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Fundamentals and Applications

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Preface

Beads made from Egyptian faience have been excavated from grave deposits (c. 4000–3100 BC), together with beads of glazed steatite (a soft rock) and of semi-precious stones such as turquoise, carnelian, quartz, and lapis lazuli. Information on these and many more ancient beads used for ornaments and jewelry, ritual ceremonies, as art artifacts and gifts for amorous women throughout history, and descriptions of the raw materials (e.g., glass, bone, precious and other stones) and manufacturing technologies used for their production can be located in many references. Many books are devoted to the description of beads that are not of water-soluble polymer origin, techniques for their production, their art, value, and distribution, reflecting the wealth of information existing in this field of science and art. On the other hand, there are no books fully devoted to the fascinating topic of hydrocolloid (polymeric) beads and their unique applications. A few books contain scattered chapters and details on such topics, while emphasizing the possibility of locating fragments of information elsewhere; however, again, there is no book that is solely devoted to hydrocolloid beads and their versatile applications. In the meantime, the use of water-soluble hydrocolloid beads is on the rise in many fields, making a book that covers both past and novel applications of such beads, as well as their properties and ways in which to manipulate them, crucial. The aim in writing this volume was to present, in an easy-to-follow sequence, a description of bead production methods and of techniques which can be used to estimate, and modify, their physical and chemical properties. This book offers a full description of not only traditional and recent developments and applications of beads in the fields of agriculture, biotechnology, environmental studies, medicine, and food, but also topics which have never been covered in the literature, making it of the utmost importance to industry and academia.

Chapter 1: Physical Properties of Beads and Their Estimation

In Chapter 1, the criteria used to describe the shape and size of beads are explained. In particular, sections on roundness, sphericity, measurement of axial dimensions, and resemblance to geometric bodies are included. A special section is devoted to

the methods used to estimate average projected area, volume, and density, including specific gravity balance and pycnometric methods. Other sections are devoted to bead surface area and specific surface in porous media, e.g., dried beads. Also covered are image processing and its utilization for hydrocolloid beads. Finally, the chapter discusses the structure of hydrocolloid beads, their density, and their porosity.

Chapter 2: Bead Formation, Strengthening, and Modification

This chapter begins with a brief overview of the typical polymeric materials used for bead creation and their limitations. A full description is then provided of procedures to construct different bead forms, e.g., from cylindrical to almost perfectly spherical, by changing both the molds and the media into which the molten or dissolved hydrocolloid preparation is dropped or transferred. Also, some information on dropping methods, changing drop size and distribution, and liquid sprays is provided, affording a measure of control over bead size and distribution. The various water-soluble polymers that can be used for bead formation are discussed at length. The properties of gel beads prepared from agar/agarose κ-carrageenan, alginate, celluloses, chitosan, and to a lesser extent polyacrylamide and other synthetic polymers, among many others, are described. The use of crosslinking agents for both creation and strengthening of several bead types is thoroughly covered. Special methods to modify the porosity of the formed beads are also described, as are methods of slow dissolution of crystals by acid to facilitate better growth of embedded cells via pH regulation. A special section is devoted to beads prepared from proteins, ways to increase their stability (with, for example, glutaraldehyde), and their influence on the cells embedded within them. Since a combination of alternative methods may well provide a good means of overcoming the evident shortcomings of current bead-formation techniques, at the end of this chapter, a few approaches are presented, such as adding epoxy-resin reagent and curing agent to alginate for matrix stabilization, and other less known approaches for bead stabilization, as well as less traditional ways of producing and modifying beads.

Chapter 3: Methods and Mathematical Models for the Drying of Polymeric Beads

Water-soluble polymer beads can be dried for a variety of purposes (described in full in Chapter 6). In general, after drying, the texture is porous. In many cases, the bead is capable of retaining its integrity even after immersion in water for long periods. In addition, and in contrast to wet gel beads, porosity facilitates the liberation of gases during fermentation without harming the dried bead's integrity. This chapter covers methods for drying polymeric beads, including air-drying, fluidized-bed and

microwave-assisted fluidized-bed drying, and freeze-drying, and freeze-dried biological products are fully described. Sections also include drying of dosage forms made of drug dispersed in a polymer, mathematical and numerical models to analyze the drying, and a discussion of special cases such as drying a polymer bead with shrinkage.

Chapter 4: Food and Biotechnological Applications for Polymeric Beads and Carriers

The immobilization of microorganisms or cell suspensions in beads for a variety of biotechnological and food purposes is described—information which is hard to find in currently available books. Examples include amino acid (e.g., L-aspartic acid, L-alanine, and L-phenylalanine) production, organic acid (e.g., citric acid, malic acid, gluconic acid, lactic acid) fermentation and conversion, special uses in ethanol, wine, vinegar, and sake production such as malolactic fermentation, removal of urea from sake and wine by immobilized acid urease, beer brewing using an immobilized yeast bioreactor system, and uses in soy sauce production. Other uses related to miscellaneous flavor materials and aroma compounds are also discussed. These include, but are not limited to, biotransformation from geraniol to nerol, production of limonin, β-ionone, naringin, blue cheese flavor, vanillin, and Japanese seasoning. Special beads that serve for immobilization and are used in the milk industry, e.g., for hydrolysis of lactose in milk, are also detailed. Miscellaneous applications also include production of oligosaccharides, preservatives and bacteriocins, xylitol, carotenoids and leucrose, and *cis,cis*-muconic acid. Less known uses of enzymes immobilized within beads for food applications are also described. Various industrial options such as fuel ethanol production, application of gels for separation matrices, bioartificial organs, and insect-cell immobilization are included. In general, the chapter attempts to touch upon all of the novel applications of bead-immobilized cells for the food and biotechnology industries, such as the production of aroma compounds, the microbial production of bioflavors and their biotransformation.

Chapter 5: Medicinal Applications of Hydrocolloid Beads

This chapter gathers together information culled from many sources. It describes the use of cells encapsulated in hydrogels, stem cells in bead environments, charged hydrogel beads as new microcarriers for cell culture, as a potential support for endothelial cells, and for vaccine delivery. Other sections provide information on crosslinked chitosan beads for different medicinal purposes: mucoadhesive beads and their applications for eyes and the alimentary system and polyelectrolyte complexes. Additional sections describe novel approaches to cell encapsulation for improved biocompatibility and immunoisolation. Emphasis is placed on methods

using alginate–polylysine alginate for encapsulation, and a glimpse is provided of the art and science of artificial cells, encapsulated enzymes for the clinical laboratory, and encapsulation of living cells and tissues for biomedical purposes.

Chapter 6: Dry Bead Formation, Structure, Properties, and Applications

The drying of hydrocolloid beads results in cellular moieties, and this chapter therefore deals with cellular solids. A few manufacturing methods for hydrocolloid cellular solids are described. They include, but are not limited to, drying bicarbonate-containing gels after acid diffusion and cellular solids produced by fermentation and enzymatically. A special section deals with the inclusion of oils in gels and their influence on the properties of the resultant dried cellular solid. Several methods, e.g., compression studies, are described for evaluating the mechanical properties of the dried beads. The chapter also details the models used for describing these beads' stress–strain behavior. The structure and acoustic properties of such cellular solids as a result of production method are also addressed. The applications of dried beads have never been thoroughly reviewed. This chapter attempts to redress this by describing their use as carriers for vitamins, as study models, and for separation and includes special dry beads for water treatment and matrices entrapping hydrocolloid cellular beads. Hydrocolloid cellular carriers for agricultural uses are also presented, e.g., the preservation of biocontrol agents in a viable form by dry cellular bead carriers and the carriers' capacity to protect these agents against UV radiation. The chapter ends with a discussion on the textural features of dried hydrocolloid beads.

Chapter 7: Liquid-Core Beads and Their Applications in Food, Biotechnology, and Other Fields

In 1971, Maddox's patent on soft gelatin capsules was approved. In 1980, Lim and Sun published their hallmark study in which microencapsulated islets were used as a bioartificial pancreas. In that manuscript, alginate–polylysine liquid-core capsules were produced and described. In the food area, Sneath's patent (1975) and later our group contributed to the manufacture and study of liquid-core hydrocolloid capsules. This chapter describes these liquid-core capsules, both natural and synthetic, and the procedures used to produce them. Methods for including oil within these capsules are also provided, along with an overview of their biotechnological and special food applications. Additional biotechnological applications of liquid-core capsules include growth of microorganisms and activity of enzymes within them, and food applications include the manufacture of unique specialty foods and fruit products and the encapsulation of aroma and health compounds, among others. The chapter also describes agricultural and environmental uses of liquid-core capsules

and illustrates some special applications: aids to quitting smoking, in the beauty industry for removal of body hair, and in the paper industry.

Chapter 8: Beads as Drug Carriers

Beads are often used as drug carriers in passive, as well as active drug targeting, making this a highly relevant topic in today's research. Major general topics covered in the first part of this chapter include controlled drug release, gels in drug-delivery systems, dual drug-loaded beads, and drug release from the beads. Throughout, issues such as methods of drug incorporation, bead properties, extent and nature of crosslinking and the physicochemical properties of the drug, interactions between the drug and the matrix material, concentration of the matrix material and release environment (e.g., the presence of enzymes) are discussed. Described beads include albumin beads, alginate beads, alginate beads reinforced with chitosan, calcium alginate/PNIPAAM beads, different chitosan beads (e.g., chitosan–tripolyphosphate beads, chitosan microspheres in treating rheumatoid arthritis, carboxymethyl chitosan beads), gelatin beads and those crosslinked with dextran, modified starch microspheres, dextran beads, gellan beads, guar beads, pectin beads for colon-specific drug delivery, pectin–chitosan beads, and modified poly(vinyl alcohol) microspheres. The chapter also summarizes information on achieving controlled production of the beads, such as preparation of biodegradable hydrogels based on polyesters, hydrogels with degradable crosslinking agents and those crosslinked with small molecules, azo reagents or albumin, and hydrogels with biodegradable pendant chains. The chapter finishes with a description of the more unique beads, such as those with floating ability and those made from xyloglucan.

Chapter 9: Beads and Special Applications of Polymers for Agricultural Uses

The concept of bead encapsulation has become highly relevant to agriculture. Beads can encapsulate microorganisms for use in the field of bacterial inoculation technology. Immobilized plant cell suspensions and single seed products have proven to be easy to produce, store, and handle during industrial operation. This chapter describes the goals of encapsulation in agriculture, e.g., to temporarily protect the encapsulated microorganisms from the soil environment and microbial competition and to release them gradually for the colonization of plant roots. Special cases for enlarging populations in which the entrapped bacterial biomass is low are described; other cases in which, for example, immobilized fungi are used as biocontrol agents against soil-borne pathogens are thoroughly detailed; survival of bead-entrapped populations is compared with that of populations encapsulated in peat, and the influence of special additives on bacterial survival is described. In addition, timing and methods for the application of bacterial inoculants are delineated. In particular, topics

such as carriers for the slow release of bacteria that affect plant growth, inoculation of seedlings and plants with beads containing fungal inoculum, joint immobilization of plant growth-promoting bacteria and green microalgae, cryopreservation by encapsulation/dehydration technique, and controlled release of agricultural chemicals are discussed at length. The chapter also supports the reader with a list of biotechnological applications such as gene-delivery systems using beads, bioactive bead methods for obtaining transgenic plants and in synthetic seed technology and describes unique applications of polymers, including superabsorbent polymers and seed coating.

Chapter 10: Beads for Environmental Applications

This chapter focuses on the use of beads to immobilize microorganisms for pollutant biodegradation. Special emphasis is placed on chemically contaminated water, soil, and air. Water treatments are reviewed, and wastewater treatment by anaerobic fixed bed reactor or using immobilized microorganisms is discussed, along with the more specific examples of arsenic removal from water, chitosan and the removal of heavy metal ions, and water denitrification. The chapter lists the advantages of using encapsulated bacterial cells for soil applications, describes the preparation of such beads, and gives information on the protection of encapsulated cells from environmental stress. Another topic is the use of beads to protect against toxicity and the related issues of soil treatments, agrochemicals, controlled release of pesticides into soils, and sustained release of fungicide. Because these beads are introduced into soils, a special section is devoted to release from these beads in the soil environment. In addition to discussing the advantages of beads for environmental applications, the chapter tries to account for the limitations of such technologies, such as the problem of substrate diffusion into immobilized preparations. Other covered issues include air pollution and sampling and the determination of trace contaminants in air by concentration on porous polymer beads. Finally, the chapter discusses miscellaneous applications such as biodegradation and removal by microalgae.

In addition to covering the numerous types of hydrocolloid beads, this book describes their many traditional and non-traditional uses, developed in many hydrocolloid R&D laboratories all over the world, including ours. The book addresses many important industries and as such is designed to capture the interest of those who are looking for new applications in the fields of agriculture, food, environmental quality control, biotechnology, and medicine; it will be of interest to the polysaccharide chemist, as well as to academic and other researchers and authorities in the fields of food, chemistry, medicine, and biotechnology.

My hope is that this book will assist all levels of readers. It is dedicated not only to the academic community but also to the broader population of industrialists and experimenters who will find this book to be not only a source of knowledge but also a launching pad for novel ideas and inventions. In particular, this book

is expected to be of interest to personnel involved in food formulation, food scientists, food technologists, industrial chemists and engineers, pharmaceutical staff and medical doctors, and those who deal with drug delivery from beads. Potential readers also include both professional and dedicated non-professional environmentalists, farmers, agriculturalists, and those working on the development of novel beads and their applications. Finally, it is hoped that this book will find a prominent place in the traditional university and research institute libraries where food science, chemistry, agriculture, environmental studies, and other theoretical and practical industrial topics are taught and studied.

Rehovot, Israel Amos Nussinovitch

Acknowledgments

This book was written over the course of 2 years. It contains a description of polymer macro- and micro-gel beads, their properties, and applications. It also includes their many traditional and non-traditional uses, developed in our and many other hydrocolloid laboratories worldwide. My hope is that this manuscript will assist readers who are in search of comprehensive knowledge about the fascinating field of beads, as well as those seeking up-to-date information on the very different current and past uses and applications of polymer beads in many areas. Comments and questions from these readers will be very much appreciated.

I wish to thank the publishers for giving me the opportunity to write this book. Special thanks to David Parsons and Susan Safren for their efficient contribution to the formation and processing of this manuscript. I wish to thank my editor, Camille Vainstein, for working shoulder to shoulder with me when time was getting short. Hanna Ben-Or's help in locating and rectifying the many old and inaccurate references was above and beyond the call of duty. The permissions that we obtained from different publishers are warmly acknowledged. The many pictures adapted from Wikipedia are acknowledged in their turn, but I feel that it is equally appropriate here to recognize the many who contributed to this gigantic educational achievement. I particularly want to thank my family, Varda, Ya'ara, Eran, and Yoav, for their love, patience, and support during these last few difficult years, in which we were under huge pressure from many different directions. Last, but not least, I wish to thank the Hebrew University of Jerusalem for being my home and refuge for the last 20 years of very extensive research and teaching.

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About the Author

Professor Amos Nussinovitch was born in Kibbutz Megiddo, Israel. He studied Chemistry at the University of Tel Aviv and Food Engineering and Biotechnology at the Technion-Israel Institute of Technology. He has worked as an engineer at several companies and has been involved in a number of R&D projects in both the United States and Israel, focusing on the mechanical properties of liquids, semi-solids, solids, and powders. He is currently in the Biochemistry, Food Science and Human Nutrition Department of the Robert H. Smith Faculty of Agriculture, Food and Environment of the Hebrew University of Jerusalem, where he leads a large group of researchers working on theoretical and practical aspects of hydrocolloids. Prof. Nussinovitch is the sole author of the following books: *Hydrocolloid Applications: Gum Technology in the Food and Other Industries*; *Water-Soluble Polymer Applications in Foods*, and *Plant Gum Exudates of the World: Sources, Distribution, Properties, and Applications*. He is the author or co-author of numerous papers on hydrocolloids and on the physical properties of foods, and he has many patent applications. This book is devoted specifically to polymer macro- and micro-gel beads. The author has been working in this area for many years and has studied the structure and texture of wet and dry beads; he has developed liquid-core capsules, different polymeric beads for water denitrification, beads for biocontrol agent encapsulation, and beads as part of novel cellular solids, among many other applications.

