncontrolled flask cultures (Remminghorst

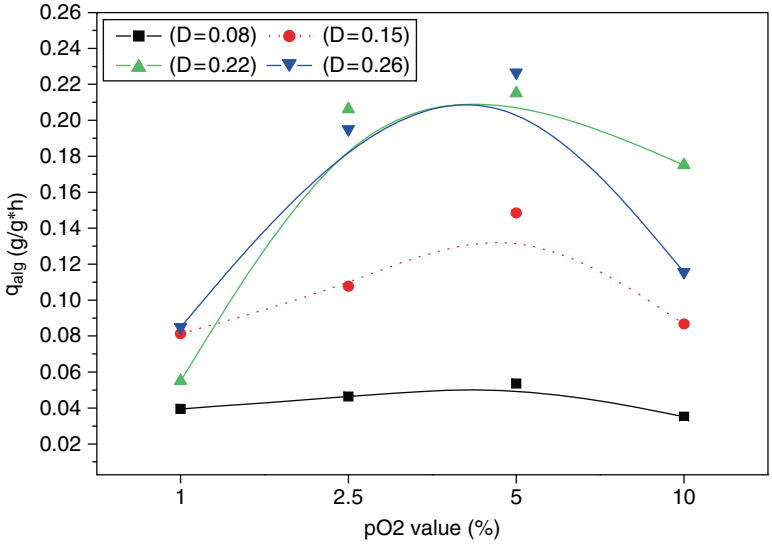

Fig. 7 Dependence of the specific alginate production rate (q_{alg}: grams of alginate per gram of biomass per hour) on pO_2 at the same growth rate of *A. vinelandii* grown in phosphate-limited chemostat culture

and Rehm 2006; Qiu et al. 2008; Stehling et al. 2008). Indeed, there is a lack of kinetics data, especially for those clinical isolates of *Pseudomonas*. The instability of alginate production in vitro by most *Pseudomonas* strains represents a major burden for data evaluation or kinetic analysis (Schurr et al. 1994). It was earlier reported that a mucoid strain of *P. aeruginosa* converted to a non-mucoid phenotype upon prolonged cultivation in continuous culture (Krieg et al. 1986). Recently, Sabra and Hassan (2008) examined the behaviour of alginate production kinetics by a stable mucoid sputum isolate from a cystic fibrosis patient (FRD1). With use of a statistical method, through factorial experimental design and response surface methodology (Yuan et al. 2008), it was shown that alginate production by the FRD1 strain exhibits a typical secondary metabolite production pattern with the highest production rate at the lowest growth rate (Fig. 8). The diminished growth rate that favours alginate production by the clinical strain of *P. aeruginosa* may simulate the condition in the biofilm where the bacteria are most persistent.

4.4 Non-Newtonian Alginate Fluids and the Role of Fermentor Hydrodynamics

In aerobic submerged exocellular microbial polysaccharides, fermentations such as those of xanthan, dextran and alginate, oxygen supply to the media, and mass transfer of oxygen to growing microbial cells are still a major technical problem affecting

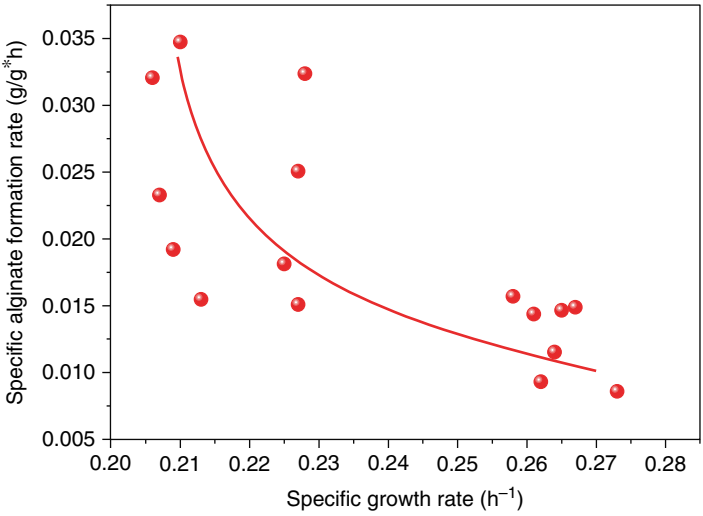

Fig. 8 Specific alginate production rate (q_{alg}) as a function of the growth rate of *P. aeruginosa* FRD1 grown in batch cultures designed according to response surface methodology

microbial productivity, since the solution becomes highly viscous and non-Newtonian during fermentation owing to the presence of polysaccharides in the culture medium. The relationship between energy input and oxygen transfer rate in a bioreactor with pseudoplastic behaviour is not easy to establish (Dussap and Gros 1985; Pena et al. 2002; Zhang and Zhong 2004; Garcia-Ochoa and Gomez 2005). In the case of alginate production by A. *vinelandii*, the low oxygen solubility in fermentation media coupled with the high oxygen consumption rate make the task of ensuring there is sufficient oxygen supply (aeration) difficult.

Changing the rheological properties of the fluid will obviously affect the flow pattern created by the impeller. Around the impeller the fluid is turbulent, and becomes laminar or stagnant when the shear stresses are below the yield stress of the polymer. The impeller creates a cavern in which the fluid moves relatively fast and where the flow is turbulent. However, in the bulk of the fluid, where the shear stresses are below the yield stresses, the fluid does not move at all (McNeil and Harvey 1993; Mancini et al. 1996; Amici et al. 2008).

Preliminary experiments in laboratory bioreactors used for the production of microbial alginate were typically restricted to aerated, mechanically agitated vessels (Deavin et al. 1977; Sabra et al. 2000; Trujillo-Roldan et al. 2001). This gave the turbulence necessary for small bubble formation and mixing and hence good mass transfer. However, for the oxygen-sensitive A. *vinelandii*, the necessity of accurately controlling the pO_2 in large-scale bioreactors remains a challenging problem. The pseudoplastic behaviour of alginate solution results in an exponential increase in viscosity as the fluid slows down after it leaves the impeller and hence the reduction in the pO_2. On the other hand, the relatively low viscosity in the

region near the impeller tends to promote channelling of gas up the centre of the vessel, creating a highly oxygenated zone. To our knowledge, experiments with other types of reactor, especially bubble column and air lift reactors, have not been done for alginate and are worth testing. In fact, for xanthan fermentation, a bubble column turned out to be more efficient than a stirred tank bioreactor on a large scale (Deckwer 1992; Suh et al. 1992). There are very few reports covering aspects of scale-up of the alginate production process (Trujillo-Roldan et al. 2001; Reyes et al. 2003). Understanding the operational parameters involved in scaling-up of alginate production is important. The viscosity and the molecular weight of the polymer produced by *A. vinelandii* in shake flasks were reported to be greater than those of the polymer produced in a pO_2-controlled bioreactor at the same polymer concentration (Pena et al. 2000). Recently Pena et al. (2008) succeed in achieving a high molecular weight alginate polymer in a stirred bioreactor similar to that obtained in flasks by simulating the evolution of actual power input occurring in shake flasks.

5 Conclusion

Commercial opportunities seem to exist for bacterial alginates, especially in the biomedical and pharmaceutical fields. The high price of alginates applied in these fields may open up a new market for polymers with a high degree of purity and defined chemical compositions. Firm knowledge of the relationship between alginate structure and function is needed to produce a polymer with the required structure for specific applications. In this regard and in view of possible industrial production of alginate, there are several aspects which need further investigation. For A. *vinelandii*, the problem of oxygen sensitivity represents a challenging task for large-scale production in a bioreactor. The effect of reactor design on the production and the quality of alginate is still not clear. An alternative physiological solution is to decrease the extreme oxygen sensitivity of this bacterium. This could enhance the feasibility of commercialized production of alginate from *A. vineladii*. It can be stated that the microbial production of high-quality alginate is technically and ecologically more favourable than the algal route. In this respect, understanding the mechanisms of epimerization, acetylation, and degradation, as well as their regulation in both *A. vinelandii* and *P. aeruginosa* is crucial for obtaining a high-quality product for a potentially new market.

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Alginate-Based Blends and Nano/Microbeads

Kamalesh Prasad and Jun-ichi Kadokawa

Contents

Abstract Usually alginates are the sodium or calcium salts of alginic acid. Alginic acid is extracted from certain species of brown seaweeds. Sodium alginate is water-soluble, while calcium alginate is water-insoluble. Apart from their sourcing from the renewable resources in the nature, they have the added advantages of biocompatibility, biodegradability and ability to form a gel, which makes them suitable candidates for various applications. Alginates are mainly processed as capsules, beads, fibres and blends with other natural and synthetic polymers and are widely used in various fields. Developments made in the field of alginate-based blends and beads in recent years are summarized here. The method of preparation of the micro- and nano-sized beads, blends and their important applications are discussed, focusing on their recent uses in membrane science, drug slow release, biomedicine, process technology including enzyme immobilization, and pollution control. Recent applications of the beads in stem cell research are also briefly discussed.

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1 Introduction

Alginates are typically the sodium and calcium salt derivatives of alginic acid, extracted from some species of brown algae. Although initially alginic acid was thought to consist of a uniform polymer of mannuronic acid, later studies showed the presence of both guluronic and mannuronic acid blocks. More precisely, it consists of (1→4)-linked β-D-mannuronic acid and α-L-guluronic acid (Fischer and Dorfel 1955). In alginic acid, both residues exist in the form of a homopolymer block or a heteropolymer block as shown in Fig. 1 (Haug et al. 1967). Because alginates are extracted from certain brown seaweeds, depending on the seaweed source and growing conditions the ratio of mannuronic acid to guluronic acid can vary in various samples of alginates. They have reversible gel-forming ability in the

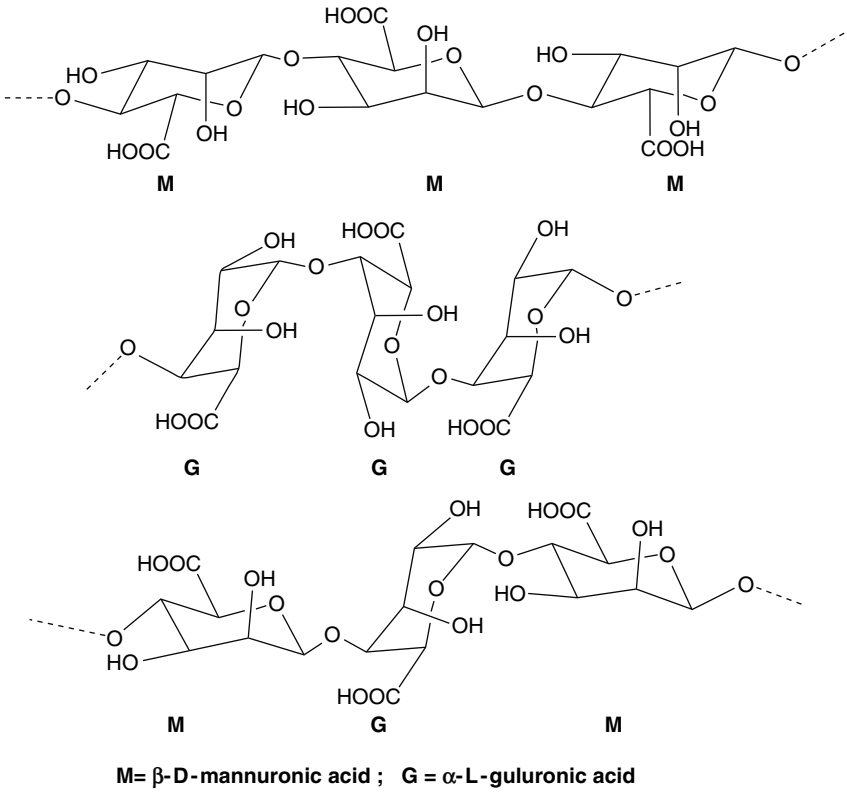

Fig. 1 Repeating D-mannuronic, L-guluronic and β-D-mannuronic-α-L-guluronic acid blocks in alginic acid

presence of calcium. It has been found that the poly(guluronic acid) blocks can bind calcium ions more effectively than the poly(mannuronic acid) blocks (Grant et al. 1973). Owing to their biocompatible nature, alginates are well adapted for tissue engineering, as delivery vehicles for drugs and cells, for enzyme entrapment as well as in biomedicine as wound-dressing material (Sussman 2006). Apart from these applications, they are also used as chelators for pulling radioactive toxins such as iodine-131 and strontium-90 from the body and as food additives to provide structures to some food materials, including marine and freshwater products (http;// www.wikipedia.org; Llaneras 2000). Alginates can be processed as capsules, beads and fibres, blends with other natural and synthetic polymers or films. Some alginate blends and films are commercialized as haemostatic wound-dressing materials. Researchers are continuing their search for suitable materials useful in many newer applications, and consequently beads and blends obtainable from alginic acid and alginates are being successfully used in a variety of applications. On the basis of the above viewpoints, the recent advances made in the research related to beads and blends are reviewed in this chapter. A simple representative structure of the alginates will be used throughout the chapter.

2 Alginate Blends

Like many other natural polymers, alginates are also used for preparation of blends by conjugating them with other compatible synthetic and natural polymers or materials. The blends thus obtained show the properties of both the ingredients and sometimes such blends have properties superior to those of many other biopolymer blends and are useful for many applications.

2.1 General Methods for the Preparation of Alginate Blends

Cross-linking of alginates with suitable materials is the most popular method for the preparation of alginate blends. There are numerous examples of blends prepared by this technique. A blend composed of sodium alginate and hydroxyethylcellulose (HEC) ionically cross-linked with phosphoric acid was prepared by Kalyani et al. (2006) and its potential use as an evaporation membrane was tested and will be discussed later in this chapter. Blends of sodium alginate with other natural polymers are also found to have improved properties compared with the properties of the individual constituents. Among the natural polymers, chitosan has been widely used for preparation of such blends as the cross-linking is easier owing to presence of amino groups. Sodium alginate–gelatin and chitosan–sodium alginate biopolymeric blend materials cross-linked with glutaraldehyde were prepared by Fadnavis et al. (2003). Xu et al. (2007) have demonstrated the preparation of alginate-chitosan blend gel beads based on Ca^{2+} or dual cross-linking with various

proportions of alginate and chitosan. Another seaweed-based polysaccharide, carrageenan, was also blended with sodium alginate via cross-linking with the water-soluble carbodiimide moiety to prepare a membrane (Xu et al. 2003) and details will be discussed later. Simple blending or mixing was also found to be successful for the preparation of alginate blends. Blending of 84% deacetylated chitosan and sodium alginate biopolymers by cost-effective and simple fabrication techniques for direct methanol fuel cell application was investigated by Smitha et al. (2005). To introduce the antimicrobial property of chitosan into alginate, Ariyakriangkrai et al. (2005) blended sodium alginate with *O*-carboxymethyl chitosan or *N*-(carboxyacyl) chitosan. More recently, a spinning technique was employed for the preparation of effective blends of alginates. Wang et al. (2006) reported the preparation of alginate and soy protein isolate blend fibres by spinning their solution through a viscose-type spinneret into a novel coagulating bath containing aqueous $CaCl_2$, HCl and ethanol. Alginate and gelatin blend fibres were also prepared by a spinning technique in the same manner (Fan et al. 2005). The same researchers have also reported the preparation of alginate-carboxymethyl chitosan blend fibres by this technique (Fan et al. 2006). They showed a good miscibility between alginate and carboxymethyl chitosan owing to the existence of a strong interaction from the intermolecular hydrogen bonds. The introduction of carboxymethyl chitosan into the blend fibre was found to improve the water-retention properties of the material in comparison with those of pure alginate fibre.

2.2 Applications of Alginate Blends

2.2.1 Membrane Science

Owing to the excellent film-forming ability of the alginates as well as that of their blends, their performances in thin-film form are being widely investigated by many researchers as potential membranes. As a result of the hydrophilic nature of sodium alginate, its applications in the separation of non-aqueous solvent mixtures by the perevaporation technique have been more successful. Hence attempts have been made by many researchers to introduce hydrophobicity into the sodium alginates by reacting or blending them with suitable hydrophobic substrates. Improved membrane performance of sodium alginate blended with HEC over sodium alginate film for 2-propanol dehydration was reported by Naidu and Aminabhavi (2005). They further found the highest selectivity for water using such membranes by incorporating zeolite [ZSM-5(40)] in the membrane, which showed enhanced flux. Further, the same group has reported the compatibility of sodium alginate and HEC and perevaporation separation of water + 1,4-dioxane and water + tetrahydrofuran (Naidu et al. 2005a, Naidu et al 2005b). The performance of the blend membranes of sodium alginate and HEC, ionically cross-linked with phosphoric acid for perevaporation separation of a *t*-butanol-water mixture, was evaluated by Kalyani et al. (2006). The blended polymer thus obtained was found to have good potential for breaking

the azeotrope of 88 wt% *t*-butanol with high selectivity. Yang et al. (2000) studied the perevaporation separation of ethanol–water mixtures through blend membranes prepared with 8 wt% cellulose cuoxam and 8 wt% aqueous sodium alginate solution cross-linked by using a Ca_2C bridge in 5 wt% $CaCl_2$ aqueous solution. They studied the effect of Ca_2C on the flux and other parameters. A hollow-fibre composite membrane made up of a poly(vinyl alcohol)–sodium alginate blend along with maleic acid, supported by a polysulfone hollow-fibre ultrafiltration membrane was prepared to study the perevaporation dehydration from 2-proanol, butanol, *t*-butanol and ethanol aqueous solutions. The results of the perevaporation experiment showed high selectivity and promising permeability for the blended membrane cross-linked with 1.5 wt% maleic acid (Dong et al. 2006a). Apart from perevaporation applications of the alginate blend membranes, sodium alginate–poly(vinyl pyrrolidone) blend membranes were used for the separation of aqueous dimethylformamide solutions in the concentration range 0–100 wt% (Solak et al. 2008). Phisalaphong et al. (2008) have used bacterial cellulose and alginate in an aqueous NaOH–urea solution as substrate materials for the fabrication of a novel blend membrane using supercritical CO_2 drying. The supercritical CO_2 drying provided a nanoporous structure that had better water adsorption capacity and water vapour transmission rate than the conventional blend membranes prepared without using supercritical CO_2 drying. Poly(ion complex) membranes made by blending 84% deacetylated chitosan and sodium alginate biopolymers followed by cross-linking with glutaraldehyde were tested for the separation of ethanol-water mixtures. These blend membranes (Fig. 2) were found to have good potential for breaking the azeotrope of water (Kanti et al. 2004).

2.2.2 Slow-Release Applications

Owing to the biocompatible nature of the alginates and their blends with natural polymers, they are being investigated as potential candidates for various drug slow-release applications. Chitosan–alginate blend microcapsules were evaluated as a method for oral delivery of egg yolk immunoglobulin Y antibodies. It was found that the stability of immunoglobulin Y in simulated gastric fluid was greatly improved by encapsulation in chitosan–alginate blend microcapsules (B. Li et al. 2007). Sarmento et al. (2007) have demonstrated that nanobeads obtained from an alginate–chitosan blend were effective for oral insulin delivery for rats. Alginate-chitosan blend gel beads based on Ca^{2+} prepared by dual cross-linking with various proportions of alginate and chitosan as stated earlier were used for the sustained release of bovine serum albumin, a model protein drug in simulated gastric, intestinal and colonic fluid by Xu et al. (2007). It was observed that the dual cross-linking promoted the stability of the beads under gastrointestinal tract conditions. Films of alginate and gelatin, cross-linked with Ca^{2+}, with ciprofloxacin hydrochloride as a model drug incorporated in different concentrations were obtained by Dong et al. (2006b). Slow-release profiling of an anti-inflammatory drug (diclofenac sodium) from the polymeric blend beads of poly(vinyl alcohol)-*g*-polyacrylamide with

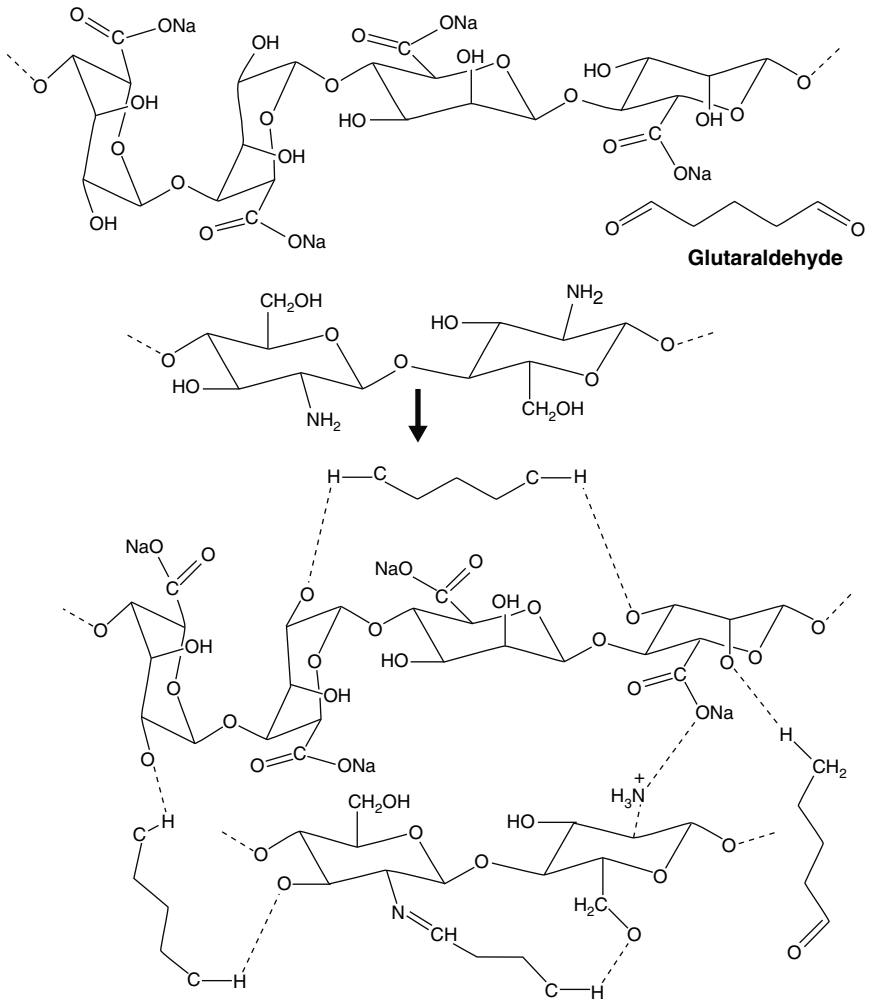

Fig. 2 Formation of blend films of sodium alginate and chitosan cross-linked with glutaraldehyde

sodium alginate cross-linked with glutaraldehyde was studied by Sanli et al. (2007), and they observed a decrease in the rate of drug release with increasing poly(vinyl alcohol) to sodium alginate ratio, drug to polymer ratio and extent of cross-linking. Polymeric blend microspheres consisting of sodium alginate and methylcellulose were prepared by a water-in-oil emulsion method followed by cross-linking with glutaraldehyde and loaded with nifedipine, an anti-inflammatory drug, and the release characteristics of the drug were evaluated by Ramesh Babu et al. (2007). Poly (DL-lactic acid) was combined with calcium alginate fibres using supercritical

CO_2 to form dual protein release scaffolds and subsequently more protein release from the hydrophilic alginate fibres was observed by Ginty et al. (2008). A blend of sodium alginate and hydroxypropyl methylcellulose was used as the candidate for modulating barrier material for pulse drug release by B. Li et al. (2008). The results revealed that programmed drug delivery to achieve pulsatile drug release three times daily can be obtained from these blend tablets in a capsule system by a systemic formulation approach. Almeida and Almeida (2004) reported preparation of pindolol (a pulsatile drug)-loaded alginate–gelatin beads using a solvent-free technique that involves a cross-linking reaction. Composite beads consisting of xanthan, a branched biopolymer, and sodium alginate were prepared using an ionotropic gelation method by Pongjanyakul and Puttipipatkhachorn (2007) and subsequently studies on the entrapment efficiency of diclofenac sodium, the thermal properties, water uptake, swelling and release in various media were carried out. They also established the molecular interactions between xanthan and sodium alginate, which modulates the physicochemical properties and drug release. Guar gum was also used to prepare blends with alginates. A pH-sensitive alginate–guar gum hydrogel cross-linked with glutaraldehyde was designed for the controlled delivery of protein drugs. The presence of guar gum and glutaraldehyde cross-linking increases the entrapment efficiency and prevents the rapid dissolution of alginate in the high pH of the intestine, ensuring a controlled release of the entrapped drug (George and Abraham 2007). Sodium alginate–carboxymethyl guar gum biopolymeric beads cross-linked with Ba^{2+} were prepared with the intention of achieving greater stability in the gastrointestinal tract (Bajpai et al. 2006).

2.2.3 Miscellaneous Applications of Alginate Blends, Including Biomedical Applications

The ideal cell-carrier material for cartilage regeneration should be one that closely mimics the natural environment in a living articular cartilage matrix. Alginate-based chitosan hybrid biomaterials could provide excellent supports for chondrocyte adhesion. To test this assumption, Iwasaki et al. (2004) investigated the behaviour of adhesion of rabbit chondrocytes onto an alginate polymer compared with the behaviour of adhesion of the chondrocytes onto some alginate-based chitosan hybrid polymer fibres in vitro. It was found that the alginate-based chitosan hybrid polymer fibres had much improved adhesion capacity with chondrocytes in comparison with alginate polymer fibre alone, showing its viability as a desirable material for cartilage tissue scaffolds. Rinaudo (2008) recently summarized some important biomedical applications of alginate blends. One of the applications described in the review involved the thermosensitive gel of calcium alginate and poly(*N*-isopropylacrylamide) (PNIPAAm). The characteristic lower critical solution temperature of this blend was tailored to be close to human body temperature by controlling the amount of *N*-isopropylacrylamide and calcium alginate during the synthesis. New biomaterial for surgical applications based on viscoelastic blends of alginate–hyaluronate that merge the gel-forming properties of alginate

with the healing properties of hyaluronate was also proposed. Another biomaterial discussed was the self-curing hydroxyapatite/alginate mixture, which has the potential for use in plastic and reconstructive as well as in oral and maxillofacial surgery. Targeting to develop biologically active scaffolds from polysaccharide mixtures, Donati et al. (2007) reported the preparation of alginate and a lactose-modified chitosan mixture under physiological conditions and at a semidilute concentration avoiding associative phase separation. A series of blended films based on poly(ethylene oxide) and sodium alginate were prepared by a solution casting method (Fig. 3). These blends were found to have mechanical properties superior to those of poly(ethylene oxide) films alone (Caykara et al. 2005). Chitosan–alginate–hyaluronate complexes modified by an arginine–glycine–aspartic acid-containing protein as tissue-engineering scaffolds for cartilage regeneration was evaluated by Hsu et al. (2004).

