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Alginates and Their Biomedical Applications

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Preface

Alginates are polysaccharides, which are naturally produced by seaweeds and bacteria, and they have been known by humans for more than a century. Mannuronic acid and guluronic acid are the basic constituents of these polymers. Alginates possess unique properties, which are harnessed for various applications such as in foods, cosmetics, and fabric products as well as pharmaceutical/biomedical and other industrial purposes. From being harvested as nutritional source from seaweeds in the oceans to being applied in the biomedical field, alginates have been extensively researched for almost a century. Owing to the revolution of scientific methods, technological advancements, and interdisciplinary approaches, the application of alginates has been revolutionized in the recent two decades because their properties and modification capabilities have been better understood for adjusting them to our needs. Nowadays, it is well established that alginates are suitable for pharmaceutical and biomedical engineering approaches. Historically, supplying alginates via harvesting seaweeds imposed ecological concerns, while increasing oceanic impurities and limited chemical modification methods could not extend alginate applications beyond traditional usage. In the last decade, scientific efforts have described in more detail the physicochemical properties and molecular interaction of alginates with other polymeric and non-polymeric substances. Furthermore, technological and engineering advancements in the modification and fabrication of biopolymers have extended alginate applications into advanced biomaterial engineering for the production of high-value pharmaceutical and biomedical products. Current research outputs demonstrate that alginates and associated derivatives are invaluable components in therapeutic developments such as tissue engineering, cell therapy, cancer treatment, drug delivery, and treatment of cardiovascular diseases and metabolic disorders. However, the current biomedical applicability of alginates has been based on algal alginates, while bacterial alginates which display different physicochemical properties have remained unharnessed. Indeed, alginate-producing bacteria are cell factories with the potential of supplying such biopolymers for biomedical purposes. More importantly, in contrast to alginates originated from seaweeds, our understanding of alginate biosynthesis pathways has been based on alginate-producing bacteria. Hence, extensive molecular studies on alginate

biosynthesis in bacteria have paved the path toward biotechnological alginate production and showed that this is a promising path for tailoring alginates to exhibit novel and reproducible compositions and properties for high-value purposes. Therefore, we believe that the application of alginates can go further and beyond current biomedical applications by harnessing various algal and bacterial alginates in combination with advanced technological and biotechnological techniques. While the number of scientific studies and published data on alginates and their biomedical and pharmaceutical applications are enormous, they have not been reviewed and covered in a single book before.

This book consists of 11 chapters and presents recent advances on the characterization and production of alginates from seaweeds and bacteria, as well as it outlines applications of alginates for advanced biomedical and pharmaceutical purposes. Chapter 1 highlights our understanding of alginate biosynthesis in bacteria and algae toward biotechnological production. Chapter 2 focuses on algal alginates and their farming which is accepted as an alternative to utilizing natural resources. Chapter 3 distils recent advances by which alginates are considered as an important biopolymer in cell microencapsulation technology and as a platform for controlled drug and therapeutic factor delivery through cell encapsulation. This chapter provides the state-of-the-art technologies and current research strategies by which alginates have been employed in formulations for treating prevalent human diseases and disorders. Chapter 4 focuses on the processing techniques mainly used for manufacturing and processing products of alginates and their potential applications in biomedical science, tissue engineering, and drug delivery. This chapter also suggests future perspectives for their novel applications in the biomedical field. Chapter 5 provides a comprehensive overview of the applications of alginate-based hydrogels to design various forms of constructs and scaffolds for tissue engineering, for example, via bioprinting, an area of high-tech research that has created a substantial foundation for treating and curing many prevalent diseases. Chapter 6 reviews application of alginates based on 3D in vitro models in the field of tumor and cancer research. Chapter 7 describes the versatile biomedical applications of alginates in creating solutions for treatment of heart and cardiovascular diseases. Chapter 8 discusses the potentials of alginates for designing dressings for the treatment and management of wounds. Chapter 9 highlights different strategies by which alginates have been introduced in formulations for treating metabolic syndromes such as gastrointestinal tract disorders, obesity, type 2 diabetes, hypertension, nonalcoholic fatty liver disease, and dyslipidemia. Chapter 10 outlines the research performed to date for introducing alginate oligomers in designing effective formulations with multiple therapeutic applications such as the management of chronic lung diseases, biofilm infections, and antibiotic use. Chapter 11 highlights the application of mannuronic acid as one of the safest drugs with potent anti-inflammatory and immunosuppressive properties.

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Chapter 1
Alginate Biosynthesis and Biotechnological Production

M. Fata Moradali, Shirin Ghods, and Bernd H.A. Rehm

Abstract Alginates are natural exopolysaccharides produced by seaweeds and bacteria belonging to the genera *Pseudomonas* and *Azotobacter*. Due to exhibiting unique physicochemical properties, they have been widely applied for various industrial purposes such as in food, agricultural, cosmetic, pharmaceutical, and biomedical industries. In the last two decades, they have found their way into the advanced pharmaceutical and biomedical applications, owing to their biocompatibility and non-toxicity as well as versatility in view of modifications. So far, algal alginates have been the sole commercialized products applied for various purposes, while the potential uses of bacterial alginates remain unharnessed. Importantly, algal and bacteria alginates differ substantially from each other with respect to their composition, modifications, molecular mass, viscoelastic properties, and polydispersity. Indeed, bacterial alginates may meet current needs in the field of advanced pharmaceutical and biomedical engineering. In this chapter, after a brief overview of alginate discovery, general properties, applications, and comparative assessment of algal and bacterial resources, current findings about the biosynthesis of alginates, mainly in bacteria, will be discussed. Furthermore, we will discuss the current understanding of alginate polymerizing and modifying enzymes and their structure-function relationship. Knowledge about alginate biosynthesis/modification enzymes provides foundation for rational design of cell factories for producing tailor-made alginates. As a conclusion, advanced understanding of alginate biosynthesis pathway and involved enzymes creates an opportunity for bioengineering and synthetic biology approaches toward the production of alginates exhibiting desired material properties suitable for pharmaceutical and biomedical applications.

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1.1 An Overview of Alginate Discovery and Application

Alginates were first discovered by E.C.C. Stanford, an English chemist, in 1883 [1]. While working on improving iodine dietary needs from seaweeds growing along the Scottish coast, he became interested in exploring the byproducts of seaweeds and their usefulness. He isolated a mucilaginous material with 2% sodium carbonate followed by acidifying solution for precipitation of this material which was named "algin." Algin displayed colloid properties while became viscose in combination with salts such as sodium and potassium and also had gelling properties [2, 3]. Stanford patented the first procedure for extraction of this material from seaweeds [4]. Years later in 1896, Krefting was able to patent a procedure for purification of align [5, 6]. In 1928, W. Nelson and L. H. Cretcher (Mellon Institute of Industrial Research, Pittsburgh, Pennsylvania) presented "Naturally occurring acidic polysaccharides" in the Fourth Annual Meeting of the Pennsylvania Academy of Science at Pittsburg. In this paper, they characterized alginic acid as purified from *Laminaria agardhii* and *Macrocystis pyrifera* which was chemically unique and made up completely of polyuronic acids. While part of this polysaccharide was readily hydrolysable, another portion displayed extensive resistance to hydrolysis (1927–1928: v.2 – Pennsylvania Academy of Science). However, between 1926 and 1930, independent groups attempted to understand alginic acid composition, and then uronic acid derivatives were proposed as constituting molecules [7–12]. In 1929, Nelson and Cretcher showed that alginic acid was a polymer made of one or more aldehyde sugar acids and at least D-mannuronic acid takes part in the composition [8]. One year later, they could isolate D-mannuronic acid in the form of crystalline lactone via hydrolyzing an alginic acid extracted from *Macrocystis pyrifera* followed by hydrolysis of alginic acid extracts from the algae *Fucus serratus* and *Laminaria saccharina* [9, 10].

In 1933, Schoeffel and Link (University of Wisconsin, Madison) explained that D-mannuronic acid was known only in the form of its lactone, similar to the parent sugar *d*-mannose. They could isolate the α form of D-mannuronic acid which was extremely soluble in the solvents similar to α,*d*-mannose property, while β variety of D-mannuronic acid existed less in equilibrium and was less soluble than the α form [13]. By 1945, particularly by means of X-ray, the formula $(C_6H_8O_6)_n$ was generally accepted for β-$_D$-mannuronic acid as main constituents of alginic acid [14, 15]. Fischer and Dorfel [16] explained that guluronic acid is another constituting component of alginic acid whose occurrence percentage and ratios to mannuronic acid varies depending on seaweed resources [16].

For the first time, alginate production from bacteria was reported by Linker and Jones in 1964. By analysis of a *Pseudomonas* bacterium isolated from sputum of

cystic fibrosis patient, they found that this bacteria form an unusually large mucoid colonies on plates and constituting polysaccharides was reported resembling alginic acid [17]. Later in 1966, they reported that alginate from this *Pseudomonas* isolate was acetylated contrary to algal alginates while acetyl groups were lost during alkaline extraction [18]. In the same year, Gorin and Spencer reported alginic acid production by *Azotobacter vinelandii* [19]. In 1981, Govan and coworkers introduced other alginate-producing bacteria including *Pseudomonas fluorescens*, *P. putida*, and *P. mendocina* [20].

Regarding earliest applications of alginate, although some UK companies such as British Algin Company Ltd (1885), Blandola Ltd (1908), and Liverpool Borax Ltd (1909) were initially established to harness alginates, the first successful company in producing large and commercial scale pure alginates was Kelco Company established by F.C. Thornley in San Diego, USA, in 1929 [21].

In 1934, Cefoil Ltd was established in the UK to exploit alginates extracted from seaweeds for producing sodium alginate fiber applicable in camouflage netting and other military items [21]. During that period of time, C. W. Bonniksen from the Chemistry Department of University College in London attempted to investigate alginic acid for production of cellophane-like material. For this purpose, Bonniksen and his colleagues established a small company and named it The Kintyre factory. In 1939, they relatively achieved the production of cellophane-like material from alginic acid [3]. But, their operation was coincident with triggering World War II, and the fate of alginates application was directed by the war. Accordingly, requested by the Government, Bonniksen and his colleagues were responsible for establishing more factories in that region in order to extract alginic acid for production of camouflage textile. Also, in parallel, other research groups were pursuing the same purposes in several other laboratories in the UK [3]. The Kintyre factory was closed in 1942, but its production was transferred to newly established factories. On the other hand, after the war (1945) Cefoil Ltd was changed to Alginate Industries Ltd. Later, the two largest alginate producers, i.e., Kelco Company (USA) and Alginate Industries Ltd (UK), were acquired by Merck and Co. Inc., USA, respectively, in 1972 and 1979, becoming the largest alginate producer worldwide [21]. In the 1980s and 1990s, more companies in other countries such as Norway, Germany, France, Japan, and China were established in particular close to abundant natural seaweed resources.

1.2 Alginate Properties and Natural Occurrence

1.2.1 Alginate Structure and Physiochemical Properties

Alginates are unbranched polysaccharides produced by seaweeds and bacteria belonging to *Pseudomonas* and *Azotobacter* genera. Basically, the structure of the alginates consists of two uronic acid residues including β-$_{D}$-mannuronic acid (M)

and its C5 epimer α-$_L$-guluronic acid (G) linking via 1,4-glycosidic bonds (Fig. 1.1). In nature, alginates are usually found with heteropolymeric structure, i.e., combination of both M and G residues, while production of monopolymeric structure (polyM) has been reported at initial stage of alginate polymerization in bacteria and genetically manipulated *P. aeruginosa* via inactivating catalytic domain of alginate epimerase (e.g., PDO300Δ*algG* (pBBR1MCS-5: *algG* (D324A)) [22, 23]. The occurrence of M and G residues in polymeric structure varies significantly among alginates as variable numbers and lengths of M blocks, G blocks, and MG blocks. Composition of alginates and molecular mass may differ significantly depending on the source of production and growth condition of the producer. However, while algal alginates usually show a high content of G blocks, alginate produced by *P. aeruginosa* does not possess G blocks. Another significant structural modification is natural acetylation of alginates at *O*-2 and/or *O*-3 positions which have been so far reported only in bacterial alginates (Fig. 1.1), while acetylating algal alginates via chemical treatments has also been reported [24, 25].

Composition of polymers determines their physicochemical properties. The intrinsic viscoelasticity of alginates depends on the frequency of constituting blocks as flexibility decreases in the order MG block > MM block > GG block. The most important features of alginates are related to its ability to efficiently and selectively bind divalent cations leading eventually to hydrogel formation and crosslinked polymeric scaffolds [26] (Fig. 1.1). The affinity of alginates toward different divalent ions was found to increase in the order $Mg^{2+} << Mn^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+} < Cu^{2+} < Pb^{2+}$ [27, 28]. The strength, dimension, stability, and mechanical property of resulting hydrogels differ based on the type of interacting cation, the G content, and variability of G blocks in the polymer [26, 29–31].

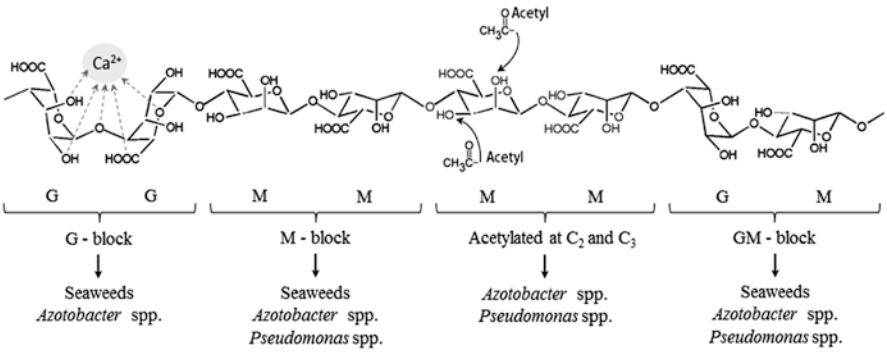

Fig. 1.1 Chemical structure of alginates produced by various organisms including seaweeds and bacteria belonging to the genera *Pseudomonas* and *Azotobacter.* G blocks occurring only in algal alginates and *Azotobacter* spp. bind selectively with divalent cations such as calcium causing hydrogel formation. Only bacterial alginates are being acetylated at C_2 and C_3 positions, leading to increasing the interaction of polymer with water molecules and increasing water capacity and polymer extension

The presence of *O*-acetyl groups in bacterial alginates notably changes the properties of the alginates reflecting at polymer conformation, chain expansion, solubility, water capacity, viscoelasticity, and molecular mass. An acetylated alginate absorbs more water due to the better interaction of chains with water molecules, leading to chain expansion and better solubility [22, 24, 32, 33].

Unique composition and properties of alginates led to their wide applications in various industries including agriculture, food, textile, cosmetic, and pharmaceutical/biomedical industries. These natural polymers have been considered as thickeners, stabilizers, viscosifiers, additives, gel and film formers, and fertilizers [2, 34–37]. Owing to non-toxicity, biocompatibility, non-immunogenicity, hydrophilicity, and biodegradability, alginates have been extensively applied for biomedical and pharmaceutical uses [38–40]. They have been traditionally applied for generating materials in dental impression and wound dressing [41–44]. However, technological advancements such as the materials fabrication and processing technologies have enhanced their biomedical applications via modifications and tailoring of alginate compositions including interactions with other polysaccharides. Alginates have been processed into nanoparticles, nanotubes, microspheres, microcapsules, sponges, hydrogels, foams, elastomers, fibers, etc. [45–51]. Nowadays, various types of alginate derivatives are considered as one of the highly valuable and biocompatible biopolymers for drug delivery [2, 37]; immobilization of enzymes [52–54]; cancer therapy by functionalizing polymeric scaffolds for controlled release of anticancer drugs [55, 56]; therapeutic cell entrapment [57–59]; protection of transplanted cells from the host immune system [60–62]; tissue engineering [63–65]; generation of three-dimensional cell culture matrices for different laboratory assessments such as cell-drug interaction, cell growth, and cell biology [66–68]; and alginate formulations for preventing gastric reflux [69, 70]. The next chapters will present the current state-of-the-art review for biomedical application of alginates and their derivatives with regard to drug delivery (Chaps. 3 and 4); tissue engineering and cell therapy (Chaps. 4 and 5); tumor studies (Chap. 6); heart and cardiovascular diseases (Chap. 7); dressings and wound management (Chap. 8); metabolic syndromes (Chap. 9); chronic lung diseases, biofilm infections, and antibiotic use (Chap. 10); and developing anti-inflammatory drugs (Chap. 11).

1.2.2 Algal Alginates

To date, seaweeds have been the sole and relatively low-cost alginate producers for all commercial purposes. These natural resources have been mainly brown algae from the genera *Laminaria*, *Macrocystis*, *Ascophyllum*, *Ecklonia*, *Lessonia*, and *Durvillaea*.

Variable composition of alginates is linked to their natural biological role for producer (Table 1.1). Hence, seasonal and growth condition as well as geographical distribution is critical for determining the composition of alginates as well as the percentage of alginates in various parts of the algae. Generally speaking, apparently

Table 1.1 Composition of various alginates produced by different organisms[a]

Species	F_G	F_M	F_{GG}	F_{MM}	$F_{GM/MG}$	Act. (%)	References
Algae							
Laminaria hyperborea	0.49–0.63	0.37–0.51	0.31–0.52	0.26–0.32	0.11–0.19	–	[130]
Laminaria japonica	0.35	0.65	0.18	0.48	0.17	–	[130]
Laminaria digitata	0.41–0.59		0.25	0.43	0.16	–	[130]
Ascophyllum nodosum			0.22	0.38	0.21	–	[130]
Macrocystis pyrifera			0.20	0.37	0.21	–	[130]
Lessonia nigrescens			0.22	0.40	0.19	–	[130]
Durvillaea Antarctica	0.32	0.68	0.16	0.51	0.17	–	[130]
Bacteria							
P. aeruginosa	0.3	0.7	–	0.40	0.30	28	[22]
P. fluorescens	0.40	0.60	–	0.2	0.4	12–17.5	[130–132]
	0.27–0.35	0.65–0.73		0.30–0.46	0.40		
P. putida	0.22–0.37	0.78–0.63	–	0.56–0.26	0.22–0.37	18–21	[131, 132]
P. mendocina	0.26	0.74	–	0.48	0.26	nd	[132]
A. vinelandii	0.25–0.75	0.75–0.25	0.07–0.65	nd	nd	nd	[130, 132]
	0.45–0.94	0.55–0.06	0.43–0.93	0.52–0.04	0.02–0.01	nd	

[a]Values reported in this table may change depending on various environmental factors and growth conditions
nd not determined

seaweeds growing near coastal areas require a composition of alginate which confers high mechanical rigidity compared to those floating on streaming waters with higher flexibility [71–73].

To date, the biosynthesis pathways of alginates in algae and controlling mechanism of their composition remain largely unknown, while almost all of the efforts have been put into analyzing the composition of various algal alginates and laboratory modifications in order to expand their industrial applications. However, one of the earliest analyses of alginate biosynthesis in brown algae showed that equivalent biochemical reactions may exist in both brown algae and bacteria in regard to polymerization [74, 75], while apparently significant differences exist at the modification level and within the protein interaction network constituting the alginate polymerization/modification/secretion multiprotein complex (Fig. 1.2).

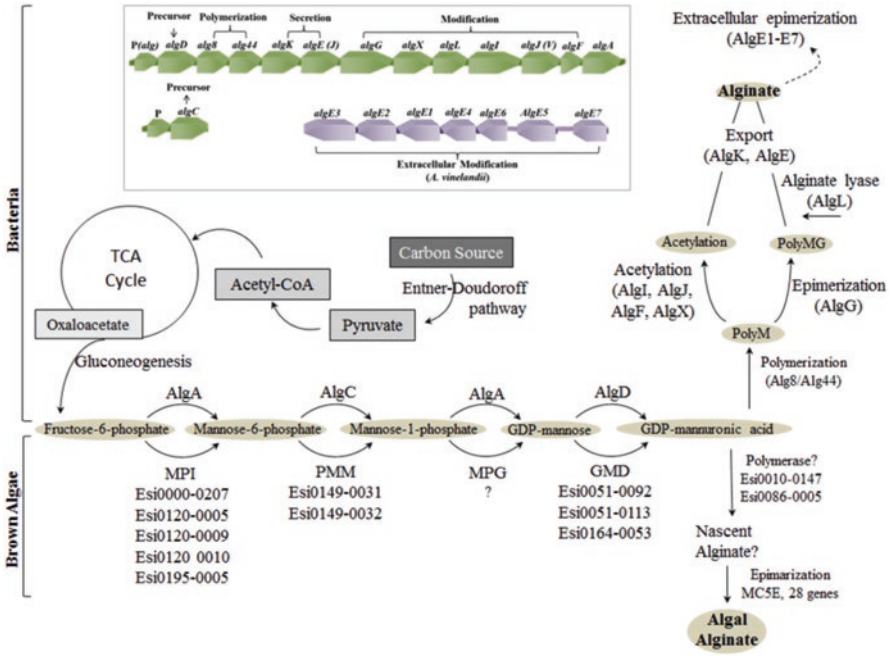

Fig. 1.2 Biosynthesis pathway of alginates in bacteria and algae. This pathway is mainly understood in bacteria *P. aeruginosa* and *A. vinelandii*, and some homologous genes which have been hypothetically reported in brown alga *Ectocarpus siliculosus* (based on reference [113]) are presented. Gene clusters encoding different proteins accomplishing different steps of alginate biosynthesis are presented and functionally assigned in the frame (*TCA cycle* the tricarboxylic acid cycle, *MPI* mannose-6-phosphate isomerase, *PMM* phosphomannomutase, *MPG* mannose-1-phosphate guanylyltransferase, *GMD* GDP-mannose 6-dehydrogenase, *MC5E* mannuronate C5-epimerase, *PolyM* poly-mannuronate, *polyMG* poly-mannuronate/guluronate)

1.2.3 Bacterial Alginates

Bacterial species belonging to the genera *Pseudomonas* and *Azotobacter* produce various alginates with different properties from each other as well as algal alginates (Table 1.1). Alginate production is mainly considered as a survival advantages by which bacteria can survive unfavorable and harsh conditions. In the case of the opportunistic human pathogen *P. aeruginosa*, alginate predominantly constitutes mucoid biofilms referred to the cell aggregations embedded in extracellular polymeric substances [76–78]. For the nitrogen-fixing soil bacterium *Azotobacter vinelandii*, alginate either takes part in the encystment process as a protective component of the cyst coat in metabolically dormant cysts or is produced as an extracellular polysaccharide by vegetatively growing cells for attachment to the surfaces [79, 80].

To date our knowledge about biosynthesis of alginates is established on the bacterial model organism *P. aeruginosa* whose alginate production is the hallmark of chronic infections in cystic fibrosis patients. Alginates produced by bacterial species

are variably acetylated, and contrary to *Azotobacter* alginates which contain all types of block structures, *Pseudomonas* alginates only possess M and MG blocks, but not G blocks, indicating their different biological role for different species (Fig. 1.1, Table 1.1).

1.3 Alginate Biosynthesis, Modification, and Secretion

For many years, understanding the biosynthesis of alginates has been of great importance for scientific community in order to inform drug development for treatment of *P. aeruginosa* infections exacerbated by alginate overproduction as well as establishing the production of bacterial and tailor-made alginates. This is particularly important for high-value purposes which require defined properties of alginates. Therefore, in contrast to biosynthesis of algal alginates, alginate biosynthesis, modification, and secretion events are relatively well understood in bacteria.

Generally, *P. aeruginosa* and *A. vinelandii* share a similar biosynthesis gene cluster which is conserved among alginate producing bacteria (Fig. 1.2). However they differ in regard to epimerization as well as regulatory mechanisms. Most genes involved in alginate production are clustered in bacterial genomes. Except for *algC*, the genes including *algD*, *alg8*, *alg44*, *algK*, *algE* (*algJ*), *algG*, *algX*, *algL*, *algI*, *algJ* (*algV*), *algF*, *algA* are clustered within the alginate operon [81, 82] (gene names in parentheses correspond gene names in *Azotobacter*). Transcription of these genes is under the tight control of a promoter upstream of *algD* (Fig. 1.2) [83, 84] and two internal promoters [85]. Hence, resulting differential transcription of downstream genes to internal promoters was proposed as a mechanism which may control the stoichiometry of protein subunits within the multiprotein complex resulting in varying composition of produced alginates under different environmental conditions [85].

1.3.1 Biosynthesis of the Alginate Precursor

The synthesis of alginate starts with the provision of the active precursor guanosine di-phosphate (GDP)-mannuronic acid. This requires a series of cytosolic enzymatic steps mediated by AlgA (phosphomannoseisomerase/GDP-mannose), AlgC (phosphomannomutase), and AlgD (GDP-mannose dehydrogenase) which catalyze four biosynthesis steps to convert fructose-6-phosphate originating from the gluconeogenesis pathway to GDP-mannuronic acid [86–90] (Fig. 1.2). The last enzymatic event catalyzed by AlgD, leading to GDP-mannuronic acid formation, is irreversible and is thought to be a key rate-limiting reaction in the alginate synthesis pathway (Fig. 1.2) [91–94].

1.3.2 Protein-Protein Interactions Constituting Alginate Biosynthesis/Modification/Secretion Multi-protein Complex

Protein-protein interaction studies demonstrated that bacterial alginate polymerization, translocation across the cytoplasmic membrane, and secretion through the outer membrane are mediated by a membrane-spanning multi-protein complex (Fig. 1.3). Briefly, this multi-protein complex involves (1) alginate-polymerizing unit (Alg8-Alg44); (2) a proposed periplasmic protein scaffold (Alg44-AlgG-AlgX-AlgK) responsible for protecting nascent alginate (polyM) against lyase activity of AlgL and translocating polymer across membrane coincident with modifications (i.e., epimerization (AlgG)/acetylation (AlgX)); and (3) secretion part (AlgK-AlgE) responsible for completing the translocation of modified alginate across the outer membrane of bacteria [22, 95, 96]. Other subunits such as AlgI, AlgJ, and AlgF have been proposed as part of periplasmic scaffold while they are necessary for acetylation event probably by providing acetylation precursor for terminal acetyltransferase AlgX [97] (Fig. 1.3). However, exact function of AlgI/J/F and their functional and structural relevance to the multi-protein complex have not been assigned, yet.

1.3.3 Alginate Polymerization and Mechanism of Activation

Alginate polymerization is mediated by two interacting membrane-anchored proteins Alg8 (polymerase) and Alg44 (co-polymerase) (Fig. 1.3). Alg8 belongs to the glycosyltransferase family 2, catalyzing the transfer of a sugar molecule from an activated donor, i.e., GDP-mannuronic acid, to an acceptor molecule which is a growing carbohydrate chain. However, activation of alginate polymerization is regulated at posttranslational level through sensing the second messenger bis-(3′, 5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) by Alg44 [98–101]. Alg44 consists of a cytoplasmic c-di-GMP sensing PilZ domain, a transmembrane region which extends into the periplasm of the bacteria and interacts with other periplasmic subunits. In other word, c-di-GMP binding to Alg44 is necessary for activation of alginate polymerization which itself interacts with Alg8 [98, 99]. Due to the difficulty of Alg8 and Alg44 purification, polymerization mechanism of alginate had been remained poorly understood for many years; until recently, our group could shed light on this mechanism. Through generation of various in silico models of Alg8, Alg44, and the PilZ domain as well as site-specific mutagenesis studies, evidence was provided that c-di-GMP binding to Alg44 targets the catalytic sites of Alg8 probably by inducing a conformational change. The activation mechanism involves the engagement of some highly conserved amino acid residues of Alg8 at two predicted loops surrounding the catalytic site of Alg8. Previously, it was demonstrated that alginate polymerization is impacted by cellular level of c-di-GMP. This is mediated by one particular c-di-GMP-synthesizing protein, MucR,

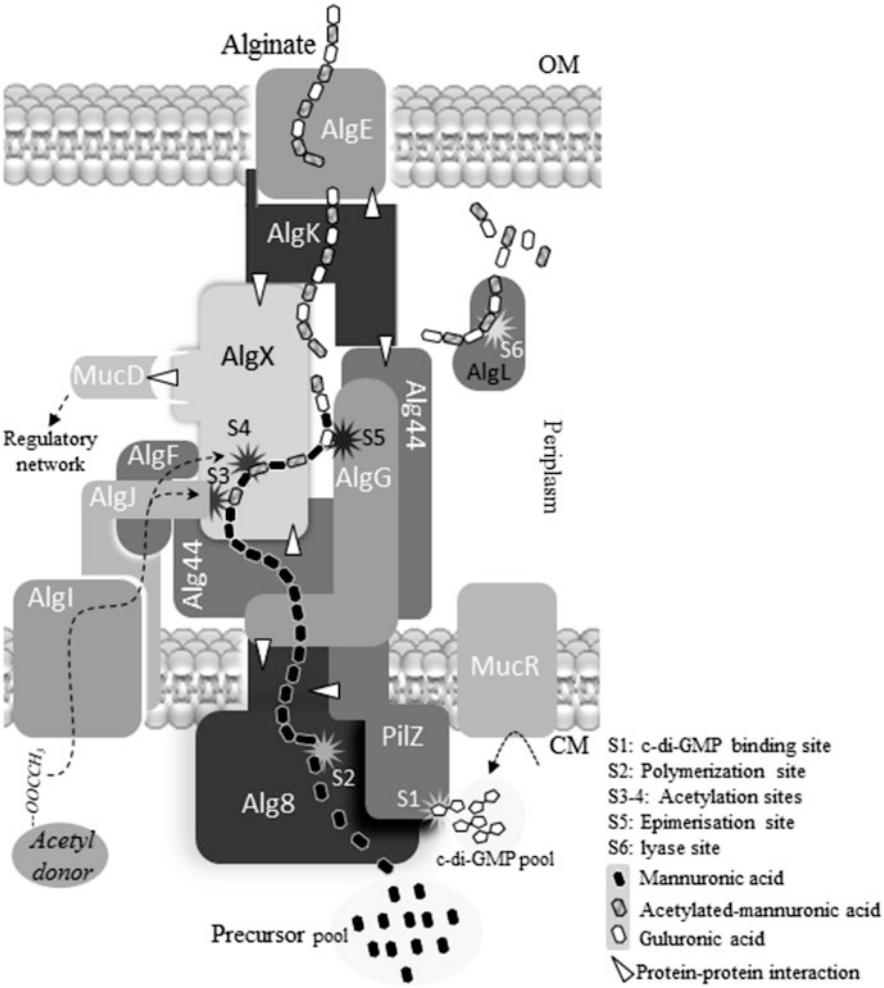

Fig. 1.3 Proposed model of alginate biosynthesis machinery complex and experimentally demonstrated protein-protein interactions (marked with white triangles). This model shows alginate production is positively regulated by c-di-GMP binding to Alg44 (S1) which targets the catalytic site of Alg8 polymerase (S2). Then, translocation across the periplasmic scaffold is coupled with modification events (S3 to S5). AlgL is responsible for degrading misguided alginate accumulating in the periplasm (S6). MucD protein links the complex with the posttranslational alginate regulatory network via an interaction with AlgX (*OM* outer membrane, *CM* cytoplasmic membrane (Adapted from Ref. [22]))

which specifically influences the level of alginate production in *P. aeruginosa* presumably by generating a localized c-di-GMP pool in proximity to the alginate polymerase (Fig. 1.3) [102, 103].

1.3.4 Epimerization

Modification of alginate in bacteria consists of epimerization and acetylation of polymerized chain. Epimerization of M residues to G and also acetylation leads to changes in material properties of alginates (Figs. 1.2 and 1.3). Generally, the presence of G residues in alginates allows for the formation of gels in the presence of divalent cations such as Ca^{2+} and acetylation increases interaction of the chain with water and cause chain expansion. A polymannuronate alginate is a fairly stiff polymer due to the di-equatorial linkages of M blocks, while equatorial-axial bond of MG blocks increase the flexibility of the chain [104].

AlgG specifically catalyzes the epimerization of M residues to G via protonation-deprotonation of C5 on the M residue in the alginate (Fig. 1.3). All alginate-producing bacteria possess AlgG-type epimerases. However, AlgG cannot generate two consecutive G residues; therefore resulting polymers are devoid of G blocks [23, 105, 106]. This is so far observed for alginate producers possessing only AlgG-type epimerases such as *P. aeruginosa*. However, there are other types of mannuronan C-5-epimerases which act extracellularly and in a Ca^{2+}-dependent manner (so-called the Ca^{2+}-dependent AlgE-type). These enzymes further catalyze the conversion of M to G residues leading to increasing G content as well as the formation of G blocks. At least seven extracellular AlgE-type epimerases (AlgE1-E7) have been reported from *A. vinelandii* with differing specificities and nonrandom epimerization patterns [75, 107, 108] (Figs. 1.2 and 1.3). Furthermore, a gene designated as PsmE was found in *P. syringae* pv *glycinea*, enabling to generate G blocks in vitro in alginate. PsmE is similar to the secreted epimerases from *A. vinelandii* [109, 110], but in contrast to them, PsmE is a biofunctional deacetylase/mannuronan C-5-epimerase protein and is able to epimerize acetylated M residues. It was shown that this protein removes acetyl groups prior to epimerization by the involvement of a module predicted belonging to the SGNH hydrolase superfamily which also comprises the *O*-acetyltransferases AlgX and AlgJ [110]. Homologous genes to PsmE are found in the genomes of many strains of *P. syringae* and related species such as *P. savastanoi*, *P. amygdali*, *P. fluorescens*, and *P. avellanae* [111].

Interestingly, a large family of mannuronan C-5-epimerases has been reported from brown algae (Fig. 1.2) which are related to the bacterial AlgG-type epimerases, and presumably they confer differentially distinct G-distribution patterns in alginates among and within algal species [112–114].

1.3.5 Alginate Acetylation

So far, acetylation of alginates is limited to bacterial alginates. Investigation of the acetylation mechanism in *P. aeruginosa* showed that AlgX binds to polymannuronic acid in a length-dependent manner and acts as a terminal acetyltransferase. AlgX adds *O*-acetyl ester linkages at the C2 or C3 position of M residues [115]. Furthermore, AlgI, AlgJ, and AlgF were shown to be essential for acetylation [97, 116] (Fig. 1.3), while their exact role and their functional and structural association with the multi-protein complex remain unknown. One proposed explanation is that they may mediate transportation of acetyl group for accomplishing acetylation by AlgX (Fig. 1.3). One proposed model is providing an acetyl group from a cytoplasmic source such as acetyl-coenzyme A. The transport of the acetyl group across the cytoplasmic membrane into the periplasm might be mediated by membrane-bound AlgI [97, 117]. AlgJ is a periplasmic protein associated with the cytoplasmic membrane which shows high homology to AlgX, both belong to the SGNH hydrolase superfamily. As it was demonstrated that AlgX is the terminal acetyltransferase, it is not known how the *O*-acetyltransferase activity of AlgJ may play a role within multi-protein complex. The periplasmic subunit AlgF is also essential for acetylation [118] (Fig. 1.3), while it does not have sequence homology to other proteins involved in *O*-acetylation.

1.3.6 Alginate Lyases

Alginate lyases are a group of alginate-degrading enzymes acting as either mannuronate or guluronate lyases which has not been classified yet due to their structural diversity [111, 119]. Natural occurrence of these alginate-modifying enzymes is very wide as they have been isolated from many organisms, including algae (but not brown algae), marine invertebrates, and terrestrial microorganisms [111, 119]. Wide natural distribution of these enzymes implicates their possible role in digesting alginates for utilizing as carbon source, while no bacterial alginate producer has been found to be able to utilize alginate as carbon source. Therefore, other biological roles for specific alginate lyases are assumed. For example, the alginate lyase, AlgL, is encoded within the alginate operon in *P. aeruginosa* [120]. It is a periplasmic protein which is functionally associated with existing alginate biosynthesis/modification/secretion multi-protein complex [121] (Fig. 1.3). AlgL was demonstrated to be essential for degrading misguided alginate trapped in the periplasm, leading to releasing free uronic acid oligomers to avoid the lethal effect of accumulated alginate on cells [122]. The deletion of genes encoding AlgK, AlgX, or AlgG, respectively, destabilized the multi-protein complex and led to formation of uronic acid oligomers. This is presumably due to misguided translocation of alginate across the bacterial envelope and hence leading to AlgL-mediated alginate degradation and production of unsaturated oligouronides [105, 106, 123, 124].

1.3.7 Alginate Secretion

The mechanism of alginate secretion is well-understood in *P. aeruginosa* as two interacting proteins AlgE and AlgK mediate alginate secretion across the outer membrane [95]. AlgE is an outer membrane protein acts selectively for secretion of the negatively charged alginate polymer upon possessing a highly electropositive pore constriction formed by an arginine-rich channel [125, 126]. On the other hand, proper localization of AlgE is facilitated by the lipoprotein AlgK at the outer membrane [127]. AlgK possesses a tetratricopeptide repeat (TPR) protein-protein interaction motif possibly mediating the interaction of AlgK with other subunits of the multiprotein complex [22, 95].

1.4 Alginate Polymerizing and Modifying Enzymes and Their Uses for Tailor-Made Alginate Production

Hitherto, alginates extracted from brown algae have been solely applied for generating desired alginate derivatives mainly via in vitro chemical modifications and treatments. However, since such approaches are often less controllable, achieving the alginates with specific and defined physicochemical properties is hard and sometimes impossible. Furthermore, in vitro chemical approaches may result in undesired changes in other polymer characteristics such as degradation of polymer chain. Hence, understanding molecular mechanisms of alginate polymerization/modification and unraveling their correlation as well as functional relationships of involved proteins can build up a critical foundation for production of tailor-made alginates. In a recent study, the functional and structural relationship of these mechanisms was investigated and resulted in the production of various alginates from engineered *P. aeruginosa* (Fig. 1.4) [22]. For many years, it was known that Alg8 (polymerase)/Alg44 (co-polymerase) and AlgX (acetyltransferase)/AlgG (epimerase) are responsible for alginate polymerization and modifications, respectively, while their possible interaction to constitute the proposed multi-protein complex remained unclear. However, functional relationships of these mechanisms and proteins were based on assumptions and indirect evidences. In recent years, studies have shed light on functional and structural relationships of proteins involved in constituting the alginate biosynthesis/modification/secretion multiprotein complex. Studies showed that the processivity of alginate polymerization was interrupted by epimerization, resulting in alginates with lower molecular mass (2755 kDa) (Fig. 1.4, Table 1.2) [22]. Upon removal of epimerization by generating a catalytically inactive variant of AlgG (Fig. 1.4 No. 8, Table 1.2), processivity of alginate polymerization was increased leading to a very high molecular mass alginate (4653 kDa) (Figs. 1.4 and 1.5, Table 1.2) [22]. Furthermore, the productivity of alginate (yield) was about threefold higher when epimerization was eliminated when compared with the presence of epimerization. Hence, it was concluded that polymerization has a negative

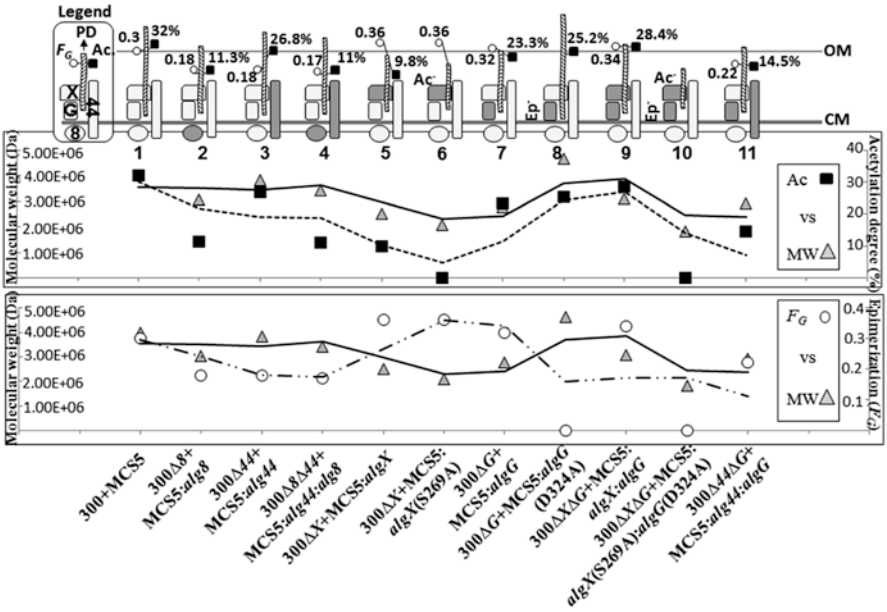

Fig. 1.4 Impact of putative alginate polymerase subunits on alginate polymerase activity, alginate polymerization, and composition and correlation between polymerization and modification. (**a**) The values of molar fraction of G residue (*FG*), acetylation degrees (Ac. %), mean molecular masses, and alginate yield are aligned with the strains producing the respective alginates. (**b**) Correlation between degree of acetylation, epimerization, and molecular mass of alginate. Presumable features (No. 1 to 11) show protein complexes constituted by Alg8, Alg44, AlgG, and AlgX (see the legend at the top left corner of the plot). The subunit produced upon in *trans* complementation is shown as darker shape(s). Inactive AlgX(S269A) and AlgG(D324A) proteins are labeled as (Ac) and (Ep), respectively. The length of various alginates (PD) with respect to acetylation (Ac. %) and epimerization (*FG*) degrees are presented and proportionally illustrated for each feature (*300* PDO300, *MCS5* pBBR1MCS-5, *PD* polymerization degree, *OM* outer membrane, *CM* cytoplasmic membrane (Adapted from Ref. [22]))

correlation with epimerization event [22]. Furthermore, evidence was provided that elimination of acetylation by producing a catalytically inactive variant of AlgX (i.e., AlgX (S269A)) resulted in lowering the molecular mass (2086 kDa) (No. 6 in Fig. 1.4, Table 1.2), while upon its presence higher molecular mass (2460 kDa) was produced [22]. This result showed that acetylation did not disrupt processivity of alginate polymerization indicative of a positive correlation. Importantly, epimerization and acetylation did not show any competitive relationship, contrary to previous assumptions, and removal of one (i.e., replacement with catalytically inactive variants of AlgG or AlgX) did not impact the other one (Table 1.2) [22]. Interestingly, AlgX and AlgG proteins showed mutual auxiliary behavior as the overproduction of AlgX boosted epimerization (F_G = 0.36; acetylation = 9.8%) and AlgG overproduction increased the acetylation degree (F_G = 0.32; acetylation = 23.3%) (Table 1.2). However, the elimination of two modification events resulted in the alginate with

Table 1.2 Composition and molecular mass analyses of alginates produced by different *P. aeruginosa* (*Pa*) PDO300 mutants complemented with plasmid-borne genes encoding Alg8 (polymerase), Alg44 (co-polymerase), AlgX (acetyltransferase), AlgG (epimerase), and inactive variants[a]

	Mutant	F_G	F_M	$F_{GM/MG}$	F_{MM}	Ac.%	$\overline{M_w}$ (kDa)	$\overline{M_n}$ (kDa)	pI	Alginate yield (g)/ CDM(g)
1	Wild type (*Pa*) +empty plasmid	0.3	0.7	0.29	0.41	32	3927 (±0.864%)	3832 (±0.842%)	1.025 (±1.2%)	1.3 ± 0.03
2	*Pa* Δ*alg8*+ MCS5:*alg8*	0.18	0.82	0.17	0.65	11.3	3045 (±0.556%)	3037 (±0.551%)	1.003 (±0.7%)	12.8 ± 1.03
3	*Pa* Δ*alg44*+ MCS5:*alg44*	0.18	0.82	0.18	0.64	26.8	3831 (±0.963%)	3650 (±0.950%)	1.05 (±1.3%)	8.7 ± 0.53
4	*Pa* Δ*alg44*Δ*alg8*+ MCS5:*alg44:alg8*	0.17	0.83	0.17	0.66	11	3369 (±0.839%)	3352 (±0.821%)	1.005 (±1.1%)	41.5 ± 4.9
5	*Pa* Δ*algX*+ MCS5:*algX*	0.36	0.64	0.36	0.28	9.8	2460 (±0.932%)	2447 (±0.913%)	1.005 (±1.3%)	104.1 ± 5.5
6	*Pa* Δ*algX*+ MCS5:*algX*(S269A)†	0.36	0.64	0.36	0.28	0	2086 (±0.960%)	2065 (±0.944%)	1.010 (±1.3%)	125.8 ± 9.9
7	*Pa* Δ*algG*+ MCS5:*algG*	0.32	0.68	0.32	0.36	23.3	2755 (±1.041%)	2726 (±0.986%)	1.011 (±1.4%)	2.6 ± 0.04
8	*Pa* Δ*algG*+ MCS5:*algG*(D324A)‡	0	1	0	1	25.2	4653 (±1.097%)	4575 (±1.117%)	1.017 (±1.5%)	7.6 ± 0.57
9	*Pa* Δ*algX*Δ*algG*+ MCS5:*algX:algG*	0.34	0.66	0.34	0.32	28.4	3076 (±1.051%)	3044 (±1.029%)	1.011 (±1.4%)	67.42 ± 4.8
10	*Pa* Δ*algX*Δ*algG*+ MCS5:*algX*(S269A): *algG*(D324A)	0	1	0	1	0	1811 (±0.884%)	1716 (±0.888%)	1.055 (±1.2%)	8.7 ± 0.42
11	*Pa* Δ*alg44*Δ*algG*+ MCS5:*alg44+algG*	0.22	0.78	0.22	0.56	14.5	2907 (±0.966%)	2861 (±0.944%)	1.016 (±1.3%)	9.0 ± 0.3

300 PDO300 strain, *MCS5* pBBR1MCS-5 plasmid, F_G molar fraction of guluronate (G) residue, F_M molar fraction of mannuronate (M) residue, $F_{GM/MG}$ molar fraction of two consecutive G and M residues, F_{MM} molar fraction of two consecutive M residues, *Ac.* acetylation, $\overline{M_w}$ weight-average molecular weights, $\overline{M_n}$ number-average molecular weights, pI polydispersity index, *CDM* cell dry mass. † catalytically inactive variant of AlgX acetyltransferase, ‡ catalytically inactive variant of AlgG epimerase

[a]Adapted from Ref. [22]

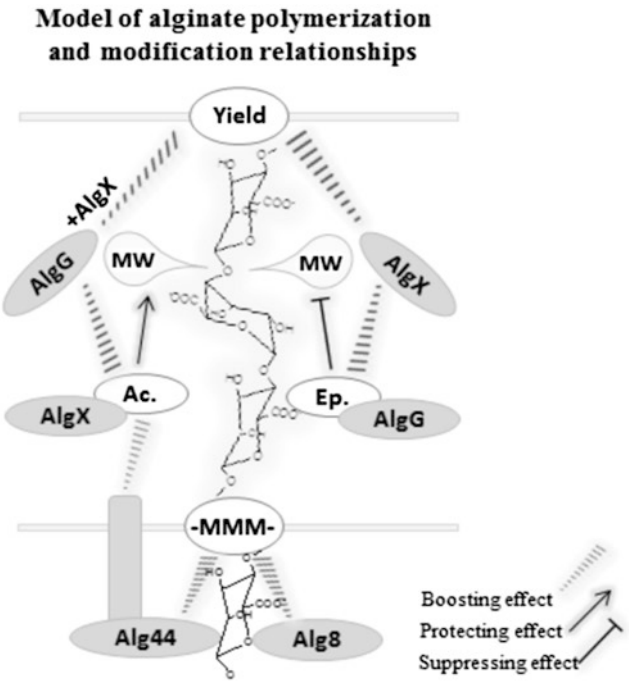

Fig. 1.5 Based on experimental results, interactive performances of protein functionality over alginate polymerization, acetylation, epimerization, and length determination are modeled. In this model, translocation of polymerized nascent alginate across the periplasmic scaffold is coupled with interactive functional performances of modification events where alginate molecular mass or polymerization is inversely correlated with alginate epimerization but positively correlated with acetylation. Also, Alg44 boost acetylation and AlgG and AlgX proteins display mutual auxiliary function for each other (Adapted from reference [22])

the lowest molecular mass (1811 kDa vs. 3076 kDa when both modification events present) (No. 10 in Figs. 1.4, 1.5, and Table 1.2).

Furthermore, the overproduction of Alg8 and Alg44 impacted on polymerization event by increasing the ratio of M residue and M blocks, while Alg44 boosted the acetylation degree (Figs. 1.4, 1.5, and Table 1.2) [22, 103].

Importantly, all tested strains harboring various combinations of Alg8, Alg44, AlgG, and AlgX and their catalytically inactive variants produced monodisperse alginates (a monodisperse polymer has a polydispersity index equal or close to 1.0) [22]. This study showed that bacterial alginate is an ideal source of monodisperse alginates versus algal alginates which are polydisperse.

Also, particle tracking microrheology was applied to assess the viscoelastic properties of the various resulting alginates [22]. All alginates showed viscoelastic properties in which the solid-like elastic modulus G' was greater than the liquid-like viscous modulus G'' ($G' > G''$), but significantly different from each other. Generally, the alginates without G residues or with the highest molar fraction of MM blocks

(F_M = 0.82 to 1.0), which possessed higher molecular mass, showed the highest and quite similar viscoelastic properties (G' = 0.41, G'' = 0.28–0.3) [22]. Lower viscoelastic properties were found for alginates with a molecular mass of ≤2000 kDa. Surprisingly, introducing high copy of alginate acetyltransferase AlgX in *P. aeruginosa* resulted in alginate with the lowest viscoelastic property among all analyzed samples because of boosting epimerization event and higher occurrence of G residues [22]. These results suggested that viscoelasticity was positively impacted by the molecular mass combined with high M content, while the presence of G residues and acetyl groups in the alginate chain lowered viscoelasticity [22].

Overall, this study had demonstrated the production of various alginates through manipulating the activity and production of various protein subunits in bacteria. Application of these biopolymers for specific purposes particularly in the context of biomedical application and generation of high-value products may require specific and desired properties of alginates. Therefore, molar fraction of M and G residues, the ratio of M and G blocks, acetylation degree, molecular mass (or polymerization degree), polydispersity, viscoelasticity, and other physicochemical properties are critical parameters which are obtainable by engineering bacterial alginate producers.

Another important step in the production of tailor-made alginate is to understand minimal protein requirements for alginate polymerization and modifications. For example, bacterial alginate polymerization is necessarily activated by the second messenger c-di-GMP, and MucR protein is specific c-di-GMP provider in alginate biosynthesis. Our recent analysis showed that site-specific mutagenesis of Alg8 at specific amino acid residues surrounding the catalytic sites could decouple alginate polymerization from MucR activity and presumably from proposed pool of c-di-GMP in the cell [103]. This finding may facilitate the production of alginate in nonpathogenic bacteria which may not generate the required localized pool of c-di-GMP for inducing alginate production. In addition, the production of polymannuronate (PolyM) alginates may be achievable by establishing only the polymerizing unit (Alg8-Alg44) as the minimal protein requirement in suitable Gram-positive bacteria which do not possess the outer membrane and the periplasm.

Production of alginates may be achievable through in vitro settings as previously reported [128]. However, tailoring alginates by combining minimal protein requirements alone or within particular lipid scaffolds such as proteoliposomes, lipid rafts, lipid discs, and inverted membrane vesicles may be approachable. Likewise, the combination of various alginate-modifying enzymes produced by various organisms will expand their applicability as well as the production of tailor-made alginates. For example, by applying the secreted mannuronan C-5-epimerases in in vitro reactions, the production of alginates with long G blocks and/or replacing stretches of M blocks with MG blocks has been achievable [129]. To this end, understanding the molecular mechanisms of various alginate-modifying enzymes such as alginate lyases and epimerases will provide further opportunities for bioengineering toward tailored alginates.

1.5 Conclusion and Future Trends

Alginates have been one of the most widely applied polysaccharides with uses in various industries due to their unique physicochemical properties. Traditionally, they have been largely utilized in food, cosmetic, and pharmaceutical industries as thickeners, stabilizers, viscosifiers, additives, gel and film formers, and fertilizers. Of the most relevant criteria which have made alginates particularly suitable for biomedical and pharmaceutical purposes are associated with their non-toxicity, biocompatibility, inertness, and modifiability. Hence, nowadays, various types of alginates and their derivatives have earned their reputation in drug delivery, cell encapsulation, and enzyme immobilization. In addition, technological advancement in three-dimensional printing and material fabrication as well as processing technologies harnessed unique properties of alginates for expanding their application for biomedical purposes. However, development of some high-value products from alginates and their derivatives may require defined compositions and material properties which might not exist in algal alginates or may not be achievable via chemical modifications. Therefore, bacterial alginates display different characteristics from algal alginates such as acetylation, higher molecular mass, monodispersity, different viscoelastic properties, and possibility to engineer the producer to produce tailored alginates with specifications for advanced biomedical uses. Most importantly, in contrast to algal alginates whose biosynthesis pathways and production in controlled environments have not been achieved yet, obtaining desired alginates from bacteria is achievable in a controlled environment (e.g., bioreactor) without excessive effort while eliminating oceanic impurities coming from algal resources.

Understanding functional and structural relationships of various protein subunits involved in alginate polymerization and modification is important for establishing bacterial production of tailored alginates. Recently we demonstrated production of various alginates via engineering *P. aeruginosa*. The unraveled interplay of polymerization with epimerization and acetylation offers bioengineering opportunities to produce various alginate compositions, molecular masses, and viscoelastic properties, while retaining monodispersity. However, bacterial production of alginates is still at early stage, as the molecular mechanism of biosynthesis/modification pathways need to be fully elucidated. In addition, these pathways are tightly controlled by bacterial regulatory systems which may be an obstacle for establishment of biotechnological production of alginates. Therefore, understanding the minimal protein requirements for bacterial production of alginates in homologous and heterologous hosts is of particular importance.

Currently most knowledge about alginate production was obtained in opportunistic human pathogen *P. aeruginosa*. Therefore, establishing the production of various alginates by nonpathogenic bacteria and non-virulent strains must be considered. Another potential challenge could be the purification process associated with alginates produced by bacteria. Existing commercial methods applied for purification of algal alginates to eliminate unwanted and immunogenic impurities may not be sufficient for removing impurities from bacterial production process. Overall,

bacterial alginates have largely remained unexplored for advanced biomedical uses and the production of high-value products. They exhibit different material properties when compared to algal alginates and can be tailored via bioengineering/synthetic biology toward the production of novel and advanced alginates.

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Chapter 2
Alginate Production from Marine Macroalgae, with Emphasis on Kelp Farming

César Peteiro

Abstract Alginates are produced industrially from marine macroalgae (also called seaweeds) belonging to the taxonomic group of brown algae (phylum Ochrophyta, class Phaeophyceae). In particular, the seaweeds commonly known as kelps (order Laminariales) are the most widely exploited worldwide as raw materials for alginate production. Alginophytes (i.e. alginate-yielding seaweeds) are mainly harvested from wild populations, although some of the raw material that is used in the alginate industry comes from the cultivation of the kelp *Saccharina japonica*. The demand for alginate production has increased over time, and it is likely to increase significantly in the future, particularly for the use of alginates in current and future biomedical and bioengineering applications. However, alginophyte resources are limited, and the natural kelp resources have declined worldwide in recent years. One way to meet the current and future demands of alginate-using industries is to encourage alginate production via kelp farming. The mariculture of the kelp *S. japonica* has already been well developed in Asia, and the cultivation of other kelp species is currently also being attempted in Europe and the Americas. This chapter provides an overview of seaweeds as a feedstock for alginate production, with emphasis on kelp farming to ensure a sustainable supply of alginates required for many applications. It describes the major stages for the cultivation of *Saccharina* and any other kelp, as well as the economic and environmental benefits of integrated kelp aquaculture to produce alginates, in addition to other value-added products.

Keywords Alginate extraction • Alginate production • Alginophytes • Aquaculture • Brown seaweeds • Kelp farming • Marine macroalgae • Seaweed alginate • Seaweed resources

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2.1 Introduction

Alginate, also called algin, is the generic name for the salts of alginic acid or any of the derivatives of this compound. It belongs to the family of linear unbranched polysaccharides, which consists of binary copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G) units linked together by 1 → 4 glycosidic bonds (see representation of the two monomeric units of alginic acid in Fig. 2.1). The monomers are mainly arranged in sequences of homopolymeric blocks (MM and GG blocks) and heteropolymeric blocks (MG or GM blocks) [1–3]. The block types and their respective chair conformations are shown in Fig. 2.2. The monomer sequence distribution in the copolymer gives rise to a flat ribbonlike structure for the MM blocks, a buckled ribbonlike structure for the GG blocks and a helix-like structure for the MG or GM blocks. The differences in conformation are due to the existence of a linkage in diequatorial position for the MM blocks, a linkage in diaxial position for the GG blocks and an equatorial/axial or axial/equatorial linkage for the MG or GM blocks, respectively. The linkage in the block structure results in varying degrees of stiffness or flexibility in alginates due to a greater or lesser hindrance of rotation around the glycosidic bonds. The polymer chains of alginates containing predominantly GG blocks are stiffer and possess a more extended chain conformation than those containing MM blocks, which in turn are stiffer than MG or GM blocks (i.e. the relative flexibility increasing in the order G block < M block < MG or GM block) [4–6].

Alginate structure depends fundamentally on the monomer composition, sequential structure and molecular weight of the polymeric chain. These structural parameters affect the chemical and physical properties of alginate, and these properties in turn have both biological and industrial significance [7–9]. Generally, chemical structure of alginate is typically described by the frequencies of monads (one monomer unit: M or G), dyads (blocks containing two monomer units: MM, GG, or MG = GM) and sometimes triads (blocks containing three monomer units: GGG,

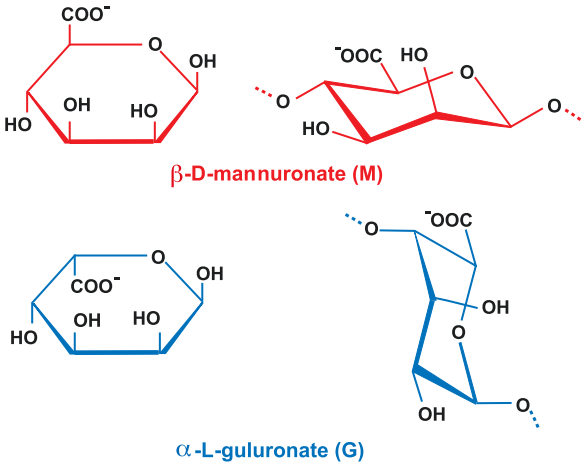

Fig. 2.1 Representation of Haworth conformation (left-hand side) and chair conformation (right-hand side) of the monomers in alginate chains

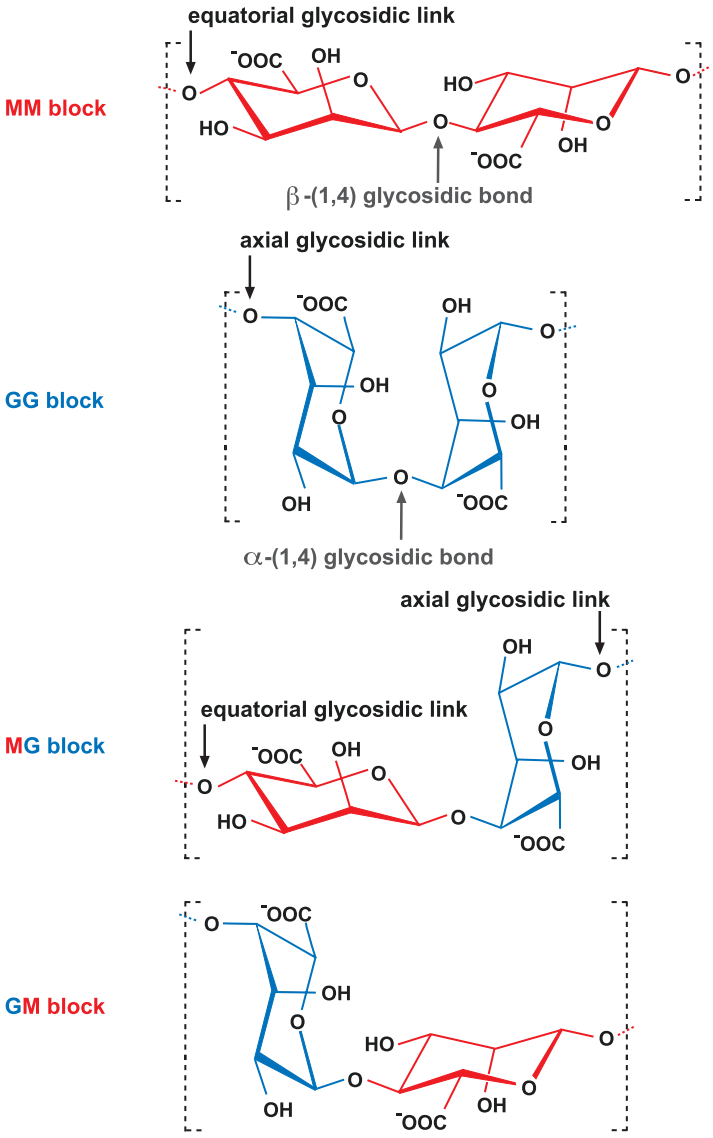

Fig. 2.2 Principal block structures in alginate chair conformation: M block, G block and MG or GM block

MGM, or GGM = MGG) [10–14]. The monad frequencies (F_M and F_G), the dyad frequencies (F_{MM}, F_{GG} and $F_{MG} = F_{GM}$) and the triad frequencies (F_{GGG}, F_{MGG} and $F_{GGM,} = F_{MGG}$) are preferably expressed as a mole fraction [15–17], although it has previously been reported as a percentage [8, 15, 18]. In addition, commercial alginate is traditionally characterized by the ratio of mannuronic to guluronic acid (M/G), which is also currently estimated from monad frequencies [3, 17, 19]. These

key structural elements of alginate are obtained by applying various methods (for more details, see review in ref. [15]). Among all techniques used for the description of alginates, proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy is the most accurate method currently employed to determine both the composition and sequential structure of alginates.

One of the most important and useful properties of alginates is their ability to form gels and stabilize emulsions in the presence of certain metal cations, particularly divalent cations such as calcium (Ca^{2+}), through a cross-linking reaction [20–22]. This ability is conventionally described in terms of the so-called "egg-box" model proposed by Grant and co-workers [20]. According to this model, the divalent cations are embedded into cavities formed naturally by two adjacent polymer chains containing GG blocks in a helical conformation. The alginate chains thereby adopt a structure that resembles an "egg-box", hence the name given to this model. The mechanism for the alginate gelation may involve the ionic-bonding interaction of cations with carboxyl groups and the hydrogen-bonding interaction of these cross-linking agents with oxygen atoms, in both cases between the guluronic acid blocks of two adjacent polymer chains [5, 21, 22]. The alginate gelation process with divalent calcium cations is depicted in Fig. 2.3.

Alginate gel formation is mainly dependent on the type and concentration of cross-linking agents, as well as the composition, sequence and polymer chain length of the alginate; these features determine the physical properties of the gels formed [23–25]. For example, the binding affinity of alginates for different divalent cations has been shown to increase in the following order: barium (Ba^{2+}) > strontium (Sr^{2+}) > calcium (Ca^{2+}) > magnesium (Mg^{2+}), as well as increasing with the density of the crosslinkers. In addition, alginates with a high guluronic acid (G) content display a higher affinity towards these crosslinkers than do alginates with high mannuronic acid (M) content [20, 25–27]. Essentially, gel strength and viscosity are the two most important physical properties used to assess the gelling capability of alginates [23, 28, 29]. While the gel strength is mainly dependent on the content and length of the guluronic acid (G) in the alginate [26, 28, 30], the viscosity of an alginate solution is directly determined by the alginate concentration and the chain length of the alginate polymer, which is proportional to its molecular weight [17, 31, 32]. Generally, alginates rich in guluronic acid are known to form strong but brittle gels, whereas those rich in mannuronic acid or mixed sequences form weaker but more flexible gels [27, 33, 34]. Thus, gel strength has also been shown to increase in the order of GG block > MG block > MM block [26, 27].

The physical and chemical properties vary considerably among different commercial alginates. This natural variability in alginates provides a wide range of functional properties that determine their use in specific applications and thus also their commercial value [8, 9, 34]. Furthermore, enzymatic and chemical modifications have been used to manipulate the composition, sequential structure and molecular weights of alginates, and their derivatives exhibit novel or improved functional properties for specific high-value applications [35–37].

Alginate was discovered in 1881 by the British pharmacist Stanford [38, 39], and it has since become one of the most useful and versatile polymers, used in a wide range of industries. Because of their gelling, thickening, emulsifying and stabilizing

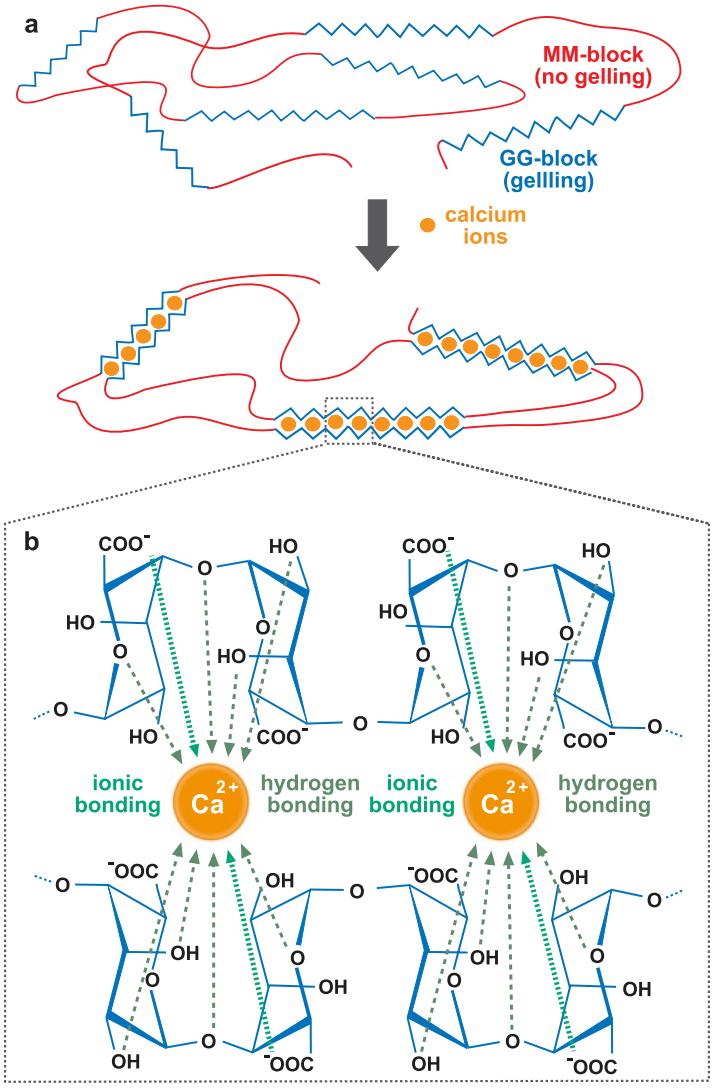

Fig. 2.3 Schematic representation of the egg-box model for calcium alginate gelation. (**a**) Illustration of the binding of polymer chains and (**b**) the formation of junction zones in alginate gels

properties, alginates have been commonly employed in the food, textile printing, papermaking and pharmaceutical industries, as well as for many other purposes. Alginates are especially important in the food and beverage industry, in which they are used as food additives or functional food ingredients in a vast array of different dairy products [9, 33, 40]. Alginates are internationally accepted food additives and are therefore explicitly listed as human food ingredients by the European Union (EU) and as "generally recognized as safe" (GRAS) by the US Food and Drug Administration (FDA), as well as being recognized as such in the United Nations

Codex Alimentarius (Latin for "Food Code") established by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). In particular, the reference codes of the European Union for the different alginates used in the food industries are E400 (alginic acid), E401 (sodium alginate), E402 (potassium alginate), E403 (ammonium alginate), E404 (calcium alginate) and E405 (propylene glycol alginate, usually abbreviated as PGA) [15, 40].

More recently, the alginates have found a wide variety of applications in biomedical and bioengineering fields. The interest in and use of alginates for biomedical applications has expanded considerably in recent years because of alginates' unique and favourable properties such as gelling capacity, biocompatibility, biodegradability and lack of toxicity as well as their biological and pharmacological activities [7, 8, 41]. The current biomedical applications of alginates are the focus of this book, and an updated and detailed review of the subject may be found in the different chapters. Although the food and textile uses are still the most important markets worldwide for alginates, there are growing markets in the bioscience, bioengineering and medical fields. The demand for alginate production has increased during recent years, and it is likely to increase significantly in the future, particularly for their use in current and future biomedical and bioengineering applications worldwide [42–44].

Alginates occur naturally as a major structural component in marine macroalgae (also called seaweeds) belonging to the taxonomic group of brown algae (phylum Ochrophyta, class Phaeophyceae) [42, 44, 45] and are also produced as extracellular polysaccharides (exopolysaccharides) by some bacteria belonging to the genera *Pseudomonas* and *Azotobacter* [46–48]. Currently, all commercial alginates are produced solely from brown seaweeds [43, 44] because most species contain large amounts of alginate [44, 49, 50] and because of the availability of seaweed resources, as they can be harvested from natural populations and farmed in the sea [42, 51, 52]. The industrially most important seaweeds used worldwide for alginate production are the species commonly known as kelp (order Laminariales) [42–44]. This review focuses on the source of seaweed alginate, the process for alginate extraction and the availability of seaweed resources and their exploitation. It also summarizes the techniques that have been developed for the commercial-scale farming of kelps as well as describes the important environmental benefits associated with their cultivation.

2.2 Alginate Production from Marine Macroalgae

2.2.1 Seaweeds Used as Alginate Sources

The term algae (singular, alga) is commonly used to refer to a large and diverse group of aquatic photosynthetic organisms that can grow in marine, brackish and freshwater environments. Based on morphology and size, algae are generally grouped into two categories: macroalgae and microalgae. Macroalgae are

multicellular forms, often with plant-like structures, ranging in length from a few millimetres up to 50 m, which typically live on hard-bottom substrates (i.e. benthic) of coastal marine habitats. In contrast, microalgae are unicellular or simple forms with a size range of a few micrometres up to hundreds of millimetres, which typically grow suspended in water [53, 54]. Marine macroalgae, so-called seaweeds, are classified primarily on the basis of their photosynthetic pigment composition into three different phyla (taxonomic groups): Ochrophyta (brown algae), Rhodophyta (red seaweed) and Chlorophyta (green algae) [55]. For example, the presence of the pigment fucoxanthin is responsible for the characteristic yellow-brown colour of brown algae. In addition, these taxonomic groups also differ in many ways, particularly in their morphology, life history, storage compounds and cell wall polysaccharides [53, 54].

Alginate is characteristically present in most or all species of brown algae, which belong to the class Phaeophyceae (phylum Ochrophyta, formerly named Phaeophyta), as a structural component of the matrix of the cell wall and intercellular regions. In these seaweeds, alginate is found in the form of insoluble mixed salts of alginic acid, mainly with calcium and to a lesser extent with sodium, potassium, and magnesium, strontium and barium, among other ions naturally found in seawater [1, 56, 57]. Its biological function is primarily skeletal, giving the algae both the mechanical strength and the flexibility necessary to withstand the force of the sea. Indeed, functional differences in the alginate content and structure of seaweeds have been reported. For example, the seaweeds growing in more wave-exposed habitats have alginates with higher mannuronic acid content than those in wave-sheltered habitats, providing greater flexibility to withstand the wave action [58–60]. It has also been observed that the part of the thallus (plural, thalli) that attaches the algae to a hard substrate (the so-called holdfast) contained more guluronic acid than in the rest of the thallus, giving it more rigidity and thereby affixing it more firmly to the rock [59–61]. In addition, alginate plays important roles in high ion-exchange equilibrium with seawater as well as functioning in retarding desiccation when the seaweeds are exposed to the air during low tide [56, 57, 62].