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Chapter 1
Physical Properties of Beads and Their Estimation

1.1 Introduction

Accurate estimates of shape, size, volume, specific gravity, surface area, bulk density, and other physical characteristics are important in the study or consideration of hydrocolloid bead properties. There is not much information in the literature on the physical properties of hydrocolloid beads, other than their mechanical properties, which are discussed in Chapter 2. The aim of this chapter is to provide the reader with simple tools for the physical characterization of beads.

1.2 Bead Size and Shape

1.2.1 General

Most hydrocolloid beads are produced in a spherical shape. However, deviations from sphericity occur, with spheroid and ellipsoid beads being quite common. In addition, beads that pass through a drying process for the manufacture of dried carriers (see Chapters 3 and 6) may undergo a change in their spherical or rounded shape. Therefore, shape and size are essential parameters for adequately describing hydrocolloid beads. Moreover, the characterization of an object's shape entails the need to measure other dimensional parameters. Similarly, an increase in bead shape irregularity entails an increase in the number of measurements needed to define its form.

1.2.2 Size of Drops and Beads

Size is a measure of an object's physical magnitude (how big or small it is). When a size is measured, it is given a number. Size is an important attribute of beads in heat- and mass-transfer calculations, and when beads are transferred in fluid flow, their average size must be known. It is easier to specify the size of a regularly

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shaped bead (i.e., spherical or ellipsoid); for irregularly shaped beads, the term size should be arbitrarily defined. Beads can be formed in the millimeter, micrometer, or nanometer size ranges. Size can be determined using the projected-area method, in which three characteristic dimensions are defined: the major diameter (i.e., the longest dimension of the maximum projected area), the intermediate diameter (i.e., the minimum diameter of the maximum projected area or the maximum diameter of the minimum projected area), and the minor diameter (i.e., the shortest diameter of the minimum projected area) (Sahin and Sumnu 2006). The terms length, width, and thickness are commonly used for the major, intermediate, and minor diameters, respectively. They can be measured using a caliper or micrometer that is especially designed for dry non-spherical beads (Fig. 1.1). In general, particle size can be determined by sieve analysis, passage through an electrically charged orifice or settling rate (Sahin and Sumnu 2006).

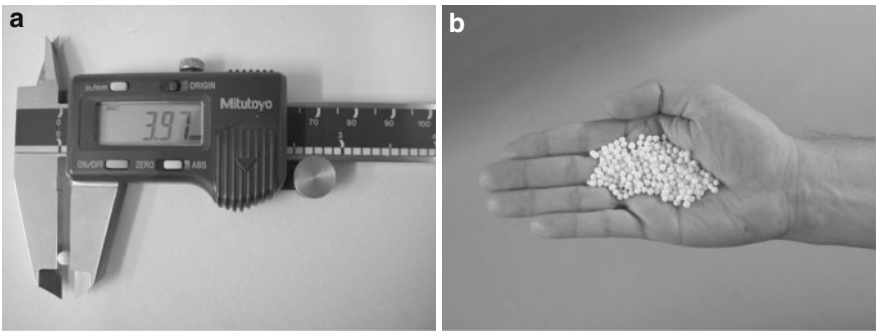

Fig. 1.1 (**a**) A caliper used especially for dry non-spherical beads. (**b**) Dry hydrocolloid bead

Due to growing interest in the field of cell immobilization, numerous techniques have been developed for bead manufacture (see Chapters 9 and 10). These techniques include dripping (Walsh et al. 1996; Romo and Perez-Martinez 1997), emulsification and coacervation (Poncelet et al. 1993, 1994; Green et al. 1996), rotating-disc atomization (Bégin et al. 1991; Ogbonna et al. 1991), air jet (Klein et al. 1983; Levee et al. 1994), atomization (Siemann et al. 1990), electrostatic dripping (Bugarski et al. 1994; Halle et al. 1994; Poncelet et al. 1994), mechanical cutting (Prüsse et al. 1998), and the vibrating nozzle technique (Ghosal et al. 1993; Brandenberger et al. 1997; Seifert and Phillips 1997). Some of these methods suffer from limitations, particularly with respect to difficulties in achieving the simultaneous production of beads at a high production rate with a satisfactory level of material utilization under mild and non-toxic conditions or under completely sterile conditions, in the ability to scale up the process and, in particular, in obtaining beads with a narrow size distribution.

To calculate the diameter of a fluid drop, prior to its transformation into a bead by further chemical/physical manipulation, a theory which was first developed to

explain the jet breakup of Newtonian fluids must be considered (Rayleigh 1879). This theory proposed an equation relating the wavelength of the perturbation to the jet diameter:

$$\lambda_{\text{opt}} = \pi\sqrt{2}d_{\text{jet}} \tag{1.1}$$

where λ_{opt} is the optimal wavelength for jet breakup and d_{jet} is the jet diameter. This theory was later developed by Weber (1931) to include the effect of fluid viscosity:

$$\lambda_{\text{opt}} = \pi\sqrt{2}d_{\text{jet}}\sqrt{1 + \frac{3\eta}{\sqrt{\rho\sigma d_{\text{jet}}}}} \tag{1.2}$$

where η is the fluid viscosity (Pa s), ρ is the fluid density (kg/m^3), and σ is the surface tension (kg/s^2). Since one droplet is produced by each Hertz of vibration, the drop diameter d_{d} can be calculated via a straightforward mass balance equation:

$$d_{\text{d}} = \sqrt[3]{6\frac{F}{\pi\nu}} \tag{1.3}$$

where F is the flow rate (m^3/h) and ν is the frequency of the vibration (Hz) which is itself linked to the wavelength by

$$\nu = \frac{F}{\lambda}\frac{4}{d_{\text{jet}}^2\pi} \tag{1.4}$$

As a consequence, the droplet diameter, as a function of wavelength and jet diameter, is specified by

$$d_{\text{d}} = \sqrt[3]{\frac{3d_{\text{jet}}^2\lambda_{\text{opt}}}{2}} \tag{1.5}$$

This suggests that the optimal frequency for the production of drops from a given nozzle diameter is given exclusively by the physicochemical properties and the flow rate of the extruded fluid (Serp et al. 2000).

However, when a non-Newtonian fluid, such as alginate or any other polymer commonly used for cell immobilization and encapsulation, is employed, the actual viscosity within the nozzle has to be calculated. In addition to being difficult to estimate, d_{jet} also has to be determined through preliminary experimentation (Brandenberger and Widmer 1998). As already noted, this theoretical approach only yields the optimum frequency for drop generation, which does not necessarily correspond to the optimal frequency for bead production. Indeed, if this frequency is too high for an alginate solution, for instance, doublets appear due to contact between the droplets and the beads in the hardening solution. On the other hand, when the

frequency is too low, satellites are observed. Thus only an empirical approach can be used to determine the actual conditions for optimum bead production (Serp et al. 2000).

The following is a brief description of a few techniques used to approximate a narrow bead-size distribution. A very popular and simple method for obtaining beads is crosslinking of an alginate solution. It is therefore not surprising that it is the model system of choice for many encapsulations and for bead manufacturers. The properties of different alginates, their manufacture, and their chemical and physical properties have been reviewed in many textbooks and manuscripts (among them Davidson 1980; Nussinovitch 1997; Phillips and Williams 2000) and the reader is referred to these for further details. The vibrating nozzle technique is a suitable process for cell immobilization within the requested size range for alginate beads. It can be used to produce a mono-dispersion of beads larger than 200 μm in diameter, depending on the application (Serp et al. 2000). Furthermore, their manufacture can be scaled up. In particular, reasonably high production rates, between 1 and 15 ml/min, depending upon the desired bead size and polymer solution rheology can be achieved, producing an alginate bead-size distribution of less than 5% (Brandenberger et al. 1997; Seifert and Phillips, 1997).

It is essential to distinguish between the size of an alginate drop formed by an encapsulation apparatus and the size of the alginate bead, that is, the size of the drop once it comes into contact with the hardening/crosslinking solution (Serp et al. 2000). In fact, using the jet breakup technique, it is not possible to form alginate beads of the desired size and within the required size distribution, with any of the commercially available alginates. Consequently, a detailed series of experiments to characterize the alginate is indispensable, before successful conditions for extrusion can be defined (Serp et al. 2000). A 2% solution of sodium alginate was extruded, using an encapsulation device fitted with a range of nozzles (diameters 50–500 μm), into a hardening solution of 110 mM $CaCl_2$ at 25°C, beads were sampled from the hardening solution at 2 min intervals over a period of 60 min, and bead size and size distribution were determined. The following observations were made: bead diameter decreased progressively during the first 5 min following extrusion and then remained relatively stable. However, irrespective of initial bead size (diameters within the range of 300–600 μm), this contraction represented less than 10% of the initial bead diameter. Size determinations over several days of beads incubated in a solution of 110 mM $CaCl_2$ revealed further gradual shrinkage. However, this size decrease represented only 1–2% of the diameter measured after 5 min. These results indicated that the alginate beads are rapidly saturated with Ca^{2+} ions within the first few minutes of incubation and that a slow rearrangement of the alginate chains, inducing further shrinkage, then occurs (Serp et al. 2000). Another manuscript described the use of alginate beads as a vehicle for the controlled release of entrapped essential oil as an antiviral agent via oral administration. In this case, the freshly prepared calcium alginate beads exhibited a regular, spherical shape that was maintained after drying. The bead size did not vary significantly when the exposure time to $CaCl_2$ was changed, possibly due to the relatively high concentration

of crosslinker. In particular, these freshly prepared formulations displayed a narrow size range. A decrease in bead size was noted due to water loss during the drying process (Lai et al. 2007).

Agar is another potent gelling agent composed of repeating units of D-galactose and 3,6-anhydro-L-galactose. The gelling component of agar, agarose, is an essentially sulfate-free, neutral (non-ionic) polysaccharide (Nussinovitch 1997). The effect of processing conditions on the mean size and size distribution of porous agarose beads manufactured by emulsification in a single stirred vessel was investigated. Agarose beads can be successfully manufactured in a single stirred vessel by emulsification of hot agarose solution and subsequent freezing/gelling of the aqueous drops into soft solid particles. The particle size and size distribution can be controlled by selection of energy dissipation rate and/or surfactant concentration (Mu et al. 2005). Despite the fact that flow in a stirred vessel is transitional, the relationship between drop/particle mean size and energy dissipation rate is similar to that for turbulent flow (Baldyga et al. 2000).

Another common technique for producing spherical polymer beads that affords control of particle size is suspension polymerization. In typical suspension polymerization processes, microscopic droplets of monomer stabilized by small amounts of surface-active polymers are suspended in a continuous non-solvent medium, most commonly water, and polymerization is carried out by the addition of monomer-soluble initiators. The advantages of suspension polymerization are good temperature control and ease of handling of the final product (O'Connor and Gehrke 1997). Suspension crosslinking technique was used to produce thermally responsive gels of hydroxypropylmethylcellulose (HPMC) in a spherical form. Suspension crosslinking of HPMC with divinylsulfone was accomplished by dispersing aqueous polymer droplets, containing all of the reactants, in a continuous organic phase. The spherical beads had diameters ranging from 500 to 3000 μm. Bead size generally decreased with the use of a larger impeller, swinging at high stirring speeds, or at a lower phase ratio. As bead size decreased, the size distribution also narrowed. The gel beads exhibited the same swelling properties and crosslinked network formation as bulk HPMC gels (O'Connor and Gehrke 1997).

1.2.3 Bead Shape

The shape of an object located in a space is the part of that space occupied by the object, as determined by its external boundary—abstracting from other properties such as color, content, and material composition, as well as from the object's other spatial properties (position and orientation in space, size). Another definition of shape is the geometrical information from an object that remains when location, scale, and rotational effects are filtered out (Kaye 1993). Simple two-dimensional shapes can be described by basic geometry, such as points, lines, curves, and planes. A shape whose points all belong to the same plane is called a *plane figure*. Most shapes occurring in the physical world are complex. Some, such as plant structures and coastlines, may be so arbitrary as to defy traditional

mathematical description—in which case they may be analyzed by differential or fractal geometry.

Describing the shape of an object is difficult in a three-dimensional space: we are often obliged to be satisfied with a two-dimensional description. A shape index is a dimensionless number that is used to characterize a shape. The word index comes from the Latin word "indicare" meaning "to show," in a way similar to a book's index which shows one where to find a particular topic. To create a shape index for a non-circular shape, it is necessary to specify the diameters of the profile (Kaye 1993). The word diameter originates from the Greek, "dia" meaning through and "metron" a measure. The diameter of a circle is measured and defined as the line moving through the center of the circle. An ellipse is always defined by its major and minor profile diameters. Shape indices of irregular profiles can be obtained by constructing an ellipse of equal area and then calculating the ratio of the minor and major axes of that equal area. However, this can sometimes be problematic due to possible difficulties in measuring the area of the profile and the subsequent calculation of the dimensions of the ellipse of equal area (Kaye 1993).

If a profile is irregular, it can be enveloped by a profile curve termed *convex hull* of the profile. *Convex* means "bulging outward" and *hull* means "an outer covering." For such a profile, the shape of the convex hull is used to generate a shape index using the length and breadth of the profile. A smooth profile can be achieved by modifying the concept used to generate π for a circle and denote the perimeter divided by the profile's maximum diameter. This shape factor is 3.14 for a circle and decreases to 2 for a very long fiber. However, this approach is not widely used since it is difficult to measure the perimeter of a profile, and for very rugged profiles, the real perimeter of the profile approaches infinity and the modified shape index becomes indeterminate and not a good indication of structure (Kaye 1993).

The sphere is one of the most commonly occurring shapes in nature (Musser and Burger 1997). Many sphere-like objects that are of interest in the life sciences can be easily located, from stones (geology, mineralogy), to oranges, watermelons, and tomatoes (food science), from soap bubbles (children's games and surface research), to frog eggs (biology and genetics), moving even further to the earth and moon (astronomy and physics), not to mention other fields. The sphere's ability to enclose the greatest volume for a given surface area is a good explanation for the considerable frequency of its appearance in nature. In other words, a sphere requires the least amount of material to surround a given volume. This may help explain why animals curl up in a ball when it is cold outside (Musser and Burger 1997).

Because most beads are produced in a sphere-like shape, a brief review of the basic formulas related to their surface area and volume estimation is warranted. Archimedes (287–212 BC), considered to have been the greatest mathematician in antiquity and ranked by many with Sir Isaac Newton and Carl Friedrich Gauss, observed that the surface area (volume) of a sphere is two-thirds the surface area (volume) of the smallest cylinder containing that sphere. Figure 1.2 shows a sphere of radius r contained within a cylinder with radius base r and height $2r$. The surface area of the cylinder is $2\pi r^2 + 2\pi r(2r) = 6\pi r^2$. Based on Archimedes' observation, the formula for the surface area of a sphere of radius r is $2/3(6\ \pi r^2) = 4\pi r^2$. The

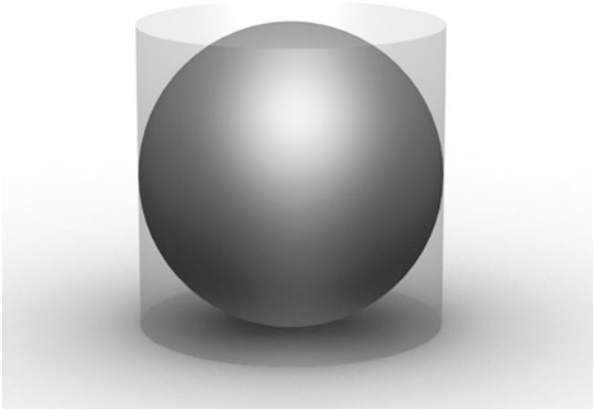

Fig. 1.2 A sphere of radius r contained within a cylinder with radius base r and height $2r$ (courtesy of André Karwath at http://commons.wikimedia.org/wiki/File:Archimedes_sphere_and_cylinder.png)

volume of the sphere is $2/3(2\,\pi r^2) = 4/3\,\pi r^3$. Of course, the r in $4\pi r^2$ is squared, a unit of area, whereas the $4/3\pi r^3$ is cubed, a unit of volume (Musser and Burger 1997).