Alginate blends were also found to be suitable for artificial organ manufacturing, e.g. bioartificial pancreas (BAP), which is a medical device enclosing insulin-secreting cells in a semipermeable membrane that is expected to physiologically control the glucose levels and thus to be able to inhibit development of serious chronic complications in diabetic patients. Sakai et al. (2002) utilized a sol–gel reaction to prepare an immunoisolatable membrane for a microcapsule-shaped bioartificial pancreas derived from two precursors, 3-(aminopropyl)trimethoxysilane and tetramethoxysilane, formed on calcium alginate gel beads via electrostatic

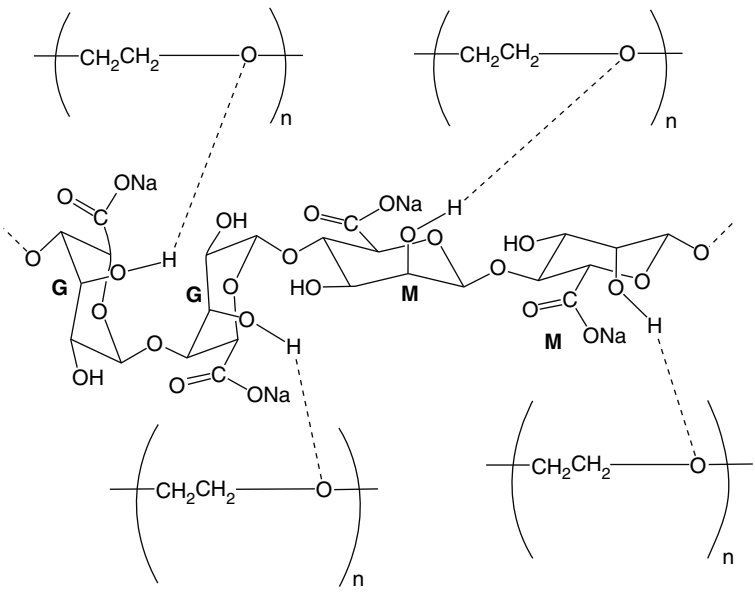

Fig. 3 Blends based on poly(ethylene oxide) and sodium alginate

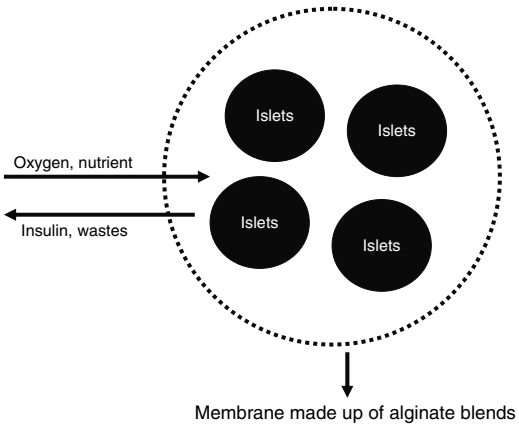

Fig. 4 Layout of bioartificial pancreas

interaction. Iwata et al. (2004) also demonstrated the preparation of a bioartificial pancreas from alginate blends (Fig. 4).

α-Galactosidase from *Aspergillus oryzae* was effectively immobilized in gelatin-blended alginate hydrogel fibres and glycerol hardened by cross-linking with glutaraldehyde. It was observed that thus immobilized α-galactosidase was more stable at higher temperature and lower pH (Naganagouda and Mulimani 2006). A strong hydrogel system based on the blends of polyacrylamide, agar and sodium alginate with water absorbency and pH resistance superior to those of their unblended counterparts was developed by Meena et al. (2008). Roy et al. (2005) utilized cross-linked alginate–guar gum beads to fluidize the bed affinity media for purification of jacalin (a lectin present in the seeds of the jackfruit). The preparation of an alginate–polymethacrylate hybrid material by radical polymerization of the cationic methacrylate monomer (2-aminoethyl methacrylate hydrochloride) in the presence of sodium alginate in water was described by Kadokawa et al. (2005). The insoluble material was formed with progress of the polymerization as shown in Fig. 5.

3 Alginate Microbeads and Nanobeads

3.1 Methods for Preparation of the Beads

Alginate beads have been popularly used for the last several decades for immobilization of enzymes and were prepared conventionally by gelation of sodium alginate solution in the presence of calcium salt (Fig. 6). The beads obtainable by this technique are normally large and their sizes are controlled by the orifice diameter

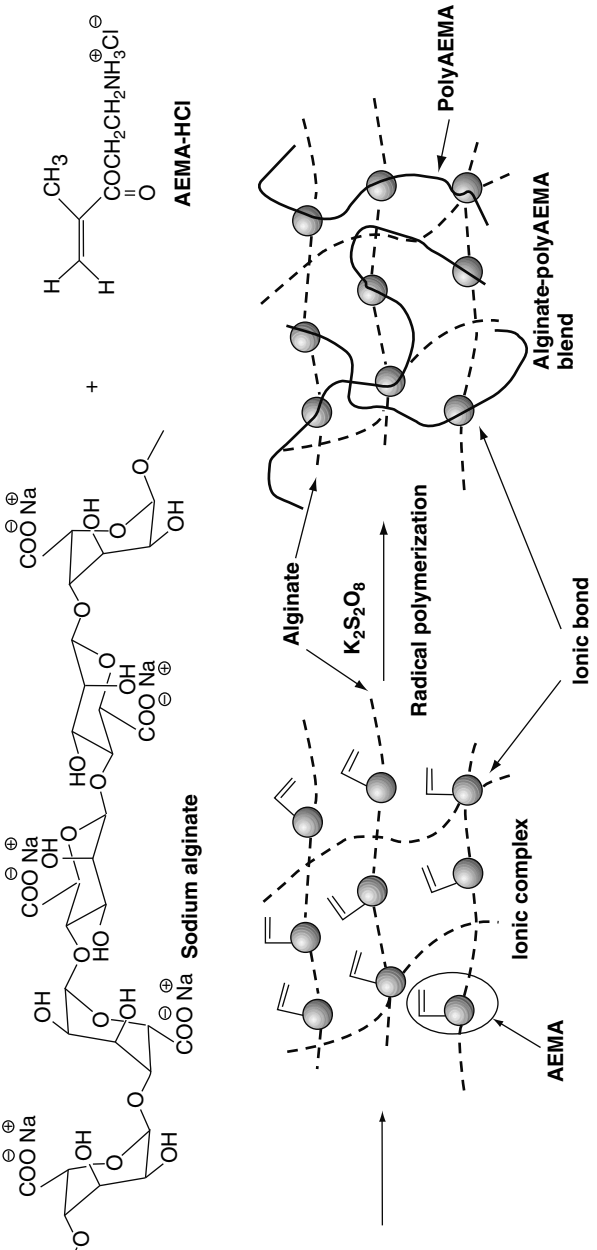

Fig. 5 Preparation of hybrid material by polymerization of 2-aminoethyl methacrylate hydrochloride in the presence of sodium alginate

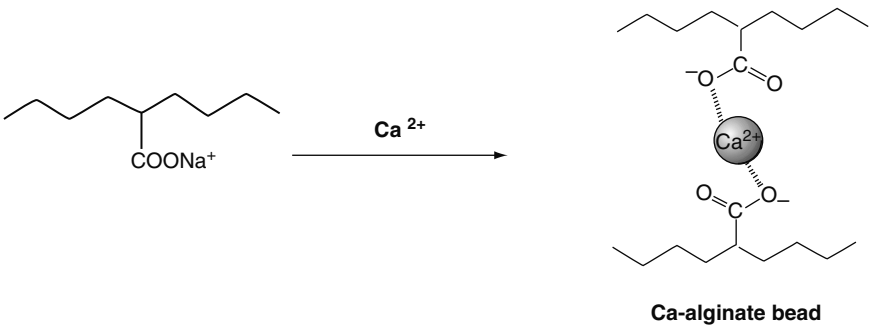

Fig. 6 Basic mechanism for the formation of calcium alginate beads

employed in their preparation. The diameters of the beads thus obtained were generally 2–5 mm (Mofidi et al. 2000). A schematic diagram of the conventional bead-forming apparatus is depicted in Fig. 7.

Owing to the demand for micro- and nano-sized beads for a variety of applications, the methods for the preparation of the beads were improved by employing various techniques. For generation of beads with smaller diameter, Zhao et al. (2007) developed an electrodispersion reactor to prepare calcium alginate microgel beads suitable for microencapsulation. In the electrodispersion reactor, pulsed electric fields were utilized to atomize aqueous mixtures of sodium alginate and $CaCO_3$ nanoparticles (dispersed phase) from a nozzle into an immiscible insulating second liquid (continuous phase) containing a soluble organic acid. The sizes of the beads obtained by this technique changed from 412 ± 90 to 10 ± 3 μm as the applied peak voltage was increased (Fig. 8).

Calcium alginate–silica dehydrogenase hybrid biocompatible beads were prepared by the polymerization of silica particles. The catalytic activity of the enzyme entrapped in such beads was investigated by Xu et al. (2006). The catalytic activity of yeast alcohol dehydrogenase entrapped in alginate-silica beads was found to be superior to that of yeast alcohol dehydrogenase entrapped only in alginate beads. The schematic layout for such bead formation is depicted in Fig. 9.

Production of small and monodispersed alginate beads (diameter 0.5–1 mm) for cell immobilization by destabilizing a viscous jet with a controlled disturbance technique in a bioreactor for high-density operations without the difficulties of cell separation was described by Seifert and Phillips (1997). Nedovic et al. (2001) were also successful in preparing alginate microbeads of diameter 250 μm–2.0 mm loaded with brewing yeast by an electrostatic technique. Laminar jet break-up technology was also utilized to prepare sodium alginate microbeads by Del et al. (2005). Alginate beads with ionic properties were synthesized through the conjugation reaction of alginate and tyramine (a monoamine compound derived from the amino acid tyrosine) by immersing an aqueous solution of the alginate containing horseradish peroxidase into a solution containing H_2O_2, caused the solution to gel

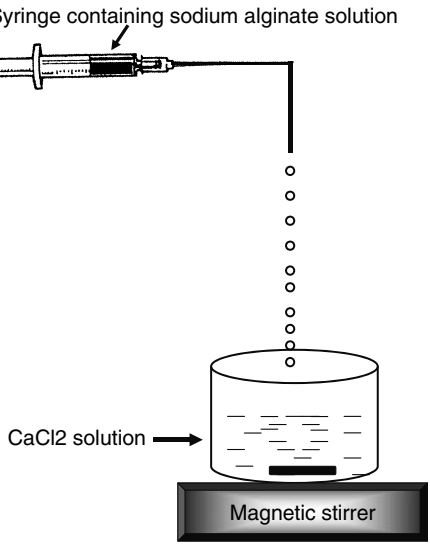

Fig. 7 Conventional apparatus used for the preparation of alginate beads

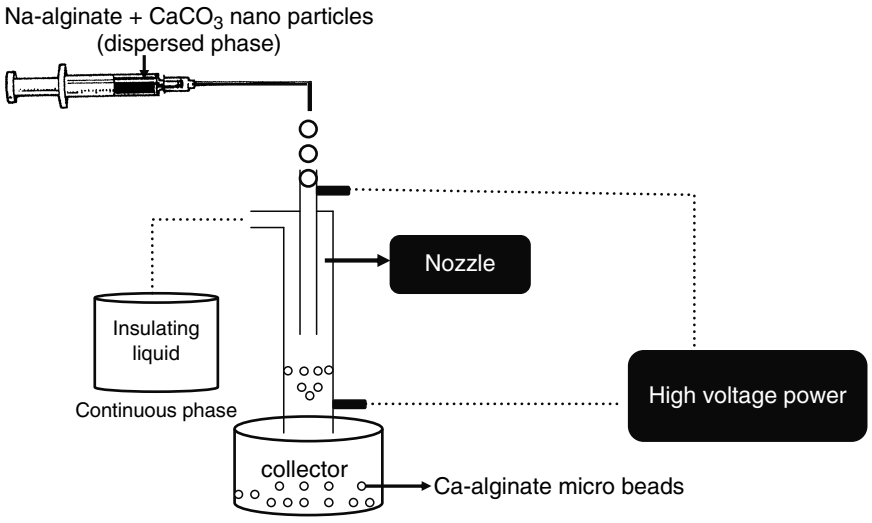

Fig. 8 Generation of calcium alginate beads by an electrodispersion technique

via peroxidase-catalysed oxidative coupling of the phenols as shown in Fig. 10 (Sakai and Kawakami 2007).

Owing to the importance of small beads for specific uses in biomedicine and bioengineering, Sugiura et al. (2005) utilized a novel microfluidic device, where a silicon micronozzle array was used to produce 50–200-μm calcium alginate beads

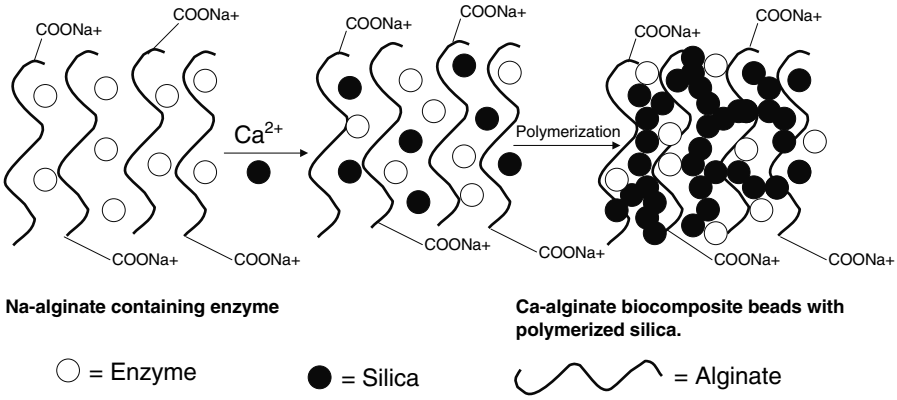

Fig. 9 Preparation of alginate beads by an in situ polymerization technique

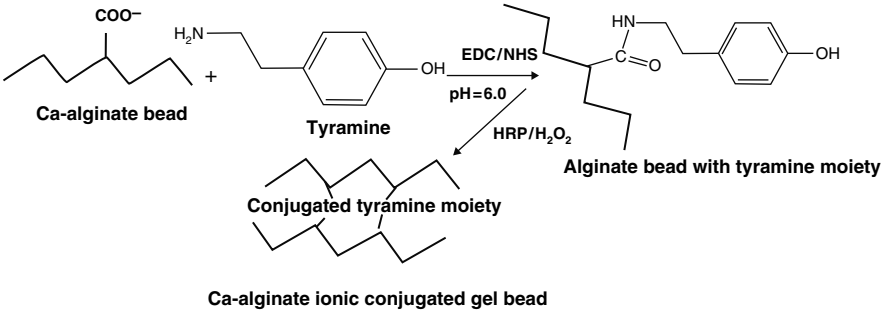

Fig. 10 Synthesis of tyramine-conjugated alginate gel beads

with narrow size distribution. They were successful in encapsulating living cells in such small calcium alginate beads. Continuous generation of hydrogel beads smaller than 100 μm using a double T-junction in a microfluidic channel for alternate injection of aqueous fluids inside a droplet unit carried within immiscible oil was obtained by Um et al. (2008). They encapsulated biological materials in such beads using a microfluidic droplet-merging channel. A novel polymer gel having characteristic temperature and magnetic field sensitivity was prepared by incorporating magnetite nanoparticles into PNIPAAm hydrogels synthesized in calcium alginate beads (Xulu et al. 2000). The enzymatically modified alginate beads prepared by Morch et al. (2007) were found to be more elastic, compact, less permeable and extremely stable under physiological conditions, offering significant advantages over native alginates. A chemoenzymatic reaction was used to prepare tailor-make alginate-bearing galactose beads by the reaction of 1-amino-1-deoxygalactose with

a mannuronan sample via an *N*-glycosidic bond involving the carboxyl group of the mannuronic acid residues, resulting in the formation of modified epimerized material with the ability to form stable gels (Donati et al. 2005). Alginate–glycerol–chitin beads with improved antifungal activity were synthesized by Zohar–Perez et al. (2004). Kim et al. (2007) demonstrated the preparation of alginate-quaternary ammonium complex beads with antimicrobial activity by the reaction of sodium alginate with 3-(trimethoxysilyl)propyl octadecyldimethylammonium chloride (TSA) in acid solution. The sodium alginate–TSA complex beads obtained were a new type of insoluble cationic polymer which can kill or remove microorganisms in water by mere contact without releasing the reactive agent.

Covalently linked alginate beads with high stability and cell compatibility were achieved by grafting of mannuronan (alginate with 100% mannuronate) with methacrylate moieties by photoinitiated polymerization in the alginate beads (Rokstad et al. 2006). The stability and the cell compatibility of the beads thus obtained may make them attractive as bioreactors for delivering therapeutic proteins.

3.2 Application of Alginate Beads

3.2.1 Process Technology Employing Immobilization on Alginate Beads

Processes or reactions yielding enantiomeric pure compounds are very important in pharmaceutical industries. Many reactions catalysed by enzymes immobilized in alginates beads have been found to provide such pure compounds. The asymmetric bioreduction of ethyl 3-halo-2-oxo-4-phenyl butanoate catalysed by several microorganisms, especially by *Saccharomyces cerevisiae* immobilized in calcium alginate beads with a double gel layer, was carried out by Milagre et al. (2006) (Fig. 11). They reported the highest chemical yield for the above-mentioned bioreduction as 90%, with 70% diastereomeric excess and 96–99% enantiomeric excess. They further stated that the entrapment of cells with double gel layers was fundamental to achieve high enantioselectivities and diastereoselectivities.

Hann et al. (2002) reported *Acidovorax facilis* 72W nitrilase catalysed hydrolysis of 2-methylglutaronitrile to 4-cyanopentanoic acid (as the ammonium salt), an intermediate in the preparation of 1,5-dimethyl-2-piperidone, an important commercial

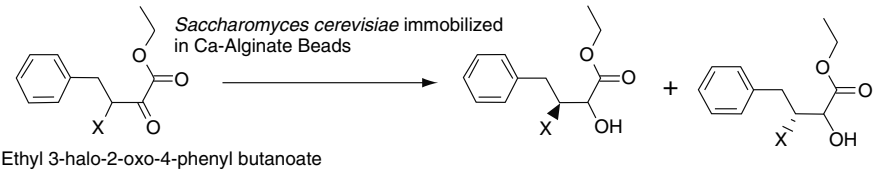

Fig. 11 Bioreduction of ethyl 3-halo-2-oxo-4-phenylbutanoate

precision cleaning solvent (Fig. 12). The immobilization of the enzyme cells was done in alginate beads instead of carrageenan gel followed by cross-linking with glutaraldehyde and polyethyleneimine. The catalyst thus formed was stable in reaction mixtures containing high concentrations of 4-cyanopentanoic acid ammonium salt.

Evidence for Fenton photoassisted processes mediated by encapsulated Fe ions in alginate beads at biocompatible pH was demonstrated by Fernandez et al. (2000). They stated that the iron carboxylate complex was active during the Fenton-enhanced decolouration/degradation of orange II via the encapsulated Fe catalyst. Baker's yeast cells entrapped in alginate beads were shown to catalyse reactions in organic solvents when a cofactor regeneration scheme was implemented. Reduction of acetophenone to 1-phenylethanol, a chiral alcohol with high enantiomeric excess using baker's yeast as well as a cosubstrate to regenerate the cofactor was reported by Griffin et al.(1998) (Fig. 13).

Owing to its ability to preserve natural colours in juice and health drinks, L-malic acid is considered to be very important in the food processing industry and thus various methods to prepare pure L-malic acid have been attempted by many researchers. Bressler et al. (2002) converted fumaric acid to L-malic acid in a bioreactor catalysed by *S. cerevisiae* immobilized in small glasslike beads of an alginate-silicate sol-gel matrix (Fig. 14). The conversion reported by them was almost 100%, which was above the equilibrium value of approximately 84% and higher than that of the industrial process. They were also able to obtain pure L-malic acid in contrast to the existing industrial biocatalytic process, which yields L-malic acid salts.

Gervais et al. (2000) studied the effect of aeration during cell growth on the subsequent reduction of 2-hexanone and 2-octanone by yeast cells entrapped in calcium alginate beads. They used 2-propanol as a sacrificial substrate to regenerate the cofactor NAD(H). The use of strictly aerobic conditions when growing the cells resulted in the highest initial reaction rates, as well as the production of only a single product, i.e. the enantiomeric excess of the (*S*)-alcohols was 100% (Fig. 15).

Some more important chemical reactions catalysed by enzymes entrapped in alginate beads have been studied. Hydrolysis of starch was carried out by a blend of individually entrapped glucoamylase and pullulanase in calcium alginate beads in a ratio of 3:2 (activitywise), the individually entrapped enzymes showed enhanced thermostability at 55°C and effective hydrolysis was observed (Roy and Gupta 2004). Won et al. (2005) entrapped lipase from *Candida rugosa* by dropwise addition of an aqueous mixture of sodium alginate and putting the biocatalyst into a hardening solution of a Ca^{2+} salt. They observed a higher rate of reusability of the catalyst coated with alginate beads in comparison with the non-coated ones. Yadav and Jadhav (2005) preimmobilized the biocatalyst *Candida antarctica* lipase B in hexagonal mesoporous silica followed by encapsulation in calcium alginate beads for the transesterification of *p*-chlorobenzyl alcohol with vinyl acetate to give *p*-chlorobenzyl acetate at 30°C. They found excellent reusability of the catalyst with a decrease of only 4% in the overall conversion of the transesterification reaction even after the fourth reuse. A microbial bioreactor based on calcium alginate immobilized *Lactobacillus* cells coupled to a pH electrode was developed for quantitative estimation of carbohydrate fermentation activity. The procedure developed

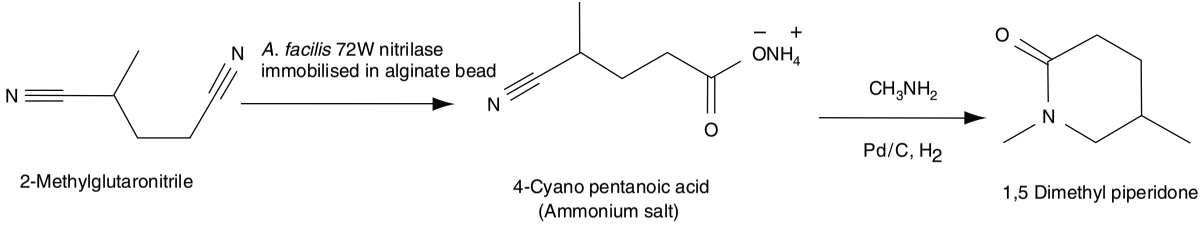

Fig. 12 Nitrilase-catalysed hydrolysis of 2-methylglutaronitrile

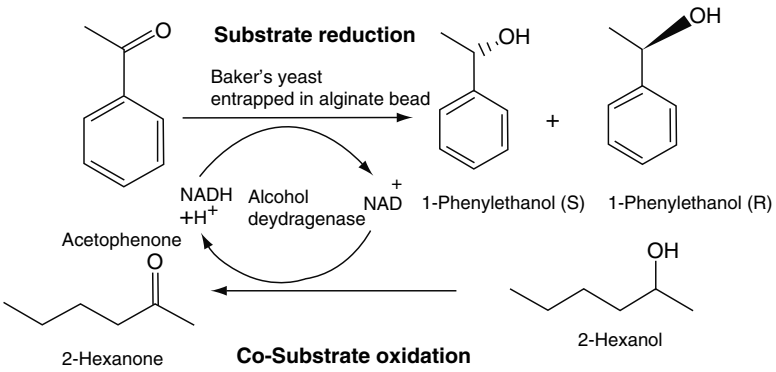

Fig. 13 Reduction of acetophenone by baker's yeast immobilized in alginate beads in the presence of a cosubstrate, 2-hexanol

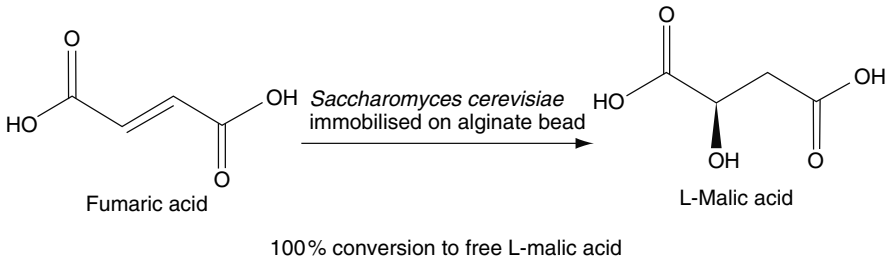

Fig. 14 Conversion of fumaric acid to L-malic acid

was simple, being based on pH variation that can give quantitative results, in comparison with other existing techniques for the carbohydrate fermentation pattern from which only qualitative results could be obtained. It also offers reduction in time and costs and is a suitable tool for the rapid analysis of isolated strains and in studies of modifications of sugar metabolism in mutants (Corton et al. 2000). An immobilization culture of *Nicotiana tabacum* cells on calcium alginate beads to produce scopoletin (a naturally occurring coumarin) was achieved by adding coconut oil as an extractive solvent (Iizuka et al. 2005). D-Tagatose (an epimer of D-fructose) was continuously produced using thermostable L-arabinose isomerase immobilized in alginate beads with D-galactose solution in a packed-bed bioreactor (Ryu et al. 2003). Production of a versatile chiral building block, (*R*)-(−)-mandelic acid, by dynamic resolution of mandilonitrile by nitrilase from *Alcaligenes faecalis* immobilized in alginate beads was described by Kaul et al. (2006) (Fig. 16). They reported reusability of the biocatalyst up to 35 batches by the immobilization as compared with nine batches for free cells, and cross-linking extended it further to 40 batches. Acetophenone, a useful solvent for many chemical syntheses and an

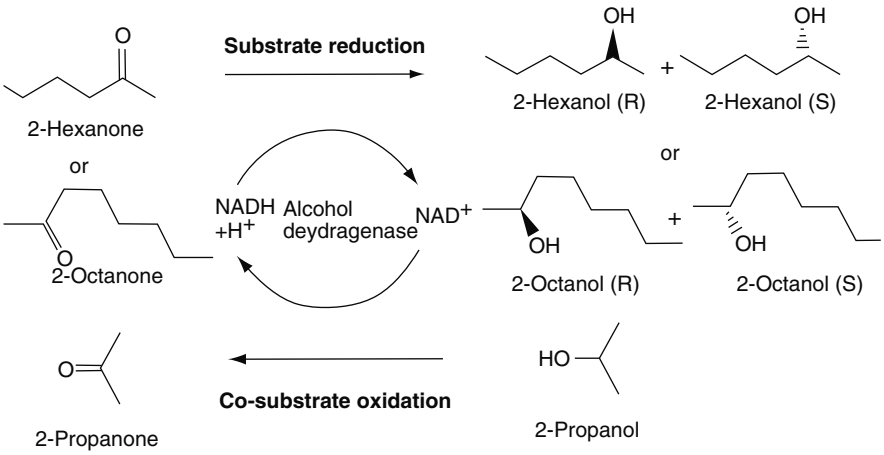

Fig. 15 Reduction of 2-hexanone or 2-octanone to the corresponding alcohols in the presence of a cosubstrate, 2-propanol

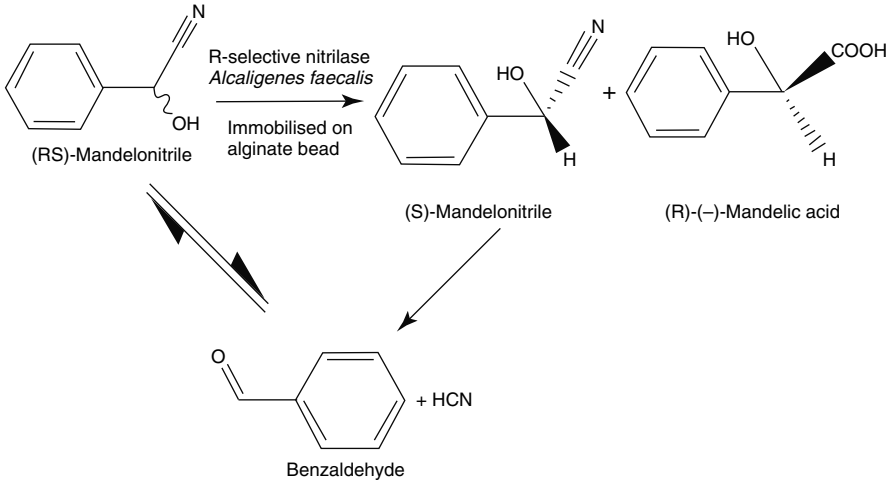

Fig. 16 Dynamic resolution of mandilonitrile by nitrilase

important chemical in the perfume industry, was obtained by the transamination of (S)-(−)-α-methylbenzylamine by (*S*)-aminotransferase immobilized in alginate beads by Martin et al. (2007) (Fig. 17).