Brown seaweeds of the class Phaeophyceae (Ochrophyta) and in particular some species of the orders Laminariales and Fucales (commonly known as kelps and fucoids, respectively) have large amounts of alginate, comprising up to 55% of their dry weight (Table 2.1). Both kelps and fucoids are the largest and most structurally complex brown seaweeds. Generally, and in particular in kelps, the body or thallus of the macroalgae consists of a holdfast (root-like), stipe (stem-like) and blade (leaf-like) (see Fig. 2.4, in which some kelp species are illustrated). At present, commercial alginates are produced mainly from brown seaweeds of the genera *Laminaria*, *Saccharina*, *Lessonia*, *Macrocystis*, *Durvillaea*, *Ecklonia* and *Ascophyllum* [42, 43] (Fig. 2.4). Specifically, the industrially most important alginate-yielding species (alginophytes) are currently the kelps (Laminariales) *Macrocystis pyrifera*, *Laminaria hyperborea*, *Laminaria digitata*, *Saccharina japonica*, *Lessonia nigrescens* species complex, *Lessonia trabeculata*, *Ecklonia arborea* and *Ecklonia radiata* as well as the fucoids (Fucales) *Durvillaea potatorum* and *Ascophyllum nodosum* [42, 43] (more information on these alginophyte resources will be described in Sect. 2.2.3).

Table 2.1 Alginate yields from the brown seaweeds used for industrial production

Seaweed species	Alginate content (DW)		Country of origin	Sampling month	References
	Range	Mean			
Macrocystis pyrifera	26–37%	n.d.	Mexico	Feb.–Nov. (monthly)	[63]
	18–45%	n.d.	Chile	Year-round	[50]
Laminaria digitata	18–26%	n.d.	United Kingdom	Year-round	[60]
	16–36%	n.d.	Denmark	Year-round	[64]
Laminaria hyperborea	14–21%	n.d.	United Kingdom	Year-round	[60]
Saccharina japonica	15–20%	n.d.	China	Mar., Apr., May	[65]
	17–25%	n.d.	Japan	Mar.–Oct. (monthly)	[66]
Saccharina latissima	16–34%	n.d.	Denmark	Year-round	[64]
Lessonia trabeculata	13–29%	n.d.	Chile	Jul.	[67]
Ecklonia arborea	24–28%	n.d.	Mexico	Feb., May., Aug., Nov.	[63]
Durvillaea potatorum	n.d.	55%	Australia	Mar.	[68]
	n.d.	45%	New Zealand	n.d.	[69]
Ascophyllum nodosum	12–16%	n.d.	Russia	Apr., Aug., Dec.	[70]

Alginate yield based on the dry seaweed weight (DW). Data obtained from the seaweed thallus and using similar alginate extraction methods
n.d no available data

Tables 2.1 and 2.2 present the alginate yield and chemical composition of the main kelp and fucoid species used worldwide for alginate production. These parameters vary considerably between and within brown seaweed species. Based on these data, the highest levels of alginate are found in *Durvillaea potatorum* and *Macrocystis pyrifera*, in which alginate represents up to 55% and 45% of the dry seaweed weight, respectively, while the lowest levels are in *Ascophyllum nodosum* and *Laminaria hyperborea*, in which alginate represents 12% and 13% of the dry weight. Regarding structural parameters, *Lessonia trabeculata* and *Laminaria hyperborea* have the highest fraction of guluronic acid (M/G ratio of <1), while the lowest proportions of guluronic acid are observed in *Durvillaea potatorum* and *D. antarctica* and to a lesser extent in *Saccharina japonica*, *Macrocystis pyrifera* and *Ascophyllum nodosum* (all with M/G ratios of >1.5). Brown seaweeds are well known to exhibit some seasonal variation both in alginate chemical structure and alginate content, which can be higher or lower depending on the species [49, 50, 75]. For example, the content of alginate in *Macrocystis pyrifera* is highly variable throughout the year, in contrast to that in *Laminaria digitata*, which is much more stable (Fig. 2.5). Similarly, there may also be seasonal differences in the structural characteristics of alginate, as in the case of *Saccharina latissima*, whose M/G ratio varies seasonally. However, there is hardly any variation in the M/G ratio over the

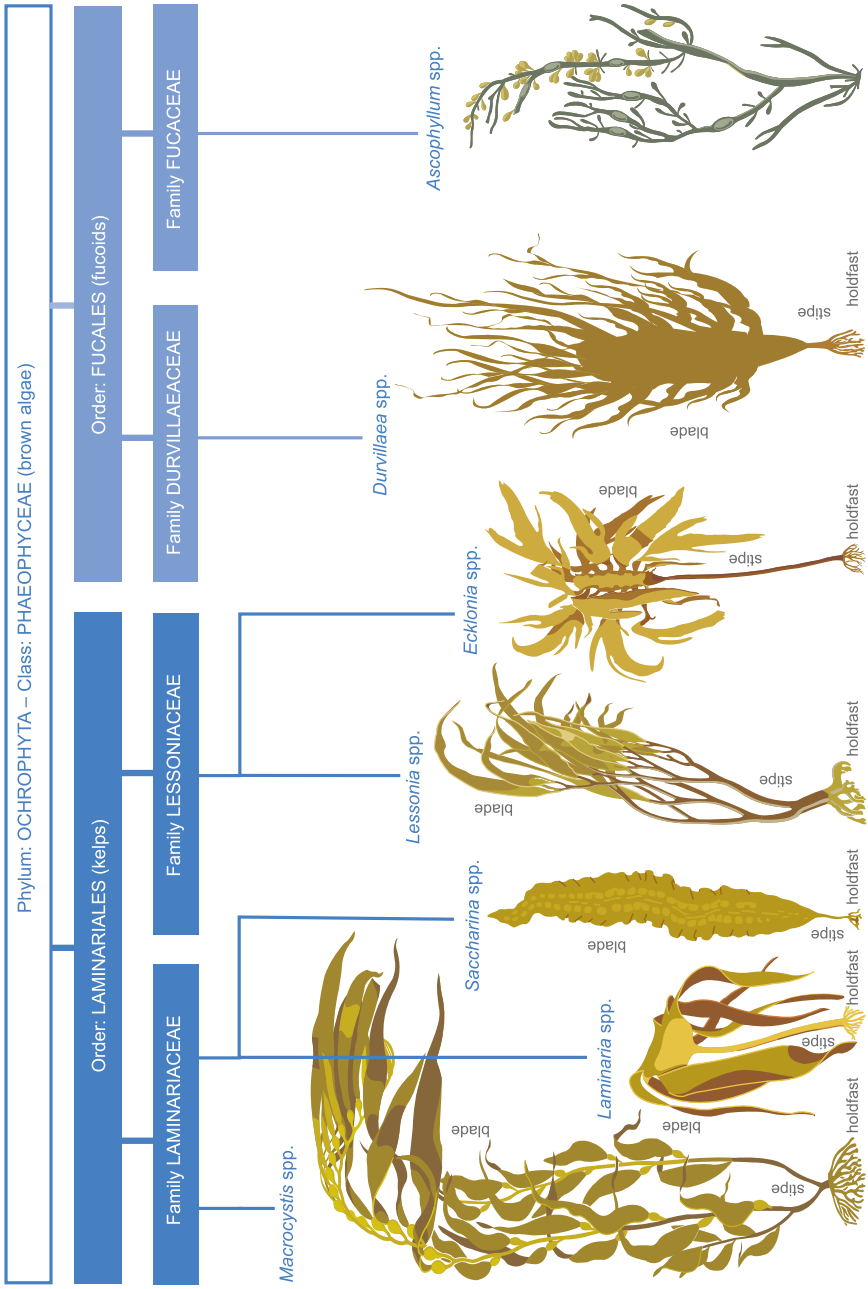

Fig. 2.4 The industrially most important alginate-yielding seaweeds worldwide (Data from Refs. [42, 43]. Taxonomic classification based on Algaebase [55]. The drawings are not completely to scale between the different seaweeds in order to improve the view. Note that, when referring collectively to some or all of the species in a genus, the generic name is followed by spp)

Table 2.2 Composition and sequence of alginates obtained from various brown seaweeds

Seaweed species	Composition			Sequence			Country of origin	References
	F_M	F_G	M/G	F_{MM}	F_{GG}	$F_{MG,\ GM}$		
Macrocystis pyrifera	0.62	0.38	1.63	0.42	0.18	0.20	Mexico	[10]
Laminaria digitata	0.59	0.41	1.43	0.43	0.25	0.16	Norway	[17]
Laminaria hyperborea	0.45	0.55	0.81	0.28	0.38	0.17	n.d.	[17]
Laminaria hyperborea (stipe)	0.32	0.68	0.47	0.20	0.56	0.12	n.d.	[17]
Saccharina japonica	0.65	0.35	1.85	0.48	0.18	0.17	China	[71]
Saccharina latissima	0.45	0.55	0.82	0.33	0.43	0.12	Norway	[72]
Saccharina longicruris	0.41	0.59	0.69	0.07	0.25	0.34	Canada, May	[16]
Lessonia nigrescens species complex	0.59	0.41	1.43	0.40	0.22	0.19	n.d.	[13]
Lessonia trabeculata	0.38	0.62	0.61	0.21	0.47	0.15	Chile	[58]
Lessonia trabeculata (stipe)	0.22	0.78	0.28	0.10	0.67	0.11	Chile	[73]
Ecklonia arborea	0.52	0.48	1.08	0.37	0.33	0.15	Mexico	[10]
Ecklonia maxima	0.55	0.45	1.22	0.32	0.22	0.32	n.d.	[15]
Durvillaea potatorum	0.76	0.24	3.17	0.58	0.06	0.18	New Zealand	[69]
Durvillaea antarctica	0.68	0.32	2.15	0.51	0.16	0.17	n.d.	[13]
Ascophyllum nodosum	0.61	0.39	1.56	0.46	0.23	0.16	n.d.	[74]

Structural parameters determined by proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy. Data were obtained from the same tissue type, the thallus (except where specified), and using similar alginate extraction methods

course of a year in species such as *Laminaria digitata* (Fig. 2.6). The seasonal variability of alginate is related mainly to seasonal changes in temperature as well as to nutrient and light availability, most often influencing seaweed growth [76–78]. It has been reported that the highest values of alginate in some kelps and fucoids occur in the summer months [49, 50, 70]. However, in general, it appears that there is no overall pattern of seasonal variation, and the same applies to the composition of alginates. Thus, it is essential to know the seasonal composition of alginates in brown seaweeds in order to determine the optimal harvesting time by which to obtain not only higher quantities but, above all, alginates of better quality, i.e. those with high G content.

Nevertheless, alginate content and structure also depend on the age and part of the seaweed used [50, 59, 61] as well as on the environmental conditions of the habitat in which the seaweed grew [58, 59, 64]. In general terms, thalli from older seaweeds are richer in mannuronic acid than those from younger specimens [17, 33]. In addition, compared with the blades, the stipes of kelp species generally contain a higher amount of alginate rich in guluronic acid [62, 75, 79]. Indeed, the highest content of guluronic acid in alginate is obtained from stipes of the kelps

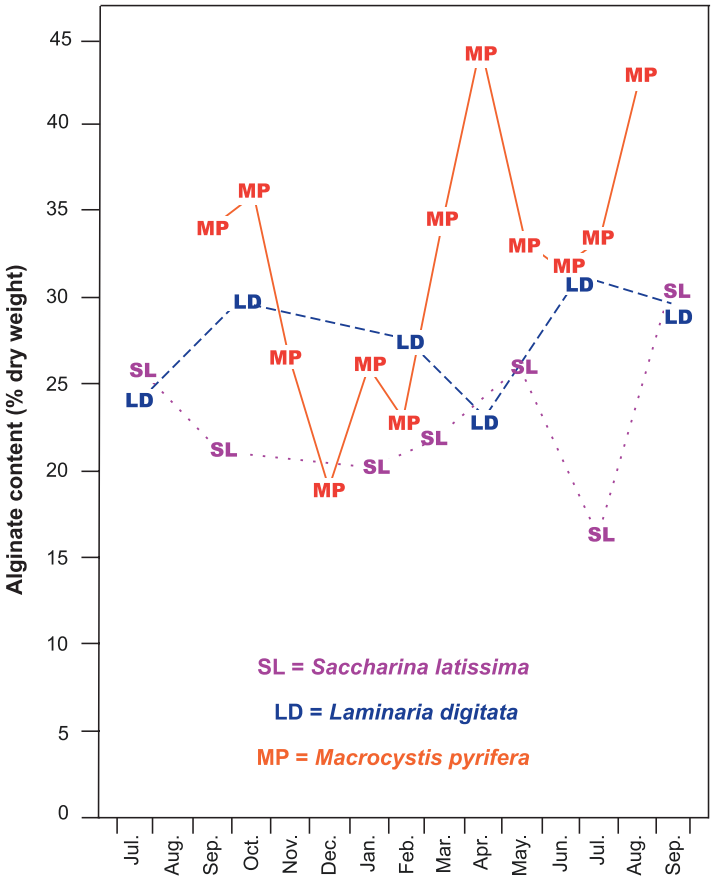

Fig. 2.5 Seasonal variation in alginate content of the brown seaweeds *Macrocystis pyrifera* (MP), *Saccharina latissima* (SL) and *Laminaria hyperborea* (LH) (Data from Refs. [49, 50])

Lessonia trabeculata (F_G of 0.78) and *Laminaria hyperborea* (F_G of 0.68) (Table. 2.2). Thus, alginate companies produce guluronic acid-rich alginates (designated high G) prepared from stipes of these species [43]. As mentioned above, differences in alginate composition in relation to the hydrodynamic environment of the seaweeds have also been reported [58–60].

2.2.2 *Alginate Extraction from Seaweeds*

Various commercial types or forms of alginate (sodium alginate, potassium alginate, ammonium alginate, magnesium alginate, calcium alginate and propylene glycol alginate, commonly abbreviated as PGA) are prepared from brown seaweeds

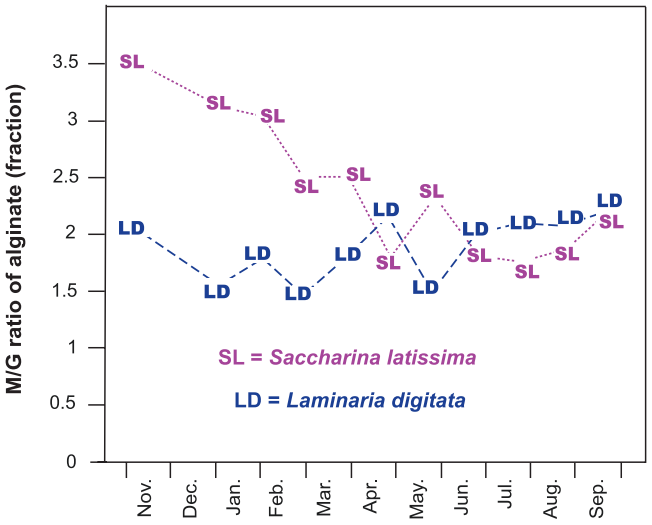

Fig. 2.6 Seasonal variation in mannuronic to guluronic acid ratios (M/G) of alginate from the brown seaweeds *Saccharina latissima* (SL) and *Laminaria digitata* (LD) (Data from Ref. [64])

[80]. All these derivatives of alginic acid are industrially produced following the same manufacturing process that will be described here based on sodium alginate extraction. The process of alginate extraction from seaweeds is based on the conversion in an alkaline medium of the water-insoluble mixed salts of alginic acid from algal cell wall matrix to water-soluble salts, normally sodium alginate, followed by precipitation and purification [42, 81, 82]. This conventional procedure for alginate extraction has been well studied during the last decade to optimize the yield and quality of alginate for various applications [81, 83, 84]. Today, it is commonly used in the industry to produce alginates from brown seaweeds [42, 80].

The following will present in detail each of the steps constituting the process of producing seaweed alginate. The alginate extraction procedure is schematically illustrated in Fig. 2.7. Generally, it consists of three major steps: (1) pre-extraction, (2) neutralization and (3) precipitation/purification [42, 81, 82].

In the first step, the seaweeds (usually dried) are washed with distilled water and then ground to speed up the chemical reactions for the extraction of alginic acid. Further, 0.1% formaldehyde solution may be added in order to avoid pigments in alginate; this has been seen to increase the alginate yield. The milled algal biomass is then dissolved and stirred with a dilute mineral acid up to pH 4 (usually hydrochloric acid (HCl) or calcium chloride ($CaCl_2$) at 0.1–0.2 M) to remove counter ions (Ca^{2+}, Na^{+}, Mg^{2+}, Sr^{2+}, etc.) of algal alginate by ion exchange with protons from the acid [81, 85]. The acid treatment is also effective in removing potential contaminants or impurities (fucoidans, laminarins, proteins and polyphenols), leading to a higher final yield and purity of alginate. In addition, 85% ethanol can be used to extract pigments and proteins in this process [16]. This pretreatment is often repeated several times to ensure full extraction of alginic acid. At the end of this process, the supernatant (residual algal particles) is eliminated [81, 85].

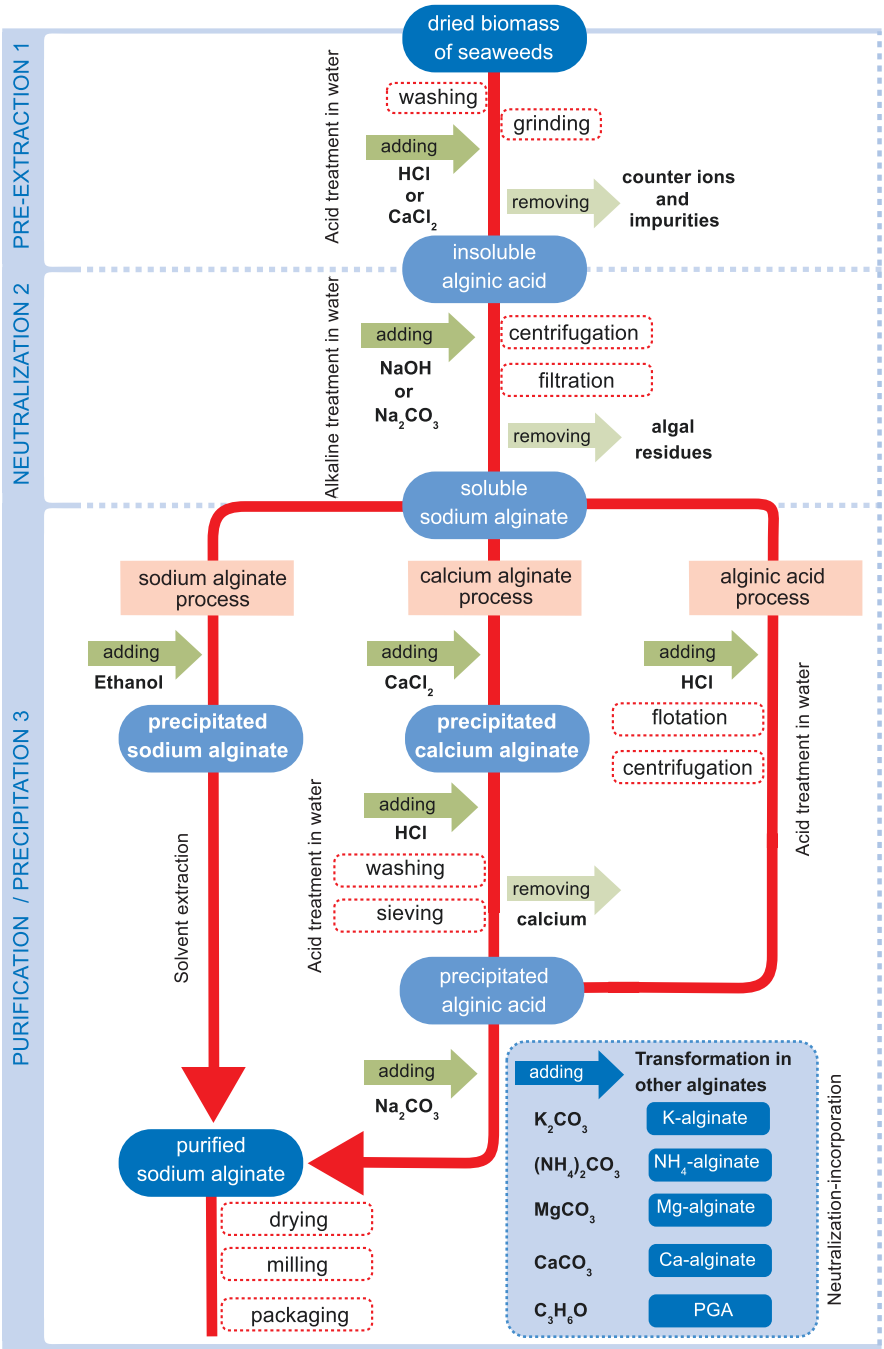

Fig. 2.7 Schematic flow chart of the process of extracting sodium alginate and other forms of alginates from seaweeds

In the second step, the insoluble alginic acid in the seaweed-water mixture is brought to pH 9–10 with an alkaline solution (usually sodium carbonate (Na_2CO_3) or sodium hydroxide (NaOH)) to form water-soluble sodium alginate. In this process, the mixture is mechanically stirred, and the temperature is maintained at 60–80 °C. The insoluble algal residues are removed by extensive centrifugation and subsequent filtration (up to 0.2 μm pore size), thereby obtaining the sodium alginate in aqueous solution [16, 81].

In the third step, the sodium alginate solution can be precipitated into sodium alginate, calcium alginate or alginic acid by the addition, respectively, of alcohol (usually ethanol (C_2H_6O)), calcium chloride ($CaCl_2$) and hydrochloric acid (HCl). These three methods are therefore known as the sodium alginate process or ethanol route, the calcium alginate process or $CaCl_2$ route and the alginic acid process or HCl route [42, 81, 85]. The sodium alginate precipitated via the ethanol route is obtained directly by the addition of ethanol, and it is separated by solvent extraction/evaporation. The $CaCl_2$ route first produces a precipitated calcium alginate that is isolated by sieving and rinsed with distilled water to remove the excess calcium. It is then converted to alginic acid by acid treatment, generally using hydrohydrochloric acid (HCl) as described above in the first step. The HCl route directly yields alginic acid, which is separated from the solution by simple flotation and centrifugation. The resulting alginic acid can also be reconverted by alkaline neutralization to any of the commercial forms of alginate in the same manner as described in the second step. Specifically, sodium carbonate (Na_2CO_3), potassium carbonate (K_2CO_3), ammonium carbonate ($(NH_4)_2CO3$), magnesium carbonate ($MgCO_3$), calcium carbonate ($CaCO_3$) or propylene oxide (C_3H_6O) is added in order to obtain the following alginates, respectively: sodium alginate (Na-alginate), potassium alginate (K-alginate), ammonium alginate (NH_4-alginate), magnesium alginate (Mg-alginate), calcium alginate (Ca-alginate) and propylene glycol alginate (PGA). Finally, all alginates form a paste that is separated, dried and milled. Commercial alginates produced specifically for biomedical purposes (e.g. ultrapure and amitogenic alginates) are prepared using more rigorous extraction processes to remove any biological and inorganic impurities, and companies consider these processes confidential. However, the alginates obtained from the described methods usually contain some impurities, making them unsuitable for some biomedical applications. In this case, an alternative extraction method using barium ions (Ba^{2+}) is used in the precipitation process due to their high binding affinity and selectivity towards alginates. Subsequently, the Ba^{2+} from the alginate is exchanged for sodium ions to form sodium alginate, which can be precipitated using ethanol [36].

It is known that the alginate extraction process can influence the yield and chemical compositions as well as rheological properties of the isolated alginates [42, 81, 85]. To illustrate these effects, Table 2.3 summarizes the comparative results of three precipitation methods in the process of alginate extraction from the brown seaweed *Macrocystis pyrifera*. According to these data, the sodium alginate process or ethanol route gives the highest yield and rheological properties of alginates, although very similar results can be obtained from the alginic acid process or HCl route. Clearly, the calcium alginate process or $CaCl_2$ route results in an alginate with poor viscoelastic properties and low toughness [85].

Table 2.3 Yields and properties of alginate obtained from the seaweed *Macrocystis pyrifera* using three precipitation methods in the alginate extraction process

Precipitation methods	Extraction yield (% DW)	Composition M/G	Sequence F_{GG}	Molar mass M_w (kg/mol)	Viscosity [ŋ] (mL/g)
$CaCl_2$ route	28	1.045	0.730	75	160
HCl route	27	1.295	0.675	220	505
Ethanol route	33	1.170	0.690	297	575

Data from Ref. [85]. Alginate yield based on the dry weight of the seaweed samples (DT). If there are several values from different samples, the average is shown

Overall, it is also important to control both pH and temperature during all extraction processes to improve the yield and quality of the alginate obtained. For example, it is well known that acid treatment at a pH lower than 4 may break bonds of the polymer chain, decreasing the alginate viscosity [81, 84, 85]. In addition, it has been shown that high temperatures (higher than 80 °C) and longer extraction processes increase yield but decrease viscosity [81, 84].

2.2.3 Alginophyte Resources Worldwide

Brown seaweeds have been worldwide resources for alginate extraction since industrial alginate production began in 1929 in California, USA, shortly thereafter, beginning in 1939 in several European countries and Japan and, more recently in the 1980s, in China [42, 43, 81]. Currently, the alginate industry is concentrated into 15 factories in 6 different countries (China, the USA, the United Kingdom, Japan, Chile and Germany), and most of these factories are now in China (see compilation in ref. [86] for more details on commercial companies selling alginates). According to the latest available data for 2009, the world market for alginates is approximately 26,500 tons (dry weight), with an estimated sale value of US$ 318 million annually [43]. Alginate production worldwide is derived from seaweed resources, most of which are harvested from the wild, except in China, where seaweed is sourced mostly from aquaculture [42, 43, 81].

Although there are over 2000 species of brown seaweeds (phylum Ochrophyta, class Phaeophyceae) [55], only some species of the orders *Laminariales* and *Fucales* (kelp and fucoid) are exploited worldwide as raw material for alginate production. It is estimated that there currently are at least 38 species of kelps and fucoids grown in 24 countries that are used worldwide to produce alginates [51, 52, 87]. A list of alginate-yielding seaweeds (or alginophytes) and their countries of origin is provided in Table 2.4, which includes the full names of the species and their taxonomic classifications (updated). However, many of these seaweeds are harvested from small local stocks and are therefore not available on the international market. In addition, some seaweed species (e.g. *Sargassum* species) are only used occasionally for alginate production, when the main commercial sources are unavailable, because their alginate is usually judged to be of "borderline" quality [42, 43]. Thus, the

Table 2.4 Species of brown seaweeds used in various countries for alginate production

Seaweed species (arranged by order and family)	Country
Order: Laminariales	
Family: Laminariaceae	
Macrocystis pyrifera (Linnaeus) C.Agardh 1820	PE, CL, MX, US, CA, NZ
Laminaria digitata (Hudson) J.V.Lamouroux 1813	FR, IS, DK
Laminaria hyperborea (Gunnerus) Foslie 1884	ES, FR, IE, NO, RU
Laminaria longipes Bory 1826	RU
Laminaria ochroleuca Bachelot de la Pylaie 1824	ES
Saccharina angustata (Kjellman) C.E.Lane, C.Mayes, Druehl & G.W.Saunders 2006	JP, RU
Saccharina bongardiana (Postels & Ruprecht) Selivanova, Zhigadlova & G.I.Hansen 2007	RU
Saccharina cichorioides (Miyabe) C.E.Lane, C.Mayes, Druehl & G.W.Saunders 2006	RU
Saccharina gurjanovae (A.D.Zinova) Selivanova, Zhigadlova & G.I.Hansen 2007	RU
Saccharina japonica (Areschoug) C.E.Lane, C.Mayes, Druehl & G.W.Saunders 2006	CN, KR, JP, RU
Saccharina latissima (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders	ES, FR, RU, CA
Family: Lessoniaceae	
Lessonia nigrescens Bory 1826	CL, PE
Lessonia trabeculata Villouta & Santelices 1986	CL
Lessonia berteroana Montagne 1842	CL, PE
Lessonia spicata (Suhr) Santelices 2012	CL, PE
Ecklonia arborea (Areschoug) M.D.Rothman, Mattio & J.J.Bolton 2015	MX
Ecklonia maxima (Osbeck) Papenfuss 1940	ZA
Ecklonia radiata (C.Agardh) J.Agardh 1848	AU, NZ
Order: Fucales	
Family: Durvillaeaceae	
Durvillaea potatorum (Labillardière) Areschoug 1854	AU
Durvillaea antarctica (Chamisso) Hariot 1892	CL
Family: Fucaceae	
Ascophyllum nodosum (Linnaeus) Le Jolis 1863	FR, IE, IS, NO, US, CA
Fucus serratus Linnaeus 1753	IE
Fucus vesiculosus Linnaeus 1753	IE
Family: Sargassaceae	
Sargassum swartzii C.Agardh Saunders 1820	IN
Sargassum aquifolium (Turner) C.Agardh 1820	PH
Sargassum cinctum J.Agardh 1848	PH
Sargassum hemiphyllum (Turner) C.Agardh 1820	PH

(continued)

Table 2.4 (continued)

Seaweed species (arranged by order and family)	Country
Sargassum ilicifolium (Turner) C.Agardh 1820	PH
Sargassum feldmannii Pham-Hoàng Hô	PH
Sargassum paniculatum J.Agardh 1848	PH
Sargassum polycystum C.Agardh 1824	CN, PH, ID, TH
Sargassum siliquosum J.Agardh 1848	PH, VN
Sargassum graminifolium C.Agardh 1820	VN
Sargassum henslowianum C.Agardh 1848	VN
Sargassum mcclurei Setchell 1933	VN
Family: Sargassaceae	
Turbinaria conoides (J.Agardh) Kützing 1860	IN
Turbinaria decurrens Bory 1828	IN
Turbinaria ornata (Turner) J.Agardh 1848	IN

Data from Refs. [42, 51, 52, 80, 87, 88]. Full names of species and their taxonomic classifications were validated with AlgaeBase [55] and updated when necessary. Country abbreviations: Australia (AU), Canada (CA), Chile (CL), China (CN), Denmark (DK), Spain (ES), France (FR), India (IN), Indonesia (ID), Ireland (IE), Iceland (IS), Japan (JP), Korea (KR), Mexico (MX), NO (Norway), New Zealand (NZ), Peru (PE), Philippines (PH), Russia (RS), Thailand (TH), United Kingdom (UK), United States of America (US), Vietnam (VN) and South Africa (ZA)

global alginate production worldwide comes from a small number of seaweed species, specifically the kelps *Macrocystis pyrifera*, *Laminaria hyperborea*, *L. digitata*, *Saccharina japonica*, *Lessonia nigrescens* species complex, *L. trabeculata*, *Ecklonia arborea*, and *Ecklonia radiata* and the fucoids *Durvillaea potatorum* and *Ascophyllum nodosum* [42, 43, 80]. These alginophytes, in addition to containing large amounts of alginate [43, 44, 51], form dense stands on shallow rocky shores commonly referred to as forests or beds. These seaweed species are distributed in cold-temperate waters around the world, and as photosynthetic organisms, they are restricted to habitats with appropriate light levels, primarily from the intertidal zone to a depth of 50 m in the sublittoral zone.

The quantities of alginophytes harvested worldwide in 2009 are summarized in Table 2.5, including the main producer countries and the quality or type of alginate obtained from seaweed species. These statistics are based on the most up-to-date and reliable estimates available [43], but some species names have been updated to the current taxonomy [55]. Indeed, recent revision of kelps based on the application of molecular techniques has greatly improved the taxonomic understanding of this group, resulting changes in the taxonomic identity and nomenclature of some commercialized species. For example, some species of the former *Laminaria* sensu lato have been transferred to the new genus *Saccharina*, including the cultivated *Laminaria japonica* (now *Saccharina japonica*) [89]. *Lessonia* species have also undergone taxonomic changes, and the former *Lessonia nigrescens* is now a species complex, i.e. encompassing multiple species that were hidden under a single name. Currently, there is genetic evidence of the presence of at least two cryptic species: *Lessonia berteroana* and *Lessonia spicata* [90, 91]. Moreover, *Lessonia flavicans*,

Table 2.5 Alginate production from brown seaweeds (alginophytes) in the world

Major alginophytes (species)	Alginate type[a]	Producer countries	Harvested (tonnes DW)	Contribution to total (%)
Macrocystis: *M. pyrifera*	Low G	US, MX, CL	5000	5
Laminaria: *L. digitata* and *L. hyperborea*	Med/high G	FR, IE, UK, NO	30,500	32
Saccharina: *S. japonica*	Med G	CN, JP	20,000	21
Lessonia: *L. nigrescens* species complex and *L. trabeculata*	Med/high G	CL, PE	31,000	33
Ecklonia: *E. maxima*	Med G	ZA	2000	2
Durvillaea: *D. potatorum*	High G	AU	4500	5
Ascophyllum: *A. nodosum*	Low G	FR, IS, IE, UK	2000	2
World total production			95,000	100

Quantities harvested of seaweeds are expressed on the basis of their dry weight (DW). Data yield for the year 2009 from Ref. [43] and commercial species used for alginate production from Refs. [42, 43, 80]. Seaweed groups and species names were updated to current taxonomy

[a]Type of alginate by measuring the guluronic acid content: low, medium and high. For country abbreviations, see Table 2.4

which has been reported traditionally in the alginate marketplace [43], actually corresponds to either *Lessonia nigrescens* or *L. trabeculata* [88, 92]. Finally, all species of the genus *Macrocystis* are now considered taxonomic synonyms of *Macrocystis pyrifera* [93, 94]. Independent of the current taxonomic status of seaweeds, the commercial companies selling seaweed or algal products generally continue to maintain the commercial names of their raw materials or products [92]. However, an adequate specific identification of seaweed species used as alginate sources is particularly important because of effects on chemical compositions and properties of the isolated alginate (see Sect. 2.2.1).

Based on data for 2009 from Table 2.5, the world harvest of alginophytes is estimated to be approximately 95,000 tonnes (dry seaweed weight) annually. The main alginophytes harvested worldwide are *Lessonia* and *Laminaria*, accounting for 65% of the total production, followed by *Saccharina* with 21% of the total. It is worth highlighting the drastic reduction in the harvest of *Macrocystis* and *Ascophyllum*, which were previously important raw materials for alginate production.

In the year 2009, these seaweeds supplied only 2% of the worldwide production, down from 58% in 1999 [43]. The reason for this change is the marked decrease in the use of these species because their alginate has low guluronic acid (G) content, while the current market is demanding alginates with intermediate or high G content [43, 81]. These commercial-grade alginates are now mainly used in biomedical applications and novel therapies [7, 36, 37], which have increased considerably in the last decade and are currently the most profitable, selling at the high end of the alginate market [43]. Regarding the kelp *Macrocystis*, harvesting has also been curtailed for ecological reasons due to concern about potential environmental effects of exploitation of kelp forests that provide habitat for many species [43].

Fig. 2.8 Worldwide distribution of seaweed resources harvested industrially for alginate production (Data from Refs. [43, 81])

The world alginate production is mainly produced from seaweed resources in 13 countries (Table 2.5, Fig. 2.8). Global distribution of alginophytes harvested industrially harvested from wild populations to meet the global demand for the alginate industry. Traditionally, harvesting of natural resources has been performed by hand, but the demand for larger quantities of seaweeds for alginate production at lower costs has led to the development of mechanized harvesters in some areas of California, Norway and France. However, alginophyte exploitation is still performed manually in most countries, such as Chile, Peru, Ireland, the United Kingdom, South Africa, New Zealand and Australia [42, 88, 95–98]. Macroalgae can be harvested manually on the shore by cutting attached seaweeds as well as by collecting drift or beach-cast seaweeds. For example, in some parts of Europe, the fucoid *Ascophyllum nodosum* and the kelp *Laminaria digitata* are cut by hand using a small knife at low tide while the seaweed is uncovered or while it remains submerged in less than a metre of water [42, 97]. *Durvillaea* and *Ecklonia* in Australia, and New Zealand and *Lessonia* in Chile are collected as drift or beach-cast, where they are washed ashore in large quantities by tides and waves [42, 88, 95, 98]. Mechanical harvesting using specially designed boats is done in Southern California (USA) and Baja California (Mexico) with *Macrocystis pyrifera*, in Norway with *Ascophyllum nodosum* and in France with *Laminaria digitata* [42, 99]. The *Macrocystis* forests are harvested using mowers from special ships that are fitted with underwater cutter bars at the front or rear that cut the seaweeds at 1 metre below the surface and a conveyor belt that moves the biomass into the hold of the vessel (Fig. 2.9) [42]. Small, flat-bottomed boats equipped with suction cutters and driven by paddlewheels or water jets are used for harvesting *Ascophyllum nodosum* in Norway [42, 99]. Exploitation of *Laminaria hyperborea* on the coast of Norway

Fig. 2.9 Vessel for harvesting the kelp *Macrocystis pyrifera* for alginate production along the coast of California (Photo by C. Peteiro)

is performed using trawler boats with a cutting dredge that is towed through the kelp beds to cut the seaweeds. In France, *Laminaria digitata* is harvested by boats with a hydraulic arm fitted with an iron hook on the end (called a "scoubidou") that rotates to wrap the kelp around itself [42, 99].

It has been demonstrated that kelp harvesting in various parts of the world may cause deterioration of natural resources or habitats or disturbance of species [100–102]. Kelps act as ecosystem engineers or foundation species, providing habitat, protection and food for numerous organisms in coastal ecosystems, in the same way as terrestrial forests [103, 104]. Over the last several years, there has been an increase in governmental control over the exploitation of natural seaweeds populations to limit their misuse. Some countries, depending on the state of the specific resource, allocate harvest quotas and/or establish different management and control measures to ensure the conservation of kelp forests and to lower the impact of their exploitation on marine ecosystems. Such measures may include establishing fallow periods of several years for areas subject to harvest, allowing only the collection of the upper part of the thallus from perennial seaweeds, limiting harvesting during non-reproductive periods, or even prohibiting the exploitation of endangered species and/or those with high ecological value [102, 105, 106]. In addition, kelp forests are also exposed to a range of disturbances of natural and/or anthropogenic origins. Particularly in recent years, kelp populations have declined in many areas of the world due to environmental stress caused by climate change, among other factors, especially by the increase in sea temperature and disruption in the natural nutrient availability patterns [107, 108].

Seaweed exploitation in Asia intended for alginate production mainly comes from commercial cultivation of the kelp *Saccharina japonica* (kombu) in China [42–44]. Kelp cultivation techniques have been well developed in Japan and China, where several Asian species have been cultivated on a large scale since the 1960s

[109, 110]. Today, aquaculture in Asia provides almost all of the global production of *S. japonica*, reaching over 900 thousand tonnes dry weight of seaweeds (estimated from fresh weight data) in 2014 [111], of which until now only a small part has been used for alginate extraction, but, as we have seen (Table 2.5), accounting for more than 20% of world alginate production [43]. Current practices of kelp farming in Asia have contributed not only to significantly increasing production to meet commercial demands for various uses, including alginate production but also to conserving natural populations and the ecosystems that they produce. Recently, mariculture of kelp species has also generated great interest in Europe and the Americas, as it may lead to increased production for commercial uses and potential applications; in addition, it may help protect the kelp forests from overharvesting [112–114]. The current limitations on the availability and use of natural kelp resources are expected to become the major driving force for the growth and development of farming of kelp species for alginate production, as has already been the case in Asia. In fact, it is now widely recognized that a transition from seaweed extraction to aquaculture is needed to meet the growing demand and to avoid the decline or loss of natural populations [115, 116]. At present, cultivation of kelp species is currently also being attempted in several countries of Europe and the Americas [112–114]. The techniques and biological basis required for full-cycle cultivation of *Saccharina*, as well as for other kelp species, will be described in the next section.

2.3 Cultivation of *Saccharina* and Other Kelp Species

2.3.1 Key Biological Aspects

Kelps are characterized by a heteromorphic life cycle that alternates between a haploid generation formed by microscopic filaments (known as the gametophyte because it produces gametes) and a diploid generation formed by a macroscopic thallus (called the sporophyte because it produces spores) [117]; the different life-history stages are shown in Fig. 2.10. Most kelp species have a perennial (i.e. lasting several years) sporophytic phase, during which the sporophyte may reach a length of several metres depending on the specific seaweed taxon (e.g. up to 50 m in *Macrocystis*, up to 15 m in *Ecklonia*, up to 10 m in *Durvillaea*, up to 4 m in *Lessonia*, up to 5 m in *Saccharina japonica*, up to 2 m in *Laminaria hyperborea* and up to 1 m in *Laminaria digitata* [55]).

Large sporophytes are commercially exploited for different uses, such as the extraction of alginates [42, 43, 99]. Sporophyte morphology of kelps varies depending on the species and the environmental conditions in which they grow. However, three parts are typically recognized: the holdfast, stipe and blade (described in Sect. 2.2.1; see Figs. 2.4 and 2.10) [55, 118]. The gametophytic phase, in contrast, consists of slightly branched male and female filaments composed of round-shaped

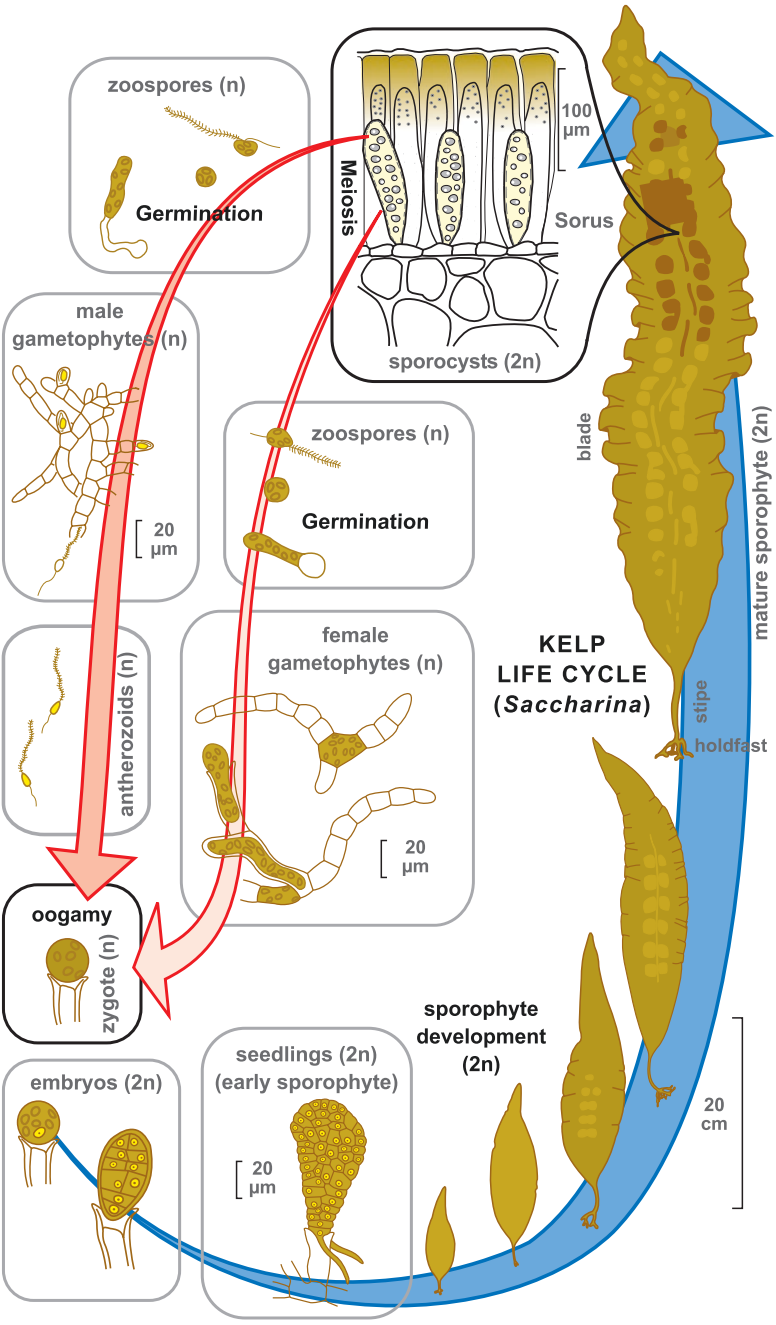

Fig. 2.10 *Saccharina* life-history stages, which are the same for all brown seaweeds of the order Laminariales (kelp)

cells smaller than 50 μm in diameter. Microscopic gametophytes are a survival strategy for the sporophyte, enabling long-term resistance to adverse environmental conditions while it waits to reproduce and form new sporophytes. The filaments of gametophytes may remain dormant or grow vegetatively, although their growth is generally much reduced [119–121].

Most kelps are distributed along the rocky shores of the Arctic and the cold-temperate regions of the Northern and Southern Hemispheres, where temperatures are generally below 20 °C. Temperature is therefore a key environmental factor that affects not only the distribution of kelp species but also their growth [122–124]. The perennial sporophytes of kelps generally exhibit strong seasonality in their development with a period of rapid growth during winter and spring and a period of minimal growth during the summer and fall, which coincides with the seasonal temperature and nutrient cycles in cold-temperate waters. In winter, temperatures are lower, and nitrogen levels are higher. In contrast, temperatures are higher and nitrogen levels are often negligible during summer [118, 125]. Another important environmental factor influencing the development of kelp sporophytes is water movement, which affects nutrient assimilation and gas exchange by determining passive transport across the diffusion boundary layer of the algal surface [126].

2.3.2 Background of Kelp Farming

Cultivation practices are rooted in Asia in the eighteenth century, during which different methods were used to expand the populations of edible kelps as a natural resource [127, 128]. However, these practices depended entirely on the natural environment since there was no control over the biological cycle of these seaweeds. The scientific basis for the development of the full-cycle cultivation of *Saccharina* and other kelp species was first established in the middle of the twentieth century, and since then, techniques have been established in Asia to obtain seedlings from spores under more-or-less controlled laboratory conditions. This Asian technique of seedling production enabled the subsequent development of different types of floating rafts for cultivating kelp in the sea, and beginning in the 1960s, commercial kelp mariculture extended to different regions of Japan, China and Korea, where it was promoted by different governments to meet the demand for human consumption in a context of insufficient natural resources [110, 127–129]. Currently, *Saccharina japonica* is the most extensively cultivated kelp species in these countries.

In Europe and the Americas, different cultivation practices were initiated in the 1980s and 1990s to study the viability of native kelps [120, 130–133]. As an alternative to the Asian method of seedling production, a European technique was developed to produce kelp seedlings from gametophyte cultures [120, 134]. Research showed that kelp cultivation using simple, relatively low-cost techniques was biologically and technically feasible, but these early attempts at cultivation did not continue due to a lack of interest since the available wild kelp stocks were sufficient

to meet commercial demand and their exploitation was considered more profitable than cultivation. However, there is currently a growing interest in the development and optimization of kelp species cultivation in several European and American countries; in fact, early cultivation practices have already begun on a commercial scale. This change is due to the growing demand for these species for different high-value commercial uses as well as the important environmental benefits that their cultivation would provide [112, 113]. Marine macroalgae use carbon dioxide and nutrients to grow and may thus contribute to the reduction of atmospheric CO_2, which is a contributing factor to climate change [135–138], and of the amount of inorganic waste that is discharged into marine coastal areas [139–142]. In particular, seaweed farming is considered to be the basis for the development of sustainable aquaculture because they can absorb some of the inorganic nutrients that are produced, for example, in the aquaculture of mussels and fish [114, 143–148] (Fig. 2.11). To date, sea farming has been tested with success for the following kelp species: *Macrocystis pyrifera* in Chile [149]; *Laminaria digitata* in Ireland [150]; *Saccharina latissima* in several countries in Europe [113], Canada [142] and the USA [139]; *Saccharina longicruris* in Canada [151]; *Lessonia trabeculata* in Chile [152]; *Alaria esculenta* in Canada [142] and in Ireland [153]; and finally *Undaria pinnatifida* in Japan, China and Korea [110] and in France and Spain [154]. In general, the techniques developed for the commercial-scale farming of the kelp *Saccharina* in Asia [109] have been adapted to the cultivation of other kelp species in Europe and the Americas [113, 149].

2.3.3 Cultivation Steps and Methods

As with other kelps, full-cycle cultivation of *Saccharina* consists of two very different phases associated with their characteristic life cycle (Fig. 2.10). In the first step (laboratory-culture stage), kelp seedlings are produced on strings (commonly

Fig. 2.11 A bay along the European coast where kelp cultivation (*1*) occurs along with the cultivation of mussels (*2*) and fish (*3*) (Photo by C. Peteiro)

known as seed-strings) under controlled environmental conditions in the laboratory, and in the second step (sea-culture stage), the seed-strings are attached to ropes on floating rafts for cultivation at sea until the sporophytes reach a certain size and are harvested. The main steps in kelp farming are summarized schematically in Fig. 2.12 and described in more detail.

2.3.3.1 Laboratory-Culture Stage

The traditional Asian seedling production method is performed by sowing strings of spores extracted from mature kelp sporophytes from natural populations [110, 129]. However, an alternative method was developed to culture kelp seedlings from "free-living gametophytes" [120, 134], which has important advantages over the traditional approach, such as the possibility of genetic selection, the creation of clones, the cryopreservation of strains and the generation of large quantities of

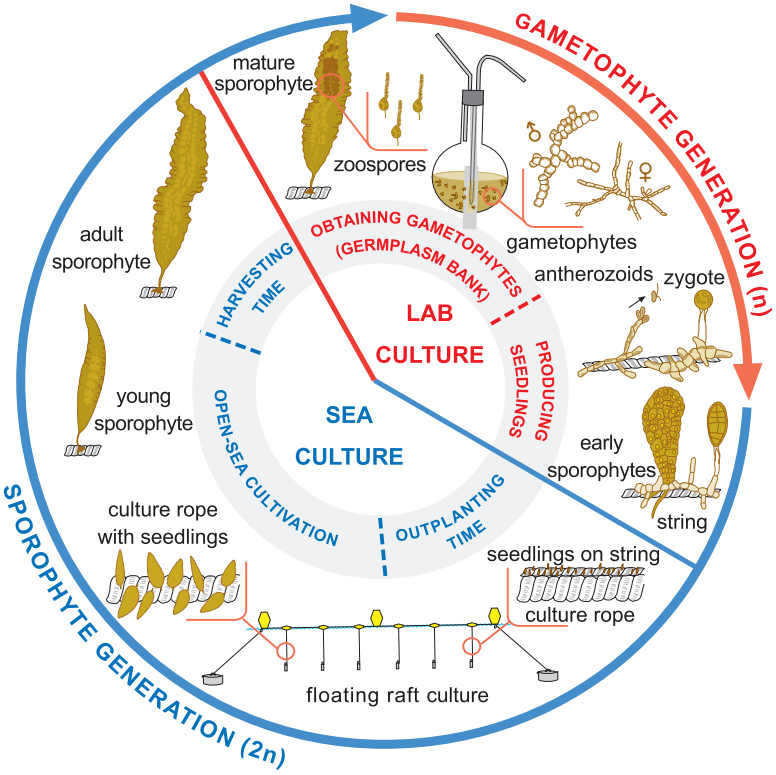

Fig. 2.12 Diagram summarizing the steps in kelp farming, which include the production of seedlings on strings (laboratory-culture stage) and their subsequent attachment to culture ropes for growth in a floating raft culture (sea-culture stage) (Adapted and reprinted from Ref. [113], Copyright 2016, with permission from Elsevier)

gametophytes through vegetative growth that can produce seedlings at any time of the year [119]. This method is currently being successfully used to cultivate different kelp species in Europe [119, 150, 153], the Americas [151, 155] and in Asia, albeit in a more limited way [156, 157]. Given its extensive role in cultivation, the production of kelp seedlings from gametophyte cultures is specifically described here.

The production of seedling strings under laboratory conditions is divided into two phases: a first phase to create a culture of free-living gametophytes maintained with aeration under controlled environmental conditions (Fig. 2.13) and a second phase where gametophytes are sown on strings and grown in tanks, in which gametogenesis is induced so that, after sexual reproduction, seedlings develop (Fig. 2.14).

Gametophyte cultures are obtained from the germination of spores extracted from the fertile parts (i.e. reproductive structures) of mature sporophytes that are generally obtained from natural populations or cultures. The formation of reproduc-

Fig. 2.13 The environmental culture chambers or incubators (above) used to maintain culture flasks (bottom left) containing microscopic kelp gametophytes (bottom right), growing under free-living conditions (Photos by C. Peteiro)

tive structures (called sori or sporophylls) in kelp sporophytes can also be induced in some species under short-day or long-day photoperiods [158, 159]. Spore germination and the subsequent development of gametophyte cultures are performed in culture flasks containing sterile, nutrient-enriched seawater under environmental conditions specific to each kelp species [119, 149, 150, 156]. The entire process is carried out in environmental culture chambers or incubators that are designed to rigorously control temperature and light (considering irradiance, the light spectrum and photoperiod) and to aerate the cultures (Fig. 2.13).

Adequate aeration of free-living gametophytes is maintained by bubbling within the culture flasks (Fig. 2.13), which homogenizes light and promotes nutrient availability in the culture medium. Gametophytes are usually conserved in their dormant or slow-growth states, although vegetative growth can be promoted by filament fragmentation under specific environmental conditions to increase their biomass. However, the growth rate of kelp gametophytes is generally very low, so large quantities of spores are usually obtained and germinated to have sufficient reserves of gametophytes [119]. It is important to note that the gametophyte culture also acts as a germplasm bank for ex situ kelp conservation, as it can be indefinitely maintained in vivo under suitable environmental conditions [119, 121, 160]. Seedlings or early sporophytes can be obtained from the gametophyte collections of a germplasm kelp

Fig. 2.14 Images of the embryogenic tanks (above) where gametophyte reproduction is induced after the fertilization of seedling strings (bottom) (Photos by C. Peteiro)

bank for sea culture and for the repopulation of coastal areas that have been degraded by human activities and/or natural processes [161–164].

For indoor production of seedlings, a selection of cultures with a suitable proportion of male and female gametophytes (generally a 1:1 sex ratio) are sprayed onto strings that are wound around a rigid coil or frame called a collector. To promote the attachment of gametophytes, the strings are pretreated by boiling followed by successive washes with distilled water, bidirectional sanding and, finally, a surface burn using hot air guns to remove any filaments produced by sanding [119, 165]. The collectors with the strings seeded with gametophytes are immersed in embryogenesis tanks, in which temperature, light (irradiance, the light spectrum and photoperiod) and water movement (by aeration) are under absolute control (Fig. 2.14). In these tanks, which contain sterile seawater enriched with nitrates and phosphates, sexual reproduction is induced under environmental conditions specific to each kelp species [119, 149, 150, 156], which allows zygotes to be fertilized, first giving rise to embryos and later to seedlings (i.e. early sporophytes) (Figs. 2.10 and 2.14). Generally, seedlings are embryos with polystromatic thalli (i.e. composed of many layers of cells) that are normally more than 2 mm long, in which the stipe-blade area begins to differentiate.

2.3.3.2 Sea-Culture Stage

To grow young sporophytes in the sea, the seedling strings are primarily attached to the culture ropes by two methods (Fig. 2.15). In the first, a continuous string is helically wound around the culture rope, while in the second, pieces of cut string are woven into the structure of the culture rope at regular intervals.

The culture ropes are deployed in the sea in floating culture rafts, the main elements of which include an anchoring system, a floating structure and culture lines. Figure 2.16 shows the different culture rope arrangements that are usually used in kelp mariculture [110, 113, 127–129, 149]. The hanging rope culture is used in protected areas, while the horizontal rope culture is used in the most exposed areas, as it better resists strong waves and currents. In horizontal culture, the light is homogeneous along the rope; thus, production is greater, and it may be used in shallow areas and highly turbid conditions. In the hanging culture, light decreases with depth, so growth is irregular. To minimize this effect, this approach is usually used in clear water and within the optimal depth range of the species. Culture rafts may be configured to regulate the depth of the culture ropes and thus control light conditions, so rafts can be adapted to the needs of the kelp species or the individual culture.

In *Saccharina* mariculture in Asia, three different methods have been used: two-year cultivation, forced cultivation and cultivation by transplanting [128, 129, 166]. Due to the natural biannual growth cycle of these kelps, two-year cultivation requires approximately 20 months of cultivation in the sea to obtain sporophytes of commercial size. An alternative method has been developed in Asia that involves the production of seedlings during the summer, allowing earlier cultivation and thus reducing the growth period in the sea to 10 months to obtain adult sporophytes. This method of sea cultivation, termed forced cultivation, expanded rapidly and became the main method for the commercial cultivation of *Saccharina* in Asia. In cultivation by transplanting, young sporophytes obtained by thinning cultures are normally

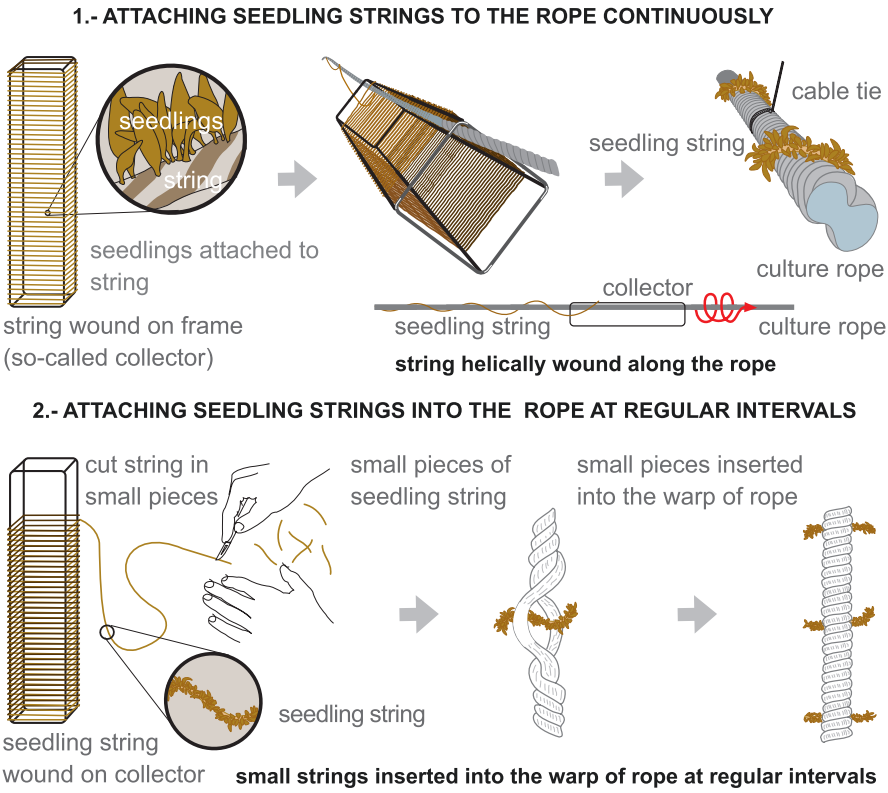

Fig. 2.15 Methods of attaching kelp seedling strings to culture ropes for later sea culture

used in combination with the above cultivation methods to increase *Saccharina* production [128, 129, 166].

The outplanting and harvesting times of kelp cultures vary by species but are mainly related to the temperature and nitrogen concentration in the sea (Fig. 2.17). In addition, there are important differences in these environmental factors between regions (and even localities), which cause variability in outplanting and harvesting periods between different areas for the same species. Generally, the cultivation period in cold waters starts earlier and has a longer duration with several possible harvests; the cultivation period in temperate waters starts later and has a shorter duration with a single final harvest [113, 127, 128]. Moreover, in some regions of China where seawater nitrogen concentrations are very low, sea fertilization is performed as part of the commercial cultivation of *Saccharina* [167]. Furthermore, the hydrodynamic conditions of the growing site are an important factor to consider when determining the most suitable locations for kelp cultivation, as they affect culture growth and production. Moderately exposed conditions tend to favour increased kelp production [113, 154, 168, 169].

Cultured sporophytes are grown in the sea until they reach commercial size, which varies by kelp species (e.g. up to 5 m in length for *Saccharina japonica*), at which time the crop is usually harvested from boats. Harvesting can be performed by means of several collections of the larger sporophytes (thinning) or by partially cutting the

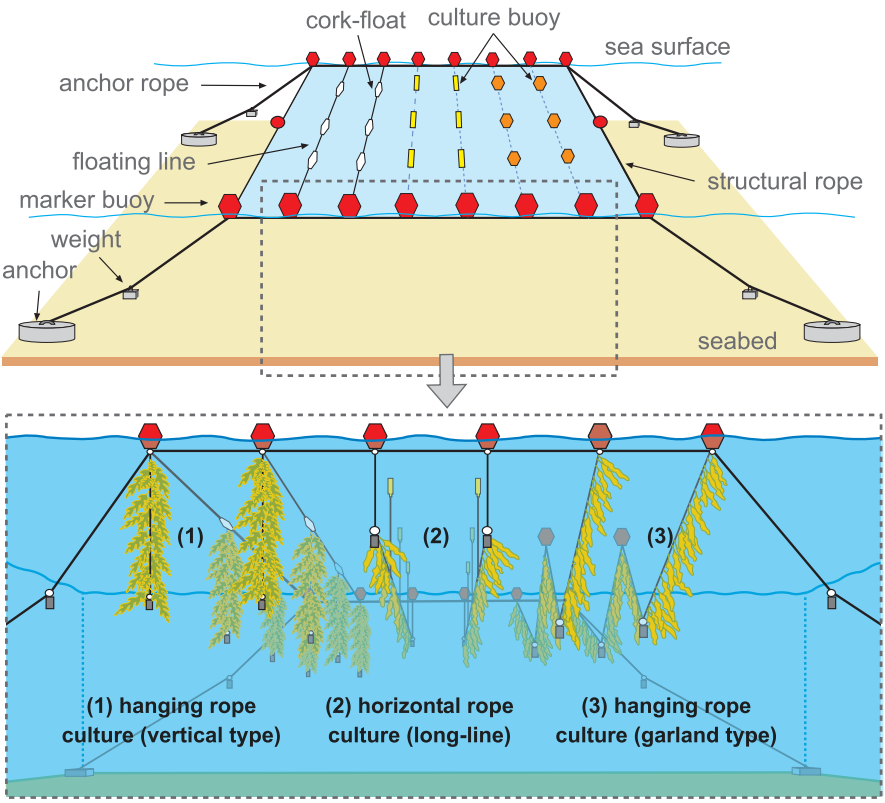

Fig. 2.16 Floating raft culture with different rope arrangements: hanging rope method (vertical type or garland type) and horizontal rope method (long-line type) (Adapted and reprinted from Ref. [113], Copyright 2016, with permission from Elsevier)

apical part of the blade, leaving the basal part, which may regrow apically from the basal blade meristem. However, the most common harvesting method is the collection of all sporophytes when most have reached commercial size [113, 127, 128].

The productivity of kelp cultures varies with the species, cultivation method, growing season, environmental conditions at the cultivation site and many other factors. However, the approximate average wet weight biomass yield per hectare of cultivation on a commercial farm has been, for example, 70 tons fresh weight for *Macrocystis pyrifera* [149], 26 tons for *Saccharina japonica* [129] and 25 tons for *Saccharina latissima* [169].

2.4 Conclusions and Perspectives

The commercial alginates that have numerous applications are exclusively extracted from brown seaweeds, and it is estimated that the global production of alginates primarily involves the exploitation of only 9 seaweed species, of which kelps *Lessonia*,

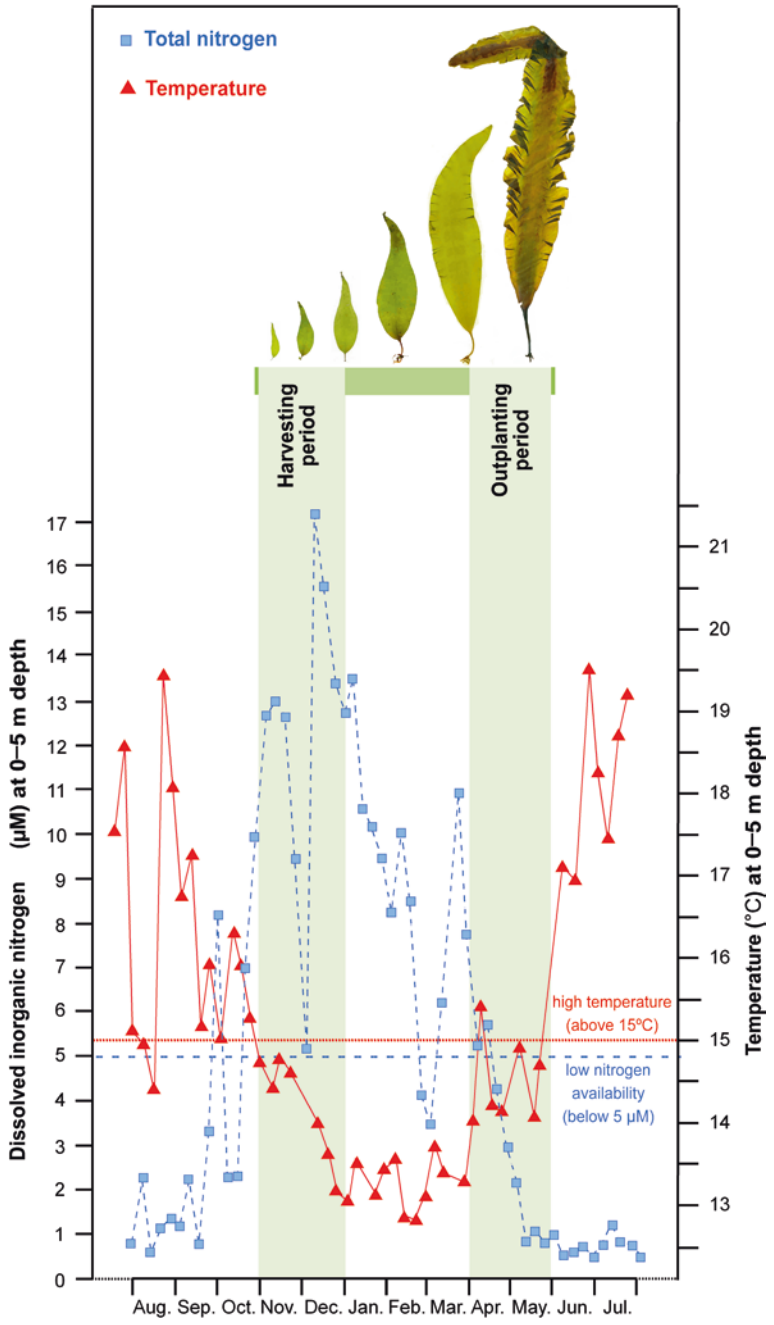

Fig. 2.17 Time frames for *Saccharina latissima* mariculture in southern Europe. As with perennial kelp species, sporophyte growth occurs when the sea temperature is lowest and nitrogen availability is high

Laminaria and *Saccharina* are the most commercially important, accounting for 86% of the worldwide production. Most of these marine macroalgae are currently harvested from native populations (over 80%), while *Saccharina* farming in Asia provides the rest of the resources (approximately 20%) for alginate production.

The demand for alginates is expected to increase in the future; however, natural resources are limited, and kelp forests are decreasing worldwide. Nevertheless, it is expected that the contribution of kelp cultures to global alginate production in the coming years will increase in volume and in the number of species used for this purpose, which would also provide greater security and stability to the market supply of alginates, as it would no longer depend on natural populations. Additionally, this cultivation would enable alginates of commercial interest to be obtained from species whose extraction has not been previously possible due to a lack of available natural resources.

Finally, kelp farming provides significant environmental benefits by capturing atmospheric carbon and recycling inorganic nutrients from the marine environment. Additionally, since kelps have many other applications, the uses of the biomass harvested in cultures could be integrated in biofactories so that other products of commercial value, besides alginates, could be obtained (Fig. 2.18).

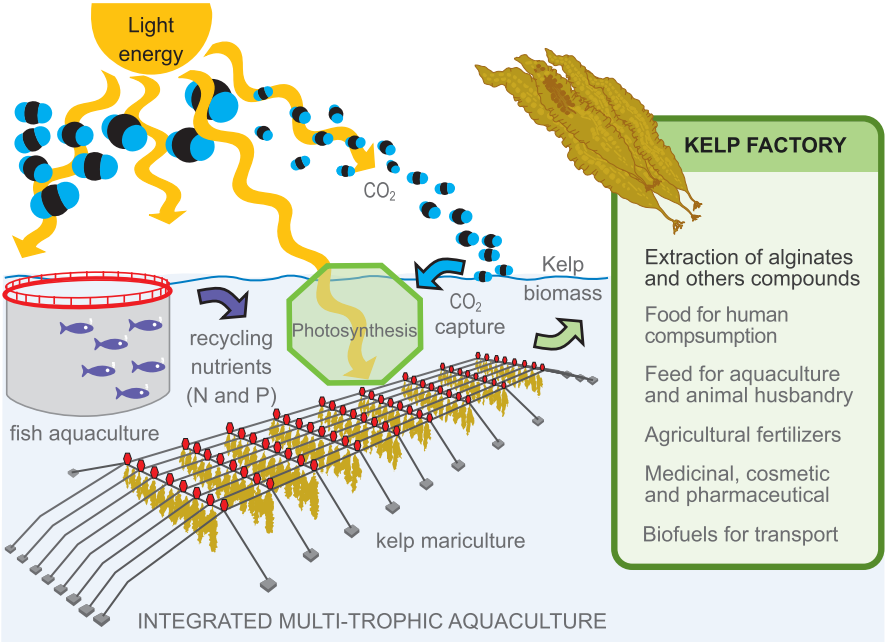

Fig. 2.18 Kelp sea farming scheme to produce alginates as well as other value-added bioproducts from the integrated use of the kelp biomass harvested in biofactories (Adapted and reprinted from Ref. [113], Copyright 2016, with permission from Elsevier)

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Chapter 3
Alginate Microcapsules for Drug Delivery

Ainhoa Gonzalez-Pujana, Gorka Orive, Jose Luis Pedraz, Edorta Santos-Vizcaino, and Rosa Maria Hernandez

Abstract Currently, conventional drug delivery systems do not provide adequate therapeutic profiles for the management of multiple diseases. In this regard, cell encapsulation technology emerges as a suitable alternative. Undoubtedly, one of the most employed biomaterials for this purpose is alginate, since it presents multiple advantages that favor the development of this technology. Importantly, the thorough study concerning the purification and modification of the polymer has led to biocompatible alginates, a vital advancement for the correct function of the system. Furthermore, the possibility to entrap different cell types together with the plausibility of engineering cells to produce disparate therapeutic biomolecules has given rise to numerous applications. That is the case of relevant and prevalent diseases nowadays such as diabetes, cancer, or neurological diseases. Intensive research in the field has resulted in promising preclinical studies in animal models that have instigated the conduction of several clinical trials. Nonetheless, addressing some current challenges regarding aspects such as biosafety or biofunctionalization seems to be a prerequisite before the clinical translation.