The shape of a bead can be described by borrowing terms from different fields. These include round—approaching spheroid; oblong—vertical diameter greater than the horizontal diameter, ovate—egg shaped, obvate—inverted ovate, elliptical—approaching ellipsoid; truncate—having both ends squared or flattened; ribbed—in cross section, sides are more or less angular; regular—horizontal cross section approaches a circle; irregular—horizontal cross section departs considerably from a circle (Mohsenin 1970).

Let us define some of the most commonly occurring bead shapes. A *spheroid* is an ellipsoid (Weisstein 2003). It is a quadric surface obtained by rotating an ellipse about one of its principal axes; in other words, it is an ellipsoid with two equal semi-diameters. If the ellipse is rotated about its major axis, the result is a *prolate* (elongated) spheroid, like a rugby ball. If the ellipse is rotated about its minor axis, the result is an *oblate* (flattened) spheroid, like a lentil (Fig. 1.3). If the generating ellipse is a circle, the surface is a sphere. Images of cut surfaces of beads often resemble a circular shape. A circle is the set of all points in a plane that are at a fixed distance from a given point (called the center). The distance from the center to a point on the circle is called the radius. Any segment whose endpoints are the center and a point of the circle is also called a radius. The length of a line segment whose endpoints are on the circle and which crosses the center is called the diameter (Musser and Burger 1997). Many properties of a circle, including its area, are obtained by comparing the circle to regular polygons with increasingly large values of n. The *perimeter* of a circle, namely the length of the circle, is given the special

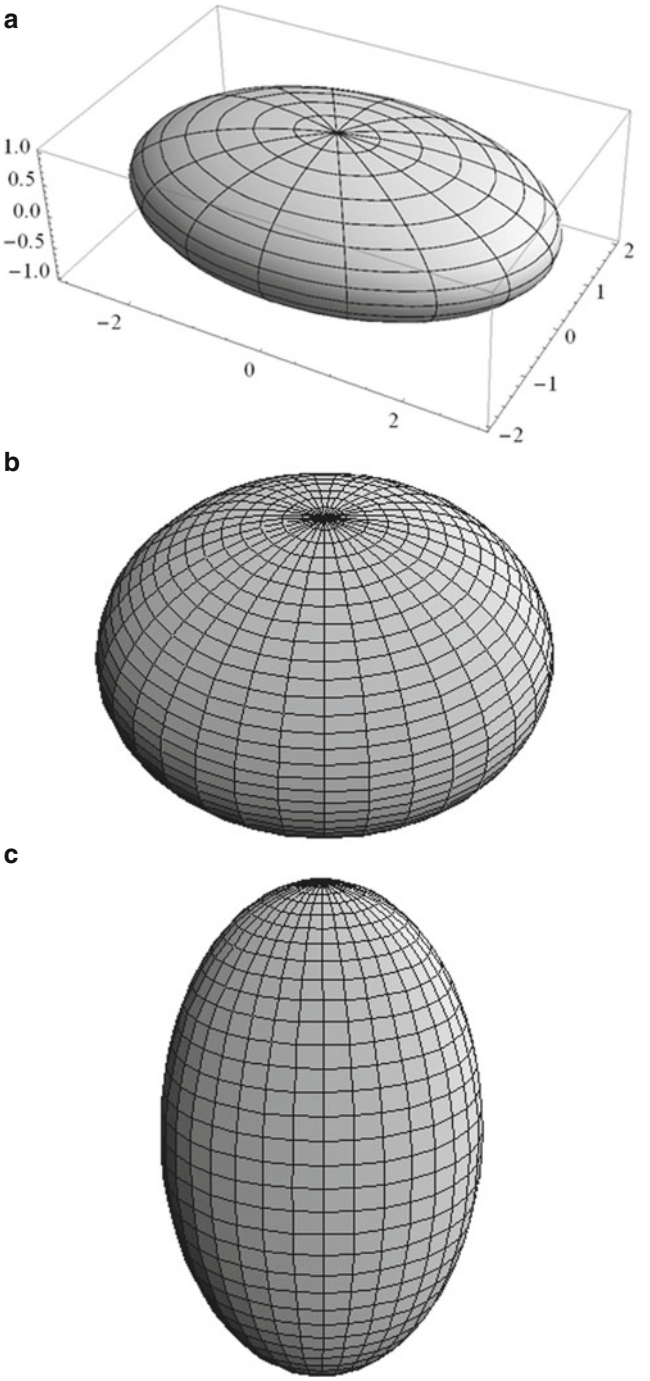

Fig. 1.3 (**a**) Ellipsoid (http://en.wikipedia.org/wiki/File:Ellipsoid_321.png), (**b**) oblate and (**c**) prolate spheroids

name of circumference. It is important to mention that the ratio between the circumference and the diameter is a constant called π, which is the Greek letter "*pi.*" The circumference of a circle is $2\pi r$ or πD, where r and D are the radius and diameter of the circle, respectively. The area of a circle, $A = \pi r^2$, was first estimated by inscribing a circle within a regular polygon and calculating its area. A rigorous verification of this formula can be achieved using calculus (Musser and Burger 1997).

In view of the above knowledge, it is not surprising that many bead manufacturers want to create beads in a spherical or sphere-like shape. For example, gellan beads can be formed in a spherical shape and thus mathematical modeling studies of their spherical geometry can be performed by solving Fick's equation to compute concentration profiles and diffusion coefficients. To produce such shapes, a dispersion of gellan gum was extruded into a solution containing a mixture of calcium and zinc ions (counter ions). By changing the experimental variables, for instance pH, of the counter ion solution and the amount of drug (cephalexin) loading, process variables could be optimized to achieve control over the final percentage of drug entrapment efficiency and release rates, as well as over bead size and morphology (Agnihotri et al. 2004). The average bead size ranged from 925 to 1183 pm as measured by laser light scattering, and up to 69.3% cephalexin entrapment was achieved (Agnihotri et al. 2004). Different bead shapes are sometimes the result of ingredients used in the manufacturing process, as well as of the entrapped moieties. Dropping a 3.5% κ-carrageenan-containing papain solution into 0.5 M potassium chloride using the ionotropic gelation method, with a hardening time of 20 min, it was possible to obtain beads characterized by a spherical disc shape with a collapsed center and an absence of aggregates (Sankalia et al. 2006a).

Bead manufacturers often need to consider whether loading a drug into a bead or chemical changes (oxidation) in its hydrocolloid composition will influence the bead's shape. In the case of ionic binding of drugs to beads made of cellulose and its derivatives, the physical properties of the beads, such as spherical shape and porosity, were not significantly influenced by oxidation of cellulose or by drug loading (Wolf and Finke 1992). Controlling formulation and process variables in order to achieve a particular bead shape may also change the bead's density, porosity, and friability (Agrawal et al. 2004).

Different combinations of water-soluble polymers and production technologies can lead to control of bead size and porosity (Agrawal et al. 2004). For instance, beads containing chitosan, fine-particle ethylcellulose, HPMC, and caffeine as the model drug were manufactured. Bead size, yield, shape, friability, density, and porosity were determined, and release studies performed. Spherical beads with good mechanical properties could be created without microcrystalline cellulose (Agrawal et al. 2004). Furthermore, beads with high percentage yield and high sphericity could be obtained at high chitosan content, and low HPMC content, water content, spheronizer speed, and extruder speed. Less friable beads were obtained at high levels of the studied formulation's variables and low levels of the studied process's variables. Beads of high density and low porosity could be manufactured at high levels of the studied formulation's and process's variables (Agrawal, Howard and Neau 2004).

Gelling agents (i.e., agar–agar, alginate, carrageenan, gellan, LMP pectin) can be used to create various gel shapes. For instance, κ-carrageenan's property of being easily induced into a gel form by contact with metal ions, amines, amino acid derivatives, and water-miscible organic solvents was used to tailor the gel into various shapes such as beads, cubes, and membranes (Tosa et al. 1979). Another example is the production of alginate hydrogel microbeads of various shapes with narrow size distributions (94–150 μm) in non-silanized/silanized poly(dimethylsiloxane) devices (Tan and Takeuchi 2007). The narrow size distribution of these alginate hydrogel beads had a coefficient of variation (CV) <2.9%. The CV is defined as the ratio between the standard deviation and the mean, and a CV of less than 5% is the commonly accepted definition of monodispersity (Tan and Takeuchi 2007). The method combined an internal gelation method with T-junction droplet formation in a microfluidic device (Okushima et al. 2004). The use of calcium carbonate nanoparticles enabled the use of an internal gelation method for microscale production. The method allowed easy control over bead size by varying flow parameters, and better monodispersity and control of the shape of the hydrogel beads compared to conventional external gelation methods performed in microfluidic devices (Tan and Takeuchi 2007). It may be that modifications to the design (e.g., shortening the channel length) or switching to devices that can withstand higher pressures (e.g., channels in a solid slab of non-silanized/silanized poly(dimethylsiloxane) or silicon-glass-based devices) will enable the production of smaller hydrogel beads (Takeuchi et al. 2005).

A novel solvent-based method for the preparation of alginate beads with improved shape was developed. An aqueous solution of 2% alginate in water was injected into a solvent consisting of layers of hexane and *n*-butanol, *n*-butanol with 1% $CaCl_2$, or *n*-butanol with 2% $CaCl_2$ in water. Beads of up to 3.5 mm in diameter obtained with this method had a roundness (see Section 1.2.3.1) which was similar or up to 5% better than comparable beads prepared by dropping alginate solution into a $CaCl_2$-hardening bath (Buthe et al. 2004). In addition, the novel solvent-based method allowed for highly reproducible preparation of alginate beads with precisely predictable sizes. The biggest beads obtained with this method were 9 mm in diameter. Thus, with the solvent-based preparation of alginate beads, it is possible to obtain beads of exactly the type needed for specific analytical purposes (Buthe et al. 2004). The method of shape analysis employed in the latter study quantified the elongation of beads, but did not account for irregularities such as the pear shape observed for several beads prepared by standard methods. The deviations from perfect bead shape were in good agreement with previous results (Nussinovitch and Gershon 1996). The latter authors investigated the shape of alginate beads prepared with a technique equivalent to the standard method, using a similar experimental setup: pictures of single beads were taken from different angles and used to determine bead dimensions. Bead roundness was described by the ratio of apparent diameter, which in this case was the diameter of a sphere of the same volume as the investigated object, and major axis length. This gave a mean deviation from a perfect bead shape of 7%. The different way of calculating bead roundness also had an influence on the final results: the ratio of bead diameter to major axis

length (as a measure of sphericity) was always higher than the ratio of minor axis length to major axis length (elongation as a measure of roundness in this method), since the diameter is always longer than the minor axis. Thus, the bead will always appear rounder when calculated with Nussinovitch and Gershon's (1996) definition than with that of Buthe et al. (2004).

The elongation ratio (ER), which is the quotient of the bead's length to width (Wong and Nurjaya 2008), was calculated to characterize the shape of the calcium pectinate beads. An ER value of unity represents a perfect sphere while higher values represent greater elongation or more divergence from a spherical shape (Das and Ng 2010). The tested beads included the following parameters: crosslinking with calcium chloride at a concentration of 2.5–20.0%, crosslinking pH of 1.5–5.5, crosslinking time of up to 24 h, pectin concentration of 3–6%, and a pectin-to-drug ratio of 1:1–4:1. Almost all of the calcium pectinate beads (except the dried beads prepared with 3% pectin) were spherical in shape (i.e., ER <1.25) (Das and Ng 2010).

Further reports dealt with the shape of chitosan-coated calcium alginate beads designed for drug-delivery purposes. Calcium alginate, chitosan-coated calcium alginate, and chitosan–calcium alginate complex gel beads, in which an oil-in-water emulsion containing allyl isothiocyanate was entrapped, were prepared and characterized for efficient oral delivery of allyl isothiocyanate. The freeze-dried beads maintained their original shape. The SEM micrographs of the gel beads treated in simulated gastric fluid for 4 h at 37°C showed that all three types of gel beads shrink into a similar spherical shape without notable degradation of bead matrix (Ostberg et al. 1994; Bajpai and Sharma 2004). For chitosan–calcium alginate complex gel beads, no notable disintegration was observed in the micrographs, and their spherical shape was well retained, even after 12 h incubation. The results indicated that the calcium alginate and chitosan-coated calcium–alginate gel beads exposed to simulated intestinal fluid (SIF) swell initially, although bead degradation is occurring simultaneously, and then rapidly disintegrate. The swelling and subsequent degradation of calcium–alginate gel beads in SIF has two main causes: (1) displacement of crosslinking calcium ions by sodium ions and (2) sequestering effect of phosphate on calcium ions, which renders the calcium alginate gel structure loose and soluble (Ostberg et al. 1994; Bajpai and Sharma 2004).

The production method, ingredients and additives, can influence the final shape and the presence of dimples on the manufactured bead. For example, carbonaceous gel beads were manufactured by the addition of polymers using ammonia water of amphiphilic carbonaceous material through a water-in-oil emulsion. The addition of poly(vinylalcohol) (PVA) created carbonaceous gel beads with dimples, the number and size of the indentations being dependent on the additive amount and the molecular weight of the PVA (Sughi et al. 1995). However, only beads with smooth surfaces were obtained with the addition of poly(vinylpyrrolidone) and poly(ethyleneglycol). Bead morphology was significantly influenced by interfacial tension at the water–oil interface. The surface area of beads prepared by the addition of polymers increased remarkably with heat treatment from 5 to 289 m^2/g and a pore size of about 20 Å was predominantly produced in the case of PVA. In addition, the carbonaceous gel

prepared by addition of PVA exhibited high graphitizability, similar to that of green coke (Sughi et al. 1995).

In some cases, the shape of the resultant polymer beads can be studied indirectly. As an example, the photo-isomerization effect of azo groups on the shape of submicron-sized liquid crystalline polymer beads containing azo groups and mesogenic groups was studied in films of the polymer beads prepared by casting the suspension liquid on a glass plate; the film was then irradiated with polarized light (488 nm) (Kim et al. 2005).

1.2.3.1 Roundness

The sharpness of the corners of a solid is defined as *roundness* (Curray 1951; Mohsenin 1970).

$$\text{Roundness} = \frac{A_\text{p}}{A_\text{c}} \tag{1.6}$$

where A_p is largest projected area of an object in its natural resting position (m^2) and A_c is the area of the smallest circumscribing circle (m^2) (Mohsenin 1970).

Roundness can also be estimated by

$$\text{Roundness} = \sum_{i}^{n} \frac{r}{NR} \tag{1.7}$$

where r is the radius of the curvature of a corner of the particle surface, R is the radius of the maximum inscribed circle in the longitudinal section of the particle, and N is the number of corners. If the corners are worn down, then r approaches R, and roundness $= 1$ (when $r \to R$) (Mohsenin 1970).

Furthermore:

$$\text{Roundness ratio} = \frac{r}{R} \tag{1.8}$$

where R in this case is the mean radius of the object and r is the radius of the curvature of the sharpest corner (Mohsenin 1970).

1.2.3.2 Sphericity

Sphericity is a measure of how spherical (round) an object is. Sphericity can be estimated by different methods. For instance:

$$\text{Sphericity} = \frac{d_\text{e}}{d_\text{c}} \tag{1.9}$$

where d_e is the diameter of a sphere of the same volume as the object and d_c is the diameter of the smallest circumscribing sphere or, usually, the longest diameter of the object (Curray 1951; Mohsenin 1970). The advantages of using such an expression are that it relates the shape of the solid to that of a sphere of the same volume.