Ascorbic acid is a very important antioxidant and plays a very crucial role in human metabolism. However, it has a tendency for oxidation, causing its degradation, and hence to stop the oxidation, suitable chemical modification of the structure of the antioxidant was performed by the effective utilization of some biocatalysts. Hsieh et al. (2007) used mycelium-bound enzymes of *Aspergillus niger* as a biocatalyst

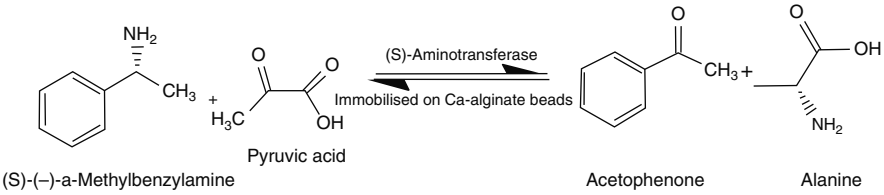

Fig. 17 Production of acetophenone by transamination process

entrapped in calcium alginate beads for the glucosylation of ascorbic acid using maltose as an acyl donor to yield ascorbic acid glucoside. They found exceptional reusability and storage stability of the entrapped mycelia. They reported thr final yield of ascorbic acid glucoside as 75%. Xytitol, obtainable from xylose, is a commercially important sweetener and is widely used in food and pharmaceutical industries. *Candida guilliermondii* FTI 20037 cells were entrapped in calcium alginate beads used as a biocatalyst for xylose to xylitol bioconversions during five successive batches in a stirred tank reactor by Carvalho et al. (2008). A gram-negative, rod-shaped, aerobe, *Alcaligenes faecalis*, entrapped on calcium alginate beads was used to convert 2-propanol to acetone by Mohammad and Bustard (2008). Owing to the increased amount of research into the preparation of environmentallly friendly polymers, the biodegradable lactic acid based polymers have been drawing attention for a long time. Apart from such applications, lactic acid is also part of many commercial foodstuffs. Research is also going on into the production of lactic acid by various methods, including enzyme-catalysed ones. In this direction, Givry et al. (2008) reported the production of lactic acid by the consumption of hexoses/pentoses by *Lactobacillus bifermentans* immobilized in alginate beads in optimized culture medium. Hydrogen, a future fuel, was produced under anaerobic conditions from municipal sewage sludge immobilized on calcium alginate beads (Wu et al. 2002). Urease, the enzyme which hydrolyses urea to CO_2 and ammonia, is very susceptible to chymotrypsin and other proteases. DeGroot and Neufeld (2001) encapsulated urease in alginate beads coated with chitosan, poly(L-lysine) or poly(methylene-*co*-guanidine) membrane and were able to substantially protect urease from these proteases.

3.2.2 Slow Release of Drugs and Agrochemicals

Alginate beads being biocompatible are widely used in drug slow-release applications. There have been many reports of such applications in the last decade. We discuss the developments in this field in recent years by mentioning the most important works.

Calcium alginate, owing to the strong gel network, can hold drugs in the gel matrix and slowly releases them in suitable environments. Murata et al. (2000) prepared calcium alginate gel beads with vegetable oil or chitosan, capable of floating in the gastric cavity. They studied the behaviour of the release of metronidazole

(an anti-infective drug) entrapped in such beads into artificial gastric juice. They observed not only sustained release but also gastric-mucosa-specific drug release from both types of bead. Later, the same research group prepared alginate gel beads containing weak acid salts of chitosan and ascorbic acid having rapid bile acid uptake ability (Murata et al. (2002). An anti-hapten immunoglobulin G (antibody) was covalently immobilized on glutaraldehyde-activated alginate–chitosan gel beads by Albarghouthi et al. (2000). It was concluded that antibodies could be successfully immobilized on alginate–chitosan gel beads. Such systems can be applied for the development of immunoaffinity purification and immunoassays. New interpenetrating polymer networks of sodium alginate with gelatin or egg albumin cross-linked with glutaraldehyde beads were prepared by Kulkarni et al. (2001) and the in vitro release of the broad-spectrum antibiotic cefadroxil incorporated in such beads was studied. To study the release profile of sodium diclofenac, alginate–chitosan particles were prepared by ionic gelation (Ca^{2+} and Al^{3+}) by Gonzalez-Rodriguez et al. (2002). The alginate to chitosan ratio and the nature of the gellifying cation were found to control the rate of release of the drug. The release profile of a model drug (brilliant blue) entrapped in chitosan-coated calcium–alginate gel beads was studied by Shu and Zhu (2002). Alginate–chitosan beads cross-linked with a natural cross-linker, genipin, were prepared by Mi et al. (2002) and the rate of release of indomethacin (an anti-inflammatory drug) from these beads was studied. It was observed that the rate of release of the drug from the beads increased with the decrease in pH or concentration of alginate in the gelling solution owing to the decrease in the cross-linking density of the beads. Furthermore, good cellular compatibility for the beads was established by cytotoxic examinations. Time-programmed release of the macromolecule dextran was achieved by Iskakov et al. (2002) by utilization of calcium alginate gel beads modified with coated copolymer layers by varying the coating thickness. Choi et al. (2002) demonstrated a novel technique for the preparation of floating alginate beads from a sodium alginate solution using $CaCO_3$ or $NaHCO_3$ as gas-forming agents and investigated the profile of the release of riboflavin from these floating beads. 5-Fluorouracil, an anticancer drug, encapsulated in alginate beads was prepared and the release characteristic of 5-fluorouracil were established by Arica et al. (2002). The influence of microwave irradiation on the drug release properties of alginate, alginate–chitosan and chitosan beads was studied and it was observed that the release-retarding properties of alginate and alginate–chitosan beads were significantly enhanced by subjecting the beads to microwave irradiation (Wong et al. 2002). Ueng et al. (2004) investigated the lyophilized poly(L-lysine)-coated alginate antibiotic delivery system in vivo for the treatment of musculoskeletal infections. Biodegradable beads with a uniform diameter of 3 mm were prepared by mixing sodium alginate with vancomycin, coated with poly(L-lysine), followed by lyophilization. This study offered a biodegradable delivery system for antibiotics to treat musculoskeletal infections. Calcium alginate encapsulated vascular endothelial growth factor, which is a potent signal transduction molecule that acts specifically on vascular endothelial cells, was prepared by Gu et al. (2004). They further investigated the release of the entrapped vascular endothelial growth factor in phosphate-buffered saline and serum media. Sodium polystyrene sulphonate is a medicine used to treat

high levels of potassium in the body and is normally administered in conjunction with sorbitol. The release of this drug from calcium alginate hydrogel beads was studied by Rousseau et al. (2004) and it was found that the structure of the cross-linked calcium alginate network was important for the retention and/or release of the sodium polystyrene sulphonate. To establish the merit of multilayered alginate beads in certain slow-release applications, Anal and Stevens (2005) prepared multilayer alginate beads by mixing chitosan, Ca^{2+} (a cationic component) and sodium alginate, and polyphosphate (anionic component) in various proportions. The multilayered bead thus obtained showed 20–30% release of ampicillin in comparison with 70% release by single-layered alginate beads. Kim et al. (2005) prepared the beads of the graft copolymers of alginate and PNIPAAm by dropwise additions of the aqueous solutions of the copolymers in Ca^{2+} solutions. They further studied the release of blue dextran from such beads. It was observed that owing to the presence of a temperature-sensitive component in the beads, the beads showed a higher release rate at high temperature and a lower release rate at low temperature. Diclofenac calcium alginate beads containing glyceryl palmitostearate were prepared by an ionotropic gelation method. The effect of the amount of glyceryl palmitostearate and heat treatment on characteristics of the diclofenac calcium alginate beads was investigated by Pongjanyakul et al. (2006). Alginate-whey protein isolate microspheres of different whey protein isolate to alginate ratio, particle diameter and concentration of polymer-bead-forming solution were prepared to develop a biocompatible vehicle for oral administration of bioactive compounds by Chen and Subirade (2006). They carried out studies on the release of riboflavin from these beads in simulated gastric fluid and found a satisfactory retarding property for the drug. Swelling studies and in vitro release of verapamil (a drug used for hypertension) from calcium alginate and calcium alginate–chitosan beads were investigated by Pasparakis and Bouropoulos (2006) and 80% encapsulation of the drug in both calcium alginate and calcium alginate–chitosan beads was observed. They further observed retardation of the drug release in the presence of chitosan in wet beads. A general schematic approach of entrapment of an active agent (e.g. drug) in alginate beads and subsequent coating with another natural polymer is shown in Fig. 18.

To design oral delivery vehicles for peptide or protein drugs, pH-sensitive hydrogels such as alginate and chitosan have attracted increasing attention. Most of the synthetic polymers are immunogenic, and the incorporation of proteins into these polymers requires a harsh environment which may denature and inactivate the desired protein. The favourable properties such as biocompatibility, biodegradability, pH sensitivity, mucoadhesiveness, etc. have enabled these natural polymers to become the choice of pharmacologists as oral delivery matrices for proteins (George and Abraham 2006). Emulsan is a polyanionic heteropolysaccharide bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1 and has the natural ability to bind protein. This nature of emulsan was utilized by Castro et al. (2005) to prepare emulsan–alginate beads to study triggered release of protein. They were successful in demonstrating the assay of protein release of azo-bovine serum albumin using lipase and subtilisin (a protease). A schematic diagram for protein release from emulsan–alginate beads using protease is shown in Fig. 19. Alginate beads were prepared in conjugation with

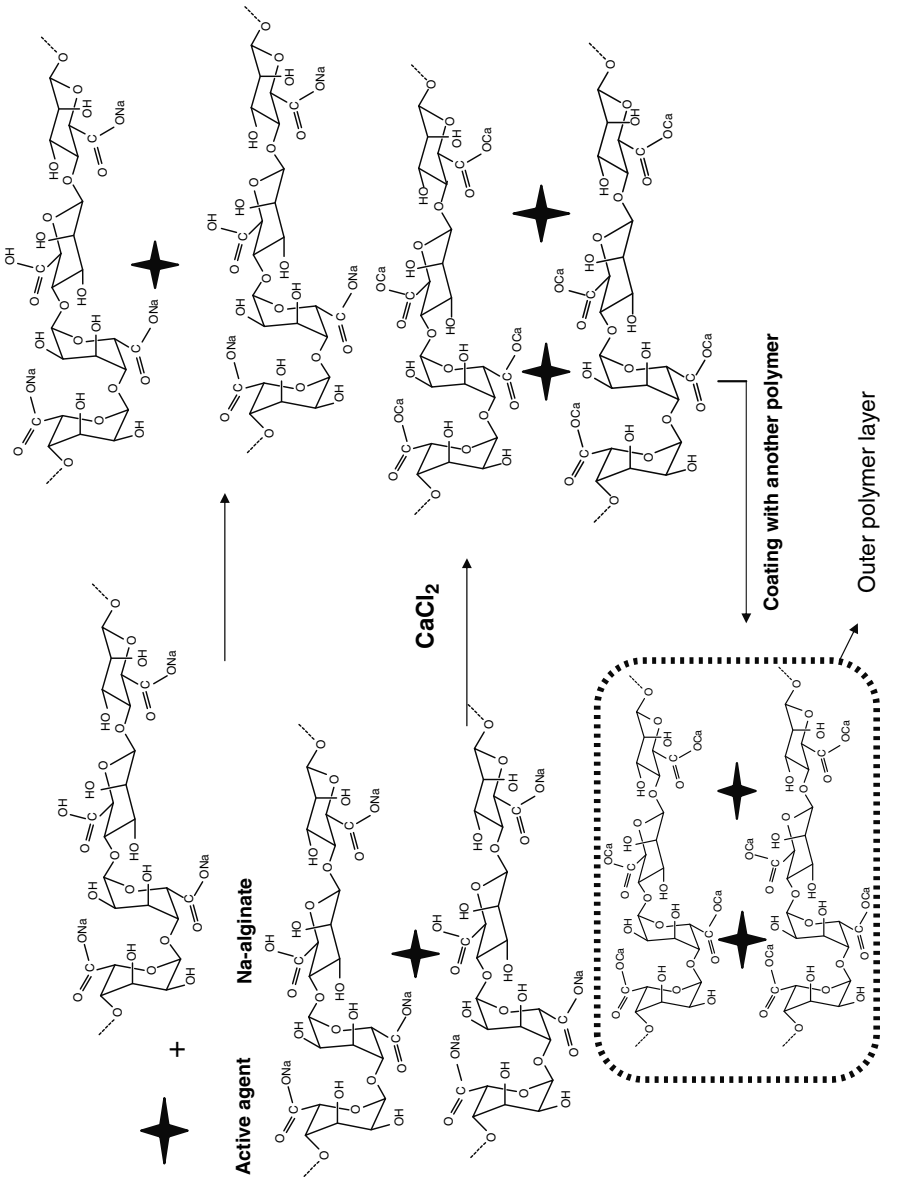

Fig. 18 Entrapment of an active ingredient in alginate beads coated with another biopolymer

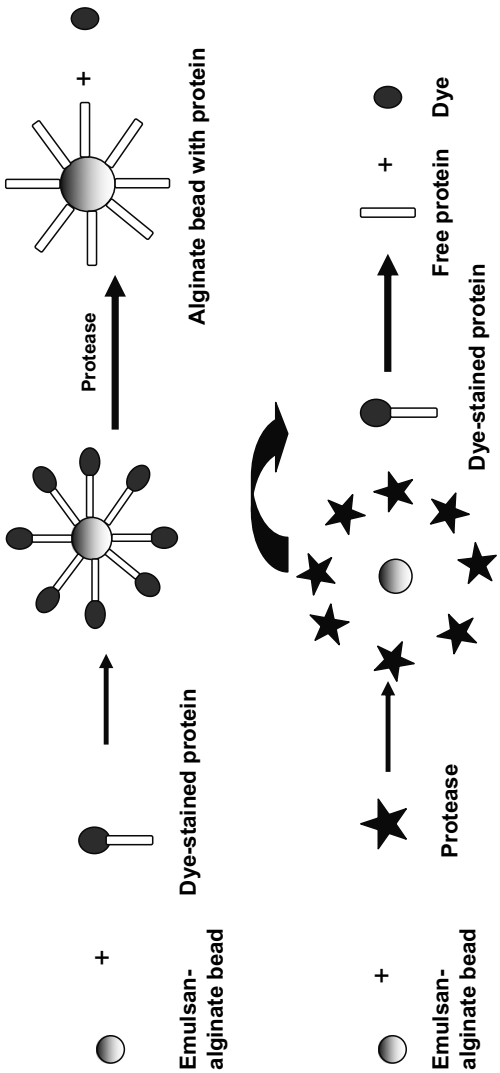

Fig. 19 Release of protein from emulsan–alginate beads

other biopolymers, konjac glucomannan and chitosan, and their in vitro assessments were done for release of two protein drugs, bovine serum albumin and insulin. It was observed that diffusion of protein was related to the viscosity and swelling properties of konjac glucomannan (Wang and He 2002). Numerical modelling on the release profile of insulin hexamer and amyloglucosidase entrapped in spherical calcium alginate beads was done by Rasmussen et al. (2003). They also modelled the effect of swelling and the size of beads on the protein release rate. Such numerical models are useful for designing desired drug release systems. Xing et al. (2003) evaluated the suitability of coated calcium alginate gel bead entrapped liposome and bee venom peptide as a model drug for colon-specific drug delivery in vitro. They concluded from various studies that the coated calcium alginate gel bead entrapped liposome could be a potential system for colon-specific drug delivery.

Apart from the sustained release of drugs as discussed above, the use of alginate beads in slow release of agrochemicals and fertilizers has also been popular in recent years. The release of alachlor (a herbicide from the chloroacetanilide family) from controlled-release formulations based on alginate-montmorillonite bead matrices into aqueous poly(ethylene glycol) solutions and soil at different moisture contents was studied by Nasser et al. (2008). The moisture content of soil, bead diameter, bead weight and the water content were found to affect the release rate. Kenawy and Sakran (1996) used calcium alginate beads for controlled release of 1-naphthalene acetic acid (a plant growth regulator) and of pentachlorophenol (a herbicide) in alkaline and acidic media. They further investigated the role of polyethyleneimine coating of the beads on the release profile. An increased release rate was monitored owing to the polyethyleneimine coating and a slow rate of release from such beads in acidic media was observed. Kulkarni et al. (2000) successfully encapsulated a natural liquid pesticide 'neem (*Azadirachta indica* A. Juss) seed oil' in glutaraldehyde cross-linked sodium alginate beads. They studied the chemical interaction between the ingredient and alginate as well as the effect of cross-linking on the release rate.

3.2.3 Use in Pollution Control

Heavy metal contamination is often found in polluted water and the removal is generally done by various chemical means, including passing through resins and precipitation. There are certain types of biomass present in nature which can form complexes with heavy metals such as Cd(II) through the functional groups present at their surface. Alginic acid and chitosan gel beads have been recognized as one of the most effective adsorbents for eliminating low levels of heavy metal ions from wastewater stream (Gotoh et al. 2004a). Bayramoglu et al. (2002) were successful in achieving the biosorption of Cd(II) by using calcium alginate beads entrapped with a white rot fungus species *Lentinus sajor-caju* biomass. They reused the biosorbent in three consecutive adsorption-desorption cycles without significant loss of the biosorption capacity. Interestingly, the adsorption of the heavy metal by alginate beads alone was also observed. Later, the same research group revealed the

potential use of immobilized microalgae in calcium alginate beads of *Chlamydomonas reinhardtii* in biosorption of Hg(II), Cd(II) and Pb(II) ions from aqueous solutions using bare calcium alginate beads as a control system (Bayramoglu et al. 2006). Another group attempted to remove Cd(II) ion from aqueous system by using dry biomass of cyanobacteria immobilized in calcium alginate beads (Katircioglu et al. 2007). Owing to the complex formation ability of As(III) with Fe, Zouboulis and Katsoyiannis (2002) modified alginate beads by doping them with hydrous ferric oxides followed by passing As(III)-contaminated water through the bed of the modified beads. Fair removal of As from the contaminated water was observed. Banerjee et al. (2007) used Fe-doped alginate beads for removal of As(III) and As(V). Chen et al. (1997) carried out equilibrium and kinetic studies on Cu ion uptake by calcium alginate beads by varying the pH, ionic strength, initial concentration of Cu ion and the amount of calcium alginate. The ion uptake efficiency was found to increase with increasing pH and decreasing ionic strength. Marine invertebrates and fish are susceptible to nitrate and removal of nitrate from a marine aquarium is essential for the health of the fish and invertebrates. Biological denitrification of nitrate to nitrogen gas in freshwater and a marine aquarium was studied by Tal et al. (2003). The denitrifiers for nitrate removal were successfully immobilized in freeze-dried alginate-starch matrix beads. The ability of protonated alginate beads to take up several heavy metal ions was investigated by Ibanez and Umetsu (2002). The uptake of trivalent Cr, Cu, Zn, Ni and Co was found to be approximately 75, 77, 46, 43 and 35 mg g^{-1} of beads, respectively. Subsequently, they also studied the uptake of trivalent Cr from aqueous solutions by the protonated dry alginate beads (Ibanez and Umetsu 2004). Calcium alginate beads containing humic acid were found to be efficient for removal of the heavy metals Cd, Cu, Cr, Ni, Mn, Fe and Zn from leachates of industrial waste and tannery effluent (Pandey et al. 2002, Pandey et al. 2003a, Pandey et al. 2003b). Gotoh et al. (2004b) studied the adsorption of Cu and Mn on covalently cross-linked alginate gel beads. Coimmobilization of the freshwater microalga *Chlorella vulgaris* in alginate beads with the microalgal growth promoting bacterium *Azospirillum brasilense* under semicontinuous synthetic wastewater culture conditions significantly increased the removal of ammonium and soluble phosphorus ions compared with immobilization of the microalgae alone as observed by Bashan et al. (2002). A methodological approach using mathematical modelling was developed by Veglio et al. (2002) to study adsorption and biosorption of heavy metals. They characterized the behaviour of calcium alginate beads with respect to Cu biosorption. Park et al. (2002) were able to successfully remove H_2S using *Thiobacillus* sp. encapsulated in calcium alginate beads in a continuous H_2S-removal bioreactor. Growth of some species of marine microalgae immobilized in calcium alginate beads as well as short term accumulation of heavy metals by them was reported by Moreno-Garrido et al. (2005). Steffan et al. (2005) reported the biodegradation of an azo dye (ethyl orange) by microbial cultures immobilized in alginate beads. Goethite (an Fe-bearing oxide mineral found in soil and other low-temperature environments) is known to bind with Cr(VI). Lazaridis and Charalambous (2005) prepared goethite-conjugated calcium alginate beads to remove Cr(VI) and Cr(III) ions from binary aqueous

solutions. Fiol et al. (2006) also reported the removal of Cr(VI) using 2% grape stalks encapsulated in calcium alginate beads. Calcium alginate prepared from alginic acid having a high β-D-mannuronic acid to α-L-guluronic acid ratio, extracted from *Laminaria digitata*, was found to adsorb Cu^{2+}, Cd^{2+} and Pb^{2+} in acidic solutions (Papageorgiou et al. 2006). The yeast *S. cerevisiae* immobilized in calcium alginate beads was used for the separation and subsequent determination of Pt in environmental samples by flow-injection chemiluminescence and electrothermal atomic absorption spectrometry by Godlewska-Zylkiewicz et al. (2008).

Organic pollutants are also being treated by alginate beads. *p*-Chlorophenol and gallic acid are the major organic pollutants present in wastewater. The alginate-activated carbon beads prepared in the presence of $CaCl_2$ were found to adsorb *p*-chlorophenol selectively from a humic acid solution. Selective adsorption of gallic acid by the beads prepared in the solution of $FeCl_3$ was also observed (Lin et al. 2005). Removal of nitrophenol contaminants using alginate gel beads was reported by Peretz and Cinteza (2007). Niladevi and Prema (2008) carried out the removal of phenol by laccase from *Streptomyces psammoticus* immobilized on alginate beads. Teerapatsakul et al. (2008) were able to decolorize the dye indigo carmine by laccase entrapped in copper alginate. A selective herbicide, atrazine, was successfully degraded by *Rhodococcus erythropolis* NI86/21 bacteria encapsulated in alginate beads (Vancov et al. 2005). Polyphenols present in olive mill wastewater were biodegraded with immobilized *Candida tropicalis* in alginate beads under metabolic induction (Ettayebi et al. 2003). Y. G. Li et al. (2008) found that *Pseudomonas delafieldii* R-8 immobilized on calcium alginate beads can improve biodesulfurization activity in oil-in-water biphasic systems.

3.2.4 Miscellaneous Use of Alginate Beads Including in Biomedical

Apart from the applications of alginate beads discussed already, research is also going on to explore more application areas for the beads. In this direction, Keshaw et al. (2005) demonstrated the delivery of angiogenic growth factors by incorporating bioactive glass and fibroblasts in alginate beads. Agarose and calcium alginate beads have been used for the crystallization of low molecular mass (14.4 kDa, hen egg-white lysozyme) and high molecular mass (636.0 kDa, alcohol oxidase) proteins by Willaert et al. (2005). Probiotics are living microbial supplements and upon ingestion in certain numbers can exert health benefits. Krasaekoopt et al. (2006) observed survival of probiotics by encapsulating them in chitosan-coated alginate beads in stirred yoghurt from UHT and conventionally treated milk during low-temperature storage. Similarly, Lee et al. (2004) observed survival of the microorganism *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles in simulated gastric and intestinal juices. Nisin, a bacteriocin, was immobilized into palmitoylated alginate based Wlms or beads. Wlms or beads incorporating various amounts of nisin could be used efficiently to control the growth of pathogens or microorganisms responsible for spoilage at the surface of round beef or other meat products (Millette et al. 2007). *N*-(3-Aminopropyl)pyrrole

was covalently coupled with alginate in an aqueous-phase reaction by means of a carbodiimide-mediated activation reaction to provide a pyrrole–alginate conjugate for subsequent use in biosensor applications (Fig. 20). The new pyrrole–alginate conjugate was used for the immobilization of polyphenol oxidase onto an electrode surface by physical entrapment resulting from the gellification process and electrochemical polymerization of the pyrrole groups. In addition, biosensors were prepared by entrapment of the polyphenol oxidase in polypyrrole–alginate and regular alginate matrices and their performance for the amperometric determination of catechol chosen as a model analyte was examined by Abu-Rabeah et al. (2005).