Keywords Alginate • Encapsulation • Drug-delivery • Cell therapy • Hydrogel • Microcapsule • Protein release • Microbead • Biomolecules • Xenotransplantation

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3.1 Introduction

Noncommunicable diseases (NCDs), also known as chronic diseases, are those not passed from person to person and usually associated with long duration and slow progression. NCDs include cardiovascular diseases, cancer, chronic respiratory diseases, diabetes, or neurodegenerative diseases. According to the World Health Organization, NCDs represent one of the major health challenges of the twenty-first century, considering both patient suffering and the subsequent socioeconomic burden [1, 2].

Their management is complex, as many of them demand a tight regulation of therapeutic factors based on physiological requirements. Hence, conventional drug administration offers poor control over this type of pathologies, leading in many cases to non-efficient treatments and undesirable effects. Consequently, over the last decades, new strategies have been thoroughly studied in order to develop new drug delivery systems.

One of the concepts that have shown a high potential to become a viable therapeutic option for chronic diseases is cell microencapsulation. In this strategy, cells that produce therapeutically active biomolecules are enveloped in a polymeric matrix. The resulting microspheres are usually coated with a polycation in order to form a semipermeable membrane. Thus, the system allows the ingress of nutrients and oxygen and the egress of therapeutic factors. Furthermore, the passage of immune cells and antibodies is restricted, leading to immunoprotection of the encapsulated cells (Fig. 3.1). Hence, the transplantation of encapsulated cells may

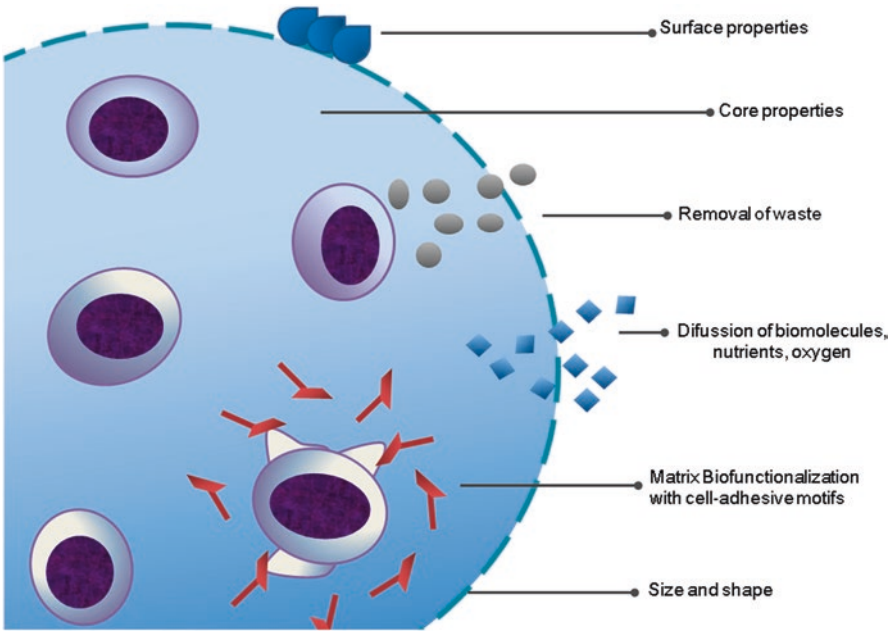

Fig. 3.1 Scheme representing the main properties of alginate cell microcapsules

represent a valuable approach to enable sustained and controlled delivery of therapeutic biomolecules to specific targets. Moreover, because of the permselective membrane, the co-administration of immunosuppressive therapies may be reduced, diminishing the severe side effects related to these drugs [3, 4].

This biotechnology gives rise to myriad opportunities for application and meets, a priori, the requirements for an adequate treatment that would drastically improve efficacy, as well as patient compliance and comfort. Therefore, the field of cell microencapsulation is currently under intensive research to face the challenge of clinical translation.

A multidisciplinary approach is crucial for the development of the technique, given that cell encapsulation combines areas such as biology, medicine, pharmaceutical technology, or surface chemistry. Specifically, material science is of paramount importance in this field; indeed, the choice of the biomaterial may make a real difference in the eventual success of the biosystem. In this sense, it is essential that the applied material presents suitable mechanical properties as well as adequate biocompatibility in order to respect cell homeostasis and viability.

To date, many polymers have been utilized with that purpose, including natural materials such as alginate, chitosan, agarose, collagen, or cellulose and synthetic like poly(ethylene glycol) (PEG), polyurethane, or polyvinyl alcohol. However, recently concerns have arisen on the use of synthetic polymers due to their impossibility to trigger physiological cellular responses and their difficulty to form gels in situ following cell-friendly protocols. This fact has resulted in a shift of interest to natural materials [5]. In particular, alginate remains the most widely employed polymer nowadays. Although other biomaterials may become a good alternative in the future, currently there is a consensus that only alginate has been exhaustively studied so as to be qualified as safe for application in humans [6]. Moreover, the intrinsic properties of alginate make it suitable for the needs of this biotechnology and confer multiple advantages to the system. Thus, alginate represents a promising tool for drug delivery via cell microencapsulation.

3.2 Cell Microencapsulation Technology

Cell microencapsulation emerged in 1933 when Bisceglie demonstrated that tumor cells remained viable after being encapsulated and transplanted in pig's abdominal cavity [7]. Three decades later, in 1964, Chang and colleagues encapsulated erythrocyte hemolysates in nylon microspheres and proposed the use of semipermeable membranes as immunoisolating barriers [8]. It was not until 1980 that Lim and Sun proved the therapeutic application of the method by transplanting alginate microcapsules containing Langerhans cells in diabetic rats and thereby returning the animals to normoglycemia for 2–3 weeks [9].

Since then, the use of a great variety of suitable biomaterials has been studied in detail. Nevertheless, after almost four decades of investigation, sodium alginate is still today the most widely used biopolymer for cell encapsulation. Alginate is a

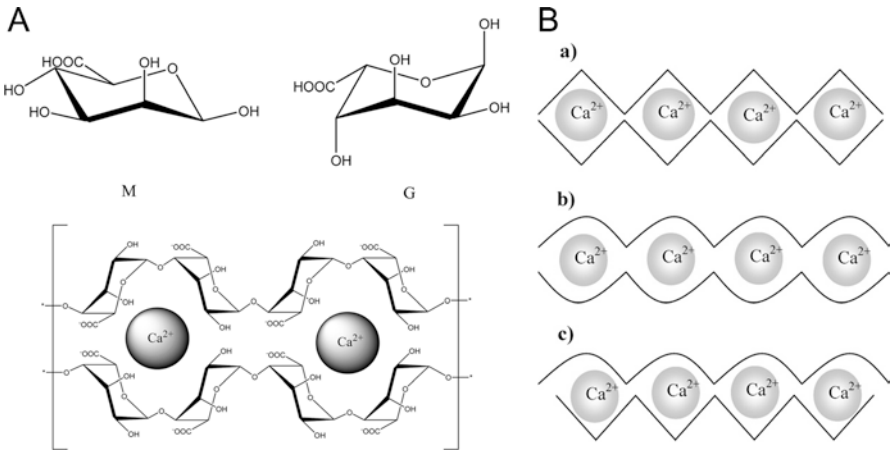

Fig. 3.2 (**a**) Structure of β-D-mannuronic acid (M) (top left), structure of α-L-guluronic acid (G) (top right). Representation of "egg-box" model binding of α-L-guluronic acid blocks to calcium ions in alginic acid (bottom) (Reproduced from Ref. [11] by permission of the Royal Society of Chemistry). (**b**) Graphical description of the three possible junctions in alginate gels. (*a*) GG/GG junctions, (*b*) MG/MG junctions, and (*c*) mixed GG/MG junctions (Reprinted with permission from Ref. [13]. Copyright 2005 American Chemical Society)

linear unbranched block copolymer composed of (1,4)-linked β-D-mannuronate (M) and its C5-epimer α-L-guluronate (G) arranged to form homopolymeric (GGG-blocks and MMM-blocks) or heteropolymeric block structures (MGM or GMG-blocks).

Alginates present the advantageous property of easily forming hydrogels by ionic cross-linking with divalent cations. The process occurs following the so called egg-box model [10]: the divalent cation binds to the G-blocks of two adjacent alginate polymer chains forming a three-dimensional gel network (Fig. 3.2a) [11]. It was believed that G-blocks were the only to participate in the cross-linking reaction [12]; however, MGM-blocks may also contribute to hydrogel formation [13] (Fig. 3.2b). Nonetheless, G content is still considered as the major factor involved in determining gel elasticity, porosity, and stability [14].

Moreover, alginates have different affinity for cationic cross-linkers, increasingly: calcium ($Ca2^{+}$), strontium ($Sr2^{+}$), and barium ($Ba2^{+}$) [15]. Consequently, the gel-forming ion may also influence final properties of microcapsules such as swelling and stability [13]. Following this premise, it is possible to choose the adequate agent to obtain microcapsules with the desired properties. The most frequently used cross-linking agent is Ca^{2+} for its non-toxicity compared with other cations. In particular, calcium chloride ($CaCl_2$) is usually employed as a Ca^{2+} source since its high solubility in aqueous solutions leads to a rapid ionotropic gelation [16]. Barium is also often used as a cross-linker, as it is known to form microcapsules with high mechanical strength [17].

Alginates also form gels by photo cross-linking [18], thermal gelation [19], or even a combination of these methods with cationic cross-linkers [20]. However, these

procedures may not tolerate cell survival to the same extent ionic cross-linking does. Hence, ionotropic gelation represents a good alternative to entrap cells in alginate hydrogels under mild conditions [21].

Consequently, sol-gel processes based on the original extrusion method developed by Lim and Sun [9] are the most widely employed to manufacture alginate capsules. Cell entrapment within the hydrogel starts by suspending cells in an aqueous solution of the polymer, giving rise to the sol flowing phase. The suspension is then extruded into a solution of the cross-linking agent, with the subsequent sol-gel transition that forms the microbeads.

Although the obtained beads may protect allogeneic cells, alginates are too porous to provide xenograft immunoprotection [6, 22]. Therefore, microbeads are usually coated with a polycation that controls the molecular weight cutoff of the biosystem by forming a semipermeable membrane. This permselective barrier allows the diffusion of the therapeutic factor, oxygen, nutrients, and cellular waste while it protects the implant from the host immune system and mechanical stress. In this regard, poly-L-lysine (PLL) [23, 24] and poly-L-ornithine (PLO) [25, 26] are the most frequently employed polycations. As positively charged ions, when implanted in the organism, they may trigger a strong immunological reaction [27–30]. For this reason, a second coating of diluted alginate is usually added with the aim to mask the positive charges and improve graft biocompatibility. Therefore, when performing both coatings "alginate-poly-L-lysine-alginate" and "alginate-poly-L-ornithine-alginate" (APA), microcapsules are obtained. Nevertheless, it has been reported that those two coatings are not multilayered but instead blend forming an external layer of PLL and alginate that surrounds the inner calcium-alginate core [31]. If the degree of interaction between the molecules is not sufficient, unbound PLL may be exposed at the surface of microcapsules, fact that might explain the immune response that APA microcapsules cause and opens the debate about the importance of the second coating based on alginate [32, 33].

Therefore, these coatings are still nowadays one of the most limiting factors of cell microencapsulation technology due to the poor biocompatibility, mechanical strength, and stability that PLL and PLO provide [34]. Being the latter a relevant drawback, it has widely been suggested that other coatings should be tested in order to overcome these limitations [35]. Chitosan [36–38], modified chitosan [39], poly(methylene-co-guanidine) [30, 40], and the application of diblock copolymers of PEG and PLL [41, 42] have been some of the alternative approaches.

3.3 Alginate Microcapsules as Platform for Controlled Drug Delivery

Cell microencapsulation within alginate matrices represents a promising tool for secretory cell dysfunction management. When implanted in the body, the tissue and vasculature that surrounds the capsule provide the cells with oxygen and nutrients,

supporting cell viability and, as a result, functionality. Thus, the immobilized cells are able to produce the therapeutic factor de novo in a sustained way from weeks to months, which may match treatment duration with disease longevity [43]. Consequently, the single application of the treatment would remarkably improve patient comfort and compliance. Hence, it may overcome the huge problem of adherence, particularly important in diseases where an exhaustive regulation of drug delivery is mandatory to achieve treatment efficacy.

This implantation of cells is possible because of the immunoisolation that the semipermeable membrane confers to the system. Indeed, it has been widely demonstrated that microencapsulation protects allografts [44]. Furthermore, due to the shortage of donor tissues for patients, xenograft transplantation has emerged as a proper alternative. Despite being known that the host's immune response for xenogeneic cells is usually more aggressive, cell encapsulation has been able to prolong xenograft survival [45, 46]. However, there are still unsolved issues such as the release of xenogeneic epitopes, which induce the formation of encapsulated tissue-specific antibodies [47] or pro-inflammatory cytokines [48] that could lead to graft rejection. For this reason, different approaches have been carried out in order to find a solution. Examples are incorporating to the alginate the CXCL12 chemokine, which can repel effector T cells while recruiting immune-suppressive regulatory T cells [49], and conjugating to the hydrogel a peptide inhibitor for the cell surface IL-1 receptor (IL-1R) thus blocking the interaction between the entrapped cells and cytokines [50].

May these issues be overcome, chronic co-administration of immunosuppressant drugs could be significantly reduced [51]. This fact would have a major impact from a therapeutic standpoint since the severe side effects produced by these drugs may be avoided. To date, almost 50 significant adverse effects have been related to the use of immunosuppressant therapy, so immunoisolation may improve significantly the life quality of patients [52].

In addition, microcapsules are three-dimensional (3D) scaffolds that mimic more efficiently the tissues of the body than two-dimensional (2D) hydrogels. Another advantage of this 3D structure is that the surface to volume ratio is increased compared to conventional bulk hydrogels. This improves nutrient and oxygen supply [53], overcoming a problem that in many cases leads to graft failure [54].

3.3.1 Advantages Alginates Offer in Cell Encapsulation

3.3.1.1 A Natural and Biocompatible Polymer

One of the advantages of using alginate for cell microencapsulation is the abundance because of its natural origin. In particular, the most commonly applied alginates for this purpose are derived from brown algae such as *Laminaria digitata*, *Laminaria hyperborea*, *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Laminaria japonica*, *Durvillaea Antarctica*, *Ecklonia maxima*, *Lessonia nigrescens*, and

Sargassum spp. [55]. Alginates with more defined chemical structures and, consequently, physical properties may be obtained by bacterial biosynthesis. In particular, alginate is produced by two genera: *Pseudomonas* and *Azotobacter*. The relatively easy modification of bacteria and the recent advances in regulation of the polymer biosynthesis may enable manufacture of tailor-made alginates with high-value biomedical applications [56].

As natural polymers, alginates may incorporate contaminants such as heavy metals, proteins, endotoxins, or polyphenolic compounds. Moreover, additional impurities could be introduced during the industrial extraction processes of raw alginates [57]. Their presence compromises the biocompatibility of the graft. In particular, a recent study pointed to endotoxins, or in other words pathogen-associated molecular patterns (PAMPs), as responsible for the pro-inflammatory responses in the host when microcapsules are implanted [58]. PAMPs are small molecular motifs found on pathogens that initiate immune responses to eliminate pathogenic bacteria and thus protect the host from infections. Cells of the innate immune system recognize PAMPs via toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). Some of the PAMPs that raw alginates contain are peptidoglycan, lipoteichoic acid, or flagellin, and they predominantly activate TLR2, 5, 8, and 9 [58]. When present in alginates, PAMPs lead to immune activation with the subsequent cytokine release. The majority of them are small enough to pass the membrane, having a deleterious effect on cell function and viability. They may also limit the diffusion of nutrients, oxygen, therapeutic molecules, and the waste products of metabolism [59]. As a consequence of cell death, intracellular components are released. These molecules are damage- or danger-associated molecules (DAMPs) which also evoke an inflammation cascade when recognized by PRRs, mainly via TLRs [53]. Therefore, it is believed that DAMPs also play a role in the responses against encapsulated cells (Fig. 3.3a) [6].

In order to avoid these problems, purification of alginates has been intensively studied. In fact, it has been reported that for manufacturing biocompatible alginate microcapsules that are suitable for cell transplant, the use of ultrapurified alginate is mandatory [60]. The efforts have given rise to different methods to obtain pure alginates that meet clinically useful criteria [61].

This ultrapurified, "clinical-grade" alginate has been proven to reduce the foreign body reaction in many in vivo studies (Fig. 3.3b) [62–64]. Indeed, nowadays there are commercially available highly purified alginates that evoke no immune reaction when injected subcutaneously to mice [12]. Moreover, the modification of alginates has been suggested as a tool to mitigate the foreign body response and achieve a better biocompatibility [65]. In particular, recently, the modification of alginates with triazole-thiomorpholine dioxide (TMTD) was able to achieve a long-term functionality of the graft in immunocompetent animals with no need of immunosuppression [66]. Going further, in a recent study, a technology platform has been designed to predict whether the purification is efficacious [67]. Finally, it is relevant to cite that alginate purification is not only beneficial to avoid the immune rejection of the implant but also to improve viability of the encapsulated cells [68].

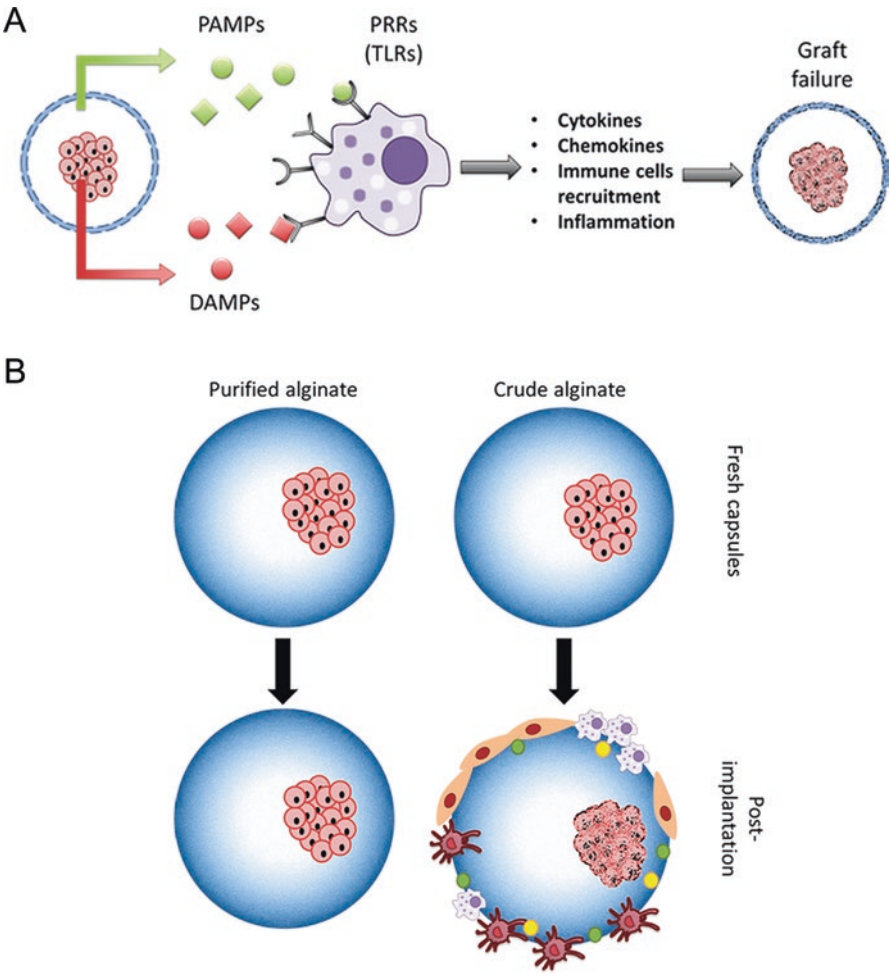

Fig. 3.3 (**a**) PAMP and DAMP release from microencapsulated cells. (**b**) Microcapsules produced with purified or crude alginate (Figure as originally published in [53])

3.3.1.2 Rapid Hydrogel Formation and Scalability

As previously mentioned, alginates form hydrogels by ionic cross-linking with divalent cations such as Ca^{+2}. This property makes alginates the most widely employed biomaterial for cell microencapsulation for two main reasons: (1) the synthesis of the capsules under mild conditions is enabled, and (2) the fact that it results in a hydrogel provides the encapsulated cells with an adequate microenvironment.

Unlike many other polymers, alginates present advantageous gelling properties. Being a thermally stable polymer, the hydrogel formation occurs at room temperature, allowing the immobilization of cells under safe and mild conditions that promote their viability, and, thus, ensures the production of the therapeutic factor [16]. In contrast to alginate, a large number of polymers are not suitable for this technology as harsh chemicals or high temperatures are required for the gel forming, leading to death of the implanted cells [69]. Moreover, the rapid gelation shortens the encapsulation process, avoiding the manipulation of the encapsulated cells for long periods of time. This advantage, together with the relatively uncomplicated method and the simple equipment that is required, gives rise to an easy and fast manufacture of the capsules.

For this reason, alginate microcapsules present the additional advantage of scalability. The high throughput of encapsulation systems facilitates the production of a vast number of microcapsules for biomedical applications [70–73]. Nonetheless, for a further development of the methodology and a better understanding of the lab-to-lab variations in the process, it is mandatory to define and standardize some particular characteristics. To date, five different parameters that influence the final properties of the capsule have been claimed to be compulsory for an adequate description of the system: the applied polymer, permeability, surface properties, biocompatibility, and storage conditions [74].

On the other hand, the resulting hydrogel forms a three-dimensional network that is capable of mimicking the basic three-dimensional properties of the natural extracellular matrix (ECM). Indeed, the high water content, necessary for physiological processes, and mechanical properties closely match the ones of soft tissues in the body [75]. Moreover, the biological interaction between hydrogels and cells can be easily modified through multiple extracellular matrix peptides or proteins (see Sect. 3.5.1). Therefore, biological, chemical, and mechanical properties and even the degradation kinetics can be tailored depending on the application. As a result, this type of hydrogels has been recognized to meet the requirements of bioencapsulation [76, 77].

3.3.2 Therapeutic Factors Delivered Through Cell Encapsulation

The major advantage of cell encapsulation lies beneath the adaptability of the biosystem. The optimization of this technology is giving rise to promising treatments for multiple disorders, since the secretion of a wide range of therapeutic factors is possible by simply selecting the appropriate cell type or by bioengineering cells so that they produce the biomolecule of interest. To date, many studies have focused their attention in specific therapeutically active molecules and their delivery through cell microcapsules. Table 3.1 classifies some of the most studied ones according to their nature.

Table 3.1. Examples of therapeutic factors delivered through cell encapsulation

Recipient	Administration site	Application	References
Insulin			
Xenogeneic	Peritoneal cavity	Diabetes	[79]
Isogeneic/allogeneic	Omentum pouch	Diabetes	[26]
Allogeneic	Peritoneal cavity	Diabetes	[80]
Xenogeneic	Peritoneal cavity	Diabetes	[83]
Allogeneic/xenogeneic	Peritoneal cavity	Diabetes	[85]
EPO			
Allogeneic	Subcutaneous tissue	Chronic anemia	[88, 90]
GLP-1			
Xenogeneic	Brain	Alzheimer's disease	[92]
Xenogeneic	Brain	Amyotrophic lateral sclerosis	[93]
Xenogeneic	Coronary artery branches	Heart failure	[94]
Xenogeneic	Brain	Traumatic brain injury	[95]
BDNF			
Xenogeneic	Cochlea	Auditory neuron degeneration	[99, 100]
GDNF			
Allogeneic	Brain (striatum)	Parkinson's disease	[102]
VEGF			
Xenogeneic	Brain	Alzheimer's disease	[106]
Therapeutic antibodies			
Allogeneic	Subcutaneous tissue	Cancer	[113]

EPO erythropoietin, *GLP-1* glucagon-like peptide-1, *BDNF* brain-derived neurotrophic factor, *GDNF* glial cell line-derived neurotrophic factor, *VEGF* vascular endothelial growth factor

3.3.2.1 Hormones

Hormones are secretory products that act in specific target cells where they elicit physiological, morphological, or biochemical responses. Since they regulate fundamental bodily and biochemical processes, their use in clinic is extensive. Indeed, being considered as important biological molecules in terms of clinical utility, hormones as therapeutics are currently under intensive research, and cell encapsulation represents a valuable strategy for their delivery.

Undoubtedly, one of the most thoroughly studied hormones in cell encapsulation is insulin. Type 1 diabetes requires a strict exogenous insulin supplementation. However, the current treatments, such as insulin injections or subcutaneous pumps, are not able to reproduce physiological profiles of the protein, which results in secondary complications. With the aim to overcome this issue, multiple studies have analyzed the possibility of cell microencapsulation as a tool for insulin delivery [62, 78]. Encapsulation of β-cells from human and even xenogeneic donors (pigs, rats) has given rise to intensive research. A recent study showed that alginate-encapsulated

human islet cells implanted in the peritoneal cavity of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice corrected hyperglycemia immediately [79]. This metabolic correction was maintained until the end of the study (5-week posttransplantation). Moreover, the majority of the implanted capsules were retrieved and their functionality analysis revealed a rapid, potent, and dose-dependent insulin response (Fig. 3.4). Another study demonstrated the long-term viability of encapsulated rat islets transplanted in the omentum pouch [26]. This site was suggested as a viable transplantation site because of its high vascularization, which provides a suitable environment for an adequate supply of nutrients and oxygen. Furthermore, the co-encapsulation of islets with mesenchymal stem cells (MSCs) has resulted in an improved islet function [80]. MSCs are known to elicit beneficial effects on the graft by reducing inflammatory damage and immune rejection and, thus, improve the islet transplantation outcome [81]. Nonetheless, the shortage of deceased human donors together with the risk of retroviral transmission and the more aggressive immune rejection from xenogeneic donors [82] represent a drawback. For this reason, some alternative sources have been investigated. An intriguing alternative is the encapsulation of non-endocrine cells that have been genetically engineered to produce insulin [83]. Another interesting approach is the use of stem cells, considering their ability to differentiate toward insulin-producing β-cells. The stem cell-derived insulin-producing cells may overcome the issue of donor limitation, and its combination with alginate encapsulation holds great promise for type 1 diabetes treatment [84–86]. In fact, when compared to 2D constructs, the 3D alginate capsules have been proven to promote the expression of primary maturation markers and the insulin delivery [71]. In a promising study, glucose responsive mature β-cells derived from human embryonic stem cells were able to provide a long-term glycemic control in diabetic immunocompetent C57BL/6 J mice [66]. The functionality of the graft continued for the 174 days of study, when implants were retrieved. Interestingly, the analysis of explants revealed viable insulin-producing cells.

Erythropoietin (EPO) is a glycoprotein that enhances the stimulation and maintenance of erythropoiesis (red blood cells maturation) as well as erythrocyte differentiation. Consequently, its delivery has gained relevance in the treatment of chronic anemia, and cell encapsulation has emerged as novel technology with this purpose [87]. This approach eliminates the need for repeated parenteral administrations of EPO, while it allows the continuous secretion of the drug, and, thus, avoids instability. An optimized capsule model achieved a controlled EPO delivery during 300 days in vivo without the implementation of immunosuppressive therapies [88]. Posterior studies confirmed the efficacy of the system by showing the maintenance of implanted cell viability and the sustained delivery of EPO, which resulted in the elevation of hematocrit levels in animal models [89, 90].

Glucagon-like peptide-1 (GLP-1) is an endogenous insulin-stimulating hormone secreted in response to food intake from the gastrointestinal tract. Furthermore, GLP-1 receptors are present in the mammalian brain and their activation leads to neuroprotective and neurotrophic effects. Thus, this factor may be effective for the treatment of multiple disorders. Considering its high potential, numerous studies

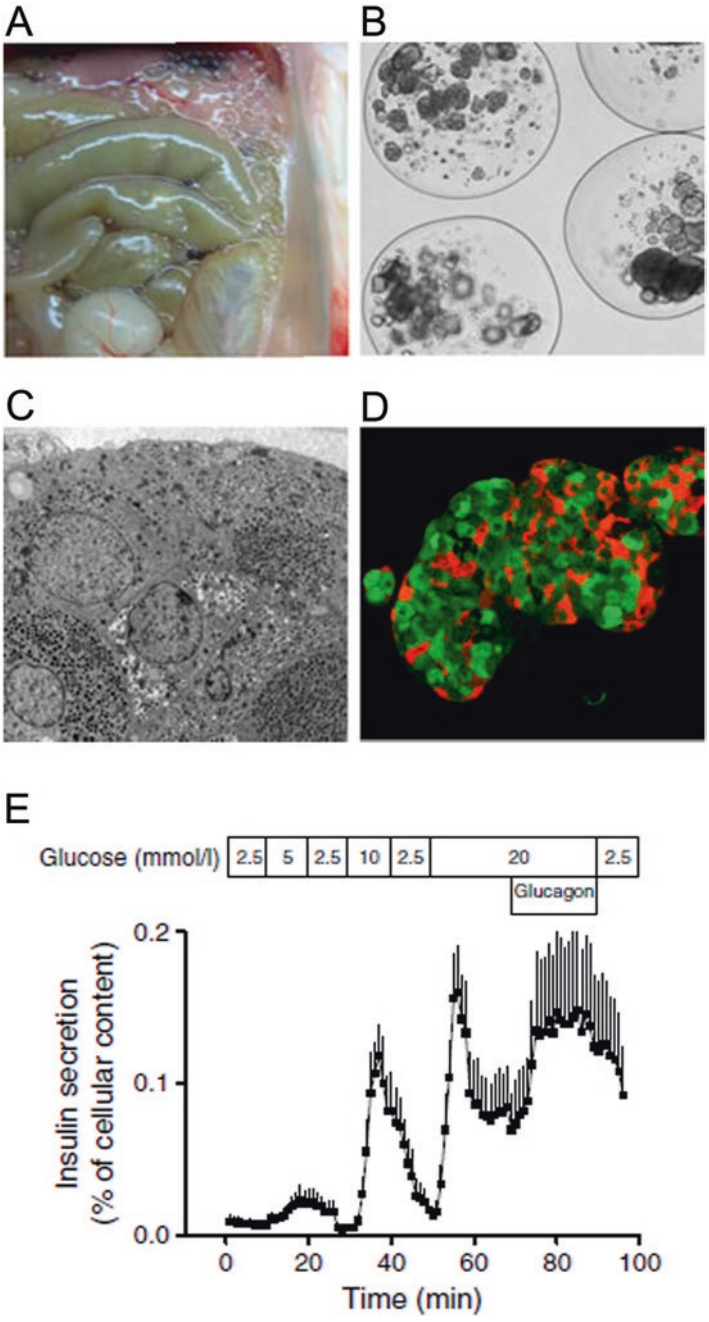

Fig. 3.4 Analysis of encapsulated human beta cell implant in NOD/SCID mice at posttransplant week 5. Free-floating capsules (arrow) in the peritoneal cavity (**a**) were retrieved as single units without fibrosis or cellular overgrowth (**b**). They contained well-granulated endocrine islet cells (**c**) that stained positively for insulin (green) and glucagon (red) (**d**). Their insulin release was examined in perifusion at 10-min episodes of varying glucose concentration with and without 10 nmol/l glucagon. The rate of insulin release is expressed as a function of the cellular insulin content before perifusion (**e**). Data represent means ± SEM from five independent experiments (Reprinted from Ref. [79], with kind permission from Springer Science + Business Media)

have investigated the entrapment of GLP-1-producing cells in alginate matrices. The applications are diverse: Alzheimer's disease (AD) [91, 92], amyotrophic lateral sclerosis (ALS) [93], damaged myocardium [94], or brain injury [95]. The latter has gained especial interest because of the recently performed clinical trials addressing it.

3.3.2.2 Neurotrophic Factors

Neurotrophic factors play a key role in the maintenance of normal neuronal function in adults and in neuronal survival and differentiation during the stages of development. The local upregulation of these factors has been detected close to the site of lesions such as acute brain injury and neurodegenerative diseases [96]. Since direct injections of different factors have been demonstrated to improve recovery, the transplantation of neurotrophic factor-producing cells close to the affected area may serve as an attractive treatment for these pathologies.

An interesting approach has been the implantation of choroidal plexus (CP) cells. CP cells are known to secrete multiple biologically active neurotrophic factors, and, thus, their encapsulation has derived promising systems that enable the local delivery of these biomolecules for restoration of brain tissue [96, 97]. This strategy has been successful in pathologies such as AD. AD is the most common form of dementia, characterized by the presence of extensive deposition of amyloid-β peptide (Aβ), abnormally phosphorylated tau, and neuronal loss. In a recent study, alginate microcapsules containing CP were implanted overlying the cerebral cortex of rats with exogenously induced Aβ memory impairment. Animals presented a significant recovery, which was attributed to the decrease in apoptosis, and the increase in neurogenesis, resulting in improved long-term memory [98].

Another example is the brain-derived neurotrophic factor (BDNF) delivery to prevent deafness-induced auditory neuron degeneration. As it has already been proven, the administration of exogenous neurotrophins to the deaf cochlea is a successful treatment; nevertheless, the benefits are rapidly lost when the therapy stops. Therefore, alginate microcapsules have been studied as a good alternative for achieving a sustained release of the factor. In a recent research work, encapsulated BDNF producing Schwann cells showed significant survival-promoting effects on the auditory neurons of deaf guinea pigs. Moreover, it was suggested that this treatment in combination with a cochlear implant might enhance and extend the benefits of the latter [99]. This advantageous effect was demonstrated in a posterior work. Specifically, it was observed that when cell-based therapy is combined with a cochlear implant, the enhanced auditory neuron survival effects are translated in important benefits with respect to electrical stimulation thresholds [100]. Altogether, the results suggest that this technology may have important clinical benefits in this area.

Another promising strategy has been the encapsulation of glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) producing cells for the treatment of neurodegenerative diseases such as Parkinson's disease (PD). PD is a

neurodegenerative central nervous system (CNS) disorder that is characterized by a debilitating motor impairment. This affectation is closely related to the gradual loss of dopaminergic neurons in the substantia nigra, which leads to a progressive decrease in dopamine levels in the striatum. PD is currently treated with levodopa o dopaminergic agonists; however, their effectiveness is temporary and the course of the pathology leads to a point where the loss of neurons is so remarkable that these therapies become ineffective. A valuable therapeutic alternative might be directly treating the cause of decreased levels of dopamine, which is the loss of dopaminergic neurons, instead of balancing them with the administration of the neurotransmitter. For this reason, encapsulation of trophic factor-releasing cells may allow the local and sustained delivery of these neurotrophic factors that protect neurons, avoiding their dysfunction and loss [101]. With this aim, different studies have obtained promising results. In particular, cell encapsulation enabled the continuous delivery of GDNF in the striatum of parkinsonian rats over 6 months. Therefore, a significant behavioral improvement was observed in the animals [102]. Moreover, the brain implantation of cell microcapsules for the delivery of neurotrophic factors has been shown to promote the survival of co-grafted cells for long time periods. In particular, baby hamster kidney (BHK) cells modified to release NGF were implanted approximately 1.5 mm away from co-grafted unencapsulated rat chromaffin cells in hemiparkinsonian rats. The survival of chromaffin cells was significantly enhanced, resulting in important improvements in animal rotational behavior [103, 104].

Vascular endothelial growth factor (VEGF) has also been delivered through alginate microcapsules for its application in PD concerning its neuroprotective effects [105]. VEGF is a potent angiogenic factor; for this reason, encapsulated cells secreting it have been employed with many other goals. One of the approaches has been the use of microencapsulated VEGF secreting cells in AD. Results in mice indicated that the therapy improved cognition as well as reduced apoptotic cell death. Aβ clearance was promoted, as a consequence of the neovascularization produced by VEGF, and hyperphosphorylated tau expression decreased, being both the main factors associated with this degenerative dysfunction [106]. In addition, this treatment enhanced cellular proliferation in the hippocampal dentate gyrus, representing a novel strategy for the treatment of brain amyloidosis [107]. On the other hand, it is important to note that this neurotrophic factor has also been employed in many other applications. For instance, a completely different strategy is to use the VEGF secretion as a therapy in bone defects, since this growth factor enhances osteoblast differentiation, and moreover, angiogenesis seems to be a prerequisite for bone rehabilitation. In this sense, a recent study showed that VEGF delivery promoted the differentiation of bone marrow MSCs and thus potentiated bone regeneration [108]. VEGF secreting alginate capsules have also been applied to improve wound healing and angiogenesis in xenogeneic acellular dermis matrix (ADM) transplants [109] or as a promising therapy for the survival of the ischemic skin flaps [110]. Considering the wide variety of applications, an angiogenesis-on-a-chip system has been proposed for the evaluation and quantification of the pro-angiogenic potential of factors such as VEGF secreted from encapsulated cells. Thus, the platform emerges as a potentially valuable preclinical tool that provides quantitative information on

encapsulated cell behavior, giving rise to an easier and less time-consuming method in comparison to in vivo angiogenesis assays [111].

3.3.2.3 Antitumor Factors

Therapeutic antibodies are nowadays employed for the treatment of multiple diseases. Therefore, encapsulated cells that produce them hold a great potential for many applications in drug delivery. A current approach is the utilization of these systems for cancer management, since the interaction of antibodies with cells from the immune system modulates their response. This modulation may occur by tampering with receptors involved in immune inhibition or by overstimulating receptors that promote the cellular immune response. In particular, the encapsulation of hybridoma cells has been widely studied as a platform for antibody delivery [112]. For instance, microcapsules containing hybridomas that produced anti-CD137 and anti-OX40 antibodies elicited an efficacious antitumor response by enhancing tumor-specific cellular immunity [113]. Recently, the capacity of encapsulated cells producing bispecific antibodies against carcinoembryonic antigens, which are present in most colon carcinomas, has been proven [114].

Another alternative is the secretion of endostatin, which is an endogenous anti-angiogenic peptide that has shown potent antitumor activity. The approach of encapsulating endostatin-producing cells has been studied for more than 15 years. In fact, in 2000, a promising study already showed the efficacy of the treatment obtaining considerable survival benefits in the immunocompetent BT4C brain tumor model [115]. Posterior studies confirmed the anti-angiogenic effect of endostatin by showing a significantly reduced tumor vascularization. Nonetheless, the effect on tumor growth was not observed [116].

The hypoimmunogenic and immunomodulatory properties of stem cells make them an interesting alternative for cancer management. In particular, alginate-encapsulated MSCs showed a threefold decrease in cytokine expression compared to entrapped cell lines [117]. Furthermore, stem cells have inherent tumor-trophic migratory properties, and the possibility to be modified to express diverse therapeutic factors renders them as optimal vehicles for the targeted delivery to isolated tumors and metastatic disease [118]. In a relevant example, MSCs were modified to secrete the angiogenesis inhibitor hemopexin-like protein (PEX). Their administration adjacent to glioblastoma tumors resulted in a relevant reduction not only in tumor volume (87%) but also in tumor weight (83%) [117].

3.4 Clinical Trials

The promising results obtained in experimental animal models have led to the conduction of several clinical trials in order to move this technology toward clinical translation. In this regard, this section gathers some relevant examples of clinical trials concerning cell encapsulation.

3.4.1 Diabetes

Overall, the results obtained with insulin-producing cell encapsulation hold a great potential. For this reason, this platform has been studied in clinical trials over the last 20 years. The first human clinical trial in alginate-encapsulated islet transplantation dates back to 1994. In this work, encapsulated cadaveric human islets were implanted intraperitoneally. Capsules were able to establish a glycemic control for 9 months in a type 1 diabetes patient who was on antirejection medications [119]. Since this successful approach, different clinical trials have been performed. Such is the case of Calafiore et al. who carried out a study where alginate-PLO microencapsulated islets were intraperitoneally transplanted without the use of immunosuppression. The results did not show side effects of the grafting procedures or any evidence of immune sensitization, confirming the technique as a powerful tool for immunoprotection. Moreover, patient's necessity of exogenous insulin intake decreased approximately to half of the pretransplantation consumption levels [120, 121].

Tuch and colleagues carried out a trial with four patients in which allogeneic islets were transplanted without immunosuppression. Neither insulin requirement nor glycemic control was altered, and C-peptide, an indirect measurement of insulin levels, was undetectable by 1–4 weeks. However, in the particular case of a recipient that received three separate islet infusions, the analysis of C-peptide revealed its presence up to the next 2.5 years [122].

Recently, Jacobs-Tulleneeers-Thevissen et al. transplanted allogeneic islets in a patient by means of Ca^{+2}/Ba^{+2} alginate microcapsules. Three months after transplantation capsules were retrieved and cells remained glucose responsive. However, the transplant showed insufficient biocompatibility [79]. In another report, Sernova Corp. announced the Cell Pouch® System, a commercial product of the macroencapsulation device that is currently ongoing a Phase I/II clinical trial in diabetic patients where measures of safety and efficacy are the primary and secondary endpoints [123].

Likewise, the company Living Cell Technologies (LCT) offers the DIABECELL® product, which is currently in late-stage clinical trials. In 1996, LCT initiated a novel study addressing the efficacy of xenogeneic islets. In particular, porcine islets were encapsulated and implanted in the peritoneal cavity of patients without the use of immunosuppressive therapies. Nine and a half years after transplantation, laparotomy of one of the patients showed the presence of microcapsules in the peritoneal cavity. Although the majority contained necrotic islets, impressively, some of them still remained viable [124]. This fact demonstrated the potential of the approach, and in consequence, the company has continued the research to achieve a correct glycemic control without the need immunosuppressants [125]. In a Phase I/II safety study, tolerability of the implant was confirmed [126]. Moreover, the trial showed proof of principle of efficacy demonstrating improvement in blood glucose control, even permitting the discontinuation of insulin injections entirely for up to 32 weeks. Later, a Phase IIa dose-finding trial was performed [127], followed by a

Phase IIb safety and efficacy study [128], which resulted in a clinically significant reduction of insulin dose and unaware hypoglycemia. As of now, a recent newsletter from the website announced the launch of Phase IIb/III clinical trials [48].

3.4.2 Intracerebral Hemorrhage (ICH)

Regarding ICH, a Phase I/II clinical trial has been conducted to evaluate the safety of its treatment with encapsulated cells that secrete GLP-1 [129]. The goal of this therapy is to improve the outcome after ICH surgery by enabling the local delivery of the neuroprotective and anti-inflammatory factor. Thus, the treatment may promote the healing of the secondary neuronal injury that occurs in the first week after the bleeding. With this purpose, stroke patients with space-occupying intracerebral hemorrhage were selected. After surgical evacuation of the hematoma, alginate microcapsules containing allogeneic mesenchymal cells transfected to produce GLP-1 were implanted in patients. Grafts were removed by a second surgery after 14 days of treatment. Preliminary results revealed that neither side effects from the surgical intervention nor implant-related side effects were shown in the interim evaluation of the first 11 patients. Furthermore, 30% of the implanted cells were viable and maintained their secretory capacity after explantation [130].

3.4.3 Neurological Diseases

The LCT company is also carrying out clinical trials regarding neurological diseases. In particular, the aim is to encapsulate clusters of neonatal porcine CP cells in alginate matrices. As already mentioned, CP cells produce a wide variety of neurotrophic and neuroprotective factors that support brain health. This product, branded NTCELL®, is intended for the treatment, without the implementation of immunosuppression, of different neurological diseases such as PD, AD, ALS, or Huntington disease. After obtaining promising preclinical data in a model of PD [131], the company conducted a Phase I/IIa clinical trial [132]. The study, completed in June 2015, investigated the safety and clinical effect of the capsules in four patients that had been diagnosed with PD at least 5 years before. The implants were safe and well tolerated and improved the clinical symptoms of PD in all of the patients, maintaining the effect for 26 weeks post-implant. In March 2016, a Phase IIb study commenced with the purpose of confirming the most effective dose [133]. The company has claimed that if the obtained results are positive, they will apply for provisional consent to launch NTCELL as the first disease-modifying treatment for PD in 2017 [134].

3.5 Complementary Strategies

The multiple clinical trials conducted in the field demonstrate the applicability and potential of cell encapsulation. Nonetheless, the sole alginate capsule does not fulfill all the requirements the cells need to survive and accomplish a correct physiological activity. The same happens with crucial aspects such as the host immune response and the safety issues of the implant. Consequently, supplementary strategies need to be implemented to broaden the possibilities of the system and thus develop a technology that succeeds in the clinical area.

3.5.1 Matrix Biofunctionalization

In their natural niches, cells receive and process information from the ECM [135]. Specifically, cells bind to ECM molecules via integrins giving rise to biochemical and mechanical signals that regulate myriad cellular processes such as proliferation, migration, or differentiation. Regarding cell encapsulation in alginate matrices, it is noticeable that this polymer by itself does not promote cell adhesion. Thus, controlling the intracapsular microenvironment by the inclusion of appropriate ligands is of paramount importance to maintain the viability and correct function of the entrapped cells.

The biofunctionalization of alginates with ECM motifs can be achieved by incorporating full-length ECM molecules as fibronectin, collagen, or laminin [34]. Nonetheless, a promising alternative involves the isolation of functional domains from these large ECM molecules and their use as short peptide sequences to form biofunctional matrices. These cell adhesive peptides have the advantage of being relatively stable and can be easily attached to the hydrogel [136]. On this point, carbodiimide chemistry has been the most commonly applied method for alginate functionalization. However, lately, new options have been suggested as efficient procedures for coupling bioactive molecules to alginates, such as the partial periodate oxidation followed by reductive amination [137]. The most widely employed motif is the arginine-glycine-aspartic acid (RGD), present in fibronectin, laminin, or collagen, among other ECM proteins. RGD has been extensively used in the field of cell microencapsulation with promising results [138–141]. Interestingly, these studies have highlighted critical aspects such as the ligand density, which has been described as a crucial factor to consider when elaborating the biomimetic capsules. Indeed, cytoskeleton organization may differ when encapsulating cells in alginate matrices with differing degree of substitution (DS). Since DS is defined as the total number of RGD peptides per alginate chain, different alginate types can be obtained. In particular, Fig. 3.5 shows a study in which cells were entrapped in four different alginates: DS 0 (No modified alginate), DS 1 (0.112 mM), DS 5 (0.5 mM), and DS 10 (1.12 mM) [141]. Moreover, the necessity of evaluating the role of RGD for each cell line has also been stated, since the effects of the peptide sequence may vary.

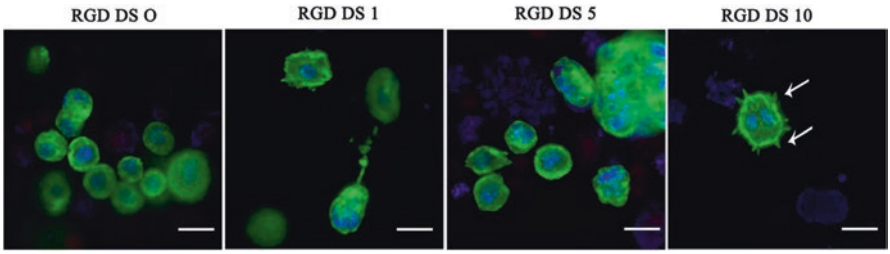

Fig. 3.5 Cytoskeleton organization of myoblasts and fibroblasts encapsulated in microcapsules elaborated with four different types of alginate in vitro. The cells inside APA microcapsules were stained with phalloidin Alexa Fluor 488 for F-actin (green) and Hoechst (blue) for nucleus. Scale bars = 520 μm (Reproduced from Ref. [141] by permission of John Wiley & Sons Ltd)

Other isolated ECM moieties that have been employed in alginate encapsulation are the laminin-derived peptides YIGSR (Tyr-Ile-Gly-Ser-Arg) and IKVAV (Ile-Lys-Val-Ala-Val) [73, 142].

Considering the importance of matrix functionalization, several novel approaches are being carried out [143, 144]. An interesting example is the introduction of galactosylated chitosan (GC) in the alginate core. GC provides cells with multiple binding domains that promote the cell-matrix interactions, improving viability and specific cell functions [144].

A different concept is the inclusion of growth factor-binding domains. Multiple signaling molecules that elicit essential cellular responses are present in the ECM given their non-covalent interactions with heparin sulfate proteoglycans. Therefore, the covalent tethering of heparan sulfate-containing molecules provides the hydrogel with binding domains that may sequestrate different growth factors for their posterior-controlled release [145]. Therefore, the encapsulated cell microenvironment may mimic more efficiently the ECM and, thus, improve cell fate.

3.5.2 Reducing Inflammation

The implantation of cell microcapsules involves a surgical procedure that in all cases is followed by a tissue repair response. This wound healing process is intensified due to the introduction of a foreign material that contains alien cells. Consequently, inflammation arises as a protective attempt to eliminate the prejudicial stimuli and initiate a healing process. Inflammation comprises vasodilatation, augmented blood flow, and increased permeability, which permits the migration of proteins and blood cells from the circulation to the damaged tissue. This immune response may limit the success of the system by leading to the pericapsular fibrotic overgrowth (PFO), a fibrotic cell layer that surrounds the system and, thus, reduces nutrient and oxygen supply endangering cell survival.

One of the strategies to mitigate this unsolved issue has been the co-administration of anti-inflammatory drugs. The in vivo screening of different anti-inflammatory molecules has pointed out to dexamethasone (DXM) and curcumin as the most effective drugs to inhibit reactive oxygen species (ROS) and early inflammatory proteases. Indeed, the co-encapsulation of cells with the latter has shown to reduce fibrosis and improve cell function [146]. The effect of DXM has also been studied. In an interesting approach, different composite drug delivery systems have been designed to combine APA cell microcapsules with DXM-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres. The combination has shown an enhanced performance of the system [147, 148], resulting in a promising alternative to prevent inflammation and, thus, improve the long-term efficacy of the graft, even when implanting xenogeneic cells [148]. More recently, the co-encapsulation of pancreatic islets with the anti-inflammatory drug pentoxifylline (PTX) was demonstrated to improve the resistance of these cells against host immune cells such as lymphocytes [149]. Similarly, antagonists of inflammation-mediators have also been entrapped in cell microcapsules. With the aim to reduce the inflammation induced by the high-mobility group box 1 (HMGB1), an antagonist of its receptor in immune cells, HMGB1 A box, was co-encapsulated. Results revealed that the amount of TNF-α secreted from macrophages was significantly attenuated. Moreover, an in vivo study showed a significant improvement in the survival rate of implanted cells [150].

Another alternative is the modification of the capsule with different motifs that exert an anti-inflammatory effect. That is the case of sulfated alginates, which are used as a secondary coating on alginate-PLL microcapsules or mixed in the gel core of non-coated microbeads, resulting in the reduction of inflammatory cytokines such as TNF, IL-6, IL-8, or IL-1b [151]. In another example, the attachment of an inhibitory peptide for cell surface IL-1β enabled the maintenance of cell viability in the presence of a combination of different cytokines including IL-1β or TNF-α. Contrarily, cells encapsulated in unmodified hydrogels were unable to survive to the exposure of these cytokines [50].

A newer strategy to reduce inflammation, and consequently PFO, is the co-encapsulation of effector-target cells with MSCs. MSCs are known to be hypoimmunogenic and exert an immunomodulatory effect because of the production of factors such as prostaglandin E2 or nitric oxide that modulate the immune response [152–154]. A recent study demonstrated the beneficial effects of co-encapsulated MSCs in an aggressive xenotransplantation model of mice by showing a dose-dependent reduction of PFO with the subsequent improvement on graft survival [155]. This approach has resulted beneficial in different applications including pancreatic islet transplantation, where MSCs present a high potential to overcome some of the current limitations of the system by suppressing inflammatory damage and immune-mediated rejection [81]. Moreover, it is possible to take advantage of the important characteristics of MSCs to modulate the neuro-inflammatory response. Therefore, it has been suggested that alginate encapsulation of MSCs may not only provide an auxiliary strategy to improve biocompatibility when co-encapsulated with other effector cells but also a valuable treatment for CNS trauma [156].

Equivalently, Sertoli cells also present similar immunoregulatory properties. In fact, they have demonstrated to deliver biomolecules associated with trophic and anti-inflammatory effects that synergistically act on multiple fronts [157]. Altogether, these factors have been proven to normalize glucose homeostasis in an experimental diabetes model [46] or to reduce striatal inflammation in Huntington disease, prolonging mice life span and improving quality of life [158].

3.5.3 Enhancing Biosafety

Despite cell encapsulation holds a great potential, the fact that it implies the use of living cells leads to safety concerns, particularly considering that in many cases these cells have been genetically modified. Moreover, once microcapsules are administered, the determination of their position and integrity is complex. In order to address this issue, different approaches have been proposed in order to monitor or inactivate the implants.

Diverse noninvasive imaging techniques have been developed with the aim of tracking the implants. Cell encapsulation is an asset for monitoring, as the contrast agents are contained in the hydrogel instead of directly labeling the cells. This reduces toxicity and promotes cell survival and function. Widely used modalities are X-ray/computed tomography (CT) [159, 160], magnetic resonance imaging (MRI) [161], and ultrasound imaging [162]. For instance, in a recent study, alginate microcapsules with a self-assembled gold nanoparticle (AuNPs) coating resulted in distinctive contrast and enabled the identification by using a conventional small animal X-ray micro-CT scanner. In particular, AuNPs were modified with the cationic 2-(methacryloyloxy)ethyl trimethylammonium chloride (METAC) polymer in order to enable the electrostatic interaction of AuNPs with the negatively charged alginate microcapsules. The resulting PMETAC_SH-Au nanoparticles were coated onto preformed alginate microcapsules (PMETAC_SH-Au MCs) with successful imaging results (Fig. 3.6a) [160].

The limitation of these techniques is that they do not provide information about cell viability or functionality. To overcome this issue, reporter genes have been employed [163–165]. They are typically based on bioluminescence and/or fluorescence and provide us with a helpful tool to perform noninvasive, quantitative, and real-time live image determinations. Regarding imaging techniques, recent approaches have also studied the possibility of analyzing the host immune response while the capsules are still implanted [166, 167]. This progress may represent a pivotal advantage, as it would permit to adjust the therapy if needed. In other words, in cases when a significant immune response is detected, the pertinent therapy could be administered in order to alleviate acute inflammation. Thus, the transplantation regimen would improve, restricting the possibilities of graft failure. This advance is especially relevant considering that nowadays it is not possible to analyze the host immune response unless biopsies are obtained.

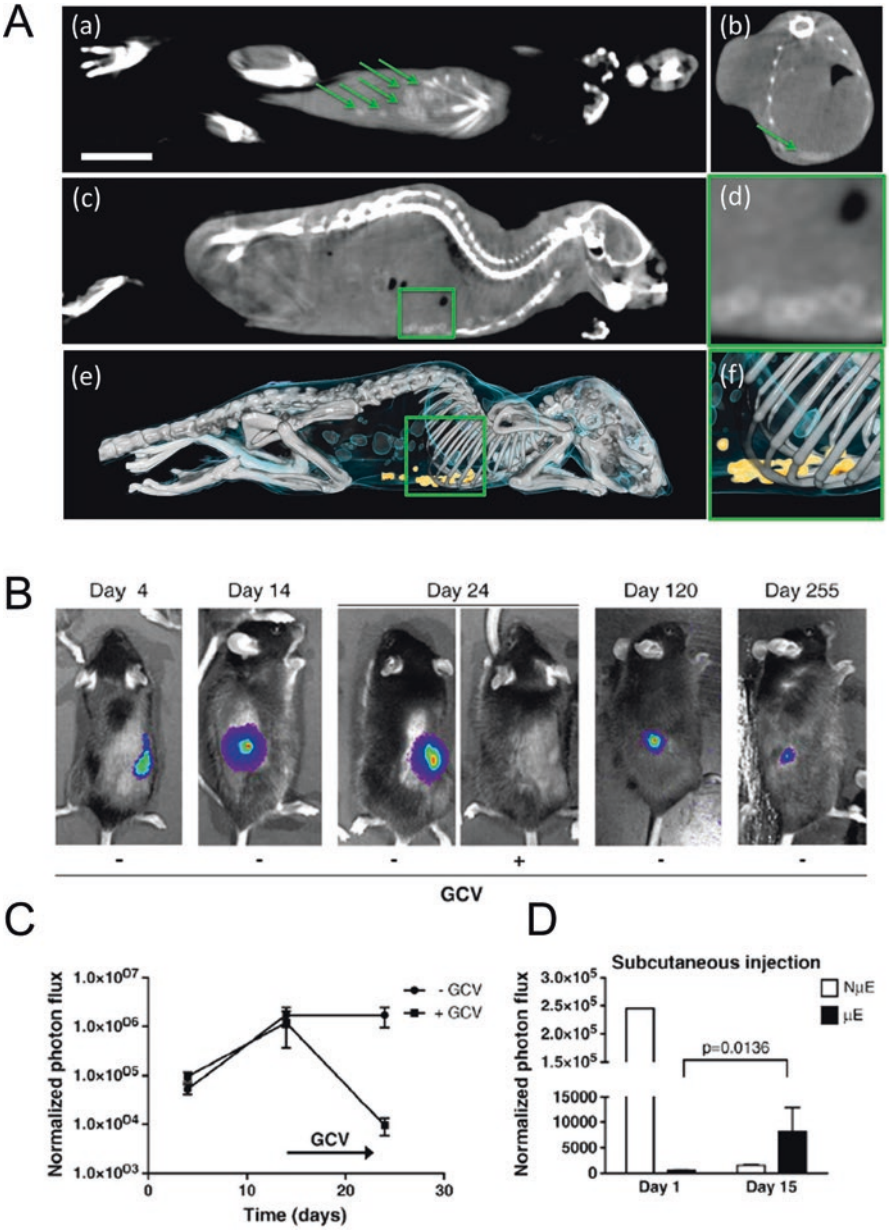

Fig. 3.6 (**a**) X-ray micro-CT reconstructed data of a mouse injected with PMETAC_SH-Au-MCs (4.7 g L^{-1}) (CTDI 28 mGy) in coronal (*a*), transverse (*b*), and sagittal (*c*) views with the magnification of the detail in the square shown in (*d*). Arrows indicate the MCs. 3D rendering of the same mouse with the MCs artificially colored in *yellow* (*e*) and the detail in the square magnified in (*f*). Scale bar 1 cm (Reproduced from Ref. [160] by permission of the Royal Society of Chemistry. (**b**–**d**) Behavior of C2C12-TGL microencapsulated cells in vivo. Microencapsulated C2C12-TGL cells exhibited light emission after being injected subcutaneously in C57BL/6J mice. Mice treated

A determinant aspect regarding the safety of the system is the possibility to inactivate the graft. This inactivation might be useful when the therapy reaches its final goal or when the system causes undesirable effects. To this end, an interesting approach is the inclusion of suicide genes in the genome of the encapsulated cells. These suicide genes induce apoptosis upon external drug administration, which allows the inactivation of the implant when necessary. Interesting studies were carried out regarding ganciclovir (GCV)-mediated inactivation in encapsulated cells bearing TGL triple fusion reporter gene, which codifies for the suicide gene herpes simplex virus type 1 thymidine kinase (HSV1-TK), green fluorescent protein (GFP), and firefly luciferase ($SFG_{NES}TGL$) [163, 165]. The in vivo studies demonstrated the potential of the procedure by providing information about the enclosed cells at desired time points in a noninvasive way, including the additional advantage of cell inactivation if required (Fig. 3.6b–d). The design of inducible systems may also dramatically improve safety. Recently, inducible hepatocyte growth factor (HGF)-secreting human umbilical cord blood (hUCB)-derived MSCs were produced via TALEN-mediated genome editing. The TetOn-HGF/hUCB-MSCs were encapsulated in alginate microcapsules and demonstrated an improvement in angiogenesis in a mouse hind limb ischemia model, proving that these inducible cells are able to deliver the therapeutic factor in a controlled and effective manner [168]. Therefore, these strategies may take control over the therapeutic effects exerted by the implanted cells leading to safer treatments.

3.5.4 Improving Administration and Extraction

The retention of microcapsules within the tissue they are implanted and the suitable retrieval of the whole implant are necessary premises in this technology. For this reason, several attempts have been carried out in order to optimize administration and extraction protocols. Different systems have been proposed to envelop the alginate cell microcapsules, such as calcium phosphate cements [169, 170], mesh bags [130], or hydrogel-based scaffolds [90]. The latter presents advantageous properties that permit to select between an invasive administration (as a preformed scaffold) and a noninvasive injection (as an in situ formed scaffold). Moreover, the scaffolds may be multifunctionalized by combining in the hydrogel the alginate microcapsules and other systems containing molecules such as anti-inflammatory drugs [148].

Fig. 3.6 (continued) with 150 mg/kg/day ganciclovir (GCV) for a week showed almost no signal (**b–c**). Luciferase activity could be found in cells within the microcapsules 255 days after injection (**b**). Quantification of light emission demonstrates an increase in the normalized photon flux during the first 2 weeks after implantation (**c–d**), probably due to vascularization of microcapsule plugs. Non-microencapsulated C2C12-TGL cells displayed a marked decrease in light emission between days 1 and 15 (**d**). Conversely, microencapsulated myoblasts increased the emission during that period of time (**d**). μE microencapsulated cells, $N\mu E$ non-microencapsulated cells (Reprinted from Ref. [163], Copyright 2010, with permission from Elsevier)

In addition, the area of implantation is generally a highly hypoxic and pro-inflammatory zone that jeopardizes the graft survival. On this point, the β-air device has emerged as an ingenious approach to solve this problem [171]. Preliminarily, a rat-type β-air device was designed to be implanted under the skin or into the pre-peritoneal cavity, areas of easy access. It consisted of two main components separated by an oxygen-permeable membrane: an islet module that contained the alginate-encapsulated cells and a gas chamber connected to an external air pump and an outlet port by two transcutaneous silicone tubes. When implanted in rodents, the macrochamber, refueled with oxygen, normalized glycemic control for periods up to 6 months. Moreover, the tissue surrounding the implant did not present signs of inflammation and showed visual evidence of vasculature. The success of the approach led to a porcine model of the β-air device. Equipped with xenogeneic rat islets, the device was implanted in diabetic Sinclair minipigs. Once again, the restoration of normoglycemia was achieved [172]. Altogether, the hallmarks of the β-air device are the sufficient oxygenation of the implant, a substantial immune barrier, especially relevant for xenotransplants, and a minimally invasive surgical procedure.

3.6 Concluding Remarks

Alginate is the most widely employed biomaterial for cell encapsulation as it provides the technology with pivotal advantages. In particular, the intrinsic characteristics of alginates make them biocompatible and allow the rapid and simple gelation under mild conditions, giving rise to scalable encapsulation methods. The obtained alginate microcapsules represent a potential alternative for the treatment of multiple chronic diseases that nowadays lack an adequate management with conventional drug delivery systems. That is the case of disorders with a high prevalence in our society such as diabetes, Alzheimer's disease, Parkinson's disease, chronic anemia, or cancer.

Over the last decades, important advances have been achieved in the field; however, there are still major challenges to face in order to move toward a definitive clinical translation. To this end, several attempts are being carried out, which include the search for suitable and biocompatible coatings [35], the biofunctionalization of the matrices, the optimization of safety measures such as inducible Tet-on/off systems [168], the synthesis of capsules with suitable size and shape [173], or the selection of the most appropriate administration site [34]. May these hurdles be overcome, therapeutic cell encapsulation would significantly evolve becoming an invaluable tool in the medical practice.

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Chapter 4
Alginate Processing Routes to Fabricate Bioinspired Platforms for Tissue Engineering and Drug Delivery

Vincenzo Guarino, Rosaria Altobelli, Francesca della Sala, Assunta Borzacchiello, and Luigi Ambrosio

Abstract Alginate is a water-soluble polymer which has gained much attention in the last 20 years as suitable biomaterial for numerous applications in biomedical science and engineering. The strong biocompatibility in cell microenvironment and the possibility to process alginate solution by safe conditions to reach a stable form after polymer gelation – via ionic, chemical, or thermal route – make them useful to design different types of devices (i.e., injectable gels, porous scaffolds, micro-/nanoparticles) which are attractive for wound healing, cell transplantation, drug delivery, and three-dimensional scaffolds for tissue engineering applications.

In this chapter, current potential applications of alginates in biomedical science, tissue engineering, and drug delivery will be discussed. After a brief overview of general properties of polymer and its hydrogels, we will focus on the processing techniques mainly used for their manufacturing, also suggesting, in the last part, potential uses and future perspectives for their novel applications in biomedical field.

Keywords Phase separation • Electrofluidodynamics • Emulsion • Layer by layer • Porous scaffolds • Micro-/nanoparticles • Drug delivery • Tissue engineering

4.1 Introduction

Alginates are natural polysaccharides typically obtained from brown seaweed which exhibit excellent biocompatibility and biodegradability that can be useful for many applications in the field of biomedicine. They mainly work as ionic polymers derived by the presence of divalent cations such as Ca^{2+}, which confer them

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interesting properties to fabricate micro- and nanostructured devices with improved molecular transport, low cytotoxicity, and relatively low-cost production, suitable for large applications in tissue engineering and drug delivery [1]. They are typically used in the hydrogel form, due to the peculiar organization of linear chains containing hydrophilic/hydrophobic ((1,4)-linked β-$_{D}$-mannuronate (M)/ α-$_{L}$-guluronate (G) blocks) [2] which concur to the assembly of stable three-dimensionally cross-linked networks with high capability of water retention, highly scalable swelling properties, and molecular release kinetics. In particular, only the G-blocks of alginate are mainly involved in the intermolecular cross-linking with divalent cations (e.g., Ca^{2+}) to form hydrogels with different properties [3]. However, relative M/G ratio, M and G sequence and block length, and molecular weight are thus critical factors affecting the physical properties of alginate and, ultimately, its resultant biological properties of hydrogels [4].

Besides, hydrogel-like behavior of alginates mainly contributes to the scaffold biomimesis, being structurally similar to the macromolecular-based components in the body. Their peculiar chemical properties assure a full compatibility in biological microenvironment, by minimizing inflammatory reactions after their administration into the body [5]. However, similarly to other biologically recognized hydrogels, alginate gels have very limited mechanical stiffness. Alginate can be easily modified via chemical and physical reactions to obtain derivatives having various structures, properties, and functions. A fine control of the structure and biological properties such as biodegradability, mechanical strength, gelation property, and cell affinity can be achieved through the combination with other biomaterials, immobilization of specific ligands such as peptide and sugar molecules, and physical or chemical cross-linking [6]. For instance, the mechanical properties of alginate gels typically may be enhanced by modifying the length of G-block and molecular weight. For example, gels prepared from alginate with a high content of G residues exhibit higher stiffness than those with a low amount of G residues [7]. However, an alginate solution formed from high molecular weight polymer generally tends to be too much viscous, showing several limitations in terms of processing [8]. In this case, proteins or cells mixed with an alginate solution of high viscosity may be more easily damaged under the effect of high shear forces generated during mixing and injection into the body [9]. Hence, the elastic modulus of alginate gels can be increased significantly, not drastically changing the solution viscosity, by using a combination of high- and low-molecular-weight alginate polymers [20]. Alternatively, structural properties of the alginates may be varied by different chemical ways. They may include natural treatments based on the use of Azotobacter species for the fabrication of bacterial alginates with higher concentration of G-blocks and, consequently, relatively higher stiffness [10] that confers a better control of the gel stability, drug release rates, and the cell phenotype functions. The most frequently used strategy still is based on the chemical cross-linking, acting directly on chemical groups of hydrophilic blocks [11]. Today, the current challenge is how to match the physical properties of alginate gels to the main requirements of specific applications, by properly setting chemical operational parameters and processing modes generally used in cross-linking strategies – i.e., various chemical

structures of molecules as cross-linking functionality, molecular weights, cross-linking density will often yield gels suitable for each application. In this context, the implementation/revisit of manufacturing processes is a key point to design innovative devices and platforms able to valorize the opportunity to manipulate alginate materials by green processing conditions [12] – for the safe release of molecules and/or cells in vitro and in vivo.

In the last years, alginate has been variously processed to fabricate injectable hydrogels, microspheres, microcapsules, sponges, foams, and fibers to be used as cell or molecular carriers suitable for drug delivery systems or tissue engineering. In this chapter, we aim at overviewing all the main processing techniques used for the manufacturing of alginates and their derivatives, basically remarking the relationships among process conditions, structure, and functions of the final devices.

4.2 Scaffolds for Tissue Engineering

A great challenge of current tissue engineering consists in controlling the delivery of biologics – namely, cells, genes, and proteins – via a degradable scaffold, to promote and support in vitro tissue regeneration. In this context, scaffold design plays a pivotal role to properly address in vitro properties of forming tissue toward an effective use in clinical application. Hence, manufacturing techniques must be sagely optimized to manipulate scaffold properties to trigger selected mechanical, mass transport, and surface functionalities in order to match environmental conditions to efficaciously work as in vivo models. [13]. Indeed, cell-loaded scaffolds have been successfully used as artificial ECM (extracellular matrix) analogue to provide a temporary environment able to support the cell infiltration, adhesion, proliferation, and differentiation [14]. In this case, they have to furnish the required structural support at the beginning of tissue growth and retain cells in the defective area for cell growth, metabolism, and matrix production, thus playing an important role during the development of engineered tissues. In the last years, many polysaccharides including alginates [15], chitosans [16], hyaluronic [17], and cellulose derivatives [18] have been investigated as highly porous, biomimetic scaffolds to overcome the limitations of two-dimensional (2D) culture systems. In particular, hydrophilic alginate hydrogels have raised special interest as a means to provide a temporary support for a variety of cell types including osteoblasts, chondrocytes, fibroblasts, and embryonic stem cells, showing minimal or negligible cytotoxicity and histocompatibility. All these results confirmed the ability of alginate-based scaffolds to properly degrade in vitro, promoting vascularization and elicited low inflammatory responses after transplantation, suggesting them as efficient cell and drug carrier for tissue regeneration.

4.2.1 Phase Separation

Traditional methods for producing porous biopolymer scaffolds include gas foaming, phase separation (freeze-drying), solvent casting, and particulate leaching [19]. In general, a polymer is first dissolved in a suitable solvent and subsequently placed in a mold that will be rapidly cooled until the solvent freezes. The solvent is then removed by freeze-drying, and pores will be left behind in the polymer. Different types of phase separation techniques are available, including thermally induced, solid-liquid, and liquid-liquid separation mechanisms [20–22].