The volume of a solid can be equal to the volume of a *triaxial ellipsoid* which has diameters equivalent to those of the sample. Such an ellipsoid is a type of quadric surface that is a higher dimensional analogue of an ellipse. The equation describing a standard axis-aligned ellipsoid body in an *xyz*-Cartesian coordinate system is

$$\frac{x^2}{a^2} + \frac{y^2}{b^2} + \frac{z^2}{c^2} = 1 \tag{1.10}$$

where a and b are the equatorial radii (along the x and y axes) and c is the polar radius (along the z axis), all of which are fixed positive real numbers determining the shape of the ellipsoid. If all three radii are equal, the solid body is a sphere; if two radii are equal, the ellipsoid is a spheroid, in other words if $a = b = c$ (sphere); $a = b > c$ (oblate spheroid, i.e., disk-shaped); $a = b < c$ (prolate spheroid, i.e., like a rugby ball). Finally, if $a > b > c$, the solid is a scalene ellipsoid (i.e., it has three unequal sides). Scalene ellipsoids are frequently called "triaxial ellipsoids" the implication being that all three axes need to be specified to define the shape.

As stated, if the volume of the solid is equal to the volume of the triaxial ellipsoid with intercepts a, b, c, and in addition the diameter of the circumscribed sphere is the longest intercept a of the ellipsoid, then the degree of sphericity can be calculated in accordance with

$$\text{sphericity} = (\text{volume of solid/volume of circumscribed sphere})^{1/3} = \frac{(abc)^{\frac{1}{3}}}{a} \tag{1.11}$$

in other words, the geometric mean diameter divided by the major diameter, where a is the longest intercept, b is the longest intercept normal to a, and c is the longest intercept normal to a and b (Mohsenin 1970). It is important to note that the intercepts need not intersect each other at a common point. Another definition of *sphericity* is given by d_i/d_c, where d_i is the diameter of the largest inscribed circle and d_c is the diameter of the smallest circumscribed circle (Mohsenin 1970).

Sphericity can also be defined as the ratio of the surface area of a sphere having the same volume as the object to the actual surface area of the object (Sahin and Sumnu 2006):

$$\varphi = \frac{\pi D_p^2}{S_p} = 6\frac{V_p}{D_p S_p} \tag{1.12}$$

where ϕ is the sphericity, D_p is the equivalent or nominal diameter of the bead (m), S_p is the surface area of one bead (m^2), and V_p is the volume of one bead (m^3)

(Sahin and Sumnu 2006). The equivalent diameter is regarded as the diameter of a sphere that has the same volume as the bead (particle) (Sahin and Sumnu 2006).

Another approach to estimating sphericity is

$$\varphi = \frac{\sum (D_i - D_{\text{ave}})^2}{(D_{\text{ave}} N)^2} \tag{1.13}$$

where D_i is any measured dimension (m), D_{ave} is the average dimension or equivalent diameter (m), and N is the number of measurements (an increase in N increases the accuracy of the result (Sahin and Sumnu 2006). In accordance with this formula, if the sample sphericity value is close to zero, it can be considered spherical (Sahin and Sumnu 2006).

Many reports have dealt with spherical beads: for example, one study described the instantaneous formation of homogeneous spherical gel beads from various chitin solutions and non-solvents upon contact of the chitin solutions with dimethylacetamide (DMAc)/lithium chloride (LiCl) or *N*-methyl-pyrrolidinone (NMP)/LiCl solvents and non-solvents such as water, ethanol, or acetone. According to this manuscript, ethanol was found to be the optimal non-solvent (Yilmaz and Bengisu 2003). Introduction of a mixture into a hydrophobic phase such as soya oil in parallel to adequate cooling is a technique suitable for thermo-gelling agents. However, if a perfect sphere is required, a non-toxic decane layer is introduced into the KCl solution (Buitelaar et al. 1988).

A more recent manuscript claimed that the optimum conditions for calcium alginate bead sphericity were a concentration of 2.24% sodium alginate, a flow rate of 0.059 ml/s for the sodium alginate solution, and a 459 rpm rotation for the calcium chloride solution. The predicted and experimental bead sphericities under optimum conditions were 94.5 and 96.7%, respectively, showing close agreement (Woo et al. 2007). Immersion in hot water slightly decreased bead size and rupture strength. NaCl treatment increased bead size and decreased rupture strength. While the pH of the calcium chloride solution had little effect on bead sphericity, bead sizes and gel strengths decreased with longer times in each pH solution. Beads coated with pectin and glucomannan showed no significant changes in sphericity, but their sizes decreased with time. The coated beads showed higher rupture strengths than the non-coated beads (Woo et al. 2007).

In the case of κ-carrageenan, the sphericity of the formed beads was calculated as the ratio of the surface area of an equivalent sphere to the surface area of the bead, which in the case of oblate spheroids can be measured (Keppeler et al. 2009). When the conventional bead manufacture method of dripping was used, significant spherical beads with a homogeneous surface were observed within a carrageenan concentration range of 1–6% (w/w). Higher carrageenan concentrations did not produce spherical beads, probably due to the high viscosity of the dropping solution. By adjusting the dripping rate (10 drops/min), the carrageenan solution temperature (50 ± 4°C), and the distance between the syringe tip and the hardening solution (optimal at 2 cm), a constant bead size could be obtained for the entire range of carrageenan concentrations, with a diameter of 3.1 ± 0.1 mm and excellent

sphericity. As already noted, an increase in carrageenan concentration led to a significant increase in dropping solution viscosity and to larger beads (Sankalia et al. 2006b). Nevertheless, the bead size was not significantly different among concentrations used, in agreement with a previous report (Sipahigil and Dortunc 2001). The explanation for this phenomenon is probably the constant surface tension of the dripping solution, which leads to homogeneous bead drops independent of carrageenan concentration in the solution (Keppeler et al. 2009).

1.2.3.3 Average Projected Area and Sphericity

Another method of estimating the degree of sphericity of a convex body was developed almost 50 years ago when sizing machines for fruits were developed (Houston 1957). A new criterion for size, the average projected area, was proposed. It is based on the theories of convex bodies. For such bodies

$$\frac{V^2}{S^3} \geq \frac{1}{36\pi} \tag{1.14}$$

where V is the volume and S is the surface area of the convex body. A_c, the average projected area of the convex body, is one-fourth of the surface area. Substituting $4A$ for S in Equation (1.14), and after some manipulation, gives

$$A \leq KV^{2/3} \tag{1.15}$$

and $K = (9\pi/16) = 1.21$ in the case of a sphere, when equality in Equation (1.15) is achieved. In other words, this method makes it possible to estimate the degree of sphericity of a bead and to check how K changes when the bead is not spherical. A few further conclusions can be derived: the dimensionless K constant varies with the characteristic dimensions of the object; the variation of A_c for unrestricted orientation is too great for a satisfactory measure of volume, but this variation can be reduced to an acceptable level (Houston 1957).

Not many manuscripts have dealt with roundness and sphericity of hydrocolloid beads. In fact, they are small objects; therefore, axial dimension measurements can be derived from the outlined projections of the bead. Tracings need to be made of both maximum and minimum projected areas, and the axis dimensions can be derived from those drawings (Nussinovitch and Gershon 1996). The same results can be achieved by shadowgraph (Mohesenin 1970). An estimate of the deviations of hydrocolloid beads from sphericity is important in studies of mass and heat transfer. The sphericity of alginate beads was estimated by two techniques (Nussinovitch and Gershon 1996): a method originally developed for quartz grains which expresses the shape character of a bead relative to that of a sphere of the same volume and a method previously developed for convex bodies which calculates a dimensionless constant that relates surface area to volume. Deviations from sphericity were easily estimated by the former method. The latter method, although more laborious, gave measurements that influenced the results less. The difference

between the two procedures for estimating bead sphericity was not more than 5%. Deviations from perfect sphericity were ~7% for the tested alginate beads under the examined conditions (Nussinovitch and Gershon 1996).

1.2.4 Bead Volume and Surface Area

Volume can be defined as how much three-dimensional space a solid, liquid, plasma, vacuum, or theoretical object occupies. One- and two-dimensional shapes are assigned zero volume in a three-dimensional space. Volume is commonly presented in units of milliliters or cubic centimeters. For simple shapes, volume can be easily calculated. For a more complicated shape, volume can be calculated by integral calculus if a formula exists for its boundary. The volume of any shape can be determined by displacement (i.e., measuring the volume of fluid displaced when an object is immersed in it). In differential geometry, volume is expressed by means of the volume form and is an important global Riemannian invariant. Volume is a fundamental parameter in thermodynamics where it is conjugated to pressure (http://en.wikipedia.org/wiki/Volume_form).

Volume can be expressed in different forms as follows: *solid volume* is the volume of the solid material (including water) excluding any interior pores that are filled with air (Sahin and Sumnu 2006); *apparent volume* is the volume of a substance including all pores within the material (internal pores); *bulk volume* is the volume of a material when packed or stacked in bulk. It includes all of the pores enclosed within the material (internal pores) as well as the void volume outside the boundaries of the individual particles when stacked in bulk (external pores; Sahin and Sumnu 2006).

An approach proposed for describing pebbles' volume (Griffiths and Smith 1964) can also be applied to beads. In geological work, visual comparison and description of shapes, sizes, colors, and various other physical properties is common (Curray 1951). For pebbles, the volume of the object is related to its axial dimensions, using the following relationship:

$$V = a_1^{b_1} a_2^{b_2} a_3^{b_3} \ldots a_n^{b_n} \tag{1.16}$$

where V is the volume of the object and $a_1, a_2, a_3, \ldots, a_n$ are the diameters of the considered object. If logs are taken of both sides of the equation, and multiple linear regression procedure is used (Goulden 1952; Quenouille 1952), the volume can be related to the axial dimensions, and the contribution of each axis (an axis being the line with respect to which a curve or figure is drawn, measured, or rotated; Weisstein 2003) to volume can be determined. For pebbles, a linear relationship was found to exist between log volume and log axial dimension. Furthermore, about 93% of the volume's variation could be accounted for by the three mutually perpendicular axes, while the intermediate axis contributed only 4% to the volume prediction, suggesting that measurements of only the major and minor axis (two axes) can provide nearly complete information on log volume variations.

If a bead is sphere-like, its volume can be estimated by $4/3\pi r^3$. If the bead is not spherical but has the shape of a spheroid, then in general, the volume of a spheroid (of any kind) is $4/3\pi a^2 b$, where a is the horizontal, transverse radius at the equator and b is the vertical, conjugate radius. For a prolate spheroid the surface area is

$$S = 2\pi b^2 + 2\pi \left(\frac{ab}{e}\right) \sin^{-1} e \tag{1.17}$$

where a and b are, respectively, the major and minor semi-axes of the ellipse of rotation and e is eccentricity, given by

$$e = \left[1 - \left(\frac{b}{a}\right)^2\right]^{1/2} \tag{1.18}$$

The surface area of an oblate spheroid is

$$S = 2\pi a^2 + \pi \left(\frac{b^2}{e}\right) \ln\left[\frac{1+e}{1-e}\right] \tag{1.19}$$

In parallel to volume and surface area estimations by formulas (Table 1.1), actual volume and surface area can be determined experimentally and a correction factor can be established, if needed, for a particular shape and manufacturing procedure (Mohsenin 1970).

Table 1.1 Volume, area, and sphericity of round shapes

Name	Volume	Area	Sphericity
Sphere	$4/3\ \pi r^2$	$4\pi r^2$	1
Ideal torus ($R = r$)	$2\pi^2 r^3$	$4\pi^2 r^2$	≈0.894
Ideal cylinder ($h = 2r$)	$2\pi r^3$	$6\pi r^2$	≈0.874
Half-sphere	$2/3\pi r^3$	$3\pi r^2$	≈0.840
Ideal cone ($h = 2\sqrt{2}r$)	$2\sqrt{2}/3\ \pi r^3$	$4\pi r^2$	≈0.794

1.2.4.1 Specific Surface Area

When a bead is spherical, then its specific surface area (m^2/kg) is

$$A_{sm} = \left(\frac{1}{\rho_{ap}}\right)\left(\frac{3}{r}\right) \tag{1.20}$$

where ρ_{ap} is the apparent density and r is the radius of the bead. If a bead is non-spherical, then its specific area can be estimated by

$$A_m = \left(\frac{1}{\rho_{ap}}\right)\left(\frac{3}{r\varphi}\right) \tag{1.21}$$

where ϕ is a correction factor which considers the shape of the bead. Thus, the sphericity of an irregular object can be defined as the ratio between the surface area of a sphere having the same volume as the object and the surface area of that object (Shafiur 1995). Some shape factor values for non-spherical particles can be found in the literature (Perry and Chilton 1973).

1.2.4.2 Volume Estimation Methods

Provided a solid (or semi-solid) does not absorb liquid rapidly, a liquid-displacement method can be applied to estimate its volume. A few weight determinations need to be made: the weight of an empty and dried specific-gravity bottle or graduated cylinder, the weight of the same container filled with a liquid of known density, and the weight of the container when the solid particles (beads) are placed in it. The volume of the particles can then be determined by V_s, the weight of liquid displaced by the solid divided by the density of the liquid, i.e.,

$$V_s = \frac{\left[\left(W_{pl} - W_p\right) - \left(W_{pls} - W_{ps}\right)\right]}{\rho_l} \tag{1.22}$$

where V_s is the volume of the solid (m^3), W_{pl} is the weight of the specific-gravity bottle (pycnometer) filled with liquid (kg), W_p is the weight of the empty pycnometer (kg), W_{pls} is the weight of the pycnometer containing the solid sample and filled with liquid, W_{ps} is the weight of the pycnometer containing solid sample with no liquid (kg), and ρ_l is the density of the liquid (kg/m^3) (Sahin and Sumnu 2006). The liquids used for measuring volume by the displacement method should have a low surface tension and should be only slowly absorbed, if at all, by the sample: water, ethanol, toluene, and tetrachloroethylene are commonly used (Sahin and Sumnu 2006). Volume can also be estimated, especially for samples with irregular shapes, by displacement of gas or air in the pycnometer (Karathanos and Saravacos 1993). The volume of irregular solids can also be measured by sand-, glass bead-, or seed-displacement methods (Sahin and Sumnu 2006).

1.3 Bead Density, Porosity, and Structure

Density is unit mass per unit volume:

$$\text{density} = \frac{\text{mass}}{\text{volume}} \tag{1.23}$$

This property is important in evaluating transport properties and for process calculations (Shafiur 1995). The SI unit of density is kg/m^3. Different forms of density are used in process calculations. *True density* is the density of a pure substance or material calculated from its component densities considering mass and volume

conservation (Shafiur 1995). If the densities and volume or mass fractions of the constituents are known, density can be determined from

$$\rho_{\mathrm{T}} = \frac{1}{\left[\frac{\sum X_i^v}{\rho_i}\right]} \tag{1.24}$$

where X_i^v is the mass fraction of ith component and ρ_i is the density of the ith component (kg/m^3) (Sahin and Sumnu 2006). *Solid density* is the density of the solid material (including water), excluding any interior pores that are filled with air. *Apparent density* is the density of a substance including all pores remaining in the material. *Bulk density* is the density of a material when packed (or stacked) in bulk. *Substance density* (material density) is the density measured when a substance has been thoroughly broken into pieces small enough to guarantee that no pores remain. *Particle density* is the density of a sample including the volume of all closed pores but not of the externally connected pores (Shafiur 1995).

Porosity is defined as the ratio between the air or void volume and the total volume. It can be defined in different ways. *Apparent porosity* is the ratio of total enclosed air space or void volume to the total volume of a material (Shafiur 1995). *Open-pore porosity* is the ratio of the volume of pores connected to the outside to total volume. *Closed-pore porosity* can be calculated by subtracting open-pore porosity from apparent porosity (Shafiur 1995). There are different methods of determining porosity: a direct method whereby porosity is determined from the difference between the bulk volume of a piece of porous material and its volume after destruction of all voids by means of compression. This method is applicable if the material is soft and no attractive or repulsive forces are present between the solid particles (Sahin and Sumnu 2006); an optical method, whereby porosity is determined from the microscopic view of a section of porous medium. Such a method can be of value if the section indeed represents the porosity of the whole sample (Abramoff et al. 2004). The third method is a density method, in which the apparent porosity (i.e., porosity due to the enclosed air space within the particles) can be calculated if the solid and apparent densities are known:

$$\varepsilon_{\mathrm{apparent}} = 1 - \left(\frac{\rho_{\mathrm{apparent}}}{\rho_{\mathrm{solid}}}\right) \tag{1.25}$$

Bulk porosity ($\varepsilon_{\mathrm{bulk}}$), also called external or inter-particle porosity, includes the external void volume.