Similarly, like *N*-3-aminopropyl pyrrole, biotin (vitamin H or B7) was covalently coupled with alginate in an aqueous-phase reaction by means of a carbodiimide-mediated activation reaction to provide a biotin–alginate conjugate for subsequent use in biosensor applications (Fig. 21). The biotin–alginate conjugate was used for the encapsulation of bioluminescent reporter cells into microspheres. A biosensor was prepared by conjugating these biotinylated alginate microspheres to the surface of a streptavidin-coated optical fibre and the performance of the biosensor was demonstrated in the determination of the antibiotic mitomycin C as a model toxin as revealed by Polyak et al. (2004). Desille et al. (2001) fabricated extracorporeal bioartificial liver for pig containing alginate bead entrapped hepatocytes.

Calcium alginate beads were utilized to prepare porous keratin sponges by Hamasaki et al. (2007). To prepare such sponges, aqueous solutions of reduced

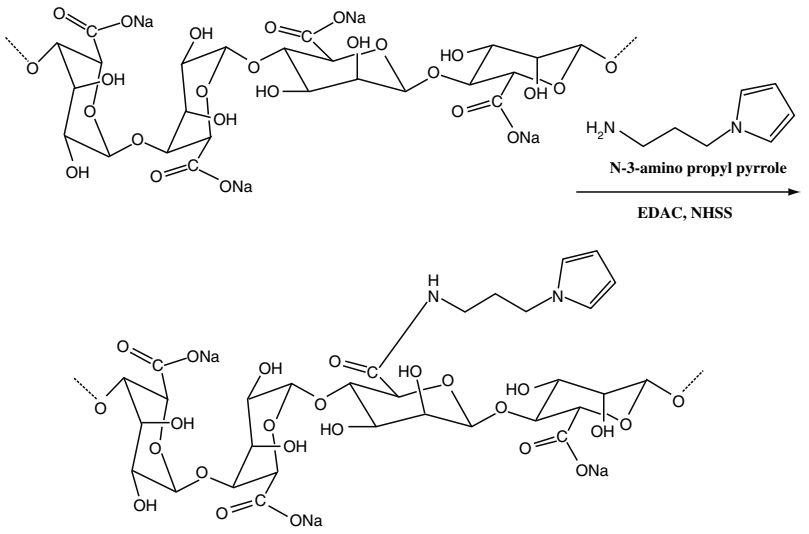

Fig. 20 Synthesis of pyrrole-coupled alginate beads

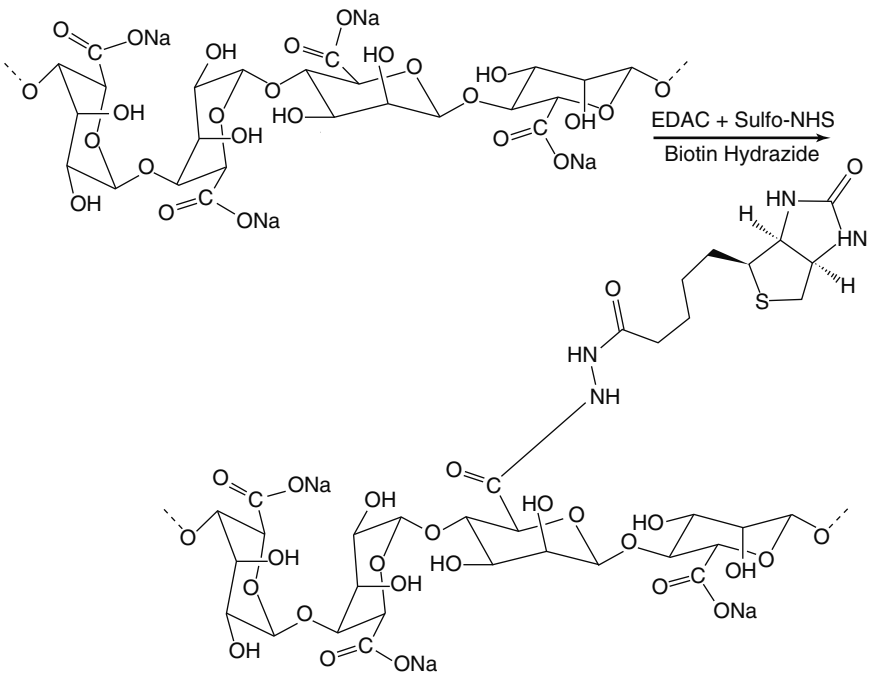

Fig. 21 Synthesis of biotin-coupled alginate beads

keratin and dried calcium alginate beads were mixed, followed by lyophilization to yield the keratin–calcium alginate complexes. These complexes were further treated with EDTA solution, causing calcium alginate beads to leach out, leaving behind the highly porous keratin sponges. In vitro culture of enzymatically isolated chondrons in sodium alginate beads as a possible model for the initiation of osteoarthritis was reported by Ross et al (2006). Rahman et al. (2005) observed that cotransplantation of encapsulated HepG2 (human hepatocellular liver carcinoma cell) in calcium alginate beads and rat Sertoli cells improves outcome in a thioacetamide-induced rat model of acute hepatic failure. Mishra and Kar (2003) were able to hydrolyse more than 83% of naringin, responsible for the bitter taste of grapefruit juice, by alginate-entrapped naringinase enzyme. Alginate microbeads have recently been introduced in stem cell research. In vitro separation of embryoid-body cells derived from embryonic stem cells into hepatocytes in alginate microbeads was studied by Fang et al. (2007). Ichinose et al. (2005) carried out the detailed examination of cartilage formation and endochondral ossification using human mesenchymal stem cells maintained in alginate beads. The effects of high molecular mass hyaluronan on the distribution and movement of proteoglycan (a type of glycoprotein)

formed around rabbit chondrocytes cultured in alginate beads were evaluated and it was found that extrinsic hyaluronan could affect the movement of newly synthesized proteoglycan both in alginate beads and in cartilage tissue (Kikuchi et al. 2001). Sakai et al. (2001) developed a novel type of immunoisolation membrane for a microcapsule-shaped bioartificial pancreas using sol-gel-synthesized silicate. They prepared an aminopropylsilicate membrane derived from 3-aminopropyltrimethoxysilane and tetramethoxysilane on calcium alginate gel beads via electrostatic interaction, where blood islets could be encapsulated and they retain their ability to secrete insulin. Masuda et al. (2003) studied the possibility of the effectiveness of human osteogenic protein-1 in promoting matrix synthesis and matrix formation by rabbit nucleus pulposus and annulus fibrosus cells cultured in alginate beads. The results which they found provided evidence of the ability of human osteogenic protein-1 to stimulate the metabolism of both rabbit annulus fibrosus and nucleus pulposus cells cultured in alginate beads. Grunder et al. (2004) found that bone morphogenetic protein-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads.

4 Conclusion

Developments made in the field of alginate-based blends and beads in recent years have been summarized in this chapter. The various recent techniques employed for the preparation of the micro- and nano-sized beads, blends and their important applications in different fields were discussed. The application areas covered for the blends and beads were membrane science, slow release of drugs and agrochemicals, biomedicine, process technology including enzyme immobilization, and pollution control. Some advance applications of these systems in research areas such as stem cells and bioartificial organs were also briefly discussed.

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Applications of Alginates in Food

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Contents

Abstract Alginates are a group of viscous polysaccharides derived from brown seaweeds and produced as an extracellular matrix by some bacterial species. Traditionally, alginates have been used as thickeners, emulsifiers and stabilising agents in a number of food applications. A number of new food applications are emerging for alginates, based on their unique biochemical and biophysical properties.

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These applications are discussed within this review, as are the nutritional properties of dietary alginates, and their physiological actions in humans.

1 Introduction

Alginate is a polyuronan that is isolated from the cell walls of a number of brown algal and bacterial species around the world, and is also produced as an extracellular matrix by certain bacteria (Stokke et al. 2000). Alginates are used in a number of large-scale industrial processes (e.g. as an ingredient in shoe polish and as an important factor in the dye industry (Jensen 1993; Yamamoto et al. 1992) and have medical uses, such as cell microencapsulation (Uludag et al. 2000), as microsphere vectors for drug delivery (Skaugrud et al. 1999), for making dental impressions (Ertesvag and Valla 1998), and as the active ingredient in absorbent dressings (Ingram et al. 1998) and antireflux therapies (Bryan et al. 2001; Mandel et al. 2000). However, more than 6 times the amount of income from these applications is generated worldwide by the use of seaweed and seaweed products in the food industry (Jensen 1993). While large quantities of seaweed are eaten in eastern Asia, the inclusion of algal phycocolloids, (e.g. alginate, carrageenan and agar) as additives is how they are commonly consumed in the Western diet. (Ertesvag and Valla 1998; Moe et al. 1995). As food additives that have been demonstrated as safe and approved for use throughout the EU, alginates are given E numbers (Food Standards Agency 2002). Alginic acid is E400, while the sodium, potassium, ammonium and calcium salts are E401–E404, respectively (Smith and LilyHong 2003). The most widely used of these in the food industry is the sodium salt (Mancini et al. 2002). Esters of alginic acids, propylene glycol alginates (PGAs), are produced industrially with various degrees of esterification and viscosity, and are used for food uses similar to those of other forms of alginate, but at a lower concentration (Baeza et al. 2004). They are identified with the number E405 (Smith and LilyHong 2003).

Food industry applications of alginates are entirely dependent on the biophysical properties of the alginates. These properties are derived from the unique chemical structure of alginates. Alginates consist of 1→4-linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) pyranose residues in an unbranched chain. These residues can combine to form G-rich (G blocks) or MG areas (MG blocks), as well as M-rich areas (M blocks), which are especially predominant in bacterial alginates. G blocks are believed to be important to alginate structure as a result of their Ca^{2+}/H^{+} binding capability, which allows alginates to form gels in the presence of Ca^{2+} or H^{+} ions at low temperatures. MG blocks allow polysaccharide chain flexibility and therefore tend to reduce alginate solution viscosity (Smidsrod and Draget 1996). These unique gelling properties of alginates are vital to many of their applications in the food industry.

Table 1 compares a number of polysaccharides commonly used in thickening applications and their biophysical properties.

The major uses of alginates in the food industry are discussed in the following sections, along with potential applications as suggested by food science literature. Finally, the effects that dietary alginates have on human physiological processes are discussed in Sect. 6.

Table 1 Comparison of the biophysical and biochemical properties of a range of viscous polysaccharides used by the food industry. (Data collected from Smith and LilyHong 2003; De Ruiter and Rudolph 1997; de Vries 2004; Sworn 2004; Ward 2004

	Source	Constituent sugars	Properties in gel or solution
Alginate	Brown seaweed	Mannuronic acid Guluronic acid	Forms heat-stable gels rapidly at low temperature without prior heating. Gels at low pH or high Ca^{2+} concentration
Propylene glycol alginate	Brown seaweed	Mannuronic acid Guluronic acid	Does not gel. Useful in stabilising foams
Pectin	Citrus fruit, apples, sugar beets, sunflowers	Galacturonic acid Rhamnose Galactose Arabinose	Acid-stable gel. Requires acid/sugar/calcium presence for gelling
Carrageenan	Red seaweeds	Sulphated D-galactose L-Anhydrogalactose	Form gels at low concentrations compared with other gelling agents Easily soluble in sugar solutions. Gels are degraded at low pH and high-temperature conditions
Guar gum	*Cyamposis tetragonolobus* seeds	L-Mannose D-Galactose	Hydrates rapidly in cold water, forming highly viscous aqueous solutions. Gels in the presence of other gums (e.g. locust bean gum, xanthan)
Gum arabic	Acacia sap	D-Galactose D-Glucuronic acid L-Rhamnose L-Arabinose	Has extremely high solubility (up to 50% aqueous solutions) with low viscosity in cold water

2 Gelling Agents

Restructuring of foods is commonly used by the food industry as a means of using less aesthetically appealing produce (e.g. cuts of meat with high fat or connective tissue content, or misshapen or missized fruits). By increasing the aesthetic quality of foods, restructured forms can often offer novel products that help meet customer demand. Restructured foods can be produced in any shape or size, which allows production of foodstuffs that are of a more uniform or attractive aesthetic nature. At the same time, the texture and structural properties of the foods can be controlled to produce more desirable products (Ranken 2000). Postharvest losses of fruits and vegetables can be high, so development of processes that can produce value-added, shelf-stable products that can be sold throughout the year is desirable (Mancini and McHugh 2000). Alginates are available at a wide range of viscosities, offer stability to foodstuffs under both high and low temperatures, and therefore have a wide range of uses as gelling agents. Alginate gelation rates and gel strength can be

controlled by the concentration of Ca^{2+} or H^{+} in solution. Alginate gel strength is also governed by the number of G blocks within the polyuronate chain. As alginates form gels at low temperatures, this is particularly useful in the restructuring of foodstuffs that may become damaged or oxidised under high temperatures (e.g. meat products, fruits and vegetables).

The most common restructured foods produced using alginates are reconstituted onion rings and pimento sections for use in olives (Brownlee et al. 2005). For both of these products, alginates allow the production of products of uniform size and consistency. In these two cases, restructuring also greatly facilitates mass production of these products.

Alginates also have a number of similar applications in meat (Ranken 2000;Cong-Gui et al. 2006; Devatkal and Mendiratta 2001; Boles and Shand 1999), seafood (Suklim et al. 2004), fruit (Mancini and McHugh 2000; Das Gupta et al. 2007; Grizotto et al. 2006, 2007), vegetable (Manjunatha and Gupta 2006) and some extruded food products (e.g. pastas and noodles (Brownlee et al. 2005).

3 Thickening, Stabilising and Emulsifying Agents

Alginates are commonly used as thickening and agents in jams, marmalades and fruit sauces, as alginate–pectin interactions are heat-reversible and give a higher viscosity than does either individual component (Brownlee et al. 2005). Alginates are also used to thicken desserts and savoury sauces, including mayonnaise (Mancini et al. 2002; Wendin et al. 1997; Gujral et al. 2001). PGAs are often used for this application, but at concentrations lower than those of standard alginate salts. This is due to their high stability and relatively low viscosity. Use of alginates on their own or in conjunction with other thickening agents has been shown to improve the acceptability of a number of low-fat processed foods (Mancini et al. 2002; Wendin et al. 1997; Kumar and Sahoo 2006; Kumar et al. 2007; Lin and Keeton 1998). Hydrocolloid use in this manner aids retention of moisture and improves food texture. This can result in an improvement to the organoleptic qualities of the food products, thus improving consumer acceptance.

Alginates form stable gels at high and low temperatures and at low pH (Smidsrod and Draget 1996). As a result, they can be used for a number of stability applications in food processing (Paraskevopoulou et al. 2006, 2005; Gomez-Diaz and Navaza 2004; Ferreira et al. 2005; Hubbermann et al. 2006). Routine use of alginates in bakery creams endows the cream with freeze/thaw stability and reduces separation of the solid and liquid components (syneresis) (Brownlee et al. 2005). Alginates are used in combination with other hydrocolloids to thicken and stabilise ice cream. While this allows control of the product's viscosity, it also increases heat-shock resistance, reduces shrinkage and ice crystal formation, and endows the ice cream with the desired melting characteristics (Regand and Goff 2003).

PGAs are commonly used to maintain foam stability, including applications in mousse and other desserts (Baeza et al. 2004; Sarker and Wilde 1999). The largest

food use of PGA is within the brewing industry, where PGA added to different beers and lagers stabilises the froth head when poured, while also protecting it from foam-negative contaminants (Ferreira et al. 2005).

By definition, hydrocolloids are water-soluble and very hydrophilic. However, previous studies have suggested that, within their use as stabilisers in food oil–water emulsions, they exhibit the ability to precipitate/adsorb onto oil droplets and sterically stabilise emulsions against flocculation and coalescence (Huang et al. 2001). PGAs have previously been demonstrated to be better emulsifiers than methylcellulose compounds and locust bean and guar gums, and to be comparable with other commonly used plant-derived polysaccharides (i.e. pectin and gum arabic) on a weight-for-weight basis (Huang et al. 2001; Gaonkar 1991). These factors are useful in simple food emulsions in which PGA and alginates are utilised, such as mayonnaise and other dressings (Mancini et al. 2002; Wendin et al. 1997; Paraskevopoulou et al. 2005).

4 Encapsulation and Immobilisation

One of the more recent uses of alginates is in encapsulation technologies. Outside the food industry, these technologies have been utilised in a wide range of drug delivery applications (George and Abraham 2006; Lee et al. 2003). Microencapsulation of insulin-producing cells for injection into type I diabetics has also been shown to be an effective treatment (de Vos et al. 2006). For this role, alginate is used to immunoisolate injected cells, thereby acting as a physical barrier against host immune sampling and response. This technology, therefore, has potential for a range of other uses in cell transplantation therapies (Uludag et al. 2000).

Within the food industry, alginate encapsulation and immobilisation technologies are used for a variety of purposes, including food processing, food functionality and product acceptability. These immobilisation technologies can enhance productivity as a result of continuous operation and reuse of the entrapped cells or enzymes (Groboillot et al. 1994). Immobilisation or encapsulation technology is used to produce a wide range of bacterial metabolites, including enzymes, amino acids, organic acids (e.g. acetic acid) and alcohols (Norton and Vuillemard 1994).

Alginates are commonly used as an immobilisation medium for whole cell or isolated enzyme preparations in industrial processes requiring enzymatic activity. Immobilisation in this manner is a simple means of reducing cell or enzyme loss, and can improve the heat stability of entrapped enzymes. As alginates (particularly their calcium salts) form ionotropic gels spontaneously under low-temperature conditions, they are ideal for entrapment of whole cells or enzymes, which would otherwise be damaged under more stringent conditions (Navratil et al. 2002). However, as alginates or other viscous entrapment media act as a diffusion barrier, they may also reduce cell/enzyme access to the substrates or decrease the rates of release of end products. Therefore, this may result in a reduction in the efficiency of this type of process (Mishra and Kar 2003). This must be taken into account when choosing an immobilisation medium for large-scale industrial processes.

While enzyme immobilisation is suitable for a number of applications, the use of whole cell immobilisation is necessary to carry out multistep, multienzyme reactions, especially those dependent on the presence of enzyme cofactors. Alginate is the most frequently described immobilisation agent in previous literature (Boyaval and Goulet 1988; Brandenberger and Widmer 1998). Table 2 shows some examples of alginate immobilisation technologies used in food production.

One of the more recent developments in alginate use by the food industry has been in the encapsulation of live cells (probiotics) for delivery in foods to the human large bowel. A probiotic has been defined as: "A preparation of or a product containing viable, defined micro-organisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host, and by that exert beneficial health effects in the host" (Schrezenmeir and De Vrese 2001). Probiotic cultures routinely contain lactic acid producing species such as lactobacillus, or bifidobacteria. While a number of benefits have been hypothesised for probiotic cultures in animal experiments and in vitro, there is still no clear-cut evidence of a beneficial effect on health of such preparations from human intervention studies (De Roos and Katan 2000; Allen et al. 2004; Honeycutt et al. 2007; Mallon et al. 2007).

While the benefits to health of probiotics are likely to be a bone of contention for a considerable time, it is apparent that formulation of probiotic bacteria in foods

Table 2 Examples of whole-cell and enzyme alginate immobilisation technologies in the food industry

Whole-cell application	Cells immobilised	Reference
Starter cultures for dairy industry	Various lactic acid producing bacteria	Champagne et al. (1994)
Milk lactose hydrolysis (production of low-lactose milk)	*Kluyveromyces lactis*	Genari et al. (2003)
Production of lactic acid from cheese whey	*Lactobacillus helveticus*	Boyaval and Goulet (1988)
Production of fermented beverages	Various yeast cultures	Navratil et al. (2002)
Production of isomaltulose from sucrose	*Protaminobacter rubrum* *Erwinia rhapontica* *Erwinia* sp. D12 *Serratia plymuthica* *Klebsiella planticola* *Klebsiella* sp.	Kawaguti et al. (2006)
Bioindicators of thermal sterilisation	*Bacillus subtitles* *Bacillus stearothermophilus*	Serp et al. (2002)
Enzyme application	**Enzymes immobilised**	**Reference**
Reduction of fruit juice bitterness	Naringinase	Puri et al. (1996)
Glucose determination of liquid foodstuffs	Glucose oxidase	Serp et al. (2002)
Production of inverted sugar syrups	Inulinase	Catana et al. (2005)
Dextran synthesis	Dextransucrases	De Segura et al. (2004)
Increasing n-3 polyunsaturated fatty acid concentrations in sardine Oil	Lipase	Okada and Morrissey (2008)

must ensure adequate delivery of viable cells to the large bowel for maximal effect. Simple and inexpensive technologies to form gel microcapsules from alginates under conditions which will not cause damage to the bacterial cells contained within are readily available.

Alginates with a high G block content are the best alginates for microcapsule formation, owing to their high mechanical stability, high porosity and tolerance to salts and chelating agents (Kailasapathy 2002). Alginates act to increase survival of bacterial cells during food storage (Kailasapathy 2006; Krasaekoopt et al. 2006), and also reduce cell destruction under the inhospitable conditions of the stomach and small intestine (Kim et al. 2008; Ding and Shah 2007; Muthukumarasamy et al. 2006). In addition, encapsulation was shown to increase cell survival through a number of food processes, such as freezing (Kailasapathy 2002) and freeze-drying (Kailasapathy 2002; Capela et al. 2006).

Microencapsulation has a number of further applications in the food industry, including encapsulation of reactive or volatile molecules, such as acidulants, fats and flavours (Desai and Park 2005). However, alginates are hydrophilic by nature, and therefore tend to be immiscible with these types of hydrophobic compound. Further from this, microencapsulation of low molecular weight components requires low matrix porosity so that entrapped compounds do not leach out. As alginates form highly porous gels, they may not be suitable for this type of application.

5 Food Coating

Edible films and coatings for foodstuffs are developing technologies that have high potential, owing to the current call for reduction or replacement of non-biodegradable or non-recyclable food packaging (Wang et al. 2007). As with conventional food packaging, these new technologies must heighten product stability, safety and shelf life.

Alginates are one of a wide range of polysaccharides and proteins that have been used as an edible coating for a number of foodstuffs. Food coatings formed from sodium alginate have been shown to have excellent tensile strength, flexibility and resistance to tearing, and are impermeable to oils. However, owing to the porous nature of alginate gels, these coatings tend to have high permeability to oxygen and water (Wang et al. 2007). Alginate food coatings can also be formed ionotropically at room temperature, and therefore their use may be advantageous in a number of food applications.

Incorporation of antimicrobial agents in the alginate gel has been demonstrated to be an effective barrier to microbial surface spoilage of vegetables, meat (Oussalah et al. 2007, 2006; Mehyar et al. 2007; Natrajan and Sheldon 2000) and fish products (Datta et al. 2008). The cold formation of the alginate gel coating reduces damage both to the antimicrobial entities and to the foodstuff itself. This property has also been demonstrated to be useful in the coating of a number of fresh fruit and vegetable products, such as lettuce (Tay and Perera 2004) and freshly cut apple and melon sections

(Oms-Oliu et al. 2008; Rojas-Grau et al. 2008), where the coating increased shelf life, reduced browning, maintained crispness and texture and reduced vitamin C loss.

6 Physiological Effects of Dietary Alginates

As alginates are polysaccharides that are indigestible in the human stomach and small intestine, they would fit any of the variety of definitions that are available to describe dietary fibre (Brownlee et al. 2006; Champ et al. 2003; DeVries et al. 2001; McCleary 2003; Ha et al. 2000; Prosky 2000). As such, they exhibit many of the physiological characteristics commonly associated with dietary fibre, such as laxation and stool bulking. At the same time, alginates also exhibit a range of unique physiological actions. As with many of their food applications, the physiological effects of alginates are dependent on their biochemical and biophysical properties. Further understanding of health benefits of alginate ingestion, and particularly how these are associated with the biochemical and biophysical properties of alginates, is likely to open up new product opportunities for the food industry.

6.1 Alginate Safety

Alginates have been classified as safe for additive-level use both in the EU and in the USA. Previous studies into the dietary effects of sodium alginate and PGA have shown no adverse effects (i.e. no effect on haematological indices, plasma and urine biochemistry and breath hydrogen concentration) of ingestion of 175 mg kg^{-1} of body weight for 7 days followed by 200 mg kg^{-1} for a further 16 days in small groups of male volunteers ($n = 5$ for both compounds) (Anderson et al. 1991a, b).

6.2 Effect on Colonic Microflora

Incubation of human faecal inocula with alginates did not produce short-chain fatty acids for over 6 h (Michel et al. 1996). After 24-h incubation, between 50 and 80% of the alginate had been degraded under these conditions (Michel et al. 1996; Bobin-Dubigeon et al. 1997). These data suggest that alginates are slowly fermented by the colonic microflora in humans, although it must be noted that faecal inocula will not necessarily be indicative of the colonic microfloral population. In animal studies, the fermentability of dietary alginates increased over time of feeding, suggesting a shift of the colonic microflora to one that was more capable of degrading alginate polysaccharide chains (Suzuki et al. 1993a). From these previous studies, there is the suggestion that M-rich alginates are less well digested than G-rich ones, and that increasing chain length of alginates results in reduced fermentability (Michel et al. 1996, 1999; Suzuki et al. 1993a).

Owing to the complexity of the human colonic microflora, it is always difficult to predict how changes to specific bacterial species will affect the microflora as a whole, or the knock-on physiological effects these changes may have on the host. To date, very few data are available on the effects of alginates on bacterial species. The ability of 21 strains of authentic human intestinal bacteria to grow on alginate-based media has been tested under in vitro conditions. Only *Bacteroides ovatus* demonstrated the capability to thrive under these conditions (Fujii et al. 1992). One previous study where human participants were fed 10 g of alginates per day demonstrated an increase in faecal bifidobacterial cultures, and a decrease in both some potentially pathogenic bacterial strains (e.g. *Enterobacteriaceae* and lecithinase-negative *Clostridia*) and the levels of faecal toxins produced by putrefaction (e.g. ammonia and sulphides) (Terada et al. 1995).

Table 3 compares the effects of a range of polysaccharides commonly used as thickeners, stabilisers and emulsifiers in the food industry on the colonic microflora.

6.3 Reduction of Toxicity of Colonic Luminal Contents

Bulking of the colonic contents (and eventually the passed stools) results in a dilution of any damaging agents in the colon, thereby effecting a reduced mucosal exposure to these factors.