Porous alginate-based scaffolds, foams, and sponges with interconnected porous structures and predictable shapes can be easily manufactured by a regular thermally induced phase separation (Fig. 4.1) [18]. When the temperature is low enough to allow freezing the solution, the phase separation mechanism induces the solid-liquid demixing, therefore forming frozen solvent and concentrated polymer phases. By adjusting the polymer concentration or varying the cooling rate, phase separation could occur via different mechanisms, resulting in the formation of scaffolds with various morphologies. "Freeze-drying" is one of the most extensively used methods that produce matrices with porosity greater than 90%. The pore sizes depend on the growth rate of ice crystals during the freeze-drying process. After the

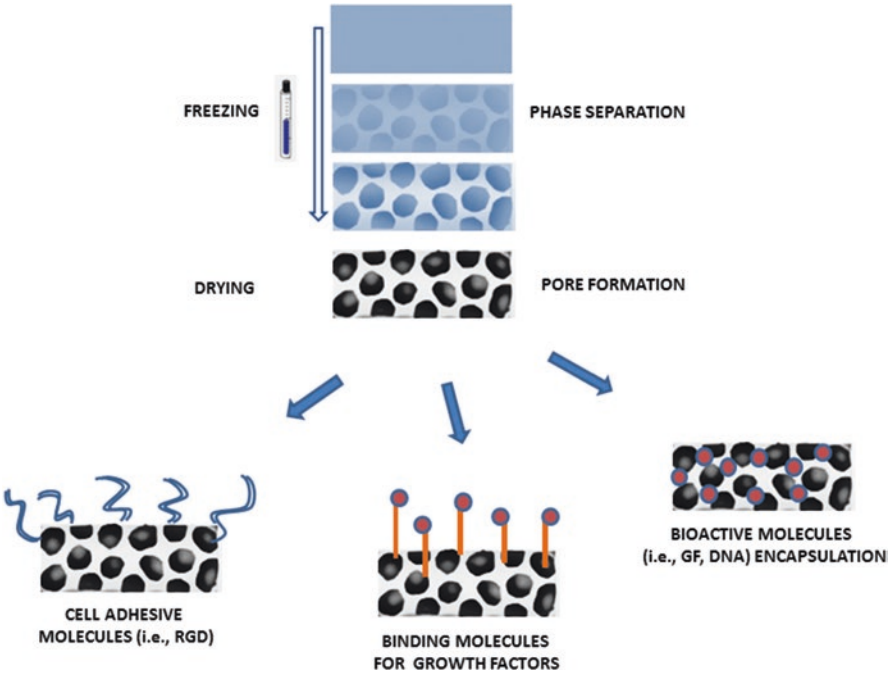

Fig. 4.1 Alginate porous foams fabricated via freeze-drying technique and different bioactivation procedures for improving cell recognition

removal of the liquid or frozen solvent contained in the demixed solution, the space originally occupied by the solvent would become pores in the prepared scaffolds. Obviously, in the stage of solvent removal, the porous structure contained in the solution needs to be carefully retained. Without freeze-drying, a rise in temperature during the drying stage could result in remixing of the phase-separated solution or remelting of the frozen solution, leading to destruction of the porous structure. In the case of hydrogels, the freeze-dry processing does not require additional chemicals, relying on the water already present in hydrogels to form ice crystals that can be sublimated from the polymer, creating a particular micro-architecture. Because the direction of growth and the size of ice crystals are a function of the temperature gradient, linear, radial, and/or random pore directions and sizes can be produced with this methodology [23]. The mechanical properties and biodegradation rate of freeze-dried scaffolds can be simply modulated by changing the relative parameters of the polymers. Porous scaffolds formed by pure alginate are unable to provide enough bioactive properties to support cell metabolism due to the lack of cellular interaction in the molecular structures [8, 18, 24, 25]. Therefore, alginate has been blended with collagen and/or gelatin to enhance cell ligand-specific binding properties to fabricate hybrid scaffolds, which showed better properties for supporting cells [18, 26, 27]. Recently, other efforts were made to enhance the biological properties of alginate porous scaffolds. In this way, alginate was irradiated and oxidized to modify its degradation and covalently grafted with growth factors, lectins, and peptides containing a RGD (arginylglycylaspartic acid) sequence to promote cell adhesion and proliferation [18, 28–30]. As we have seen, the porosity and pore sizes of the scaffolds fabricated through this method are largely dependent on the parameters such as the ratio of water to polymer solution and viscosity of the emulsion. This technique also does not necessitate an extra leaching step, but the addition of organic solvents such inhibits the incorporation of bioactive molecules or cells during scaffold fabrication. In addition, the small pore sizes obtained are another limiting factor of scaffolds fabricated by phase separation [22].

4.2.2 Atomization

The ultimate goal of regenerative medicine is to produce bioinspired scaffolds able to meet specific functionalities of native microenvironment by replying their specific chemical (i.e., composition), physical (i.e., fluid transport), and morphological (i.e., shape, porosity) features [13]. Several investigations have been performed to design alternative systems able to conjugate main advantages and, simultaneously, remove major drawbacks of traditional porous systems. For example, micro- and nanoparticles have been integrated to polymer gels to realize injectable or moldable systems (i.e., gels, cements) able to better support cell activities, i.e., adhesion, proliferation, and mineralization – in the three-dimensional (3D) cavities [31]. These systems offer the possibility of injecting isolated polymeric particles as vehicles to deliver encapsulating bioactive agents or in vitro pre-seeded cells in the defect [32,

33]. Alternatively, different types of micro-sized polymeric particles have been extensively used to assemble porous scaffolds with interconnected porosity by particle agglomeration via sintering or solubilization methods [34], mainly due to relevant benefits associated with high surface area for cell expansion.

More recently, the use of polymeric microgels – i.e., sized from 10 to 1000 μm – is rapidly diffusing for different applications in tissue engineering and nanomedicine [35]. Hydrogels in the form of capsules or particles have been largely used to deliver active molecules or living cells for therapeutic and cell-based disease treatments [36]. Their peculiar capability to generate a highly hydrated microenvironment also allows for protecting sensitive drugs, thus preserving molecular stability prior to the delivery at the site of injury [37]. Meanwhile, they may assure an efficient transport of biological substances, such as nutrients and products from cell metabolism, in and out of the hydrogels, which are fundamental to protect and sustain cell viability during the regeneration processes [38, 39].

In this way, several strategies have been implemented as the in situ formation of scaffolds by cell-induced aggregation of injected or cell-encapsulated hydrogel particles to promote the cell delivery for new scale-up biofabrication methods, such as organ printing [33, 40–43]. The wide versatility of these systems is mainly associated with the variables that can be tuned to obtain an optimal system for a specific application, such as the particle composition, size and shape, existence of porosity, cell culture conditions, or incorporation of bioactive agents.

During the last decades, several techniques have been used to process microparticles for different applications. The preparation of microgels or particles requires several considerations about their manufacturing, modification, and manipulation and should also ideally allow the production of large quantities of particles with a narrow size distribution [33].

Among them, atomization techniques are the most viable way to create systems that have complicated external anatomic shapes and complex internal porous architectures. In this method, an electric field is applied to a polymeric solution extruded from a syringe. The applied high voltage potential forces the polymer to form a jet that, using specific parameters, enables the formation of micro-/nanoparticles [32]. The great advantage of this technique over other commonly used methods is the fact that it is a one-step process that does not make use of organic solvents or cross-linking agents [13]. Their ability is to create devices to achieve both a wide range of effective mechanical and mass transport properties as well as a low variation in those properties. Further the much finer feature resolution that these techniques can generate is beneficial for cell seeding and growth factor delivery [13, 44, 45].

The encapsulation of living cells in a variety of soft polymers or hydrogels is important, particularly, for the rehabilitation of functional tissues capable of repairing or replacing damaged organs (Fig. 4.2). Generally, cells are mixed with the encapsulation material before gelation occurs. Diverse hydrogel membranes have been popularly used as encapsulating materials and permit the diffusion of gas, nutrients, wastes, and therapeutic products smoothly. Microtechnologies have been adopted to precisely control the encapsulated cell number, size, and shape of a cell-laden polymer structure [22, 46]. Many biomaterials have been proposed as

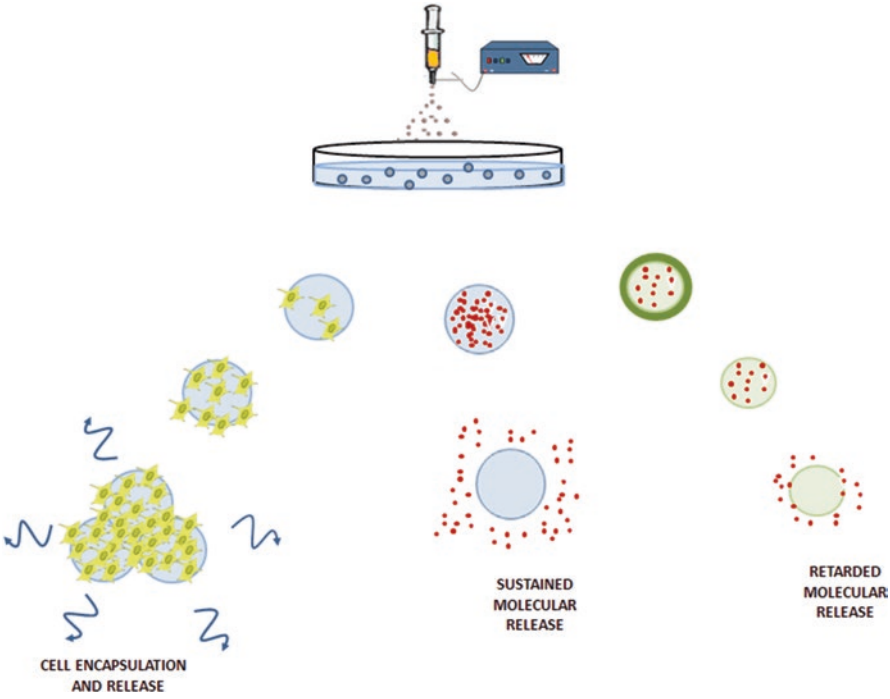

Fig. 4.2 Fabrication of alginate micro-carriers by electrohydrodynamic atomization: schematic approach for cell and molecular release

entrapping matrix to fulfil the specific requirements. Alginate is by far the most studied material for cell encapsulation, and it has been adopted for many biomedical applications. This material has historically been used as a protective barrier to enhance cell therapies, for immunoprotection of pancreatic islets, treatment of brain tumors, treatment of anemia, and cryopreservation [47–49]. The main benefit of using water-absorbable polymers for the preparation of these systems is not only to develop a confined barrier to entrap living xenogeneic or allogeneic cells to be transplanted but also to prohibit the entry of the host antibodies and immune cells. Besides, encapsulated cells generally show limited interactions, and the physical barrier concurs to mask them from the immune surveillance at a local level, thereby assuring a reduction of the inflammatory response after transplantation [50]. Microcapsule and micro-carrier systems provide a larger surface area for cellular attachment, provide cell protection against excessive mechanical stresses, and simulate an in vivo environment [51, 52]. Recent improvements in fabrication technologies allow generating matrices with different conformations – i.e., core shell with liquid or hollow core and/or multicomponent coatings – which may optimally be adapted to co-culture of single/multiple cell types for molecular guided tissue engineering and "bio-organs" manufacturing [52, 53].

Among them, electrofluidodynamic atomization (EFDA) is a promising technology capable of generating micro-sized scaffolds, due to the opportunity to fabricate variously sized particles from micro- to sub-micrometric scale, by controlling a large set of parameters including applied voltage, biomaterial properties (e.g., concentration/viscosity, conductivity), and needle geometry, in order to design structures with growing complexity [54–56]. Recently, electrofluidodynamics has been successfully used as low-cost, high-throughput, controlled technology for the production of full or hollow spheres from a polymer solution, by applying a high-voltage electric field [57]. The principle of the electric field-assisted atomization is based on the ability of electric forces to charge solution droplets by deforming their interface until breaking them into smaller droplets in the micrometric/sub-micrometric range. The jet deforms and disrupts into droplets due mainly to electrical forces by the competition between coulomb forces related to surface charge and cohesive forces inside the droplet, without the administration of additional mechanical energy to reach the liquid atomization [58]. Atomization process can be distinguished in electrodynamic spraying (EDS) and electrohydrodynamic atomization (EHDA), respectively, as a function of the collector used: EDS and EFDA [59, 60].

The former one involves the deposition of charged droplets on a grounded plate, by the breaking of polymer jet into nano-droplets under the solution overcharging conditions to form individual nanoparticles or agglomerates as a function of the local surface charge. The latter one is based on the deposition of charged droplets in a cross-linking agent solution – i.e., calcium chloride ($CaCl_2$) for alginate particles – prior to the solution overcharging, by the perturbation and cutting of polymer jet until the formation of micro-sized particles.

Future directions should veer on the development of technically advanced systems to satisfy several demands as controlled systems, operational rigor, and improved quality control. Cryopreservation, banking, and subsequent culture of cells encapsulated in microcapsule delivery systems represent an alternative to the economically attractive “off-the-shelf” micro-carrier concept. With long-term delivery of therapeutic agents, the cost of encapsulated cells is offset by the cost of short half-life therapeutic peptides and the costs involved in organ transplantation. This favorable pharmaco-economics of electrodynamic atomization-based technologies may impact insurance companies who may favor such an approach over traditional therapies, creating a paradigm shift in regenerative medicine [52].

4.3 Design of Carriers for Drug Delivery

4.3.1 Emulsion

Alginate has many possible applications in the area of drug delivery due to their low cost, low toxicity, biocompatibility, and biodegradability [61, 62]. Alginate microspheres have been widely used as carriers for the controlled release of active agents

due to their low immunogenicity and their muco-adhesive properties [63]. In addition, alginate matrices have the ability to encapsulate protein and DNA while maintaining their biological activities and have shown very strong bioadhesive abilities making alginate a promising candidate for site-specific mucosal delivery [64–66]. Moreover, the alginate polymer also finds application as a surfactant stabilizer in oil-water emulsions in microbead preparation. Alginate surfactants serve to lower the interfacial energy between the phases, thereby increasing the stability and lifetime of the emulsion. In fact, an emulsion is a thermodynamically unstable system consisting of at least two immiscible liquid phases, one of which is dispersed as globules in the other liquid phase, stabilized by the presence of an emulsifying agent, as can be observed in Fig. 4.3. Emulsions fall into a greater class of two-phase systems known as colloids, with the special characteristic that both the dispersed and continuous phases are liquid. Depending on the volume fraction of the phases, both water-in-oil and oil-in-water emulsions can be formed. There is a rule which governs the emulsion formation, known as the Bancroft rule: emulsifiers and emulsifying tend to promote dispersion of the phase in which they do not dissolve well; for example, proteins dissolve better in water than in oil and therefore tend to form oil-in-water emulsions, promoting the dispersion of oil droplets throughout a continuous phase of water. Emulsions can be prepared through various methods of agitation, as the two phases are immiscible and droplets will not form spontaneously, such as sonication, which can produce droplets of 100–400nm. Alginate microbeads for drug delivery can be easily prepared by emulsion-gelation technique. As reported by Putta S.K. et al. [67], an example of alginate microbead preparation consists of sodium alginate dissolution in purified water to form a homogeneous polymer solution. The drug is added to the solution and mixed thoroughly with a stirrer to form a viscous dispersion. The dispersion is then added in an oil phase such as heavy liquid paraffin while stirring at sufficiently high speed to emulsify the added dispersion as fine droplets. Then appropriate amount of calcium chloride solution (15% w/v) is transferred into the emulsion while stirring to complete the gelation reaction and to

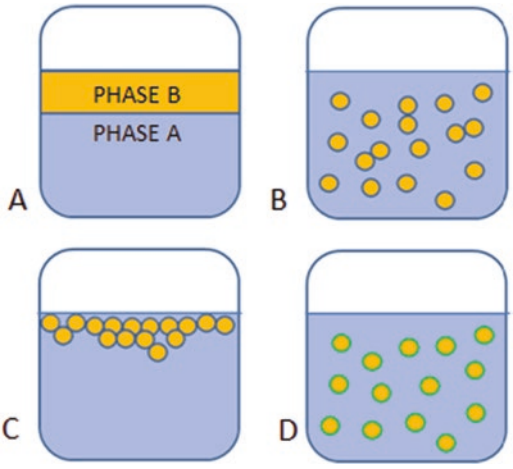

Fig. 4.3 (**a**) Two immiscible liquids not emulsified. (**b**) An emulsion of phase B dispersed in Phase A. (**c**) The unstable emulsion. (**d**) The green surfactant as an emulsion stabilizer

produce spherical rigid microbeads. The microbeads are collected by decantation and washed repeatedly with petroleum ether. The product is then air dried overnight at room temperature to obtain discrete microbeads. This method of preparation shows many advantages [68]; first of all it is easy, mild, and inexpensive preparation technique, it is stable, and it is suitable to encapsulate broad categories of drugs such as macromolecules, protein, and others.

By emulsion technique various alginate microbeads have been prepared with different therapeutic applications, such as the encapsulation of a water-soluble drug as ranitidine hydrochloride (peptic ulcer drug) in sufficient amount and that could be delivered in the stomach for a prolonged period of time without using any organic solvent and any time-consuming step in the preparation, so opening new treatment of gastric acidity and ulcer [69]. Furthermore, for the delivery of acyclovir, antiviral medication, oil entrapped acyclovir floating alginate beads used as floating controlled drug delivery systems have been also realized. The beads showed the excellent sustaining properties as compared to the conventional dosage form. The designed therapeutically efficacious gastro-retentive formulation of acyclovir, combining an excellent buoyant ability and a suitable drug release pattern, could possibly be advantageous in terms of increased bioavailability of acyclovir [70]. Therefore, alginate microspheres appear, technologically, as a promising antigen delivery system; in fact, the alginate microspheres were easy to prepare, and the mean diameter of beads increased with the increase in the amount of the oil phase. The encapsulation efficiency of bovine serum albumin (BSA), chosen as model antigen, was very high. The beads showed excellent sustaining properties as compared to the conventional beads [71]. As regards the treatment of diabetes, insulin, an antidiabetic drug, has been formulated in the alginate microspheres intended for oral administration in order to directly deliver them in the intestinal region without drug degradation in the stomach. Process and formulation variables were of utmost importance for size and encapsulation properties. Encapsulated insulin produced the same bioavailability in diabetic rats as short-acting insulin formulation showing that insulin integrity was maintained during microspheres manufacturing and recovery [72]. Besides, the emulsion-gelation method was successfully utilized for the formulation of floating alginate beads of clarithromycin, antibiotic used to treat various bacterial infections, including strep throat, pneumonia, skin infections, *Helicobacter pylori* infection, and Lyme disease, among others. The adopted method for the estimation of clarithromycin showed good linearity. The formulated floating alginate beads have shown high percentage of drug loading, encapsulation efficiency, particle size, and very low moisture content. In vitro dissolution study showed that, among the formulations, the formulation containing 2 percent sodium alginate solution and 5 percent calcium chloride solution along with 500 mg hydroxypropyl methylcellulose (HPMC) and 5 ml of sunflower oil released clarithromycin for prolonged time (12 h) [73].

4.3.2 Layer by Layer

Alginate microbeads can be also prepared by layer by layer technique, generally to make multilayered beads. An advantage of these multilayered alginate beads can be due to their ability to allow simultaneous encapsulation of cells within the alginate core and delivery of other factors/cells from the outer layer of alginate. Growth factors can be encapsulated and delivered from the outer layer with release kinetics determined by its composition [74, 75].

Coating the alginate core with a perm-selective layer polymer may reduce or possibly completely eliminate the requirements of immunosuppressive drugs when delivering allogeneic or xenogeneic cells. Alginate microcapsules are typically coated with a polycation, such as poly-L-ornithine (PLO) or poly-L-lysine (PLL). The positive cationic chains interact with the negative alginate core to form a polycation-polyanion complex that coats the entire alginate surface. This layer influences the rate of diffusion of solutes and is typically designed to limit the immune system from recognizing the cells contained within the core. PLO and PLL play an important role in transport, but their positive charge results in inflammatory response following implantation, so to avoid this the surface is coated with an outer monolayer of alginate to prevent direct contact of cells from the host with the polycation.

Delivery of fibroblast growth factor-1 (FGF-1) from multilayered alginate beads, made of two alginates layers with in between a PLO layer, has been shown to stimulate blood vessel formation around encapsulated cells [76, 77]. In order to achieve the success in islet trasplantation, the local tissue response, including vascularization and modulation of the foreign body response, is essential. These multilayer structures allow for delivery of factors directly around the encapsulated cells in order to provide both therapeutic cells and local control of tissue development. The procedure for generating multilayer alginate microbeads, shown in Fig. 4.4, involves first the synthesis of uncoated alginate microbeads followed by coating of the polycation layer and, finally, the generation of an outer alginate layer. Briefly, to prepare uncoated alginate microspheres, low and high viscosity alginate solution is extruded in a flask containing the cross-linking solution (such as $CaCl_2$ or Ba) for a defined period of time with continuous stirring. The resulting gelled spherical microbeads to be covered by a polycation layer (e.g., PLL or PLO) are transferred into the polycation solution. After, the polycation solution is removed and washes are performed, an alginate solution of desired concentration is transferred onto the alginate microbeads previously created and incubated for a defined time to allow for alginate to interact with polycation layer. Then, the microbeads are transferred into the cross-linking solution to allow formation of outer layer [78].

Furthermore, multilayer sodium alginate beads have been successfully developed to improve the oral availability of conventional anticancer drug [79, 80]. To improve these, nanoparticles have been immobilized on multilayer alginate beads, by dropping aqueous chitosan/carboxymethylchitosan (CS/CMS) blended with alginate into CaCl2 solution [81]. The cross-linker between $–COO^-$ groups on alginate and Ca^{2+} occurred from external of alginate matrix to form the compact core of

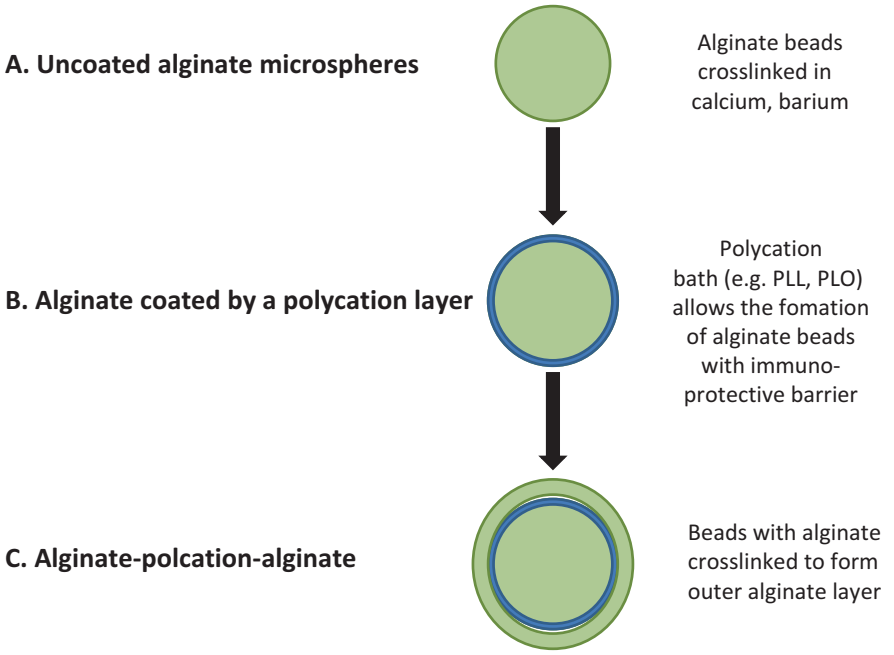

Fig. 4.4 Multilayered alginate microbeads consist of a perm-selective membrane around the alginate core (**a**–**b**) followed by an outer layer alginate (**c**). The outer layer alginate allows for encapsulation of proteins and growth factors while also maintaining biocompatibility

multilayered bead. The alginate matrix could protect encapsulated doxorubicin (DOX) loaded CS/CMS nanoparticles from undesirable drug release in gastric juices but too rapidly releases the intact DOX:CS/CMS nanoparticles in the small intestine. To overcome this limitation, it has been constructed a porous core of multilayer beads by internal gelation method to improve their ability of drug control release. By dropping aqueous CS/CMS nanoparticles blended with alginate and $CaCO_3$ into hydrochloric acid, the cross-linking between Ca^{2+} and $–COO^-$ groups on alginate occurred from interior of alginate matrix to form a compact core with porous structure in multilayer bead. The cross-linking effect is strengthened in the core of porous multilayer beads, and it may overcome undesirable drug release encountered in nanoparticle multilayer beads, to prolong the contact time between the formulation and the small intestinal mucosa, thereby potentially enhancing drug delivery efficacy. Concluding, alginate beads loaded with drug are important in the drug delivery by oral route as well as other routes, as sustained and controlled release formulations. As these microbeads are biocompatible, nontoxic, and biodegradable, so they may be better used and, i.e., they have paved a better way for controlled/sustained release of drug through the use of natural, biodegradable material.

4.4 Applications in Regenerative Medicine and Industrial Pharmacology

Industrial applications of alginates, based on their gelling, viscosifying, and stabilizing properties, account for the quantitatively most important uses of alginates. In comparison, emerging and knowledge-demanding specialty applications within biotechnology and medicine are based on biological effects of the alginate molecule, of alginate molecular building blocks, or of alginate's unique, gentle, and almost temperature-independent sol/gel transition in the presence of multivalent cations (e.g., Ca^{2+}), which makes alginate highly suitable as an immobilization matrix for biocatalysts such as living cells [82]. The conventional role of alginate in pharmaceutics includes serving as thickening, gel forming, and stabilizing agents, as alginate can play a significant role in controlled release drug products. Oral dosage forms are currently the most frequent use of alginate in pharmaceutical applications, but the use of alginate hydrogels as depots for tissue-localized drug delivery is growing [53].

Alginate gels have been investigated for the delivery of a variety of low-molecular-weight drugs and are likely most useful when a primary or secondary bond between the drug and the alginate can be exploited to regulate the kinetics of drug release. Alginate gels are typically nanoporous, leading to rapid diffusion of small molecules through the gel. However, incorporation into beads formed from partially oxidized alginate in the presence of both calcium ions and adipic acid dihydrazide (combination of ionic and covalent cross-linking) led to a prolonged release due to the increased number of cross-links and resultant reduced swelling [83–85]. The controlled and localized delivery of antineoplastic agents has also been achieved using partially oxidized alginate gels. Multiple drugs can be loaded into alginate-based gels for simultaneous or sequential delivery, as the chemical structure of the drug and mode of incorporation will dramatically alter the release kinetics [53, 86–88]. Alginate has also been widely exploited in many drug delivery applications in combination with chitosan, as the combination forms ionic complexes [44, 53, 88].

Alginate gels are increasingly being utilized as a model system for cell culture in biomedical studies. These gels can be readily adapted to serve as either 2D or more physiologically relevant 3D culture systems. RGD-modified alginate gels have been most frequently used as in vitro cell culture substrates to date. Further, the number of cells adherent to the gels, as well as the growth rate, was strongly dependent on the bulk RGD density in the gels. The length of the spacer arm between the RGD peptide and the alginate chain is a key parameter in regulation of cellular responses. Recent studies utilizing alginate gels as 3D cell culture substrates have revealed key insights regarding stem cell and cancer biology. The fate of mesenchymal stem cells was demonstrated to be controlled by the elastic modulus of the RGD-alginate gels in which they were encapsulated, as differentiation down fat and bone pathways was promoted at different values of gel stiffness. Strikingly, and in contrast to 2D culture systems used in previous mechanotransduction studies, the control over stem cell fate was related to the number of adhesive bonds formed between the gel and the cells, as well as alterations in the receptors cells utilized to adhere to the RGD pep-

tides in 3D versus 2D culture. The cells actively reorganized on the nanoscale the adhesion ligands presented from the gels. Alginate gels have also been used to examine how a 3D culture microenvironment influences cancer cell signaling and tumor vascularization [53, 89–92]. Alginate has also been combined with inorganic materials to enhance bone tissue formation. Alginate/hydroxyapatite (HAP) composite scaffolds with interconnected porous structures were prepared by several methods to enhance the adhesion of bone cells. Also cell-encapsulating alginate gel beads were introduced into calcium phosphate cement and demonstrated potential for bone tissue engineering under moderate stress-bearing conditions [53, 93–96].

In this context, alginate has demonstrated great potential for the fabrication of micro-sized devices for many applications in drug delivery, tissue engineering, and cancer therapy. Most attractive features of alginate in biomedical field include biocompatibility, mild gelation conditions, and simple modifications to prepare alginate derivatives with new properties. Alginate has a track record of safe clinical uses as a wound healing dressing material and pharmaceutical component; it has been safely implanted in a variety of applications, including islet cell transplantation [50, 53]. As one looks to the future, the alginate-based materials used in medicine are likely to evolve considerably. In wound healing, and more generally drug delivery applications, precise control over the delivery of single vs. multiple drugs or sustained vs. sequential release in response to external environmental changes is highly desirable. Dynamical control over delivery can potentially improve the safety and effectiveness of drugs, providing new therapies. On-demand drug release from alginate gels in response to external cues such as mechanical signals and magnetic fields can be used to design active depots of many drugs, including therapeutic cells. The introduction of appropriate cell-interactive features to alginate will also be crucial in many tissue engineering applications. The type of adhesion ligands and their spatial organization in gels are key variables, as they can regulate cell phenotype and the resultant function of regenerated tissues. Further, current understating of fundamental properties of alginate and developing of new types of cell and tissue-interactive alginate gels may enable future advances in biomedical science and engineering [53].

4.5 Conclusions and Future Trends

Alginate is one of the most frequently used polysaccharides in biomedical field, to fabricate building blocks or scaffolds for tissue repair and regeneration as well as micro-sized devices for drug delivery applications. The peculiar hydrogel-like structure makes them promising as scaffolds able to integrate either cells or bioactive molecules for tissue engineering. The success of tissue constructs is highly dependent on the design of the alginate-based scaffolds including the physical, chemical, and biological properties. In this context, physical and/or chemical modifications have been carried out to design *ex novo* polymers with the desired properties and functions, despite several limitations still concern the chance to meet all the design parameters simultaneously (e.g., degradation, swelling, and mechanical properties).

In the future perspective, alginate-based materials used in medicine are likely to evolve rapidly. To date, alginate gels played a fairly passive role in the clinical use for wound healing applications. Future dressings will likely play a much more active role of alginates in the controlled delivery of more bioactive agents to facilitate wound healing. Indeed, the use of alginate gels to incorporate bioactive molecules may assure the preservation of local concentrations of biological factors, such as proteins, for extended time periods. Besides, in the current drug delivery applications, a precise control over the delivery of single vs. multiple drugs or sustained vs. sequential release in response to external environmental changes is highly desirable. So, the use of active alginate gels may contribute to dynamically control the delivery mechanisms by improving the safety and effectiveness of drugs, for the discovery of new therapies. On-demand drug release from alginate gels in response to external cues (i.e., mechanical [97] and/or electromagnetic [98] stimuli) can be used to design active depots both for therapeutic drugs and cells.

Moreover, they will allow incorporating cell induction ligands such as growth factors directly to the scaffold surfaces for a controlled delivery of bioactive signals such as functional DNAs or siRNAs by appropriate spatial and temporal way. For this purpose, the introduction of appropriate cell-interactive features to alginate will also be crucial in many tissue engineering applications. The type of adhesion ligands and their spatial organization in gels are key variables, as they can regulate cell phenotype and the resultant function of regenerated tissues. In the place of RGD peptides extensively exploited as a cell adhesion ligand in the last years, multiple ligands and/or a combination of ligands and soluble factors could be alternatively considered to properly address specific biomolecular mechanisms for the replacement of tissues and organs. In this context, a deeper understating of fundamental properties of alginate as well as the development of new tissue-interactive alginate gels with novel chemical and physical moieties could enable future advances in biomedical science and engineering. For instance, it could be possible to design interpenetrating polymer networks by using alginates as building blocks for the design of pharmaceutics and drug delivery vehicles (i.e., drugs, biomolecules, proteins) in the form of films, microspheres, and depot matrices. By their assembly, the peculiar gelling ability with divalent cations offers the unique opportunity to fabricate smart systems able to change and/or adjust their mechanical and drug release properties in response to an external stimulus. For instance, new interpenetrated systems based on the combination of alginates with other natural polymers, mainly including polysaccharides with intrinsic bioactivity such as chitin or cellulose derivatives, have been recently exploited to improve the performances of multiple drug delivery systems. In this case, the synergistic behavior of interacting polysaccharide networks able to exert new features with respect to the single ones will concur to the design of novel in vitro models for fundamental studies to understand and tailor their physicochemical and biological properties, as well as for development of smart systems that fulfil unmet medical and pharmaceutical needs.

Meanwhile, the design and creation of new alginate polymers with better or distinct properties can potentially be achieved using genetic engineering techniques to control bacterial synthesis. Various polypeptides and proteins with improved struc-

tural properties and novel functions have already been prepared by this approach and explored for biomedical applications [99]. The ability to chemically or physically engineer new classes of alginates with precisely controlled functionalities, unlike the limited repertoire available from natural sources, designed for a specific application, paves toward an enormous revolution for the future use of alginate in biomedical field.

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Chapter 5
Alginate Utilization in Tissue Engineering and Cell Therapy

Bapi Sarker and Aldo R. Boccaccini

Abstract Due to the structural similarity to the extracellular matrix, nowadays, hydrogels are widely used for tissue engineering applications. Among the various hydrogels, alginate is considered a very useful biomaterial that has found numerous applications in the biomedical field due to its favorable properties, including biocompatibility and ease of gelation. It has been used to design tissue engineering constructs of various structures, such as porous scaffolds, microspheres, films, and microcapsules for drug and cell delivery and various tissue engineering applications particularly for bone, cartilage, muscle, and vascular tissue engineering. This chapter will provide a comprehensive overview of the applications of alginate-based hydrogels as various forms of constructs and scaffolds for tissue engineering.

Keywords Alginate • Hydrogel • Tissue engineering • Scaffold • Bioprinting

5.1 Alginate: A Promising Biomaterial

Regarding biocompatibility, cytocompatibility, and biodegradability, naturally derived hydrogel-forming materials are an attractive choice as an exogenous extracellular matrix (ECM) for applications in tissue engineering. Hydrogels derived from natural materials exhibit a similar structure to the ECM of many native tissues. The polymeric structure of these hydrogels is similar to the biological macromolecules engineered by nature to perform specific functions in the human body [1, 2]. A wide variety of naturally derived hydrogel-forming materials is used for tissue engineering applications.

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Among the most popular hydrogels, alginate, an anionic polymer, is receiving increasing attention in a variety of tissue engineering applications. Alginate is biocompatible, nontoxic, and non-immunogenic. In addition to its biocompatibility, low cost and availability make alginate one of the widely used materials in tissue engineering. The main advantage of alginate is its rapid ionic gelation which occurs at mild pH and temperature conditions, suitable for living cells and also for sensitive biomolecules like proteins and nucleic acids. Due to the rapid ionic gelation and the mild gelation conditions, alginate is widely used for the encapsulation of cells, drugs, enzymes, and biomolecules as well as for the biofabrication of tissue or organ-like structures. Alginate is an excellent material for performing two-dimensional (2D) and three-dimensional (3D) cell study in order to investigate cell-matrix interaction and other aspects of cell behavior, like proliferation, migration, and differentiation. The dimension of the cell culture microenvironment is one of the most important factors for cell-material interactions influencing the phenotypic morphology of the cells [3]. 2D cell culture systems which have been widely used in the field of tissue engineering are being increasingly replaced by 3D systems in the current era of regenerative medicine in order to closely mimic the in vivo environment of the native ECM. Alginate is extensively used as hydrogel in 3D scaffold for investigating cell behavior in the 3D environment since it embodies tissue-like flexibility while possessing viscoelastic properties, interstitial flow, and diffusive transport characteristics similar to the native ECM. In the hydrogel state, alginate composes a huge amount of water, which provides a tissue-like environment to the embedded or encapsulated cells. Moreover, due to the inherent porosity of the hydrogel, the alginate matrix possesses semipermeability that allows the exchange of oxygen, nutrients, and metabolites and simultaneously protects the encapsulated cells from the toxic foreign bodies. Alginate is an ideal scaffold-forming material, which can also be modified with proteins, peptides, and inorganic and other organic biomaterials, opening multiple possibilities for tissue engineering applications.

5.2 Alginate in Tissue Engineering: Opportunities and Limitations

Though alginate has a lot of advantages, it possesses some major limitations for the applications in tissue engineering, which will be discussed in this section. Alginate hydrogels do not degrade but rather disintegrate when the coordinated divalent cations are replaced by monovalent cations present in the surrounding fluids. The interactions between the alginate chains and the monovalent cations lead to the dissolution of the gels. The unbounded polymer chains cannot be degraded by the biological activity of the mammalian hosts. Even if the gel dissolves in the physiological environment of mammals, the molecules of alginate cannot be completely removed from the body since the average molecular weights of commercially available alginates are higher than the renal clearance threshold of the kidneys [4]. However, alginates can be degraded by the specific enzymes, alginate lyases also known as

alginases, which are not present in mammals [5]. In addition, another drawback of alginate is its lack of cell adhesion motifs and the resulting failure of cell attachment leading to very poor cell-material interactions both in 2D and 3D environments [6, 7]. Moreover, alginate hydrogels promote minimal protein adsorption due to its hydrophilic nature. Consequently, mammalian cells are unable to interact with the hydrogel through serum proteins [8]. Cell anchorage or cell-material interaction is the key factor for cell survival in 2D and 3D cultures and orchestrates most of the cellular functions including migration, proliferation, differentiation, and apoptosis [6, 9]. Moreover, a high mechanical stress is exerted on the cells embedded in alginate hydrogel that impedes elongation and migration of active viable cells. Therefore, since *alginate* hydrogels do not promote cell adhesion and migration, the cells cultured on the surface (2D) or inside (3D) of alginate hydrogel form multicellular aggregates [10]. The drawbacks of alginate as an emerging biopolymer for biomedical applications can be overcome by some approaches, such as by enhancing its degradation through chemical modification, like partial oxidation [2, 11] or gamma irradiation [12], and by introducing cell-binding motifs, like RGD (Arg-Gly-Asp)-containing peptides or proteins, which can be conjugated with alginate to promote cell adhesion [6]. However, designing an alginate-based hydrogel system which can support cell anchorage and promote typical cellular functions including elongation, migration, proliferation, and differentiation in 3D environments suitable for tissue engineering applications remains challenging.

These limitations of alginate can be overcome by incorporation of gelatin through covalently crosslinking with alginate dialdehyde (ADA) [13, 14]. ADA is a partially oxidized product of alginate which facilitates the covalent crosslinking with gelatin through the Schiff's base formation due to the reaction of free amino groups of lysine or hydroxylysine amino acid residues of gelatin and available aldehyde groups of ADA [13, 15]. The partial oxidation cleaves the carbon-carbon bond of the cis-diol group in the uronate residue of alginate and alters the chair conformation to an open-chain adduct, which facilitates degradation of the alginate [16]. Moreover, the biodegradability of the covalently crosslinked hydrogel can be tuned by using ADA of different degrees of oxidation which can control the hydrolysis property of alginate [16, 17] and also by changing the ratio of ADA and gelatin [18, 19].

5.3 Tissue Engineering Constructs and Their Applications

As mentioned above, alginate is being increasingly utilized in tissue engineering. Alginate hydrogels and hydrogel-derived scaffolds are the model system for cell culture, especially for mammalian cell cultures, and it is an ideal material for physiologically relevant 3D culture systems. Among the various models of 3D cell culture systems, alginate-based microspheres, porous scaffolds, and bioplotted scaffolds are extensively used.

Compared to 2D cell culture, 3D culture systems simulate natural cell adhesion, proliferation, migration, and differentiation, resulting in an increasing shift in cell

culture research, where 3D cell culture systems are replacing 2D cell cultures. In vitro 3D cell culture models span the gap between conventional in vitro 2D cell culture models and in vivo animal models [20, 21]. Alginate-based 3D cell cultures are performed in multiple ways. Among them, (1) encapsulation of cells in alginate beads, (2) fabrication of scaffolds by plotting cell-loaded hydrogel precursors with subsequent gelation of the precursors, and (3) seeding cells in prefabricated porous scaffolds are the most used techniques. Alginate has been used in advanced research in various tissue engineering application fields, e.g., bone, cartilage, dental, cardiac, muscle, adipose, vascular, neural, and retinal tissue engineering.

5.3.1 Porous Scaffolds

Since the last decades, porous biomaterials-based constructs with a high degree of porosity and an interconnected pore structure are considered for tissue engineering scaffolds. Several techniques including gas foaming, solvent casting and salt leaching, freeze-drying, electrospinning, and rapid prototyping (bioprinting) are applied to fabricate 3D hydrogel-based polymeric scaffolds [22]. Among the conventional techniques, freeze-drying is widely used for fabrication of tissue engineering scaffolds from hydrogel-based materials, like alginate. Porous solid free-form scaffolds can be fabricated from an alginate solution by simple two steps: freezing followed by freeze-drying, as shown in Fig. 5.1. The method is based on rapid cooling of the material under freezing temperature to generate thermal instabilities within the system which cause phase separation and sublimation of the solvent under vacuum resulting in voids in the space it previously occupied [23]. Among the various relevant processing parameters, freezing temperature, freezing rate, and freezing process before the lyophilization have a major impact on the pore structure (overall porosity, pore size, and pore morphology) of the resulting scaffolds [23–25]. Generally, the porosity and pore size of freeze-dried scaffolds increase with increasing freezing temperature. This phenomenon can be attributed to the formation of ice crystals which are larger in size and lower in number at higher freezing temperatures than at lower freezing temperatures. During the freeze-drying

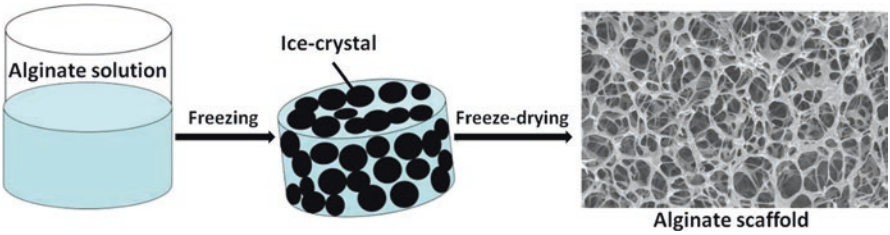

Fig. 5.1 Schematic diagram showing the fabrication technique of porous alginate-based scaffolds using the freeze-drying method (Reproduced with permission from Ref. [27]. Copyright 2013, MDPI publishing)

process, the larger ice crystals push and expand the biopolymer to a greater extent leading to the formation of highly porous scaffolds with large pore sizes [26]. Scaffolds with open-pore structures are generated at a high freezing temperature (between −20 °C and −80 °C), whereas scaffolds with parallel sheet-like morphology are obtained at a very low freezing temperature (−196 °C in liquid N_2) [23].

This technique is generally used to fabricate porous scaffolds with interconnected pores from ionically crosslinked alginate hydrogels [24, 27, 28]. However, it is well known that the scaffolds from pure alginate do not provide enough biocompatibility to support cell adhesion and cell metabolism due to lack of cell adhesion motifs in the alginate network [27, 29]. Therefore, in order to promote cell adhesion and cell metabolism, different polymers, proteins, or peptides are combined with alginate, such as chitosan [30, 31], gelatin [32], especially peptides having sequences like G_4SPPRRARVTY, G_4SPPLLALVTY, G_4RGDY, etc., which are known to promote cell adhesion [29]. Alginate hydrogel-based porous freeze-dried scaffolds have been mostly used for bone and cartilage tissue engineering research, which will be discussed in the next paragraphs.

5.3.1.1 Bone Tissue Engineering

A porous biodegradable and biocompatible scaffold serves as a temporary structure in bone tissue engineering (BTE), which is enabling cells to grow and form new bone tissue at the defect site while the implanted scaffold gradually degrades and is replaced by the new bone tissue [31].

Due to the complexity of the bone structure, the ideal scaffold for BTE should meet the following requirements:

- Biocompatible, nontoxic to the host tissue and implanted cells
- Adequate and controlled biodegradability that should match the rate of tissue regeneration
- High porosity, adequate pore size with interconnected pore structure, which can facilitate cell growth, migration and proliferation, and most importantly vascularization
- Suitable mechanical strength that can provide initial support at the implanted site
- Osteoconductivity and osteoinductivity

Although alginate does not exhibit any toxic effect to cells or host tissues, it lacks cell adhesion properties. Therefore, proteins or peptides are generally used with alginate to design tissue engineering scaffolds to support bone tissue generation. Peptides are generally conjugated with alginate by carbodiimide chemistry treatment. However, this method was not implemented for combining bone-forming peptide-1 (BFP-1), derived from bone morphogenetic protein-7 (BMP-7) with alginate to design BFP-1-incorporated porous freeze-dried alginate scaffolds for promoting bone-repairing ability [33]. In this study, the peptide was simply blended with alginate. As expected significantly higher cell activity and alkaline phosphatase (ALP) expression of human osteoblast-like MG63 cells were observed for BFP-1-containing alginate scaffolds compared to pristine alginate scaffolds.

Sustained release of BFP-1 from the scaffolds was observed that promoted the osteogenic differentiation of osteoblasts. Among the three concentrations of BFP-1, optimal results regarding cell viability and osteogenic activity were obtained for the hydrogel composing 10 μg/mL peptide. Furthermore, the incorporated peptide significantly enhanced osteo-regeneration in vivo when the scaffolds were implanted into Beagle calvarial defects. Osteoid, the organic portion of the bone matrix that is composed of collagen-I and chondroitin sulfate, deposited in the calvarial defects where BFP-1-containing alginate scaffolds were implanted. In another study, carbodiimide chemistry was used to crosslink gelatin with alginate, in which stable amide bonds formed between alginate and gelatin in the presence of EDC and NHS through the three steps of reaction [34]. Microwave-vacuum drying technique was used to fabricate porous scaffolds from low and high crosslinked alginate-gelatin hydrogels. In both conditions, in vitro and in vivo, the scaffolds exhibited excellent cytocompatibility, biocompatibility, and bioresorbability. It is important to note that a strong bond was observed between implanted alginate-gelatin scaffolds with upper subcutaneous tissue and lower muscle tissue at the implanted site of mice. Moreover, angiogenesis and neovascularization were observed and that generally help to supply nutrient to the implanted cells, which enhance tissue regeneration process. However, the degree of osteogenic, adipogenic, and chondrogenic differentiations of mesenchymal stem cells (MSCs) was observed to be lower for subcutaneously implanted scaffolds in mice compared to in vitro culture, which shows that the in vitro differentiation potential of MSCs does not always correlate with in vivo tissue regeneration. Not only proteins or peptides but also other polymers are used with alginate to design BTE scaffolds. Among the naturally derived polymers, chitosan is the most studied material with alginate because these two polysaccharides are ionically opposite in nature and therefore polyelectrolyte complex forms in their mixture that can minimize the drawbacks of both materials. Since alginate is a negatively charged polymer, no protein adsorption usually takes place on it. Due to the inclusion of positively charged polymer, chitosan, protein can be adsorbed on the polymer composite and that can improve cell adhesion [30]. Li et al. showed alginate-chitosan hybrid freeze-dried porous scaffolds supported growth and proliferation of MG63 osteoblast-like cells in vitro [31]. Moreover, calcium and phosphate-rich mineral deposition was observed on osteoblasts grown on the hybrid scaffolds, and this type of deposition was not observed on pristine chitosan scaffolds. Mineral deposition in large areas was also confirmed on the bone marrow-infused hybrid scaffolds in vivo at 4 and 8 weeks after implantation into the muscle of rats. In another study, undifferentiated rat bone marrow-derived MSCs were used to investigate comparative osteogenesis in the defect sites of calvaria of Sprague Dawley rats, which were treated separately with freeze-dried chitosan-alginate (CA) scaffolds and the scaffolds with seeded MSCs, bone marrow (BM) aspirate, and bone morphogenetic protein-2 (BMP2) growth factor [35]. Cells and growth factor were mixed with 0.5% (w/v) alginate solution prior to loading into the porous scaffolds. The cell- or growth factor-immobilized alginate solution was ionically crosslinked after loading. Among the four groups of constructs, the BMP2-loaded scaffolds exhibited the greatest defect closure (71.6 ± 19.7%) after 16 weeks of implantation. Mature lamellar bone and the greatest amount of mineralization with

denser and thicker structure were observed for BMP2-containing CA scaffolds, proving the effect of BMP2 in enhancing osteogenesis. It is important to note that native CA scaffolds showed mineralization, which was enhanced by the incorporation of MSCs, BM, or BMP2, indicating that CA scaffold is osteoconductive and that suggests CA is a promising scaffolding material for cranial defect repair.

Since the bone is composed of organic and inorganic materials (collagen and hydroxyapatite), researchers use often inorganic components with alginate for the applications in BTE. Incorporation of inorganic materials to alginate can enhance the strength of the alginate-based BTE constructs, which can mitigate the lack of mechanical strength of alginate scaffolds for mimicking the functions of the bone. Moreover, it can enhance new bone tissue formation at the defect site of the patient. Among the various inorganic materials, bioactive glass (BG) and different types of calcium phosphates, e.g., tricalcium phosphate (TCP), hydroxyapatite (HAp), are mostly used with alginate hydrogels for designing tissue engineering scaffolds.

A preliminary study was conducted with freeze-dried porous alginate-HAp scaffolds for BTE applications, where rat osteosarcoma UMR106 cells were used [36]. Due to the incorporation of HAp, the compressive strength of the composite scaffolds increased significantly. Though a significantly higher number of cells was observed in the composite scaffolds compared to the pristine alginate scaffolds, the cell morphology was found to be round since the composite scaffolds do not possess any cell-binding motifs that could provide the adhesion sites for the cells. The presence of HAp enhanced protein adsorption from culture media to the certain extent that enhanced cell adhesion to a certain degree compared to pristine alginate. Rajesh et al. developed a tri-component composite scaffold composing oxidized multi-walled carbon nanotube, alginate, and HAp by a freeze-drying technique for the applications in BTE [37]. Another alginate-based tri-component porous freeze-dried composite scaffold was designed with alginate, chitosan, and silica nanoparticles ($nSiO_2$) [38]. Incorporation of chitosan and $nSiO_2$ facilitates protein adsorption and $nSiO_2$ enhanced exogenous biomineralization on the composite scaffolds in simulated body fluids (SBF).

As a bioactive phase sol-gel-derived BG was incorporated to alginate-polyvinyl alcohol (PVA) system to develop potential composite scaffold for bone regeneration [39]. In this study, the porous scaffold was fabricated by surfactant foaming technique where sodium lauryl sulfate was used as the surfactant. Due to the presence of BG, the composite scaffolds exhibited osteoconductivity by forming HAp on the surface that mimics the ECM of bone.

In another study, mineralization on freeze-dried porous alginate scaffolds was achieved without incorporating any bioactive inorganic component to alginate [40]. The mineralization was achieved by simply incubating the pristine alginate scaffolds in modified simulated body fluid (mSBF) that had an identical composition to that of the human plasma but with double concentration of calcium and phosphate, which enhanced mineral growth onto the scaffold's surface. The composition and morphology of the deposited mineral phase were observed to be similar to that of vertebrate bone tissue and that supported the attachment and growth of hMSCs.

In the study of Cai et al. [42], tricalcium phosphate (TCP) was incorporated into the oxidized alginate-gelatin hydrogels in order to induce bone formation by osteo-

blasts for the application in BTE. It has also been proven that the TCP-incorporated oxidized alginate-gelatin hydrogels can be used as a potential drug delivery carrier. Moreover, the encapsulated osteoblasts within the TCP-incorporated oxidized alginate-gelatin hydrogels exhibited high ALP activity, proving that the encapsulated cells maintained their osteoblastic nature. Recently, Sarker et al. designed hydrogel-based freeze-dried scaffolds composed of oxidized alginate, gelatin, and BG for bone tissue engineering applications as shown in Fig. 5.2 [41]. The presence of BG (45S5) enhanced the crosslinking kinetic and crosslinking degree of the oxidized alginate-gelatin (ADA-GEL)-based hydrogel. The enhanced crosslinked structure of hydrogels ultimately contributed to the mechanical properties of the hydrogel-derived scaffolds. High BG content enhanced the crosslinking degree of the hydrogel significantly due to the alkalinity of the reaction medium, which facilitated Schiff's base bond formation between free amino groups of gelatin and available aldehyde groups of oxidized alginate. Highly crosslinked hydrogel networks enhanced mechanical properties of the scaffolds. Degradation property of the scaffolds was also successfully tailored by changing the BG content in the ADA-GEL hydrogel. Moreover, the scaffolds composed of high BG exhibited low protein release profile since the high BG-containing ADA-GEL hydrogel possessed high crosslinked structure that inhibited protein release and hydrolytic degradation. Bone marrow-derived stromal cells were cultured in the scaffolds, and the cell growth was found to be promoted in ADA-GEL scaffolds and 1% BG-containing ADA-GEL scaffolds compared to pure alginate scaffolds and 5% BG-containing ADA-GEL scaffolds. Low cell viability in 5% BG-containing ADA-GEL scaffolds could be due to the possible cytotoxic effect of a high concentration of released ions from BG particles. Scaffolds with optimum BG content (1%), adequate protein, and controlled degradation supported better cell growth, proliferation, migration, and osteogenic differentiation. Moreover, the presence of BG facilitated HAp deposition onto the scaffolds that made the constructs osteoconductive.

5.3.1.2 Cartilage Tissue Engineering

Due to the very limited self-healing capability of damaged or degenerated articular cartilage, millions of people suffer from degeneration of articular cartilage in primary osteoarthritis. Alginate has been widely used as a scaffolding material for cartilage tissue regeneration. As stated earlier due to some limitations of alginate, other proteins, peptides, and inorganic materials are incorporated to alginate. Similar to the study for bone tissue engineering, Li and Zhang used chitosan-alginate system to develop porous freeze-dried scaffolds for the purpose of articular cartilage regeneration [43]. HTB-94 chondrocytes in pure chitosan scaffolds exhibited fibroblast-like morphology prove that cells dedifferentiated in chitosan scaffolds. However, the typical chondrocytic morphology (spherical) of the cells was found to be retained in alginate-chitosan composite scaffolds. Moreover, over the culturing period, the expression of the cartilage-specific collagen-II marker was found to be increased in the composite scaffolds and decreased in chitosan scaffolds. It is well established that the

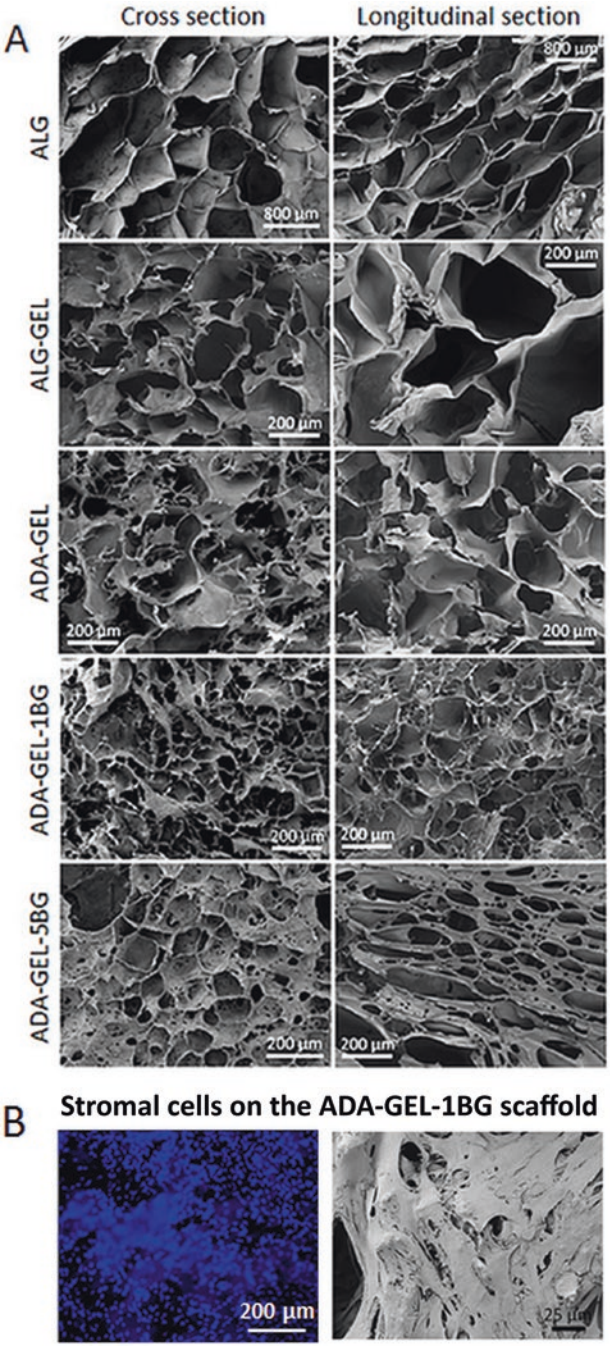

Fig. 5.2 (**a**) Scanning electron microscopy (SEM) images of the freeze-dried scaffolds from different hydrogels composing pure alginate (ALG), oxidized alginate (alginate dialdehyde, ADA), gelatin (GEL), and bioactive glass (BG), exhibiting their morphologies in cross-section and longitudinal section. (**b**) Growing bone marrow-derived stromal cells (ST-2) on the ADA-GEL-1BG scaffolds after 21 days. Cell nuclei are stained with DAPI, appeared in blue on the left and SEM image on the right, showing ST-2 cells with flattened morphology (Reproduced with permission from Ref. [41]. Copyright © 2016 American Chemical Society)

chondrocytes with elongated fibroblastic morphology (especially in 2D culture) produce less collagen-II and proteoglycans compared to the spherical chondrocytes [44]. Chitosan-alginate composite scaffolds promoted the growth of chondrocytes with characteristic morphology and exhibited better productivity in the biosynthesis of collagen-II than pure chitosan scaffold, which is due to the ability of alginate to induce redifferentiation of 2D culture-expanded dedifferentiated chondrocytes as revealed by Homicz et al. [45]. To enhance cell-material interaction, RGD peptide was conjugated to chitosan-alginate-hyaluronate (CAH) complexes, which have been evaluated as scaffolds for cartilage tissue engineering [46]. As expected the highest attachment and proliferation of primary rabbit articular chondrocytes, as well as higher glycosaminoglycan and collagen contents, were observed in RGD-conjugated CAH freeze-dried scaffolds compared to CA, CAH scaffolds. However, the typical spherical morphology of chondrocytes was observed in all scaffolds with and without RGD. In the animal study, though the cartilage defects of rabbits were found to be completely repaired where CA and RGD-conjugated CAH scaffolds were implanted along with allogenic rabbit chondrocytes, RGD-conjugated CAH scaffolds demonstrated better cell growth with lacunae and neocartilage formation.

Apart from the chondrocytes, MSCs are also promising candidates for cartilage regeneration, where appropriate microenvironment is necessary to stimulate chondrogenic differentiation. In a study, RGD peptide was conjugated to alginate to design such an appropriate microenvironment, which can stimulate MSC chondrogenesis [47]. It is well established that RGD enhances cell adhesion to the matrix that allows better accessibility to the surrounding microenvironment. In that study, the immobilized RGD peptide enhances the accessibility to the chondrogenic-stimulating molecule, transforming growth factor-β1 (TGF-β1) in the surrounding environment by inhibiting cell aggregation in the macroporous freeze-dried scaffold that eventually stimulates chondrogenesis of human bone marrow-derived mesenchymal stem cells (hBMSCs). The chondrogenic differentiation of MSCs in RGD-conjugated alginate scaffolds was confirmed with the upregulation of the chondrocytic gene markers, Sox9 and collagen-II.

5.3.1.3 Soft Tissue Engineering

Alginate-based freeze-dried scaffolds have been developed for the applications in various tissue engineering fields. Bone tissue engineering and cartilage tissue engineering are the two major application fields of the porous tissue engineering constructs that are discussed in the previous sections. Alginate-based scaffolds are not often used for soft tissue engineering applications. Among the few soft tissue engineering applications, muscle and liver tissue engineering applications will be discussed in the next paragraphs.

In the study of Wang et al., a partially crosslinked alginate scaffold with shape-memory properties was designed by mixing low and high molar mass partially oxidized alginate conjugated with RGD peptides for muscle tissue engineering applications [48]. It is well known that degradation properties of alginate can be tuned by changing the molar mass or by scissoring the polymer chain of alginate. The combination of both strategies was applied in this study to design the matrix

with required degradation properties. The same strategy was also applied to design the scaffolds with optimal shape-memory properties that enable in vivo scaffold implantation via a minimally invasive manner. Moreover, the molar mass and partial oxidation of alginate are the tuning tools of mechanical strength of scaffolds, which controls the mechanotransduction of cell that controls myoblast functionality such as adhesion, proliferation, and differentiation. Partially oxidized alginate has also been used to design suitable exogenous matrix for liver tissue engineering applications [49]. In this study, the partially oxidized alginate was covalently crosslinked with galactosylated chitosan via Schiff's base reaction since hepatocyte surface receptors, asialoglycoprotein (ASGP), recognize galactose moieties, which promotes hepatocytes adhesion. Due to the presence of galactose, the water solubility of chitosan increases, which is a major limitation of using galactosylated chitosan alone in liver tissue engineering applications. High water solubility of galactosylated chitosan was reduced and controlled by crosslinking with partially oxidized alginate, a biocompatible material. Moreover, the mechanical strength and degradation properties of the covalently crosslinked freeze-dried scaffolds were easily tuned by changing the ratio of oxidized alginate and galactosylated chitosan, which changed the degree of crosslinking of the synthesized hydrogel. The covalently crosslinked composite scaffolds supported hepatocytes adhesion and growth. Moreover, hepatocytes exhibited typical spheroidal morphology and formed multicellular aggregates, which prove the alginate-based composite matrix is a favorable niche for the growth of hepatocytes.

One of the major challenges in tissue engineering is insufficient nutrient supply to the distant cells in the tissue engineering constructs due to the lack of vascularity. Freeze-dried porous alginate scaffolds have been employed to induce vascularization via physical stimulation to endothelial cells using magnetic nanoparticles [50]. The aortic endothelial cells in magnetite-impregnated alginate scaffolds were stimulated with an external magnetic field during the first 7 days of culture, which exhibited high metabolic activity. Most importantly, a capillary-like organization of endothelial cells was observed in the magnetite-impregnated alginate scaffolds that were exposed to an alternating magnetic field. It is believed that the mechanical stimulation with magnetic field enhances the organization of endothelial cells and eventually the capillary-like structures form.

5.3.2 Bioprinted Scaffolds

Bioprinting is a new manufacturing technology under the banner of rapid prototyping, which is well known for fabrication of structures resembled in architecture to native biological tissue. Bioprinting can be described as robotic additive biofabrication, which has emerged as a potential tool in regenerative medicine. In this technique, a computer-aided 3D printing device is used to precisely plot cells and biomaterials into predesigned geometry that can mimic native biological tissue construct and later can be matured into a tissue or organ [51]. Selecting or designing a perfect ink for printing tissue engineering scaffolds remains a challenge. Hydrogel-forming materials are generally used to plot scaffolds since hydrogels mimic the structure of

the native biological tissue. The hydrogel must be biocompatible and non-immunogenic and support appropriate cellular activities, such as migration, proliferation, and differentiation of embedded cells. The hydrogels with suitable mechanical properties and swelling and degradation characteristics are desirable properties for printing tissue engineering scaffolds to promote cell proliferation, migration, and other important cellular functions. Among the various naturally derived hydrogel-forming materials, alginate is the most used material for printing tissue engineering scaffolds because of its biocompatibility, non-immunogenic property, and most importantly excellent ionic gelation property.

5.3.2.1 Bone Tissue Engineering

Majority of the works on bioprinted alginate-based scaffolds have been conducted for the applications in bone tissue engineering. Since the bone is a composite of collagen and HAp, a significant number of researches have been conducted using a mixture of polymer/HAp. In this field, a pioneer research group introduced alginate/nano-HAp (nHAp) composite scaffolds with tailored pore parameters and core/shell structures, which were fabricated using 3D plotting technique [52]. Alginate/nHAp scaffolds were designed in two different ways: mixing HAp nanoscaled powders with alginate and in situ mineralization. In situ mineralization is a simple and effective method for designing organic/inorganic composite scaffolds for bone tissue engineering applications. In the study of Luo et al., alginate paste was prepared by mixing sodium alginate powder in aqueous 500 mM Na_2HPO_4 solution at a concentration of 15.4 w% and transferred into a plotting cartridge of a bioplotter. 3D scaffolds were fabricated by extruding the alginate ink through a needle layer by layer as programmed in the integrated software. The dimensions of the scaffold's strut can be tailored by changing the needle's diameter, air pressure, and plotting speed. The plotted alginate scaffolds were crosslinked with 1 M $CaCl_2$ solution, in which the pH value was adjusted to 5 and 9.5. In situ mineralization on the alginate scaffolds was achieved at both pH. Brushite ($CaHPO_4{\cdot}2H_2O$) crystals formed on the scaffold's surface at pH 5, whereas the scaffolds were completely covered with a layer of nHAp at pH 9.5. Most of the minerals were observed on the surface. When the plotted alginate scaffolds (alginate paste in Na_2HPO_4) came in contact with $CaCl_2$ solution, a chemical reaction occurred between Ca^{2+} and PO_4^{3-} at the surface of the scaffolds leading to HAp formation. Protein adsorption or binding, which is a major factor for cell adhesion, strongly depends on the surface chemistry. Formation of nHAp on the surface changed the chemistry and topography of the scaffold's surface that enhanced protein adsorption and therefore promoted attachment of hBMSCs. On the other hand, very few cells were observed on the pure alginate scaffolds. It is important to note that much rounder and small cells were found on the mineralized scaffolds compared to that on pristine alginate scaffolds, which suggests that the cells on mineralized scaffolds tend to differentiate more to osteogenic lineage [52]. In another study of the same research group, mesoporous bioactive glass (MBG) was mixed with alginate to design an ink for the plotting of 3D scaffolds for bone tissue engineering [53]. The ink composed of alginate/MBG

exhibited good processability for plotting scaffolds with tailored architecture using a bioplotter. MBG with a pore size of 5 nm was mixed with alginate at various concentrations and added into polyvinyl alcohol (PVA) solution to prepare the ink for plotting scaffolds. The scaffolds were plotted in layer-by-layer deposition of the prepared ink and the models of the scaffolds were designed by CAD. The composite ink was extruded through a nozzle by a specific dosing air pressure at a constant plotting speed at room temperature. The strut width and pore size of the scaffolds can be tailored by changing the inner diameter of nozzle, dosing pressure, and plotting speed. The pore structure and pore size can also be tailored by optimizing the plotting pattern. In the study of Luo et al., two different plotting patterns, named XY and XXYY, were employed to design the scaffolds with different pore sizes and pore structures. For XY pattern, the first layer was plotted in the X direction, and the second layer was plotted in the Y direction, and this plotting fashion was repeated until the whole scaffold was completed. For XXYY pattern, the ink was first plotted in the X direction for two layers, and the next two layers were plotted in the Y direction, then repeated until the scaffold was completed. The scaffolds that were plotted in XXYY pattern exhibited significantly improved pore interconnectivity compared to the scaffolds plotted in XY direction. Mechanical properties of the scaffolds were modulated by changing the amount of the MBG content in the plotting ink. Due to incorporation of 30% and 50% MBG into the alginate ink, mechanical strength and modulus of the plotted scaffolds have increased significantly. Apatite mineralization was observed on the scaffolds that contain MBG after soaking in SBF, which confirms the bioactivity of the scaffolds. It is well established that apatite mineralization enhances the cell-material and tissue-implant interactions and improves osteoblastic activity, which was also observed in the study of Luo et al. Improved cell-material interaction with high ALP activity of hBMSCs was found on the mineralized MBG/alginate scaffolds compared to the pristine alginate scaffolds. Due to mineralization, protein binding was improved and bioactive silicate ions released from MBG, which might be the possible reason of high cell-material interactions and improved osteoblastic activity of hBMSCs. The designed MBG/alginate scaffolds were also used to deliver drug, where Dexamethasone was used as the model drug, which is generally used for treatment of rheumatoid arthritis. Much controlled and sustained release of the drug was found when the drug was loaded to MBG particles prior to fabrication of MBG/alginate scaffolds compared to the pure alginate scaffolds. The release kinetic of the drug can be further controlled by changing MBG content in the plotting ink, which is very important for biomedical applications. In another study, the effect of BG on growth and mineralization of human osteogenic sarcoma cells, SaOS-2 cells, immobilized into a printable alginate-gelatin hydrogel, was investigated [54]. Furthermore, the hydrogel was supplemented either with polyphosphate (as polyP·Ca^{2+} complex) or silica, or as biosilica that was enzymatically prepared from ortho-silicate by silicatein. The cell-embedded bio-ink was loaded into the printing cartridge, connected with a needle, and mounted in the preheated printing head of a 3D bioplotter. The scaffolds were plotted in a meander-like pattern by changing the directions of the consecutive layers. The strut width of the plotted scaffolds was 300 μm, and the struts were arranged in a perpendicular orientation. In the presence of BG nanoparticles with a size of 55 nm and a molar

ratio of 55:40:5 of SiO_2:CaO:P_2O_5, encapsulated cell proliferation was not affected, which proves no adverse effect of BG particles on cell growth. However, inclusion of BG to the alginate-gelatin hydrogel significantly enhances the mineralization potency of the embedded SaOS-2 cells. When cells were entrapped in BG-containing hydrogel together with 100 μmoles/L polyP·Ca^{2+} complex, mineralization activity increased from 2.1 to 3.9 folds, and the metabolic activity increased from 2.7 to 4.8 folds with 50 μmoles/L biosilica, which was found to increase from 4.1 to 6.8 folds when both components (polyP·Ca^{2+} complex and biosilica) were used in the hydrogel system. The deposited minerals were found to be composed of Ca, P, O, and C, indicating that the nodules contained calcium phosphate and calcium carbonate. Alginate can also be used in a drug delivery system to control drug release for bone tissue engineering applications. In a study of Lee et al., alginate was used to coat 3D bioplotted highly porous collagen scaffolds to control drug release without loss of the original biological properties of collagen scaffolds [55]. Collagen scaffolds were plotted using a nozzle of 300 μm diameter, connected with a three-axis robotic bioplotting system supplemented with a cryogenic plate (−30 °C). The scaffolds were plotted in a layer-by-layer fashion with the pore and strut sizes of 250–350 μm and 250–300 μm, respectively. The width of the struts can be controlled by changing the plotting speed and the temperature of the cryogenic plate. After plotting, the collagen scaffolds were crosslinked with EDC and lyophilized. Drug was loaded into the scaffolds, which were then coated with different alginate solutions (5, 10, 20 wt% of alginate) and crosslinked with 1 wt% $CaCl_2$ solution for 1 h. Alginate coating influenced the porosity of the collagen scaffolds. Porosity of the alginate-coated collagen (CAC) scaffolds was found to be decreased with increasing of volume percentage of coated alginate. Pristine collagen scaffolds exhibited very poor mechanical properties, which were increased significantly by coating the scaffold struts with alginate layer. Since collagen is one of the major components of the native ECM, the highly porous lyophilized collagen scaffolds are considered to be very promising for tissue engineering applications including drug delivery. However, controlled drug release from pristine collagen scaffolds is a major challenge because of the highly porous structure and high degradability. Alginate coating reduced the rapid drug release behavior of collagen scaffolds, and the release behavior was controlled by applying coating of alginate with various volume percentages. Both pure collagen scaffolds and alginate-coated collagen scaffolds supported attachment, growth, and proliferation of MG63 cells. Moreover, calcium-rich minerals were identified in the growing cells on the scaffolds coated with high volume percentages of alginate. Moreover, osteogenic activity of MG63 cells was also observed in pure collagen and CAC scaffolds.