Porosity is an important aspect in many fields. For example, porous materials are believed to be common in the asteroids and satellites of outer planets; in order to study the relationship between the structure of small bodies and their thermal and collisional evolution, impact disruption experiments have been performed on porous sintered glass beads (Setoh et al. 2007). These glass beads can be prepared with various porosities and compressive strengths (Setoh et al. 2007). Porous hydrocolloid beads are very common and can serve for many biotechnological operations. As such, their porosity is important and the pore size of

gel beads was investigated. It was observed that the pore size of a gel matrix is small enough to prevent enzymes from leaking out from the gel lattice, whereas substrates and products easily pass through the gel wall (Smidsrod and Skjak-Braek 1990). In calcium alginate gels, the open lattice structure provides high porosity for the efficient exchange of large substrates and products. An electron microscopy study of 2% calcium alginate beads showed pores of 5–200 nm in diameter (Smidsrod and Skjak-Braek 1990). Addition of low molecular weight linear polymers such as poly(*N*-vinylpyrrolidone) [PVP; $(C_6H_9NO)_n$; poly(1-(2-oxo-1-pyrrolidinyl)ethylene); molar mass 2500–25,000,000 g/mol; appearance: white to light yellow, hygroscopic, amorphous powder; melting point 110–180°C (glass point temperature), density 1.2 g/cm^3; Fig. 1.4] or poly(ethyleneglycol) [PEG; also known as poly(ethyleneoxide) (PEO) or polyoxyethylene (POE); the most commercially important type of polyether; molecular formula $C_{2n+2}H_{4n+6}O_{n+2}$; molar mass $44n + 62$; flash point 182–287°C; Fig. 1.5] in the range of 1–5% of the organic phase for the entrapment of enzymes and cells in poly(2-hydroxyethylmethacrylate) led to the production of a more porous support for better mass transfer of both reactants and products (Cantarella et al. 1988). Another report claimed that the structure and porosity of alginate gel beads are dependent on whether the calcium source (used to crosslink the alginate) is internal or external. Careful experimentation showed that beads with an internal calcium source had a looser structure and larger pore size than those with an external calcium source due to different gelation mechanisms. Furthermore, the diffusion rate of hemoglobin within the beads with an internal calcium source was faster than that in beads with an external source, which was consistent with the observation of their structures (Liu et al. 2002).

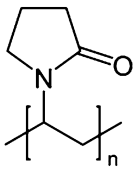

Fig. 1.4 Structure of polyvinylpyrrolidone (PVP) (courtesy of NEUROtiker, http://en.wikipedia.org/wiki/File:Polyvinylpyrrolidon.svg)

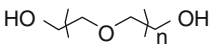

Fig. 1.5 Structure of polyethylene glycol (created by Sensei, http://en.wikipedia.org/wiki/File:Polyethylene_glycol_chemical_structure.png)

It is not surprising that cellulose (or treated cellulose), which is the most abundant naturally occurring organic substance in nature making up approximately one-third of the world's vegetative matter, is also used for bead preparation. Macropore cellulose beads with various pore sizes have been prepared from cellulose diacetate in the presence of pore-forming agents, the latter crosslinked with epichlorohydrin.

The reaction conditions and the amount of pore-forming agent can be optimized to control these beads' pore volume, pore structure, porosity, and swelling behavior in water (Bai and Li 2006). Another report described the fabrication of a superporous cellulose (SC) matrix by water-in-oil emulsification and thermal regeneration using granules of calcium carbonate as the porogenic agent. The matrix bead included more "craters" of several microns scattered over the surface of the beads than the control microporous cellulose bead that was fabricated in the absence of calcium carbonate. The presence of these craters resulted in a higher water content and effective porosity of the SC medium (Wang et al. 2007). Selected modifiers (such as montmorillonite) are commonly used in bead production from other edible hydrocolloids such as carrageenan and guar gum to reduce their solidification temperature and swelling ratio and to form beautiful three-dimensional reticulated structures and improve their stability (Zhang et al. 2007).

Beads with controlled porosity and structure can be formed from synthetic materials as well. A few examples are suspension polymerization for the synthesis of various glycidyl methacrylate (GMA) copolymers, using pentaerythritol triacrylate, trimethylolpropane triacrylate, and trimethylolpropane trimethacrylate as crosslinking comonomers: broad pore-size distribution in the range of 5–300 nm can be achieved in parallel to increased surface area and pore volume with an increase in the mole fraction of crosslinking comonomers (increased crosslinker density). The pore volume of the copolymers was more than doubled by including lauryl alcohol as a porogen (Kotha et al. 1998). Inverse suspension polymerization can also serve as a tool for producing macroporous beads composed of thermosensitive copolymers of poly(*N*-isopropyl acrylamide)-*co*-2-hydroxyethyl methacrylate macroporous resins. The porosity parameters, such as true density, apparent density, pore volume, and porosity of the resin were measured by pycnometry (Ni et al. 2004). While selecting a carrier for a given system, factors such as enzyme properties, substrate(s), and product(s) under the conditions of catalysis, which contribute to the economics of the process, have to be taken into account. The design of optimal porous and beaded copolymers for enzyme immobilization necessitates an in-depth examination of the effect of synthesis variables, such as type and mole fraction of crosslinking comonomer, diluent type, and volume. The interrelation between synthesis variables and material properties, such as surface area, porosity, and mechanical strength of the carrier, was thoroughly examined for beaded GMA–ethylene glycol dimethacrylate (Lukas et al. 1981) and GMA–divinyl benzene (Kotha et al. 1996a, b) copolymers. It was shown that porosity and surface area are influenced by the crosslinker density, the type and volume of porogen, the initiator concentration, and the polymerization temperature. Macroporous reactive copolymers comprise three-dimensional structures formed by coupling vinyl monomers with crosslinking multifunctional (di- or trivinyl) comonomers. In non-swelling systems, the desired macroporous structure is formed at a very high relative concentration of crosslinker. The hydrophilic/hydrophobic character of the crosslinker dictates the properties of the copolymer. Therefore, the crosslinking comonomer will influence the extent of enzyme immobilization.

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Chapter 2
Bead Formation, Strengthening, and Modification

2.1 Introduction

This chapter provides a brief description of the typical materials used today for bead formation and their limitations. These include gel beads prepared from agar/agarose, κ-carrageenan, alginate, and celluloses after special dissolution, chitosan, and to a lesser extent polyacrylamide and other synthetic polymers. Every section includes a short description of the natural or synthetic polymer followed by a description of the crosslinking agents used for both the creation and strengthening of several types of beads. In many cases, special methods to modify the porosity of the formed beads are also described. The chapter also covers procedures for the construction of different forms of carriers—from cylindrical to almost perfectly spherical—by changing both molds and the medium into which the molten or dissolved hydrocolloid, polymer or preparation/mixture is dropped or transferred. Information can also be found on means of dropping, changing drop size and distribution, and liquid sprays. The chapter also includes more than a few examples of these beads' uses. For the most part, however, uses are covered in detail in other chapters of this book, devoted to the beads' major applications in the food and biotechnological fields (Chapter 4), in medicine (Chapter 5), for drug delivery (Chapter 8), in agriculture (Chapter 9), and in environmental fields (Chapter 10). Applications of unique carriers, such as dried beads and liquid-core capsules, are described in Chapters 6 and 7.

2.2 Entrapment

Cells are entrapped by gels that permit the diffusion of small molecules, both substrate and product, at rates that are adequate for the cells' viability and functioning. In general, there are two types of cell entrapment. The first includes preparations in which cell viability is the primary concern. Viability is defined here as the cells' ability to increase in size and undergo nuclear and, where possible, cytoplasmic division (Tampion and Tampion 1987). If this occurs, intensification of the process after immobilization can be expected, i.e., the loosely entrapped cells will proliferate to very high densities, dependent upon the suitable addition of culture medium. Thus,

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the cell density in the matrix can be higher than that generated in free culture. The second type of immobilization involves entrapment of non-viable cells. The loss of viability may be intentionally induced or occur as a result of the techniques and materials used for the entrapment. In some cases, this provides an operational benefit since permeability barriers are removed and competitive biocatalytic pathways are destroyed. Of course, another option is to use activated supports that will couple covalently with purified enzymes, but this is beyond the scope of our discussion and in general, entrapment substances are less costly (Tampion and Tampion 1987).

2.3 Single-Step Methods

The major types of entrapment have been thoroughly reviewed (Cheetham 1980; Bucke 1983; Mattiasson 1983; Nussinovitch 1994, 1997). One of the most frequently used methods is single-step entrapment. This involves the simple gelation of macromolecules by lowering or raising temperatures, using hydrocolloids such as agar, agarose, κ-carrageenan, and chitosan and proteins such as gelatin and egg whites. Although quite simple to achieve, these preparations commonly suffer from heat damage and low mechanical strength. An alternative single-step method consists of ionotropic gelation of macromolecules such as alginate and low-methoxy pectin by di- and multivalent cations, but these systems suffer from low mechanical strength and breakdown in the presence of chelating agents. Jen et al. (1996) provide a summary of the hydrogels used for cell immobilization. They also review current developments in the immobilization of mammalian cells in hydrogels and discuss hydrogel requirements for use in adhesion, matrix entrapment and microencapsulation, the respective processing methods, and existing applications (Jen et al. 1996).

2.3.1 Agar

Agar was discovered in Japan in the mid-seventeenth century (Yanagawa 1942; Hayashi and Okazaki 1970; Matsuhashi 1978). One of the sources for traditionally manufactured agar in Japan is the thalloid alga *Gelidium*, with as many as 124 local species (Segawa 1965; http://en.wikipedia.org/wiki/Gelidium). The members of this genus are known by a number of common names. Specimens can reach ~2–40 cm in length; branching is irregular or occurs in rows on either side of the main stem, and the alga produces tetraspores. As stated, many of the algae in this genus are used to make agar (Guiry and Guiry 2008). An additional resource is the genus *Gracilaria*, which became significant after the discovery of alkali pretreatment (Funaki 1947; Matsuhashi 1972; Armisen and Kain 1995; Murano and Kaim 1995). *Gracilaria* is a genus of the red algae (Rhodophyta) which is noted for its economic importance as an agarophyte, as well as its use as a food for humans and various species of shellfish (Davidson 2004). Various species in this genus are cultivated in the developing world, including Asia, South America, Africa,

and Oceania (Steentoft and Farham 1997; http://en.wikipedia.org/wiki/Gracilaria). Numerous reviews contain information on the collection and processing of agar seaweed, the gelation mechanism, the effects of adding other materials on agar properties, and its applications, and the interested reader is referred to these references (Nussinovitch 1997). Agar is unique among gelling agents in that gelation occurs at temperatures below the gel's melting point (Nussinovitch 1997). Agar produces rigid gels at a concentration of ~1% (w/w) (Davidson 1980; Nussinovitch 1997). The sol sets to a gel at about 30–40°C. After setting, the gel maintains its shape. Self-supporting shapes are formed with 0.1% agar (the rest being water). In the past, the word "brittle" best described agar gels; today, however, elasticity or rigidity can be achieved using different agars. The gel is melted by heating to ~85–95°C (Nussinovitch 1997). Agar is used by microbiologists as an inert growth support because it is resistant to degradation by most microorganisms (Tampion and Tampion 1987).

To achieve entrapment by agar, a solution of 2–4% gum is prepared. Preference may be given to preparing the agar in a medium that is suitable for the particular cells being entrapped. To avoid damaging the entrapped cells, the temperature of the agar solution is reduced as close as possible to its setting point. It is important to note that the concentrated cell suspension is not preheated but added to the gum solution during mixing (Tampion and Tampion 1987). Brodelius and Nilsson (1980) reported slight heat damage to plant cells during a preparation procedure at 50°C. The shape can be custom designed by using a mold, by cutting, or via the use of various techniques and apparatuses. A sheet or slab with a predetermined thickness can be cut up into smaller pieces. Cylindrical beads can be produced by using a perforated Teflon mold with 3 mm diameter holes (Brodelius and Nilsson 1980). Spherical beads can be produced by either dropping the molten preparation into ice-cold fluid or pouring into a preheated vegetable oil to produce an emulsion that is cooled to 5°C with stirring (Wikstrom et al. 1982). Instead of injecting drops of warm agar solution into a cold oil bath, the warm solution may be dripped onto the surface of a cold oily medium. A particularly efficient process involves mechanically dispersing the warm solution in a cold immiscible oil or the like using an agitator. The rate or degree of agitation determines the size of the resultant gel beads (Delrieu and Ding 2001). Another report describes dropping agar solution at 45–50°C into an ice-cold mixture of toluene and chloroform (3:1, v/v), followed by washing with phosphate buffer and air-drying to produce further mechanical strength (Banerjee et al. 1982). Centrifugation may also be involved. In this case, the agar solution (or bacteriological medium that contains agar and is mixed with bacterial inoculum) is pipetted at ~50°C into mineral oil and stirred for 6 min at room temperature. The oil is cooled to 4°C with continuous stirring for 20 min, and then the oil–agar mixture is centrifuged at 4000 rpm for 20 min to sediment the beads (http://pen2.igc.gulbenkian.pt/cftr/vr/f/bragonzi_establishment_paeruginosa_airway_chronic_infection_agar_bacteria.pdf). Preferred agar beads are complexes of a continuous phase of agar gel in a self-supporting solid or semi-solid form with a restraining polymer. Entrapped in and dispersed randomly throughout each agar bead is a water-soluble, preferably polar restraining, polymer, preferably a

quaternized cationic polymer such as polyquaternium or steardimonium hydroxyethylcellulose. Various active agents may be bound to the restraining polymer, for example, ascorbic acid, lactic acid, or papain (Delrieu and Ding 2001). It is important to note that today, agar and other beads can be purchased from various companies, which offer a wide range of activated agar and agarose beads for cosmetics, personal care, and neutraceutical, affinity chromatography, and immobilization processes. Furthermore, to support the customer with a reliable product, these companies have total control over the supply chain, from collecting the seaweed, to having a profound knowledge of the raw materials, through the final manufacture of the finest beads.

2.3.2 Agarose

Two groups of polysaccharides—agarose, the gelling component which is an essentially sulfate-free, neutral (non-ionic) polysaccharide (Fig. 2.1), and agaropectin, a non-gelling ionic (charged) polysaccharide—are contained within the agar extract (Nussinovitch 1997). The basic sugar units of agarose (which consist of a linear structure with no branching) are D-galactose, 3,6-anhydro-L-galactose, and d-xylose. The percentage of agarose in agar-bearing seaweed is 50–90 (Araki 1937). Agarose gel is formed by lowering the temperature of the heated agarose to under 40°C. Its melting point is ~90°C and its molecular weight 10^5 Da. Agarose is more costly to use than agar, except in biotechnological applications as a base material for electrophoresis and as a filter for gel filtration (Osada and Kajiwara 2001). Low-melting agarose is used in a manner analogous to agar. There are many reports of using agarose as an entrapment medium, for example, for the entrapment of *Escherichia coli* and anucleate minicells produced by a mutant with defective cell division (Khachatourians et al. 1982). Special grades of agarose with lower gelling temperatures are used for the immobilization of *Catharanthus roseus* (Brodelius and Nilsson 1980). Not only bacteria are entrapped in agarose: examples include the photosynthetic alga *Chlorella vulgaris* (unicellular strain from 5 to 10 μm in size, a known source of chlorophyll and a resource for the production of biodiesel) and the blue-green bacterium *Anacystis nidulans* (Wikstrom et al. 1982) in research designed to study the oxidative deamination of amino acids. Such deamination can be fortified by co-immobilization of *C. vulgaris* and *Providencia* sp. PCM 1298.