Seven-day supplementation of the diet of 5 healthy adult males with sodium alginate (175mg/kg body weight) significantly increased faecal wet and dry weights

Table 3 Effect of a range of viscous polysaccharides commonly used in the food industry on the colonic microflora. (Data collated from Bobin-Dubigeon et al. 1997; Fujii et al. 1992; Terada et al. 1995; Cherbut et al. 2003; Bliss et al. 1996; Barry et al. 1995; Bourquin et al. 1996; Ferguson and Jones 2000; Gulfi et al. 2005, 2006, 2007; Gibson et al. 1991; Macfarlane et al. 1998)

		Approximate percentage small-chain fatty acid production			Effects on microfloral strains
	Fermentability (%)	Acetate	Propionate	Butyrate	
Alginate	50–80	66	19	15	↑ *Bacteroides* ↑ *Bifidobacteria* ↓ *Enterobacteriaceae* ↓ *Clostrida*
Pectin	>83	77	8	14	↑ *Enterobacteriaceae* ↑ *Bacteroides*
Carrageenan	24	75	10	14	↑ Sulphate-producing bacteria ↓ *Bacteroides*
Guar gum	70–95	53	33	14	↑ *Bifidobacteria*
Gum arabic	77	69	18	11	↑ *Bifidobacteria* ↑ *Lactobacilli*

Small-chain fatty acid data typically do not add up to 100% owing to the production of other, minor fatty acids in fermentation

compared to outputs during a normal diet. A similar supplementation regime with PGA had no effect on these parameters (Anderson et al. 1991a, b). In porcine studies, 5% alginate in the feed increased the volume of colonic luminal contents. This effect was reported to be higher than that with cellulose, xylan and carageenan at the same concentration (Hoebler et al. 2000). A range of damaging agents which originate from food, microflora and the gastrointestinal tract itself that occur in the colon have been shown to be directly adsorbed to dietary alginates in a way similar to that of other dietary fibres (Brownlee et al. 2005).

6.4 Effect of Alginate on Satiety

Unlike other viscous polysaccharides, which need to be administered in gel form to be a gel in the stomach, alginates have the unique ability to form gels spontaneously at low temperature in the presence of acid or calcium ions. High viscosity or gel strength in the mouth is well associated with poor organoleptic acceptability of foods (Wolf et al. 2002). At the same time, high viscosity in the stomach is linked to increased gastric distension and thereby increased satiety (Hoad et al. 2004). Previous research has suggested that, as alginates can be administered in a low-viscosity form and gel spontaneously in the stomach, they represent a good candidate for formulations targeted at increasing satiety.

In a participants study ($n = 12$) addition of either alginates or guar gum to a milk based liquid meal replacement resulted in participants reporting an increased feelings of fullness compared to a control (meal replacement only). Alginates with high gel strength significantly prolonged a postprandial feeling of hunger compared with both control and guar gum meal replacements. Alginate increased the volume of stomach contents, but did not affect gastric emptying rates (Hoad et al. 2004).

A two-part beverage that formed a calcium alginate/pectin gel in the stomach was used in a participant study measuring dietary restraint in overweight and obese, non-dieting female volunteers (the beverage was taken twice daily). This beverage increased dietary restraint in participants with low initial dietary restraint, but not in those who already had high restraint levels. The beverage significantly reduced total energy intake, seemingly as a result of reducing carbohydrate intake (no significant effect was seen on total protein or fat intakes) (Pelkman et al. 2007). A snack bar formulation including alginate and guar gum had no reported effect on appetite compared with control snack bars (Mattes 2007).

6.5 Effect on Intestinal Absorption Rates

Alginates appear to have some inhibitory effects on a range of digestive enzymes in vitro. This is likely due to their high viscosity, which in turn reduces the availability of substrate for enzyme action. These effects are reviewed in detail elsewhere (Brownlee et al. 2005).

As with other viscous fibres, inclusion of high levels of alginate in the diet has been linked with a reduced bioavailability of certain beneficial dietary components,

including β-carotene (Riedl et al. 1999), and minerals, such as calcium (Bosscher et al. 2001), iron, chromium and cobalt (Harmuth-Hoene and Schlenz 1980). This reduced nutrient and mineral absorption might suggest that the inclusion of high levels of alginate in the diets of at-risk individuals (e.g. the elderly, pregnant women and infants) may outweigh any potential health benefits. However, presaturation of alginate formulations with calcium and other divalent cations could reduce the possibility of these negative side effects.

6.6 Effect on Plasma Cholesterol

Increased intake of viscous dietary fibres has been linked to a reduction in plasma cholesterol concentrations (Dikeman and Fahey Jr. 2006). A meta-analysis of 67 controlled trials suggested that pectin had the greatest effect on lowering total cholesterol concentrations per gram of four common viscous fibre sources (reduction in cholesterol of 70 $\mu M\ L^{-1}$ plasma g^{-1} fibre, compared with 37 $\mu M\ L^{-1}\ g^{-1}$ for oat products, 28 $\mu M\ L^{-1}\ g^{-1}$ for psyllium and 26 $\mu M\ L^{-1}\ g^{-1}$ for guar gum). These effects appeared to be almost entirely due to a reduction in LDL cholesterol, rather than HDL cholesterol (Brown et al. 1999). This style of analysis does not account for variations in the study populations used, and currently no single study has adequately compared the cholesterol-lowering effects of dietary fibre types with each other within the same population. To date, relatively few studies have considered the effects of alginates in lowering cholesterol.

Alginate (7.5 g day^{-1}, M to G ratio 1.5) supplementation of a low-fibre diet has previously been shown to more than double (140% increase) mean fatty acid excretion in the digesta of a small cohort ($n = 6$) of human ileostomy patients (Sandberg et al. 1994). A number of animal model studies have also demonstrated that the presence of alginate in the small intestinal lumen decreases uptake of fats and reduces plasma cholesterol under a range of different diets (Brownlee et al. 2005; Jimenez-Escrig and Sanchez-Muniz 2000). These effects are likely to be due to the increased levels of faecal bile and cholesterol excretion that have previously been reported (Seal and Mathers 2001; Kimura et al. 1996).

Levels of 1 and 3% sodium alginate were shown to have hypocholesteraemic effects (mean reduction in plasma cholesterol from fibre-free controls of 8.5 and 20.5%, respectively) similar to those of the algal polysaccharide funoran (7.3 and 20.9%), but less than those of carrageenan (14.6 and 29.9%) (Jimenez-Escrig and Sanchez-Muniz 2000; Ito and Tsuchiya 1972) in rats fed 1% dietary cholesterol. All of these fibre types had a much larger effect than agar (3% in diet gave a reduction of only 1.8% in total plasma cholesterol). Within this study, it was also shown that low molecular weight alginates do not appear to have these hypocholesteraemic effects. In rat diets with higher total cholesterol and fat content, sodium alginate inclusion had effects on total cholesterol reduction similar to those of inclusion of a range of other algal polysaccharides (sulphated glucuronoxylohamnan, porphyran and furonan), and reduced blood cholesterol concentrations more than fucoidan and agar (Jimenez-

Escrig and Sanchez-Muniz 2000; Ren et al. 1994). Feeding of a cholesterol-free diet to rats containing 5 or 10% sodium alginate or guar gum caused a similar drop in plasma cholesterol concentrations over a 21-day period (Seal and Mathers 2001).

Increased G-block content will lead to an increased gel-forming capacity of the alginate, which would be expected to reduce intestinal absorption. The effects of alginate on cholesterol uptake into the bloodstream have previously been suggested to be increased with a lower M to G ratio (Suzuki et al. 1993b), but this was believed to be due to the effects of the high-viscosity alginate on depression of appetite (i.e. lower levels of ingested cholesterol), rather than effects on intestinal absorption and/or increased cholesterol excretion.

6.7 Effect on Glycaemic and Insulinaemic Responses

Glucose absorption rates have also been shown to be reduced in the presence of alginate. A 5-g supplement of sodium alginate to test meals containing similar levels of digestible carbohydrates, fats and proteins was given to a cohort of diabetes type II patients. This low level of dietary alginate caused a reduction in blood peak glucose and plasma insulin rise (by 31 and 42%, respectively), as well as prolongation of postprandial emptying (Torsdottir et al. 1991). Similar results of blunting postprandial plasma glucose and insulin elevation have also been reported upon feeding a low dose of alginate (1.5 g sodium alginate within a liquid drink) to healthy human subjects (Wolf et al. 2002). Within this study, the 1.5 g alginate drink reduced both peak postprandial glucose concentrations and total glucose uptake over 3 h significantly more than a comparable drink containing 1.2 g of a mixture of gum arabic and 0.3 g of guar gum in the same population of 30 healthy adults. Snack bars including alginate also reduced postprandial peak glucose concentrations and total glucose uptake (over 3 h) when compared with snack bars containing guar gum (Williams et al. 2004).

7 Summary

Alginates are being used or have the potential to be used for an increasing variety of applications in the food industry. Their unique gelling abilities at low temperature alongside good heat stability make them ideal for use as thickeners, stabilisers or restructuring agents. In addition to this, alginates also have uses in a myriad of newer food applications, from encapsulating active enzymes and live bacteria, to acting as the carrier for protective coating of prepacked, cut or prepared fruits and vegetables. A wide range of alginates are commercially available with various bioactive properties, mainly dependent on polysaccharide chain length and the ratio of mannuronate to guluronate sugars, thus allowing for a large scope of potential uses.

Alginate intake in the diet has a number of physiological effects both in the gastrointestinal tract and systemically that are relatively similar to those of a range of

other viscous polysaccharides. Once again, the unique biophysical properties of alginates separate them from other similar dietary fibres. Alginates can be given in low-viscosity formulations, which firstly allows both increased palatability and the possibility of a higher oral dose of alginates being delivered than is possible for other viscous polysaccharides. Secondly, as alginate formulations gel in the stomach, in the presence of either acid or calcium ions, they can be used to increase satiety and reduce absorption and digestion rates of other macronutrients in the diet. Although alginates have been demonstrated to be beneficial in this way in a number of human participant studies, current legislation worldwide only allows for their use in food as an additive (as opposed to an ingredient). This means that alginate used in foods will remain at a low concentration for the foreseeable future. However, this still allows the possibility of novel applications of specific alginates in the food industry that have high bioactivity at low concentration. Therefore, food manufacturers, alginate producers and food and nutrition scientists will need to work more closely together if novel uses for these unique algal and bacterial polysaccharides are to be identified.

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Alginate and Its Comonomer Mannuronic Acid: Medical Relevance as Drugs

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Contents

Abstract The therapeutic and anti-inflammatory properties of sodium alginate and one of its components, β-D-mannuronic acid, were tested in various experimental models. On the basis of its chemical structure, β-D-mannuronic acid had been conceived as an anti-inflammatory drug. β-D-Mannuronic acid is a constituent of alginate but is not available commercially; hence, polymannuronate was obtained from an epimerase (AlgG) negative mutant of *Pseudomonas* sp. and the monomer was isolated by acid hydrolysis. The oral and intraperitoneal administration of alginate gels in experimental models of ulcerative colitis and glomerulonephritis as well as the oral administration and intraperitoneal injections of β-D-mannuronic acid in different models of rheumatoid arthritis, multiple sclerosis, glomerulonephritis, and nephrotic syndrome showed that alginate as well as β-D-mannuronic acid are able to exhibit

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their therapeutic efficacy in inflammatory diseases. Recent experimental evidence has revealed that β-D-mannuronic acid represents a novel nonsteroidal anti-inflammatory drug with a low molecular weight and which provides a new therapeutic option in attenuation of inflammatory reactions and autoimmune diseases.

1 Introduction

Alginates are natural macromolecules composed of β-D-mannuronate and α-L-guluronate linked by 1→4 glycosidic bonds. They are synthesized by bacteria and/or brown seaweeds, and the mannuronate residues of the bacteria, but not seaweed polymers, are acetylated at positions 0–2 and/or 0–3 to a variable extent (Skjak-Braek et al. 1986; Bucke 1987; Rehm 1998).

The variability in monomer block structures and acetylation which are associated with the source of alginate strongly affect the physicochemical and rheological properties of the alginate polymer (Rehm and Valla 1997).

The alginate gels are well known as being biocompatible, degradable, and nontoxic; thus, they are widely used as carriers for drug delivery, hemostatic wound dressing, and immunoisolation systems for transplantation using uncoated alginate microspheres and devices anastomosed to the vascular system as arteriovenous shunts such as alginate-impregnated polyester vascular graft (Odell et al. 1994; Agren 1996; Murata et al. 2007; Lee et al. 1997; Efentakis and Buckton 2002; Li et al. 2006; Selmi et al. 2008; Sevgi et al. 2008). On the other hand, inhibitory effects of various types of alginic acid on hyaluronidase and mast cell degranulation were examined, and it was found that alginic acid with a mannuronate to guluronate ratio of 1.0 exhibited the strongest inhibition of both activities (Asada et al. 1997). Moreover, the protective and reparative effects of sodium alginate on radiation stomatitis and suppression of radioactive absorption by this compound in animals and human subjects were investigated (Hasegawa et al. 1989; Oshitani et al. 1990; Gong et al. 1991; McGlashan et al. 2009). The effect of alginic acid on an allergy model was analyzed by anaphylaxis, a histidine decarboxylase assay, and a histamine assay. Alginic acid dose dependently inhibited systemic anaphylaxis and passive cutaneous anaphylaxis and also decreased histamine release from serum and peritoneal mast cells. All these effects were stronger than those of disodium cromoglycate, the reference drug tested. In addition, alginic acid significantly inhibited the expression level of nuclear factor (NF)-κB and some proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor α (TNF-α) (Jeong et al. 2006). Although (Yang and Jones 2008) have reported that sodium alginate causes innate immune responses through NF-κB activation, the majority of investigations have demonstrated the biocompatibility and anti-inflammatory effects of alginate and one of its components, β-D-mannuronic acid (M2000), in various experimental models and/or patients (Siebers et al. 1997; Kammerlander and Eberlein 2003; Ohsumi et al. 2005; Hori et al. 2008). In this chapter, our aim is to show the

tolerability and therapeutic potency of alginate and M2000 in different disease models as well as various pharmaceutically relevant investigations.

2 Therapeutic Effects of Alginate on Experimental Acute Ulcerative Colitis

The potential therapeutic effect of low-viscosity sodium alginate (LVA) was studied in a rat model of acute colitis induced by intracolonic administration of acetic acid (Mirshafiey et al. 2005a). To induce the experimental model of acute ulcerative colitis, the method of (Morris et al. 1989) and (Yamada et al. 1991) was used. This experimental model produced a significant ulcerative colitis. Induction of colitis also significantly enhanced the levels of serum and colonic mucosal cytokines (interleukin-6, IL-6; and TNF-α) and eicosanoids (leukotriene B_4, LTB4; and prostaglandin E_2, PGE2), which paralleled the severity of colitis. For treatment, LVA was dissolved in water and adjusted to a concentration of 0.5% (w/v). The prophylactic and therapeutic rat groups received 0.5% LVA solution as drinking water ad libitum for 7 days. The onset of oral LVA administration for prophylactic and therapeutic groups was 24 h before and after the induction of colitis, respectively. The rats were killed at the end of the week.

The results showed that intracolonic administration of 4% acetic acid resulted in acute inflammatory bowel disease (IBD) in control nontreated rats. The control rats developed colonic macroscopic damage such as diffuse hyperemia and ulcerations. Daily oral treatment with LVA (drinking 35.5 ± 2.8 ml of 0.5% w/v LVA solution per rat, which was equivalent 0.65–0.77 mg kg^{-1} LVA per day) significantly reduced the colonic damage score in prophylactic and therapeutic rat groups (with mean scores of 5.0 and 4.6, respectively) when compared with control rats (mean score 9.4; $P < 0.05$). The microscopic examination of colonic specimens revealed a marked infiltration of inflammatory cells into both mucosa and submucosa, ulceration, and fibrosis. The most pronounced lesions were observed in a nontreated (control) group. Oral administration of LVA significantly inhibited acetic acid induced mucosal injury in both prophylactic and therapeutic groups in comparison with control rats (with mean scores of 4.2 and 4.2 vs 10.3, respectively; $P < 0.05$). Moreover, the data obtained showed that LVA therapy could significantly reduce serum and colonic mucosal IL-6, TNF-α, LTB4, and PGE2 levels in treated groups compared with nontreated controls.

Figure 1 shows that LVA therapy is able to reduce significantly the serum level of TNF-α in prophylactic and therapeutic groups compared with control rats ($P < 0.001$); however, although there was a significant difference between prophylactic and control groups in the serum IL-6 level ($P < 0.003$), the difference between therapeutic and control groups was not significant ($P < 0.058$). Moreover, as shown in Fig. 2, LVA administration could significantly diminish the serum concentration of eicosanoids (LTB4 and PGE2) in prophylactic and therapeutic groups compared

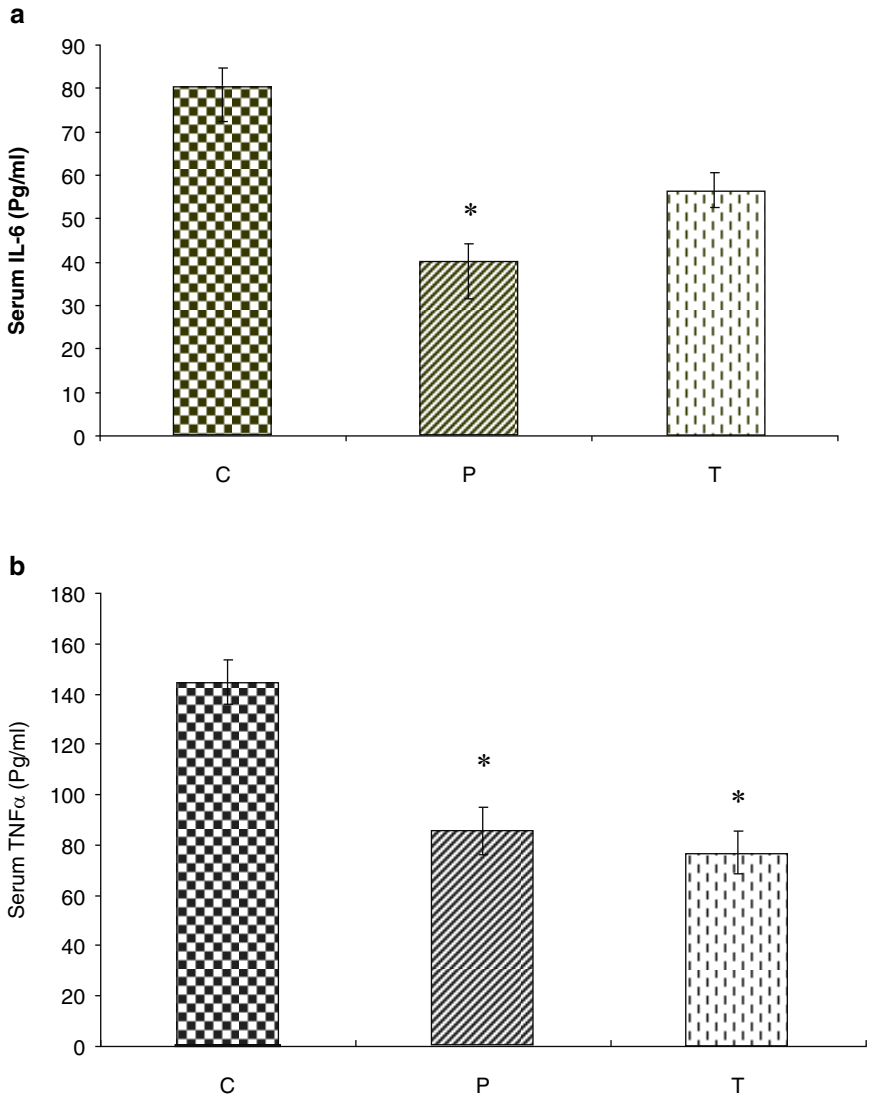

Fig. 1 Effects of low-viscosity sodium alginate (LVA) on serum levels of interleukin-6 (IL-6) (**a**) and tumor necrosis factor α (TNF-α) (**b**), 1 week after induction of colitis. *C* control rats, *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± the standard deviation (SD). *Asterisks* denote a significant difference between C and P and/or C and T groups, $*P \leq 0.05$ (Mirshafiey et al. 2005a)

with the control group ($P < 0.01$). On the other hand, with use of the supernatant of homogenized colonic tissue samples, it was found that there was a significant difference between prophylactic and therapeutic groups compared with the control group in connection with the amounts of IL-6, TNF-α, LTB4, and PGE2 ($P < 0.05$).

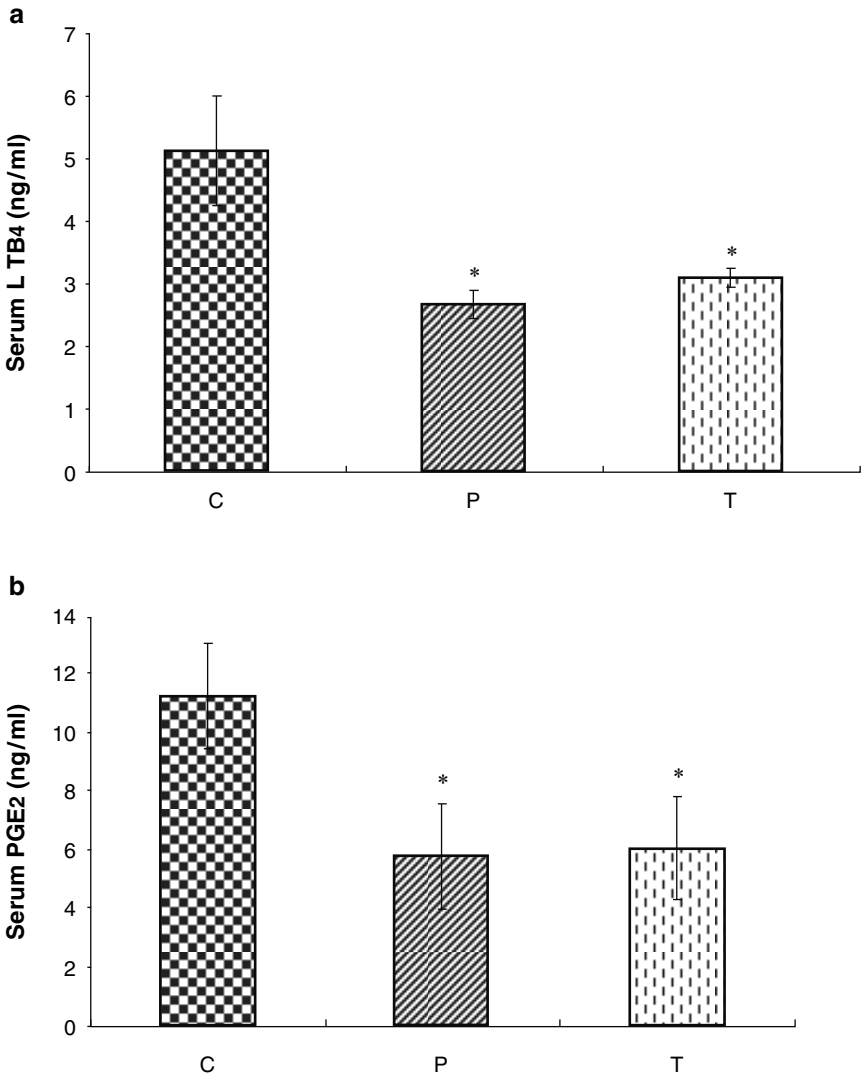

Fig. 2 Effects of LVA on serum levels of leukotriene B_4 (LTB4) (**a**) and prostaglandin E_2 (PGE2) (**b**), 1 week after induction of colitis. *C* control rats, *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± S.D. *Asterisks* denote a significant difference between C and P and/or C and T groups, $^*P \leq 0.05$ (Mirshafiey et al. 2005a)

Figures 3 and 4 show the effect of a therapeutic protocol on the reduction of the production of cytokines (IL-6 and TNF-α) and eicosanoids (LTB4 and PGE2). Taken together, the results of this study demonstrate that LVA therapy palliates the progression of colonic inflammatory lesions in an experimental model of IBD. The

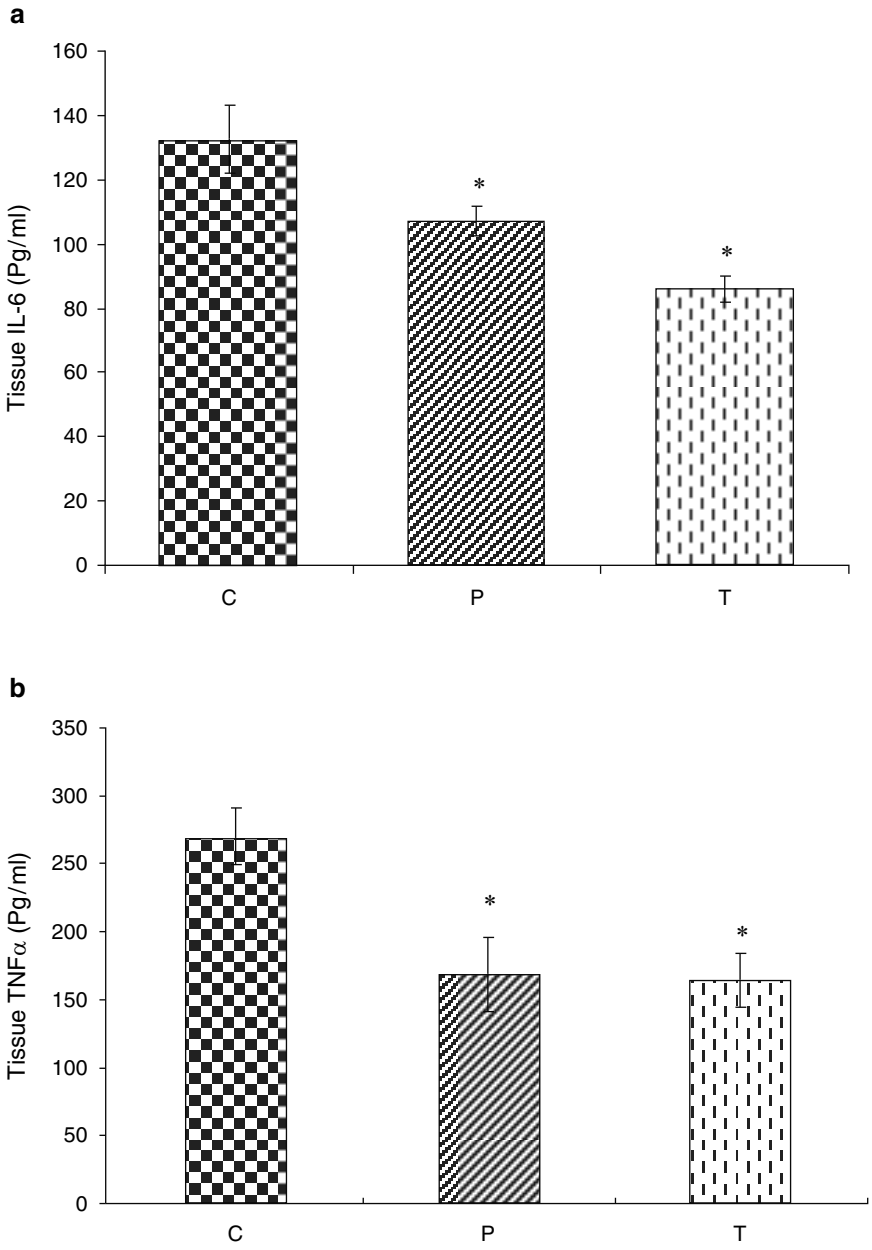

Fig. 3 Amounts of IL-6 (**a**) and TNF-α (**b**) in colonic homogenate following 1 week of oral administration of LVA. *C* control rats, *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± S.D. *Asterisks* denote a significant difference between C and P and/or C and T groups, $*P \leq 0.05$ (Mirshafiey et al. 2005a)