Most of the works on alginate-based bioplotted 3D scaffolds were conducted using very high concentration of alginate to achieve the scaffolds with high Z height. The major drawback of this technique is the alginate hydrogel matrix with very high concentration is not suitable for cell immobilization since embedded cells experience high mechanical stress by the surrounding alginate matrix. Moreover, alginate is not suitable for cell growth and attachment since it does not contain any cell adhesion ligand. Therefore, a very suitable alginate-based hydrogel for cell growth in 3D,

which was developed by Sarker et al. [18], was used to fabricate scaffolds using a 3D bioplotting technique [56]. In this study oxidized alginate-gelatin covalently cross-linked hydrogel was used in which osteoblast-like MG63 cells were immobilized prior to plotting. Over the incubation period, an increasing trend in metabolic activity of embedded MG63 cells in the plotted scaffolds was observed. Moreover, the release of vascular endothelial growth factor (VEGF) was observed, and the release trend was found to be increased over the incubation period. The upregulation of VEGF expression of embedded osteoblast-like cells in ADA-GEL matrix proved the ongoing angiogenesis, which is very important for all types of tissue engineering including bone tissue engineering. The embedded cells were observed to grow out of the ADA-GEL hydrogel strut and covered the whole scaffold. This result confirms the migration of the embedded cells in the hydrogel matrix, which occurred due to the high matrix porosity, low stiffness, and high degradability of the ADA-GEL hydrogel as stated elsewhere [57–59]. Moreover, cells exhibited spreading morphology with high cell-material interaction, which has never been observed for alginate-based bioplotted scaffolds. The outcomes demonstrated that ADA-GEL hydrogel is a superior material in the context of bioplotting compared to pure alginate hydrogel. However, designing scaffolds with tailored height in Z direction was found to be very challenging to achieve with the ADA-GEL hydrogel, which is the bottleneck of this matrix system for biofabrication in the field of tissue engineering. In another study, strontium-doped bioactive glass nanoparticles (BGNPs) were incorporated into ADA-GEL hydrogel to fabricate bioplotted scaffolds with improved bioactivity for bone tissue engineering applications [60]. Growth of bone-like apatite layer on the surface of the plotted scaffolds occurred when the constructs were immersed in SBF. The embedded MG63 cells exhibited high viability, and no difference was found in cell viability between pristine ADA-GEL scaffolds and the BGNP-incorporated ADA-GEL constructs, proving that the addition of BGNPs did not affect cell viability.

5.3.2.2 Cartilage Tissue Engineering

Alginate-based hydrogels are widely used as therapeutic materials for cartilage tissue regeneration [61]. However, the major challenge of using alginate hydrogels in biofabrication technique to design 3D scaffold is their inability to maintain a uniform 3D structure. To overcome this problem, alginate hydrogel was integrated with a synthetic polymer, polycaprolactone (PCL) [62]. In this study, additive manufacturing (AM) technique with a multihead deposition system (MHDS) was used to fabricate 3D scaffolds by plotting primary nasal septal cartilage chondrocytes and/or TGF-β-embedded alginate hydrogel in between the plotted PCL struts using a layer-by-layer plotting approach. Using the MHDS, multiple biomaterial inks can be dispensed to plot complex 3D scaffolds, which can closely mimic our tissue structure. In vitro cell studies showed that cell viability was reduced to about 85% when multiple PCL layers were plotted compared to the single-layer constructs, where cell viability was found to be 95–97%. Though the cell viability reduced due to plotting more PCL matrix in the constructs, the reduction was not significantly

low, which suggested that the shear stress due to dispensing cell-containing alginate matrix did not have a significant impact on cell viability. In vitro biochemical assay indicated that more glycosaminoglycan (GAG) and total collagen were expressed by the cells in the constructs having TGF-β-containing alginate. Moreover, more cartilaginous tissue formation was observed for the constructs having 4% alginate gels compared to the 6% alginate gels. For in vivo study, three different types of scaffolds were printed and that were composed of PCL+ alginate gel (no cells) named as group I;, PCL+ alginate gel (chondrocytes), group II; and PCL+ alginate gel (chondrocytes + TGF-β), group III, which were implanted into the dorsal subcutaneous spaces of a 7-week-old female nude mice. After 4 weeks, accumulation of more GAG and formation of cartilaginous tissue and type II collagen were observed in the constructs of group III compared to that in the constructs of other two groups. Type II collagen exhibits a fibrillar structure of healthy cartilage tissue that maintains the mesh structure of cartilage and takes water-retentive proteoglycan into its pores. In order to recapitulate the nanofibrous matrix constitution of the native musculoskeletal soft tissue, a fibrous bio-ink composed of alginate and polylactic acid (PLA) nanofibers was used to print tissue engineering constructs [63]. Human adipose-derived stem cells (hADSCs) containing alginate-PLA nanofibers bio-ink was plotted to fabricate the construct that can mimic the structure of human medial knee meniscus, which was digitally modeled using magnetic resonance imaging. Viability study showed that the PLA nanofibers-containing alginate constructs promoted higher cell viability and proliferation compared to the constructs having alginate only. At day 7, metabolic activity of the hADSCs was found to be 28.5% higher in the nanofiber-containing constructs compared to the constructs without nanofiber. Most importantly, collagen and proteoglycans were found to be prominent in the areas surrounding the hADSCs in the nanofiber-containing constructs which confirmed the ability of bioprinted hMSC to differentiate down the chondrogenic pathway. In another study, nanofibrillated cellulose (NFC) was mixed with alginate to design a printable bio-ink by taking the advantage of shear-thinning properties of NFC [64]. The low zero-shear viscosity of alginate makes the pristine alginate ink a poor shape fidelity when printing. To improve the shape fidelity of the alginate bio-ink, NFC was used with alginate in the bio-ink formulation, which combines the high rheological properties of NFC and the good ionic gelation ability of alginate. Human nasoseptal chondrocytes (hNC) were used for 3D bioprinting of gridded constructs using the NFC-alginate bio-ink for cartilage tissue engineering. The cytotoxicity and live/dead assays showed no potential detrimental effect of the bio-ink on embedded cell viability. These preliminary results demonstrate that NFC-alginate bio-ink is a biocompatible hydrogel well suited for 3D bioprinting for designing cartilage tissue engineering constructs.

Highly organized alginate-based 3D scaffolds were developed using a microfluidic technique [65]. The microfluidic device that has been used is a two-channel fluid jacket microencapsulator for bubble formation equipped with a micropipette (inner diameter 45 μm and outer diameter 95 μm). A 1.5% alginate solution with 1% Pluronic® F127 (surfactant) was fed into the outer channel, and nitrogen gas was fed into the inner channel as shown in Fig. 5.3a. Hollow alginate microcapsules were

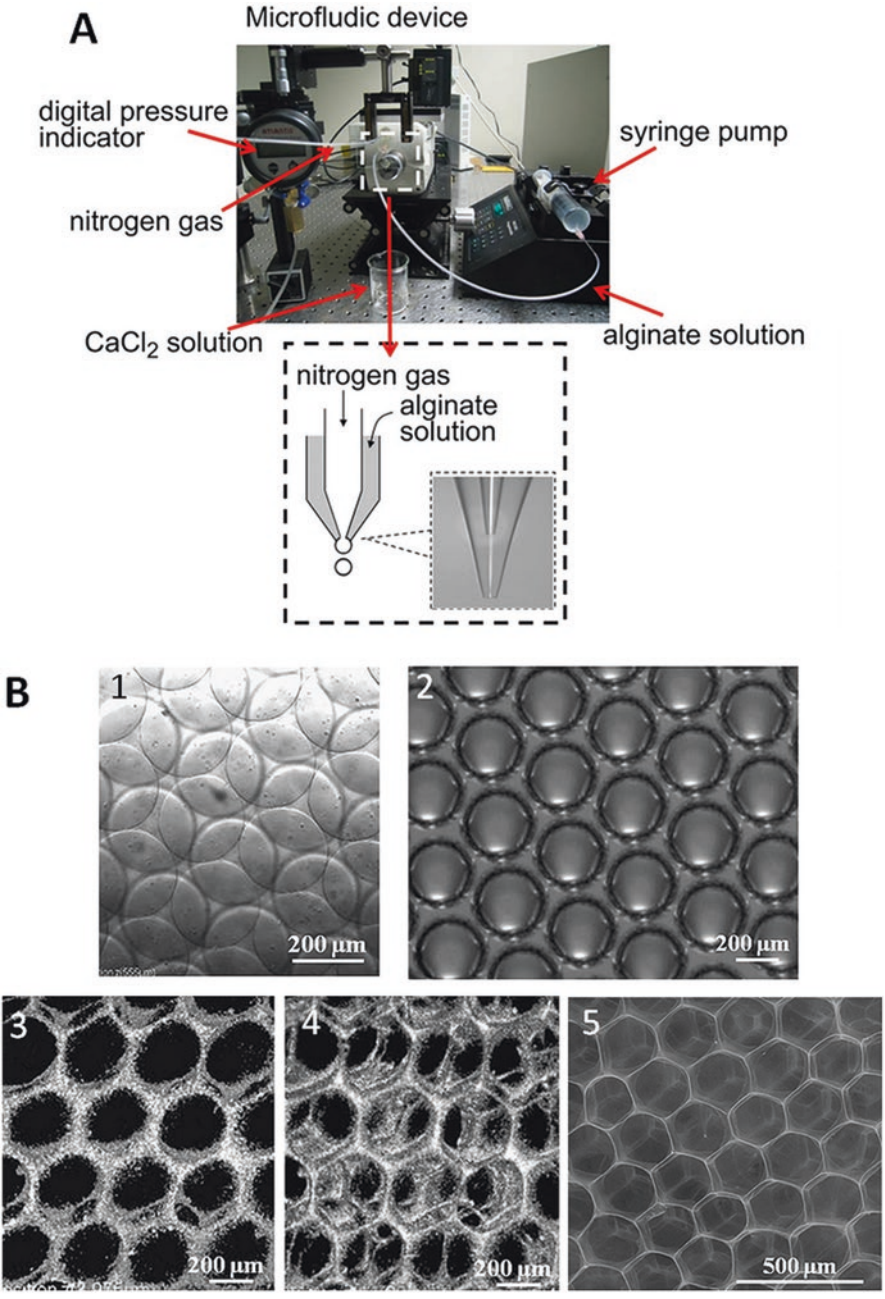

Fig. 5.3 (**a**) The microfluidic device and the scheme of the dispenser tip, in which the surfactant-containing alginate solution and nitrogen gas were fed separately to generate (**b**1) bubble-like alginate microcapsules, which formed (**b**2) a honeycomb structure after gelation. (**b**3 and **b**4) A 3D ordered array structure with high and precise organization is shown in the confocal microscopy images. (**b**5) SEM image of the 3D alginate scaffold with a highly interconnected porous structure, which was achieved after vacuum degassing (Reproduced with permission from Ref. [65]. Copyright 2011, Elsevier)

generated at a specific flow rate and gas pressure. The microcapsules were kept in a gelation bath with 2% calcium chloride solution to facilitate ionic gelation. The microcapsules formed a honeycomb structure (Fig. 5.3b2) when the array was adequate with the high organization. After proper gelation, the structure was put in the vacuum system overnight for removing air bubbles and fabricating the highly organized porous scaffold with high interconnected porosity as shown in Fig. 5.3b5. The pore size of the scaffolds is influenced by the viscosity of alginate solution, nitrogen gas pressure, and the inner diameter of the micropipette. Inconsistence in pore size of the scaffolds fabricated by other conventional methods (e.g., freeze-drying) is a major drawback, which is overcome by this technique. The seeded porcine chondrocytes in the scaffolds showed excellent viability and proliferation, revealing that the highly porous and organized scaffold has good biocompatibility. GAG content was found to be increased over the culture time. In addition, the expression of collagen type II and aggrecan increased. On the other side, a decreasing trend for collagen type I and X was observed, which proved that the chondrocytes maintained their normal phenotype without differentiating toward osteogenesis in the alginate scaffolds.

Apart from the specific tissue engineering purpose, various biofabrication strategies have been utilized to design biocompatible alginate-based scaffolds, which show excellent viability of the embedded cells. Park et al. used the combination of low molecular weight (LMW) alginate and high molecular weight (HMW) alginate at various ratios as the bio-ink to investigate the effect of the composition of alginate bio-ink on the printability of the scaffolds and on the embedded cell viability. As shown in Fig. 5.4, alginate bio-ink composed of LMW and HMW alginate in the ratio of 1:2 with a concentration of 3 w% showed the best performance, including good printability and a suitable environment for cell growth and proliferation. In another study, a cell-laden alginate hydrogel-based 3D constructs were designed that contain hollow calcium alginate filaments, which were fabricated by using a coaxial nozzle [67]. Using this bioprinting method, the hollow calcium alginate filament-based scaffolds were fabricated by controlling the crosslinking time to allow fusion of adjacent hollow filament as shown in Fig. 5.5. Porous 3D hydrogel constructs with various sizes and shapes and with tunable mechanical properties can be fabricated using this approach. The viability of L929 mouse fibroblasts in the constructs with built-in microchannels was found to be higher than that in alginate structures without microchannels, which proves that the microchannels in the constructs can facilitate transporting of nutrients and oxygen to the embedded cells.

Fig. 5.4 (continued) with concentration of 3%), Ink 5 (LMW alginate: HMW alginate 1:2 with concentration of 3%), and Ink 8 (HMW alginate with concentration of 3%) before and after ionic crosslinking with calcium ion, and thick 3D porous constructs are fabricated using the three inks. (**c**) Fluorescence live/dead images of cells printed in the alginate bio-inks (Ink 2, Ink 5, and Ink 8) after 3 h and 7 days of culture, where living and dead cells are appeared in green and red, respectively (scale bar: 500 μm) (Reproduced with permission from Ref. [66]. Copyright 2017, Elsevier)

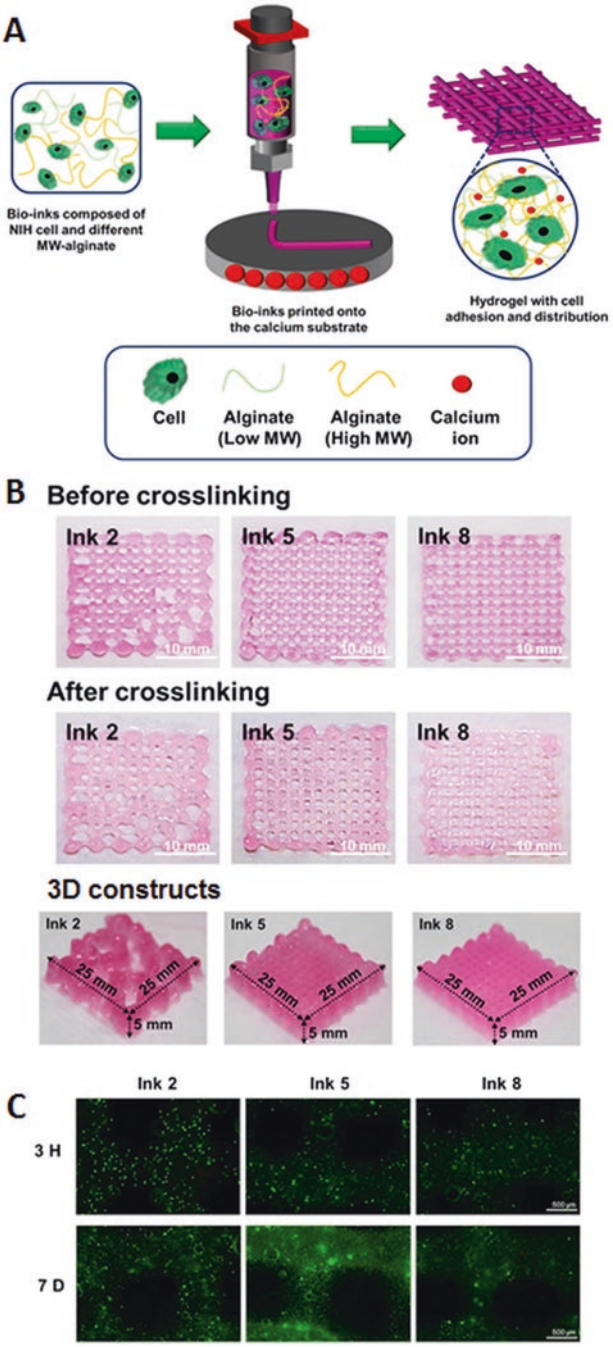

Fig. 5.4 (**a**) Schematic presentation of 3D bioprinting process using the bio-inks with various ratios of LMW alginate and HMW alginate. The fibroblasts-embedded scaffolds were plotted in a layer-by-layer printing sequence, followed by subsequent gelation with calcium ions. (**b**) Photographs of the 3D porous scaffolds printed with Ink 2 (LMW alginate: HMW alginate 1:1

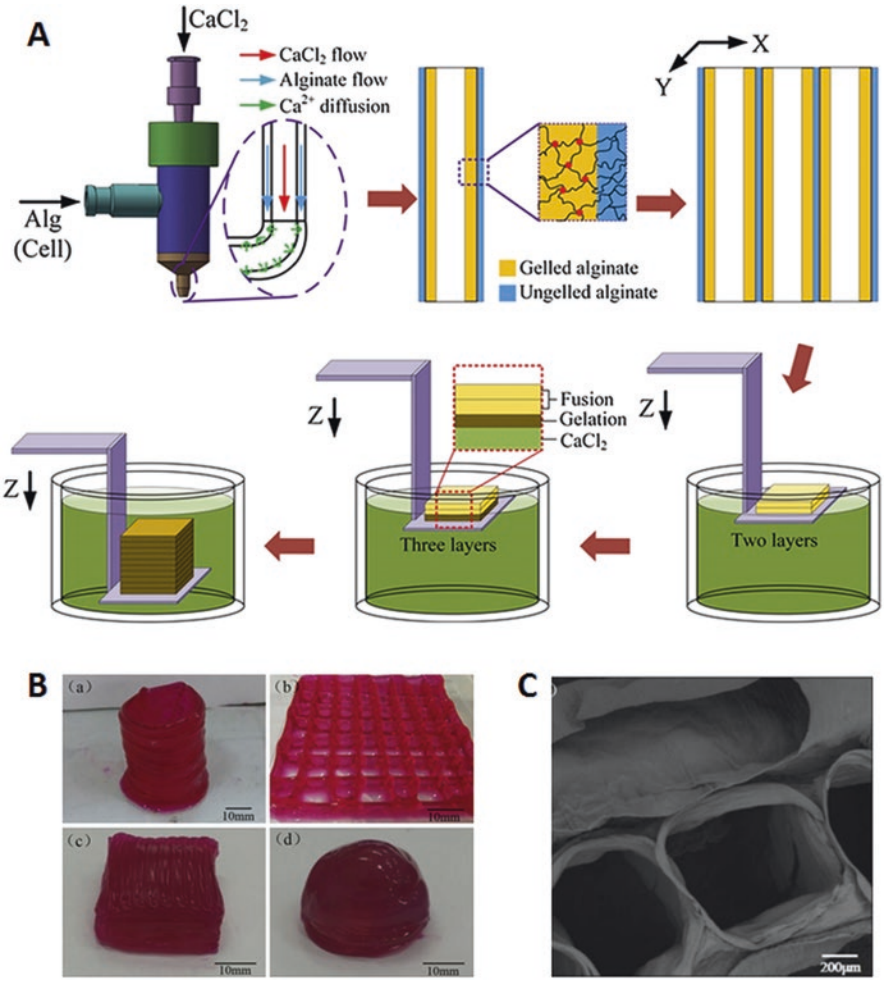

Fig. 5.5 (**a**) Schematic showing the fabrication process of a 3D alginate structure with built-in microchannels that was fabricated using a coaxial nozzle-assisted 3D bioprinting system. (**b**) Printed 3D alginate porous structures with built-in microchannels: (*a*) hollow cylinder, (*b*) grid, (*c*) cuboid, and (*d*) hemispheroid. (**c**) SEM image of the alginate hollow filaments in a printed cuboid structure consisting of six layers of hollow filament (Reproduced with permission from Ref. [67]. Copyright 2015, Elsevier)

5.3.3 Microspheres

In tissue engineering field, microspheres are generally used for encapsulation of cells, proteins, drugs, and other biomolecules. Microencapsulation technique is an attractive approach to delivering cell biomolecules by injecting constructed microspheres with minimal invasion. Microspheres are also often used as a controlled

release system of growth factors and drugs [68]. In this section, applications of cell encapsulation will be highlighted. Cell encapsulation technique aims to embed viable and functional cells within a biocompatible matrix in order to provide the embedded cells with a 3D tissue-like environment, mimicking in vivo condition. In addition to biocompatibility, a suitable matrix for cell encapsulation must possess semipermeability that allows the exchange of oxygen, nutrients, and metabolites and simultaneously protects the encapsulated cells from the toxic foreign bodies [2, 69]. Due to their ability to protect the encapsulated cells from antibodies and the host immune system, this technique has drawn attention in clinical applications as a delivery system enabling the transport of specific cells to the target site in vivo.

Designing matrix for encapsulation of cell or tissue is the major challenge in this field. Initially, this approach was proposed as a mean to protect the encapsulated cells from the external environment, thereby preventing the rejection by the host immune system [70]. For the applications in tissue engineering, this property is not only the major requirement. The permeability of smaller molecules like oxygen, nutrients, growth factors, and metabolites are equally important for cell survival inside the matrix, and these properties must be possessed by ideal microsphere used for tissue engineering applications, especially for cell encapsulation. Furthermore, equilibrated mass transfer through the whole microcapsule is very important. If the dimension of the pores and the porosity of matrix are not sufficient for the diffusion through the matrix, the cells that are far from the surface of the microsphere may receive a lower amount of nutrients at a given time [2]. Moreover, the matrix of the microspheres must be biodegradable to provide enough space for migration and proliferation of cells, leading to build the ECM [71]. Another important factor is the mechanical stiffness of the matrix that should be optimized to protect the encapsulated cells from external stress. However, the matrix should not exert high mechanical stress to the embedded cells, which can inhibit normal cellular behavior [72].

Hydrogels are commonly used as matrices for cell encapsulation since they provide a number of features which are advantageous for the biocompatibility. Among the various synthetic and naturally derived hydrogels, alginate is widely used for the encapsulation of cells and biomolecules due to its excellent ionic gelation properties with divalent cations which occurs under mild, nontoxic conditions for the encapsulated cells. Microencapsulation of cells using alginate has been originally done by Lim and Sun [73]. Cell encapsulation strategy has been used mostly for the applications in bone, cartilage, vascular, muscle, adipose, neural, and other soft tissue engineering, which will be discussed in the next paragraphs.

5.3.3.1 Bone Tissue Engineering

Since alginate is nontoxic and non-immunogenic to the encapsulated cells, numerous studies have been conducted for the applications in the field of bone tissue engineering using mostly stem cells. Though alginate does not possess any cell adhesion ligand, several studies have been performed on growth and osteogenic differentiation of stem cells in pristine alginate microsphere without further

modification. In one of the studies, murine-derived adipose tissue stromal cells have been encapsulated to investigate growth and osteogenic differentiation in comparison to the conventional 2D culture [74]. Cells were found to be grown in clusters with rounded and cuboidal morphology in alginate microspheres during osteoinduction whether cells exhibited elongated fibroblastic morphology in 2D culture. Compared to 2D culture, encapsulated cells exhibited significant osteogenic activity, which was revealed by a high expression of ALP and osteocalcin mRNA, and prove that alginate hydrogel provides a niche for osteogenic differentiation of stem cells.

Local microenvironment, like the presence of cytokines, immune cells, and growth factors, controls the stem cell-mediated bone tissue regeneration. Among the various growth factors, bone morphogenetic protein-2 (BMP2) is often used to induce osteogenic differentiation of stem cells [75]. As an alternative to using recombinant BMP2, an anti-BMP2 monoclonal antibody (mAb) was used in RGD-conjugated alginate microspheres to design an appropriate microenvironment for osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs). Anti-BMP2 mAb can trap endogenous BMP ligands, which activate BMP receptors of hBMSCs and thus induce osteogenic differentiation of the stem cells [75, 76]. An in vivo study was conducted using this approach, in which hBMMSCs-encapsulated anti-BMP2 mAb-preloaded RGD-alginate microspheres were implanted in a calvarial defect of immunocompromised mice. A significant amount of bone repair in the presence of hBMSCs and anti-BMP2 mAb was confirmed by micro-CT and histological analyses compared to the groups with hBMSCs or anti-BMP2 mAb alone or even recombinant human BMP2 (rhBMP2) (Fig. 5.6). In addition, in vitro model was used to investigate the molecular mechanism governing the osteogenic differentiation of hBMSCs, which also confirmed that the osteogenic differentiation is modulated by the BMP signaling pathway through capturing BMP2 ligands by the incorporated anti-BMP2 mAb. The use of RGD-conjugated alginate as an exogenous ECM matrix makes the system simpler with it and can be easily modified to provide a perfect 3D niche to the encapsulated hBMSCs.

Efficient mass transfer throughout the microsphere is very challenging in conventional static in vitro culture. To facilitate nutrient and osteogenic supplement supply throughout the microsphere for enhancing viability, proliferation, and osteogenic differentiation of embryonic stem cells (ESCs), a study was performed using an efficient rotary cell culture microgravity bioreactor integrated with a cell encapsulation unit [77]. High viability and osteogenic differentiation of ESCs and mineralization were observed throughout the microspheres. In bone tissue engineering, mineral deposition is specifically controlled by osteoblasts or differentiated osteogenic cells rather than nonspecific mineral precipitation. This phenomenon can be confirmed by the presence of collagen type I and osteocalcin within the mineralized constructs since type I collagen is the most abundant protein and osteocalcin is the most abundant non-collagenous protein in bone. The presence of collagen type I and osteocalcin in the cell-encapsulated microspheres was confirmed by immunocytochemistry. Moreover, the composition of the deposited minerals throughout the constructs is very important to understand the nature of the mineralization, which is generally

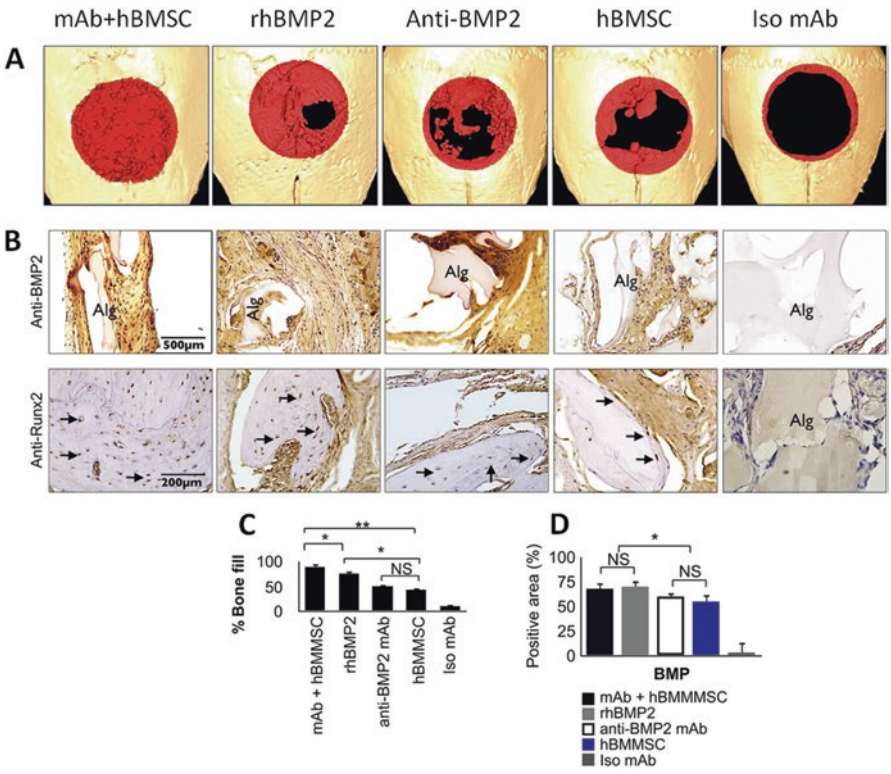

Fig. 5.6 Contribution of anti-BMP2 mAb to bone regeneration in a critical size mouse calvarial defect, where hBMSC-encapsulated RGD-alginate microspheres loaded with or without anti-BMP2 mAb or Iso mAb were implanted. (**a**) Micro-CT results of bone repair in mouse calvarial defects, where regenerated bone is denoted with pseudo-colored red. (**b**) Characterization of the hBMSCs after transplantation: cells and alginate positive for BMP2 epitopes are stained brown (middle panel), and cells expressing the bone-associated transcription factor Runx2 are stained brown (black arrows) shown in the bottom panel. (**c**) Semiquantitative analysis of bone formation based on micro-CT images. (**d**) Histomorphometric analysis of calvarial defects showing the relative amount of bone formation in the critical size calvarial defects. $*p < 0.05$, $**p < 0.01$ (Reproduced with permission from Ref. [75]. Copyright 2013, Elsevier)

accomplished by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and energy dispersive X-ray spectroscopy (EDS). Calcium- and phosphate-based mineralization was confirmed by FTIR analysis in the study of Hwang et al. Calcium and phosphate are the major inorganic components of the bone. The nature of calcium- and phosphate-based minerals can be further confirmed by analyzing Ca/P molar ratio as such 1.00 in dicalcium phosphate dihydrate, 1.33 in octacalcium phosphate, 1.50 in tricalcium phosphate, and 1.67 in hydroxyapatite [78]. Though the mass transfer could be improved through dynamic in vitro culture, insufficient oxygen and nutrient supply remains a major challenge in vivo due to poor vascularization. Angiogenesis is a complex process; it involves endothelial cells and pericytes,

which is affected by multiple growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF), and angiopoietin- and platelet-derived growth factor (PDGF). Since combining all these growth factors is very cumbersome and expensive to achieve, platelet-rich plasma (PRP), a blood-derived product containing various autogenic growth factors, was used to induce angiogenesis of adipose-derived stem cells (ADSCs) in alginate microspheres [68]. First of all, PRP induced osteogenic differentiation of encapsulated ADSCs. A significant mineralization with a considerable amount of small capillaries was achieved for the ADSC-encapsulated alginate microspheres having 10% and 15% PRP in the in vivo study, which was performed in an 8-week-old athymic nu/nu mice, which underwent subcutaneous injection of the microspheres on the dorsum. Though enhanced osteogenic differentiation was observed in this study, the effect of PRP in osteogenesis is contradictory to date [79, 80]. Regarding vascularization, though small capillaries formed for the alginate microspheres containing 10% and 15% PRP, the number of capillaries was not significant enough for regeneration of sufficient vascularized bone tissue. Lower vascularization might occur due to inadequate degradation of alginate microspheres. To achieve adequate degradation of alginate microspheres in vivo, a study was conducted using a strategy of covalent crosslinking between partially oxidized alginate and gelatin [81]. Due to the absence of alginate-degrading enzyme in mammals, alginate does not degrade in vivo and cannot be eliminated by the kidney. Partial oxidation of alginate allows hydrolytic degradation of oxidized alginate due to cleavage of vicinal glycols of polysaccharide structure [18]. Gelatin is generally degraded in vivo by matrix metalloproteinase-2 (MMP-2) and MMP-9. Degradation of the subcutaneously implanted oxidized alginate-gelatin microspheres was observed, and that facilitated ingrowth of connective tissue into microspheres. A significant number of capillary structures were observed in the connective tissue between microspheres, which is the sign of ongoing angiogenesis. Most importantly tubular structures were formed within the connective tissue inside the microspheres in the absence of encapsulated cells. Oxidized alginate-gelatin covalently crosslinked hydrogel microspheres originally fabricated by Sarker et al. [18], and that was used to investigate encapsulated cell behavior [59]. It is well known that due to the absence any cell adhesion ligand, alginate does not promote cell adhesion, proliferation, and migration. Due to the presence of RGD peptide sequence, gelatin is used with alginate to promote cell-material interaction [82]. However, a question was aroused whether the presence of gelatin in alginate hydrogel would be sufficient for cell adhesion, proliferation, and migration. It was revealed that covalently crosslinked oxidized alginate-gelatin hydrogel supported high viability, proliferation, and migration of encapsulated osteoblast-like MG63 cells compared to the blended alginate-gelatin hydrogel and pristine alginate hydrogel [59]. As shown in Fig. 5.7, cells spread throughout the covalently crosslinked hydrogel (ADA-GEL-x) microbeads. Cells migrated out of the microbeads and formed a bridge between the neighboring ADA-GEL-x microbeads. Higher degradability, highly porous structure, and lower gelatin release make the oxidized alginate-gelatin hydrogel superior for encapsulated cell growth over the blended hydrogel, even over RGD-conjugated alginate

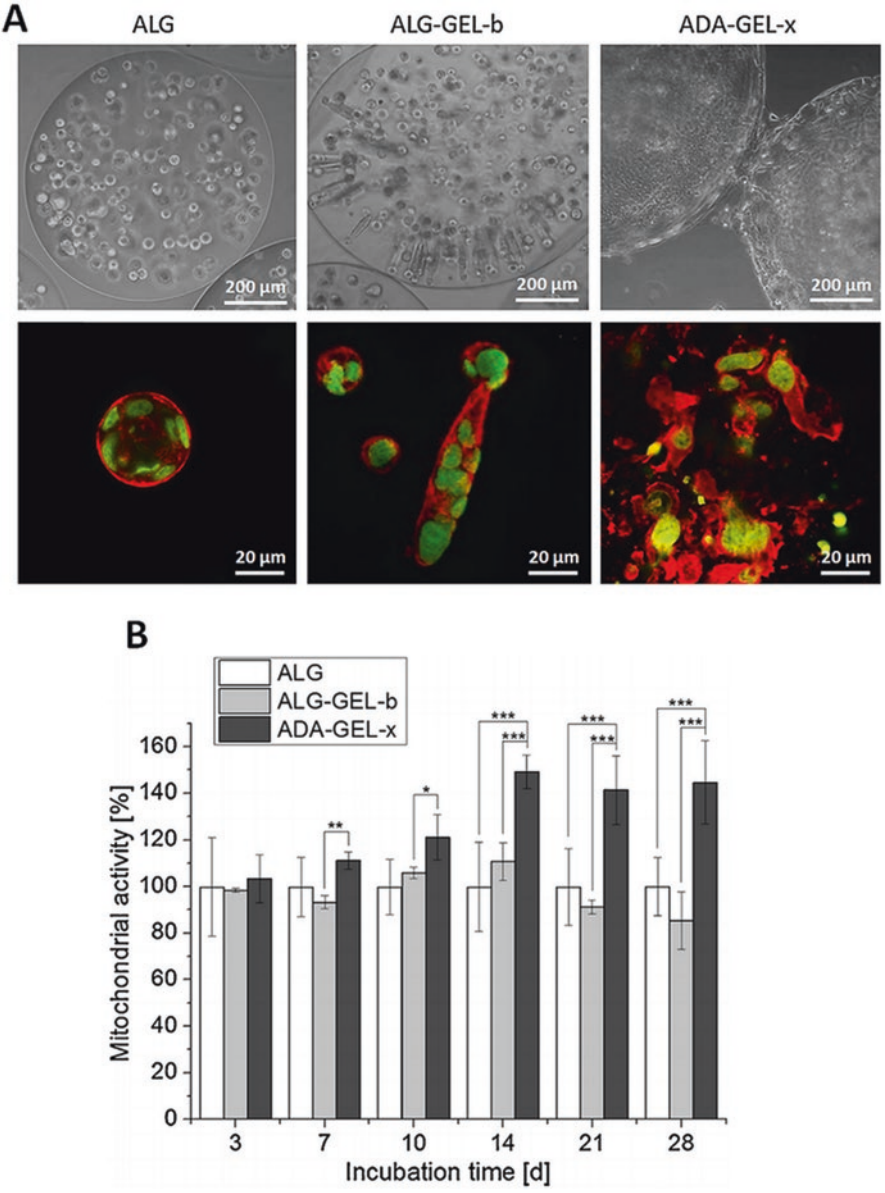

Fig. 5.7 Viability and morphology of encapsulated osteoblast-like MG63 cells in the microbeads of various alginate-based hydrogels. (**a**) Bright field (top row) and confocal microscopy (bottom row) images of cell-loaded microbeads made of pure alginate (ALG), physically blended alginate-gelatin (ALG-GEL-b), and chemically crosslinked oxidized alginate-gelatin (ADA-GEL-x) hydrogels after 28 days of incubation. Cells were stained for the nucleus (green) and F-actin (red). (**b**) The mitochondrial activity of the encapsulated cells over the incubation period showing higher cell activity in the chemically crosslinked composition compared to pure alginate and blended alginate-gelatin hydrogel. Asterisks denote significant difference, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ (Bonferroni's post hoc test was used) (Reproduced with permission from Ref. [59]. Copyright 2015, Elsevier)

hydrogel [57]. Apart from gelatin, other proteins, e.g., keratin [83], silk fibroin [84], fibrin [85], have been used with alginate hydrogel to encapsulate cells, which proves that alginate is a suitable material that can be modified chemically or with different proteins and peptides to promote cellular functions in 3D. Utilizing degradation characteristic of oxidized alginate, a fast cell release study was designed by encapsulating human umbilical cord mesenchymal stem cells (hUCMSCs) into oxidized alginate-fibrin microbeads [85]. A fast degradation of oxidized alginate-fibrin microbeads was observed, and that facilitated the fast release of hUCMSCs, which showed osteogenic differentiation with elevated bone marker gene expressions of ALP, osteocalcin (OC), collagen type I, and Runx2. Moreover, an increasing trend of hUCMSC-synthesized bone mineral formation over the culturing period was confirmed by Alizarin Red S (ARS) staining.

As an inorganic phase, BG was introduced into alginate to generate composite hydrogel microspheres as cell career for bone tissue regeneration [86]. As stated earlier due to the presence of BG, apatite phase deposition was induced on the surface of the microspheres after being soaked in SBF. Higher proliferation and stimulated osteogenic differentiation of encapsulated preosteoblastic cell line, MC3T3-E1, were observed in BG-incorporated alginate microspheres compared to the pristine alginate microspheres. Some studies revealed that the released ions from BG, especially silicon and calcium, change the local environment and stimulate osteogenesis and angiogenesis in vitro and in vivo [87, 88].

5.3.3.2 Cartilage Tissue Engineering

Cell encapsulation is a very useful strategy whereby living cells are entrapped within biocompatible, biomimetic hydrogel-based matrix, such as alginate. Cell encapsulation within alginate microbeads has been extensively used for cartilage regeneration using various cell types, e.g., chondrocytes, stem cells, and progenitor cells. In order to effectively differentiate stem cells and progenitor cells into chondrocytes, an appropriate microenvironment and signaling molecules are required in alginate-based hydrogel systems. It is well known that growth factors such as TGF-β1, BMP-4, and FGF-2 are often used to induce chondrogenesis. Moshaverinia et al. [89] developed alginate hydrogel-based co-delivery system, in which dental MSCs such as periodontal ligament stem cells (PDLSCs) and gingival mesenchymal stem cells (GMSCs) were encapsulated within TGF-β1-loaded RGD-conjugated alginate hydrogel. Comparative chondrogenic differential potentiality of the two dental MSCs was investigated over bone marrow MSCs (BMMSCs) in this hydrogel system. Successful chondrogenesis of encapsulated PDLSCs and GMSCs was observed in vitro and in vivo, confirmed by histochemical analysis and immunofluorescence staining. Moreover, the two major matrices of articular cartilage, proteoglycan and type II collagen, were produced for the groups having PDLSCs and GMSCs, and the synthesis of the two matrices was found to be induced in the presence of TGF-β1. More chondrogenesis was observed for PDLSCs than BMMSCs. The results showed that the presence of TGF-β1 and RGD peptides in alginate

matrix had great importance on chondrogenic differentiation of dental stem cells. Alginate hydrogel possesses microporosity that allows the penetration or transportation of macromolecules with a molar mass of less than 49 kDa and therefore various growth factors (e.g., TGF-β1) can transport through alginate matrix and induce differentiation of encapsulated stem cells [90, 91]. In another study, Endres et al. [92] investigated chondrogenic differentiation of human subchondral corticospongious progenitor (CSP) cells in Ca-alginate microbeads. The permeability of the alginate microbeads was sufficient for nutrient and growth factor supply to the encapsulated cells and therefore, transforming growth factor beta-3 (TGF-β3) could induce chondrogenic differentiation. After 14 days, chondrogenic marker genes aggrecan, type II collagen, and cartilage oligomeric matrix protein were found to be upregulated when CSP cells were encapsulated in alginate microbeads with a mean diameter of 600–700 μm and stimulated with TGF-β3. It is important to note that no upregulation of adipogenic and osteogenic marker genes was observed, which proves that the alginate microbeads can be used to achieve differentiation of progenitor cells into a specific lineage. Since the dynamic regulation of integrin-binding peptides is crucial for chondrogenic differentiation of stem cells, Chang et al. [93] used modified RGD by embedding RGD-chimeric protein (CBD-RGD) with the cellulose-binding domain (CBD) in alginate beads to induce chondrogenesis of ADSCs. In the absence of chondrogenic supplements, higher expressions of chondrogenic marker genes (Sox9, Col-II, and AGG) were observed when ADSCs were encapsulated in CBD-RGD-containing alginate microbeads compared to the pristine alginate microbeads. However, enhanced expressions of those genes were observed in the absence of CBD-RGD when cultured in the chondrogenic medium. This effect of chondrogenic media was further enhanced in the presence of CBD-RGD, which however was found to be dose-dependent of CBD-RGD and its release profile over the time. At the early stage of cell differentiation, the fibronectin expression of the encapsulated cells markedly increased but the RhoA activity was inhibited for the dose of 10 mg/g CBD-RGD in alginate. In the presence of TGF-β3, CBD-RGD-mediated suppression of RhoA activity enhanced the chondrogenic differentiation capacity of ADSCs at the concentration of 10 mg/g CBD-RGD. However, the higher loading of CBD-RGD (20 mg/g) in alginate impaired chondrogenesis of encapsulated ADSCs. In normal culture condition, the RGD peptides could release rapidly from alginate matrix because of its more acidic nature, which is in contrast to the CBD-RGD protein that had prolonged retention and could mimic more dynamic niche of stem cells.

Chondrogenesis can also be promoted by employing Co^{2+} ion in the alginate matrix, without using costly growth factors to direct stem cell differentiation into cartilage-generating chondrocytes [94]. Cobalt chloride in different concentrations along with calcium chloride was used as hardening solution to prepare Co^{2+} ion-containing alginate beads. Cell viability assay showed a dose-dependent relationship with Co^{2+} ion, where 1.25 and 2.5 mM of Co^{2+} ion in the hardening solution were found to be optimal concentrations for encapsulated human adipose-derived mesenchymal stem cells (hADSCs) viability. Chondrogenic differentiation of hADSCs was analyzed by quantifying the expression level of various chondrogenic

markers. Chondrogenic differentiation markers at early stages (Sox9 and VCAN) were observed to be upregulated in Co2.5 and Co5 samples (alginate beads generated in hardening solutions containing 2.5 and 5 mM Co^{2+}, respectively). However, the lower concentration of Co^{2+}-containing alginate beads (Co1.25) did not promote upregulation of VCAN, which prove that lower Co^{2+} concentration in alginate bead is not sufficient for chondrogenic differentiation of hADSCs. The alginate beads with an optimal concentration of Co^{2+} ions herein provide a favorable environment for chondrogenic differentiation of stem cells. Barium alginate was also used to develop a 3D coculture system of ADSCs and nucleus pulposus (NP) cells for regeneration of the human degenerated intervertebral disk (IVD) [95].

5.3.3.3 Soft Tissue Engineering

Though the microencapsulation of cells within alginate hydrogel is mostly used for regeneration of bone and cartilage, a significant research has been carried out for regeneration of soft tissues, like muscle, nerve, ovarian follicle, cardiac, and vascular tissues [82, 96–99]. One of the major challenges in the tissue regeneration using stem cells is the design of a suitable microenvironment, which can support cell attachment and promote proliferation and differentiation of stem cells into the required lineage. Differentiation of stem cells is generally controlled by the ECM microenvironment, including the presence of cell adhesion ligands, growth factors, cytokines, etc. [96]. Moreover, mechanical cues of ECM also direct stem cell differentiation lineage [100]. Ansari et al. used RGD-conjugated alginate hydrogel to design niche for growth and differentiation of encapsulated GMSCs [96]. This microsphere system has been used to deliver multiple growth factors, (Forskolin (FSK), 6-Bromo-1-methylindirubin-3′-oxime (MeBIO), and basic fibroblast growth factor (bFGF)) to the encapsulated GMSCs to differentiate to myogenic lineage in vitro and in vivo, where GMSC-encapsulated RGD-coupled alginate microspheres were transplanted subcutaneously into immunocompromised mice. The presence of the myogenic growth factors doubled the expression of myogenic-specific genes (MyoG, MyoD, and Myf5) compared to non-encapsulated GMSCs. The porous microstructure of alginate hydrogel synergistically facilitates cell growth and differentiation due to the availability of nutrients and growth factors. It has also been revealed that the mechanical properties of RGD-conjugated alginate hydrogel control the fate of encapsulated GMSCs. Highest myogenic differentiation of GMSCs was observed within the hydrogel with an intermediate modulus of elasticity (10–16 kPa) in comparison to softer (<5 kPa) or stiffer (>20 kPa) hydrogels. It is interesting to note that greater myogenic differentiation was observed for GMSCs compared to the positive control, hBMMSCs. Moreover, the implanted GMSCs encapsulated in RGD-conjugated alginate microspheres loaded with multiple myogenic growth factors showed increased neovascularization and local angiogenesis compared to the microspheres containing hBMMSCs. Taking into account the outcomes of the study, it can be concluded that GMSCs have better muscle tissue regeneration capacity compared to hBMMSCs in an appropriate

environment with suitable inducing signals. GMSCs and other dental-derived stem cells, PDLSCs, have also been used for tendon tissue regeneration, where a co-delivery system based on TGF-β3-loaded RGD-conjugated alginate microspheres was developed for encapsulation of the two stem cells along with hBMMSCs as a positive control [101]. Since collagen bundle structure in the periodontal ligament is similar to that of tendon tissue, it has been hypothesized that PDLSCs could be a suitable stem cell for tendon regeneration. All three stem cells encapsulated in RGD-coupled alginate exhibited high levels of mRNA expression for gene markers, Scx, DCn, Tnmd, and Bgy, which are related to tendon regeneration. However, it is interesting to note that expression levels of the gene markers were found to be higher for PDLSCs compared to the other two cell types. In the in vivo study, where the MSCs-encapsulated TGF-β3-loaded RGD-coupled alginate microspheres were transplanted subcutaneously in mice, histological and immunohistochemical staining confirmed the regeneration of ectopic neo-tendon tissue in the implanted constructs. Similar to the in vitro outcomes, significantly higher tendon tissue regeneration was observed for PDLSC-encapsulated microspheres compared to the microspheres containing GMSCs or hBMMSCs in the in vivo study. The unique porous structural property of alginate and the presence of conjugated RGD tripeptide and suitable signal molecule (TGF-β3) facilitated cell-matrix interactions, leading to enhanced MSC adhesion, proliferation, and differentiation to a specific lineage. The porous structural properties, ease of conjugation or loading of peptides, proteins, and growth factors, and non-cytotoxicity make alginate hydrogel a highly suitable biomaterial for tissue engineering. Cell encapsulation strategy was also used to grow and expand neural stem cells (NSCs), where alginate-gelatin microbeads were designed with tuned internal pore size and structure, which provided a suitable 3D environment supporting NSC proliferation [82].

5.4 Conclusions

Since hydrogels mimic the native extracellular matrix, hydrogel-forming biomaterials, especially naturally derived materials, are extensively used for biomedical applications. This chapter highlighted the applications of one of the most used naturally derived hydrogel-forming biomaterials, namely, alginate, in the field of tissue engineering. The three major types of tissue engineering constructs (porous solid scaffolds, bioprinted scaffolds, and microbeads) fabricated from alginate-based hydrogel matrices are discussed here in the context of various tissue engineering applications, such as bone, cartilage, and vascular tissue engineering. All three systems provide a 3D environment to the embedded cells that can closely mimic the native tissue environment. Since pristine alginate does not provide cell adhesion sites, most of the works have been done with modified alginate rather than pure alginate. Cell adhesion motifs are generally introduced into the alginate system by incorporating RGD peptides or various proteins such as gelatin and keratin. Few studies have been also conducted to tailor the degradability and porosity of the

alginate hydrogel matrix by chemical modification. The alginate-based hydrogels that are chemically modified and having incorporated peptides or proteins showed better cell growth, migration, and proliferation compared to non-modified alginate-based hydrogels. Porous scaffolds made of alginate-based hydrogels are mostly studied for applications in bone tissue engineering because of their suitable mechanical properties. Nowadays, additive manufacturing techniques, such as bioprinting, are considered as biofabrication methods to design and fabricate tissue engineering scaffolds in very precise dimensions according to the critical defect size that needs to be regenerated. Alginate-based materials showed great potentiality for designing such scaffolds using the bioprinting technique for various tissue engineering applications. The third system, alginate-based microbeads, is generally used to encapsulate cells in order to provide a tissue-like environment to the encapsulated cells and to protect the cells from external mechanical stresses and the host immune system during in vitro and in vivo studies. This chapter discussed key research studies that highlighted the various tissue engineering applications and cell therapeutic applications of microbeads made of alginate-based hydrogels. All types of tissue engineering constructs made of alginate-based hydrogels showed excellent biocompatibility and potentiality in tissue engineering applications.

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Chapter 6
Alginate-Based Three-Dimensional In Vitro Tumor Models: A Better Alternative to Current Two-Dimensional Cell Culture Models

Amit Khurana and Chandraiah Godugu

Abstract The conventional two-dimensional (2D) cell culture models, although providing considerable information about the cellular dynamics, often fail in vivo. In the area of oncology drug discovery and development process, the tumor microenvironment poses a significant challenge owing to the complexity of tumor stroma, and the traditional 2D in vitro systems fail to mimic the extracellular matrix (EC) and cell-to-cell interaction-based modulations. Three-dimensional (3D) cell culture systems/matrices, also designated as scaffolds, offer an excellent platform to study the tumor microenvironment in a more realistic way. Alginate matrices are widely used for cellular encapsulation, cell transplantation, and tissue engineering. Alginate-based 3D gels and scaffolds have emerged as a prime matrix to simulate tumor microenvironment closer to physiological condition. Alginate being hydrophilic provides uniform matrix for growth and proliferation. The alginate scaffolds and hydrogels have been used to investigate the cytotoxicity, apoptosis, and penetration of conventional drugs as well as various formulations into the in vitro tumor scaffolds/spheroids. Another advantage of alginate-based matrices for 3D cultures is that the cancer stem cell (CSC) niches can be better understood owing to the inherent 3D nature of CSCs. Moreover, the use of 3D systems gives a better impression of the physiological architecture; unique cellular interactions can occur and improve the functional properties. In this chapter, initially we compare and contrast 2D and 3D cell culture systems and the pitfalls of the conventional in vitro tumor models. Then a brief introduction of alginate-based 3D scaffolds is provided. Various in vitro models based on alginate scaffolds (AlgiMatrix™) and hydrogels are briefed with the parameters studied and the associated advantages. Future improvements in the 3D cell culture based on alginate matrices are thought through, and the possible future directions are provided. In conclusion, results from different

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studies give an indication that high-throughput in vitro 3D tumor models based on alginate can be prepared to study the effect of various anticancer agents and various molecular pathways affected by the anticancer drugs and formulations.

Keywords Alginate • AlgiMatrix • Spheroids • Tumor • Anticancer • Tumor microenvironment

6.1 Introduction to 3D Cell Culture Models and Problems Associated with 2D Monolayer Models

The advancements in the biological sciences resulted in the origin of cell culture-based experimental models to study various biological phenomena. Most of the initial animal-based experiments are performed on in vitro cell culture models. Currently, in vitro cell culture-based models play a crucial role in new drug discovery and developmental process. The cell culture models have important role in cancer research. The initial anticancer effects of new chemical entities and novel anticancer formulations are performed on in vitro cancer cell lines [1]. Although these cell culture-based models offer numerous advantages in various stages of drug discovery and developmental process, they also suffer from several shortcomings. Most of the cell culture studies are based on the traditional two-dimensional (2D) models where the cells are grown on a polystyrene plastic surface. In these models, the plastic cell culture surfaces are coated with biocompatible matrix which supports the cell attachment and growth [2]. Though 2D models offer the advantage of ease of subculture and cell isolation for further molecular studies, they often produce excessive positive response in such a way that these effects are observed only in in vitro cell culture models and may never translate those effects while performing the in vivo animal models for confirmation of observed in vitro effects. When 2D-based models are used, generally there is found to be poor in vitro and in vivo correlation. The in vitro and in vivo correlation was one of the biggest challenges while developing the new drugs and formulations. Experimental outcomes when performed on 2D cell culture models are in general found to produce increased sensitive responses, and questions on relevance of conventional 2D grown cancer cells have been raised [3–5]. That means 2D models suffer from several disadvantages including (1) lack of cell–cell interactions, (2) lack of cell–extracellular matrix (ECM) interactions, (3) lack of permeation barriers to hinder the diffusion of drugs or agents into cells, (4) lack of attainment of original shape, etc. [6, 7]. Owing to multiple shortcomings associated with 2D cell culture-based models, scientists felt the need to develop new in vitro models with better correlation with in vivo condition. As a result of the conquest for finding better alternatives, a multiple three-dimensional (3D) cell culture-based models were proposed with reproducibility and integrity across a wide array of variables including the matrix used and the type of culture system employed (adherent and suspension) [8, 9]. The 3D cell culture-based systems/matrices, also

called as 3D scaffolds, include the multicellular spheroids, microcarrier beads, scaffold-based culture, and organotypic explant culture [10–14].

The conventional 2D cell culture models although providing considerable information about the cellular dynamics may often fail in vivo. In the area of oncology drug discovery and development process, the tumor microenvironment poses a significant challenge owing to the complexity of tumor stroma, and the traditional 2D in vitro systems fail to mimic the extracellular matrix and cell-to-cell interaction-based modulations. 3D cell culture systems/scaffolds offer an excellent platform to study the tumor microenvironment in a more realistic way. 3D cell culture systems address the concerns associated with 2D cell culture and mimic the intercellular and cell–matrix interactions in a fashion much closer to the real-time in vivo condition, the key behind deciphering the true mechanistic profile. In the area of anticancer drug discovery, a large number of compounds may show promising cytotoxicity in 2D systems, but the same compounds may fail to elicit similar pharmacological activity in 3D systems owing to enhanced cellular interactions and the active role played by the matrix interactions in 3D systems [15]. The monolayer 2D systems fail to mimic the cellular interactions as approximately 50% of the cell surface is in contact with the plastic surface, and approximately the same proportion of cell surface contacts the media for nutrient supply. Therefore, a meager proportion is in contact with other cells, a condition very unlikely to happen in in vivo conditions as the organs grow in 3D. However, in 3D cell culture systems, the cell-to-cell and cell–matrix interaction are much superior, which makes them much closer to the in vivo conditions. The extracellular matrix (ECM) plays an integral role in the normal growth, differentiation, and homeostasis of a tissue, a condition mimicked during 3D cell culture and absent in case of 2D culture. The routinely employed multicellular spheroid-based cell culture models mimic the in vivo ellipsoidal cellular shape in contrast to the relatively flat shape in the case of 2D cell culture models. Furthermore, 3D-cultured cells mimic in vivo morphology, proliferation, metabolism, gene expression, and viability in a much better physiological way.

Another major area of debate in cancer drug discovery is the large-scale use of nude animal models. Using nude animals is costly on one side and of ethical concern on the other. Thus, the adoption of 3Rs in oncology drug discovery is the need of the hour. The use of 3D cell culture systems may replace the 2D systems and the use of animals to a significant extent. Secondly, the use of 3D tumor models refines the hitherto used physiologically quiet irrelevant 2D systems and gives a better option to understand the real-time in vivo pharmacodynamics with much superior integrity. Last but not the least, the 3D systems may significantly reduce the time, the money, and the labor involved in pursuing in vivo anticancer research on nude animal models [16]. Moreover, large-scale adoption of 3D cell culture technique imbibes not only the 3Rs but, on a long haul, adds multiple advantages including the more reliable mechanistic data, much superior replication of cell–cell and cell–matrix interaction, a prerequisite and must needed to solve the issues associated with tumor microenvironment heterogeneity, in particular the tumor fibrosis which cannot be mimicked in 2D systems but can be decently mimicked in 3D cell culture-based tumor systems. Thus, the use of 3D cell culture systems reduces the

discrepancies associated with 2D cell culture and provides a better platform for oncology drug discovery and development process at a wide array of closets including the preclinical cytotoxicity screening, screening of pharmacological activity, and toxicological evaluation. It is expected that the development of 3D tumor models will reduce animal testing, yield more predictive data, improve cell culture efficiency, reduce cost and time to identify new drug candidate, and reduce development time to market [17, 18].

6.2 Problem with In Vitro and In Vivo Correlation Based on 2D Cell Culture Models

As discussed in the introduction, there are multiple areas where 2D and 3D cell culture systems differ including the cellular interactions, morphology, and cellular dynamics. Another important area of relevance is how much we can correlate these systems with in vivo conditions be it the 2D or the 3D systems. In general, owing to the multiple advantages associated with 3D system, one can easily judge 3D systems to be much superior to the 2D systems. Moreover, in terms of clinical and in vivo correlation, 3D systems stand way ahead of 2D systems. For in vitro to in vivo translation of results for successful cancer chemotherapy, it is essential to mimic the in vivo environment in the in vitro models of screening. The rationale behind using a 3D system in vitro is to mimic the 3D tumorlike microenvironment which gives better correlation with in vivo results. For example, multiple research findings report that 3D cell culture systems are much more resistant to anticancer drugs compared to 2D systems [5, 19]. The difference in sensitivity may be attributed to the poor diffusion of drug across the tumor. Another explanation may be that, owing to 3D microenvironment, the tumor mass may become hypoxic which may upregulate some of the important genes responsible for drug resistance [20]. Imamura et al. showed that multicellular spheroids of breast cancer cells become hypoxic causing an increase in the cells in G_0 phase and/or suppression of caspase-3, and the results were in line with the in vivo patient-derived tumors, in contrast to the results obtained with 2D monolayer of the same cells [21]. It is almost impossible to study tumor fibrosis in case of 2D culture, whereas the same can be decently mimicked with 3D multicellular tumor spheroids (MCTS). Hypoxia and tissue necrosis are certain issues associated with molecular complexities of the 3D microenvironment and are observed in vivo as well. Study of these events in 2D monolayer cell culture poorly correlates with in vivo microenvironment. In contrast, study of hypoxia is very much possible with 3D MCTS and better correlate with in vivo tumors. Thus, these observations serve as a warning on the relevance of 2D systems for anticancer drug screening and warrant due consideration for the use of pragmatic 3D in vitro models for anticancer drug research.

6.3 Introduction to AlgiMatrix™ Based 3D Cell Culture Models

Bridging the gap between 2D and 3D cell culture requires a base matrix which can allow the cells to sufficiently grow in an environment which mimics the in vivo conditions and at the same time protects the cellular physiology from extreme hypoxia owing to the limited fluid turbulence during 3D cell culture. Wide arrays of matrices of natural origin have been explored for 3D cell culture including collagen and alginate as the most widely studied scaffold materials. Among the natural scaffolds, alginate has received wide attention by virtue of its high biocompatibility, low toxicity, and low cost. It is an anionic polymer isolated from brown seaweeds possessing excellent gelation properties which can be controlled for generating wide variety of scaffolds with various sizes and matrix properties [22]. AlgiMatrix™ is an innovative marketed product from Invitrogen (Thermo Fisher Scientific) for development of tumor spheroids. It is a biologically inert alginate sponge-based (with pore size in the range of 50–200 μm) bioscaffold for 3D cell culture. The product is available in 6-well, 24-well, and 96-well format for growing spheroids with remarkable similarities with the in vivo phenotype of cells. AlgiMatrix™ 3D culture system can be applied to a wide number of cell-based assays, including multicellular tumor spheroid (MCTS) assays, hepatocyte and cardiomyocyte organogenesis studies, coculture studies, high-throughput drug screening, and embryonic stem cell 3D differentiation studies. These scaffolds have a unique feature of dissolution of matrix by using dissolving buffer, which is a nonenzymatic solution and solubilizes the matrix in few minutes, and the grown spheroids can be isolated for performing various assays like staining, histology, immunohistochemistry, Western blotting, RT-PCR, etc. It has been observed that the 3D alginate scaffold microenvironment promotes the development of better cellular phenotype compared to traditional sandwich culture as evident from long-term viability, toxicity assays, molecular expressions, and ultrastructural features [17].

Our group has standardized the tumor spheroids from H460, A549, and H1650 non-small cell lung cancer (NSCLC) cell lines on this unique 3D scaffold and compared the results with conventional monolayer 2D culture. The tumor spheroids were cultured in 6-well and 96-well formats. We could develop highly reproducible 3D tumor spheroids in the size range of 100–250 μm with optimized cell density. We limited the size to below 250 μm. The nutrient distribution and air circulation are compromised with larger-sized tumor spheroids, and the risk of necrotic lesions arises. The IC_{50} values showed significant differences in 2D and 3D formats for the conventional anticancer drugs (cisplatin, docetaxel, gemcitabine, 5-fluorouracil, and camptothecin) which correlate with earlier reports. There was a significant reduction in the expression of caspase-3 in the 3D culture format (2.09- and 2.47-folds, respectively, for 5-fluorouracil and camptothecin) compared to the 2D format in H460 spheroids. In addition, we could study the cancer stem cell population behavior in H1650 stem cells and parental cells where the number of spheroids was found to be higher for stem cells compared to parental population. The stem cell

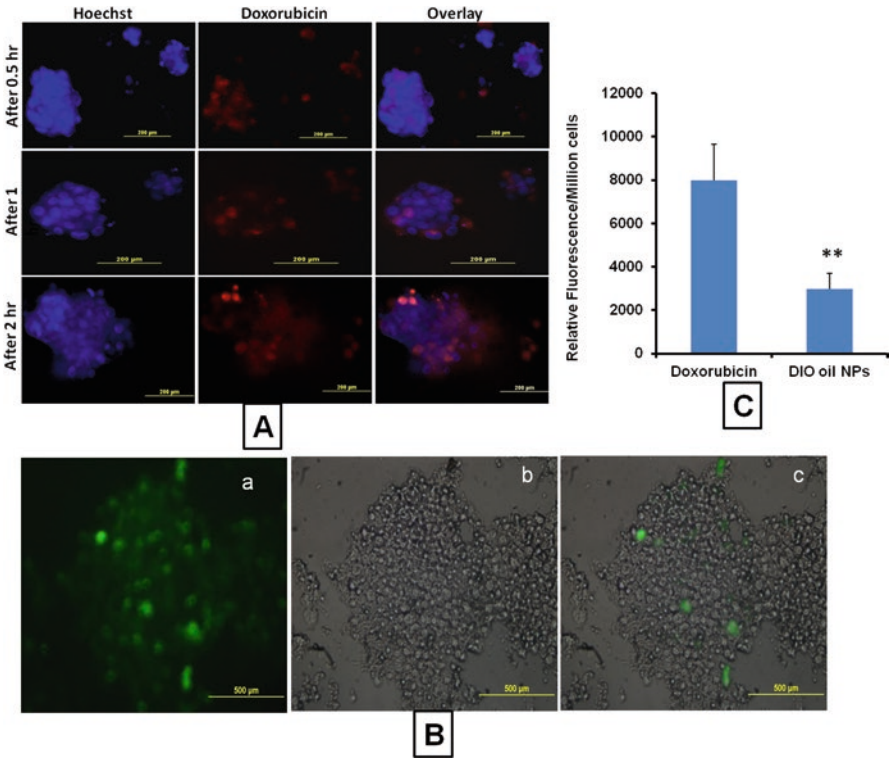

Fig. 6.1 *Drug and nanoparticles uptake by spheroids*. (**a**) Representative images of doxorubicin uptake in H1650 parental cell spheroids grown in 3D alginate scaffold system uptake images were taken at 0.5, 1, and 2 h time points. (**b**) Representative images of the nanoparticle uptake by H1650 parental cell spheroids. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DIO) oil was encapsulated in nanolipid carrier (NLC) and incubated with the spheroids for 2 h. (*a*) Fluorescent image, (*b*) DIC image, and (*c*) merged image. The fluorescent images clearly indicate the nanoparticles uptake into spheroids. (**c**) Relative fluorescence intensities of free doxorubicin uptake and DIO oil loaded NLC nanoparticles into 3D spheroids. Each data point is represented as mean±sem ($n = 3$). $^{**}P<0.01$ vs doxorubicin group (Reproduced from Ref. [17])

spheroids exhibited higher IC_{50} values, and the results correlated with previous findings [23]. The tumor penetration/uptake results also depicted the extended role of matrix proteins as evident from poor penetration of doxorubicin (10.52%) and even nanoformulations (3.41%) as shown in Fig. 6.1. Moreover, we investigated the molecular features of the spheroids by immunohistochemistry and RT-PCR as well and concluded that the tumor spheroids in 3D culture show higher chemoresistance compared to 2D monolayer culture [17].

Canine mammary tumors (CMTs) are the most common type of cancer in female dogs. Most CMT have epithelial origin and are known as simple adenoma/simple carcinoma (SC), some consist of both epithelial and myoepithelial tissues and are called complex carcinoma (CC). Cardoso et al. developed 3D tumor spheroids of

canine complex carcinoma (CC) and canine simple carcinoma (SC) cell lines derived from canine tumors in 6-well AlgiMatrix™ plates and reported spheroids in size range 50–125 μm for CC and 175–200 μm for SC. The spheroids were grown for 2 weeks and thereafter evaluated the expression of various molecular markers. 3D tumor spheroids obtained from both the cell lines showed expression of epidermal growth factor receptor (EGFR). However, there was differential expression of the same in spheroids from both the cell lines. Microarray analysis showed upregulation of crucial extracellular matrix proteins (MMP1, MMP3, MMP9, and MMP13) and downregulation of cadherin-1. The results obtained with 3D tumor spheroids were in concordance with the in vivo canine tumors showing the higher correlation of 3D tumor models with in vivo tumors. This study demonstrated the utility of AlgiMatrix™ 3D tumor models for veterinary oncology and opens new avenue for animal 3D tumor-derived cells for future research [24].

Thus, 3D AlgiMatrix can fill the gap in the area of preclinical high-throughput screening (HTS) of anticancer drugs owing to the teething discrepancies in 2D and 3D in vitro cancer models and may serve as a better method of cytotoxicity evaluation with higher correlation to in vivo results.

6.4 Alginate Scaffold as a Matrix for 3D Cell Culture-Based Tumor Model

A wide variety of matrices have been explored for developing 3D tumor models. Some of the most commonly used matrices are collagen-based scaffolds, hyaluronic acid-based scaffolds, alginate-based scaffolds, peptide-based scaffolds, hydrogels, etc. Alginate-based scaffolds have received significant attention for 3D cell culture and especially 3D tumor models for anticancer studies. Some of the advantages of alginate scaffold are animal-free product, stable at room temperature, and flexible porosity. The animal-derived scaffolds like collagen, fibrin, or hyaluronic acid possess bioactivity. In contrast, alginate is bioinert and does not contain intrinsic bioactivity; thus, its bioactive properties can be improved by modifications with peptides or by using composite hydrogels, such as alginate–collagen [25–27]. In case of cancer research, a desirable need is the formation of multicellular spheroids which can mimic the in vivo tumor microenvironment without compromising the respiratory features of the tumor owing to the fact that continuous culture for a long duration may lead to the development of hypoxia-induced necrotic regions which may lead to discrepancies in the results obtained. By virtue of its highly porous nature, alginate scaffolds allow the formation of multicellular tumor spheroids. Unique feature of alginate scaffold spurred substantial efforts toward the development of 3D tumor models, and a significant number of reports are a proof of the commercial viability of the products based on alginate matrix.

6.4.1 Alginate-Based Scaffolds for Anticancer Drug Screening

Multicellular tumor spheroids (MCTS) constitute an attractive 3D platform for anticancer drug screening and have been gaining popularity in anticancer research programs. The MCTS mimic the tumor microenvironment in a much closer to in vivo environment in terms of better cellular interactions and cell–matrix modulations. Akeda et al. proposed a 3D alginate-based cell culture system for cultivation of murine osteosarcoma cells (Dunn and LM8 metastatic clones). The cells were encapsulated inside alginate scaffold and were subsequently cultured. Both the metastatic cell lines could form nice tumor spheroids with LM8 taking lead in terms of the size of spheroids. However, detachment of cells was observed with higher rate in LM8 clones suggesting its higher metastatic potential. Thereafter the metastasis potential was evaluated in vivo by injecting five alginate beads. The alginate-encapsulated cells could form nice tumors post 7 days of inoculation with higher rate of lung metastasis in case of LM8 cells owing to their higher metastatic potential. Spheroid histology revealed uniform growth and proliferation of both the xenografts and lung metastases as well [28]. However, no mechanistic data was presented concerning the protein levels. To accelerate the rate of wide level use of alginate for 3D culture, detailed studies revealing the molecular insights are desired with a special attention on cutting down the cost of overall experimentation.

A large number of reports have raised concerns over the predictability of data obtained from 2D-cultured systems. Therefore, 3D systems with higher reproducibility have been explored for mimicking in vivo microenvironment. Chitosan is a natural polymer and has been widely used to prepare matrix for 3D cell culture with high similarity to the in vivo conditions. Chitosan–alginate combination scaffold adds the advantages of both the polymers at the same time and has been used for various applications. Leung et al. studied the role of chitosan and alginate (CA) scaffold for the culturing of HepG2 hepatocarcinoma (HCC) cells. Comparison of results with 2D and Matrigel-cultured cells revealed higher correlation with in vivo tumor microenvironment. The higher cellular interactions due to close attachment to neighboring cells via tight junctions might have promoted enhanced formation of multicellular clusters. Angiogenesis is another important phenomenon which is required for successful in vivo survival of tumors. Investigation of angiogenesis markers (IL-8, bFGF, and VEGF) showed higher expression in CA scaffold-grown tumor spheroids compared to both 2D and Matrigel-cultured cells. The in vivo metastasis study also showed rapid angiogenesis and higher malignancy of CA-scaffold cultured HepG2 cells compared to 2D and Matrigel-cultured cells. The LD_{50} study with doxorubicin revealed higher resistance of CA scaffold-grown cells suggesting higher resistance of 3D cultured spheroids which is a reported feature of tumor spheroids [29]. The CA scaffold system is a highly reproducible, versatile model and may aid in 3D culture of various cancers to better understand the tumor behavior and to explore the efficacy of novel anticancer regimen. Kievit et al. reported the use of a 3D matrix based on combination of chitosan and alginate for glioma which favors the generation of highly malignant phenotype of cancer cells

similar to in vivo conditions that promotes the conversion of cultured cancer cells to a more malignant in vivo-like phenotype. The 3D scaffolds were prepared by lyophilization and subsequent cross-linking of chitosan and alginate. The formed scaffolds were highly porous to allow the influx of cells throughout the scaffold and provide a large surface area for cell attachment and proliferation, ideal for modeling the tumor microenvironment. The tumor model was established by seeding U-87 MG and U-118 MG human glioma cells on the scaffolds and allowing the tumor cells to proliferate in vitro for 10 days. The developed 3D matrix was evaluated for human (U-87 MG and U-118 MG) and rat C6 glioma cells (cancer stem cell-like cells). The in vitro expression of VEGF, MMP-2, fibronectin, and laminin was assessed to understand the malignancy and angiogenesis potential. The electron microscopy results showed biocompatibility of the CA scaffold as the studied cell lines could grow inside the scaffold. The results were compared to cells grown in 2D (24-well plates) and in 3D Matrigel matrix. The cells growing in the CA matrix were found to grow at a slower pace. Similarity to the in vivo microenvironment conditions was speculated to be the reason behind the observed retarded growth. The scaffolds precultured with U-87 MG and C6 cells for 10 days were then implanted into nude mice to evaluate tumor proliferation and angiogenesis compared to the standard 2D cell culture and 3D Matrigel matrix xenograph controls. The study presented interesting findings as the malignancy potential of the human glioma cell lines considerably increased, whereas the results were similar for the 2D cell culture and Matrigel-implanted C6 glioma cells when compared to the CA scaffold in vivo. The results of 3D scaffolds outweighed the 2D monolayer-based results. The immunohistochemistry for CD31, an angiogenesis marker, showed notably higher expression in the CA scaffold-grown tumors suggesting the close resemblance of cancer microenvironment inside the CA scaffolds [30]. The CA scaffolds provided a 3D microenvironment for the cancer cells and were successfully grown in vivo, indicating the potential for further preclinical exploration for developing better 3D systems to reduce the use of animal experiments in anticancer drug discovery.