Agarose can be formed in the shape of threads, for example, in the immobilization of hepatocytes (Foxall et al. 1984; Farghali et al. 1994). The immobilized

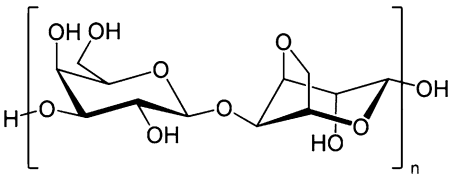

Fig. 2.1 Structure of an agarose polymer (courtesy of yikrazuul, http://en.wikipedia.org/wiki/File:Agarose_polymere.svg)

preparation can be looked upon as an intermediate step between the animal model (either whole or isolated perfused liver) or subcellular organelles and solubilized enzymes. Even though immobilization in beads and hollow fibers is also possible, the thread technique is simple and can be employed for many other cells, such as Sertoli cells [i.e., a "nurse" cell of the testes, part of the seminiferous tubule which is activated by follicle-stimulating hormone (FSH) and has an FSH receptor on its membranes], in just a few hours (Foxall et al. 1984; Farghali et al. 1993). The method involves the isolation of rat hepatocytes and mixing the agarose solution at 37°C with the cells at a density of 4–5 $\times$ 10^7 cells/ml. The threads are prepared by passing the agarose-cell mixture through cooled tubing, a step which can also be performed manually. The agarose solidifies and entraps the cells. The formed threads are compressed into a column for further experimentation and can be used as small research bioreactors (Gillies et al. 1993) and in nuclear magnetic resonance studies of cells (Caraceni et al. 1994). Agarose can be used not only for manufacturing beads for cell encapsulation but also as a bead coating (Jain et al. 2008). Coating is performed by rolling solid beads (agarose, collagen–agarose, or gelatin sponge–agarose combinations) in ~5–10% agarose, contacting the rolled beads with mineral oil and then washing the oil from the beads. Such beads, containing secretory cells, can be transplanted into mammals to treat a condition caused by impaired secretory cell function (Jain et al. 2008).

2.3.3 κ-Carrageenan

Agar, furcellaran, and three types of carrageenan (κ, ι, and λ) make up the family of gums derived from red seaweed (Nussinovitch 1997). Carrageenans are linear polysaccharides composed of alternating β(1-3)- and α(1-4)-linked galactose residues. The basic repeating unit is carrabiose (a disaccharide). The (1-4)-linked residues are commonly, but not invariably, present as 3,6-anhydride. Variations in this basic structure can result from substitutions (either anionic or non-ionic) on the hydroxyl groups of the sugar residues and from the absence of a 3,6-ether linkage (Stasnley 1990). Carrageenans are soluble in water at temperatures above 75°C. Sodium salts of κ- and ι-carrageenan are soluble in cold water, whereas salts with calcium and potassium exhibit varying degrees of swelling but do not dissolve completely. λ-Carrageenan is fully soluble in cold water (Nussinovitch 1997). Gel formation may be likened to crystallization or precipitation from solution. Both κ- and ι-carrageenans require heat for dissolution. After cooling and in the presence of positively charged ions (e.g., potassium or calcium), gelation occurs (Nussinovitch 1997). Gel preparation involves dispersing the κ-carrageenan plus the salt (potassium chloride) in distilled water and heating to ~80°C, then adding the water evaporated during the heating process and slowly cooling to room temperature to induce gelation (Nussinovitch 1997). Maximum strength is achieved for 1, 2, and 3% κ-carrageenan gels at ~1.5% potassium chloride (Nussinovitch et al. 1990). In another study dealing with the gelation of κ-carrageenan in the presence of potassium chloride (Krouwel et al. 1982), it was claimed that carrageenan gels

(beads) are superior to agar and inferior to calcium alginate. A comparison between κ-carrageenan and calcium alginate as entrapment media revealed a small advantage of the former over the latter (Grote et al. 1980).

Since gel formation is thermally reversible, immobilized carrageenan preparations are not well suited to higher temperature applications (Guiseley 1989). Moreover, the gel-inducing reagent's concentration might influence the success of the process: in very small quantities, the preparation may not be stable, whereas in excess it might inhibit some enzyme activity (Chibata et al. 1986). In addition to extrusion through an orifice or hollow needle (dripping method), dispersion in liquid or air is possible and shapes such as cubes, beads, or membranes can be produced. After its manufacture, κ-carrageenan bead strength can be increased by treatment with chlorohydrins, diepoxides, glutaraldehyde, tannin, or polyamines (Chao et al. 1986). The concentrations used should be considered in light of the possible damage to the viability/activity of the cells or enzymes (Chibata et al. 1987). Addition of Al^{3+} cations is another way in which such beads can be strengthened (Sanroman et al. 1994).

A mixture of *Saccharomyces cerevisiae* and carrageenan was pumped into a 2% (w/v) potassium chloride solution (Wang and Hettwer 1982). The immobilized preparation contained ten times more cells than the free cell suspension. The immobilized cells reached a stationary-phase plateau at a higher cell density in about twice the time taken by the free yeast cells. An increase in bead size from 3.5 to 5.5 mm had no effect on the final concentration of the cells. Further study revealed that inclusion of 5% (w/v) tricalcium phosphate (hydroxyapatite) (Fig. 2.2) crystals at sizes of up to 30 μm in the carrageenan gel can delay the dramatic drop in viable cell counts that are usually observed after 30 h (Wang and Hettwer 1982). At low pH, rapid dissolution of the crystals occurred, inducing better growth due to increased porosity, while at natural pH, better growth due to pH-value regulation was detected. When 10% (w/v) tricalcium phosphate was included, the settling velocity of the beads doubled (Wang and Hettwer 1982). Those authors also suggested separating old, less productive beads from the younger ones due to their differences in density resulting from dissolution of the included salt. An unexplored option is the questionable interaction between the tricalcium particles and the yeast within the gel as contributors to the adsorption support. Wang et al. (1982) claimed that increasing the potassium chloride concentration not only reduces cell leakage from the κ-carrageenan beads but also reduces yeast viability. κ-Carrageenan beads can be used to immobilize a very large number of species, with almost no restrictions (Mattiasson 1983). Immobilized *Saccharomyces carlsbergensis* produced ethanol continuously for over 3 months, at close to the

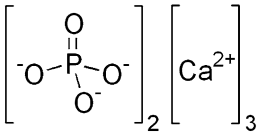

Fig. 2.2 Structure of tricalcium phosphate (courtesy of edgar 181, http://en.wikipedia.org/wiki/File:Tricalcium_phosphate.png)

theoretical conversion yield (Wada et al. 1980). A different approach to using κ-carrageenan was presented by Grote et al. (1980). They added *Zymomonas mobilis* to 2% (w/v) κ-carrageenan at 47–50°C to coat Raschig rings in a rotating flask. The rings were packed into a column and stabilized by 0.75% (w/v) potassium chloride in a 15% (w/v) glucose solution. The continuous operation was found to be successful although a 30% reduction in activity after 1 month was reported, in addition to a reduction in the void space of the column reactor (Grote et al. 1980).

Another report discussed combining locust bean gum and κ-carrageenan to immobilize *Penicillium urticae* conidia for production of the antibiotic patulin (Fig. 2.3). Germination and growth took place at 28°C for 36 h, giving better results in comparison to the free cells; this was also manifested by an extension of their half-life from 6 days (free cells) to 16 days for the entrapped preparation (Deo and Gaucher 1983). In addition to the production of antibiotics, immobilization of *Trichoderma viride* in κ-carrageenan matrix has been used for the production of extracellular enzymes (Frein et al. 1982).

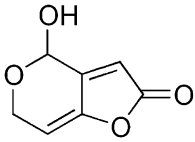

Fig. 2.3 Structure of patulin (courtesy of edgar 181, http://en.wikipedia.org/wiki/File:Patulin.png)

Crosslinked carrageenan beads can be used as a controlled-release delivery system. The influence of bulk carrageenan and crosslinker concentrations on bead size was studied to evaluate the mechanism of crosslinking between epichlorohydrin (a well-known crosslinker for polysaccharides) and the polysaccharide. The conditions were optimized with macroparticles (3.1 mm in diameter) for a better understanding of crosslink density and its effect on the morphology and surface topography of the bead (Keppeler et al. 2009). Low epichlorohydrin concentrations led to unstable and weak beads with uneven, cracked surfaces. The optimum crosslinker concentration, which resulted in smooth and stable gel beads, was applied to microparticles (76 μm in diameter). The swelling/shrinking behavior of these crosslinked microsponges in saline solution showed great potential for their application as delivery systems in food or pharmaceutical products (Keppeler et al. 2009).

2.3.4 Alginates

Alginates are perhaps the most studied and recognized component for entrapment. Alginic acid (Fig. 2.4) is a linear copolymer composed of D-mannuronic acid (M) and L-guluronic acid (G) (Whistler and Kirby 1959; Hirst and Rees 1965). Regions can consist of one unit or the other, or both monomers in alternating sequence, i.e., M blocks, G blocks, or heteropolymeric MG blocks, respectively. More information on their structure can be located elsewhere (Nussinovitch 1997;

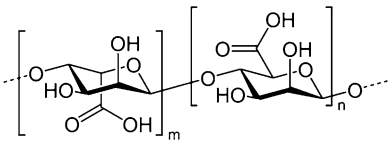

Fig. 2.4 Structure of alginic acid (courtesy of NEUROtiker, http://en.wikipedia.org/wiki/File:Algins%C3%A4ure.svg)

Phillips and Williams 2000). Alginate forms gels with a number of divalent cations (McDowell 1960). For food and biotechnological purposes, calcium is particularly suitable because of its non-toxicity. In poly-G segments (with chain lengths of over 20 residues), enhanced binding of calcium ions occurs and a cooperative mechanism is involved in the gelation. Crosslinking takes place via carboxyl groups by primary valences and via hydroxyl groups by secondary valences (Nussinovitch 1997). Coordinate bonds extend to two nearby hydroxyl groups of a third unit that may be in the same molecular chain, thereby retaining the macromolecule's coiled shape (Glicksman 1969), or in another chain, resulting in the formation of a huge molecule with a three-dimensional net-like structure (Glicksman 1969). Entrapment by alginate is a mild, safe, and simple method which is generally suitable for immobilizing any type of cell (i.e., bacteria, yeast, fungi, higher plant cells, animal cells, and even embryos) while retaining maximal biocatalytic flexibility.

When *Chlorella* was immobilized in alginate beads (Danity et al. 1986), importance was placed on stabilizing the gel structure and minimizing growth where prolonged use was required. An increase in gel strength can be achieved by using aluminum nitrate (trivalent cations) (Rochefort et al. 1986). Many procedures have been suggested to scale up the use of alginate beads, for instance, a rotating nozzle ring which sprays the gum solution–bacterium mixture into rotating vessels containing crosslinking solution (Matulovic et al. 1986). This apparatus is capable of producing beads of the requested size in large quantities per unit time. Another large-scale approach was proposed by Rehg et al. (1986), who used a dual fluid atomizer in which sodium alginate solution droplets were sheared off the tips of hypodermic needles into calcium chloride solutions to produce beads with an average diameter of 1 mm (Rehg et al. 1986).

Bead manufacture is not restricted to spherical shapes. For example, when *Z. mobilis* cells were immobilized in their late exponential stage (Grote et al. 1980), a syringe was used to inject the alginate-cell suspension into the gaps between perforated plates in a column reactor, which was filled with 0.75% (w/v) calcium chloride, forming what they described as fiber-like masses. Maximal ethanol production was obtained by diluting the substrate (glucose) and production continued for 800 h (Grote et al. 1980). Alginate beads of 1 mm in diameter were used to immobilize *Z. mobilis*, which attained a very high cell density. The reactor was operated continuously for up to 168 h. The fermentation ability was boosted by

strengthening the beads with calcium chloride during the operation (Margaritis et al. 1981).

It is well known that other divalent cations, such as Cu^{2+} or Pb^{2+}, have a greater affinity to alginate than Ca^{2+}. However, toxicity limits their use in many fields. Instead of Ca^{2+}, the use of Ba^{2+} for the immobilization of yeast cells (Chen and Huang 1988) was reported, and Cu^{2+} has been used for the immobilization of phenol oxidase (Palmieri et al. 1994). A differently shaped immobilization system for ethanol production by *S. cerevisiae* was produced by gelling alginate with calcium ions directly in a reactor around a regular pattern of rods. When the rods were removed, a gel block with internal flow channels was formed for easy liberation of the gas produced during the fermentation (Johansen and Flink 1986). Production of beads with controllable sizes in the range of 0.5–3.0 mm by a suitable apparatus was reported by Klein and Kressdorf (1983). Haggstrom and Molin (1980) immobilized both vegetative cells and spores of *Clostridium acetobutylicum* in alginate beads for the production of acetone–butanol–ethanol. Spores were activated by heating the beads to 95°C, in order to induce germination, prior to washing in non-growth medium. Anaerobic conditions were achieved by flushing with nitrogen gas. Use of a continuous stirred tank fermentor to produce isopropanol–butanol–ethanol by immobilization of *Clostridium beijerinckii* allowed for several rounds of reuse, although sudden and sometimes rapid losses in activity occurred after 15–27 days (Krouwel et al. 1983). A simple glass packed bed column was used for continuous and batch-recycled fermentation of 4.8% (w/v) glucose by *Lactobacillus delbrueckii* entrapped in alginate beads. After 40 h at 43°C, 97% of the theoretical yield was obtained. The operation could be run for 55 days with only a slight reduction in activity at first batch reuse (Stenroos et al. 1982).

Alginate can be used as a matrix for the immobilization of fungal hyphae. Pellets (2 mm) of the fungus *T. viride* were immobilized and the activity of β-glucosidase was investigated using cellbiose and salicin as substrates (Matteau and Saddler 1982). The β-glucosidase activity had a half-life of over 1000 h at 50°C, based on 340 h of continuous operation. To produce fragments of mycelium for immobilization, Livernoche et al. (1981) placed 2 cm diameter glass balls inside flasks of the fungus *Coriolus versicolor* on a shaker. The resultant fragments could easily be incorporated into alginate gel beads. Royer et al. (1983) reported on hyphal outgrowth from beads that contained viable mycelial fragments. In addition to bacterial, yeast, and fungal preparations, higher plant cells have also been immobilized in alginate. Entrapped cells of *Daucus carota* retained their ability to biotransform digitoxigenin and remained viable 24 days (Jones and Veliky 1981). Cell clumping is a problem which can be eliminated by allowing regrowth of viable cells entrapped in alginate beads (Morris and Fowler 1981). Alginate is a convenient medium for the encapsulation of mycobacteria (Brink and Tramper 1986) and the marine alga *Dunaliella tertiolecta* (Grizeau and Navarro 1986). Shapes other than beads or threads can be used: one report describes the use of a calcium alginate film formed on a stainless steel mesh and immobilizing the bacteria *Lactococcus* for both milk acidification and inoculation. The productivity of such preparations depends on the ratio between the surface area of the immobilized

biocatalyst and the bioreactor volume (Passos and Swaisgood 1993; Passos et al. 1994).

2.3.5 Chitosan

Commercial chitosan is derived from the shells of shrimp and other sea crustaceans. Chitosan is manufactured by deacetylation of the *N*-acyl group by heating chitin in a highly concentrated (40%) alkali solution or heating powdered chitin in fused calcium hydroxide at 180°C for 30 min. Chitosan is positively charged and soluble in acidic to neutral solution, with a charge density that depends on pH and the percentage degree of deacetylation (DA value). It binds to negatively charged surfaces. Chitosan is biocompatible and biodegradable. Purified chitosans are available for biomedical applications (Shahidi and Synowiecki 1991). Chitosan and its derivatives have been used in non-viral gene delivery for the transfection of breast cancer cells; with approximately 50% degree of trimethylation, the derivative is most efficient at gene delivery (Kean et al. 2005). Chitosan is a polycation that can be crosslinked with multivalent anions. This option can be used to prepare beads.