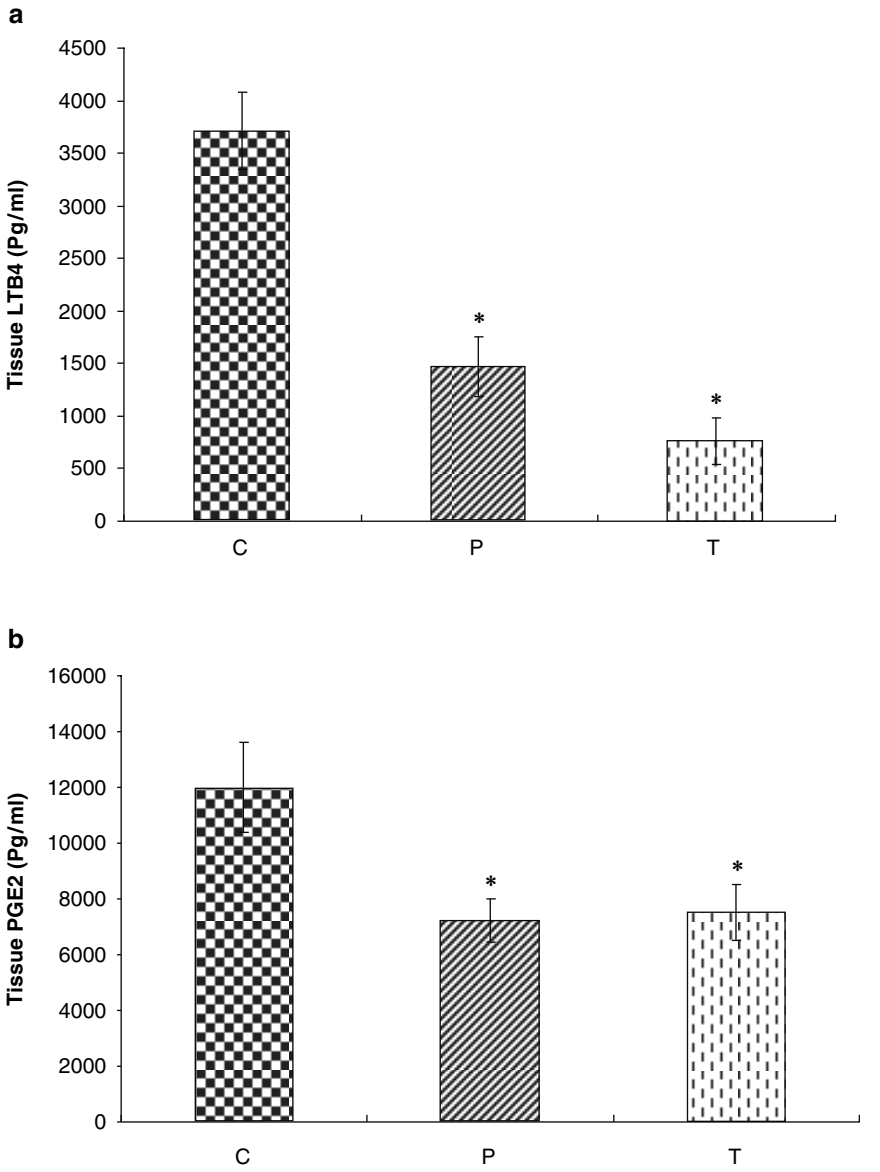

Fig. 4 Amounts of LTB4 (**a**) and PGE2 (**b**) in colonic homogenate following 1 week of oral administration of LVA. *C* Control rats *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± S.D. *Asterisks* denote a significant difference between C and P and/or C and T groups, $^*P \leq 0.05$ (Mirshafiey et al. 2005a)

beneficial effect of LVA is associated with a reduction of cytokine (IL-6 and TNF-α) and eicosanoid (LTB4 and PGE2) synthesis. Thus, LVA as a new therapeutic option can be recommended for IBD preclinical trials.

3 Treatment of Experimental Chronic Ulcerative Colitis with Sodium Alginate

The purpose of this study was to test the therapeutic efficacy of sodium alginate in a rat model of trinitrobenzene sulfonic acid (TNBS) induced IBD (Razavi et al. 2008). This experiment was carried out using 77 Sprague-Dawley rats which were divided into five groups: normal, control, prophylactic, therapeutic, and experimental groups. The rats were killed 1, 2, 3, and 6 weeks after induction of colitis. The severity of colitis was graded macroscopically and assessed using serum and colonic mucosal cytokines and eicosanoids. Intrarectal TNBS (30 mg) produced a significant chronic ulcerative colitis, on the basis of the method of (Menozzi et al. 2006). The lesions were most severe on the seventh day after TNBS instillation, and then declined, but lesions were still observed after 6 weeks. The onset of oral LVA administration for prophylactic and therapeutic groups was 24 h before and after the induction of colitis, respectively. Macroscopic findings showed that intracolonic administration of TNBS resulted in acute (week 1) and chronic (week 6) IBD in control nontreated rats. The control rats developed colonic macroscopic damage such as diffuse hyperemia and ulcerations. Daily oral treatment with LVA (drinking 35.5 ± 2.8 ml of 0.5% W/V LVA solution per rat, which was equivalent 0.65–0.77 mg kg^{-1} LVA per day) significantly reduced the colonic damage score in prophylactic and therapeutic groups in weeks 1, 2, 3, and 6 after induction of colitis. TNBS administration also significantly enhanced the levels of serum and colonic mucosal cytokines (TNF-α and IL-6) and eicosanoids (LTB4 and PGE2), which paralleled the severity of colitis, whereas pretreatment (in the prophylactic group) and treatment with LVA were significantly able to reduce the colonic damage score, serum level, and colonic mucosal production of TNF-α, IL-6, LTB4, and PGE2 in prophylactic and therapeutic rat groups compared with nontreated controls.

Figure 5a shows that LVA therapy was significantly able to reduce the serum level of TNF-α in prophylactic and therapeutic groups (during weeks 2, 3, and 6) compared with colitis induced in control rats ($P < 0.05$). Figure 5b represents a significant difference in the serum level of IL-6 in prophylactic and therapeutic groups (during weeks 1, 2, 3, and 6) compared with control rats ($P < 0.05$). Moreover, as shown in Fig. 6a, LVA administration could significantly diminish the serum concentration of LTB4 (during weeks 1, 3, and 6) in the prophylactic group and (during weeks 1 and 3) in the therapeutic group compared with the colitis-induced group ($P < 0.05$). Figure 6b shows that LVA therapy was significantly able to reduce the serum level of PGE2 in the prophylactic group (during week 6) and in the therapeutic group (during weeks 1 and 3) compared with colitis-induced rats ($P < 0.05$). In addition, with use of the supernatant of homogenized colonic tissue samples, it was found that there is a significant difference between prophylactic and therapeutic groups compared with the colitis-induced group in connection with the amounts of IL-6, TNF-α, and LTB4 (during weeks 1 and 6) (Figs. 7a, b, 8a), whereas there was no significant difference between prophylactic and therapeutic groups compared with the control group in colonic tissue PGE2 level following LVA therapy in an experimental model of colitis (Fig. 8b). Collectively, the data showed that LVA therapy is able to suppress chronic ulcerative colitis in an experimental model.

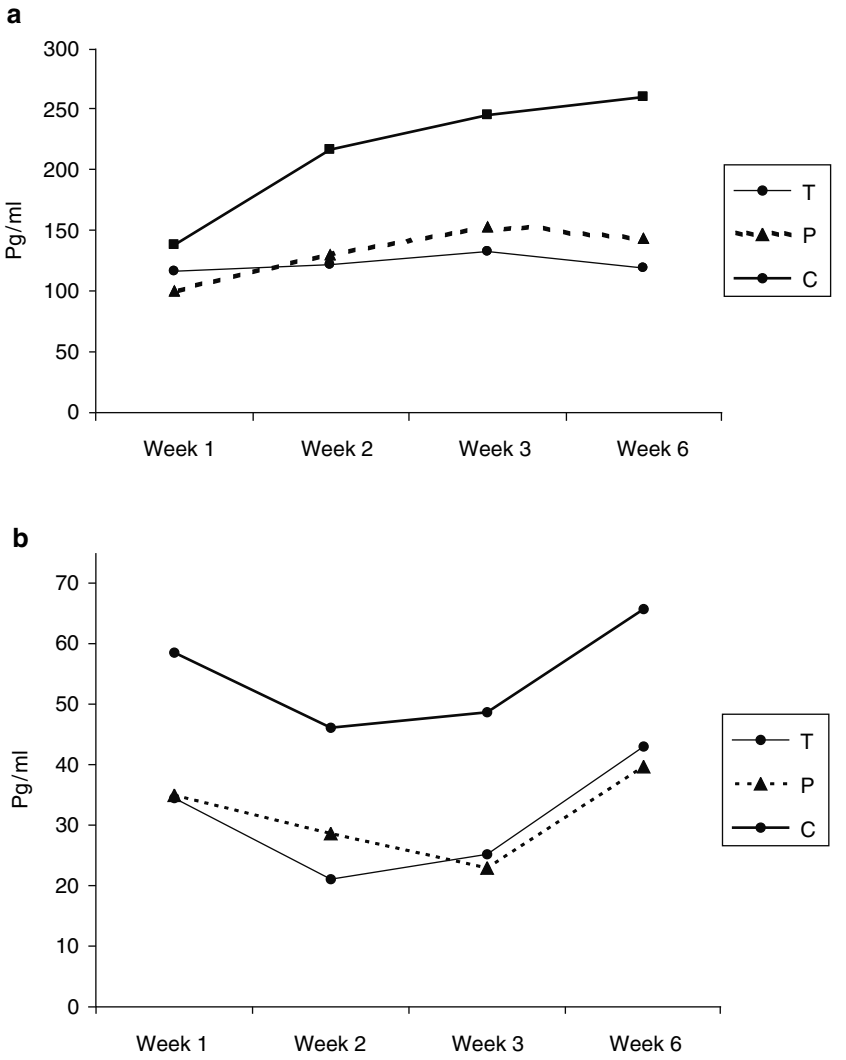

Fig. 5 Effects of LVA on serum levels of TNF-α (**a**) and IL-6 (**b**) during weeks 1, 2, 3, and 6 after induction of colitis. *C* colitis-induced rats; *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

4 Treatment of Experimental Immune Complex Glomerulonephritis by Sodium Alginate

The therapeutic efficacy of sodium alginate was studied in experimental immune complex glomerulonephritis (Mirshafiey et al. 2005). On the basis of the method of (Yamamoto et al. 1978), bovine serum albumin (BSA) nephritis as an

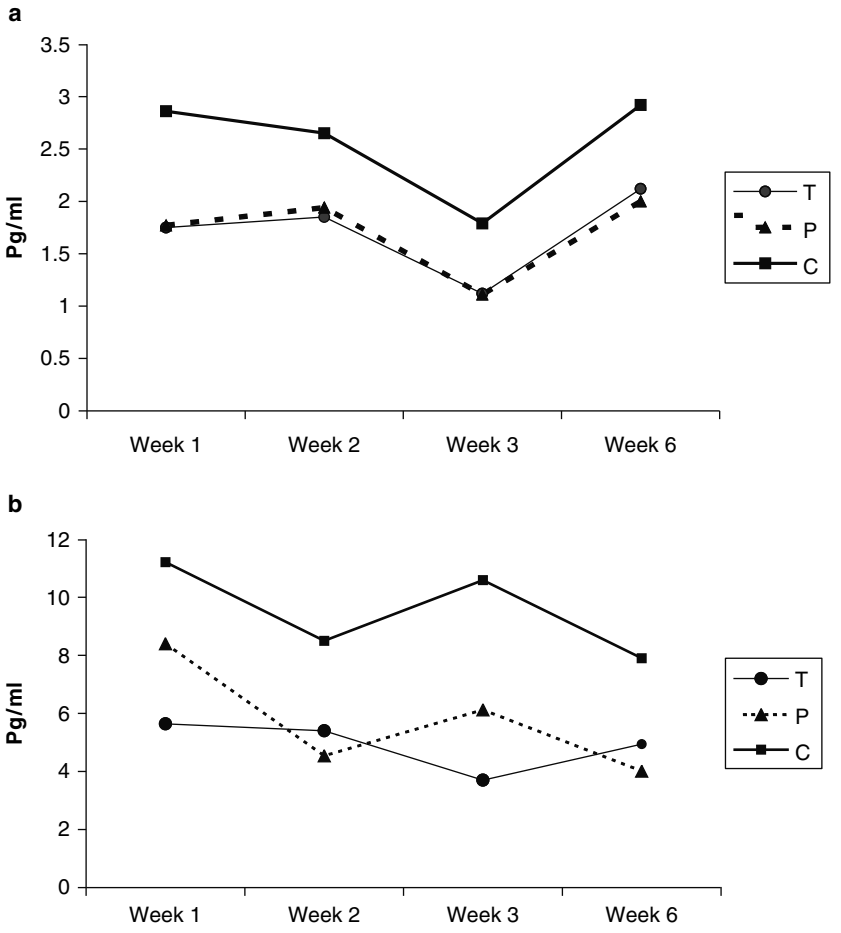

Fig. 6 Effects of LVA on serum levels of LTB4 (**a**) and PGE2 (**b**), 1 and 6 weeks after induction of colitis. *C* colitis-induced rats; *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

experimental model of glomerulonephritis was induced in rats by a subcutaneous immunization and daily intravenous administration of BSA. Sodium alginate at two different doses (25 and 50 mg kg^{-1}) was administered intraperitoneally to the treatment groups (T1 and T2) at regular 72-h intervals for 6 weeks. The onset of treatment was on day 42. Urinary protein was measured weekly and serum anti-BSA antibody was assessed by ELISA at different intervals. Rats were euthanized at the 12th experimental week and blood samples and kidney specimens were obtained. Blood urea nitrogen (BUN), serum creatinine, and serum cholesterol and triglyceride were measured at the time of death. Kidney specimens were processed for light and immunofluorescence microscopy examination.

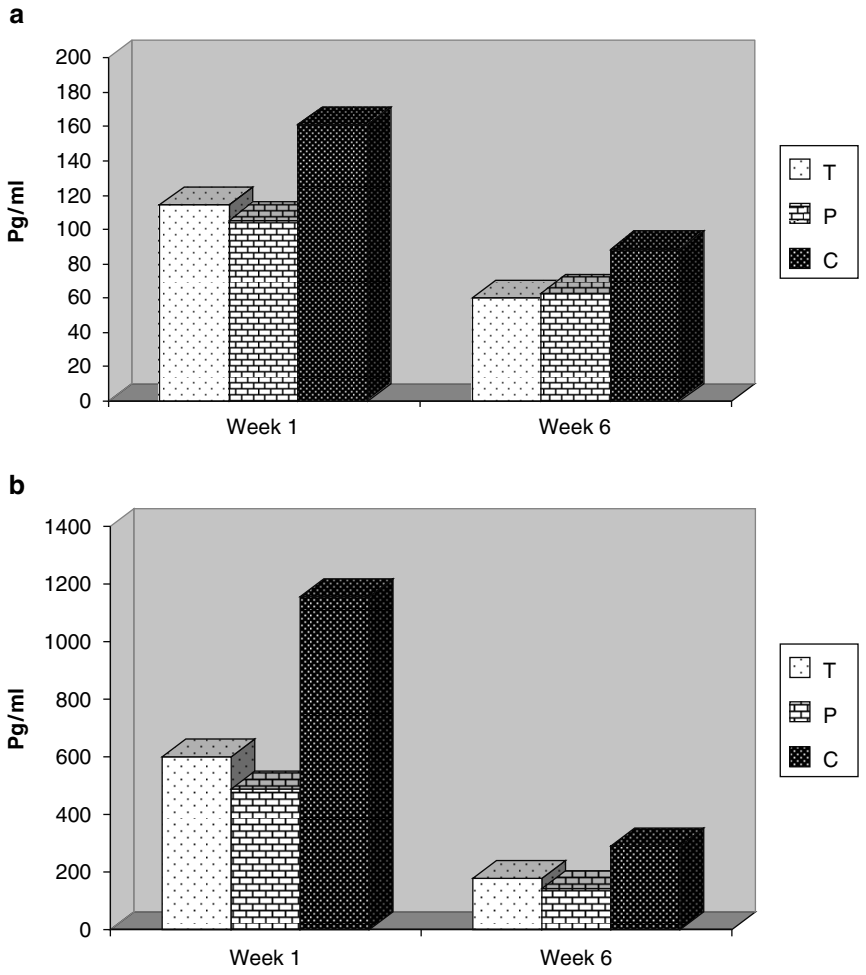

Fig. 7 Amounts of IL-6 (**a**) and TNF-α (**b**) in colonic tissue homogenate following 6 weeks of oral administration of LVA. *C* colitis-induced rats, *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

The results of this experiment showed that treatment with sodium alginate could significantly reduce the urinary protein excretion and serum creatinine in treated rats compared with nontreated controls.

Figure 9 shows the changes of the mean levels of urinary protein excretion between normal, patient, and treatment (T1 and T2) groups. The urinary protein excretion was significantly less at the end of the experiment (day 85) in LVA-treated rats (group T2, 50 mg kg^{-1}) than in the nontreated controls. Comparison of BUN and serum creatinine concentrations among various groups showed only significant reduction ($P < 0.05$) in the level of serum creatinine in LVA-treated rats (T2 group) compared with

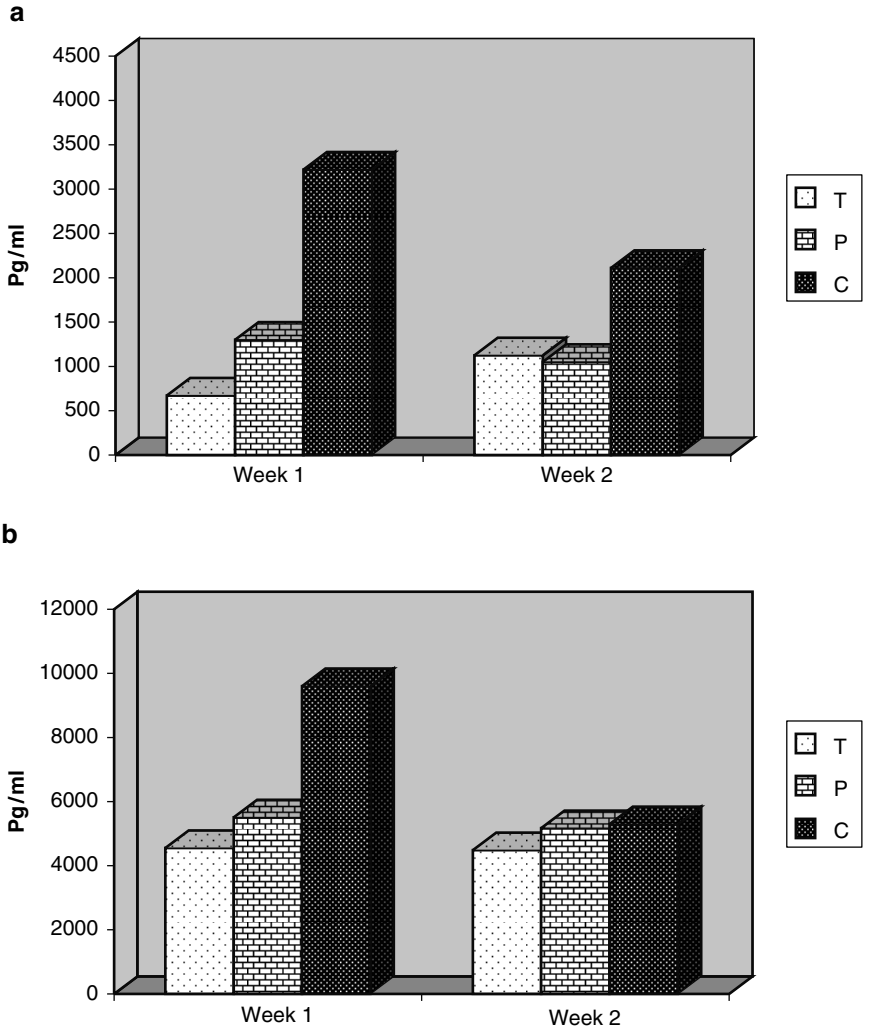

Fig. 8 Amounts of LTB4 (**a**) and PGE2 (**b**) in colonic tissue homogenate following 6 weeks of oral administration of LVA. *C* colitis-induced rats, *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

nontreated controls. Moreover, the anti-BSA antibody titers were significantly lower in LVA-treated rats (group T2, 50 mg kg^{-1}) than in nontreated controls at the 12th week after immunization. The changes of the mean levels of anti-BSA antibody titers in the third, sixth, ninth, and 12th experimental weeks are illustrated in Fig. 10.

There was no significant difference in the level of BUN and serum lipids (triglyceride and cholesterol) between different groups. The light microscopy examination of renal tissue revealed the severity of hypercellularity, glomerular infiltration of polymorphonuclear leukocytes (PMN), fibrinoid necrosis, and inter-

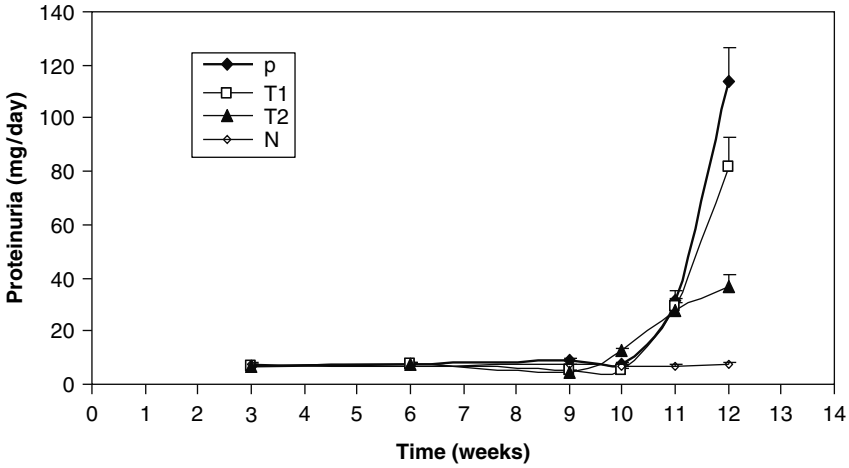

Fig. 9 Time course of the mean values of proteinuria in different groups in immune complex glomerulonephritis: *N* normal rats ($n = 10$), *P* patient rats ($n = 9$), *T1*, *T2* groups treated by intraperitoneal injections of LVA at two different doses, 25 and 50 mg kg^{-1}, respectively ($n = 2 \times 9$). The onset of intraperitoneal administration of LVA to T1 and T2 groups was on day 42. There were 14 intraperitoneal injections, the injection interval was 72 h, and the end of the therapeutic protocol was day 81. There was a significant difference between the patient group and the treated group (T2). $P < 0.05$ was considered significant (Mirshafiey et al. 2005b)

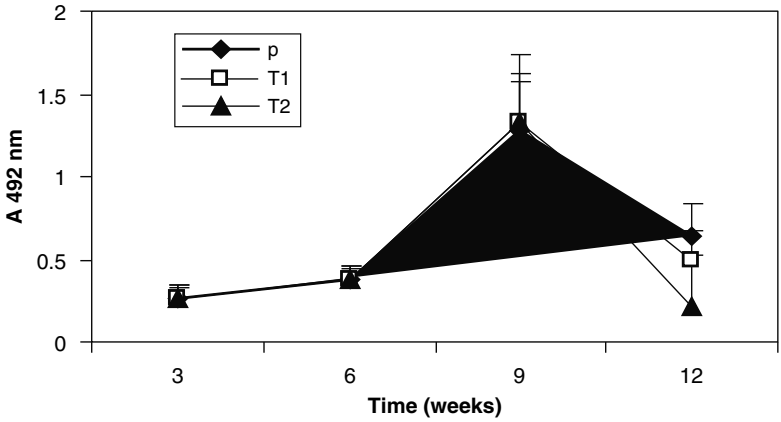

Fig. 10 Comparison of anti-bovine serum albumin (BSA) antibody titers in groups P, T1, and T2 during the course of acute serum sickness. Patient rats were divided into four subgroups ($n = 5 + 5 + 5 + 9$) and treated rats into T1 ($n = 7 + 9$) and T2 ($n = 7 + 9$) subgroups. Rats from patient subgroups were killed in four stages (third, sixth, ninth, and 12th weeks), whereas treated subgroups were killed in two stages (ninth and 12th weeks) after immunization to obtain their sera. Treated groups (T1 and T2) received intraperitoneal injections of LVA at two different doses, 25 and 50 mg kg^{-1}, respectively. The onset of intraperitoneal administration of LVA to T1 and T2 groups was on day 42. As determined by the ELISA method, the highest titers occurred in the P group, the intermediate titers were in the T1 group, and the lowest titers were in the T2 group. At the 12th week, the reduction of anti-BSA antibody in the T2 group was significant compared with that in the P group. Values are means ± S.D (Mirshafiey et al. 2005b)

stitial infiltration in various groups. Rats treated with LVA (group T2) showed a significant reduction in glomerular changes compared with nontreated controls. Immunofluorescence microscopy investigation of glomeruli revealed deposits of immune complexes in the mesangial areas and along the capillary walls of all the rats. Glomerular deposition of immune complex was significantly less intense in LVA-treated rats (group T2) than in nontreated controls. These findings suggest that treatment with sodium alginate as a new immunosuppressive agent can reduce proteinuria, and suppress the antibody production as well as the development of glomerular lesions in a rat model of immune complex glomerulonephritis.

5 Therapeutic Effects of M2000 on an Experimental Model of Rheumatoid Arthritis

This investigation was planned to explore the therapeutic potency of M2000, a novel designed nonsteroidal anti-inflammatory drug in an adjuvant-induced arthritis model (Mirshafiey et al. 2005). For this purpose, a production process for the commercially unavailable M2000 had to be established. An alginate epimerase negative *Pseudomonas* strain was engineered which synthesized homopolymeric polymannuronate. The polymannuronate was subjected to acid hydrolysis to obtain the monomer, which was further purified by gel filtration chromatography. On the basis of the method of (Cuzzocrea et al. 2005), arthritis was induced in Lewis rats by a single intradermal injection (0.1 ml) of heat-killed *Mycobacterium tuberculosis* (0.3 mg) in Freund's incomplete adjuvant into the right foot pad. Fourteen days after injection of adjuvant, the contralateral left foot pad volume was measured. The rats with paw volumes 0.37 ml greater than those of normal paws were then randomized into treatment groups. Intraperitoneal administration of test drugs (M2000, 40 mg kg^{-1} per day to group T1 and indomethacin, 2 mg kg^{-1} per day to group T2) and oral administration of M2000 (40 mg kg^{-1} per day to group T3) were started on day 15 after adjuvant injection and continued until the final assessment on day 25. The left hind limb was removed for histological evaluation. The results showed that the oral administration as well as the intraperitoneal injection of M2000 into arthritic rats induced a significant reduction in paw edema. Histopathological assessment showed a reduced inflammatory cell infiltrate in joints of treated rats, and the number of osteoclasts present in the subchondral bone, tissue edema, and bone erosion in the paws were markedly reduced following M2000 therapy.