The same group explored the utility of this versatile CA scaffold to understand the ex vivo tumor cell physiological interaction between prostate cancer cells (LNCaP, C4-2, and C4-2B) and peripheral blood lymphocytes (PBLs) to aid in understanding the immunotherapeutic behavior of castration-resistant prostate cancer. The results were found to be interesting. The growth of tumor spheroids was supported well by CA scaffolds and Matrigel. The growth could be monitored up to 15 days, whereas the in situ imaging of spheroids could be possible even up to 55 days. Live cell imaging is an excellent tool to observe and measure real-time events and provides a useful means to monitor the external surface of tumor spheroids and their interaction with surrounding matrix and other cells. Live cell imaging by using tracker dyes revealed the infiltration of immune cells within the 3D microenvironment of tumor spheroids. Scanning electron microscopy (SEM) images showed dynamic interaction of PBLs with tumor spheroids in CA scaffolds as well as Matrigel. The interaction was found to be significant on the second day. Heterogeneity in the PBL population was observed, and these immune cells were distinguished from tumor cells by their morphology and size. In addition, the

localization of specific PBLs on the cancer cells seeded scaffolds was assessed via immunohistochemical analysis of CD45R staining (for B cells and activated T cells). CD45R+ cells were observed bound to the in vitro tumors in CA scaffolds after 2 days, which is in confirmation with the SEM results. The cells could be harvested for flow cytometry-based analysis and showed promising results with CA scaffolds in terms of future therapeutic utility [31]. This study opened up a new innovation 3D culture where understanding the interaction of microenvironment cells with tumor spheroids could be possible and lays foundation to head on and develop matrices where tumor along with multiple other TME cells can be grown and simulate the in vivo tumors within the boundaries of 3D culture. The use of 3D biomaterial scaffolds for immunological or immunotherapy studies provides a more relevant model that should accelerate the successful translation of new immunotherapies into the clinic.

Gene therapy for cancer has shown promising results preclinically and offers molecular treatment of cancer. Different approaches have been used to ferry the payload to tumors. In a subsequent study with CA scaffolds, the relevance of this novel CA scaffold was investigated to mimic 3D microenvironment for prostate cancer and its utility as tumor model for nanoparticle-mediated gene therapy. The results were compared with 2D-cultured cells and were found distinguishably modulated in 3D-cultured cells. The selected TRAMP-C2 (TC-2) prostate cancer cells could form nice tumor spheroids when cultured in CA scaffolds. The harvested spheroids showed upregulation of ECM and epithelial to mesenchymal transition (EMT) gene markers as compared to standard 2D culture, indicating better mimicry of in vivo conditions. Targeted nanoparticle (NP)-mediated delivery of red fluorescent protein (RFP) plasmid DNA into TC-2 tumor spheroids was achieved with chlorotoxin (CTX) in cells cultured in CA scaffolds. However, in case of 2D culture, the delivery could not be achieved. This finding questions the utility of the 3D culture for testing cationic-targeted NPs with CA scaffolds as the standard 2D culture did not show the targeting effect of CTX. The 3D culture results could be reproduced in vivo model, thus proving the viability of this platform for wide-scale usage [32]. This scaffold may be of utility in drug delivery programs as good correlation in vitro and in vivo results could be generated as compared to standard 2D format.

Ginzberg et al. explored the use of porous 3D scaffolds of alginate for the entrapment of retroviral vector-producing cells (VPC) for producing a local and sustained release of viral particles for the treatment of murine colon cancer (MC38 tumors) in vivo. The incorporated gene codes for the prodrug-activating enzyme, which produces local cytotoxicity. In this study, PA317/STK cells were used as VPC, which carries the gene for herpes simplex virus thymidine kinase (HSV-tk) and constantly produces a local release. HSV-tk monophosphorylates the prodrugs ganciclovir and N-methanocarbathymidine (N-MCT), which are further converted to triphosphate form by kinases. The triphosphate form produces DNA adducts and leads to cancer cell death. By using the static seeding method, VPC were seeded into porous 3D alginate scaffolds at two densities 0.5×10^6 and 1×10^6. The cell-seeded 3D system was thoroughly characterized for cell number, cell leakage, and spheroid morphology. The VPC were found to form spheroids after 1 day and high seeding

density maintained a considerable number of viable cells throughout the experimental period with spheroids in the size range 120–150 μm. The viral vector activity was conducted on MC-38 cells by using the vector-conditioned media. The results confirmed effective conversion to triphosphate form of N-MCT, thus indicating successful in vitro induction. To explore the in vivo potential of this novel gene therapy module, C7 mice bearing intraperitoneal MC-38 tumors were used. Intraperitoneal implantation of the alginate-based 3D scaffold device led to significant improvement of survival and effective antitumor effect when given along with ganciclovir up to 2 weeks. However, poor survival was noted after the 2-week period. The reported system was found to be better compared to other system owing to low immunogenicity and local effect by virtue of VPC encapsulation inside the 3D scaffolds, and the scaffolds were found to maintain structural integrity up to 27 days in vivo highlighting the therapeutic relevance in terms of local and sustained effects [33]. Thus, this study opened new avenues to explore similar systems for cancer gene therapy using alginate-based 3D scaffolds.

High-throughput screening (HTS) platform for anticancer drugs based on 3D cultures is the need of the hour. Various attempts have been made, and versatile HTS platforms have been proposed for cancer drug discovery. Wang et al. developed a MCTS based on chitosan/collagen/alginate (CCA) fibrous scaffold for anticancer drug screening. The CCA scaffolds were designed by spray-spinning technique. The physicochemical characterization of various components of fibers was carried out by Coomassie blue staining, X-ray diffraction (XRD), and Fourier transform-infrared spectroscopy (FTIR). The in vitro coculture indicated that MCF-7 cells showed a spatial growth pattern of multicellular tumor spheroid (MCTS) in the CCA fibrous scaffold with increased proliferation rate and drug resistance to mitomycin-C, adriamycin, and 5-azacytidine compared to traditional 2D culture. Figure 6.2 indicates the SEM architecture of CCA scaffold and the growing tumor spheroids within the scaffold. Measurement of cell viability was used as an indicator of apoptosis for the growth inhibition of cells within MCTS in the presence of various drugs. The results indicated significant increase in the number of live cells in the MCTS grown in CCA scaffolds. Thereafter, the assessment of metabolism of MCTS was carried out by glucose–lactate analysis, which again better correlated with in vivo conditions compared to 2D culture. In addition, MCTS showed the characteristic of epithelial mesenchymal transition (EMT). Interestingly, the result comparison with 2D culture showed significant differences in the levels of vimentin and E-cadherin as shown in Fig. 6.3 [34]. Thus, this novel scaffold may be useful for HTS screening of anticancer drugs by using MCTS.

Tumor relapse is a major concern in the emerging threat of cancer drug resistance and poses a significant challenge worldwide. There is a need to develop platforms where the behavior of dormant tumors may be studied to understand the molecular pathology and the associated complications. The tumors are generally poorly vascularized, and this leads to selection pressure of unfavorable metabolic environment on cancer cells growing in such areas within the tumor. This may lead to dormancy of cancer cells distant from blood vessels owing to poor oxygenation and low nutrient supply. With three-dimensional growth conditions, multicellular

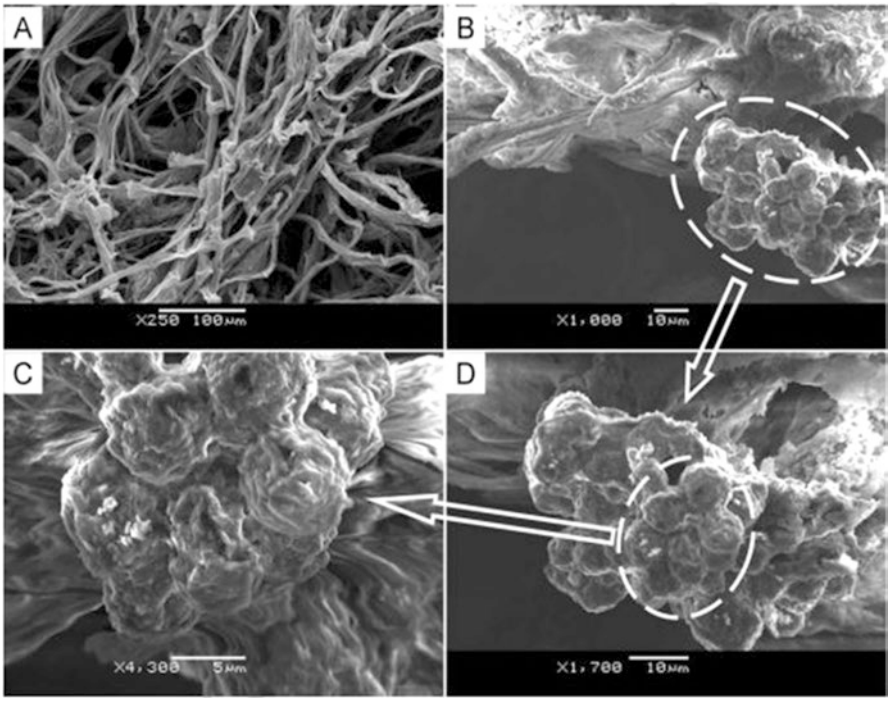

Fig. 6.2 (**a**) Chitosan/collagen/alginate (CCA) fibers under scanning electron microscope (SEM), (**b**–**d**) the cell mass 3 days after cell seeding. (**c**) and (**d**) are enlarged partial views of (**b**) (Reproduced with permission from Ref. [34])

tumor spheroids (MCTS) may replicate several parameters of the tumor microenvironment, including oxygen and nutrient gradients as well as the development of dormant tumor regions. Wenzel et al. reported the setup of a 3D MCTS assay on 384-well microtiter plates compatible for high-content screening system. The platform could exhibit the characteristics of real-time tumor with the study of inner core of tumor, which shows distinct respiration profile. The developed system was used to identify nine substances from two commercially available drug libraries that specifically target cells in inner MCTS core regions, while cells in outer MCTS regions or in 2D cell culture remain unaffected. They identified all hits as being inhibitors of the respiratory chain and further characterized their mode of action in MCTS as shown in Fig. 6.4. The cumulative data suggested that targeting cytostatic-resistant tumor cells in dormant tumor regions with respiratory chain inhibitors could be a therapeutic option that could enhance the effectiveness of cytostatic-based chemotherapy, and 3D MCTS may be a boon to screen such agents [35]. These findings could facilitate the establishment of secondary assays in more extensive screening programs for early hit classification for respiratory chain inhibition.

Microarray is a high-end technique where the genetic profile can be screened on high-throughput scale. Meli et al. developed an alginate-based 3D cellular microarray

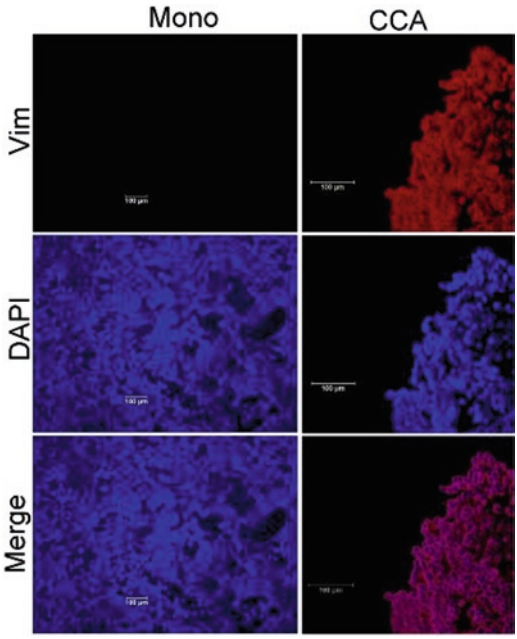

Fig. 6.3 MCF-7 cells on monolayer (Mono) or tumoroids on 3D chitosan/collagen/alginate (CCA) scaffolds immunostained with anti-vimentin (red) and DAPI (blue, for nuclear stain) (Reproduced with permission from Ref. [34])

platform for the high-throughput analysis of growth, cytotoxicity, and protein expression profile of a liver cancer cell line, HepG2. The results obtained were compared to 2D and 3D environments at the preliminary developmental scale. The antiproliferative effects of four anticancer drugs, tamoxifen, 5-fluorouracil, doxorubicin, and amitriptyline, were studied as a function of seeding density in the selected three platforms. The 3D-cultured grown liver cancer cells showed substantial resistance at high cell number seeding, whereas no seeding density dependence was observed in the IC_{50} values obtained in the 3D microarray culture platform. These results could be explained by higher confluency which limits cell growth by virtue of restricted cell–cell contact and nutrient supply. To explore the versatility of this platform, additional studies on biocompatible chips was carried out. The in-cell immunofluorescence (IF) assay provided quantitative data on the levels of specific target proteins involved in proliferation, adhesion, angiogenesis, and drug metabolism and was used to compare expression profiles between 2D and 3D environments. The upregulation of several CYP450 enzymes, β1-integrin, and vascular endothelial growth factor (VEGF) in the 3D microarray cultures suggested that this platform provides a more in vivo-like environment [36]. This approach thereby facilitates to better understand the variables that affect drug resistance such as hypoxia, cell quiescence, and cell adhesion. Moreover, the use of this platform for in-cell IF assays (on-chip) may aid in exploration of critical factors playing role in 3D-dependent cell behavior and signaling at a high-throughput scale.

As a new dimension of 3D culture, the role of 3D alginate gel beads was studied for the investigation of metastasis. In a study, the metastatic potential of two

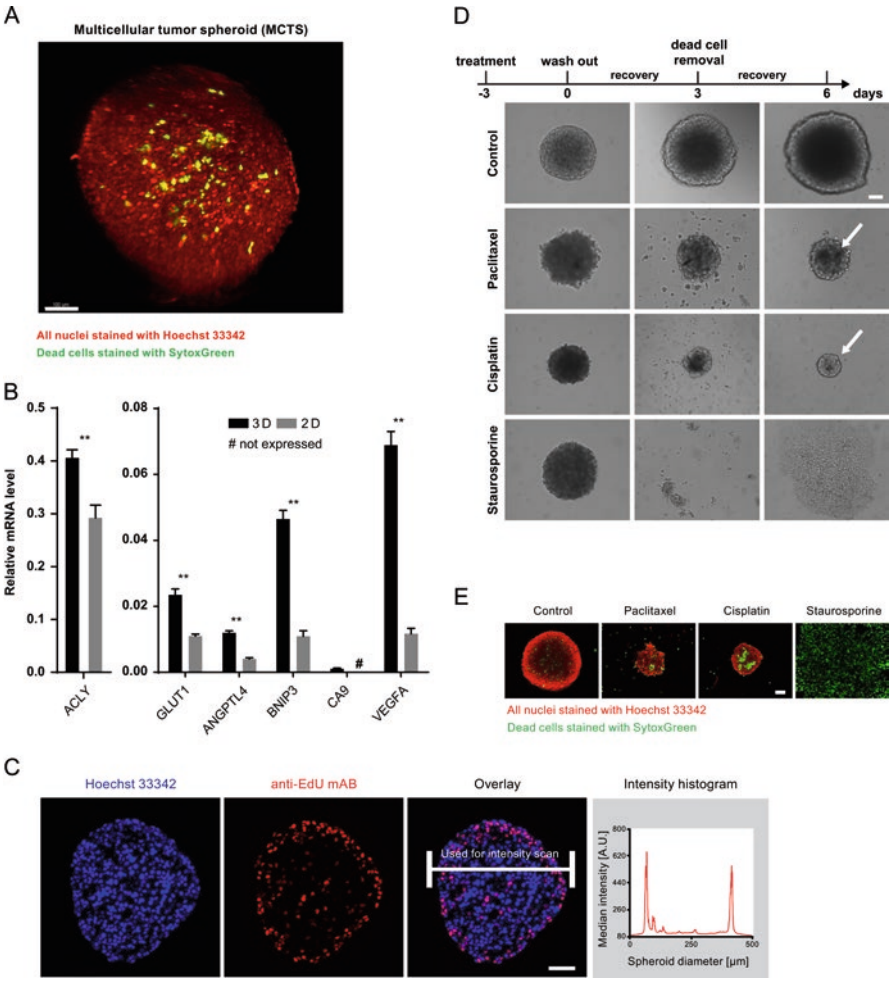

Fig. 6.4 *Multicellular tumor spheroids (MCTS) mimic several parameters of the tumor microenvironment*. (**a**) T47D breast cancer MCTS: 2D projection of a 3D image stack of 142 z-planes with 2.58 µm spacing showing the organization of T47D spheroids. Cell nuclei are stained with Hoechst (red) and dead cells are labeled with Sytox green (green). Scale bar, 100 lm. (**b**) RT-qPCR shows upregulation of hypoxia and low nutrition-responsive genes in MCTS compared to standard 2D cell culture conditions. Geometric mean of reference genes ACTB and RPLP13A was used for normalization of all RT-qPCR results. Bars show average of 3 biological replicates (+SD), $p < 0.01$. For full gene names, see material and methods. (**c**) Cryosections of an untreated spheroid cultured for 5 days, stained with Hoechst (blue) and incubated 18 h with 5-ethynyl-2′-deoxyuridine (EdU) probe (red). EdU, as a thymidine analogue, is incorporated into DNA in S-phase and indicates proliferating cells. Mainly outer cell layers of the MCTS show EdU incorporation (see histogram of EdU signal in MCTS cross section). Scale bar, 100 lm. (**d**) Cytostatics, paclitaxel (100 nM) and cisplatin (100 µM), mainly affect the outer proliferative layer in MCTS, which could be removed after 3 days of treatment and 3 days of recovery by pipetting. An inner cytostatic-resistant viable core could be isolated (arrows). The staurosporine (10 lm) cytotoxic control leads to complete disruption of the MCTS. Scale bar, 100 lm. (**e**) Sytox green staining shows that isolated cytostatic-resistant cores (see D) are viable. Scale bar, 100 µm (Reproduced with permission from Ref. [35])

hepatocarcinoma (HCC) cells (low (MHCC97L) and high (HCCLM3) metastatic potential) cultured within alginate gel (ALG) beads was investigated. In the culture system, HCC cells formed nice spheroids by proliferation within the 3D alginate gel beads. The gene and protein expression of metastasis-related molecules was increased in ALG bead-grown cells, compared with 2D culture. In addition, several gene expression levels in ALG bead culture system were found closer to liver cancer tissues (MMP-2, MMP-9, E-cadherin, N-cadherin, and vimentin) showing better mimicry of in vivo environment. Interestingly, the results of in vitro invasion assay indicated that the cells derived from ALG beads had 7.8-fold higher invasion potential than adhesion cells. Another important feature of 3D matrices is the stiffness that they impart which serves to improve the in vivo solid tumorlike stiffness effect. These results indicated that the in vitro 3D model based on ALG beads increased metastatic ability compared with adhesion culture and mimicked the in vivo liver tumor tissues [37]. The study speculated that owing to the controllable preparation conditions, stable uniformity, and production at large scale, the 3D ALG bead model may become a handy tool for HTS of anti-metastasis drugs and the metastatic dynamics research programs.

To overcome the limitations of animal-based experiments, 3D culture models mimicking the tumor microenvironment in vivo are gaining attention. In one of the studies, an alginate-based 3D scaffold was used for screening of 5-fluorouracil (5-FU) and/or curcumin on malignancy of colorectal cancer (CRC) cells. CRC cells were encapsulated in alginate. The CRC cells were able to proliferate in 3D environment in an in vivo-like fashion. The cells showed impressive invasion potential after encapsulation inside alginate scaffold. During cultivation of cells in alginate, cells were isolated in three stages for further characterization: (1) alginate-proliferating, (2) invasive, and (3) adherent cells. Tumor-promoting factors (CXCR4, MMP-9, NF-κB) were significantly increased in the proliferating and invasive compared to the adherent cells, suggesting an increase in malignancy behavior of CRC cells grown inside alginate microenvironment. Inside the alginate-based scaffold, curcumin could substantially enhance the potency of 5-FU. IC_{50} for HCT116 to 5-FU was 8 nM, but co-treatment with 5 μM curcumin significantly reduced 5-FU concentrations in HCT116 and HCT116R cells, and these effects were accompanied by downregulation of NF-κB activation and NF-κB-regulated gene products [38]. These results indicated that such 3D systems can improve the quality of in vitro drug screening, animal-free clinical treatment, and investigation of the initial steps of spontaneous carcinogenesis and metastasis. In a similar study, the effect of resveratrol in parental CRC cell lines (HCT116, SW480) and their corresponding isogenic 5-FU-chemoresistant derived clones (HCT116R, SW480R) was examined by MTT assays, intercellular junction formation and apoptosis by electron microscopy, NF-κB, and NF-κB-regulated gene products by Western blot analysis in a 3D-alginate microenvironment. Resveratrol blocked the proliferation of all four CRC cell lines. Resveratrol was found to increase the invasion inhibitory effects of 5-FU. In addition, resveratrol significantly attenuated drug resistance through inhibition of epithelial–mesenchymal transition (EMT) factors (decreased vimentin and slug, increased E-cadherin) and downregulation of NF-κB activation and its translocation

to the nucleus and abolished NF-κB-regulated gene end-products (MMP-9 and caspase-3) [39]. In continuation of the same of work, alginate beads were used to further explore the molecular mechanism of resveratrol in CRC. Role of Sirt1 was postulated behind the protection against CRC cells. Resveratrol suppressed proliferation and invasion of two different human CRC cells in a dose-dependent manner, with a significant decrease in Ki-67 expression, a critical marker of cellular proliferation. By transient transfection of CRC cells with Sirt1-antisense oligonucleotides (ASO), the anticancer effects of resveratrol were suppressed, indicating the vital role of ASO. Sirt1 was found to interact with NF-κB, and resveratrol could not inhibit Sirt1-ASO-induced NF-κB phosphorylation, acetylation, and NF-κB-regulated gene products [40]. Although a detailed mechanistic insight was presented, however, a lack of 2D comparison was a concern. Comparison with 2D culture helps in better understanding of tumor microenvironment behavior and must be considered while experimental design. However, these reports suggest that 3D cell culture models are ready to take the challenge of routine cell culture for elucidation of molecular mechanisms. The higher correlation to in vivo tumor microenvironment can be a benefit for drug discovery programs owing to the fact that, in case of traditional 2D culture, a large number of NCEs may show good activity in vitro but often fail in vivo, whereas use of 3D cell culture platforms may substantially relieve this discrepancy.

Combinatorial drug delivery is an attractive but challenging requirement of next generation cancer nanomedicines. A transferrin-targeted core-shell nano-delivery platform formed by encapsulating doxorubicin and sorafenib against liver cancer was investigated by Malarvirzhi et al. Doxorubicin was loaded in poly(vinyl alcohol) nano-core and sorafenib in albumin nano-shell, formed by a sequential freeze-thaw/coacervation method. Sorafenib inhibited oncogenic signaling involved in cell proliferation, and doxorubicin enhanced DNA damage, thus showing enhanced anticancer activity. Interestingly, the cytotoxicity assay performed in the 3D spheroids displayed a relatively limited cell death compared to that of the 2D culture showing the tissue-like physiological properties of 3D microenvironment. Studies using 3D spheroids of liver tumor indicated efficient penetration of targeted core-shell nanoparticles throughout the tissue causing effective cell death [41]. Thus, this study presented the utility of 3D alginate scaffold for novel drug delivery system screening.

Lan et al. developed an alginate hydrogel-based platform for HTS of anticancer agents. The cellular viability and metabolic capacity of the encapsulated cells in two different alginate structures SLM100 (G:M::40:60) and SLG100 (G:M::60:40) were studied. The results indicate that cells encapsulated within SLM100 and SLG100 class of alginates showed high cellular viability with >80% even after 14 days in culture. The proliferation rates of the encapsulated cells were steady. Production of liver-specific enzymes such as CYP1A1 and CYP3A4 after 14 days in culture indicated the viability and metabolic dynamics of the HepG2 cells. The encapsulated cells within the 3D gels were able to metabolize the pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin) to HFC (7-hydroxy-4-trifluoromethyl) in a time-dependent manner [42]. Thus, the model was found to be successful in deciphering the metabolizing profile of liver cancer cells. These results indicated promising findings and may aid in future improvements in material and design configurations for optimal pharmacokinetic response of in vitro tissue model systems.

6.4.2 *Alginate-Based Scaffolds for Studying Cancer Stem Cell (CSC) Biology*

Cancer stem cells (CSCs) have emerged as an important factor responsible for clinical failure of various chemotherapeutic agents. A large number of reports indicate the unprecedented role of CSCs behind the emergence of chemoresistance and associated complications with anticancer therapy in humans. There is an advent need of systems where CSCs can be isolated and cultured and concentrated to study the molecular pharmacodynamics. Study of CSCs is another dynamics of 3D cell culture and offers unique features of concentration of CSCs from a cancer cell population by inducing selection pressure. Therefore, simple, reliable, reproducible, and authentic 3D culture platforms are needed to accelerate the CSC research.

Various studies indicate the successful utility of alginate-based 3D culture for development of CSCs spheroids. In one report, human osteosarcoma cells were isolated from patients, made into single-cell suspension, and were grown on alginate gel for producing 3D microenvironment. Epirubicin was used to enrich the CSCs by killing the general cancer cell population. The study revealed that the enrichment of CSC under selection pressure and single-cell cloning spheres was obtained with the features of CSCs after 7–10 days. Confirmation of molecular features of CSCs was done by carrying immunofluorescent staining for Oct3/4 and Nanog, markers for self-renewal and multipotential differentiation of CSCs. The spheres were found to contain positive cells for both the markers indicating the development of enriched CSCs with 3D culture. Moreover, the expression was found to be more in the core compared to the periphery indicating the formation of CSCs niche. The in vivo tumorigenicity study also showed similar pattern with successful development of tumors 1 week after the inoculation. Similar characteristics of CSCs were obtained upon immunohistochemical analysis of Oct3/4 and Nanog. This study shows the importance of 3D culture for selective growth of CSCs with in vivo reproducibility [43]. Therefore, study of CSCs by 3D culture provides better correlation in vivo and needs to be further explored in this area.

The conventional methods of CSC enrichment take about 7–10 days with frequent change in media and growth factors. Cellular encapsulation inside biocompatible microcapsules is an attractive option for 3D cell culture of CSCs. Maintenance of stemness is one of the important criteria for fruitful results with 3D cell culture. Miniaturized 3D liquid core of core-shell microcapsules (CSMCs) with a hydrogel shell has been shown to significantly better maintain the pluripotency of the pluripotent stem cells compared to traditional culture [44]. In an attempt to reduce the time taken for CSC enrichment, Rao et al. developed a miniature 3D liquid core of microcapsules using alginate hydrogel, a strategy for concentration of CSCs from PC-3 prostate carcinoma cells. The results were compared with ultralow attachment plate (ULAP)-based enrichment method for CSCs. There was a significant difference in the number of prostaspheres formed by CSMCs compared to ULAP culture. Encapsulation of 40 cells per microcapsules gave the highest number of prostaspheres with larger sizes compared to ULAP. Evaluation of CSCs surface markers

(CD44 and CD133) showed the superiority of prostaspheres obtained with CSMCs with better results with prostaspheres obtained on day 2. The gene expression studies also revealed significant differences in the expression of Oct 4, Nanog, and Klf4 with day 2 prostaspheres obtained with CSMCs. In vivo xenograft studies also showed superiority of day 2 prostaspheres obtained by CSMCs compared to ULAP and parent PC3 cell inoculation as the tumors obtained by inoculation of 3000 prostaspheres from CSMCs were significantly compared to both the groups. In conclusion, this study revealed better maintenance of stemness with the proposed CSMC-based method of CSCs enrichment (due to autocrine signaling within the microcapsule) and offers unique advantages over the conventional method of CSCs enrichment by ULAP as the former is time-consuming (7–10 days) and very costly (almost ten times the cost of normal plates). Thus, the miniaturized system based on this approach may aid in CSC biology studies and may help in the elimination of CSCs, the major culprit behind chemoresistance, recurrence, and metastasis [45]. In an independent study, Xu et al. developed alginate gel bead-based 3D cell culture platform for the enrichment of CSCs from human liver (HCCLM3) and human head and neck squamous cell carcinoma cells (TCA8113). The results were compared with 2D monolayer and showed some interesting findings. The CSCs obtained with 3D alginate beads were found to possess higher chemoresistance and tumorigenicity compared to 2D culture. Both the tumor cell line-derived CSCs could thrive well in alginate beads. The findings suggested suitability of alginate beads for expanding and enriching CSCs of different histological origin and was claimed to be superior to other inventions where different cell lines of same histological origin were studied. The markers for CSCs characteristics (Oct3/4, Nanog, Notch-1, Smoothened, CD44, EpCAM, CD133, and ABCG2) were significantly modulated in both types of 3D-cultured CSCs compared to respective 2D monolayer system indicating the viability of alginate bead-based system for enrichment of CSCs and superior platform for the investigation of growth and biophysical factors like matrix stiffness (Fig. 6.5). Higher chemoresistance, metastatic potential, and tumorigenicity were observed in both the types of 3D cultured CSCs. Immunostaining for von Willebrand factor (vWF) showed the presence of this endothelial surface marker in the xenografts as shown in Fig. 6.6. Furthermore, significantly increased levels of hypoxia-inducing factors (HIF1A and HIF2A) were observed in CSCs obtained in 3D-cultured cells compared to respective 2D-cultured CSCs (grown under normal and low oxygen conditions) which might be the reason for the higher levels of CSCs marker genes Nanog and Oct3/4 in 3D-cultured CSCs [25]. Collectively, these results indicate that alginate hydrogel beads might be a better matrix compared to synthetic (polyethyleneglycoldiacrylate) and natural polymers (agarose, collagen, hyaluronic acid, etc.)-derived hydrogels by virtue of inertness, ease of dissolution and reconstitution, and high mass transfer rate which aids in better cell viability. However, despite the advantages, more research is warranted to reliably use these scaffolds for understanding CSCs biology.

Kievit et al. reported the use of CA scaffold for proliferation and enrichment of $CD133^+$ glioblastoma stem cells (U-87 MG and U-118 MG cells) for understanding CSC biology. The SEM imaging and immunostaining confirmed the selective

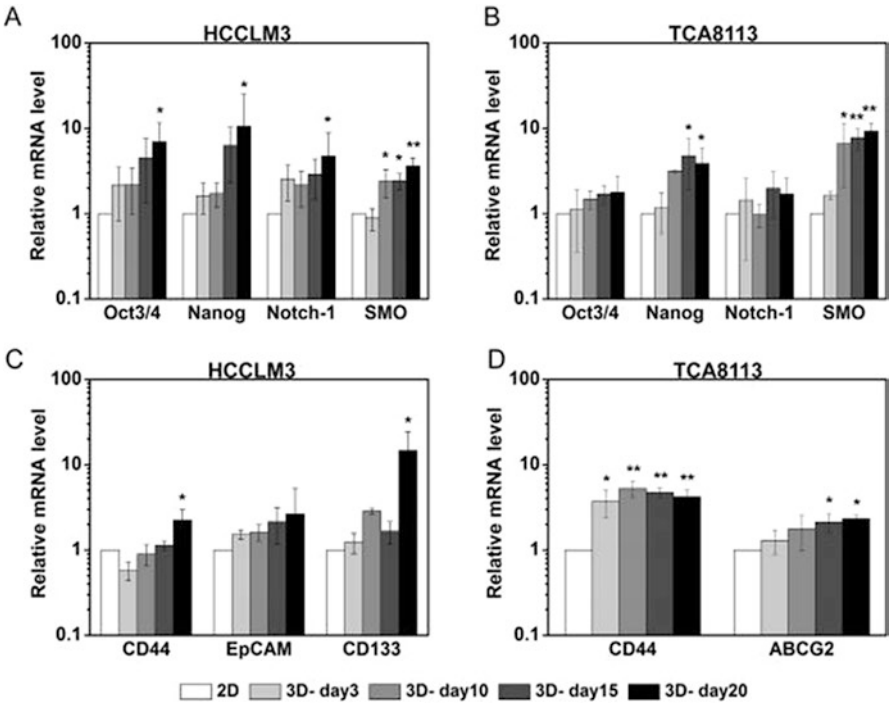

Fig. 6.5 *Gene expression of CSC-related markers in HCCLM3 cells* (**a**, **c**) and TCA8113 cells (**b**, **d**). There are three groups of genes: Oct3/4, Nanog as self-renew-related genes; Notch-1, SMO as stemness-related genes; CD44, EpCAM, CD133, and ABCG2 as CSC marker genes. β-Actin mRNA was utilized as an internal control. $*P < 0.05$ and $**P < 0.001$, compared with 2D cells (Reproduced with permission from Ref. [25])

enrichment as shown in Fig. 6.7. The expression of stemness markers (Nestin, Frizzled 4, GLI, HES, Snail, and Notch) were significantly modulated with cells grown on CA scaffolds compared to 2D culture. There was selective enrichment of $CD133^+$ CSCs by CA scaffolds as polycaprolactone-coated CA scaffolds could not enrich $CD133^+$ CSCs nor were able to promote spheroid formation, showing the uniqueness of invention. The expression of CD44 was found to be upregulated in CA scaffold cultured cells which might be the reason behind downstream promotion of $CD133^+$ enriched cells. As expected, the in vivo tumorigenicity results also indicated superiority of CA scaffold in xenografts formation [46]. These results indicated differential behavior of CSCs growth on different matrices as the surface chemistry could modify the subsequent enrichment of CSCs. Therefore, due consideration of matrix microenvironment is needed before designing scaffolds for CSC-related studies as it may badly hamper the outcomes [47].

In a recent study Dai et al. proposed a novel modified porous gelatin/alginate/fibrinogen hydrogel to mimic ECM and established a 3D bioprinted glioma stem cell model. The 3D-printed glioma CSC cell lines (U87 and SU3) could grow well in the

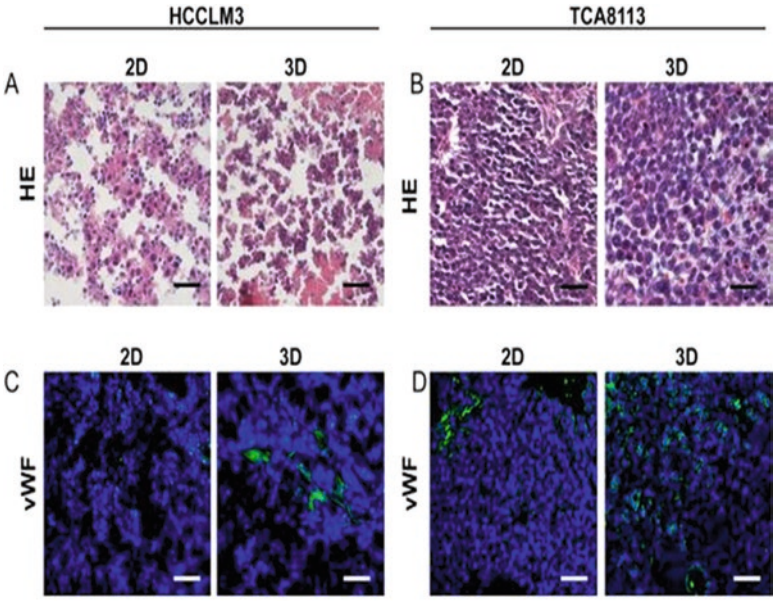

Fig. 6.6 *Morphology and von Willebrand factor (vWF) protein expression of xenografts formed by 2D- and 3D-cultured cells*. HE-stained histology slides of HCCLM3 cells (**a**) and TCA8113 cells (**b**) forming xenografts were morphologically similar to the HCC and HNSCC in vivo. Xenograft sections formed by HCCLM3 (**c**) and TCA8113 (**d**) cells in both 2D monolayer and 3D ALG beads were stained with anti-vWF antibody (green). Nuclei were stained with Hoechst 33342 (blue). Scale bar 50 μm (Reproduced with permission from Ref. [25])

bioprinted scaffolds. The CSC marker Nestin was also found to be upregulated along with enhanced levels of VEGF, a crucial angiogenesis promoter. The 3D-printed CSCs showed higher chemoresistance to temozolamide compared to 2D model. The bioprinting of cells for 3D culture may offer unique advantages like predesigned product configuration, flexible size of hydrogel filament, printing of several cell types simultaneously, and multilevel biomolecular gradient formation [48]. However, 3D bioprinting is a relatively new dimension of the well-known field of 3D bioprinting and addressal of various questions associated with tumor microenvironment needs to be thoroughly evaluated before this technique may be widely used.

Growing research evidence indicates that the CSCs also behave similarly to normal stem cells in terms of the microenvironment niche. Various systems were used to mimic the CSC niche to promote their growth and proliferation. A recent study with porous, tunable alginate hydrogels could mimic the CSC niche and promoted enrichment of CSC of mouse 4T1 breast cancer model. Roles of different parameters including mechanical properties of polymer, cytokine immobilization, and the composition of ECM on CSC niche were investigated for their role on the proliferation. Interestingly, modulation of matrix stiffness (1%-190 Pa, 1.1%-210 Pa, 1.2%-270 Pa, 1.3%-710 Pa, 1.4%-950 Pa, 1.5%-1070 Pa, and 1.6%-4700 Pa) led to significant variation in the size of tumor spheroids, and continuous growth for longer

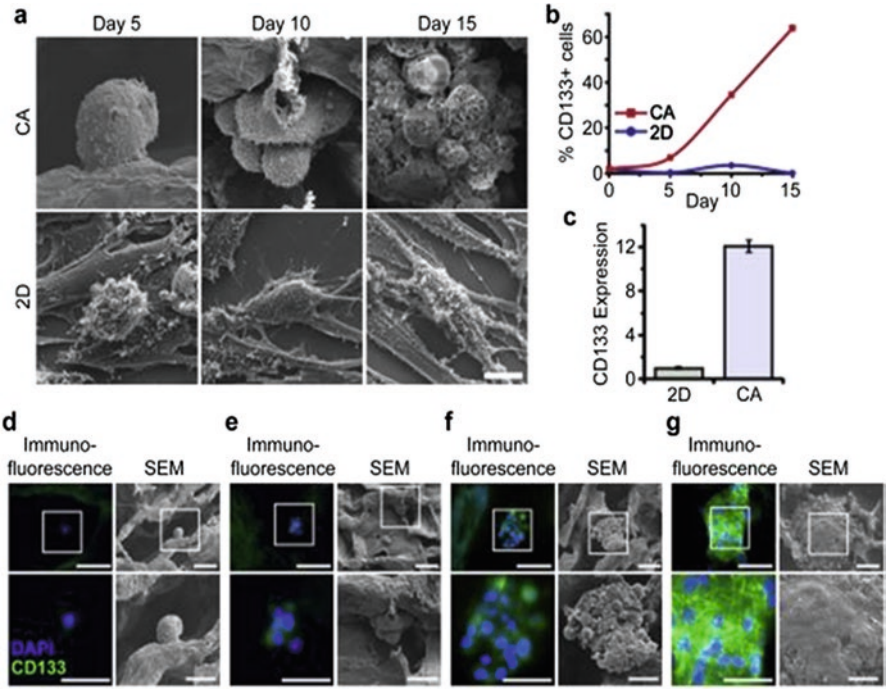

Fig. 6.7 *Growth of CD133⁺ GBM cells on CA scaffolds*. (**a**) SEM images comparing the morphology and proliferation of human U-118 MG GBM cells cultured on 3D CA scaffolds and 2D monolayers over 15 days. Scale bar corresponds to 10 mm. (**b**) Comparison of the change in fraction of CD133⁺ cells in U-118 MG cell population grown for 15 days on CA scaffolds or as monolayers. Immunopositivity for CD133 was determined by flow cytometry. (**c**) CD133 mRNA content determined by real-time PCR in U-87 MG GBM cells grown for 10 days on CA scaffolds compared to that of monolayer cultures. CD133 mRNA content was normalized to the monolayer condition. deg) Immunostaining for CD133 (green) and SEM imaging of CA-scaffold cultured U-118 MG GBM cells at day 10. The boxed regions in the top-row images correspond to the areas of the bottom images. Blue color reflects DAPI counterstaining of nuclei. (**d**) Solitary U-118 MG cells generally showed no CD133 staining. (**e**) Small clusters of U-118 MG cells showed faint CD133 staining. (**f** and **g**) Intensity of CD133 staining increases as clusters of U-118 MG cells grow larger. Scale bars for panels d-g correspond to 50 μm for the upper row and 25 μm for the lower row (Reproduced with permission from Ref. [46])

duration led to decrease in the spheroid colony numbers. Hydrogels with 950 Pa (1.4% alginate) elasticity were found to promote the number of colonies, and 27±3 colonies were formed from day 3 to 7 (Fig. 6.8). Cytokines are essential for continuous maintenance of CSC culture, and immobilization of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) was carried out on alginate hydrogels to support CSC proliferation and to produce a conducive niche for CSC enrichment. The results showed better growth characteristics of spheroids grown on immobilized cytokine hydrogels compared to control (no cytokines) and the solution group (cytokines added to the culture media). Further, the addition of low

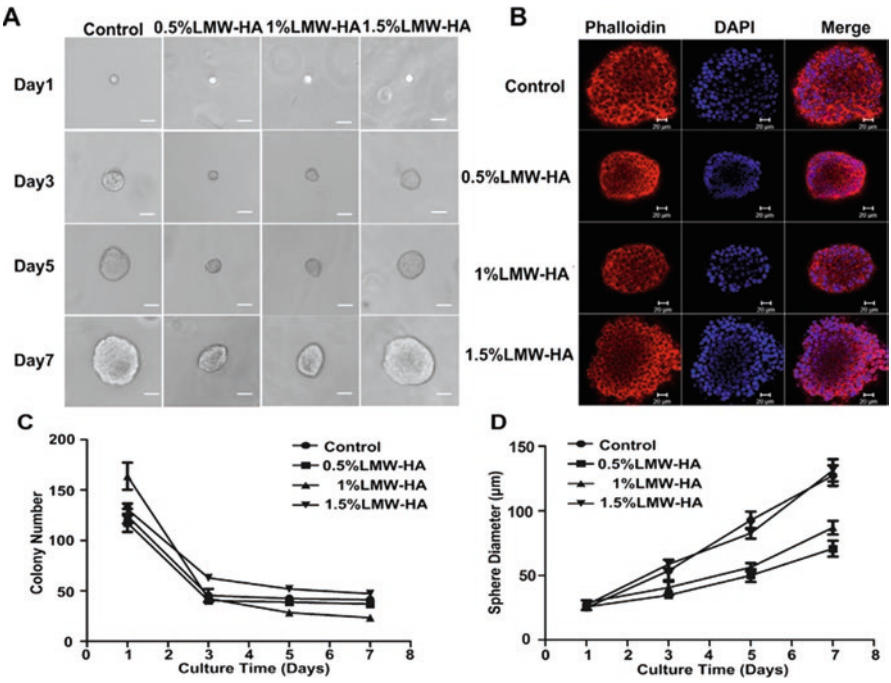

Fig. 6.8 *Tumor sphere formation in an alginate-based hydrogel with different degrees of stiffness*. (**a**) A single 4T1 breast cancer cell grew into tumor spheres in alginate hydrogels of different stiffness levels throughout the course of culture from day 1 to day 7. Scale bar: 50 lm. (**b**) The observation of the cytoskeleton of the multicellular 4T1 tumor spheroid after 7 days in culture in an alginate hydrogel with different levels of stiffness; the multicellular 4T1 tumor spheroid was released from the hydrogel and was stained with phalloidin for the cytoskeleton (red) and DAPI for the nucleus (blue); it was imaged with an inverted fluorescent microscope. Scale bar: 20 lm. (**c**) Tumor sphere (round colony) number as a function of culture time: day 1 to day 7. The 950 Pa alginate hydrogel seems to be optimal for sustaining the spheroid colony number. Mean ± SD; $n = 3$ (for the 190 Pa, 270 Pa, 950 Pa, and 4700 Pa alginate hydrogels); independent experiments. (**d**) Colony size of the tumor spheres as a function of culture time and hydrogel stiffness. Apparently, the 950 Pa hydrogel best promotes tumor growth. Mean ± SD; $n = 3$ (for 190 Pa, 270 Pa, 950 Pa, and 4700 Pa alginate hydrogels); independent experiments (Reproduced with permission from Ref. [49])

molecular weight hyaluronic acid (HA) to alginate hydrogel was found to promote the spheroid number. However, there were no significant changes in the spheroid size, whereas use of high molecular weight HA was found detrimental to growth of CSCs. The alginate scaffold enriched the 4T1 CSCs compared to 2D cultured cells as revealed by the gene expression of Nestin, Tert, Nanog, and SOX2. Immunostaining for CD44, CD24, MDR1, and Dclk1 also suggested superiority of alginate hydrogels. Moreover, the tumorigenicity study in Balb/c mice also revealed superiority of the proposed hydrogels [49]. Thus, this unique alginate hydrogel-based platform may provide a means to study the CSCs biology from preclinical and patient-derived samples. However, to harness the real potential of such CSC-based platforms,

detailed studies in different lab experiments other than proof of concept assays should be carried out to furnish robust, reliable, and reproducible results.

6.5 Advantages of Alginate-Based 3D Models Compared to Other 3D Models

Alginate is a unique biocompatible natural polymer of non-animal origin with structural similarity to extracellular matrix. The traditionally used biocompatible scaffolds are largely based on polymers of animal origin like collagen, hyaluronic acid, and gelatin. The use of such scaffolds for 3D culture leads to significant bioactivity and may hamper the outcomes, whereas alginate is an inert biomaterial with negligible bioactivity, thus making it advantageous over the use of traditional animal-derived biopolymers. Moreover, another unique advantage of alginate scaffolds is their ability to be cross-linked with different peptides and other chemical entities with desired matrix design and activity. This may help in synthesizing tailor-made microenvironment for cell lines of interest. Immobilizing cells in alginate hydrogels is a mild process wherein the physiological conditions are maintained. Another advantage is that the dissolution of alginate scaffolds and hydrogels can be carried out relatively easily which serves for hurdle-free separation of tumor spheroids for further processing. In addition, alginate is a relatively cheaper biomaterial compared to the polymers of animal origin.

6.6 Future Perspectives

Cell culture is an important tool for understanding the physiological behavior of cell pharmacodynamics. Primary cell culture and secondary cell culture serve to aid in the basic and molecular pathology for direct and indirect clinical applications. Three-dimensional cultures have significantly contributed in the overall progress of cell culture techniques for drug discovery and development. Controlling the matrix for better simulation of in vivo microenvironment is of utmost importance and needs due consideration during designing of 3D systems. Although 3D cell culture models have a plethora of applications, however, there are still certain areas where further improvement is desired. Understanding of 3D complexity of tumor must be considered before selection and designing of such scaffolds as matrix interactions in terms of the elasticity and reactivity may badly hamper the outcomes of 3D cell culture. Optimization of 3D scaffold by using high-end techniques of 3D bioprinting may advance the field and may help in growing multiple cell lines on a single bioprinted sheet. Another area of improvement includes the development of 3D friendly imaging techniques. The traditional 3D cultured cells are difficult to be imaged with the regularly used inverted microscope as the density of tumor spheroid may not be

optimally reached. The periphery and the core of tumor spheroids contain different regions of metabolism and bioactivity as the distance from nutrient supply dictates the formation of hypoxia and the subsequent necrotic lesion formation. Another dimension of 3D culture is the growth and proliferation of CSCs using special matrices. Understanding the biology of CSCs requires an ideal matrix that can mimic the tumor stroma interactions and at the same time may aid the selective proliferation of CSC population. Last, but not the least, overcoming the economical barrier to translate the wide-scale use of 3D scaffolds is of urgent need. Thus far, the techniques used to synthesize 3D matrices are largely confined to tissue engineering and biomaterial laboratories. To promote the utility of 3D scaffolds for cancer drug discovery, one needs to establish simple, robust, reproducible, economical, and commercially viable products. The 3D scaffolds available in market are relatively costly and out of reach of developing countries.

6.7 Summary

Three-dimensional cell cultures offer a versatile platform for different needs during various phases of drug discovery and development. In this chapter, we summarized a brief introduction of 3D cancer models and the advantages of 3D models over 2D cell culture models. Studies confirm regarding better in vivo correlation of results obtained with 3D cell cultures owing to better similarity to the in vivo 3D matrix. Alginate is a versatile biomaterial with novel advantages like inertness, animal-free product, no intrinsic bioactivity, and feasible chemical modifications for tailored applications. AlgiMatrix™-based 3D scaffolds are discussed with unique feature of matrix dissolution for further processing of the grown spheroids. A significant number of studies have been carried out with alginate-based matrices like 3D scaffolds, alginate beads, alginate hydrogels, alginate–collagen complexes, and chitosan–alginate–gelatin mixed scaffolds. These 3D scaffolds have been used for wide applications ranging from anticancer HTS drug screening, for understanding drug molecular mechanisms, molecular pathology of various cancers, enrichment of cancer stem cells, 3D bioprinting, and so on. In conclusion, alginate-based biomaterials can contribute significantly to expand the horizon of 3D culture and may serve to cut down the cost of commercial 3D anticancer screening platforms for wide-scale application of 3D culture models in cancer research programs.

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Chapter 7
Alginate Application for Heart and Cardiovascular Diseases

Zhengfan Xu and Mai T. Lam

Abstract Alginate biomaterial has been extensively investigated and used for many biomedical applications due to its biocompatibility, low toxicity, relatively low cost, and ease of use. Its use toward cardiovascular application is no exception. Alginate is approved by the Food and Drug Administration (FDA) for various medical applications, such as a thickening, gel forming, and as a stabilizing agent for dental impression materials, wound dressings, and more. In this chapter, we describe the versatile biomedical applications of alginate, from its use as supporting extracellular matrices (ECM) in patients after acute myocardial infarction (MI), to its employment as a vehicle for stem cell delivery, to controlled delivery of multiple combinations of bioactive molecules. We also cover the application of alginate in creating solutions for treatment of other cardiovascular diseases by capitalizing on the natural properties of alginate to improve creation of heart valves, blood vessels, and drug and stem cell delivery vehicles.

Keywords Alginate hydrogel • Extracellular matrix • Cardiovascular • Heart valves • Drug delivery • Stem cell delivery

7.1 Introduction

Alginate is a naturally occurring anionic polymer obtained from brown seaweed and has gained popularity in a wide range of biomedical applications. Alginate has many beneficial properties conducive to cardiovascular applications due to its relatively low reactivity in vivo, low cost, non-thrombogenic nature, mild gelation process, and structural resemblance to the ECM [1–4]. Two injectable alginate implants have already reached clinical investigation phase, demonstrating the promising potential of alginate-based treatments for myocardial repair and regeneration [4–8].

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The more recent evolution in cardiac application of alginate has now led to new standards in biomaterial application in the cardiac system to not only "fill the gap" in the injured area, but to act as an interface with the cardiac biological systems as well [9]. Such applications focus on four major areas: (1) using alginate hydrogels as an ECM substitute in heart tissues to promote tissue regeneration due to the structural similarity between alginate and natural heart ECM, (2) using alginate hydrogels as a delivery vehicle for cardiac stem cells or adult cardiomyocytes to the injury site to facilitate regeneration of functional heart tissue, (3) using alginate as a platform for sustained delivery of growth factors to mimic natural physiology, and (4) using alginate gels to control drug release. As a drug release vehicle, an alginate-based system can be fine-tuned to control the speed of cardiac medicine release (such as with antihypertensive or antiarrhythmia medications) by controlling the cross-linker type and the cross-linking method.

In this chapter, we present an up-to-date view of alginate-based applications in the treatment of cardiovascular diseases, with emphasis on cardiac tissue engineering, myocardial regeneration, as well as their translational status and clinical advancement.

7.2 Alginate in Treating Myocardial Infarction

7.2.1 Myocardial Infarction: Inflammation, Wound Healing, and Remodeling of the Left Ventricle

To understand the role of alginate in treatments of myocardial infarction (MI), one must first understand the underlying mechanics of the disease state. Myocardial infarction occurs when the supply of oxygen and nutrients to the cardiac muscle is impaired due to blocked coronary arteries. This process generally occurs in the left ventricle (LV). Thirty minutes following cutoff of blood perfusion, irreversible injury to the heart tissue takes place, and the cardiac muscle will start undergoing necrosis [10, 89]. The inflammatory response starts as early as the first day after MI, evidenced by infiltration of neutrophils [10, 89]. Phagocytosis starts around day 3 and reaches a maximum around day 7. Around day 10, myofibroblasts migrate into the wound, and granulation tissue starts to appear. After several weeks, the wound site is composed of a solid scar with a stable collagen structure, little overall cellularity, and few myofibroblasts remaining. Unfortunately, infarcted hearts usually undergo adverse remodeling, resulting in enlargement of the ventricular chamber and heart wall thinning, which leads to decreased functional capacity as evidenced by ejection fraction or fractional shortening (relative functional measures of the amount of blood pumped during each cycle), heart failure, and poor prognosis. Poor cardiac performance following MI is also attributed to replacement of the original contractile cardiomyocytes by fibrotic collagen deposition, severely altering mechanics of the heart and thus critically diminishing cardiac function [10, 11].

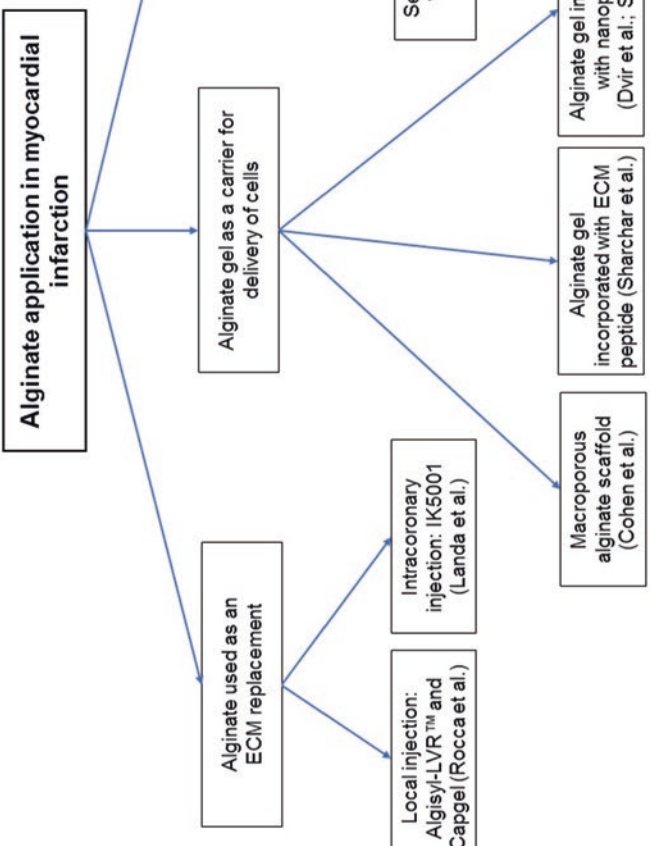

Fig. 7.1 Summary of alginate application in myocardial infarction. Alginate has been used as an extracellular matrix (ECM) replacement, for cell delivery and as a growth factor delivery vehicle

Figure 7.1 diagrams the applications of alginate in the treatment of myocardial infarction, and information is presented in detail in the following sections.

7.2.2 *Injectable Alginate Hydrogel for Replacing Damaged ECM*

In exploring options to regenerate myocardial tissue, alginate has been introduced into various tissue engineering strategies including alginate-assisted cell transplantation to improve retention and function, acellular strategies to confer mechanical support, extracellular matrix replacement, and drug delivery platforms to capitalize on alginate's ability to biodegrade [12–14].

Cardiac ECM plays a critical role in the maintenance, integrity, and function of heart tissue. In the process of MI, the ECM is excessively damaged. Changes in cardiac ECM composition will lead to (1) an increase in LV wall stress and extensive remodeling of the heart which results in detrimental effects on both systolic and diastolic function [15–17] and (2) an adverse environment which hinders cardiac stem cell regeneration through loss of physical support to the cells, loss of microvasculature, and cell damage.

In 2006, Zhang et al. confirmed that alginate-based hydrogel as an ECM substitute can promote cell survival of transplanted neonatal cardiomyocytes in a rat model of acute MI [90]. The left coronary arteries of female Sprague Dawley (SD) rats were ligated to create MI. Ventricular cardiomyocytes from 1- to 3-day-old SD rats were isolated, cultured, and fluorescently labeled. Three weeks after MI, the animals were randomized into four groups: (1) cells plus matrix (n = 12), (2) cells only (n = 12), (3) matrix only (n = 12), and (4) a control group in which neither cell nor matrix was delivered (n = 11). Four weeks after transplantation, echocardiography and the Langendorff model were used to assess heart function. Immunohistochemical (IHC) staining and polymerase chain reaction (PCR) showed that transplanted cardiomyocytes survived, formed condensed tissue, and produced connected protein in the cell plus matrix group. Heart function assessment indicated transplantation of cardiomyocytes plus matrix preserved left ventricle wall thickness, fraction shortening, and end-systolic internal diameter most effectively.

Promising studies such as that described above led to further studies through which alginate-based hydrogels were designed to mimic cardiac ECM properties to recapitulate chemical, mechanical, and morphological characteristics. Alginate can provide temporary tissue support as the heart heals, preserving cardiac function and facilitating myocardial repair [9]. The achievements of the last decade provide solid evidence confirming the efficacy of biomaterial injection into myocardial tissue for reconstruction and cardiac function preservation [18–20]. In these studies, alginate-based biopolymer injection led to increased scar thickness, infarct zone stabilization, and increased physical support to the healing of the left ventricle. By serving as a substitute for the damaged ECM, wall stress was reduced and LV dilatation prevented.

LV dilatation is enlargement of the LV wall, creating a stretched and thin heart wall. Decreasing the degree of LV dilatation is of utmost importance, as it is considered to be one of the most important predictors of mortality in patients with MI.

7.2.2.1 Alginate Hydrogel for Treating MI and Congestive Heart Failure Patients

In a study by Yu et al., injections of fibrin or alginate gel were made directly into infarcted myocardium 5 weeks after induced infarction in rats [21]. Echocardiographic results at 5 weeks after injection of alginate demonstrated persistent improvement of left ventricular fractional shortening and prevention of continued enlargement of left ventricular dimensions indicating dilatation, whereas a comparison of fibrin glue demonstrated no progression of left ventricular negative remodeling. Following that study, the same research group conducted in vitro cell culture and in vivo rat studies to evaluate efficacy of RGD peptide-conjugated alginate hydrogel [22]. RGD peptide-conjugated alginate improved human umbilical vein endothelial cell (HUVEC) proliferation and adhesion in culture. Injection of the alginate hydrogel into the infarct area of rats 5 weeks post-MI demonstrated that while alginate hydrogel could reshape a dilated aneurysmal LV and lead to improved LV function, a RGD modified alginate enhanced angiogenesis. Both modified and non-modified alginate improved heart function, while LV function in the control group deteriorated. Both the RGD modified alginate and non-modified alginate increased the arteriole density compared to control, with the RGD modified alginate resulting in the greatest angiogenic response. These results suggest that in situ use of modified polymers may influence the tissue microenvironment and serve as potential therapeutic agents for patients with chronic heart failure.

Next, Sabbah et al. tested the efficacy of Algisyl-LVR™ in dogs with heart failure (HF) [23]. The heart failure was induced by repetitive coronary microembolization in dogs. The final injection mixture was prepared by combining sodium-alginate aqueous solution mixed with calcium cross-linked alginate hydrogel. The material was applied at least 2 weeks after the last coronary microembolization through 7 injections (0.25–0.35 ml each) directly into the LV wall. Injections were made circumferentially along the LV mid-ventricular level halfway between the apex and base. The treatment was well tolerated, and ambulatory 24 h Holter electrocardiography showed no significant differences between groups with respect to heart rate and frequency and severity of ventricular arrhythmias. Seventeen weeks post treatment, histological analysis showed that pockets of the implant material were still within the LV free wall and were encapsulated by a thin layer of connective tissue with no evidence of inflammation. Compared to saline-treated animals, the alginate implant significantly reduced LV end-diastolic and end-systolic volumes and end-diastolic pressure, improved LV sphericity, and increased wall thickness. The treatment also significantly increased ejection fraction (EF) from ~26% at baseline to ~31% after treatment, compared to decreased EF from~27% at baseline to ~24% after saline injection seen in control dogs [23]. These promising results led to clinical trials.

Lee et al. combined coronary artery bypass grafting (CABG) with Algisyl-LVR™ injection to treat three congestive heart failure patients (NYHA class 3) [24]. Magnetic resonance images obtained before treatment (n = 3) and at 3 months (n = 3) and 6 months (n = 2) afterward were used to reconstruct the LV geometry. The LV became more ellipsoidal after treatment, and both end-diastolic volume (EDV) and end-systolic volume (ESV) decreased substantially 3 months after treatment in all patients. Ejection fraction increased from 32 ± 8% to 47 ± 18% during that period. Volumetric-averaged wall thickness increased in all patients. These changes were accompanied by about a 35% decrease in myofiber stress at end-diastole and at end-systole. Although the statistical power of this finding is limited by the small sample size, the concept of this treatment is novel and the effects are interesting and remarkable.

In a study by Lee et al., eleven male patients aged 44–74 years with advanced heart failure (NYHA class 3 or 4), a left ventricular ejection fraction (LVEF) of < 40%, and a need for conventional heart surgery received Algisyl-LVR delivered into the LV myocardial free wall [25]. Serial echocardiography, assessment of NYHA class, Kansas City Cardiomyopathy Questionnaire (KCCQ), and 24-hour Holter monitoring were obtained at baseline; 3–8 days (for echocardiography and Holter monitoring); and at 3, 6, 12, 18, and 24 months. A total of 9 (81.8%) patients completed 24 months of follow-up. There were no adverse events attributed to the use of Algisyl-LVR. Improvement in LV function was evidenced by mean LVEF, improved NYHA class, as well as improved KCCQ summary scores. Holter monitor data showed a significant decrease in the episodes of non-sustained ventricular tachycardia following administration of Algisyl-LVR. This first-in-man study confirmed feasibility and safety of administering Algisyl-LVR to patients with heart failure at the time of open-heart surgery. Thus, a randomized controlled study to evaluate Algisyl-LVR™ solely as a method of LV augmentation for patients with severe heart failure (AUGMENT-HF) has been initiated and should provide definitive conclusions about the clinical benefits of Algisyl-LVR™ therapy [26].

Recently, Rocca et al. developed a novel hydrogel composed of gelatinized alginate with parallel capillary-like channels, termed Capgel [27]. To make this hydrogel, a solution of 2% (w/v) alginate and 2.6% (w/v) oligo-gelatin was poured into an alginate-coated glass petri dish. The mixture was then submerged in a tank of 0.5 M $CuSO_4$ and undisturbed for at least 36 h. The gel was then rinsed and cross-linked using N-ethyl-N′-(3-dimethylamino propyl) carbodiimide hydrochloride and N-hydroxysulfosuccinimide. The modulus of elasticity of the resultant material was around 4 kPa, above the baseline modulus shown to support cardiomyocyte function (1–3 kPa, [28–31]) and below the fibrotic modulus shown to induce dysfunction (40–70 kPa). Angiotensin-(1–7) is a small peptide previously shown to be cardioprotective for infarcted myocardium, although the main disadvantage is its short half-life. Angiotensin was incorporated into Capgel to sustain local molecule release, prolong molecule bioactivity, and reduce off-target complications. Forty-eight hours after induced anterior MI, Sprague Dawley rats received intramyocardial injection of Capgel directly into the anteroseptal wall at the infarct border zone or no injection. Echocardiograms were performed at 48 h and 4 weeks to evaluate left ventricular function. Echocardiograms showed improvement of left

ventricular systolic function over time with gel injection. Histologically, the authors observed that: (1) Capgel was present at the injection site after 4 weeks but was minimal at 8 weeks, (2) the remaining gel was populated with blood vessels to promote regeneration of cardiomyocytes, and (3) the remaining gel was heavily populated by CD 68+ macrophages with CD 206+ clusters. CD206+ macrophages are known as M2 (alternative) activation CD 206+ macrophages. This activation is more consistent with a regenerative healing response rather than the classic inflammatory response [32]. An in vitro experiment was performed and demonstrated. Angiotensin-(1–7) was released from the Capgel in a sustained manner for 90 days. Lastly, the study showed intramyocardial injection of Capgel did not result in arrhythmic events, and all animals survived after injection. Capgel injection did not change systolic or diastolic LV function indices at 4 weeks compared with the saline control group, indicating that the gel appeared safe for this intraventricular/intramyocardial application.

7.2.2.2 Intracoronary Injection of Calcium Cross-Linked Alginate Hydrogel in Treatment of Acute Myocardial Infarction

Landa et al. developed an injectable alginate biomaterial (BL 1040/IK5001) which can be delivered by intracoronary injection as a solution [34]. This solution creates a partially cross-linked alginate network, prepared by mixing a 1% (w/v) solution of 30–50 kDa sodium alginate. This solution has a high guluronic acid (G) to β-D-mannuronic acid (M) ratio, indicating that it has improved mechanical properties due to binding of guluronic acid with bivalent cation such as Ca^{2+}, Ba^{2+}, or Zn^{2+} leading to hydrogel formation. The mixture is capable of flow due to its relatively low apparent viscosity of ~10 cP. In response to the elevated calcium concentrations at the acute infarct site, and due to water diffusion from the injectable solution to the surrounding tissue, the partially cross-linked alginate solution undergoes rapid gelation and phase transition into a hydrogel [33–35]. The alginate hydrogel degrades and disappears from the infarct zone within 6 weeks after administration. At this time point, only remnants of the biotinylated alginate material remain, which initially occupied up to 50% of the scar area. The alginate is replaced by host tissue composed of myofibroblasts and is enriched with blood capillaries. The alginate hydrogel dissolution occurs via an exchange reaction between the cross-linking calcium ions and sodium ions from the surrounding tissue. This reaction occurs with time at the healing infarct due to the reduction in calcium ion concentration via this reaction: $2NaAlg + Ca^{2+} \rightleftarrows 2Na^{++}Ca(Alg)_2$.

The beneficial therapeutic effects of this novel in situ forming alginate hydrogel have been recently proven in acute and chronic models of MI in rats and in an acute MI model in pigs [34, 35]. Serial echocardiography studies performed in rats before and 60 days after injection showed that injection of alginate biomaterial into a recent (7 day) infarct led to an increase in scar thickness and an attenuation in left ventricular systolic and diastolic dilatation and dysfunction. The beneficial effects achieved by the alginate injection were comparable and sometimes superior to those

achieved by neonatal cardiomyocyte cell transplantation. Furthermore, injection of alginate biomaterial into a 60-day-old MI led to an increase in scar thickness and an improvement in systolic and diastolic dysfunction [34]. The research team then proceeded to a large animal model in pigs. Intracoronary injection of the partially cross-linked alginate solution into the infarcted pig heart of pigs is feasible, safe, and effective. Four days after the MI, which was induced by transient balloon occlusion of the left anterior descending artery (LAD), either alginate solution (2 or 4 ml) or saline was selectively injected into the infarct-related coronary artery. An additional group of animals was treated with incremental volumes of biomaterial (1, 2, and 4 ml) or 2 ml of saline and underwent serial echocardiography studies. Examination of the hearts after injection showed that the alginate crossed the infarcted leaky vessels and was deposited as hydrogel in the infarcted tissue.

Importantly, the intracoronary injection of alginate into healthy hearts did not result in any material deposition or myocardial damage, and intracoronary injection into healthy or infarcted hearts showed no evidence of infarcts or intravascular thrombi in distal organs. Sixty days after infarction, in control saline-treated pigs, an increase in LV diastolic area by 44%, LV systolic area by 45%, and LV mass by 35% was noted. Strikingly, intracoronary injection of alginate (2 and 4 ml) significantly prevented and even reversed LV enlargement. Postmortem analysis showed that the biomaterial (2 ml) increased scar thickness by 53% compared with control and was replaced by myofibroblasts and collagen. The beneficial effects of the alginate hydrogel were dose dependent and are probably due to temporary replacement of the functions of the damaged ECM, followed by increased cellular infiltration during hydrogel erosion [34, 35].

Encouraging results of intracoronary delivery of engineered alginate implant have led to the first-in-man single-arm open-label clinical trial, aimed to test the safety and feasibility of this strategy in patients recovering from an extensive MI [36]. Due to its local and strictly physical effect, the material is regulated by the FDA as a medical device. Twenty-seven patients with moderate-to-large ST-segment-elevation MI (STEMI), after successful revascularization, were enrolled. Two milliliters of alginate implant (designated as IK-5001 bioabsorbable cardiac matrix (BCM) now from Bellerophon BCM LLC, previously known as BL-1040 from BioLineRx), were administered by selective injection through the infarct-related coronary artery within 7 days after MI. The procedure was performed in the catheterization laboratory, via percutaneous radial artery access, under local anesthesia. Thus, this approach avoids the need for surgical procedure performed under general anesthesia. Coronary angiography performed 3 min after injection, confirmed that the injection did not impair coronary flow and myocardial perfusion.

Furthermore, IK-5001 deployment was not associated with additional myocardial injury or re-elevation of cardiac biomarkers. Clinical assessments, echocardiographic studies, 12-lead electrocardiograms, 24-hour Holter monitoring, blood tests, and completion of Minnesota Living with Heart Failure Questionnaires were repeated during follow-up visits at 30, 90, and 180 days after treatment. During a 6-month follow-up, these tests confirmed favorable tolerability of the procedure, without device-related adverse events, serious arrhythmias, blood test abnormali-

ties, or death. Importantly, serial echocardiographic studies showed preservation of LV indices and LVEF, as compared to previously reported progressive dilation of the LV during the 6–12 months after infarction, which is accompanied by progressive deterioration in cardiac function as determined by LVEF [37].

Based on positive preclinical data, and the encouraging findings of the pilot clinical study, a pivotal trial has been designed and launched. The PRESERVATION 1: IK-5001 for the Prevention of Remodeling of the Ventricle and Congestive Heart Failure after Acute MI is an international, randomized, double-blind, controlled trial, enrolling 303 subjects with large areas of infarction despite successful primary percutaneous coronary intervention (PCI) of ST-segment elevation myocardial infarction (STEMI) [38]. Subjects were randomized 2:1 to BCM or a saline-injected group, injected into the infarct-related artery 2–5 days after primary PCI. The primary outcome was a mean change from baseline in LV end-diastolic volume index (LVEDVI) at 6 months. Secondary outcomes included a change in Kansas City Cardiomyopathy Questionnaire score, 6-minute walk time, and New York Heart Association functional class at 6 months. The primary safety endpoint was a composite of cardiovascular death, recurrent MI, target-vessel revascularization, stent thrombosis, significant arrhythmia requiring therapy, or myocardial rupture through 6 months. The results showed that there was no significant difference ($p = 0.49$) in LVEDVI from baseline to 6 months between the BCM (14.1 ± 28.9 ml/m^2) and saline (11.7 ± 26.9ml/m^2) groups. There was also no significant difference in the secondary endpoints. The rates of the primary safety outcome were similar between the two groups (BCM = 11.6%; saline = 9.1%; $p = 0.37$). The authors thus concluded that intracoronary deployment of BCM 2–5 days after successful reperfusion in subjects with large myocardial infarction did not reduce adverse LV remodeling or cardiac clinical events at 6 months [39].