A chitosan–glycerol–water gel or gel-like membrane, useful as a carrier for medications to be applied to wounds, was prepared by dissolving chitosan in an acid–water–glycerol solution which when neutralized forms a gel upon standing (Jackson 1987). A typical simple example of gel formation was provided with chitosan tripolyphosphate and chitosan polyphosphate gel beads (Mi et al. 1999). Chitosan gel beads could also be prepared in an amino acid solution at about pH 9, despite the requirement for a pH above 12 for gelation in water (Kofuji et al. 1999). pH-sensitive hydrogels were also synthesized (Qu et al. 1999a, b) by grafting D,L-lactic acid onto the amino groups in chitosan without catalyst. Enzymatic reactions might also lead to gels (Chen et al. 2003). Glutaraldehyde was used as a crosslinking agent for chitosan. A semi-interpenetrating network was synthesized with poly(ethylene oxide) and chitosan and crosslinked with glyoxal (Khalid et al. 1999). Advances in the field of chitosan gelation promote biomedical applications that use microgel or nanogel particles for drug delivery (Oh et al. 2008). Uses include, for example, mixing *E. coli* cells into a chitosan acetate solution. The solution was dropped and left in 1.5% (w/v) sodium polyphosphate at pH 5.5 for 30 min. It was then transferred to polyphosphate at pH 8.5 for shrinking inducement. This preparation was used to study the tryptophan synthetase activity of the bacterium (Vorlop and Klein 1981). Of course drying can result in further strengthening of the beads. When dry, these beads do not decompose in the presence of phosphates as alginates do. Kluge et al. (1982) extended the afore-described work to the fungus *Pleurotus ostreatus*. Pellets composed of mycelium disrupted in dilute sodium chloride were washed and immobilized in chitosan. Polyphosphate was the best crosslinker for enzyme retention. Stocklein et al. (1983) demonstrated the efficiency of working with chitosan for conversion of phenylalanine to tyrosine by a *Pseudomonas* sp.

2.3.6 Cellulose

Cellulose is the most plentiful organic substance in nature, making up about one-third of the world's vegetative material (Zecher and Van Coillie 1992). Cellulose content in wood and cotton is ~40–50% and 85–97%, respectively (Ott 1946; Glicksman 1969; Whistler 1973). Cellulose (Fig. 2.5) is a linear polymer of D-glucose monomers joined by D-β(1,4) linkages, built from repeating units of cellobiose. The nature and structure of these molecules facilitate the formation of crystalline regions with consequent rigidity and strength (Ward and Seib 1970; Whistler and Zysk 1978). The degree of polymerization (DP) of cellulose depends on its origin (Krassig 1985; Zecher and Van Coillie 1992). The polymer has a maximum of three degrees of substitution (Greminger and Krumel 1980; Zecher and Van Coillie 1992). Products with a wide range of functional properties can be created by controlling the degree and type of substitution (Whistler and Zysk 1978). Extensive intra- and intermolecular hydrogen-bonded crystalline domains cause cellulose to be insoluble in water. Manufacture of water-soluble cellulose derivatives starts with a preformed polymer backbone of either wood or cotton cellulose (Greminger and Krumel 1980). Cellulose can be converted to a soluble compound via its derivatization and disruption of hydrogen bonds (Zecher and Van Coillie 1992). Cellulose derivatives are prepared by reacting alkali cellulose with either methyl chloride to form methylcellulose (MC), propylene oxide to form hydroxypropylcellulose (HPC), or sodium chloroacetate to form sodium carboxymethylcellulose (CMC). In the latter case a side reaction, the formation of sodium glycolate, also occurs (Stelzer and Klug 1980). Mixed derivatives such as methyl hydroxypropylcellulose (HPMC) can be formed by combining two or more of these reagents. Of the many possible derivatives investigated and manufactured, CMC, MC, HPMC, and HPC are utilized in the food industry in addition to modified forms of cellulose, which have been found to have useful functional hydrocolloidal properties and significance in several food applications. CMC is the most important cellulose-derived hydrocolloid for viscosity-forming applications, and it is used for its ability to react with charged molecules within specific pH ranges (Ganz 1966; Hercules Inc. 1978; Stelzer and Klug 1980). HPC, a non-ionic cellulose ether, is soluble in water below 40°C and in polar organic solvents such as methanol, ethanol, and propylene glycol (Butler and Klug 1980).

Microcrystalline cellulose has thickening and water-absorptive properties. MC and HPMC are soluble in cold water but insoluble in hot water. Upon heating such

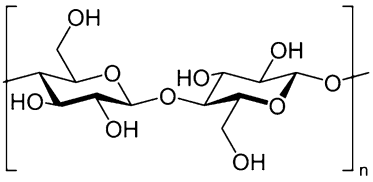

Fig. 2.5 Structure of cellulose (chain conformation) (courtesy of NEUROtiker, http://en.wikipedia.org/wiki/File:Cellulose_Sessel.svg)

a solution, gel structures can be formed at gelation temperatures ranging from 50 to 90°C (Dow Chemical Co. 1974; Greminger and Krumel 1980; Aqualon Co. 1989; Zecher and Van Coillie 1992). Solution viscosity decreases with increasing temperature to the thermal gel point, then rises sharply to its flocculation temperature (Zecher and Van Coillie 1992). Gels formed as a result of phase separation are susceptible to shear thinning. If the temperature is lowered, the original solution is restored. The thermal gel point is influenced by the type and degree of substitution. Flocculation temperature is influenced by the concentration of salts (decrease) and alcohols (increase) (Zecher and Van Coillie 1992). Microcrystalline cellulose gels are highly thixotropic and have a finite yield value at low concentrations. The formation of a network by solid-particle linkage is responsible for the produced yield value and elasticity. If shear is applied, the gel shears and thins. Resting allows the gel to reform a network. The addition of CMC reduces the thixotropic character of the gels and results in reduced yield values. Gum addition changes the rheological behavior of microcrystalline cellulose gels. Temperature has a small effect on the viscosity of microcrystalline cellulose dispersions (Nussinovitch 1997).

Cellulose is not soluble in water but dissolves in organic liquids (Tampion and Tampion 1987). Cellulose beads were produced by using *N*-ethyl pyridinium chloride and dimethyl formamide for polymer dissolution. *Actinoplanes missouriensis* was added, followed by dropping into water to produce the entrapping beads. Glutaraldehyde was used as a crosslinking agent to prevent cell leakage. Glucose isomerase retained 40–60% of its original activity before the immobilization and preparation, with a half-life of 45 days (Linko et al. 1977). Cellulose acetate was also used, but without much additional benefit (Sakimae and Onishi 1981). Cellulose can be used to create solid fibers for entrapment purposes. Wet spinning of fibers can be achieved with standard equipment (Dinelli 1972). Fibers produced from cellulose acetate were successful in entrapping *E. coli*, which exhibited 80% penicillin acylase activity when compared to free cells (Dinelli 1972). If fibers were heavily loaded, the bacteria showed reduced activity. By reducing solvent damage, better results may be achieved. Another approach, using cellulose acetate in acetone mixed with *E. coli* for the impregnation of cotton cloth, was reported. Optimal aspartase activity can be obtained by controlling porosity and degree of cell loading (Joshi and Yamazaki 1986).

2.3.7 Proteins

2.3.7.1 Collagen

Collagen is a natural animal protein. It is derived from connective tissues such as skin and cartilage (Tampion and Tampion 1987). It is manufactured by using organic solvents to extract the collagen's mixed materials, followed by a water wash and extraction in a dilute salt. The process involves using acid or alkali, which is then removed by enzyme interactions leaving the collagen as the non-solubilized material (Osada and Kajiwara 2001). Another procedure involves degrading fresh skin

extract in 0.06 M citric acid buffer (pH 4). After dialysis, extraction is performed in 0.5 M hydrogen phosphate-2-sodium. The precipitate obtained in the dialysis is again extracted using 0.2 M citric acid buffer (pH 3.8). Dialysis of this extract produces a recycled collagen (Osada and Kajiwara 2001). The hydrogel produced from collagen is a physical gel. It is formed when the concentration of the collagen solution is increased. When boiled for a long time in water, dilute acid or dilute alkali, it changes into a gelatin made of protein derivative (Osada and Kajiwara 2001). Collagen is widely used as a support for enzymes. It is often cast into a membrane form and stabilized with glutaraldehyde (Tampion and Tampion 1987). The first coagulation with the immobilized cells is probably due to hydrogen bonding forces and possibly an adsorption process in which the lysine residues of the collagen participate (Cheetham 1980). However, for stabilization, the dominant step is the extensive covalent bonding by glutaraldehyde. This step must be temporally controlled since excessive exposure can damage cell function. Successful entrapment of eight bacteria, *Aspergillus niger*, mammalian erythrocytes, and chloroplasts in a patented collagen system was reported by Vieth and Venkastsubramanian (1979). The method is based on casting the membrane from tanned collagen and winding with inert plastic spacer material. The invention has not raised a great deal of interest due to the extensive use of gelatin.

Spherical microcarriers can be used for cell culturing since they provide large surface areas for cell growth. They can be manufactured from polysaccharides, gelatin, or collagen (Cahn 1990; Altankov et al. 1991). The matrix formed by collagen has the unique characteristic of being macroporous and contains a fibrous microstructure appropriate for cell ingrowth (Langer and Vacanti 1993). Collagen microcarriers are generally prepared from a suspension of crude collagen extract. Spherical beads can be structured by discharging a suspension of collagen fibers into liquid nitrogen, followed by dehydration and crosslinking of the gel beads with formaldehyde or glutaraldehyde vapors (Yannas and Kirk 1984; Dean et al. 1989). These processes employ harsh conditions and do not appear to be suitable for the entrapment of cells in situ for subsequent cell culturing (Tsai et al. 1998). Alginate beads can be formed under mild conditions by contacting with calcium ions (Nussinovitch 1997), and this gelling property was made use of by preparing spherical gel beads of collagen/alginate: droplets of a mixture containing collagen (1.07–1.90 mg/ml) and alginate (1.2–1.5% w/v) were discharged into a 1.5% (w/v) calcium chloride solution at 4°C (Tsai et al. 1998). Collagen in the gel beads was reconstituted by raising the temperature to 37°C after alginate had been liquefied by citrate. Scanning electron microscopy of the beads revealed the characteristic fibrous structure of collagen. To demonstrate the application of this new technique in cell culture, GH3 rat pituitary tumor cells were entrapped and cultured in these gel beads (Tsai et al. 1998).

2.3.7.2 Gelatin

Gelatin is a natural organic protein that can be used to create physical hydrogels. The gelatin is manufactured by refining processed collagen. It has a molecular weight

of 100,000–250,000 (Osada and Kajiwara 2001). Gelatin is sold as a whitish to light yellow, flavorless, and odorless powder. It does not dissolve in cold water, but swells to between 5 and 10 times its size. It dissolves in warm water and becomes a homogeneous sol. Upon cooling, an elastic gel is formed. After dissolution, a clear solution is achieved, and the created gel is clear or semi-clear. An increase in concentration makes the gel stronger (Osada and Kajiwara 2001). Gelatin is used as a thickener and gelling agent for photography, cosmetics, and the food industry (Michon et al. 1997). It is an important wall material in the production of pharmaceutical microcapsules that enclose an active agent (Vandelli et al. 2001). Other uses for bacterial encapsulation, adhesives, and emulsion films are common.

The use of gelatin (10% w/v) was reported as a solution for the suspension of cells of *Arthrobacter* strain X-4 which were pre-adapted to xanthine before the immobilization, since the preparation was designed to generate high xanthine oxidase activity. Crosslinking by glutaraldehyde, freeze-drying, and then milling produced a powder with particles of 0.5, 0.7, and 1.0 mm nominal diameter (Tramper et al. 1979). Gelatin was reported as a suitable immobilization medium for *S. cerevisiae*, which was capable of sustaining more stable invertase activity than free cells, although the fermentation of glucose or sucrose was not possible (Parascandola and Scardi 1981). Gelatin and its copolymers with agarose and alginate, all crosslinked with glutaraldehyde, were used for the immobilization of *C. roseus*. The glutaraldehyde had an adverse effect on cell growth, respiration, and selectivity of the permeable membrane (Brodelius and Nilsson 1980).

Control over microcapsule size and size distribution has several important implications for controlled-release drug delivery (Berkland et al. 2001). More than a few methodologies for microcapsule preparation exist, including precipitation, spraying, phase separation, and/or emulsion techniques. Microchannel emulsification is a novel technique for preparing water-in-oil and oil-in-water emulsions (Kawakatsu et al. 2001; Sugiura et al. 2001). The microchannel plate has uniform microsized channels fabricated on a single-crystal silicon substrate using photolithographic and etching processes. Emulsions with a relative standard deviation of approximately 5% have been effectively prepared by applying this method (Iwamoto et al. 2002). Gelatin microbeads with a narrow size distribution were prepared by microchannel emulsification. An average particle diameter of 40.7 μm was prepared using this technique. Gelatin microbeads dispersed in isooctane after overnight gelation had smooth surfaces, with an average particle diameter and relative standard deviation of 31.6 μm and 7.3%, respectively (Iwamoto et al. 2002). The dried gelatin microbeads could be thoroughly resuspended in isooctane and had an average particle diameter of 15.6 μm and a relative standard deviation of 5.9%. Such a procedure is promising for the creation of monodispersed microbeads (Iwamoto et al. 2002). It should be emphasized that other proteins can be used for immobilization, and the protein need not have the ability to form a gel on its own. However, in such cases, glutaraldehyde should be used as the crosslinking agent.

2.3.7.3 Hen Egg White

Egg white is the common term for the clear liquid (albumen) in the egg. It consists mainly of ~15% proteins dissolved in water. Egg white constitutes about two-thirds of the total egg's weight, excluding the shell, with water accounting for ca. 90% of this weight. The remaining weight of the egg white comes from proteins, trace minerals, fatty materials, vitamins, and glucose (McGee 2004). Egg white contains approximately 40 different proteins, among them ovalbumin, ovotransferrin, ovomucoid, ovoglobulin G2, ovoglobulin G3, ovomucin, lysozyme, ovoinhibitor, ovoglycoprotein, flavoprotein, ovomacroglobulin, avidin, and cystatin. At 62–65°C, ovotransferrin—the most heat-sensitive protein in the egg white—starts to denature and the egg white begins to set. At 80°C, the main protein ovalbumin denatures. Denaturation and rearrangement at 80°C causes the egg white to firm up (McGee 2004).

Hen egg white can be used for entrapment after being crosslinked with glutaraldehyde. Free cells of *Calderiella acidophila*—a thermoacidophilic Archaebacterium capable of growth at 87°C at pH 3.0—were mixed with liquid egg white at 0°C and glutaraldehyde in phosphate buffer was added. This was followed by vacuum rotary evaporation at 60°C, grinding, and washing (De Rosa et al. 1981). The studied activity of β-galactosidase was ~30 times greater than that of comparable free cells. The matrix can be rendered magnetic by the addition of 0.3–0.7 μm diameter magentite particles (De Rosa et al. 1981). Egg white contains large quantities of lysozyme, which is capable of lysing many species of bacteria. This ability was utilized by D'Souza et al. (1983) to continuously self-sterilize an entrapment matrix with *E. coli* as the immobilized cells and *Micrococcus lysodeikticus* as the contaminant. During the 7 days of the study, lysis of about 30% of the cells was observed. The use of fresh egg whites instead of purified lysozyme was reported by Mattiasson (1979) to be less expensive. A thorough consideration of the glutaraldehyde concentration and conditions used is crucial to the efficiency of the process (D'Souza et al. 1983).

2.3.8 Synthetic Polymers

2.3.8.1 Polyacrylamide

The polymer polyacrylamide is manufactured from acrylamide subunits that can be readily crosslinked. Acrylamide must be handled very carefully to avoid toxic exposure. Polyacrylamide (Fig. 2.6) is not toxic; nevertheless, non-polymerized acrylamide can be present in the polymerized acrylamide. It is therefore recommended that polyacrylamide be handled with caution. In its crosslinked form, it is a soft gel that can be utilized in polyacrylamide gel electrophoresis and in manufacturing soft contact lenses. In its straight-chain form, it is also used as a suspension agent and thickener. Polyacrylamide gels are cast into slabs or blocks and mechanically converted into small particles after polymerization (Tampion and Tampion 1987).