The data in Fig. 11 demonstrate a time-dependent increase induced by adjuvant in the left hind paw volume (milliliters) of rats. The intraperitoneal injection of M2000 into arthritic rats could rapidly reverse paw edema as did indomethacin, and after 10 days of treatment paw swelling was significantly ($P < 0.05$) reduced in M2000-treated rats compared with vehicle-treated controls. On the other hand, as is apparent in Fig. 12, oral administration of M2000 (40 mg kg^{-1}) in arthritic rats induced a significant reduction in paw edema. After 10 days of treatment, paw swelling was reduced in M2000-treated rats relative to the paw volume of vehicle-treated rats. This difference was statistically significant. There was no macroscopic evidence of hind paw erythema and/or edema in the normal control rats.

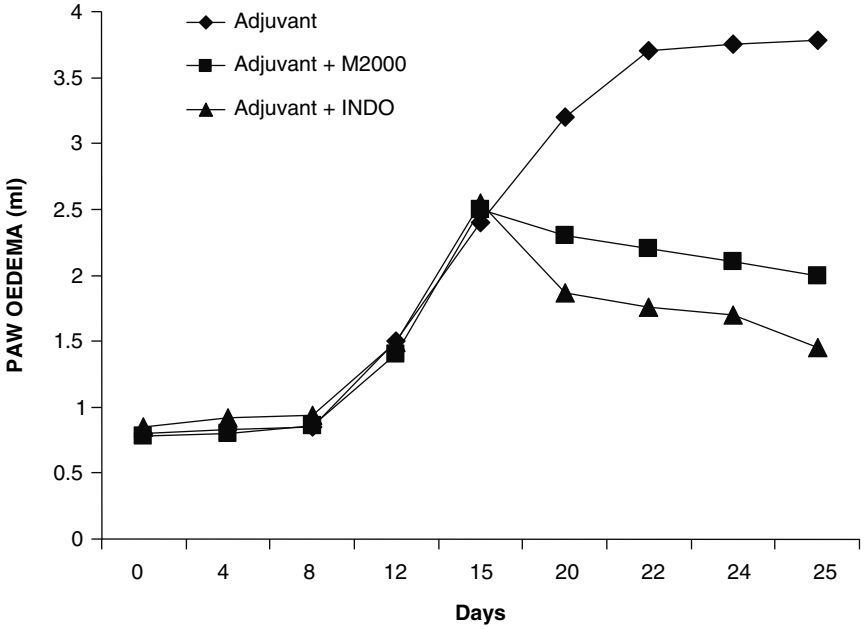

Fig. 11 Severity of arthritis score in treated groups (A + M) compared with nontreated control rats (**a**). A time-dependent increase induced by adjuvant in the left hind paw volume (milliliters) of rats is demonstrated. The intraperitoneal injection of β-D-mannuronic acid (M2000) to arthritic rats could rapidly reverse paw edema as did indomethacin, and after 10 days of treatment paw swelling was significantly ($P < 0.05$) reduced in M2000-treated animals compared with vehicle-treated controls (Mirshafiey et al. 2005c)

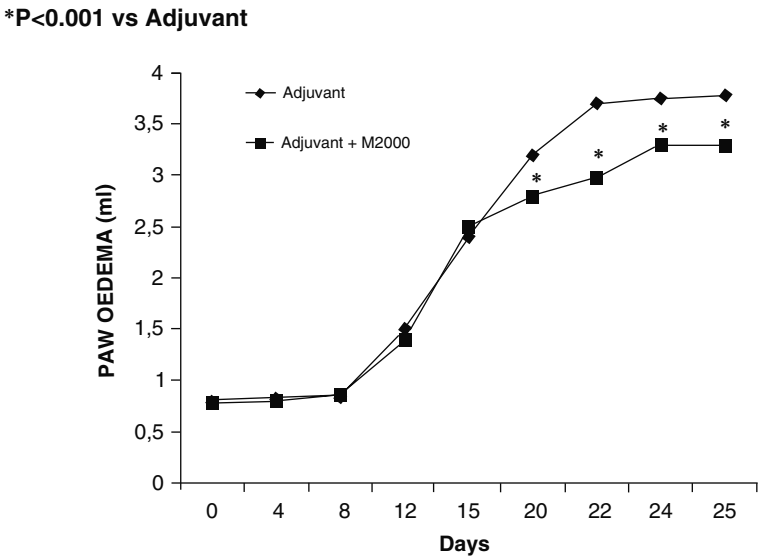

Fig. 12 The oral administration of M2000 (40 mg kg^{-1}) in arthritic rats induced a significant ($P < 0.05$) reduction in paw edema. After 10 days of treatment paw swelling was reduced in M2000-treated animals relative to the paw volume of vehicle-treated rats. This difference was statistically significant, $P < 0.05$ (Mirshafiey et al. 2005c)

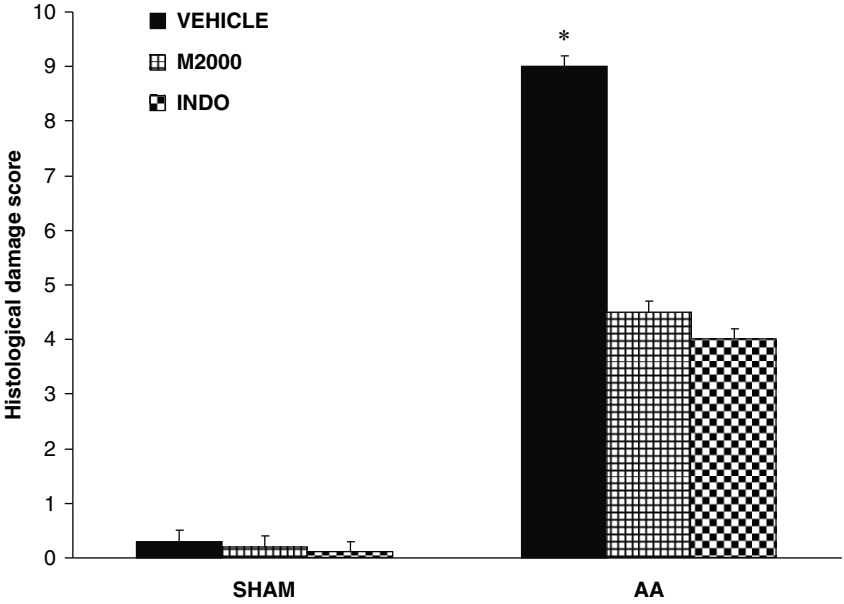

Fig. 13 Effect of M2000 treatment on histological damage score. The histological evaluation of the paws in the vehicle-treated arthritic rats reveals signs of severe arthritis along with inflammatory cell infiltrate. M2000 therapy could significantly reduce the pathological parameter compared with that of the control group. $P < 0.05$ was considered significant (Mirshafiey et al. 2005c)

As shown in Figs. 13 and 14, histological evaluation of the paws in the vehicle-treated arthritic rats reveals signs of severe arthritis along with inflammatory cell infiltrate. Histopathological assessment showed a reduced inflammatory cell infiltrate in the joints of treated rats, and the number of osteoclasts present in the subchondral bone, tissue edema, and bone erosion in the paws were markedly reduced by both treatments, indicating that the drugs tested were effective in retarding synovial inflammation and prevented joint destruction. Treatment with M2000 and indomethacin resulted in preservation of hyaline in the articular cartilage. Moreover, subchondral bone was intact and the numbers of osteoclasts in the subchondral and trabecular bone space were greatly reduced.

Taken together; the data obtained show that M2000, as a novel nonsteroidal anti-inflammatory drug, could be suggested as an anti-inflammatory drug for long-term administration.

6 Immunosuppressive Effect of M2000 in Experimental Multiple Sclerosis

The therapeutic potency of M2000 as a novel designed nonsteroidal anti-inflammatory drug with immunosuppressive property in a T-cell-mediated autoimmune disease was tested (Mirshafiey et al. 2005). The influence of M2000 on experimental autoimmune

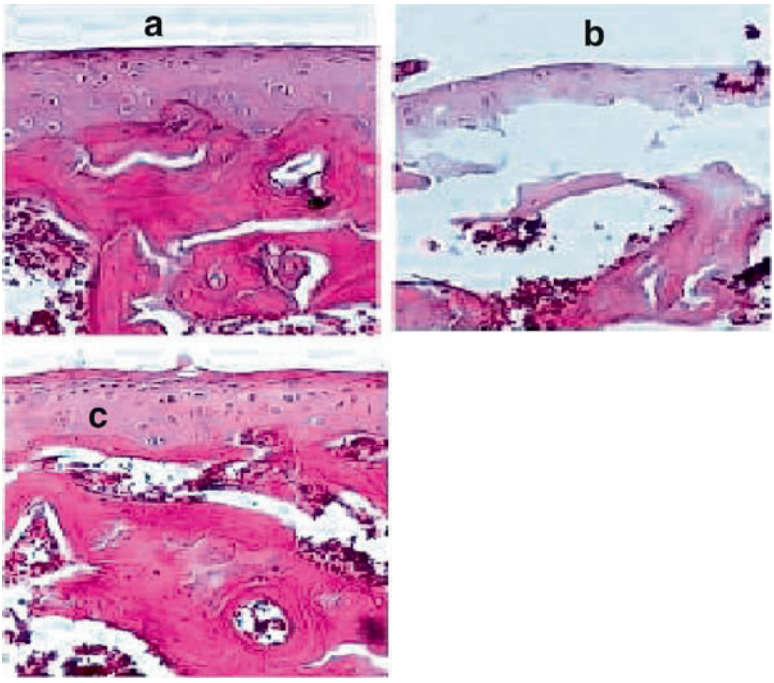

Fig. 14 Representative histopathological slides of a hind limb joint of a healthy Lewis rat (**a**), a rat with adjuvant-induced arthritis (**b**), and an arthritic rat treated with M2000 (**c**). The joint of the rat which had been treated with M2000 (**c**) shows significantly fewer signs of joint destruction (Mirshafiey et al. 2005c)

encephalomyelitis (EAE), an animal model of multiple sclerosis, induced by myelin basic protein (MBP) was assessed. M2000 at two doses, 40 and 80 mg kg^{-1} per day, was administered intraperitoneally to prevention and treatment groups, respectively. The onset of intraperitoneal injections of M2000 for prophylactic and therapeutic groups was on the first and seventh days after immunization. Rats were divided at random into a prevention group with two subgroups: M1 – M2000-pretreated rats, which received in total 18 intraperitoneal injections from 1 day before immunization (day 1) to day 16 after immunization; C1 – control patient rats, which received intraperitoneal injections of saline during the same period (from day 1 to day 16). The treatment group included two subgroups: M2 – M2000-treated rats, which received in total 14 intraperitoneal injections from day 7 to day 20 after immunization; C2 – control patient rats, which received intraperitoneal injections of saline during the same period. Rats were killed on day 21 after immunization. The results of this experiment showed that the treatment of EAE with M2000 could significantly suppress disease development both prophylactically and therapeutically; the onset and symptoms of EAE in Lewis rats could be suppressed following the administration of M2000. Clinical improvement was accompanied by a marked decrease in the mean numbers of vessels with perivascular cellular

infiltration in M2000-treated rats compared with nontreated control. Disease suppression was associated with a marked suppression of MBP-specific T-cell reactivity in vitro, without any evidence for a generalized impairment of T-cell activity.

Following the chronological changes of the clinical score of each group of the EAE rats (Figs. 15, 16), the first clinical signs appeared in some rats of each group on day 10 and then the symptoms reached their maximum level on day 13 after immunization. The maximum severity score of EAE was 5 in each group. The rats pretreated and treated with M2000 were less affected than the controls (Figs. 15 and 16). Three rats from the M1 subgroup did not develop apparent signs of EAE during the period of observation, whereas all of the rats from the C1 and C2 subgroups developed typical signs (clinical score more than 4) of EAE. As shown in Figs. 15 and 16, the mean clinical scores of rats for subgroups M1 and M2 were significantly less than those of control rats (the two-way repeated measures analysis of variance, $P < 0.05$). The mortality rate in the M1 subgroup (40 mg kg^{-1} per day) was 0/7 compared with 0/8 for the C1 subgroup, whereas, interestingly, the mortality rate in the M2 subgroup (80 mg kg^{-1} per day) was 0/6 compared with 2/8 in the C2 subgroup. The mean body weight more reduced in controls than in subgroup M1 rats after day 12 (Fig. 17). Moreover, lymph node cells from the rats treated with M2000 exhibited a reduced proliferative response to MBP in contrast to those of control patient rats, whereas the proliferative responses of lymph node cells to Con A were almost the same in both groups (Fig. 18). On the other hand, the histopathological assessment of brain and spinal cord sections in treated and nontreated groups showed that the mean numbers of vessels with perivascular cellular infiltration in brain (0.57 ± 0.79) and spinal cord (2.0 ± 2.65) in M2000-treated rats were

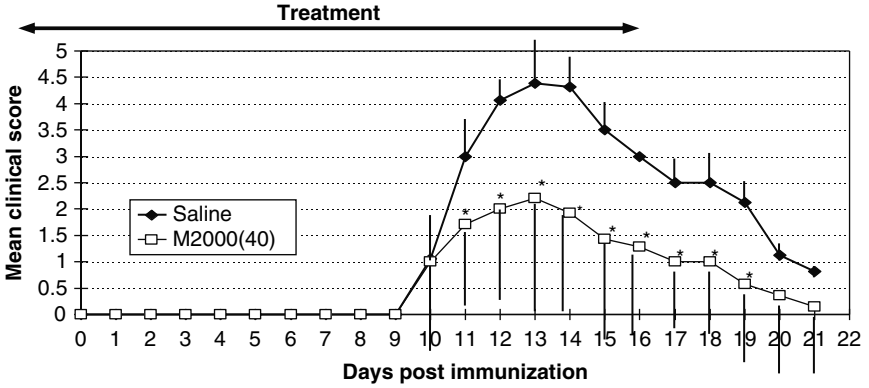

Fig. 15 Amelioration of myelin basic protein (MBP) induced experimental autoimmune encephalomyelitis (EAE) by preventive application of M2000. Fifteen Lewis rats were immunized with 50 μg MBP in complete Freund's adjuvant into one hind footpad and were then divided into two subgroups. M2000-pretreated rats ($n = 7$) received in total 18 intraperitoneal injections of M2000 (40 mg kg^{-1} per day) from 1 day before immunization to day 16 after immunization. Control rats ($n = 8$) received saline intraperitoneal injections during the same period. M2000 therapy caused a significant reduction in clinical score compared with that for nontreated controls ($P < 0.05$). *Bars* indicate ± SD (Mirshafiey et al. 2005d)

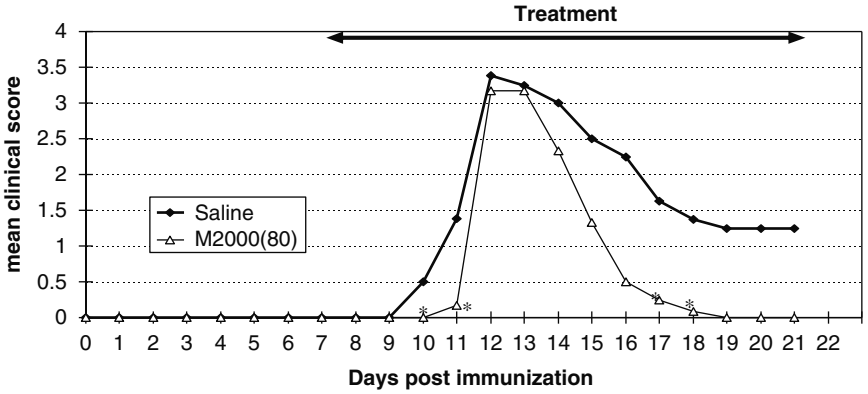

Fig. 16 Amelioration of MBP-induced EAE by therapeutic application of M2000. Lewis rats were immunized with 50 μg MBP in CFA into one hind footpad and were then divided into two subgroups. M2000-treated rats ($n = 6$) received in total 14 intraperitoneal injections of M2000 (80 mg kg^{-1} per day) from day 7 to day 20 after immunization. Control rats ($n = 8$) received intraperitoneal injections of saline during the same period. M2000 therapy caused a significant reduction in clinical score compared with that for nontreated control ($P < 0.05$) (Mirshafiey et al. 2005d)

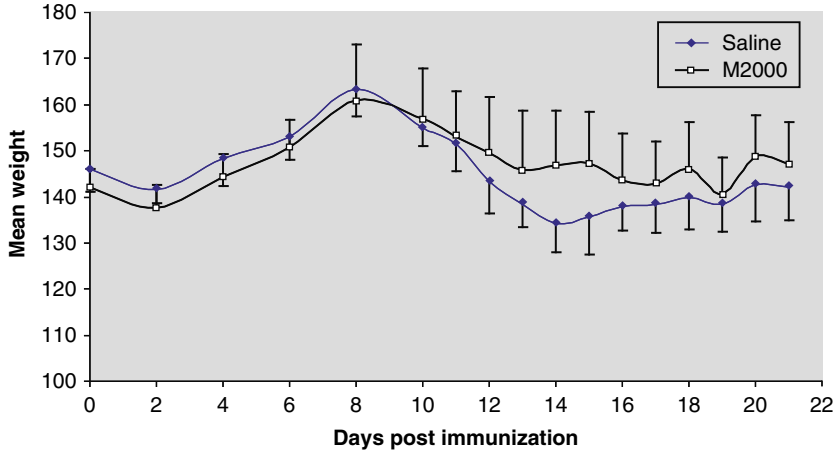

Fig. 17 The higher weight gain in M2000-treated rats compared with nontreated controls during the EAE period. M2000-treated rats ($n = 7$) received in total 18 intraperitoneal injections of M2000 (40 mg kg^{-1} per day) from 1 day before immunization to day 16 after immunization. Control rats ($n = 8$) received intraperitoneal injections of saline during the same period. *Bars* indicate SD (Mirshafiey et al. 2005d)

significantly less than in nontreated controls, with mean values of 2.43 ± 3.41 for brain and 10.71 ± 8.76 for spinal cord, respectively.

Collectively, our data suggest that M2000 may provide a novel therapeutic option for T-cell-mediated autoimmune diseases in humans.

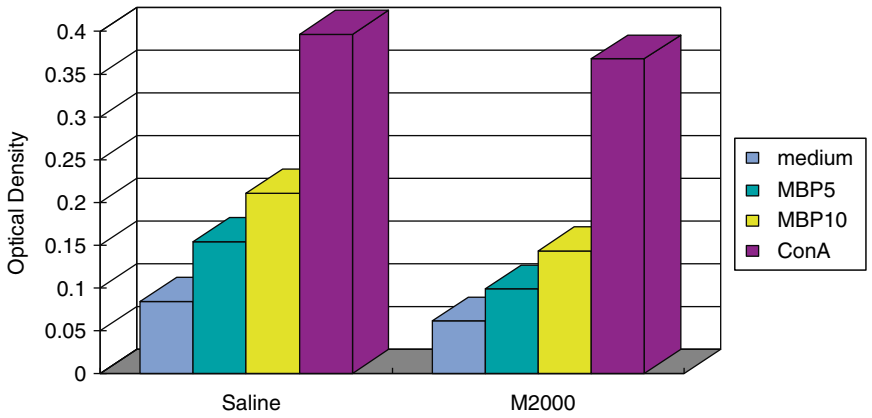

Fig. 18 The effect of M2000 at two different doses (5 and 10 μg ml^{-1}) on proliferative responses of regional lymph node cells from EAE rats, "$n = 4$, for each group, on day 21 after immunization" (Mirshafiey et al. 2005d)

7 Treatment of Experimental Nephrotic Syndrome with M2000

The therapeutic effect of the M2000 molecule was tested in adriamycin-induced nephropathy (Mirshafiey et al. 2004a). To induce the experimental nephrotic syndrome, adriamycin was given once by a single intravenous injection (7.5 mg kg^{-1}) through the tail vein. Six days after injection of adriamycin, a therapeutic protocol was developed by intraperitoneal administration of 30 mg kg^{-1} M2000 solution to the treatment 1 (T1) group and intraperitoneal injection of piroxicam (0.3 mg kg^{-1}) for the treatment 2 (T2) group. In total there were 14 intraperitoneal injections, of which five injections were performed day after day and nine injections were carried out at regular 48-h intervals. The therapeutic protocol was terminated on day 28 and the rats were killed on day 43. The treated patient rats showed a significant reduction in proteinuria, BUN, serum creatinine, and serum cholesterol, and administration of M2000 could significantly diminish the serum level of IL-6 in treated rats compared with nontreated controls. Moreover, treatment with M2000 significantly reduced the number of glomerular leukocytes, hypercellularity, and hydropic change in the capillary network within the renal cortex and decreased tubular casts.

The changes of the mean levels of urinary protein excretion between normal, patient, T1, and T2 groups are shown in Fig. 19. This experiment showed that intraperitoneal administration of M2000 (30 mg kg^{-1}) could exert its therapeutic effects on adriamycin-induced nephropathy. For exact evaluation of the effects of M2000, the experiment was terminated 14 days after the last injection of M2000. The urinary protein excretion was significantly less in M2000-treated rats than in nontreated controls and piroxicam-treated rats.

Figure 20 shows the antiproteinuric effect of M2000 therapy compared with that of piroxicam at the end of experiment (day 42). Here there was significant difference

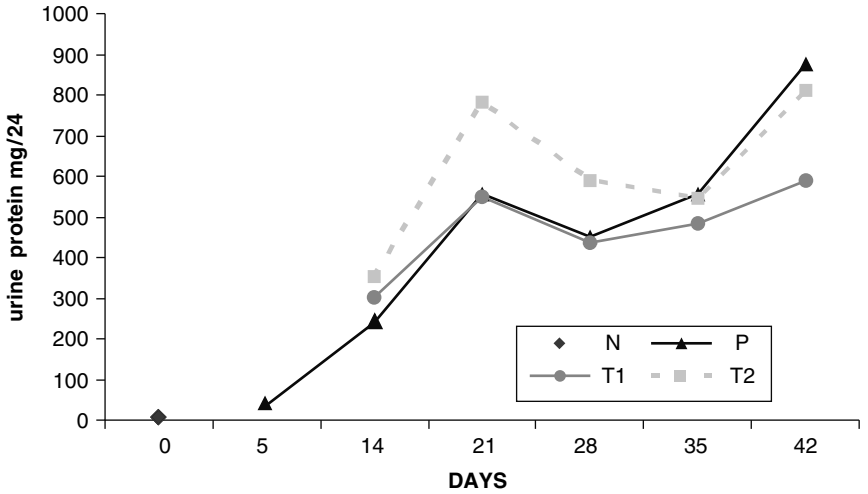

Fig. 19 Time course of the mean values of proteinuria in different groups in experimental nephrosis: *N* normal rats ($n = 8$), *P* patient (nontreated) rats that show an increase of proteinuria levels after day 5 ($n = 9$), *T1*, *T2* groups treated by intraperitoneal injections of M2000 (30 mg kg^{-1}) and piroxicam (0.3 mg kg^{-1}), respectively ($n = 2 \times 8$). Note: a single intravenous injection of adriamycin (7.5 mg kg^{-1} body weight) induced a severe nephrotic syndrome. The onset of intraperitoneal administration of M2000 and piroxicam to groups T1 and T2 was on day 6 (after development of disease).There were significant differences between nontreated (P) and M2000-treated rats (T1). $P < 0.05$ was considered significant (Mirshafiey et al. 2004a)

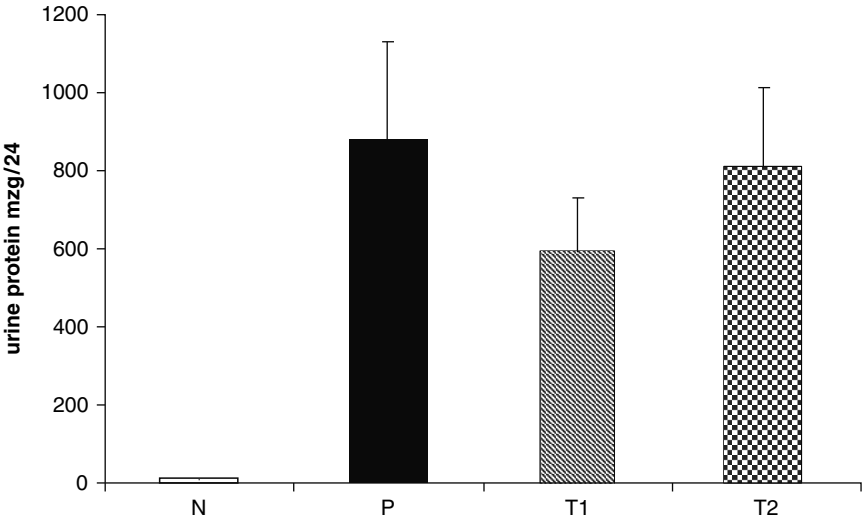

Fig. 20 Effect of M2000 on proteinuria at the end of the experiment (day 42) in different groups: *N* normal rats (8), *P* patients rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. The comparison of the antiproteinuric effect of M2000 between groups P and T1 showed a significant difference, "$P < 0.05$ (Mirshafiey et al. 2004a)

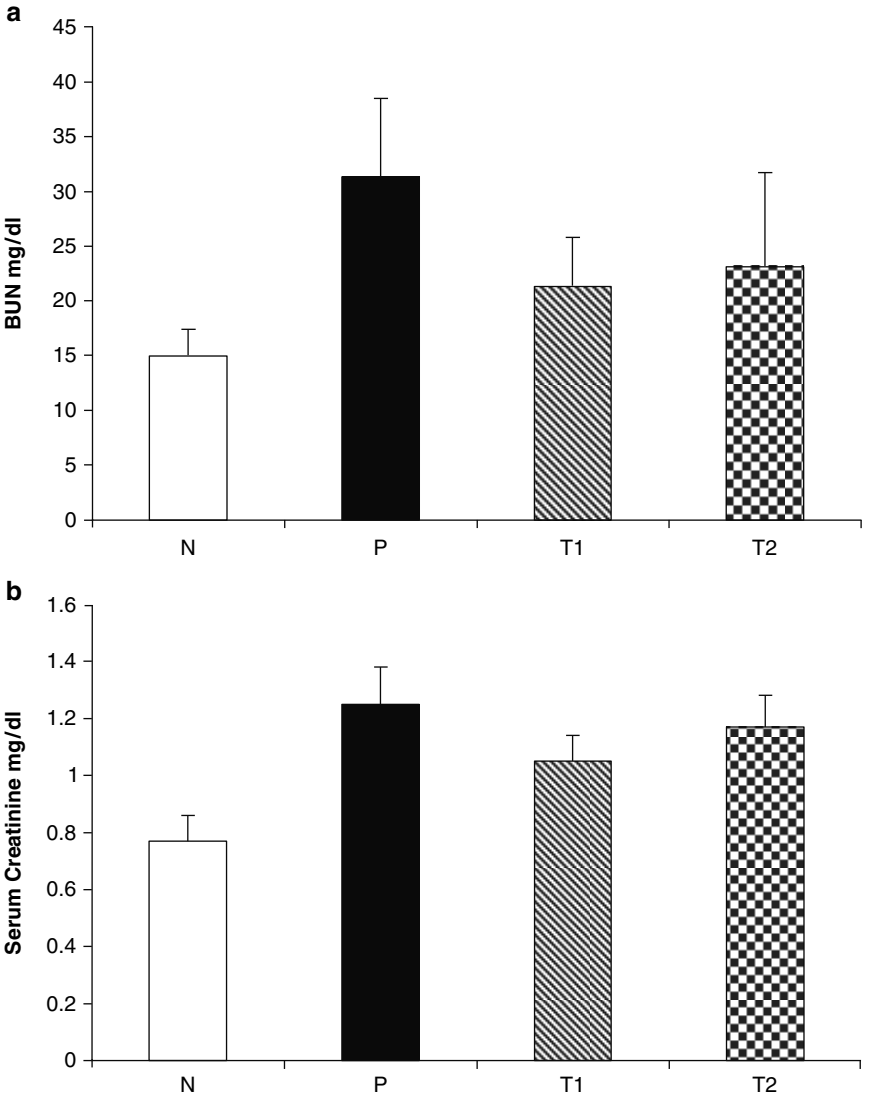

Fig. 21 Effect of M2000 on serum blood urea nitrogen and serum creatinine in different groups: *N* normal rats (8), *P* patient rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. **a** BUN concentration in groups N, P, T1, and T2. BUN concentration in group T1 vs. group P was significant, $P < 0.05$. **b** Creatinine concentration in groups N, P, T1, and T2. Creatinine concentration in group T1 vs. group P was significant, $P < 0.05$ (Mirshafiey et al. 2004a)

between T1 and patient groups. In Fig. 21a, the amounts of BUN are compared between the different groups (normal, patient, T1, and T2). This figure shows that the difference between treated rats (T1 group) and nontreated rats (patient group)

is significant. In Fig. 21b, the comparison of serum creatinine concentration among various groups shows that there is a significant difference between T1 and patient groups, whereas there were no significant differences in the levels of urinary urea nitrogen and urine creatinine concentration between M2000-treated rats and nontreated controls (data not shown).