At the same time, several limitations and factors of this study need to be taken into consideration. First, the volume of BCM might not have been high enough for the large infarctions included in this trial. The 4 ml deployment volume was based on the first-in-human study demonstrating tolerability and safety of 2 ml of BCM. Second, the population enrolled in this study might have been a limiting factor – in subjects with very large infarctions, it is possible that remodeling simply cannot be prevented. Among subjects who had infarct size measured, approximately 30% of the LV was involved. Given the large size of the infarcted myocardium, it is also possible that attendant microvascular occlusion prevented BCM from reaching the intracellular space in the infarct zone. Third, the trial did not test whether more acute deployment of BCM (e.g., during primary PCI) might vary its impact. However, although it is possible that BCM could have a benefit with earlier administration, many people with STEMI with large areas at risk do not actually go on to have large infarctions, which makes the study of the mechanistic benefit of BCM more complex in such a mixed cohort. Lastly, the study did show statistically significant improvement in functional outcomes, such as exercise capacity in the 6-minute walk test, in the BCM group compared to the saline group. Overall, BCM might be better suited for the decidedly narrower group of those with slightly smaller infarcts who are still at risk for adverse LV remodeling.

7.2.3 Alginate Hydrogel as a Vehicle for Cell Delivery into Infarcted Tissue

Given its innate physical properties, alginate hydrogel could significantly improve cell retention, survival, and function by serving as an artificial biomimetic ECM. Alginate can provide the required temporal support for cell growth and function until the cells are able to support themselves with their own cell-secreted ECM [40]. The aid of a temporary scaffold is critically important in conditions to which transplanted cells are presented, that is, the "hostile" environment of an injury site, such as in ischemic myocardium. Of note, poor cell retention is likely to be a major factor responsible for inconsistent and limited efficacy of cell transplantation studies for MI treatment thus far [41, 42]. Roche et al. compared four biomaterial delivery vehicles for improving mesenchymal stem cell (MSC) retention in a rat MI model – two hydrogels (RGD-modified alginate and chitosan/β-glycerophosphate) and two epicardial patches (RGD-modified alginate and collagen) [43]. From 0 to 24 h, an average of 50–62% cell retention in all biomaterial-treated hearts is seen, comparing to 9% cell retention in saline control-treated hearts. At the same time, no significant difference was observed between the individual biomaterials.

Alginate hydrogel is most commonly used for intramyocardial delivery of MSCs. Alginate is used to increase cell retention and enhance cell-mediated myocardial repair. In a study by Levit et al., human bone marrow MSCs were encapsulated in alginate hydrogel and then attached to the infarcted rat heart in a biocompatible poly(ethyleneglycol) (PEG) hydrogel patch to secure the encapsulated cells [44]. Transthoracic echocardiography and cardiac magnetic resonance imaging showed significantly improved cardiac function in encapsulated hMSC-treated animals, associated with reduced scar size and increased microvascular density, compared to directly injected cells. Bioluminescence imaging confirmed that encapsulation resulted in significantly greater hMSC retention over a period of 7 days.

A macroporous alginate scaffold, commercially available from Life Technologies, Inc. as the AlgiMatrix™ 3D Cell Culture System, was developed by the Cohen group [45, 46]. This scaffold is created by controlled freeze drying of calcium cross-linked alginate solution. The porous structure of the scaffold is dependent on the freezing protocol, namely, parameters of rate and spatial direction of the temperature gradient. When calcium cross-linked alginate solutions are slowly frozen at −20 °C in a nearly homogeneous cold atmosphere, the resultant scaffold has an isotropic pore structure, creating pores that are spherical and interconnected. Conversely, when the cooling process is performed under a unidirectional temperature gradient along the freezing solution, an anisotropic pore structure is attained.

Alginate scaffolds are characterized by an interconnected pore structure with large pore size (50–200 μm in diameter) and high matrix porosity (70–90%) [45, 46]. The pore size in scaffolds should ideally be at least 50 μm in diameter to allow high mass transport during in vitro culture and enable vascularization after implantation. Macroporous solid 3D scaffolds are an ideal platform for in vitro cardiac tissue bioengineering and creation of 3D tissue grafts, as they are capable of com-

pletely recreating the 3D microenvironment of the infarcted tissue, enabling vascularization and directing cell organization into tissue-like structures.

The same study group later reported successful implantation of cardiac cell-seeded macroporous alginate scaffolds into infarcted rat hearts [47]. The seeded fetal rat cardiomyocytes retained viability within the scaffolds and formed multicellular beating cell clusters within 24 h [48]. Following their implantation into the infarcted myocardium, some of the cells in the cell constructs appeared to differentiate into mature myocardial fibers, shown by the appearance of typical striation and the formation of gap junctions. The graft itself and its surrounding area were populated with a large number of newly formed blood vessels, consequently leading to attenuation in LV dilatation and improved heart function.

Through incorporation of ECM-derived peptide into alginate hydrogel, cell adhesion and other functions are facilitated, further maturing the seeded cells. Several factors facilitate incorporation of ECM-derived peptide into alginate hydrogel: its low protein adsorption capability, good biocompatibility, and the availability of carboxyl and hydroxyl groups in the alginate polymer allowing such modifications using well-established water-soluble chemical methods.

In one study, Shachar et al. attached sequence peptides (Arg-Gly-Asp) to alginate [50]. The RGD peptide sequence is a commonly used because it is derived from the fibronectin and laminin signal domain. The RGD peptide functions in the mediation of cell adhesion and signaling via binding of various ECM proteins to integrin receptors on the cell surface [49]. By conjugating RGD peptide into macroporous alginate scaffolds, they found increased formation of functional cardiac muscle tissue and better preservation of the regenerated tissue properties in long-term in vitro cultures. Another study conducted by the same group showed that the integration of the heparin-binding peptide (HBP) promotes the formation of an improved cardiac tissue in vitro [51]. HBPs, with the sequence XBBXBX and XBBBXXBX, where X is a hydropathic amino acid and B is a basic amino acid, have been shown to bind cell surfaces via the syndecans present on the cell surface, which bear 3–7 covalently attached heparin/heparan-sulfate chains [52]. Neonatal rat cardiomyocytes were cultivated within macroporous alginate scaffolds attached with both RGD and HBP, attached with single peptide (RGD or HBP) or without any modification. Fourteen days after seeding in the different scaffolds, the characteristic striated fiber organization was apparent mainly in the RGD/HBP-attached cell constructs, while no such structures were observed in HBP-attached and unmodified alginate cell constructs. The 14-day-old HBP/RGD-attached scaffolds also presented an isotropic arrangement of the fibers in the form of consistent tissue (resembling isotropic organization of native cardiac tissue), while no such arrangement was seen in the RGD-attached scaffold. Selected cardiac markers (sarcomeric α-actinin, N-cadherin, and connexin-43 (Cx-43)) were demonstrated to be preserved by Western blotting, and an increase in the expression level of Cx-43 was observed with time in the HBP/RGD-attached scaffolds, further supporting the notion of contractile muscle formation and tissue maturation [51].

Other nanocomposites have been reported to be integrated onto alginate scaffold preparation to increase cells' specific responses to various stimulation patterns. Dvir

et al. developed 3D nanocomposites of gold nanowires (NW) within macroporous alginate scaffolds, which functioned as a bridge between the nonconducting pore walls, increasing electrical signal propagation throughout the cell-seeded scaffold, and enhancing the organization of functioning cardiac tissue [53]. Such strategy resulted in an improved electrical communication between adjacent cardiac cells, better cell organization, synchronous contraction under electrical stimulation, and higher expression levels of sarcomeric α-actinin and Cx-43. Sapir et al. used alginate scaffolds with magnetically responsive nanoparticles which were exposed to an alternating magnetic field at a physiologically relevant frequency (5 Hz) to determine whether the addition of the nanoparticles would promote the formation of a functional myocardial tissue [54]. Magnetic stimulation of cell constructs resulted in a more mature cardiac culture characterized by anisotropically organized striated cardiac fibers, which preserved its features for longer times than non-stimulated constructs. This was associated with increased Troponin-T expression in the stimulated constructs. After applying a short-term 20 min external magnetic field, a high activation rate of AKT protein kinase in cardiac cell constructs was detected, indicating the efficacy of magnetic stimulation to actuate at a distance and providing a possible mechanism for its action.

7.2.4 Alginate Hydrogel as Vehicle for Delivery of Growth Factors into Infarcted Tissue

The use of bioactive molecules (growth factors, cytokines, and stem cell-mobilizing factors) has always been of interest in the field of therapeutic myocardial regeneration. The effect exerted by these molecules includes cell proliferation, vascularization apoptosis inhibition, progenitor cell migration, and progenitor cell differentiation [12, 55–60]. However, systemic cytokine or growth factor administration is currently considered unpractical because of several reasons. First, systemic administration is associated with numerous safety concerns including elevated blood pressure, increased incidence of restenosis, thrombolytic events, arrhythmia, etc. [61]. Secondly, systemic administration requires high doses of the protein due to extremely low protein stability in the circulation. Lastly, most of these molecules have pleiotropic functions. Thus, a careful, local, and time-adjusted intervention is needed for administration. Biomaterials could be engineered to produce a sustained delivery system for bioactive molecule combinations. In such a system, the biomaterial will provide structural temporary matrix support and direct the formation of functional tissue in situ. Simultaneously, it will serve as a temporary depot for sustained delivery and presentation of bioactive molecules with spatial and controlled distribution of the desired agent to induce multiple reparative/regenerative processes. In general, due to low protein adsorption and its highly porous and hydrophilic nature, the pristine unmodified alginate matrix yields fast and unpredictable

protein release kinetics. Thus, several modification designs and engineering schemes were applied for the use of alginate in protein delivery.

Hao et al. used alginate consisting of both high and low molecular weight (MW) components, otherwise known as binary molecular weight alginate. Increasing the molecular weight in alginate gel leads to enhanced mechanical properties in resultant gels, although the gel becomes more difficult to be cleared by the body [62]. The binary MW alginate also supplies a partially oxidized formulation for sequential delivery of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)-BB into the infarcted myocardium. The factors were adsorbed to the hydrogel via electrostatic interaction, and the sequential factor delivery was achieved due to the varying degradation rates of the partially oxidized alginates constituting the hydrogel. One week after MI was induced in rats, the modified alginate hydrogels loaded with VEGF and PDGF-BB were injected intramyocardially along the border zone of the infarct. Four weeks after injection, the sequential growth factor release led to a higher density of α-SMA-positive (mature) vessels compared to the delivery of single factors. Sequential delivery of VEGF and PDGF-BB increased the systolic velocity-time integral compared to delivery of VEGF alone.

Alginate has also been used as a component in composite carriers. Banquet et al. developed cross-linked albumin-alginate microcapsules that sequentially released fibroblast growth factor-2 (FGF-2) and hepatocyte growth factor (HGF) [63]. The developed microcapsules (~100 μm in diameter) contained a thin, covalently cross-linked human serum albumin and propylene glycol alginate membrane surrounding a liquid center. Both proteins were bound to a microcapsule surface layer, and FGF-2 was also found in the liquid center. The microcapsules displayed differential release profiles for FGF-2 (released first) and HGF (release delayed for >1 week) in vitro. In a rat model of MI, immediate post-MI intramyocardial injection of FGF-2/HGF alginate-albumin microcapsules stimulated angiogenesis and arteriogenesis and prevented cardiac hypertrophy and fibrosis, as determined by immunohistochemistry. Cardiac perfusion was improved after 3 months, as shown by magnetic resonance imaging. These multiple beneficial effects resulted in reduced adverse cardiac remodeling and improved left ventricular function, as shown by echocardiography.

Ruvinov et al. established an alginate-based scaffold which binds to various bioactive molecules, collectively known as heparin-binding proteins [64]. To enable this process, sulfation of the uronic acid monomers on alginate was performed, presenting affinity-binding sites for heparin-binding proteins. The sequential delivery of different growth factors was achieved by (1) the varied equilibrium binding constants of the modified alginate as determined by surface plasmon resonance (SPR) analysis and (2) manipulating the initial amount of factors added to the system. The research group chose insulin-like growth factor (IGF)-1 and HGF for the injectable alginate system. These two growth factors have established and complimentary beneficial effects on infarcted myocardium [61, 65–67]. Biologically active IGF-1 followed by HGF proteins were sequentially released from the alginate hydrogel. This sequential delivery system of IGF-1 and HGF could be applied for the proper execution of reparative processes in the infarcted myocardium and for achieving a more favorable course of repair. The faster released IGF-1 can provide

an immediate pro-survival signal to rescue the functional myocardium and reduce cell apoptosis after the initial ischemic event. Later phases of repair require processes such as angiogenesis induction, ECM remodeling, and fibrosis reduction, and these can be mediated by the slower, yet continuous, release of HGF. The efficacy of the delivery system on protein retention was tested on an immediate post-acute MI injection. In comparison to soluble HGF administered by bolus injection, which was rapidly eliminated from the infarct, HGF-bound alginate increased HGF retention and bioavailability in the myocardial tissue after acute MI [68]. Subsequently, the scientists tested the therapeutic efficacy of multiple growth factor delivery by alginate hydrogel in an acute MI model in rats. The results showed that the sequential delivery of IGF-1 and HGF from this system reduced scar fibrosis, increased scar thickness, and prevented infarct expansion 1–4 weeks after injection, compared to controls of soluble factors or saline. Furthermore, angiogenesis was induced, and cell apoptosis was reduced at the infarct. Lastly, increased incidence of Ki-67+ cardiomyocytes and GATA4+ cell clusters were observed, indicating potential endogenous regeneration of the cardiac muscle [64].

Affinity-binding alginate scaffolds were used for engineering of pre-vascularized cardiac patches. A cocktail of pro-survival (IGF-1), migratory (stromal cell-derived factor (SDF) -1), and angiogenic (VEGF) factors, together with cardiac cells, were seeded onto the alginate scaffolds. The scaffold was then implanted on the omentum for 7 days to achieve host-induced vascularization of the engineered tissue. Next, the patches were harvested, and after detecting the formation of proper networks of blood vessels by identification by positive α-SMA staining, the omentum-generated (Om+) patches were re-transplanted onto the scar tissue of the infarcted rat hearts. Four weeks after implantation, the omentum-generated patches were fully integrated into the myocardium of the host and induced the formation of thicker scars than those observed on the myocardium of control rats. Importantly, omentum-generated patches were electrically coupled with the host myocardium, as assessed 4 weeks after engraftment using Langendorff-perfused isolated heart preparations. This integration was evidenced by the higher amplitude of electrical signals in the scar zone and by the markedly lower capture threshold for pacing, compared to untreated infarcted hearts, indicating better excitability and/or electrical connectivity between the scar and healthy myocardium. Finally, omentum-generated patches were able to preserve cardiac function and prevent LV dilatation and adverse remodeling, as shown by 2D echocardiography. Similar beneficial results were obtained when the omentum-generated patch was constructed from a cell-free affinity-binding alginate scaffold, which was supplemented only with the mixture of pro-survival and angiogenic factors. After regeneration on the omentum, cell penetration and consistency in these acellular constructs was similar to that of the cardiac cell-seeded constructs, possibly explaining their beneficial effects on infarct repair [69].

Recently, Rodness et al. invented a combined strategy for VEGF delivery [70]. They produced a compacted calcium-alginate microsphere patch restrained by a chitosan sheet to deliver VEGF to the heart after myocardial injury in female Sprague-Dawley rats. Growth factors delivered by hydrogel-based microspheres have tunable degradation properties and support the prolonged release of soluble

factors. Cardiac patches provide mechanical restraint, preventing dilatation associated with ventricular remodeling. Microspheres were produced using a water-in-oil-in-water double emulsion technique. The VEGF+ beads were conjugated with 15 μg/ml VEGF-164 and the VEGF- beads were conjugated with 15ug/ml bovine serum albumin (BSA). The microbeads had an average diameter of 3.2 μm, were nonporous, and characterized by a smooth dimpled surface. Microsphere patches demonstrated prolonged in vitro release characteristics compared to non-compacted microspheres and VEGF supernatants obtained from patches maintained their bioactivity for the 5-day duration of the study in vitro. In vivo, patches were assessed with magnetic resonance imaging following MI and demonstrated 50% degradation 25.6 days after implantation. Both VEGF- and VEGF+ microsphere patch-treated hearts showed improved cardiac function than unpatched (chitosan sheet only) controls. However, VEGF+ microsphere-patched hearts had thicker scars characterized by higher capillary density in the border zone than did those treated with VEGF- patches. VEGF was detected in the patches 4 weeks postimplantation. This microsphere-composite patch prototype may enable (1) localized delivery, as it can be placed onto the surface of the heart; (2) the delivery of protein or growth factors, which can be loaded into microsphere modules; and (3) tailored protein release from microspheres or microsphere patches. Producing a protein-releasing patch from basic constituent components leads to a greater degree of control over the release properties of the patch. This proof-of-concept study has demonstrated the potential utility of an alginate-microsphere patch system. However, optimized therapy will likely need to include the use of multiple sequentially released cytokines for rapid angiogenesis and vessel maturation thereby improving cardiac function, as well as further improvement in LV morphology via patch implantation directly onto the infarct and border zone regions.

Henri et al. investigated in rats the impact of MI and subsequent chronic heart failure on the cardiac lymphatic network [71]. The lymphatic system regulates interstitial tissue fluid balance, and lymphatic malfunction causes edema. The heart has an extensive lymphatic network displaying a dynamic range of lymph flow in physiology. Myocardial edema occurs in myocardial infarction (MI) and chronic heart failure, suggesting that cardiac lymphatic transport may be insufficient in pathology. The study group evaluated the functional effects of selective therapeutic stimulation of cardiac lymphangiogenesis post-MI. The researchers investigated cardiac lymphatic structure and function in rats with MI induced by either temporary occlusion ($n = 160$) or permanent ligation ($n = 100$) of the left coronary artery. The researchers noticed, although MI induced robust, intramyocardial capillary lymphangiogenesis, adverse remodeling of epicardial precollector and collector lymphatics occurred, leading to reduced cardiac lymphatic transport capacity. Consequently, myocardial edema persisted for several months post-MI, extending from the infarct to non-infarcted myocardium. Intramyocardial-targeted delivery of the vascular endothelial growth factor receptor 3–selective designer protein VEGF-C_{C152S}, using albumin-alginate microparticles, accelerated cardiac lymphangiogenesis in a dose-dependent manner and limited precollector remodeling post-MI. As a result, improved myocardial fluid balance was evidenced by gravimetric analyses of

cardiac water indicating there is less edema in alginate particle-treated hearts. The IHC staining at week 3 post MI showed less CD 68+ macrophage at infarcted area. Picrosirius Red stain at the same time showed less collagen formation at the same area. These results indicated cardiac inflammation and fibrosis were attenuated. Finally, magnet resonance (MR) and ultrasound were conducted at week 6 post MI to evaluate cardiac function. MR showed increased cardiac perfusion in the alginate particle-treated group compared with the untreated control. Ultrasound showed significant increase in function of the treated rat hearts compared to the control. This study showed that, despite the endogenous cardiac lymphangiogenic response post-MI, the remodeling and dysfunction of collecting ducts contribute to the development of chronic myocardial edema and inflammation-aggravating cardiac fibrosis and dysfunction. Moreover, the study revealed that therapeutic lymphangiogenesis may be a promising new approach for the treatment of cardiovascular diseases.

Chang et al. produced genome-modified mesenchymal stem cells that secreted HGF upon drug-specific induction [72]. The modified MSCs were then integrated in arginine-glycine-aspartic acid (RGD)-alginate microgel. This microgel was then transplanted into a hindlimb ischemia model in rats. First, a TetOn-HGF-expression construct was generated. The drug-inducible HGF expression was evidenced by transiently transfecting the construct into HEK293t cells. Then, the TetOn-HGF-expression construct was integrated into a safe harbor site in an MSC chromosome using the transcription activator-like effector nuclease (TALEN) system, resulting in the production of TetOn-HGF/human umbilical cord blood-derived (hUCB)-MSCs. Gel migration test of the TetOn-HGF/hUCB-MSCs showed that they had enhanced mobility upon the induction of HGF expression. Moreover, long-term induction of HGF expression in TetOn-HGF/hUCB-MSCs enhanced the antiapoptotic responses of these cells subjected to oxidative stress. Long-term HGF induction in the same cells also improved the tube formation ability evidenced by a matrigel tube forming assay. Lastly, TetOn-HGF/hUCB-MSCs encapsulated by RGD-alginate microgel induced to express HGF improved in vivo angiogenesis in a mouse hindlimb ischemia model. After one week of induced hindlimb ischemia, mice were treated with phosphate-buffered saline, UCB-MSCs only, hrHGF only, an RGD-alginate microgel containing UCB-MSCs, an RGD-alginate microgel containing HGF integrated UCB-MSC with sustained inducing of HGF. After treatment, the levels of blood perfusion of the hindlimbs were measured using a Laser-Doppler flowmeter weekly for 4 weeks. Blood flow was significantly higher in the microgel group, and a statistically significant difference was observed between the microgel group and other groups. The IHC stain of the ischemic limb also showed positive stain for von Willebrand factor in microgel group, further confirming angiogenesis in the lesion. In this study, by genetically modifying mesenchymal stem cells, the researchers achieved sustained secretion of HGF and increased the viability and migration ability of the MSCs. This system also overcomes the issue of the short half-life of HGF, a major limitation for the usage of recombinant HGF. Thus, the MSCs that express HGF in an inducible manner are a useful therapeutic modality for the treatment of vascular diseases requiring angiogenesis.

7.3 Application of Alginate in Other Cardiovascular Diseases

Beyond myocardial infarction, alginate has also been used in potential treatments for other cardiovascular diseases, as summarized in Fig. 7.2 and detailed in the following sections.

7.3.1 Three-Dimensional (3D) Printed Aortic Valve and Its Potential Application in Aortic Valve Disease

Aortic valve disease usually results in prosthetic replacement. Currently, tissue-engineered living aortic valve conduits have potential for remodeling, regeneration, and growth, but fabricating the natural anatomical complexity with cellular heterogeneity remains challenging. In a study by Duan et al., alginate/gelatin hydrogel was implemented to fabricate living valve conduits through 3D bioprinting [73]. Aortic root sinus smooth muscle cells (SMC) and aortic valve leaflet interstitial cells (VIC) were successfully incorporated in a regionally constrained manner by placing the SMCs into the valve root and the VICs into the leaflets. Both cell types were viable (survival rates of 81.4 ± 3.4% for SMCs and 83.2 ± 4.0% for VICs) within 3D printed tissues. Encapsulated SMC expressed elevated alpha-smooth muscle actin when printed in a stiff matrix, while VIC expressed elevated vimentin in soft matrix.

In another study by Hockaday et al., 3D extrusion printing of aortic valve hydrogel scaffold was achieved [74]. Based on poly(ethylene) glycol-diacrylate (PEG-DA) hydrogels (700 or 8000 MW), 10–15 % v/w alginate was added to reach suitable extrusion viscosity. This technique allowed for rapid 3D printing of the scaffolds. Shape fidelity was examined by micro CT, indicating that the scaffolds had high geometric precision, but decreased accuracy with reduced size. Porcine aortic valve interstitial cells (PAVIC) were cultured on the 3D printed scaffolds and cell viability was maintained at 100% for 7 and 21 days.

3D printing of aortic valves using alginate-based hydrogel allows fabrication of complex and heterogeneous aortic valve conduits. However, further work is necessary to complete the translation of this strategy to the clinic. Continued effort must be made to understanding how cells in constructs would remodel the scaffold in simulated hemodynamic conditions.

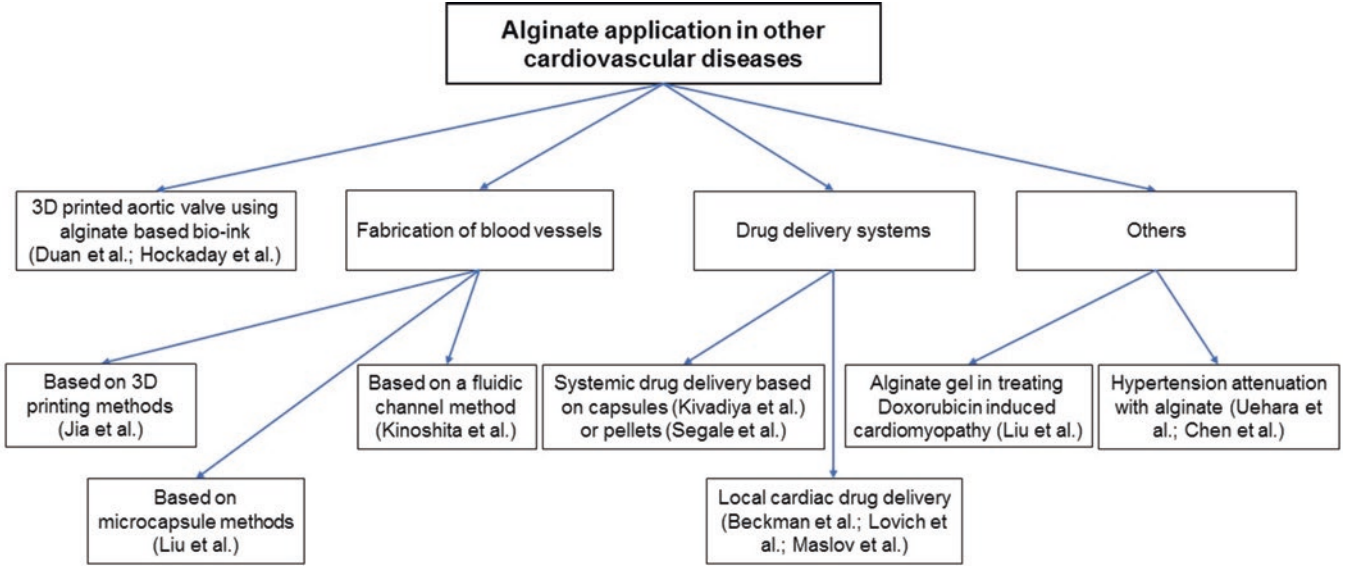

Fig. 7.2 Summary of alginate application in other cardiovascular diseases. Alginate has been explored for its utility as a bio-ink in 3D printing approaches, in the fabrication of blood vessels, in drug delivery systems, and in other applications for potential treatment in cardiovascular disease

7.3.2 Fabrication of Bioengineered Blood Vessels Using Alginate-Based Methods

Development of the technique for constructing an internal perfusable vascular network is a challenging issue in fabrication of dense three-dimensional tissues in vitro. Liu et al. reported a method for creating a perfusable network [75]. The researchers assembled HepG2 cell enclosing hydrogel microcapsules (about 200 μm in diameter) and a single hydrogel fiber, both covered with human vascular endothelial cells, in a collagen gel. The microcapsules and fiber were made from alginate and gelatin derivatives and had cell-adhesive surfaces. The endothelial cells on the hydrogel constructs sprouted and spontaneously formed a network connecting the hydrogel constructs with each other in the collagen gel. Perfusable vascular network-like structure formation after degrading the alginate-based hydrogel constructs by alginate lyase was confirmed by introducing solution containing tracer particles of about 3 μm in diameter into the lumen templated by the alginate hydrogel fiber. The introduced solution flowed into the spontaneously formed capillary branches and passed around the individual spherical tissues. This method could greatly contribute to the development of dense tissues in vitro.

In another study by Kinoshita et al., researchers proposed an efficient strategy for fabricating vascular tissue models with multilayered, branched, and thick structures through the in situ hydrogel formation in fluidic channels [76]. First, an aqueous solution of RGD-alginate containing smooth muscle cells (SMCs) is introduced into channel structures made of agarose hydrogel, forming a cell-embedding calcium-alginate hydrogel layer. The resulting thickness was around several hundred micrometers on the channel surface due to Ca^{2+} ions diffusion from the agarose hydrogel matrix. Next, endothelial cells (ECs) are introduced and cultured for up to 7 days to form hierarchically organized, multilayered vascular tissues. The researchers confirmed that increased alginate solution flow rate into agarose gel resulted in a thinner alginate hydrogel layer or larger width of flow path. IHC stain confirmed the existence of an EC layer above the SMC layer. The fabricated vascular tissue models were recovered from the channel by simply detaching the agarose hydrogel plates. In addition, the effect of O_2 tension (20 or 80%) on the viability and elastin production of SMCs during the perfusion culture was evaluated. When the perfusion culture was performed under 20% O_2 conditions, the expression of α-elastin by SMCs was confirmed, and the relative area of the α-elastin+ region to the cell nuclei by ratio was 0.57. In contrast, a considerably large amount of α-elastin was expressed by SMCs in the entire region of the cross-section in the perfusion culture at 80% O_2; the relative area of α-elastin by ratio was 1.27. This technique could provide a novel strategy for vascular tissue engineering because it enables the facile production of morphologically in vivo vascular tissue-like structures that can be employed for various biomedical applications.

Jia et al. used a 3D bioprinting technique to fabricate vascular networks to be adopted within engineered tissue constructs to overcome the perfusion limit with the tissue [77]. Through the use of a 3D bioprinting strategy, researchers employed

biomimetic biomaterials and an advanced extrusion system to deposit perfusable vascular structures with highly ordered arrangements in a single-step process. In particular, a specially designed cell-responsive bioink consisting of gelatin methacryloyl (GelMA), sodium alginate, and 4-arm poly(ethylene glycol)-tetra-acrylate (PEGTA) was used in combination with a multilayered coaxial extrusion system to achieve direct 3D bioprinting. This blend bioink could be first ionically cross-linked by calcium ions followed by covalent UV crosslinking of GelMA and PEGTA to form stable constructs. The rheological properties of the bioink and the mechanical strengths of the resulting constructs were tuned by the introduction of PEGTA, which facilitated the precise deposition of complex multilayered 3D perfusable hollow tubes. Moreover, a multilayered coaxial nozzle containing concentric channels was designed for continuous generation of perfusable constructs with hollow interiors and various diameters. Based on live/dead stain, the percentages of viable cells within the bioprinted constructs at UV exposures of 20 s and 30 s exceeded 80% after 1, 3, and 7 days of culture. IHC staining of the constructs showed hollow structure with SMA+ and CD34+ cells lining. These results confirmed the bioink displays favorable biological characteristics that supported the spreading and proliferation of encapsulated endothelial and stem cells in the bioprinted constructs leading to the formation of biologically relevant, highly organized, perfusable vessels. These characteristics make this novel 3D bioprinting technique superior to conventional microfabrication or sacrificial templating approaches (i.e., templates removed from hydrogels to generate hollow channels) for fabrication of the perfusable vasculature.

7.3.3 Alginate-Based Drug Delivery Systems in Cardiovascular Diseases

Alginate gels have been investigated for the delivery of a variety of low molecular weight drugs, including drugs used to treat cardiovascular diseases. Alginate-chitosan systems are often used to decrease release of medicine into the stomach. In one study by Kevadiya et al., antiarrhythmia procainamide (PA) was intercalated into the interlayer of montmorillonite (MMT) via an ion exchange mechanism [78]. The prepared PA–MMT composite was then compounded within an alginate (AL) and chitosan (CS) complex. The release performance of PA was found to be delayed in the PA-MMT-AL/CS composite in the gastric environment compared to the PA-MMT composite. Release of the drug in the PA-MMT-AL/CS composite in the intestinal environments exhibited a controlled manner.

Alginate-based local drug delivery systems are being tested on variant applications. In the Beckerman et al. study, alginate-based glue (SEAlantis) with amiodarone was applied pericardially to the right atrium [79]. Rapid atrial response (RAR, an abnormal rhythm associated with atrial fibrillation) to burst pacing was assessed before application and in the third postoperative day (POD3). The results yielded a

significant RAR frequency reduction of clinical importance in response to burst pacing. Additionally, such electrophysiological response was achieved while maintaining below detection systemic drug levels, which minimizes the risk of extracardiac adverse effects of amiodarone (thyroid, lung, and corneal toxicity).

Segale et al. studied the formulation and the coating composition of biopolymeric pellets containing ranolazine, an anti-angina medication under the trade name Ranexa™ [80]. Small pellets were prepared by ionotropic gelation using three concentrations of hydroxypropyl cellulose HPC (0.50%, 0.65%, and 1.00% wt/wt) and 1.5% wt/wt sodium alginate. After this, 5% ranolazine was added. The uncoated pellets were regular in shape and had a mean diameter between 1490 and 1570 μm. Eudragit L100 (EU L100) and Eudragit L30 D-55 were then coated onto the pellets at different percentages (5%, 10%, 20%, and 30% wt/wt). The researchers found that with the increase in the HPC concentration, the structure of the pellets became more compact and slowed down the penetration of fluids. Coated alginate-HPC formulations were able to control the drug release at neutral pH. A higher quantity of HPC in the system resulted in a slower release of the drug. The nature of the coating polymer and the coating level applied affected the drug release in an acidic environment – EU L100 gave better performance than Eudragit L30 D-55, and the best coating level was 20%. The pellets containing 0.65% of HPC and coated with 20% EU L100 represented the best formulation and were able to limit the drug release in an acidic environment and control to a pH of 6.8.

Another application of epicardial delivery of drugs is myocardial application of inotropes, which affect the force of muscle contractions. In a study by Lovich et al., epicardial drug-releasing platforms were constructed from calcium cross-linked alginate hydrogels and served to apply dobutamine over the anterior surface of the rat heart [81]. Pressure volume analyses indicated that while both local and systemic use of dobutamine increased stroke volume and contractility, epicardial application preserved heart load and systemic blood pressure. Epicardial dobutamine increased indices of contractility with less rise in heart rate and lower reduction in systemic vascular resistance than IV infusion. These data suggest that inotropic EC delivery has a localized effect and augments myocardial contraction by different mechanisms than systemic infusion, with far fewer side effects.

Maslov et al. used the same alginate-based delivery platform to deliver epinephrine epicardially [82]. Comparing local delivery, systemic side effects (tachycardia and loss of systemic vascular resistance) were far more profound with IV infusion. Interestingly, the contractile stimulation by epinephrine was linked to drug tissue levels and commensurate cAMP upregulation for IV systemic infusion, but not with local epicardial delivery, probably because only a small fraction of the deposited epinephrine was utilized in second messenger signaling and produced a biologic effect. The remainder of deposited drug was likely used in diffusion and distribution. Later, the same group studied alginate-based epicardial epinephrine delivery in a large mammal swine model [83]. They found that the vector of myocardial drug distribution coincides with the predominant direction of the blood flow away from the nearest large epicardial coronary arteries, suggesting locally delivered epinephrine is rapidly cleared by capillaries. Thus, coronary blood flow which drives capillary

perfusion is a plausible mechanism of both drug distribution and clearance from the myocardium. These critical insights suggest that inotropic compounds with physicochemical properties that lend themselves to tissue retention may be better suited for epicardial applications. The demonstrated local myocardial pharmacokinetics may allow for practical designs for epicardial drug therapy systems.

7.3.4 Alginate-Based Stem Cell Delivery in Reverting Doxorubicin-Related Cardiomyopathy

A study by Liu et al. showed that encapsulation of cardiac stem cells (CSCs) in superoxide dismutase (SOD)-loaded alginate hydrogel prevents doxorubicin (DOX)-mediated toxicity in vitro [84]. The study was based on the concept that DOX-induced cardiotoxicity can be viewed as a stem cell disease, whereby the formation of reactive oxygen species (ROS) by DOX is seen to predominantly hinder regenerative capability. Increased cell survival was confirmed by fluorescent microscopy and assays measuring metabolic activity, cell viability, cytotoxicity, and apoptosis. Encapsulation of CSCs in alginate alone failed to prevent apoptosis in DOX-conditioned cell culture medium while encapsulation in SOD-loaded alginate reduced apoptosis to near-normal levels, while metabolic activity was returned to baseline. Although this study explored the protective effects of CSC encapsulation against DOX, this technology may also be successfully implemented in the treatment of other diseases in which sustained oxidative stress contributes to pathology.

7.3.5 Alginate's Direct Antihypertensive Function

In several studies, alginate administration is associated with decreased hypertension in rat models [85–87]. Uehara et al. investigated the effects of sodium alginate oligosaccharides on the development of spontaneous hypertension in rats. Spontaneous hypertensive rats (SHR) treated with alginate showed both attenuated systolic blood pressure elevation and attenuated morphologic glomerular damage compared to the control group. Subsequently, the same research group tested antihypertensive efficacy of sodium alginate oligosaccharide on salt-induced hypertension in Dahl salt-sensitive (Dahl S) rats and found decreased systolic blood pressure (SBP) and less severe target organ damage (i.e., the heart and kidneys) in the treatment group. Furthermore, sodium alginate oligosaccharide treatment almost completely eliminated salt-induced hypertension with significantly attenuated hypertensive glomerular sclerosis and arterial injury in Dahl S rats fed a high-salt diet.

Chen et al. [88] reported similar antihypertensive effect of alginate. Oral low molecular mass potassium alginate (L-PA) dose dependently normalized the hypertensive changes induced by DOCA salt, evidenced by increased SBP, serum sodium,

plasma atrial natriuretic peptide (ANP) content, heart and renal weight indices, and decreased plasma aldosterone (ALD). The authors claimed L-PA offered a novel form of potassium supplementation with greater antihypertensive and sodium excretion actions than KCl.

7.4 Conclusions and Future Prospectives

Alginate has demonstrated great utility and potential as a biomaterial for use in cardiovascular diseases, particularly in the applications such as ECM replacement, 3D microenvironment design for functional cardiac tissue formation, stem cell delivery, and controlled release and presentation of multiple combinations of bioactive molecules and regenerative factors. The most attractive features of alginate for these applications include biocompatibility, mild gelation conditions, and simple modifications to prepare alginate derivatives with new properties. Moreover, based on encouraging preclinical data, acellular injectable alginate implants for myocardial repair and tissue reconstruction have already reached the clinical investigation phase in MI and HF patients.

In the near future, the use of alginate-based materials in cardiovascular diseases is likely to evolve considerably. However, several challenges and needs have still not been fully addressed. First, the design of a more cell-interactive biomaterial is required. For example, RGD peptides have been extensively exploited to date as a cell adhesion ligand. However, multiple other ligands (such as heparin binding proteins) may be required to properly produce replacement tissues in cardiac diseases. One or more bioactive agents can be incorporated into alginate hydrogel to facilitate cardiac regeneration, as these gels have demonstrated utility in maintaining local concentrations of biological factors, such as proteins, for extended time periods. Introduction of multiple signaling ligands and topographical cues and control over their spatial distribution at the nanoscale are the key variables in such design process.

Next, the development of “smart” alginate-based release systems will continue, and this will potentially improve safety, increase sustained and local effects, and provide new therapies. Precise control over the delivery of multiple drug combinations, spanning multiple drug families (proteins, nucleic acids, small molecules) of sustained vs. sequential release in response to external environmental changes (mechanical signals, electromagnetic fields, changes in cell populations, ECM degradation, etc.) is highly desirable.

It is clear that biomaterials combined with adult stem cells may become an optimal and effective myocardial recovery and usually show synergistic effects that play an important role in recovery progress of the infarcted myocardium. Results show that even the small remaining numbers of transplanted stem cells may lead to restoration of the infarcted myocardium. The majority of researchers believe it is due to the paracrine effects through which more CSCs are recruited from the host tissue. Subsequently, the CSCs undergo differentiation, restoring the cardiomyocyte compartment. The lack of true regeneration in terms of increasing the fraction of

contractile units is one of the main reasons that limit the success in clinical trials of stem cell transplantation. When alginate hydrogel is used in cell encapsulation strategies, the commonly used covalent cross-linking reactions can cause toxicity to the encapsulated cells, making the appropriate choice of cell-compatible chemical reagents (e.g., initiator), and thorough removal of unreacted reagents and by-products necessary. Future research may focus on the following areas: (1) design of a smart hydrogel that can degrade upon an activation signal or at a specific pH, (2) optimization of the hydrogel's properties to improve its resistance to cardiac cyclic loading movement, and (3) progressing in vivo studies to large animal models to create more relevancy to humans.

In the future, we expect the evolution of combinatory and more complex strategies, where combinations of the abovementioned approaches will be used together, to achieve more powerful and synergistic effects on tissue repair and regeneration.

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Chapter 8
Alginates in Dressings and Wound Management

Michael Clark

Abstract This chapter considers how wound dressings are used in the treatment of wounds identifying the ideal properties of a wound dressing. Changes in the treatment of wounds with dressings since 1980 are discussed highlighting the current availability of a wide range of advanced wound dressings that clinicians have to select from for each wound they treat. Alginate wound dressings are introduced with their chemistry briefly considered and their indications and contraindications for clinical use reported. The clinical evidence supporting the use of alginate wound dressings is discussed highlighting the generally weak evidence underpinning the use of all advanced wound dressings. Recent reviews of the effectiveness of alginate dressings noted that across all the studies, there were no statistically significant differences between the outcomes achieved using the alginate dressings and the comparison groups. It is concluded that alginate dressings are currently not in widespread use in the UK National Health Service and may now be considered as comparisons against which new technologies may be compared.

Keywords Alginate wound dressings • Properties of the ideal wound dressing • Clinical effectiveness of alginate wound dressings • Indications for use of alginate wound dressings • Contraindications for use of alginate wound dressings

8.1 Introduction

Wounds to the skin and underlying tissues are both common and costly to healthcare services. All surgical patients will have a wound and while most heal uneventfully, local infections are common with surgical site infections (SSI) ranging from 0.3 per 1000 in-patient days to 8.2 per 1000 in-patient days dependent upon the type of surgery [1]. Other wound aetiologies include burns, traumatic wounds, pressure

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ulcers (bedsores), leg ulcers and diabetic foot ulcers. The final three wound aetiologies are often known as chronic wounds with these wounds often failing to heal after 3 months of treatment [2].

There are many estimates of the number of patients with chronic wounds, for example, Posnett and Franks [3] reported that at any moment in time there would be around 70–100,000 people with leg ulcers in the United Kingdom, 64,000 with diabetic foot ulcers and 20,000 with pressure ulcers. The cost to health services due to wound treatment is high; Phillips et al. [4] calculated that in Wales (population 3,168,000), each year wound care costs 6% of all expenditure on health or £330,000,000. Wounds are not only common and costly, but also they pose serious consequences for patients in terms of increased morbidity (increased pain, malodour, reduced activity and mobility) and mortality. Between 1990 and 2001, 114,380 people in the United States had pressure ulcers noted as a cause of death, with 21,365 (18.7%) having pressure ulcers as the primary cause of mortality [5].

8.2 How Do Wounds Heal?

The detailed biology of wound healing is beyond the scope of this chapter with a recent reference covering this complex process by Martin and Nunan [2]. The healing of wounds is often very simplified into four overlapping phases [6]:

Bleeding and haemostasis

Platelets aggregate and attach to collagen surfaces and release vasoactive agents prompting blood clotting while chemokine release attracts inflammatory cells (neutrophils).

Inflammation

Initial attraction of neutrophils to kill microbes that have accessed the wound and later entry of macrophages to control repair processes and remove neutrophils and cell debris.

Cell proliferation and matrix deposition

Formation of granulation tissue and new blood vessels and formation of a new epithelial layer

Matrix remodelling

Remodelling of collagen, reduction in blood vessels and scar formation

In simplistic terms, wounds are closed by primary or secondary intention [6]. A wound healing by primary intention, for example, a surgical wound, has the two edges of the wound brought together and held in place with sutures or tape. Whereas in wound healing by secondary intention, the edges of the wound cannot be approximated, and the wound will heal through a combination of wound contraction, gen-

eration of new soft tissue and blood vessels and re-epithelialisation. Chronic wounds are examples of wounds that will heal by secondary intention (Fig. 8.1). A wound healing by secondary intention would typically be covered with a wound dressing.

8.3 Background to Wound Dressings

The management of wounds has been a common challenge to clinicians throughout recorded history with several authors reporting upon developments in wound healing over time (for example [7–9]). Much of this discussion focused upon the processes involved in wound healing including stopping bleeding from the wound, the prevention of infection and wound contraction; however open wounds were seen to require an appropriate dressing. Early wound dressings included grease, absorbent dressings and a range of natural products including lizard and donkey excrement, cobwebs, honey, boiled puppies, ground shellfish, burned bones and earthworms [8, 9]. While many of these interventions may seem strange, honey has reappeared in modern wound management [10], and todays' commonly used silver-containing dressings [11] may be the 'descendants' of preparations containing other metallic elements such as lead and copper [7].

Up to the early 1980s, very little had changed from ancient and medieval times regarding wound dressings. In 1983, David and colleagues [12] reported upon the treatment of pressure ulcers in hospitals across England. Across 961 patients with 1506 pressure ulcers, 741 (49.2%) had no wound dressing. Where dressings were used, the majority were dry gauze (n = 360) or non-adherent dressings (n = 280) with limited use of advanced wound dressings (100 wounds dressed with a film dressing and 13 other advanced dressing materials including hydrocolloids). Between 1983 and the end of the century, the use of advanced wound dressings accelerated in the United Kingdom. For example, Vowden and Vowden [13] reported the dressings used on pressure ulcers encountered in all care locations in Bradford (Table 8.1). Few pressure ulcers were now left uncovered, and most of these were

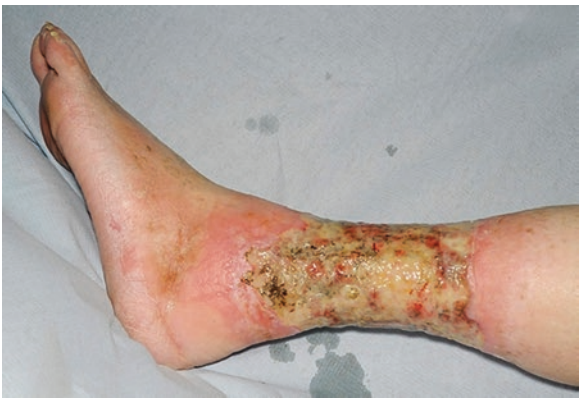

Fig. 8.1 Open wound requiring wound dressing

Table 8.1 Wound dressings applied to pressure ulcers

	Severity of pressure ulcer			
Dressing	Category I	Category II	Category III	Category IV
No dressing	12	7	1	0
Dry dressing	1	4	0	0
Film	7	5	0	0
Hydrofibre	0	7	10	10
Hydrocolloid	11	45	9	1
Antimicrobial	2	18	14	18

Reprinted from Vowden and Vowden [13], Copyright 2009, with permission from Elsevier

areas of damaged but intact skin (Category I pressure ulcer). Dry dressings, common in 1983, were only used on five wounds [13]. By 2009, clinicians appeared to be using advanced wound dressings selectively upon different presentations of pressure ulcer. Where the skin was broken, but damage restricted to the epidermis (Category II pressure ulcers), foam and hydrocolloid dressings were most prevalent. As the severity of the wound increased involving tissues below the dermis, then antimicrobial dressings were commonly used. So between 1983 and 1999, wound dressings were both more commonly used, and their complexity increased.

Today, clinicians are faced with a wide range of choices of products with 958 products listed on an on-line compendium of wound care products, most of these being wound dressings [14]. The vast range of wound dressings available to clinicians poses questions regarding how clinicians are expected to discriminate between the hundreds of wound dressings now available and to make an appropriate dressing selection for each wound they encounter.

8.4 What Is the Ideal Wound Dressing?

Turner [15] described 11 characteristics of an ‘ideal’ wound dressing:

- It should maintain a high humidity at the wound surface.
- It should remove exudate from the surface of the wound.
- It should not prevent gaseous exchange through the dressing.
- It must keep the wound surface warm.
- It should not allow bacteria to enter the wound from the external environment.
- It should not leave particles of the dressing material in the wound.
- It should be easily removed without damaging the wound and surrounding skin.
- It should be available in a range of sizes that will cover most wounds.
- It should be able to cope with dry wounds and wounds with heavy exudate levels.
- It should be conformable to the skin surface and be easy to handle when dry and wet.
- It should be sterile and stable when in storage and be easy to dispose of.

This list of the properties of the ideal dressing dates to times when few advanced wound dressings were available and concluded that the future would see dressings specified for individual wound aetiologies and for different stages of the wound healing continuum [15]. Perhaps we now practice within that 'future' where wound dressings are selected for different presentations of wound and with greater emphasis placed upon the ability of a dressing to manage specific phases of wound healing, for example, odour control, debridement and exudate management.

8.5 Alginate Dressings in Wound Healing

The preceding discussion has explored the development and increased use of wound dressings in wound management over the past 30 years. One of the earliest classes of advanced wound dressing that remains in widespread use today is alginate dressings, of which 48 types are currently available to clinicians in the UK National Health Service [14, 16]. Seaweed has long been recognised as an aid in wound healing dating back to the Romans and then in later use among sailors to stop bleeding and by doctors draining abdominal wall abscesses; however it is challenging to verify these anecdotes [17–19]. After the Second World War, alginate dressings were in use across 70 UK hospitals as haemostatic agents initially in surgical wounds with their use extending into accident and emergency departments [17]. However, the early use of alginate dressings came to a halt in the early 1970s when general alginate production reduced due to the availability of cheaper alternative products [17] with a resurgence in interest in alginate dressings in the early 1980s as interest in wound healing and the role of advanced dressing materials expanded.

8.6 How Do Alginate Dressings Work?

Alginate dressings are founded upon the mixing of sodium carbonate or sodium hydroxide with the alginic acid extracted from seaweed [17, 19] to form sodium alginate. The sodium alginate is then forced under pressure into a solution of calcium salt leading to the formation of fibres of calcium alginate. To the calcium alginate, sodium alginate may also be added to help accelerate the process of gelling when in contact with the fluid leaking from a wound (wound exudate). Commercially manufactured alginate dressings will vary both in the mix of calcium alginate and sodium alginate fibres but also in the proportion of the chains produced using the constituting monomers of the alginic acid (β-D-mannuronic acid and α-L-guluronic acid). M-group chains contain all β-D-mannuronic acid, while G-group chains contain all α-L-guluronic acid; the final chain (MG group) contains alternate units of β-D-mannuronic acid and α-L-guluronic acid [19]. The relative proportions of the M and G groups present will influence the interaction of the alginate dressing with the wound. When an alginate dressing is in contact with a wound, there is an ion

exchange between the calcium ions in the dressing and the sodium ions in blood or wound exudate; when this exchange reaches a point where sufficient calcium ions have been exchanged, the alginate fibres begin to swell and partially dissolve forming a gel. Gel formation is faster where M groups predominate with the resulting gel softer and more elastic than the gel produced by a predominantly G-group alginate dressing. While dressings with predominantly M groups gel faster, the greater dissolution of the fibres affects dressing removal which is typically undertaken through irrigation of the wound to remove the partially dissolved fibres. Predominantly G-group alginates swell less and so can be removed from the wound as an intact dressing [19].

8.7 Indications and Contraindications for Alginate Dressing Use

Advanced wound dressings, including alginate dressings, are designed to maintain a moist wound bed environment. Additionally, alginate dressings help to absorb wound exudate and stop bleeding at the wound site [17]. Further claims for alginate dressings include management of wound related pain, reduced microbial contamination of the wound, reduced odour from the wound and the absorption of proteinases [20–22].

One of the main reasons for selecting alginate dressings is their ability to absorb wound exudate with alginate dressings capable of absorbing 15–20 times their own weight in wound exudate [23]. Given the use of alginate dressings to manage exudate, it is not surprising that these dressings are used across a wide range of wound aetiologies including diabetic foot ulcers, pressure ulcers, venous leg ulcers, cavity wounds, traumatic wounds, post-operative wounds, malignant wounds, donor sites, pilonidal sinus wounds and partial thickness burns [20–22, 24–27]. Nonadhesive alginate dressings require a second dressing to be applied to hold the alginate in place over the wound with a second absorbent dressing such as a pad or foam likely to be used although semipermeable film dressings have also been used to retain alginates in place at the wound site [19]. Clark [19] discussed the current clinical uses of alginate dressings noting that these dressings are typically left in place for between 5 and 7 days. The dressing may be changed earlier if it has reached its maximum absorptive capacity and wound exudate passes through the dressing to contaminate the secondary dressing. If the alginate dressing covers both the wound and its surrounding skin, skin maceration may occur if the alginate becomes soaked in wound exudate. To prevent this maceration, it is possible to cut the alginate dressing to fit the shape of the wound, and to further safeguard the skin surrounding the wound, a peri-wound skin protectant can be applied. Over the course of wound treatment, the volume of exudate reduces, and the alginate may become adhered to the wound and surrounding skin. If this occurs, the alginate should be moistened before any attempt is made to remove the dressing and alternative dressings considered as the wound begins to reduce exudate production.

Manufacturers have produced several variants of alginate dressing ranging from flat sheets through to ribbons and ropes [17]. Alginate dressings produced as ropes or ribbons are intended to be introduced into cavity wounds, and to assist this, probes may be included with some rope and ribbon alginates to help pack the wound cavity. Flat sheet alginate dressings are typically used to cover superficial wounds. In addition to flat sheets, rope and ribbon alginate dressings may be superabsorbent (to help manage exudate) and present either as adhesive or nonadhesive dressings [17].

Beyond the management of wound exudate, alginate dressings can also be used to control bleeding at the wound site. However, once bleeding stops, the alginate dressing impregnated with blood should be removed before it dries and adheres to the wound [19]. If the dressing dries, removal of the dressing becomes challenging and is likely to be painful. While alginate dressings are intended to control bleeding, these are not indicated for heavily bleeding wounds which may require diathermy or cautery. Other contraindications for the use of alginate dressings have been reported [19] including full thickness burns, tumours with friable tissue, surgical implants, dry wounds, wounds with little exudate and where allergies to any element of the dressing are encountered.

The treatment of infected wounds often involves the use of dressings with antimicrobial properties [17], and alginate dressings have been impregnated with silver to provide antimicrobial activity [28–30] indicating that the silver-impregnated alginate dressing may be considered for use where wounds are infected although such dressing combinations should be used following the general recommendations for use for antimicrobial dressings [31].

8.8 Do Alginate Dressings Work in Clinical Practice?

There is a general lack of robust clinical evidence for the effect of wound dressings within wound healing [32]. The reasons for this are varied – among these are a lack of research funding to allow large-scale clinical trials; challenging methodological issues around masking patients, clinicians and researchers to the dressings allocated to study participants; and the strong commercial drive towards funding evaluations leading to multiple small studies which are often uncontrolled. The general weakness in the evidence base for the effect of wound dressings is equally applicable to alginate dressings.

Thomas [17] provided an excellent overview of the clinical studies of alginate use across a range of wound aetiologies including pressure ulcers, leg ulcers, burns and donor sites, foot care, surgery and dental practice. Of the 117 references cited all bar 13 dated to before 2000 and often report the use of products no longer commercially available. The Cochrane Wounds Group (http://wounds.cochrane.org) reports systematic reviews of the literature reporting controlled trials of wound healing interventions in human subjects. Since 2013, the Wounds Group have published three reviews of the role of alginate dressings in the treatment of diabetic foot ulcers [33], leg ulcers [34] and pressure ulcers [35]. Across the three reviews, there were 17 controlled studies with 1006 participants. Alginate dressings were com-

pared with a wide range of control interventions including hydrocolloid dressings (four studies), a silver-containing hydrocolloid, foam dressings, a wound contact layer, a non-adherent dressing, alternative alginates including a silver containing alginate and finally radiant heat therapy. Across all the studies, there were no statistically significant differences between the outcomes achieved using the alginate dressings and the comparison groups although the authors often noted the low to very low quality of the available studies.

Post-closure of the literature searches for the Cochrane reviews, one further clinical study of alginate dressings is available. Monsen et al. [36] reported outcomes of treatment of groin infections using negative-pressure wound therapy (NPWT) and alginate dressings in a small study (n=16 participants). The median time to re-epithelialisation was shorter in the NPWT group (57 versus 104 days), while the alginate-treated group required more dressing changes, and there was less pain affecting sleep and relationships with other people in the NPWT arm. This study suggests that the long-established use of alginate dressings now provides a potential control arm against which more recent interventions can be assessed.

8.9 Conclusions

While alginate dressings have been used for around 30 years in wound healing, their use is now relatively limited in clinical practice. Vowden and Vowden reported the use of advanced wound dressings used to treat leg ulcers [37], pressure ulcers [13] and acute wounds [38] across the population of Bradford, UK. Of the 1671 wounds reported, only 57 were dressed with an alginate dressing. The apparent currently limited use of alginate dressings may offer opportunities for a revival of alginate dressings potentially through the manipulation of the sodium and calcium alginate fibre M- and G-group composition along with the introduction of silver and other antimicrobial agents. Such refinements in alginate manufacture may lead to increased fluid handling properties combined with antimicrobial effects. Thomas [17] suggested four areas for exploration which might help revive interest in alginate wound dressings:

- Can changes in the chemical composition of alginate dressings be correlated with either wound healing or wound infection rates?
- Can the composition of alginate dressings be altered to stimulate cytokine production?
- Can alginate dressings be manipulated to increase their absorption of bacteria and proteolytic enzymes?
- Can alginate dressings be used to treat infected or malodorous wounds?

Clark [19] added a fifth question to this list:

- Can alginate dressings be used to manage wounds that have blood as part of the wound exudate?

Without investment to answer these questions, it is unlikely that the clinical use of alginate dressings will increase with other dressing technologies, also designed to manage exudate. Perhaps the time is fast approaching when alginate wound dressings will have no role within wound management having been effectively replaced by other wound technologies.

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Chapter 9
Alginates in Metabolic Syndrome

Senthil Arun Kumar and Lindsay Brown

Abstract Alginates extracted from seaweeds are widely used for nutrition, but they are underutilised for the prevention or reversal of human disease. Alginates are long chains of α-L-guluronic acid and β-D-mannuronic acid from brown seaweeds that act as readily available, low cost, non-toxic and biodegradable biopolymers. Sodium alginates are primarily used for the management of gastrointestinal tract disorders, but they are of potential use to attenuate the components of the metabolic syndrome including obesity, type 2 diabetes, hypertension, non-alcoholic fatty liver disease and dyslipidaemia. As prebiotics, alginates changed the gut microbiome to increase production of short-chain fatty acids as substrates for *Bifidobacteria*. Alginates inhibited pancreatic lipases and so decreased triacylglycerol breakdown and uptake. Treatment with alginates decreased food intake by inducing satiety and increased weight loss in patients on a calorie-restricted diet. Both glucose and fatty acid uptake were reduced. In rat models of hypertension, alginates decreased blood pressure. An alginate-antacid combination is an effective treatment of gastric reflux disease by forming a raft on the gastric contents. Alginates are important as drug carriers in microparticles and nanoparticles to increase drug bioavailability, for example, in drugs used for treatment of metabolic syndrome. Alginates are also used to protect cells during transplantation from immune responses of the host, allowing potential long-term control of some endocrine disorders such as type 1 diabetes and increased thermogenesis by brown adipocytes in obesity. There are many potential uses for these versatile biopolymers in the treatment of human disease.

Keywords Alginates • Metabolic syndrome • Gastrointestinal tract • Hypertension • Inflammation • Nanoparticle delivery • Cell transplantation

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9.1 Introduction

The human diet in Japan, Korea, China, Vietnam and the Philippines has included seaweeds for hundreds of years. As potential functional foods, seaweeds may prevent or treat disease in addition to their nutritional advantages [1, 2], but their usefulness is underestimated. Seaweeds are aquatic photosynthetic plants separated into macroalgae and microalgae, with macroalgae classified into three types: brown algae (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta) [3]. Brown seaweeds contain alginates as viscous water-soluble polysaccharides that consist of (1,4)-linked chains of α-L-guluronic acid and β-D-mannuronic acid as the major sugar residues [4, 5]. The concentration of alginates can be high in seaweeds, for example, 15–30% in *Ascophyllum nodosum* (rockweed or Norwegian kelp), 20–45% in *Laminaria digitata* (oarweed) and 21–42% in *Alaria esculenta* (dabberlocks or winged kelp) [6]. Brown seaweeds such as *Sargassum* sp. are widely used in food and have been used in Traditional Chinese Medicine for nearly 2000 years as potential anticancer, anti-inflammatory, antibacterial and anti-viral medicines [7]. *Laminaria japonica*, a source of alginate and fucoidan, as well as fat-soluble components, such as fucoxanthin and fucosterol, is widely used in Japan as a healthy food (kombu) that may prevent obesity and diabetes [8]. The physiological responses to consumption of seaweeds containing alginates mainly involve the gastrointestinal tract, including increased gastric distension, delayed gastric emptying and enhancement of satiety together with delayed postprandial glycaemia and insulin responses [9]. Sodium alginates have found applications in the management of gastrointestinal and metabolic complications, primarily of the components of the metabolic syndrome including obesity, type 2 diabetes, hypertension, non-alcoholic fatty liver disease and dyslipidaemia [10–12]. This chapter will discuss the role of alginates to improve health, primarily based on their changes in gastrointestinal function.

9.2 Alginates in the Gastrointestinal Tract

Alginates have multiple effects on gastrointestinal function including reduction of intestinal absorption rates and systemic effects, decreased uptake of fats and reduced plasma cholesterol, increased faecal bile and cholesterol excretion, reduction in blood peak glucose and plasma insulin rise, stool bulking, adsorption of toxins found within the colon, alteration of colonic microflora, direct effects on colonic mucosa and increased sensation of satiety and reduced caloric intake [10]. Some of these will now be further examined. Polysaccharides from seaweeds and microalgae such as alginates, fucoidans and carrageenans may act as prebiotics [10, 13]. Prebiotics are long-chain carbohydrates that are not broken down in the stomach but metabolised by bacteria in the colon to short-chain fatty acids such as acetate, butyrate and propionate which serve as metabolic substrates for some gut bacteria [14–16]. Treatment with alginates (10 g/day) in healthy male volunteers enhanced the

growth of beneficial gut microbes, particularly *Bifidobacterium* species, with a concomitant decrease in Gram-negative *Enterobacterium* and *Clostridium* species, with increased acetate and propionate production and decreased release of toxic metabolites such as sulphide, *p*-cresol and indole in the faecal samples [14].

Pancreatic lipase is an important enzyme in triacylglycerol breakdown in the gastrointestinal tract. Therefore, inhibition of this enzyme is a potential mechanism for the reduction of obesity as shown by orlistat, a commonly prescribed antiobesity medication. Alginates also inhibit pancreatic lipase [10]. Alginates high in guluronic acid from *Laminaria hyperborea* inhibited lipase activity more than alginates high in mannuronic acid from *Lessonia nigrescens* suggesting that guluronic acid-rich alginates could help in treating obesity by reducing dietary triacylglycerol uptake [17]. Interactions between the negatively charged alginates and positively charged proteins are more likely at low pH, as in the stomach [10]. Testing alginates in a bread vehicle using a model gut showed that alginates retained their lipase inhibitory properties despite cooking at 150°C, showing the potential for this product in obesity [18].

Altered satiety signalling plays an important role in type 2 diabetes and obesity, both key components of metabolic syndrome [19]. Treatment of healthy humans with sodium alginate treatment at 9.9–15 g/day reduced energy intake by inducing a feeling of satiety, probably caused by increased viscosity causing swelling in the gastrointestinal tract [20]. This gelling effect plays a central role in delaying the gastric emptying by increasing stomach extension in the antrum and in slowing down nutrient absorption in the small intestine. An increased guluronic to mannuronic acid ratio increases viscosity and gel strength of the sodium alginate and so could increase satiety [21]. A short-term trial using guluronic acid-enriched alginates together with calcium or pectin for 7 days increased satiety in overweight individuals [22, 23]. This protocol reduced daily energy intake by 134.8 kcal (7%) associated with reductions in mean daily intake of sugar, saturated fats and proteins [23]. In contrast, 10-day treatment with CM3 alginate, a compressed, lyophilised sodium-alginate active complex, based on the brown seaweed *Laminaria digitata*, had no effect on satiety, appetite, gastric function or gut hormone secretion [24].

There are only limited studies on weight-reducing effects of alginates, despite newspaper articles with anecdotes that alginates in seaweeds can help control obesity (e.g. http://www.telegraph.co.uk/foodanddrink/foodanddrinknews/11853491/Can-seaweed-really-help-you-lose-weight.html). The paucity of studies is surprising, given the evidence showing weight loss with administration of prebiotics [25]. There is solid evidence that alginates moderate many mechanisms that should assist in weight management [10, 26], but there are few studies demonstrating an anti-obesity effect. In a single study, patients on a calorie-restricted diet of 300 kcal/day showed a further increase in weight loss from 5.0 to 6.7 kg when given 15 g fibre as alginates three times a day for 12 weeks, mainly as a reduction of body fat, while plasma markers of glucose and lipid metabolism and inflammation were unchanged [27]. Long-term studies remain necessary to define the anti-obesity effects of alginates [26].

9.3 Alginates in Metabolic Changes

Insulin resistance or diabetes together with dyslipidaemia are used as clinical signs to define metabolic syndrome. High-viscosity dietary fibres, including guar gum and alginates, when incorporated in edible crispy bars containing 50 g carbohydrate, attenuated postprandial glycaemia without any change in gastrointestinal tolerance in healthy adults [28]. Supplementation with alginates and calcium in rats attenuated postprandial glycaemic responses in streptozotocin (STZ)-induced diabetes in rats, probably due to increased viscosity as well as calcium-induced gel formation [29]. This increased gelling is likely to delay gastric emptying and decrease nutrient absorption in the small intestine, and both changes will decrease postprandial glycaemic responses and attenuate peak insulinaemic responses [26].

In male patients, cholesterol uptake from a fixed diet increased with increasing body fat; a single administration of 1.5 g sodium alginate with calcium carbonate decreased uptake of glucose, cholesterol and triacylglycerols to the levels in healthy subjects [30]. In rats fed a high cholesterol diet, addition of 2% calcium alginate to the diet decreased plasma cholesterol concentrations, possibly due to an increased bile acid excretion due to reduced intestinal reabsorption [31]. The gelling of both high and low molecular weight alginates from *Laminaria angustata* in the stomach was proposed as the mechanism for the reduced glucose uptake and insulin response and increased cholesterol excretion from the gastrointestinal tract [32]. These studies suggest that the gastrointestinal changes induced by alginates can reduce dyslipidaemia in overweight/obese patients.

9.4 Alginates in Hypertension

Hypertension is one of the diagnostic criteria for metabolic syndrome, and, further, metabolic syndrome increases the risk of cardiovascular disease. Oral administration of low molecular weight potassium alginates (250 or 500 mg/kg body weight) extracted from brown seaweeds normalised the cardiovascular changes in DOCA-salt hypertensive rats to a greater extent than the same dose of potassium chloride [33]. Sodium alginate oligosaccharides (60 mg/kg given subcutaneously) almost completely abolished the increased blood pressure in Dahl salt-sensitive rats fed 4% sodium chloride; this response may be due to improved kidney function with decreased sclerosis and vascular injury in the kidney, together with direct effects on vascular function, rather than by reducing salt absorption [34]. Dietary sodium alginate oligosaccharides given as a 4% intervention in the diet induced small changes in systolic blood pressure in male SHR, but renal glomerular damage was markedly decreased [35]. In obese patients, sodium alginates had no effect on borderline hypertensive patients with a baseline systolic blood pressure of 132.7 ± 2.2 mmHg [27]. No studies were found that reported changes in blood pressures in hypertensive patients following alginate interventions.

Alginates derived from *Sargassum vulgare* have shown antitumour activity in mice. However, these mice developed acute tubular necrosis, suggesting intrinsic

nephrotoxicity, producing increased perfusion pressure, renal vascular resistance, glomerular filtration rate, urinary flow and sodium, potassium and chloride excretion and reduction of chloride tubular transport, possibly due to direct vascular effects [36]. These actions could be due to direct actions on the renal vasculature, as shown for mesenteric blood vessels [36]. No studies were found showing toxicity of alginates in heart or liver or in humans.

9.5 Alginates in Gastric Reflux Disease

Alginates have been given to relieve gastric reflux for many years. They precipitate upon contact with gastric acid to produce low-viscosity gels of near-neutral pH, triggering the sodium bicarbonate in the formulation to release carbon dioxide in the gel, which then floats on the stomach contents as a raft close to the oesophageal-gastric junction [37]. Combination of calcium carbonate and sodium bicarbonate with sodium alginate reduced gastric reflux episodes and increased time to reflux symptoms compared to patients given antacid only [38]. This study showed that the alginate-antacid raft was localised to below the diaphragm in these gastric reflux patients [38]. Despite a substantial placebo response, an alginate-antacid combination reduced heartburn, regurgitation and dyspepsia in a randomised trial of 1107 patients with mild-to-moderate symptoms [38].

9.6 Alginates in Liver Disease

Obesity increases the risk of developing non-alcoholic fatty liver disease (NAFLD), which may develop into non-alcoholic steatohepatitis (NASH) and then progress to hepatocellular carcinoma [39]. In monosodium glutamate-treated mice with NASH symptoms, oral sodium alginate treatment improved liver steatosis, insulin resistance and chronic inflammation, and prevented the progression to carcinoma [11]. Translation to humans with NAFLD or NASH has not been reported.

9.7 Alginates in Inflammation

Obesity is defined as a chronic inflammation [40], yet no studies have reported anti-inflammatory effects of alginates in obese rats or humans. Adjuvant-induced arthritic rats as a model of rheumatoid arthritis when treated with alginate from *Sargassum wightii* showed decreased paw oedema, reduced activities of inflammatory enzymes and reduced plasma inflammatory biomarkers [41]. However, anti-inflammatory compounds such as indomethacin will induce gastric and small intestinal ulcers. Sodium alginate has been proposed as a treatment to prevent indomethacin-induced small intestinal injury as mice showed reduced intestinal injury and reduced expression of mucin following alginate treatment [42] (Fig. 9.1).

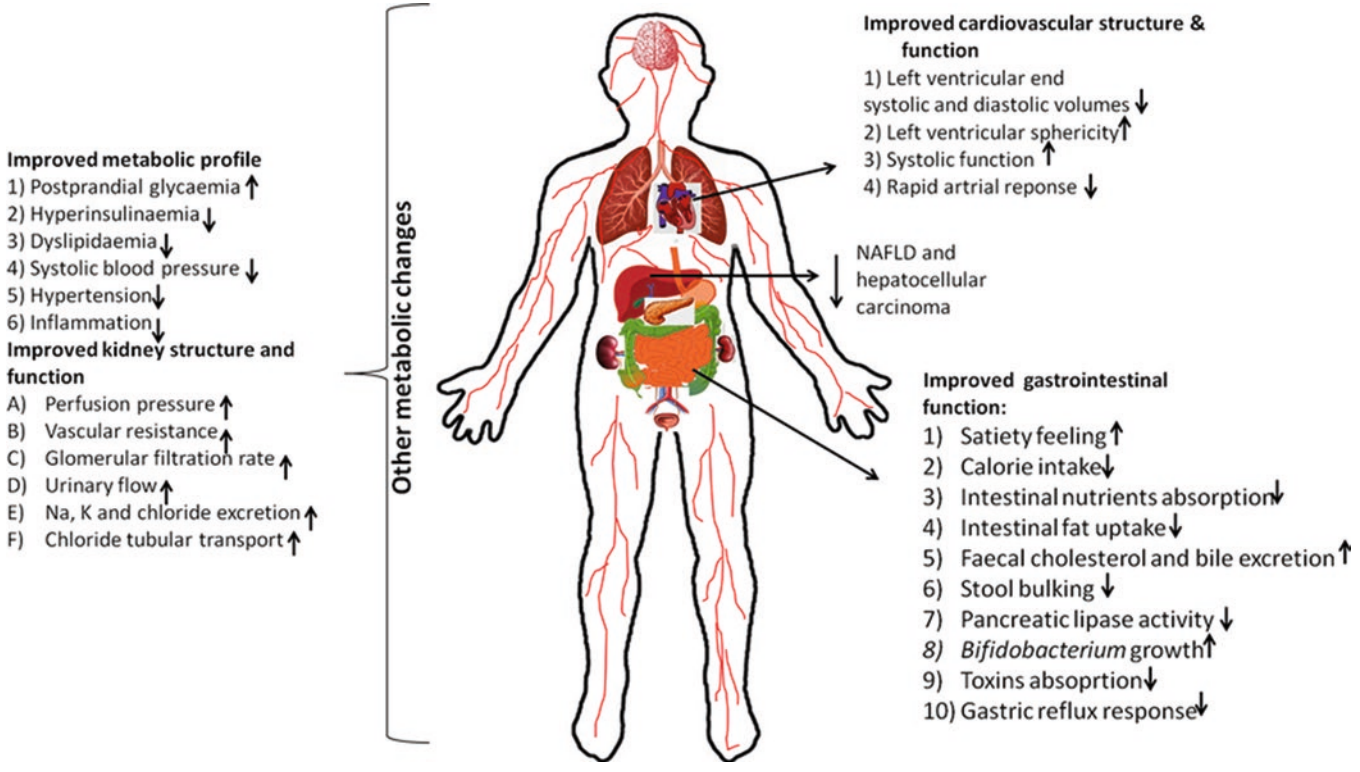

Fig. 9.1 Possible therapeutic effects of alginates in the attenuation of metabolic complications. NAFLD non-alcoholic fatty liver disease; (↑) increased response; (↓) diminished response

9.8 Alginates as Drug Carriers for Treatment of Metabolic Syndrome

Alginates are readily available, low cost, non-toxic, biodegradable and versatile biopolymers and are therefore useful as drug carriers for therapy, for example, as hydrocolloids in sustained-release products [43]. Further development to produce nanoparticles with improved drug delivery is one of the success stories in pharmaceutical technology in the last 20 years, with a wide range of techniques being available for their preparation [44]. The effectiveness of nanoparticles depends on their size and surface area, with a wide range of possible shapes giving a range of potential applications [45]. As an example, the oral bioavailability of insulin has been improved by formulation as polymer-based nanoparticles, including alginate, but these products have not reached the market [46]. The preparation of alginate microparticles and nanoparticles has been summarised, and future challenges have been outlined [47].

There is now clear evidence that alginate-containing microparticles of oral hypoglycaemic drugs could be effective in type 2 diabetic patients. Metformin encapsulated in alginate floating beads produced greater decreases in blood glucose concentrations in Sprague-Dawley rats made diabetic following injection of streptozotocin (60 mg/kg ip for 3 days) than with metformin alone [48]. Microcapsules of gliclazide prepared using taurocholic acid and sodium alginate decreased hyperglycaemic responses in alloxan-induced type 1 diabetic rats [49]. Exenatide delivered orally in microcapsules with alginates and hyaluronate to *db*/*db* mice normalised the blood glucose concentrations for 2 h; this response could be prolonged until 4 h with increased exenatide doses for effective control of type 2 diabetes [50]. These studies show the potential of micro- and nanoparticles to increase treatment options for type 2 diabetes. These techniques may also apply to insulin treatment of type 1 diabetes, now exclusively given subcutaneously. Oral administration of chitosan-alginate insulin nanoparticles reduced blood glucose concentrations in alloxan-diabetic mice more slowly than subcutaneous insulin, with bioavailability of approximately 8% [51]. Liver damage is common in diabetes. One possible alternative for treatment of liver tumours is the use of alginate microspheres with the antineoplastic drug, amonafide, that causes serious adverse effects with oral delivery, to achieve targeted delivery with reduced systemic toxicity [52]. Another option for intracellular targeting of liver tumour cells is the use of microspheres with mesoporous silica nanoparticles together with alginate providing high biocompatibility and sustained release [53].

Alginate-containing nanoparticles may also be useful to administer lipid-lowering drugs such as probucol [54]. The physical characteristics of these probucol nanoparticles were appropriate for treatment [54]; similar nanoparticles of probucol improved insulin release and decreased TNF-alpha production by pancreatic beta cells cultured in 25.5 mM glucose [55].

Hypertension is an important component of the metabolic syndrome. Many antihypertensive drugs have been formulated in alginate-containing nanoparticles for

oral administration to produce sustained-release characteristics, including nifedipine [56], diltiazem [57], carvedilol [58] and propranolol [59]. Possible alternative routes of administration include transdermal delivery of an alginate hydrogel containing prazosin [60] and buccal absorption of nimodipine [61]. There are no reports of studies specifically targeting hypertension in patients with metabolic syndrome using micro- or nanoparticles, but these formulations may offer advantages for specific drugs and patients. Intramyocardial injections of alginate hydrogel implants in dogs with cardiac failure following intracoronary micro-embolisations improved left ventricular structure and function with reduced left ventricular end-diastolic and end-systolic volumes, improved left ventricle sphericity and an improved systolic function with increased ejection fraction [62]. Local application of amiodarone in an alginate-based glue to the right atrial wall of goats markedly decreased the rapid atrial response to burst pacing, suggesting a potential use in postoperative atrial fibrillation in humans [63] .

9.9 Alginates in Cell Transplantation

Transplantation has a long history in endocrinology with recent studies using isolated β-islet cells or stem cells showing the potential of this procedure to restore the endocrine activity of the pancreas [64]. Procedures to improve success include treatment of the cells with alginates. In immune-competent STZ-induced type 1 diabetic C57BL/6J mice, transplantation of in vitro-derived glucose-responsive mature β-cells from human embryonic stem cells encapsulated using chemically modified alginates via the intraperitoneal route normalised blood glucose concentrations up to 174 days after transplantation with minimal graft rejection [65]. The development of an oxygenated chamber system with immune-isolating alginate and polymembrane covers allowed the survival and function of human pancreatic islets without immunosuppression [66]. Transplantation of these cells into a 63-year-old man with a history of type 1 diabetes for 54 years was followed by persistent graft function and regulated insulin secretion for at least 10 months, without immunosuppression [66].

As myocytes cannot replicate, cell transplantation is an attractive alternative to improve cardiac function after injury. Foetal cardiomyocytes grown on porous alginate scaffolds were transplanted into rats 7 days after myocardial infarction [67]. After 9 weeks, the transplanted cells had stimulated intense neovascularisation and attenuated left ventricular dilatation and cardiac failure [67].

Unlike the heart, the liver can regenerate, but hepatocyte transplantation may be needed in acute liver failure to provide short-term support. Further, these patients may require liver transplantation, a major challenge for the health system [68]. Transplantation of rat hepatocytes microencapsulated with alginate markedly improved liver parameters in a rat model of D-galactosamine-induced acute liver failure; further, recovery of microbeads on day 8 after transplantation showed no signs of adhesion or inflammation [69]. Alginate-polyethylene glycol microspheres

of human mesenchymal stem cells transplanted into mice delayed the development of fibrosis in bile duct-ligated or carbon tetrachloride-treated mice [70]. After partial hepatectomy in mice, the use of implanted alginate scaffolds supported the growth of the remaining kidney, decreasing liver injury and improving survival [71]. Alginate microspheres with adipose tissue-derived stem cells could be transplanted into recipient mice where the stem cells underwent hepatogenic differentiation to cells that secreted albumin in the liver [72].

In contrast to white adipocytes, brown adipocytes may help control obesity [73]. The encapsulation of mouse embryonic stem cells in alginate hydrogel microstrands allowed differentiation into brown adipocytes confirmed by the expression of uncoupling protein 1 which is characteristic of these cells, as well as increased expression with β_3-adrenoceptor agonists [74]. Cell entrapment within alginate microcapsules allows the cells to avoid the immune responses of the host; the use of this technique with catabolic cells that use lipids for thermogenesis may be applicable for the treatment of obesity [75]. Alginate-poly-L-lysine microencapsulated Chinese hamster ovary (CHO)-E3 cells secreted apolipoprotein E3 when given intraperitoneally to mice, leading to decreased cholesterol and increased HDL concentrations in the plasma [76]. This technology may be feasible to minimise atherosclerosis in obese and diabetic patients.

In conclusion, alginates are low cost, mostly non-toxic and versatile biopolymers that can be used for treatment of many gastrointestinal problems. In addition, they are useful in microparticles and nanoparticles as drug carriers and to protect cells during transplantation. However, the full potential of these natural products as functional foods needs to be further researched.

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Chapter 10
Alginate Oligomers and Their Use as Active Pharmaceutical Drugs

P.D. Rye, A. Tøndervik, H. Sletta, M. Pritchard, A. Kristiansen, A. Dessen, and D.W. Thomas

Abstract Alginate oligomers retain most of the chemical and physical properties of the higher molecular weight commercial alginates, retaining affinity towards monovalent and divalent ions, which is dependent on the chemical composition of the oligomer. However, due to their low molecular weight, they will normally not form gels in the presence of divalent cations. This property is exploited in biological systems to chelate multivalent ions and disrupt Ca^{2+}-mediated cross-linking. Studies have also identified interactions between alginate oligomers and complex mucin polymer systems, bacteria and extracellular polymeric substance (EPS), which suggests that these interactions are not simply the result of cationic chelation. By virtue of their low molecular weight, alginate oligomers stay in solution at high concentration without significant increase in viscosity and can be tailor-made to precisely defined chemical composition and molecular weight. This affords the opportunity to design effective formulations with precisely defined properties and biological effects. The properties now being identified for alginate oligomers represent a promising new approach in the management of chronic lung diseases, biofilm infections and antibiotic use. This chapter outlines the research performed to date, highlighting the excellent safety profile and novel chemical characteristics of alginate oligomers that emphasize their potential in multiple therapeutic applications.

Keywords Alginate oligomers • Biofilms • Mucus • Medical device • Infection control

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10.1 Alginate Oligomers: Composition, Structure and Production

10.1.1 Composition and Structure

Alginate is a linear anionic polysaccharide polymer that occurs naturally in a wide range of brown seaweeds and some bacteria. It is composed of β-$_{D}$-mannuronic acid (M) and its C-5 epimer α-$_{L}$-guluronic acid (G) residues that are arranged in homogenous blocks or random alternating units. Alginates from different sources vary in composition and length, which greatly influences their function in terms of gelation and affinity for monovalent and divalent cations. It is widely used in the food, textile and pharmaceutical industry. While alginates have been used in a wide variety of biomedical applications, these products have focused on the gelation properties exhibited by high molecular weight polymeric alginates. The molecular weight of commercially available alginates currently range from around 30,000 to 400,000 g/mol. However, the scope of this chapter is limited to the potential applications of the low molecular weight alginate oligomers (in the region of 2000–5000 g/mol). Although technically still polymers, the use of the term "oligomer" has been adopted to differentiate these low molecular weight alginates that have quite distinct functional characteristics from the gel-forming and thickening properties exhibited by their commercially available, larger polymeric siblings.

10.1.2 Production

Alginate oligomers, like their high molecular weight siblings, are commercially manufactured by extraction of harvested brown algae such as *Macrocystis*, *Laminaria* and *Ascophyllum* spp. The G-content of alginates derived from these seaweeds varies greatly, not only between species but also within the different parts of the plant. The G-content of alginate from the stipe and leaves of *Laminaria hyperborea* can contain 75% and 35% G residues, respectively. The specific G-content of alginates can be determined by nuclear magnetic resonance spectroscopy (NMR). Alginates are also produced by several species of the bacteria *Pseudomonas* and *Azotobacter*. The role of these bacterial producers has attracted a great deal of attention for several reasons. Initially the focus was motivated by identifying the role of alginate in the pathogenesis of *Pseudomonas aeruginosa* lung infections and developing an understanding of alginate biosynthesis and regulation [1–6]. During recent years there has been an increasing focus on microbial strain engineering to potentially utilize these bacterially produced or modified alginates for high-value applications [2, 4, 6–9].

In both algae and bacteria, the polymer is first synthesized as poly-mannuronate (polyM) chain, and then certain M residues are converted to G by mannuronan C5-epimerases [10]. Alginate from *Pseudomonas* spp. does not contain G-blocks

due to the expression of a single alginate epimerase, the intracellular AlgG, which introduces only single G residues into the alginate chain [1]. Conversely, *Azotobacter* species such as *Azotobacter vinelandii* produce and secrete several epimerases (AlgE1–AlgE7) that lead to alginates with a variety of physical properties and different structures including G-blocks [11, 12]. Bacterial mannuronan C5-epimerases have been isolated and extensively characterized, providing powerful *in vitro* tools to manipulate the structure of alginates, enabling the synthesis of new alginates with defined chemical composition [10]. These enzymes have also been further engineered for optimizing their properties in relation to the tailoring of alginates [13]. Unlike the alginates of algal origin, the bacterial alginates contain various degrees of O-acetyl groups associated with the M residues. Acetylated M residues cannot be epimerised, and thus acetylation is thought to be involved in controlling the level of G residues in the alginate.

The G-content and composition of algal alginates differ greatly depending on the species, the part of the algae used, the harvesting season and growth conditions, implying great diversity in the algal epimerases. Indeed, genomic analyses of the model brown algae *Ectocarpus siliculosus* and *Saccharina japonica* indicate the presence of 45 and 105 mannuronan C5-epimerases, respectively [14, 15]. Algal epimerases are apparently difficult to express in recombinant systems, and so far only the successful expression of one of the *S. japonica* enzymes has been reported [16]. This enzyme was produced in an insect-cell expression system and found to introduce alternating MG structures when polyM polymer was epimerised *in vitro*, thus resembling the *A. vinelandii* AlgE4.

To understand the chemical-physical properties, and thereby facilitate optimal tailoring of alginate structures, it is important to characterize the alginates at a compositional level. By combining the action of sequence-specific alginate lyases with analytical techniques like NMR, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS), it is possible to obtain information on the length and distribution of the different alginate block structures (M, MG and G-blocks) (Fig. 10.1). This approach has been used successfully to determine the block structure and more specifically the length of G-blocks in both natural and *in vitro* epimerised alginates [17, 18].

Alginate oligomers are prepared by controlled acid hydrolysis of high molecular weight alginates [19, 20], followed by purification and characterization. Characterization includes a determination of the G-content by NMR, protein and endotoxin content and inorganic elements by inductively coupled plasma mass spectroscopy (ICP-MS) and a determination of the molecular weight distribution by HPAEC-PAD and SEC-MALLS. HPAEC-PAD is a powerful tool for determining the distribution of oligosaccharides after acid hydrolysis of homopolysaccharides (Fig. 10.2) [21].

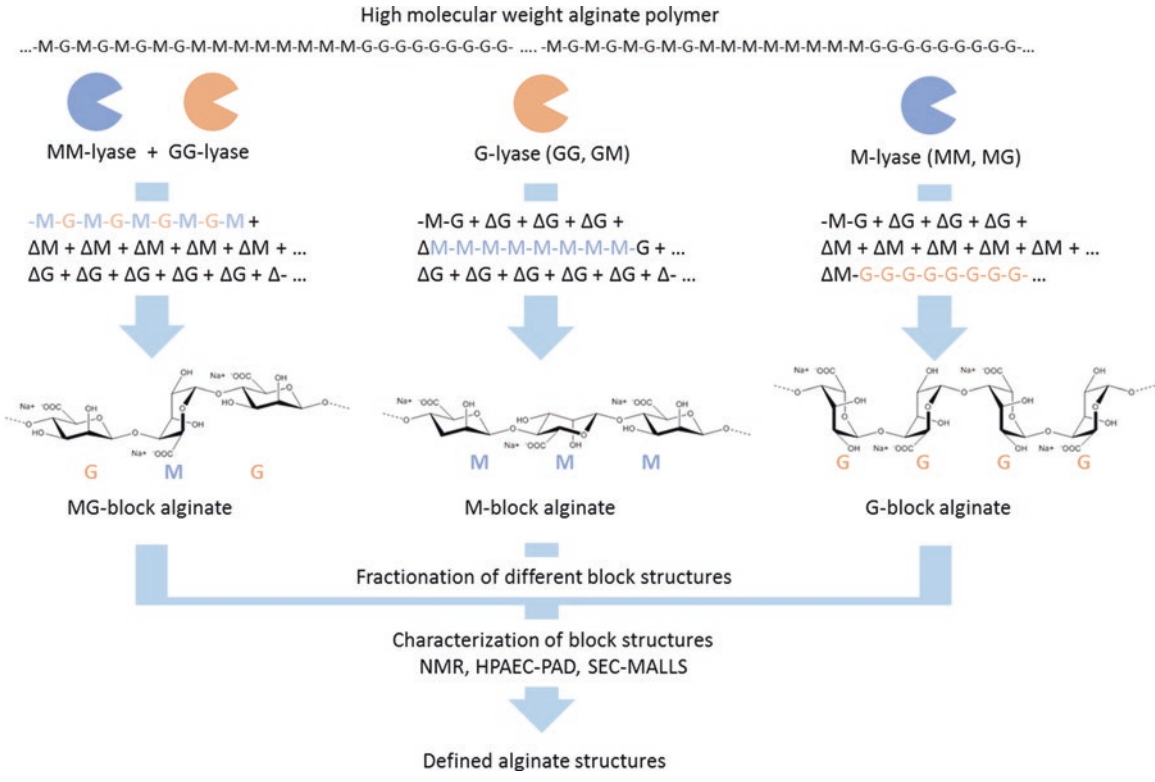

Fig. 10.1 Diagram outlining the combined role of sequence-specific alginate lyases with analytical techniques to derive alginate oligomers with defined length and distribution of block structures (M, MG and G-blocks). The lyase enzymes cleave the alginate polymer through β-elimination, resulting in unchanged saturated uronate at the reducing end and an unsaturated (4-deoxy-L-*erythro*-hex-4-enepyranosyluronate) residue (Δ) at the non-reducing end [17]

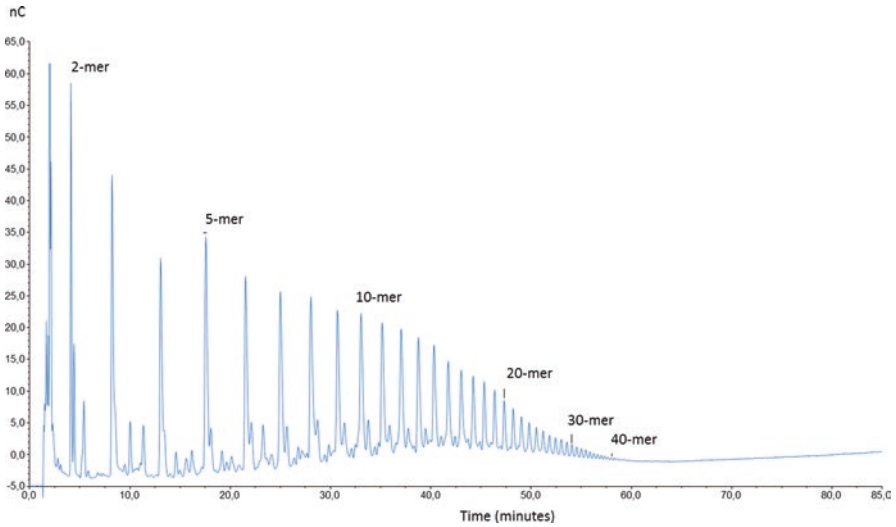

Fig. 10.2 HPAEC-PAD chromatogram of G oligomers produced by acid hydrolysis of high molecular weight G-rich alginate. The numbers above the peaks indicate oligomer degree of polymerization

10.2 Alginate Oligomer Properties

10.2.1 Chemical and Physical Properties

Alginate oligomers retain most of the basic chemical and physical properties of commercial alginates of higher molecular weight, with some exceptions. Alginate oligomers retain affinity towards monovalent and divalent ions, such affinity being dependent on the chemical composition of the oligomer. Notably, oligoguluronates can be considered as free G-blocks that readily compete for and bind to multivalent cations. However, due to their low molecular weight, they will normally not gel or introduce network connectivities [22]. This property can be exploited in biological systems by using oligoguluronates to chelate multivalent ions like Ca^{2+}, thereby disrupting Ca^{2+}-mediated cross-linking in the environment. Studies have also identified potential electrostatic interactions between alginate oligomers and complex mucin polymer systems [23, 24], bacteria [25, 26] and extracellular polymeric substances (EPS) suggesting that these interactions are not simply the result of cationic chelation. By virtue of their low molecular weight, alginate oligomers can stay in solution at high concentration without significant increase in viscosity. While alginates of higher molecular weights are usually polydisperse and contain a mixture of molecules with different chemical composition [27], alginate oligomers can be tailor-made to precisely defined chemical composition and molecular weight. This opens up the possibility of designing highly efficient formulations with precisely defined properties.

10.2.2 Biocompatibility

The biocompatibility and low toxicity of alginates have been extensively reviewed. Due to their advantageous properties and their safety profile, alginates are widely employed in the food and pharmaceutical industry. Alginate oligomers have demonstrated similar safety profiles with no safety issues reported. Alginate oligomers have been proved to be safe for inhalation, as supported by clinical safety and tolerability studies (www.clinicaltrials.gov, Identifier: NCT00970346; NCT01465529). There are currently no known mammalian enzymes that degrade alginates, and preclinical toxicity studies have shown alginate oligomers are rapidly cleared through the kidneys. The US FDA recognizes alginic acid and alginates as GRAS (generally recognized as safe) for use in foodstuff (reference no. 21CFR184.1724).

10.3 Biological Effects of Alginate Oligomers

A number of biological effects have been demonstrated with alginate oligomers that highlight their potential use in a range of clinical applications. These essentially address the challenges of mucus-biopolymer interactions such as those seen in cystic fibrosis (CF) and other related respiratory diseases and the treatment of multidrug-resistant (MDR) biofilm-related infections. The primary mechanisms of action driving these effects and their potential clinical applications are outlined in the following section.

10.3.1 Mucin, Mucus and Cystic Fibrosis

Mucins are a large family of glycoproteins which line the aerodigestive, urinary and reproductive tracks (along with DNA, lipids, proteins and cellular debris) and represent the major macromolecular component of mucus. Mucins are secreted by specialized goblet cells and rapidly assemble and "unfold" at the cell surface (in a partly Ca^{2+}-dependent process) into a hydrated, cross-linked network. Mucin production represents an effective barrier to pathogens and facilitates their removal/clearance in the respiratory tract by the action of cilia. Mucus secretion serves to protect the surface of the lung, intestine and urogenitary tract [28]. The overproduction, increased viscosity of mucus and/or failure to secrete mucus impairs this mucosal barrier function and in a range of diseases favours microbial colonization, chronic inflammation and luminal obstruction.

In cystic fibrosis (CF), a genetic disorder, the absence of a functional CF transmembrane conductance regulator (CFTR) protein leads to altered anion flow and a dehydrated luminal surface. The loss of bicarbonate secretion through CFTR further impedes normal mucus formation, which requires bicarbonate to sequester calcium

from packed condensed mucins enabling expansion and release from glands [29–31]. In CF, this altered mucus results in bacterial overgrowth and an inflammatory response with subsequent overproduction of additional viscous mucus [28] and a concomitant decrease in lung function. Dependency on chronic antibiotic therapy in CF eventually leads to the acquisition of MDR pathogens within the CF lung, with associated morbidity and mortality. The accumulation of stagnant mucus in CF patients also leads to complications observed in the CF intestine such as distal intestinal obstruction syndrome (DIOS) and small intestinal bacterial overgrowth (SIBO) [32]. The latter condition is not limited to CF but can be associated in a wide variety of conditions where an abnormal intestinal transit may result in dysbiosis such as inflammatory bowel disease (IBD). Osmotic laxatives and stool softeners are widely prescribed to treat intestinal obstructions such as those found in DIOS and constipation. However, these treatments often requiring frequent repetition are not without side-effects, including diarrhoea, vomiting and dehydration [33].

Initial *ex vivo* studies using CF sputum samples demonstrated the impressive ability of alginate oligomers to modify the shear rheology of the viscous sputum from patients with CF [26]. Subsequent studies using molecular dynamic modelling and infrared spectroscopy have shown that this is the result of specific interactions between the alginate oligomers and the mucin. Moreover, further studies of sputum from CF patients receiving rhDNase-I (a mucolytic therapy that specifically breaks up extracellular DNA in sputum) demonstrated that these alginate oligomers were also able to potentiate the activity of this mucolytic DNase. It is thought that these potentiating effects result from the direct interaction of alginate oligomers with the mucin biopolymer network in CF mucus. The respiratory effects of the alginate oligomer OligoG CF-5/20, a dry powder for inhalation developed by AlgiPharma AS, are currently being investigated in phase 2b clinical trials.

A recent study performed at Case Western Reserve University [34] used a CF mouse model to investigate the effects of an alginate oligomer in disrupting the intestinal mucus accumulation *in vivo*. While no significant changes were observed in the treated and non-treated wild-type (WT) mice, the administration of the alginate oligomer to CF mice had a significant impact on the CF intestinal phenotype. Intestinal transit times were normalized in the treated CF mice suggesting that intestinal contents could more easily move through the small intestine and consequently shorten intestinal transit time. Both short- (7 days) and long-term (25 days) treatment of CF mice resulted in a significant decrease in intestinal obstruction and dramatically improved survival to near WT levels (Fig. 10.3). The improved survival was most likely due to the reduction in accumulated mucus, which was the direct result of the alginate oligomer enabling the normal unpacking of the mucus. Although these effects have not yet been tested in other non-CF models of intestinal disease, the dramatic improvement in intestinal transit time shows promise for other intestinal complications. Indeed, evidence for the potential in other non-CF intestinal conditions is provided by the ability to (1) chelate or sequester calcium and (2) bind to mucin. Calcium is a major factor in pre-secreted mucin, and removal of calcium leads to mucin release, unfolding and expansion [35]. Several studies have shown that mucus aggregates dissolve in calcium chelators [30, 35]. In a study

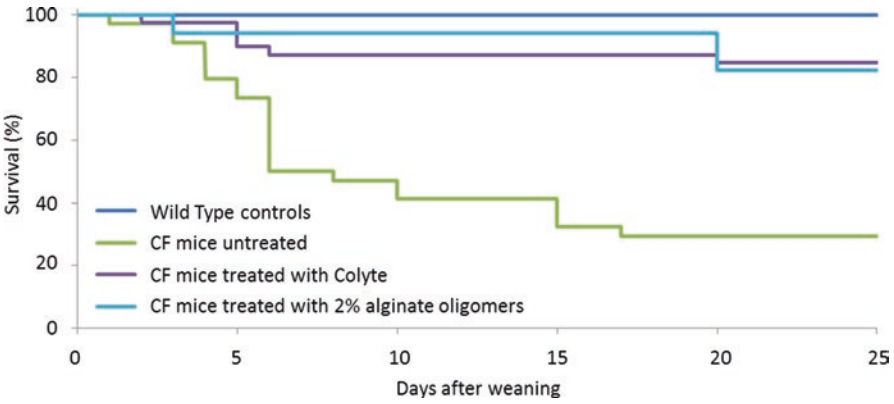

Fig. 10.3 Survival curve showing the effect of alginate oligomer treatment on the survival of CF mice [34]. Alginate oligomer administered in drinking water improved the survival of CF mice compared to untreated controls (Reprinted from Ref. [34], Copyright 2016 with permission from Elsevier)

specific to CF, the combination of bicarbonate with the calcium chelator EDTA increased the detachment of preformed CF mucus in *ex vivo* explants [36]. Although alginates in general do have calcium-chelating properties, guluronate oligomers specifically have an increased ability to bind calcium *in vitro* compared to M oligomers [37]. Several studies have demonstrated that low molecular weight guluronate oligomers are particularly proficient at sequestering calcium [22, 38] including oligoG's [39]. OligoG CF-5/20 has also been shown to act as a calcium-chelating agent which can compensate for the impaired secretion of bicarbonate in CF intestinal environment [40]. These studies showed that OligoG CF-5/20 worked by sequestering calcium away from the packed mucins that are then secreted into the intestine allowing the mucus to expand, mature and disperse [40].

Low molecular weight guluronic acid oligomers have also been shown to directly bind mucins leading to the disruption of intermolecular interactions between mucins in complex mucus polymer networks. This interaction is independent of the cation chelating ability of these G-rich oligomers, and results in an increased pore size of the mucin matrix, and altered mucus rheology [23, 24, 41]. Similarly, direct binding of OligoG CF-5/20 to mucins has been demonstrated which modifies the mucin surface charge and may explain its ability to reduce the viscoelastic properties of CF sputum [42]. Further studies are needed to examine the effect of alginate oligomers on additional intestinal alterations such as SIBO or changes in the intestinal microbiota and intestinal inflammation [43, 44].

CF is not the only disease where mucociliary clearance is impaired due to abnormal mucus viscosity. Other chronic respiratory conditions such as chronic obstructive pulmonary disease (COPD) and sinusitis are also potential application areas where the mucus rheology-modifying properties of alginate oligomers could have clinical benefit.

10.3.2 Infection Control and Antibiotic Resistance

Infection with multidrug-resistant (MDR) organisms represents a global health challenge. This is increasing annually due to (1) the inexorable rise in antibiotic consumption in medicine and animal husbandry and (2) the increasing age of the population [45]. This resistance is due to the ability of bacteria to both genetically acquire resistance mechanisms and aggregate on surfaces, forming dense three-dimensional coherent assemblies of bacteria encased within extracellular polymeric substance (EPS), which are known as biofilms. Studies have shown that bacteria within such biofilms may resist antibiotics and biocides at up to 200 times the normal therapeutic dose [46].

Surprisingly, it has been shown that alginate oligomers are able to potentiate the activity of conventional antibiotics (i.e. macrolides and β-lactams) against a range of MDR pathogens (such as *Pseudomonas*, *Acinetobacter* and *Burkholderia* spp.) by up to 512 times. These studies, with the human pathogen *Pseudomonas aeruginosa* exposed to alginate oligomers, demonstrated that the bacteria failed to develop resistance to the effects of the alginate oligomer [47].

In vivo studies, using a rodent soft tissue infection model developed at the University of Buffalo SUNY [48], showed a clear antibiotic potentiation effect for an alginate oligomer in combination with azithromycin to treat an *A. baumannii* infection. This combination treatment reduced the amount of antibiotic required from 64 µg/mL to 16 µg/mL, significantly potentiating the bactericidal activity of azithromycin in the eradication of *A. baumannii*-infected rats (Fig. 10.4).

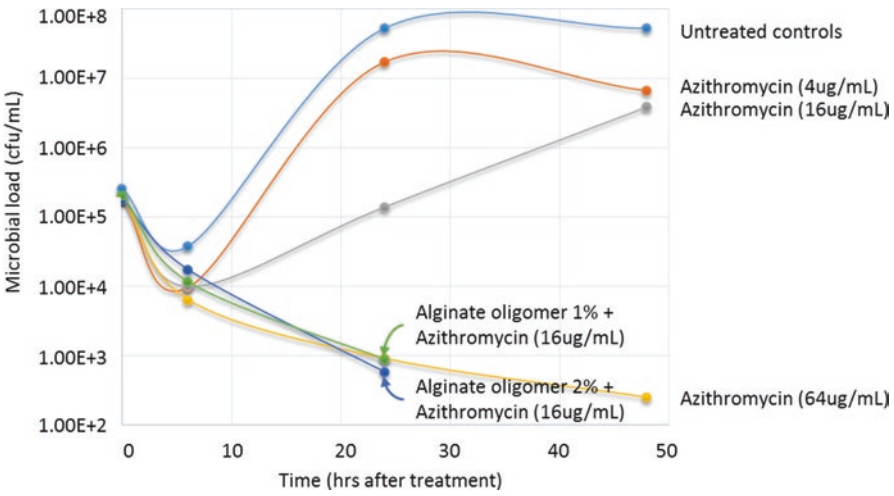

Fig. 10.4 Microbial burden in rat soft tissue animal model after treatment with antibiotic (azithromycin) alone or in combination with alginate oligomers. The infection challenge was a clinical isolate of *A. baumannii* strain 307-0294 (Unpublished data from a study performed at Prof. Russo's lab at University of Buffalo (SUNY) on behalf of AlgiPharma AS)

The increase in MDR bacterial infections has been mirrored by the corresponding rise in invasive MDR fungal infections. This represents a cause for concern since the utility of common antifungal therapy is also limited by drug toxicity. Interestingly, alginate oligomers have also been shown to modify the growth of fungal pathogens (including *Candida* and *Aspergillus* spp.), inhibiting hyphal formation [49] and invasion [50] and increasing the effectiveness of antifungal therapies by up to 16 times [51]. Alginate oligomer technology may therefore represent a considerable potential clinical benefit in addressing the challenge of bacterial and fungal infection.

10.3.3 Infection Control and Biofilms

Bacterial biofilm development is a common feature of respiratory diseases such as COPD, bronchiectasis and CF, periodontal disease, chronic skin wounds and infections associated with implanted materials.

Studies with the alginate oligomer (OligoG CF-5/20) demonstrated marked changes in microbial adherence, growth and motility, which are all key features in the development of bacterial and fungal biofilms in human disease [47]. A motility testing assay with *Proteus mirabilis* demonstrated a clear inhibition of motility in the presence of alginate oligomers (Fig. 10.5) [52]. The same group demonstrated a dose-dependent disruption in pseudomonal biofilm formation (with increased cell death). Moreover, they also showed the ability of alginate oligomers to both inhibit biofilm formation and effectively disrupt established MDR pseudomonal biofilms (Fig. 10.6). Subsequent labelling and imaging studies demonstrated the ability of these negatively charged alginate oligomers to diffuse through the dense EPS of pseudomonal bacterial biofilms [53], decreasing biofilm thickness and increasing porosity and bacterial cell death [54]. In the chronically diseased CF lung, pseudomonal Gram-negative bacteria often develop a mucoid phenotype that is characterized by the bacterial overproduction of a high molecular weight alginate [55].

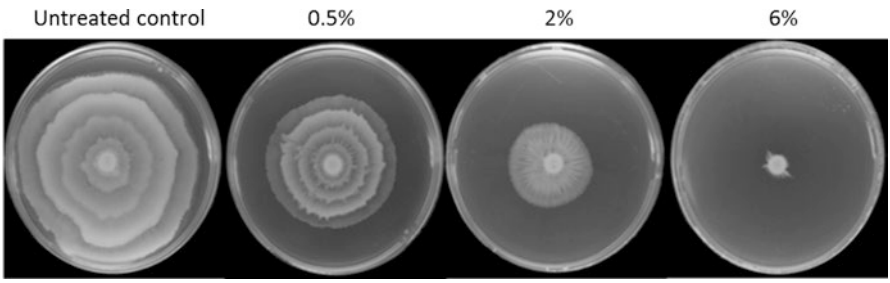

Fig. 10.5 Motility testing of *Proteus mirabilis* (NSM6) grown on ISO agar containing different concentrations of alginate oligomers (Reprinted from [47], Copyright 2012 with permission from American Society for Microbiology)

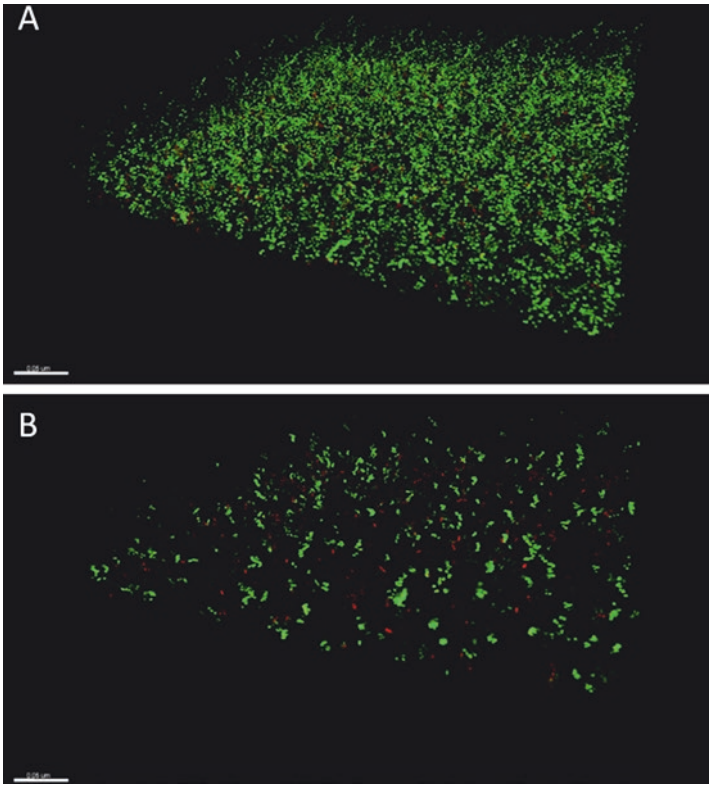

Fig. 10.6 Confocal laser scanning microscopy images of *P. aeruginosa* (NH57388A) biofilms grown for 24 h (37°C) in Mueller-Hinton broth. Cells are visualized by live/dead staining (live cells stained green, dead cells stained red). Untreated control biofilm (**a**); biofilm treated with alginate oligomer (**b**). Scale bar = 0.05 μm

Similar biofilm-disruptive effects were also observed in highly drug-resistant mucoid *Pseudomonas* spp., which is in keeping with the original report that alginate oligomers affect bacteria/alginate interactions [41, 42]. Interestingly within this mucoid biofilm model, alginate oligomer treatment was shown, both *in vitro* and *in vivo*, to potentiate the activity of the antibiotic colistin against MDR *P. aeruginosa* (by over 200 times) [46]. This group also demonstrated a dose-dependent reduction in microbial burden after treatment with alginate oligomer alone in a mouse lung infection model [46] (Fig. 10.7).

There are two potential mechanisms by which alginate oligomers might result in these biofilm changes: firstly, via a direct effect on the bacteria and, secondly, via modulation of the bacterial EPS. Since alginate oligomers were known to chelate Ca^{2+} and bind to the bacterial cell surface, it was initially assumed that they simply permeabilized the bacterial cell membrane and thus facilitated the access of antibiotics. More detailed analysis of these cell-surface interactions (using atomic force microscopy, permeabilization modelling, metabolomics and small-angle

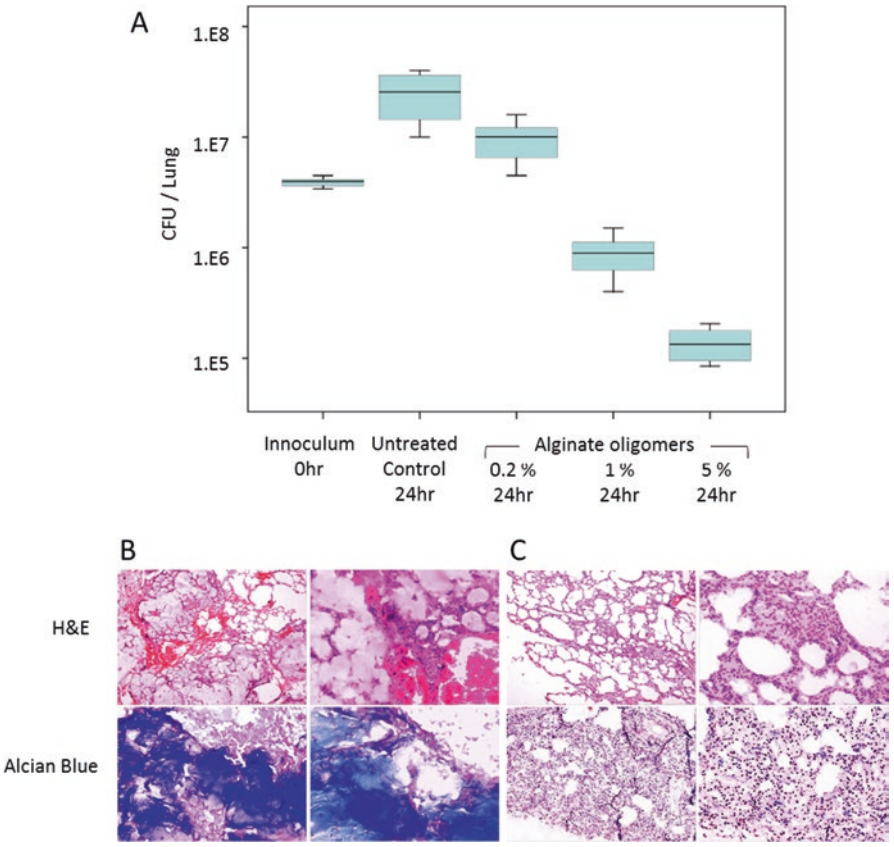

Fig. 10.7 Effect of increasing concentration of alginate oligomers on the eradication of *Pseudomonas aeruginosa* (NH57388A) biofilm-infected mouse lung. Dose-dependent reduction in microbial burden in the lungs (**a**); histology of H&E and Alcian blue staining in lung tissues of untreated (**b**) and treated mice (**c**) (magnification ×10, ×40). Alcian blue stains the biofilm EPS. (N=4 mice) (Reprinted from Ref. [46], Copyright 2016 with permission from American Society for Microbiology)

neutron scattering) demonstrated conclusively that although the alginate oligomers bind tightly to the bacterial cell surface, the binding did not induce structural rearrangement of the lipopolysaccharide membrane of the bacteria [42, 56]. Interestingly, although biofilm assembly is dependent upon local Ca^{2+} concentration, the biofilm-disrupting effects of alginate oligomers did not appear to be mediated by their Ca^{2+}-chelating properties. Within the bacterial biofilm, EPS is an extremely effective barrier to the diffusion of antibiotics and biocides, providing resistance to physical disruption. It has also been shown that alginate oligomers can induce a marked decrease in the total EPS produced and drastically decrease the production of extracellular (e)DNA in biofilms.

Many species of bacteria use a communication mechanism called quorum sensing (QS) to coordinate gene expression based on the density of the local population. This is considered an important mechanism of control in the development of cohesive populations of cells within biofilms. Since alginate oligomers were known to disrupt biofilms, there was a reasonable assumption that this would in turn influence the population balance and subsequently impact QS pathways. In *P. aeruginosa* QS is regulated by acylated homoserine lactones (AHLs). Preliminary studies appear to show that the two AHLs, 3-oxo-C12-AHL (*N*-(3-oxododecanoyl)-L-acylated homoserine) and C4-AHL (*N*-butyryl-L-acylated homoserine), are reduced after treatment with alginate oligomer [57]. As QS inhibitors target specific pathogenicity traits such as virulence determinants, there has been considerable interest in their use as novel anti-infective therapies both by screening for novel compounds and by targeted synthesis of new ligands. These studies suggest that alginate oligomers may act as QS inhibitors and may indicate a mechanistic rationale for the previously described anti-biofilm properties.

QS also plays a role in virulence factor production. In *P. aeruginosa* QS-activated virulence factors include proteases, e.g. elastase, pyocyanin, lectins, rhamnolipids and toxins. Their regulation is complex, with numerous intrinsic and environmental factors involved such as cell number, composition of the extracellular polymeric substances (EPS), matrix density and oxygen availability [58]. Pyocyanin is one of the several toxins released by *P. aeruginosa*, which not only lends the organism its characteristic colour but more importantly enables it to kill competing bacteria and mammalian cells such as in the *P. aeruginosa*-infected lungs of patients with CF [59]. Recently, alginate oligomers have shown an ability to induce changes in the expression of the virulence factor pyocyanin and elastase within biofilms [57]. Biofilm studies with *P. aeruginosa* demonstrated that alginate oligomers induced changes in QS signalling via perturbation of the N-acyl homoserine lactones signalling molecules 3-oxo-C12-AHL (*N*-(3-oxododecanoyl)-L-acylated homoserine) and C4-AHL (*N*-butyryl-L-acylated homoserine). These play a key role in controlling biofilm formation and the expression of virulence factors [57] and indicate a direct mechanistic rationale for the previously described anti-biofilm properties. As QS inhibitors target specific pathogenicity traits such as virulence determinants, there has been considerable interest in QS inhibitor development as novel anti-infective therapies [60].

10.3.4 Medical Device Coatings

Medical devices comprise a wide range of component forms, including surgical implants, catheters, endotracheal tubes, etc., and are susceptible to biofilm development with subsequent risk to the patient of infection and/or inflammation. The healthcare costs associated with infections of medical devices represents a significant burden on healthcare budgets, while the impact for the patient can mean the

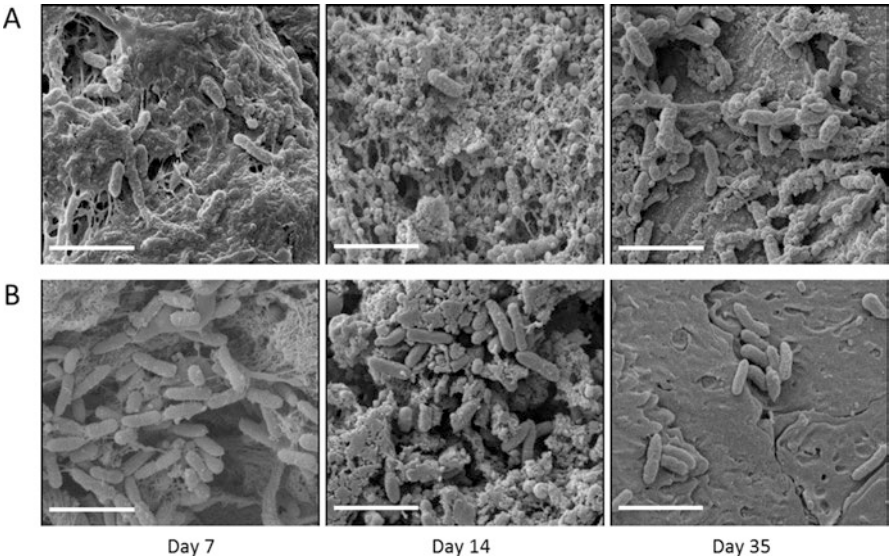

Fig. 10.8 Scanning electron microscopy (SEM) images of mixed biofilm-infected wounds, untreated (**a**) or treated with alginate oligomers (**b**). Treatment with alginate oligomers shows a marked reduction in bacteria and biofilm. SEM images were of biopsies taken at days 7, 14 and 35 postinoculation from multispecies biofilm-infected wounds. Scale bar = 5 μm. [62]

removal of the device (which is not always possible) and/or an increased use of antibiotics. Depending on the medical device, this can have life-threatening implications for the patient. Since alginate oligomers have already been shown to effectively disrupt established biofilm infections, their use in preventing biofilm contamination of medical devices represents a significant area of clinical value. Studies using alginate oligomers and their effects on oral pathogens appear to support this. Roberts *et al*. [61] showed that alginate oligomers coated onto dental materials such as titanium could inhibit the attachment of *Streptococcus mutans* and *Porphyromonas gingivalis* two important oral pathogens associated with dental carious lesions and periodontitis. This highlights the potential value of using alginate oligomers either as a preventative coating for dental implants/prostheses or simply as part of a decontamination strategy prior to device implantation.

Studies investigating a wound care application demonstrated that alginate oligomers formulated as a salve and applied to an infected wound could prevent the formation of mixed species biofilms in a pig skin burn wound model (Fig. 10.8) [62].

Clearly there are several opportunities to further exploit the properties of alginate oligomers in the coating of other medical devices, such as catheters, etc. Ventilator-associated pneumonia (VAP) is a particularly serious lung infection, and the pathophysiology of the infection is thought to be due to the endotracheal or tracheostomy

tube allowing free passage of bacteria into the lungs. *P. aeruginosa* is one of the more common organisms that are associated with ventilator-associated pneumonia as they are known to colonize and form biofilms within the endotracheal tube. Since the effects of alginate oligomers on these organisms are well established, it is not unreasonable to postulate that coating the surface of these tubes with alginate oligomers could inhibit the accumulation of biofilm on the internal surface of these medical devices, thereby reducing the risk of potentially lethal lung infections.

10.3.5 Anticoagulation

An additional property, not unrelated to the use of alginate oligomers in medical devices, is their ability to act as anticoagulants [63]. Previous studies have indicated that the high molecular weight polymeric alginates have a haemostatic effect, which is thought to be associated with their role in donating divalent cations to the coagulation process [64]. The far smaller alginate oligomer structures appear to exhibit an anticoagulant effect and may reflect an, as yet, unidentified mechanism of action. The use of alginate oligomers as a surface coating that provides not only an antibiofilm but also a haematologically compatible surface represents a valuable dual functionality for this promising technology.

10.4 Conclusions

Based on the studies to date, it is clear that alginate oligomers, with their excellent safety profiles for oral and inhaled delivery, have considerable potential in a variety of medical applications (Fig. 10.9). The indication that is most advanced in terms of clinical studies is in the symptomatic treatment of CF. It is anticipated that the combined properties of alginate oligomers will not only facilitate the clearance of the viscous sputum characteristic of the disease but also potentiate the treatment of chronic MDR lung infections that affect CF patients. The potential use of the alginate oligomers in the treatment of infections is considerable, particularly in the context of addressing MDR bacterial and fungal infections, where there are limited therapeutic options. Moreover, in contrast to antibiotic treatments that induce resistance, long-term studies with alginate oligomers have not shown any development of resistance. This application of alginate oligomer technology is of particular importance in the treatment of biofilm-related infections, such as chronic venous leg ulcers, diabetic foot ulcers and periodontal disease, where the persistence of biofilm contributes to the chronic inflammation and infection in these patients. The safety profile and chemical characteristics of alginate oligomers facilitate their application, formulation and delivery for these multiple therapeutic applications.

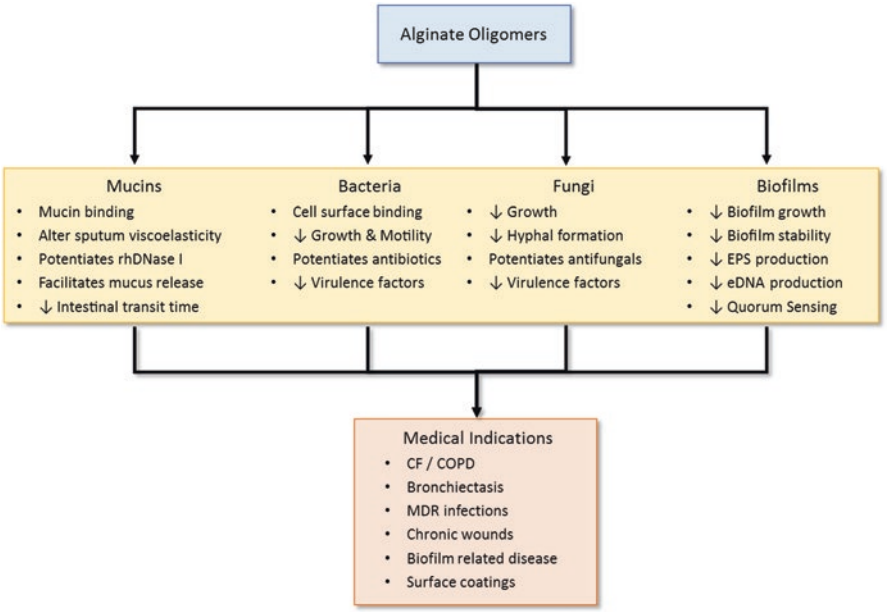

Fig. 10.9 Summary diagram of biological properties associated with alginate oligomers

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Chapter 11
Mannuronic Acid as an Anti-inflammatory Drug

Rosalia Crupi and Salvatore Cuzzocrea

Abstract Alginic acid is a linear polymer forming of β-D-mannuronic acid and α-L-guluronic acid residues that are present in the polymer chain in blocks. The D-mannuronic acid represents a newly designed nonsteroidal anti-inflammatory drug (NSAID) that has also immunosuppressive effects together with antioxidant property. D-mannuronic acid has been studied as an anti-inflammatory and novel immunosuppressive agent in several experimental models such as animal models of immune complex glomerulonephritis, nephrotic syndrome, multiple sclerosis, and rheumatoid arthritis. Both molecular mechanism and therapeutic efficacy of this new drug are based, in particular, on its inhibitory effects on matrix metalloproteinase-2 activity, immune cell infiltration in inflammatory foci, decrease of inflammatory cytokine IL-6 level, a reduction in antibody production, and induction of apoptosis. Several literature data reported no gastro-nephrotoxicity and therapeutic effects in several inflammatory diseases; for this reason it is strongly recommended as the safest drug for decreasing anti-inflammatory reactions. Moreover, recently many clinical trials were performed; results obtained support the idea that D-mannuronic acid is characterized by potent anti-inflammatory and immunosuppressive properties.

Keywords β-D-Mannuronic acid • Nonsteroidal anti-inflammatory drug (NSAID) • Toxicity

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11.1 Introduction

Alginates are natural polymers consisting of β-D-mannuronate (M) and α-L-guluronate (G) linked by 1→4 glycosidic linkage. These constitutive residues are epimers where some D-mannuronic acid residues on polymerized alginate chain are enzymatically catalyzed to convert to L-guluronic acid, hence differing at C5 position. Also, M and G residues showed different conformations within the chain structure as M residues possess $^4C_1$ conformation with diequatorial links between them and G residues are with $^1C_4$ conformation with diaxial links between them. Alginates are produced by seaweeds and some bacteria [1]. Contrary to algal alginates, bacterial alginates are *O*-acetylated at C2 and/or C3 positions of D-mannuronic acid residues [2].

Three main alginate polymers have been reported:

1. Alginates mainly consisting of D-mannuronic acid building blocks
2. Alginates mainly containing L-guluronic acid building blocks
3. Alginates composed of alternating and variable sequences of D-mannuronic acid and L-guluronic acid motifs [3]

Because of both thickening and gel-forming capacities, alginates have found vast and diverse applications as coating materials and additives in food industry. Moreover, alginates are biomaterials that have been largely employed in biomedical sciences, bioengineering, and in particular pharmaceutical applications for over 40 years without any considerable side effects [3]. They have been applied in drug delivery systems such as controlled release, film former, disintegrant, thickening, and stabilization [4]. Moreover, efficacy and safety of sodium alginate on gastric reflux control and wound care have been demonstrated [5].

The physicochemical and rheological properties of the alginates and their biological basis are determined by the variability of constitutive blocks and acetylation [6].

Alginates are common in nature since they are observed in capsular polysaccharides in several bacteria and as a structural element in some marine brown algae. Literature data showed that alginates are, in particular, synthesized by bacteria belonging to the genera *Pseudomonas* and *Azotobacter* and brown seaweeds [7, 8].

11.2 D-Mannuronic Acid (M2000): Anti-inflammatory Properties

The D-mannuronic acid, also named as M2000 and patented as 102016113018.4, is a small molecule ($C_6H_{10}O_7$) with the molecular mass of 194.139 Da (Fig. 11.1); it is a newly designed nonsteroidal anti-inflammatory drug (NSAID) with main function in controlling inflammatory diseases [9]. In the group of NSAID drugs, M2000 molecule is noted as a molecule with the lowest molecular mass and very less toxicity [10]. Numerous researches showed the importance of M2000's therapeutic and

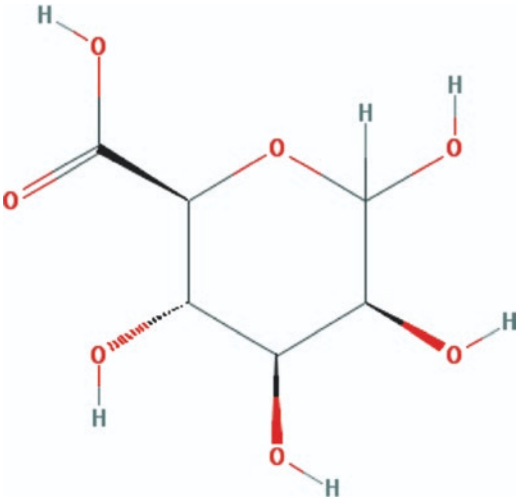

Fig. 11.1 Chemical structure of β-D-mannuronic acid

anti-inflammatory potential in different experimental animal models. Both molecular mechanism and therapeutic efficacy of this new drug are based, especially, on its inhibitory effects on matrix metalloproteinase-2 activity, infiltration of immune cells in inflammatory foci, decrease of inflammatory cytokine IL-6 level, a reduction in antibody production, and induction of apoptosis by fibrosarcoma cell line [11]. Pharmacodynamics and pharmacokinetic properties also in addition to potential therapeutic application of nonsteroidal anti-inflammatory drugs were showed by several researchers in recent years [12–16]. Especially, a major preoccupation in advising NSAIDs for therapeutic aim is their gastro-nephrotoxicity [17–21]. Moreover, dyspeptic symptoms are estimated to fall in 10–60% of NSAID users and lead to suspension of treatment in 5–15% of rheumatoid arthritis patients taking NSAIDs [22]. Literature data described that the point prevalence of peptic ulcer disease in patients treated with NSAIDs ranges between 10 and 30% [22]. Therefore, NSAIDs showed adverse effects, for example, in the gastrointestinal tract [23]. In 2000, it was described that around 165 NSAID-treated patients died because of these complications [24]. Due to the importance of adverse effects, recently several efforts have been done to create a national evidence-based guideline for studying the prevention of gastric damage provoked by usage of nonsteroidal anti-inflammatory drugs.

Moreover, NSAIDs are also characterized by renal toxicity which can limit their utility [18, 25]. The side effects reported about these organs have been ascribed in particular regarding the inhibitory effect on the cyclooxygenase activity of these drugs. However, NSAIDs' mechanism of action seems to be multifactorial and is not limited to inhibition of cyclooxygenases [26]. Other mechanisms which are possibly involved in the NSAIDs' anti-inflammatory action include inhibition of neutrophil aggregation as well as superoxide production, induction of the shedding of L-selectin, decrease in proteoglycanase activity, and gelatinase release [12, 15, 16, 27]. Matrix metalloproteases (MMPs), especially the gelatinases (MMP-9 and

MMP-2), are involved in some features of inflammatory arthritis, such as angiogenesis and bone erosions [9], as angiogenesis is a major factor of the inflammatory pannus in rheumatoid arthritis. MMP secretion through microvascular endothelial cells is an pivotal phase in angiogenesis [28, 29]. The role of M2000 in the decrease of MMP-2 activity has been thought probably similar to the efficacy of TGF-β1, sulfasalazine, and its metabolites in reducing the levels of MMP-2 activity in inflamed articulation [10, 30].

Furthermore, mesangial cells play an important role in renal inflammatory disease, and MMPs play a role in the activation of mesangial cells. Hence, increased cell proliferation amounts and extracellular matrix accumulation are focal targets in glomerulonephritis' therapy [31–33]. Therefore, MMP inhibitors deliver an innovative approach for the inflammatory therapy, maybe even beyond the field of renal disorders [33–35]. M2000 was reported to have an important advantage when compared with classical NSAIDs represented by the lowest molecular mass and no gastro-nephrotoxicity. Moreover, it was reported that M2000 possesses an antitumoral feature due to its apoptotic efficacy [36]. Based on the molecular structure, M2000 could be strongly predicted that the cell surface receptors for it may be the mannose receptor (MR), Endo 180/uPARAP from the mannose receptor family, and probably accessory receptors for M2000 would be Toll-like receptor 2 (TLR2), TLR4, CD11b/CD18 (Mac-1 or CR3), and P-selectin [37].

However, the involvement of macrophage MR (mannose receptor) in both, binding and transmission of HIV by macrophages like the fact that tissue loss during aging and age-dependent pathogenesis are the result of a disturbed regulation of proteolytic activity, and MMP-2 and MMP-9 activities especially should also be considered [38]. D-Mannuronic acid has also been tested as an anti-inflammatory and a novel immunosuppressive agent in numerous experimental models such as animal models of immune complex glomerulonephritis, nephrotic syndrome, multiple sclerosis, and rheumatoid arthritis. In particular, in the immune complex glomerulonephritis and experimental nephrotic syndrome, it has been indicated that the M2000's administration leads to a significant reduction in blood urea nitrogen (BUN), proteinuria, serum creatinine, and cholesterol or a reduction in the glomerular lesion in M2000-treated rats [11, 30]. The immunosuppressive properties of M2000 could significantly lessen clinical scoring and histological lesion in the experimental autoimmune encephalomyelitis (EAE) model [39]. The results of lymph node cell proliferation assays in this model revealed the immunosuppressive efficacy of M2000. The oral and/or intraperitoneal administration of this drug considerably decreased edema and histopathological parameters in arthritic rats [40]. In another research on Wistar rats and NMRI mice, the outcomes in chronic and subchronic toxicity studies demonstrated no significant clinical or histopathological findings, and the assessment of the hematological and biochemical indices illustrated no evidence of adverse effects systemically due to M2000 therapy. In addition, these results suggested that the oral administration of M2000 at levels up to 1250 mg/kg BW/dose did not cause any adverse effects in animals. Therefore, the use of appropriate levels of this agent can be considered relatively safe for administration in human [10]. The tolerability and biocompatibility of WHI-164, as a sensitive cell

line, against increased quantities of M2000, diclofenac, piroxicam, as well as dexamethasone have been examined. Literature data showed that 50% of cells died when piroxicam, dexamethasone, and diclofenac were added to tissue culture at doses of 25 mg/ml, 80 mg/ml, and 80 mg/ml, respectively, whereas the M2000 revealed no cytotoxic effect with 200 mg/ml if compared with steroidal and nonsteroidal drugs employed. These pharmaco-toxicological studies displayed that M2000 is the safest anti-inflammatory and immunosuppressive drug in comparison with dexamethasone and conventional NSAIDs employed [41]. Additionally, M2000 had no ulcerogenic effect on the rat stomach [41]. Moreover, using the zymoanalysis method, it was demonstrated that M2000 represents a potent MMP inhibitor, implicated in some inflammatory renal disorders and arthritis [11, 30, 40]. Another research concluded that M2000 can be tested as NSAID in LPS-induced inflammation and reduce inflammatory cytokine production by targeting the suppressor of cytokine signaling-1 (SOCS-1), phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1), and microRNA-155 in autoimmune and inflammatory diseases [42].

Recently, it was demonstrated that M2000 as a new NSAID with immunosuppressive properties is able to alter TLR signaling through suppressing the adaptor molecules IRAK1 and TRAF6, the transcription factor NF-κB, and miR-146a and finally leading to the reduction of pro-inflammatory cytokine production [33–35]. Thanks to the chemical structure of this molecule, it can be expected that it attaches to the cell surface receptors especially TLRs available on endothelial cells, monocytes, macrophages, and dendritic cells; and so this influences cell signaling. In vitro data demonstrated that following the impact of the M2000 with a low dose (5 μg/well) and high dose (25 μg/well) on HEK-Blue hTLR2 cells, it was revealed that both doses can significantly reduce miR-146a gene's expression level. Moreover, these two doses significantly reduced IRAK1 and TRAF6 gene expression levels. In addition, this study showed that the expression level of the gene NF-kB (as the main factor of the inflammatory transcription of cytokines) is significantly reduced in low and high doses. Furthermore, it was demonstrated that D-mannuronic acid downregulated MyD88 and NF-kB mRNA levels together with TNF-α and IL-6 cytokine production levels in HEK-Blue hTLR2 and HEK-Blue hTLR4 cell line in vitro [43]. This inhibitory effect is exerted via the MyD88-dependent pathway where M2000 acts as an inhibitor of TLR2 signaling [43]. Therefore, M2000 blocks signaling adaptor protein and its related transcription factor. Another important anti-inflammatory activity of M2000 was demonstrated in the management of glomerulonephritis that is the second or third most common primary renal disease type to progress to end-stage renal failure. Typically, aggressive form of the disorder is managed by the administration of steroids and a cytotoxic agent, usually cyclophosphamide followed by azathioprine [44]. Currently anti-inflammatory drugs (NSAIDs) are used extensively in clinical medicine. In spite of their therapeutic utility, they are known to provoke important gastrointestinal and renal toxicities, circumstances that limit their application [45–47]. In these organs occurred side effects that are due principally to drugs' inhibitory effect on the activity of cyclooxygenase, a key enzyme in prostaglandin synthesis. In addition to this, one of NSAIDs' mechanisms which induces renal damage is through adverse effect of

reactive oxygen species, possibly generated by activated neutrophils and mitochondrial dysfunction [47, 48]. Thus, hemodynamic renal falling may result from drugs that are able to reduce renal prostaglandins and therefore renal blood flow as well as glomerular filtration rate [49]. In contrast to piroxicam or other NSAID [47, 49, 50], M2000 did not aggravate renal damage, but, interestingly, this newly designed NSAIDs could significantly reduce renal lesions in experimental model of glomerulonephritis. However mesangial cells play an important role in renal inflammatory disease, as well as the role of MMPs in the activation of mesangial cells; in this respect, enhanced cell proliferation amounts and extracellular matrix accumulation are central targets in glomerulonephritis therapy [51–53]. Therefore, MMP inhibitors have been considered as novel approach for inflammation therapy maybe even beyond the field of renal disorders [53–55]. The literature data reported the possible therapeutic action of M2000 in experimental model of rheumatoid arthritis (RA) [56, 57]. To investigate tolerability and MMP-2 activity, the authors used a fibrosarcoma (WEHI-164) cell line, an extremely sensitive cell line to estimate cytotoxic factors/TNF from human monocyte [58]. Synovial fibroblasts concurred with chronic inflammatory responses in RA as a prominent part of invasive pannus [59]. Moreover, fibroblast cell line isolated from RA patients was able to produce matrix-degrading enzymes and some cytokines, like IL-1, IL-8, granulocyte-macrophage colony-stimulating factor, and TNF-α [60, 61]. It has been reported that MMPs, especially the gelatinases (MMP-2 and MMP-9), are implicated in some features of inflammatory arthritis including angiogenesis and bone erosions [62]. One of the prominent component of the inflammatory pannus in RA is angiogenesis, and the secretion of MMP by microvascular endothelial cells could be a fundamental factor in this process [63]. The role of M2000 in the decrease of MMP-2 activity could be probably comparable to the efficacy of TGF-β1, sulfasalazine, as well as its metabolites, in lowering MMP-2 activity's levels in inflamed joints [64, 65]. Furthermore, Gervasi et al. [66] displayed a carbohydrate-mediated regulation for MMP-2 activation in normal human fibrosarcoma cells and fibroblasts. However, the main problem in advising NSAID for therapeutic aims is their gastro-nephrotoxic effects [45, 48, 49, 67, 68], so that dyspeptic symptoms are expected to happen in 10–60% of NSAID consumers, leading to interruption of treatment in 5–15% of RA patients using NSAID [69]. It is now well known that the point prevalence of peptic ulcer disease in patient getting classic NSAID therapy ranges between 10% and 30%, representing a 10–30-fold increase over that found in the total population [69]. Thus, NSAID showed both therapeutic and also adverse effects, principally on the gastrointestinal tract [70], while oral and intraperitoneal (i.p.) administration of M2000 throughout its therapeutic effects on RA showed no toxicity on gastroduodenal tract and kidney function.

Until now, β-D-mannuronic acid has been tested as an anti-inflammatory drug in phase 1/2 clinical trial with the registered No. IRCT2013062213739N1 in ankylosing spondylitis patients and phase 1/2 clinical trial with the registered No. IRCT2014011213739N2 in rheumatoid arthritis patients without displaying side effects and without causing cardiovascular problems (these achievements will be

published in near future). It should be noted that cardiopathy could be one of the most important NSAID side effects.

In RA, the immune system is misdirected and attacks the joints. This misdirecting could promote inflammation in joints and also in other various organs and body tissues, occasionally. Generally, the conventional disease-modifying antirheumatic drugs (DMARDs) and TNF-α blockers were used to decrease both disease activity and progression in patients. In this clinical trial, patients were allowed to use their routine medications, which included methotrexate (15–20 mg weekly), hydroxychloroquine (400 mg daily), and steroids (5–15 mg daily), and also 7 out of 12 patients received a subcutaneous injection of etanercept (25 mg twice weekly). However, patients were forbidden from using NSAIDs or other pharmacologic treatment during this 12-week follow-up. Based on the preclinical assessment, a minimum dosage (18 mg/kg/d) of M2000 was provided in a gelatinized capsule (500 mg of M2000) for oral administration. Finally, the M2000 capsule (500 mg) was prescribed twice daily for 12 weeks. The patient status showed an improvement after 2 weeks and also continued during the treatment course. The mean of disease activity (DAS28), morning stiffness, rheumatoid factor (RF), and C-reactive protein (CRP) had met a significant reduction after 12 weeks of treatment. In addition, improvement was observed in other clinical laboratory tests, including anti-CCP, anti-dsDNA, and ESR levels, which returned to the normal range. After 12 weeks of therapy with M2000, IL-17 and RORγt gene expressions in patients' PBMCs were decreased by 22.39- and 2.36-fold, respectively, when compared with the gene expressions of the patients before therapy. However, several patients do not respond to these treatments and present a diminished response to them over time. Furthermore, RA patients always suffer from the adverse effects of these chemicals and biological drugs. Therefore, M2000 as a new and natural anti-inflammatory agent was used during a 3-month clinical trial and showed a suitable response in the proposed gene expression and clinical as well as paraclinical results. Findings indicated that IL-17 and its transcription factor RORγt displayed a significant diminution in PBMCs. Previous evidence indicated the critical role of IL-17 in RA and that suitable therapies were able to decrease its level. The increase of IL-17 production and upregulation of Th17 cells which was characterized by expression of the RORγt are the most common features of RA [71]. Regarding IL-17-producing $CD4^+$ T (Th17) cells as unique T-helper cells associated with T-cell-mediated tissue injury, they could promote inflammation by producing pro-inflammatory cytokines and chemokines in autoimmune disease [72, 73]. The IL-17 level after treatment with M2000 significantly decreased 22.39-fold the level of these cytokines as compared to before treatment. The results of this trial according with the other studies reported the potential role of IL-17 in mediating joint damage and the ability of IL-17 to induce collagen release from cartilage [74]. In this study, there were significant correlations between the gene expression results and clinical and paraclinical assessments. The levels of these cytokines were in conformity with the disease activity, tender joint, and swelling, all of which showed a reduction in mean after treatment. Moreover, the range of ESR and CRP, which was quite high before M2000, faced a significant decrease and was back to the normal range.

This clinical trial revealed potent anti-inflammatory and M2000's immunosuppressive properties in 12 RA patients. During this trial, owing to the good response to this natural agent by patients, the rheumatologist started to decrease the corticosteroid dosage, and after 3 months, the intake dosage of corticosteroid was decreased to half. In addition, etanercept injection was eliminated in patients. Moreover, it is now being run with two other clinical trials using M2000 on osteoarthritis and multiple sclerosis in Iranian patients. Since many studies have shown the important role of the cyclooxygenase (COX) isoforms in inflammatory and autoimmune diseases, this research aimed at studying the effects of M2000 on the gene expression and activity of COX-1/COX-2 enzymes in order to introduce a novel NSAID for treating inflammatory diseases. The results showed that the low and high doses of this drug (respectively, at 2.5 and 12.5 mMol/ml) could significantly decrease the gene expression level of the COX-2 matched to the control treated with LPS, whereas surprisingly no significant reduction was observed in the gene expression level of COX-1 confronted to the control treated with LPS. Furthermore, the results established that this drug at the three concentrations 5, 50, and 500 mMol/ml was able to strongly reduce the COX1/COX-2 enzyme's activity compared to the control treated with arachidonic acid (AA) and LPS. Collectively, many researchers, to date, have tried to lower the symptoms related to inflammatory reactions through inhibition of COX enzymes as pharmaceutical targets and have considered NSAIDs with the lowest toxicity and side effects for suppressing the inflammatory and autoimmune disease development. This research indicated that M2000 is a novel NSAID with the immunosuppressive property, which is able to strongly inhibit the activity of the COX-1/COX-2 enzymes, specifically suppressing the gene expression of COX-2.

11.3 Conclusion

Several literature data revealed that M2000 therapy represents an important tool in the management of many inflammatory diseases; moreover M2000 is the first novel designed NSAID with the lowest molecular weight and therapeutic effects, and for this reason it could be recommended in an extensive scale as the safest drug for decreasing anti-inflammatory reactions.

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