In addition, serum cholesterol and triglyceride levels were significantly elevated in nephrotic rats when compared with the healthy controls at the end of the experiment. Intraperitoneal injections of M2000 solution into patient rats significantly reduced serum cholesterol levels in nephrotic rats (Fig. 22).

Figure 23 shows the effect of M2000 therapy on the reduction of IL-6 production in treated patient rats (group T1). The difference between T1 and patient groups in terms of IL-6 concentration was significant.

Light microscopy examination of renal tissue revealed the severity of hypercellularity, glomerular infiltration of PMN, hydropic change in the capillary network within the renal cortex, and the existence of tubular casts in the various groups. The rats treated with M2000 showed a significant reduction in glomerular changes compared with nontreated controls (Table 1).

These data suggest that M2000 therapy can ameliorate proteinuria and suppress the progression of glomerular lesions in an experimental model of nephrosis.

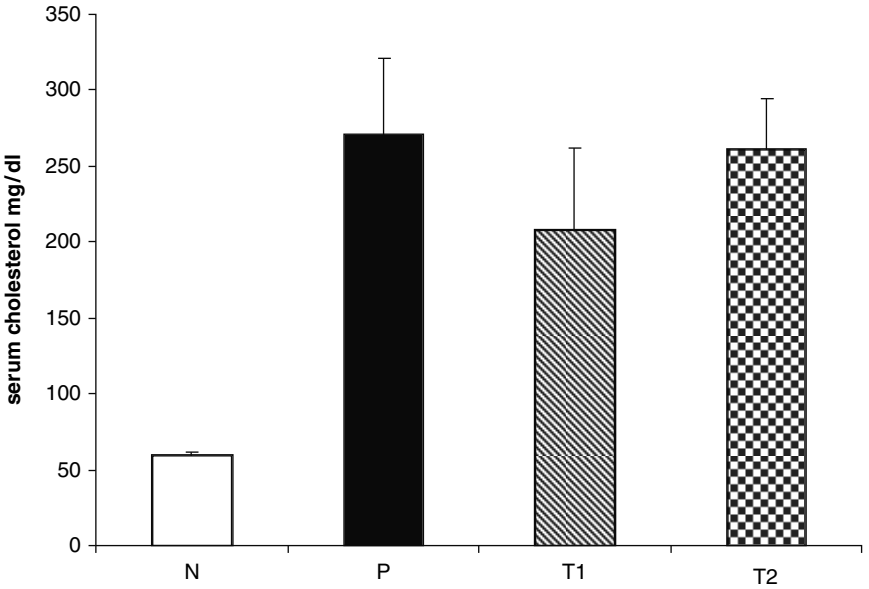

Fig. 22 Effect of M2000 on serum cholesterol level in different groups: *N* normal rats (8), *P* patient rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. Cholesterol concentration in group T1 vs. group P was significant, $P < 0.05$ (Mirshafiey et al. 2004a)

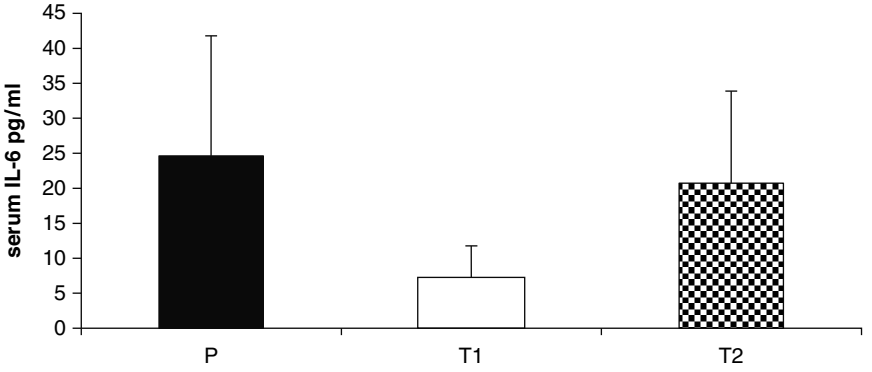

Fig. 23 Effect of M2000 on serum IL-6 level in different groups: *N* normal rats (8), *P* patient rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. The Difference between group T1 and group P in terms of IL-6 concentration was significant, $P < 0.05$, (Mirshafiey et al. 2004a)

t.1 **Table 1** Light microscopy findings of kidney histological lesions in various groups

t.2 t.3 Group	No. of rats	Hypercellularity	Polymorphonuclear leukocyte infiltration	Cast	Hydropic change
t.4 N	8	0.40 ± 0.53	–	–	–
t.5 P	9	1 ± 0.57	1.71 ± 0.48	2.14 ± 0.69	1 ± 0
t.6 T1	8	0.57 ± 0.53	1	1.14 ± 0.63	–
t.7 T2	8	0.71 ± 0.46	1.6 ± 0.76	1.87 ± 1.1	–
t.8 H	8	0.42 ± 0.53	–	–	–

t.9 Values are expressed as the mean ± the standard deviation. Semiquantitative scoring of histological
t.10 lesions shows the significant differences between groups P and T1, in hypercellularity, in glomerular
t.11 polymorphonuclear leukocyte infiltration, hydropic change in the capillary network within the renal
t.12 cortex, and in tubular casts, ($P < 0.05$). Group H was the healthy control receiving β-D-mannuronic
t.13 acid (M2000; 30 mg kg^{-1})

8 Therapeutic Effects of M2000 in Experimental Immune Complex Glomerulonephritis

The therapeutic efficacy of the novel anti-inflammatory agent M2000 in an experimental model of immune complex glomerulonephritis was evaluated (Mirshafiey et al. 2007). BSA nephritis as an experimental model of glomerulonephritis was induced in rats by a subcutaneous immunization and daily intravenous administration of BSA. Rats were divided randomly into five groups. normal; patient; patient groups treated with M2000 and piroxicam (T1 and T2, respectively); a healthy control receiving M2000. The M2000 solution (30 mg kg^{-1}) was administered intraperitoneally at regular 48-h intervals for 4 weeks. The onset of treatment was on day 56. Urinary protein was measured weekly and serum anti-BSA antibody was assessed by ELISA at different intervals. The rats were killed on day 84 and blood samples and

kidney specimens were obtained. Serum (creatinine, BUN, cholesterol, and triglyceride) and urine (protein, urea, and creatinine) determinants were measured at the time of death. Kidney specimens were processed for light and immunofluorescence microscopy examination. The results showed that M2000 therapy could significantly reduce the urinary protein excretion in treated rats compared with nontreated controls. The anti-BSA antibody titer was lower in treated rats than in controls at the 12th experimental week. There were no significant differences in the levels of serum determinants, urine urea, and creatinine between control and treated rats. PMN infiltration and glomerular immune complex deposition were less intense in treated rats than in controls. Figure 24 shows the changes of the mean levels of urinary protein excretion between various groups; normal, patient, T1, and T2. The urinary protein excretion was significantly less in M2000-treated rats than in nontreated controls.

Figure 25 shows the antiproteinuric effect of M2000 therapy compared with patient and piroxicam (T2) groups at the end of the experiment (day 84). Here, the reduction of proteinuria in T1 compared with patient and T2 groups was significant.

Figure 26 illustrates the changes of the mean levels of anti-BSA antibody titers in the sixth, ninth, and 12th experimental weeks. The anti-BSA antibody titers were significantly lower in M2000-treated rats (T1 group) than in piroxicam-treated rats (T2 group) and nontreated controls (patient group) at the end of the experiment (day 84).

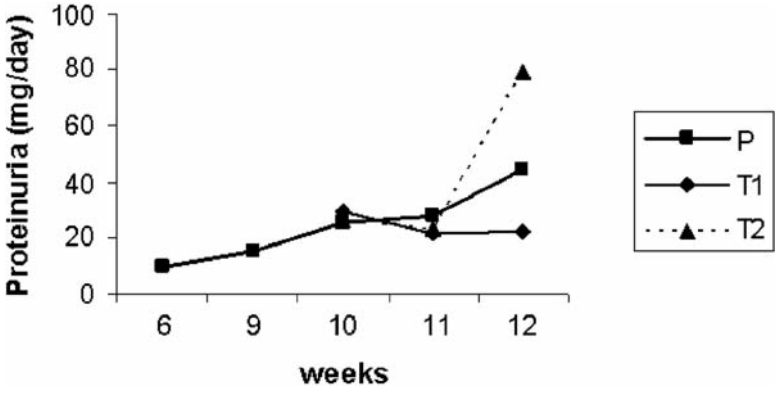

Fig. 24 Time course of the mean values of proteinuria in different groups in immune complex glomerulonephritis: *N* normal rats ($n = 8$), *P* patient rats ($n = 9$), *T1*, *T2* rats treated with intraperitoneal injections of M2000 (30 mg kg^{-1}) and piroxicam (0.3 mg kg^{-1}), respectively ($n = 2 \times 8$). The onset of intraperitoneal administration of M2000 and piroxicam to T1 and T2 groups was on day 56. There were 15 intraperitoneal injections, the injection interval was 48 h, and the end of the therapeutic protocol was day 84. There was a significant difference between the M2000-treated group (T1) and the patient group. $P < 0.05$ was considered significant (Mirshafiey et al. 2007)

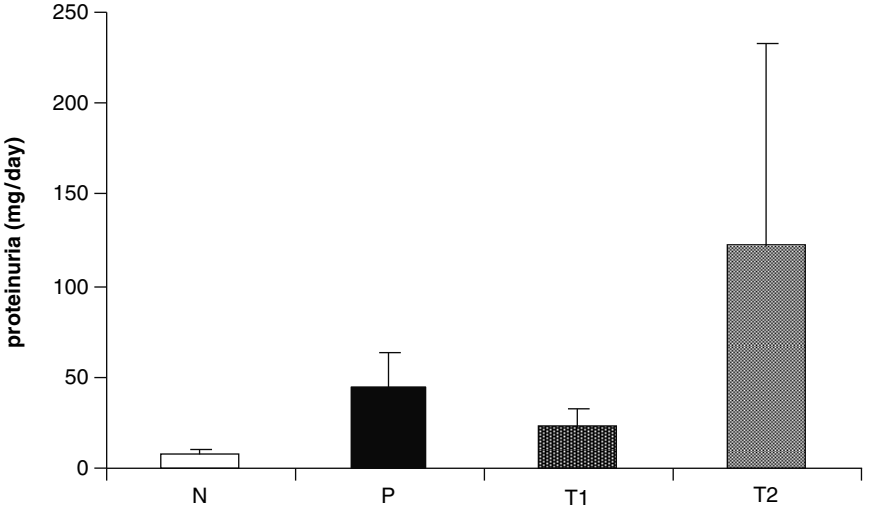

Fig. 25 Comparison of the antiproteinuric effect of M2000 between different groups at the end of experiment (week 12). *N* normal rats (8), *P* patient rats (9), *T1*, *T2* rats treated with M2000 (30 mg kg^{-1}) and piroxicam (0.3 mg kg^{-1}), respectively (2 × 8 = 16). Each *bar* represents the mean ± S.D. The antiproteinuric effect for group T1 vs. group P was significant, $P < 0.05$ (Mirshafiey et al. 2007)

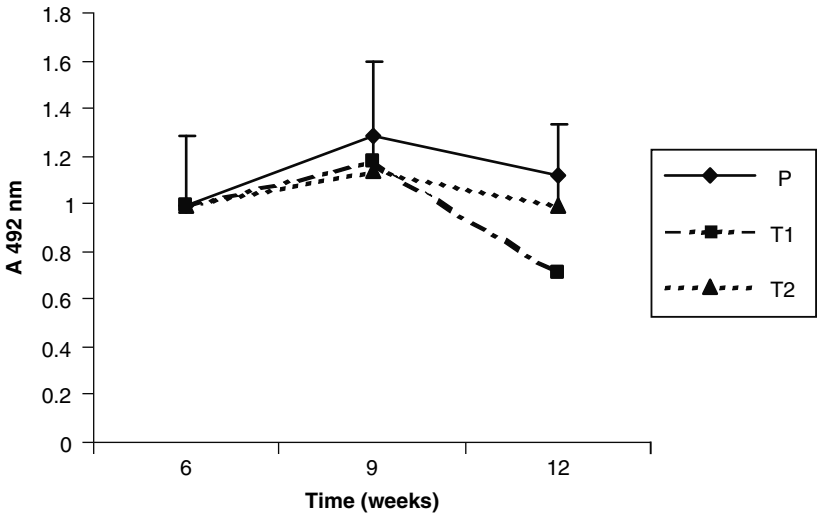

Fig. 26 Anti-BSA antibody titers in groups P, T1, and T2 during the course of acute BSA nephritis, as determined by an ELISA method. The highest titer occurs in group P, the intermediate in group T2, and the lowest in group T1. Values are means ± S.D. The difference between the M2000-treated group (T1) and the patient group (P) at the 12th week was significant. $P < 0.05$ was considered significant (Mirshafiey et al. 2007)

Light microscopy examination of renal tissue revealed the severity of glomerular infiltration of PMN in nontreated rats (patient group) and in rats treated with piroxicam (T2 group) compared with M2000-treated rats (T1 group) (data not shown). Immunofluorescence microscopy investigation of glomeruli revealed that glomerular immune complex deposition was less intense in rats treated with M2000 (T1 group) than in controls (patient group) and in rats treated with piroxicam (data not shown).

These findings suggested that treatment with M2000 ($C_6H_{10}O_7$) can reduce proteinuria, diminish antibody production, and suppress the progression of disease in a rat model of immune complex glomerulonephritis.

9 Tolerability and Pharmacotoxicology of M2000

The pharmacotoxicology study of M2000 was carried out on animal models based on the evaluation of serum and urine determinants, structure of kidney, gastrointestinal tolerability, and body temperature (Mirshafiey et al. 2004). Moreover, the WEHI-164 (fibrosarcoma) cell line was used to assay tolerability and matrix metalloproteinase type 2 (MMP-2) activity, on the basis of the method of (Heussen and Dowdle 1980). MMP-2 activity was assessed using zymography.

Toxicology studies were categorized on the basis of:

1. Serum and urine determinants: To evaluate the side effects of M2000, assessment of kidney function was performed in healthy rats receiving the test drug after 12 intraperitoneal injections of M2000 (30 mg kg^{-1} each 48 h) and was based on the measurements of serum creatinine, BUN, urinary protein excretion, urine urea, as well as the plasma concentration of triglyceride and cholesterol.
2. Histological examinations: Renal tissues were assessed using light microscopy. Glomerular lesions were graded on a scale of 0–3 (0, *negative*; 1, *mild*; 2, *moderate*; 3, *marked*) according to four parameters: hypercellularity, glomerular infiltration of PMN, hydropic change in the capillary network within the renal cortex, and the presence of tubular casts.
3. Gastroulcerogenic study: After 12 intraperitoneal injections of M2000 (30 mg^{-1} kg^{-1} each 48 h) for the group of healthy control rats, as well as oral administration of M2000 (40 $mg^{-1}kg^{-1}$ per day) for 10 days to group T3, the rats were killed and the stomach and the duodenum were dissected out. Scoring was done on the basis of at least one gastric ulcer or one hemorrhagic erosion.
4. Body temperature: The course of rectal temperature of rats which received 12 intraperitoneal injections of M2000 solution (30 mg^{-1} kg^{-1} each 48) was monitored continuously by thermoelements and compared with the baseline temperature in normothermic controls.

Cytotoxicity analysis showed a much higher tolerability for M2000 than for other drugs tested (diclofenac, piroxicam, and dexamethasone). The inhibitory effect of M2000 on MMP-2 activity was significantly greater than that of dexsamethasone

and of piroxicam at a concentration of 200 μg ml^{-1}. Moreover, data analysis showed no significant differences in the levels of serum determinants (BUN, creatinine, cholesterol, and triglyceride) and urine determinants (protein excretion and creatinine) between the normal group and the healthy experimental group challenged with M2000 (Table 2), whereas a significant increase of urine urea in the healthy group compared with the normal group revealed the advantage using M2000 in healthy controls (Table 2). The histological study of kidney specimens obtained from the normal group and healthy controls receiving M2000 showed no glomerular changes

Table 2 Toxicological inspection of M2000 administration on serum and urine determinants

Group	No. of rats	Blood urea nitrogen (mg dl^{-1})	Serum creatinine (mg dl^{-1})	Serum cholesterol (mg dl^{-1})	Serum triglyceride (mg dl^{-1})	Urine protein (mg day^{-1})	Urine urea (mg dl^{-1})
N	8	16 ± 2	0.7 ± 0.1	59 ± 3	79 ± 9	8 ± 3	939 ± 300
H	8	17 ± 4	0.8 ± 0.1	56 ± 6	89 ± 23	6 ± 2	1,284 ± 226

The healthy group receiving M2000 (*H*) versus the normal (*N*) group shows no significant difference in serum and urine determinants.

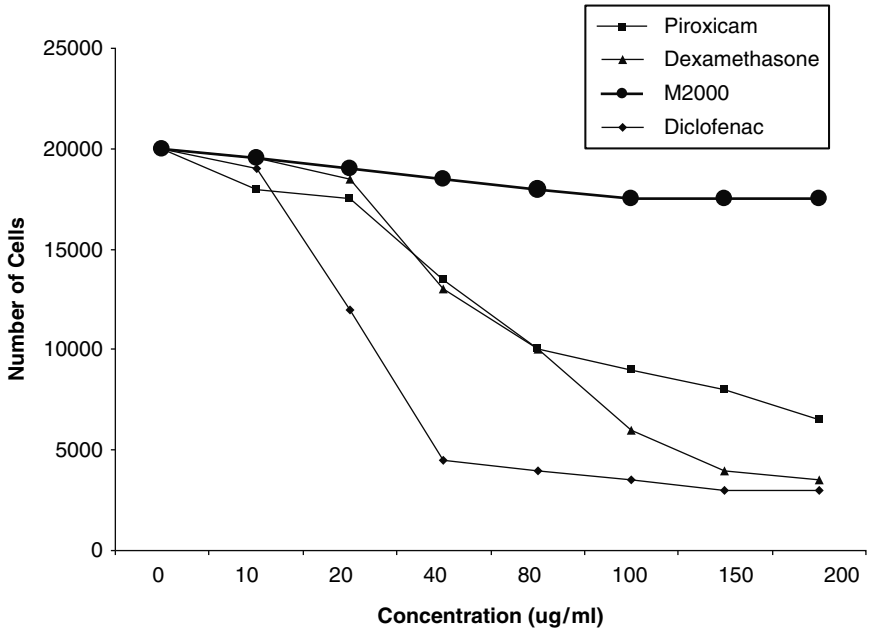

Fig. 27 Cytotoxic effect of M2000. Proliferative response of fibrosarcoma (WEHI-164) cell line to M2000 at different doses (10–200 μg ml^{-1}) compared with those of diclofenac, piroxicam, and dexamethasone. LD_{50} for diclofenac, piroxicam, and dexamethasone was 25, 80, and 80 μg ml^{-1}, respectively. In contrast, WEHI-164 as a sensitive cell line showedhigh tolerability against increasing amounts of M2000 (Mirshafiey et al. 2004b)

in the healthy group in comparison with the normal group (data not shown). On the other hand, administration of M2000 had no influence on body temperature of normothermic rats which had received the test drug. Additionally, the results of this experiment showed that 12 intraperitoneal injections of M2000 solution (30 mg kg^{-1} each 48 h) as well as oral administration of M2000 (40 mg kg^{-1} per day) for 10 days could not provoke gastromucosal lesions in the rats of the experimental group.

Figure 27 shows the proliferative response of a fibrosarcoma (WEHI-164) cell line to M2000 at different doses (10–200 μg ml^{-1}) compared with the proliferative responses to diclofenac, piroxicam, and dexamethasone. The tolerability and biocompatibility of WEHI-164, as a sensitive cell line against increasing amounts of M2000, was very high, whereas 50% of cells died when diclofenac, dexamethasone, and piroxicam were added to tissue culture at doses of 25, 80, and 80 μg ml^{-1}, respectively. M2000 showed no cytotoxic effect compared with steroidal and nonsteroidal drugs tested. Figure 28 presents the dose response analysis of the effect of M2000 on MMP-2 activity compared with the effect of various drugs. The inhibitory effect of M2000 at

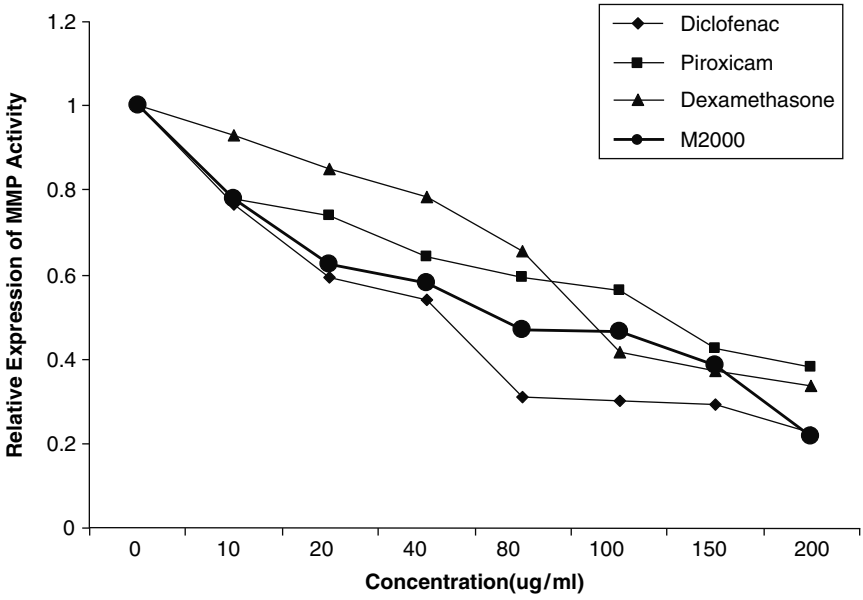

Fig. 28 The inhibitory effect of M2000 on matrix metalloproteinase type 2 activity. Fibrosarcoma cell lines (2×10^4 cell $wall^{-1}$) were incubated overnight with increasing doses of M2000. Diclofenac-, piroxicam- and dexamethasone-treated cells were used as controls. M2000-treated and nontreated cells were investigated in triplicate. The inhibitory activity of M2000 paralleledthat of diclofenac at doses of 10, 20, 40, and 200 μg ml^{-1}, whereas the inhibitory effect of M2000 at concentrations of 20, 40, and 80 μg ml^{-1} was significantly more than that of dexamethasone ($P < 0.05$). Moreover, the inhibitory activity of this novel agent at a dose of 200 μg ml^{-1} was significantly more than that of dexamethasone and that of piroxicam, $P < 0.05$ (Mirshafiey et al. 2004b)

concentrations of 20, 40, 80, and 200 μg ml^{-1} was significantly more than that of dexamethasone ($P < 0.05$), and this difference was significant between M2000 and piroxicam at a concentration of 200 μg ml^{-1}, ($P < 0.05$).In contrast, the inhibitory activity of this novel agent paralleled that of diclofenac at doses of 10, 20, 40, and 200 μg ml^{-1}.

10 Outlook

Alginates have been considered for medical applications such as drug delivery, wound coverage material, and cell encapsulation/transplantation. However, only recently have alginate and its comonomer M2000 been described as potential drugs suitable, in particular, for the treatment of inflammatory diseases. As compiled in this chapter, alginate as well as its comonomer M2000 show efficacy as anti-inflammatory drugs when tested in various inflammatory diseases. The direct application of alginate/M2000 as a medical drug requires the production of alginates with consistent composition and high purity as well as the production of tailor-made alginates such as homopolymeric polymannuronate. Hence, the use of (engineered) microorganisms as production organisms is becoming increasingly attractive and fermentative production might in future be the only feasible production process.

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