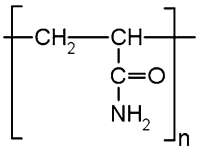

Fig. 2.6 Structure of polyacrylamide (courtesy of pulko citron, http://en.wikipedia.org/wiki/File:Polyacrylamide.png)

The polymerization conditions and their influence on the retention of β-glucosidase in an immobilized *Alcaligenes faecalis* preparation were studied by Wheatley and Phillips (1983). Highest retention of activity was observed under reaction conditions in which the maximal temperature did not rise above 40°C and its rise and fall were most rapid (Wheatley and Phillips 1983). Complete loss of viability of polyacrylamide-entrapped plant cells was reported by Brodelius and Nilsson (1980). Cells of *Corynebacterium dismutants* entrapped in polyacrylamide showed improved thermostability. Synthesis of alanine was highest in comparison with κ-carrageenan or absorption to DEAE (DE52) cellulose (Sarkar and Mayaudon 1983). Advantages of polyacrylamide over polymethacrylamide or polyepoxide for the entrapment of *E. coli* in beads for the production of tryptophan were reported by Bang et al. (1983). Suzuki and Karube (1979) studied the production of penicillin by *Penicillium chrysogenum* after its immobilization in polyacrylamide and alginate. Alginate exhibited poor mechanical properties while in polyacrylamide, the cells showed lower initial activity. To overcome the cell damage caused by the entrapment, organisms can be developed that are better suited to the immobilization than species taken directly from conventional production systems (Tampion and Tampion 1987).

2.3.8.2 Polyvinyl Alcohol (PVA)

Polyvinyl alcohol (PVA) is an odorless and non-toxic water-soluble synthetic polymer. PVA has a melting point of 230°C and 180–190°C for the fully hydrolyzed and partially hydrolyzed grades, respectively. It decomposes rapidly above 200°C as it can undergo pyrolysis at high temperatures. It has excellent film-forming, emulsifying, and adhesive properties. Phosphorylated PVA can be used for the immobilization of bacterial and yeast cells or activated sludge due to its non-toxicity and low cost (Arigo et al. 1987; Hashimoto and Furukawa 1987; Shindo and Kamimura 1990; Myoga et al. 1991; Wu and Wisecarver 1992). Spherical PVA beads are produced by crosslinking with saturated boric acid solution (for a short time to eliminate damage to the immobilized microorganisms), followed by esterification of the PVA with phosphate for further solidification. A pH range of 4–6 is suitable for both bead formation and strength. Stability and strength are better maintained at higher pHs. In the case of PVA-immobilized denitrifying sludge, the beads were observed to be stable over long periods (Chen and Lin 1994; Lin and Chen 1995). To change the poor gas permeability of PVA gels, sodium alginate is added to the PVA solution and the saturated boric solution includes calcium chloride for further crosslinking of the alginate. Later, this is removed by using the phosphate

solution, thus a more porous structure and phosphorylation are achieved simultaneously (Chen and Lin 1994; Lin and Chen 1995). The water-purification capacity of a polyvinyl(alcohol) (PVA) gel bead filtration system using photosynthetic bacteria was studied (Jeong et al. 2009). Long-term goldfish-rearing experiments were conducted using four different types of aquarium systems. Prominent decomposition of organic matter in the aquarium tank containing the PVA system, as well as less turbid aquarium water and more active goldfish, was observed. In addition, use of the PVA gel beads resulted in almost complete denitrification, even after 6 months of goldfish rearing. The results of this study indicate that this immobilized photosynthetic bacterial system has the potential for use as a component in circulating filtration systems (Jeong et al. 2009).

2.3.8.3 Other Synthetic Polymers

Other synthetic polymers can be used for cell entrapment, including copoly(styrene-maleic acid), polyethylene glycol (PEG) methacrylate, methoxypolyethylene glycol methacrylate (MPEGMA), PEG dimethacrylate, polyisocyanates, and polyurethane. Many researchers use polyacrylamide as their first choice of synthetic polymer due to the poor performance of many of the others (Tampion and Tampion 1987). Klein et al. (1979) compared the potential ability of many synthetic polymers to form an ionic network. MPEGMA polymerization by radiation was studied (Fujimura and Kaetsu 1982). Cell damage from this process can be reduced by using 0.5×10^4 rad/h, ~100 times lower than the initial radiation dose. Other beneficial actions included lowering the temperatures to –24°C and 0°C and using the monomer of MPEGMA, which is somewhat less toxic than that of 2-hydroxyethyl methacrylate (HEMA) (Fujimura and Kaetsu 1982). Entrapment of enzymes and cells in poly(HEMA) has been reported in many instances, a few examples being the entrapment of phosphatase (Cantarella et al. 1988), β-glucosidase (Alfani et al. 1987), β-fructofuranosidase, glucose oxidase, and cells of *S. cerevisiae* (Cantarella et al. 1989).

In contrast to conventional entrapping biopolymers (e.g., agar, alginate, and carrageenan), polyurethane gels demonstrate increased mechanical stability (Fukui et al. 1987); however, the isocyanate prepolymer is toxic (Klein and Wagner 1983). Blocking the isocyanates (by reaction at room temperature of the prepolymers with $NaHSO_3$) to form the polycarbamoyl sulfonate (PCS) propolymer could help solve this problem (Vorlop et al. 1992). The relationship between PCS gelation time and pH was studied (Muscat et al. 1996). At pH 8.5 and room temperature, gelation took on the order of seconds, while at pH < 5.5, it took up to 10 h. Diffusion coefficient values for a PCS hydrogel of $0.50–1.45 \times 10^{-5}$ cm^2/s for the substances glucose, ethanol, nitrate, and nitrite ion at 25°C were observed. These values were in the same range as those observed for calcium alginate (Daynes 1920). Substances with molecular weight >67,000 did not diffuse through the PCS hydrogel (Muscat et al. 1995). Entrapping nitrifying bacteria (*Paracoccus denitrificans*) in PCS versus calcium alginate beads showed almost the same activity (Muscat et al. 1995). Another study (Wilke et al. 1994) reported that during the immobilization,

reversible deactivation of *P. denitrificans* occurs. *S. cerevisiae* entrapped by PCS versus calcium alginate beads was reported to have similar activities directly after the immobilization. PCS hydrogel membranes were suitable for cell and enzyme immobilization (Kotte et al. 1995; Muscat et al. 1995).

Polyisocyanates have also been reported for the immobilization of *E. coli*, with the option of using either gels or foam beads. The foamed material is produced in stirred liquid paraffin. The foam's volume increases during the production process due to the inclusion of carbon dioxide bubbles. Low-density rigid foams are favored (Klein and Kluge 1981). Polyurethane foam was used to immobilize *C. roseus* cells. Isocitrate dehydrogenase and cathenamine reductase activity was detected in the foamed preparation by dimethyl sulfoxide (Felix and Mosbach 1982).

2.4 Two-Step Methods

Two-step methods may be used if they provide the manufacturer with improved processes or products or the possibility of overcoming evident problems. A procedure to create a more porous structure with desirable elastic behavior and mechanical stability throughout handling was reported by Klein and Eng (1979). They mixed epoxy resin reagent and curing agent with *E. coli* cells in aqueous medium. Alginate was then mixed in, followed by the traditional dropping into a calcium chloride bath to produce alginate beads. Following polycondensation of the resin, the beads were air-dried and the alginate dissolved in a phosphate buffer to produce swelling of the beads and a porous product. These moieties were advantageous over those obtained by single-step production with epoxy resin followed by grinding. The latter method did not sustain yeast cell viability and therefore, the improved two-step method was proposed (Klein and Kressdorf 1982). The selected types of resin and curing agent were those with low contents of low molecular weight components. Their 15 min reaction was followed by the addition of the alginate–yeast solution. The rest of the procedure was similar to that described previously. The results demonstrated an alcohol production activity of 21% of the original free cells and an option for more stable beads that can be successfully reactivated (Klein and Kressdorf 1982).

Three methods of alginate stabilization were proposed by Birnbaum et al. (1981). Alginate beads were treated with polyethyleneimine-HCl by infiltration of their structure for 24 h, and 1% (v/v) glutaraldehyde was applied at pH 7.0 for 1 min, followed by a wash in water. Although the glutaraldehyde was toxic to the immobilized yeast (*S. cerevisiae*) and ethanol production was reduced, the activity still remained considerable and the method was less costly than other proposed processes. A second method suggested the activation of alginate with a mixture of *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide for 1 h. The cells were added to this mixture and beads were produced by the traditional dropping of solution into a calcium salt bath (Birnbaum et al. 1981). The third suggested procedure involved the addition of sodium meta-periodate to half of the alginate for 1 h. The cells were mixed with the other, untreated half and then the two halves were mixed together and beads formed. Curing for 1 h was followed by

treatment with polyethyleneimine-HCl and a water wash. The mechanisms underlying these methods are not fully known, but they may involve some ionic or covalent binding of the cells themselves or, in the second and third procedure, direct coupling to the alginate (Birnbaum et al. 1981).

Alginate (2% w/v) and gelatin (20% w/v) were mixed with yeast cells (*S. cerevisiae* and *Saccharomyces uvarum*) before dropping into a calcium chloride solution. After formation of the beads, phosphate buffer was used to leach out the alginate and the gelatin beads were stabilized with glutaraldehyde. Since the latter is toxic, maintaining its concentration under 0.015 M results in enhancing fermentation rates (SivaRaman et al. 1982). Another method to enhance the mechanical properties of κ-carrageenan beads was reported by Chibata (1979). They were treated with hexamethylenediamine (HMDA) and glutaraldehyde, both at 85 mM. As a result, the half-life of *E. coli* aspartase activity was extended to 680 days. Other hardening agents included glutaraldehyde alone or persimmon tannin. Higher productivity (15-fold) was reported for HMDA + glutaraldehyde-hardened κ-carrageenan beads in comparison to polyacrylamide (Chibata 1979).

2.5 Cell Immobilization by Electrostatic Method

Electrical fields can be used to produce micron diameter beads (Goosen et al. 1986; Bugarski et al. 1993, 1994a, b; Goosen 1994; Sun 1994). The degree of applied electrical potential helps control bead size. During extrusion of a fluid through a positively charged stainless steel needle using a syringe pump, and upon electrification at a voltage <30 kV with low current (<0.4 mA), a charge is induced on the liquid's surface and mutual charge repulsion results in an outwardly directed force, producing a liquid spray. The drops are of various sizes and are emitted over a wide range of angles. If low-viscosity alginate is sprayed into a calcium chloride solution (contained in a grounded plate), the spontaneous crosslinking reaction produces alginate beads. Controlling liquid pressure, applied voltage, electrode spacing, and charge polarity is important for the production of regular and periodic spraying (Bugarski et al. 1993, 1994a, b; Sun 1994).

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Chapter 3
Methods and Mathematical Models for the Drying of Polymeric Beads

3.1 Introduction

Drying involves the transfer of heat into or out of a specimen. There are three ways in which heat may be transferred: radiation (heat transfer by photons), conduction (heat transfer by molecular-scale vibration and rotation), and convection [heat transfer by large-scale fluid motion (liquid or gas)] (Fig. 3.1). Steady-state conduction means that the rate at which heat is being transferred is determined by the temperature difference between the specimen and the heating or cooling medium and the total resistance to heat transfer. Under steady-state conditions, the rate of heat transfer is calculated by

$$Q = \frac{kA\,(\theta_1 - \theta_2)}{x} \tag{3.1}$$

where Q (J/s) = rate of heat transfer, k (J/m s K or W/m K) = thermal conductivity, A (m^2) = surface area, $\theta_1 - \theta_2$ (°C or K) = temperature difference, and x (m) = thickness of the material. $(\theta_1 - \theta_2)/x$ is also known as the temperature gradient. The thermal conductivity of the specimen is influenced by a number of factors including structure, amount of entrapped gas, and moisture content, as well as temperature and pressure of the surroundings (Fellows 2002). Under non-steady-state conduction and during processing, the temperature at a given point within a specimen depends on the rate of heating or cooling and the position in the specimen. Continuous changes in temperature are influenced by the temperature of the heating medium, the thermal conductivity of the specimen, and its specific heat. Thermal diffusivity is related to the thermal conductivity, specific heat, and density of the specimen by

$$a = \frac{k}{\rho c} \tag{3.2}$$

where a (m^2/s) = the thermal diffusivity, ρ (kg/m^3) = density, c (J/kg K) = specific heat capacity and k (W/m K) = thermal conductivity. The specific heats of water, water vapor, and ice are 4.18, 2.05, and 2.04 kJ/kg K, achieved at 15, 100, and 0°C,

A. Nussinovitch, *Polymer Macro- and Micro-Gel Beads: Fundamentals and Applications*, DOI 10.1007/978-1-4419-6618-6_3,

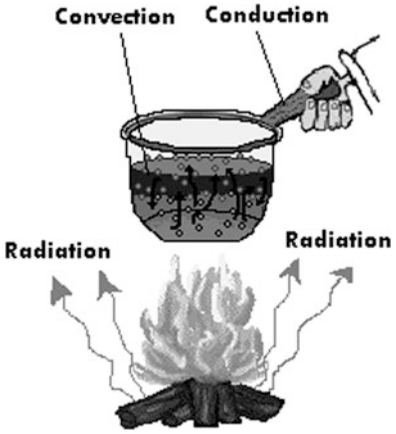

Fig. 3.1 Heat is transmitted by three distinct modes: conduction, radiation, and convection

Table 3.1 Thermal conductivity of selected materials

Type of material	Thermal conductivity (W/m K)	Temperature of measurement (°C)
Ice	2.25	0
Water	0.57	0
Poly(vinyl chloride)	0.29	20
Polystyrene foam	0.036	0
Polyurethane foam	0.026	0

respectively (Peleg and Bagely 1983). Thermal conductivities of selected materials are detailed in Table 3.1.

The basic equation for non-steady-state heat transfer in a single direction (x) is

$$\frac{\mathrm{d}\theta}{\mathrm{d}t} = \frac{k}{\rho c}\frac{\mathrm{d}^2\theta}{\mathrm{d}x^2} \tag{3.3}$$

where $\mathrm{d}\theta/\mathrm{d}t$ = change in temperature with time.

3.2 Methods for Drying Polymeric Gel Beads

3.2.1 General

Dehydration involves the simultaneous application of heat and removal of moisture. There are a large number of factors controlling the rate at which beads or other commodities dry, which can be grouped into the following categories: those related to the processing conditions; those related to the nature of the commodity; and those related to the dryer design (Fellows 2002).

3.2.2 Air-Drying

Air-drying is controlled by the quantity of water vapor already being carried in the air (expressed as either absolute humidity or relative humidity), the air temperature, and the amount of air passing over the beads. Another important factor is the air velocity. When hot air is blown over a product, a water vapor pressure gradient is established between the two, providing a "driving force" for water removal (Fellows 2002). The benefits of drying bacteria, which include ease of handling and distribution in the environment, have been previously discussed (Cassidy, Lee and Trevors 1996). Nevertheless, the drying process has also been shown to reduce the viability of encapsulated organisms (Paul et al. 1993; Cassidy et al. 1997). The challenge is to maintain high cell viability following a drying process and extended storage. One example of this is the development of a method to produce alginate beads encapsulating *Rhodococcus erythropolis* NI86/21 with high cell density, extended shelf life, ease of handling, and good atrazine-degrading capabilities in both liquids and agricultural soil. Atrazine (Fig. 3.2a, b) is a widely used herbicide that prevents pre- and post-emergence broad-leaf and grassy weeds in major crops by binding to the plastoquinone-binding protein in photosystem II, i.e., the first protein complex in the light-dependent reaction (Fig. 3.3), thereby inhibiting electron transport (Vancov et al. 2007). The beads were air-dried in a laminar flow hood for up to 24 h or freeze-dried by snap-freezing in liquid nitrogen, followed by lyophilization to complete dryness (overnight) in a freeze-dryer unit, by maintaining the pressure between 10^{-1} and 10^{-2} torr at −40°C (Vancov et al. 2007). Comparison of the different drying methods' effects on bead shrinkage demonstrated that beads dried by laminar air flow shrank substantially, while the freeze-dried beads displayed considerable weight loss compared with wet beads (Vancov et al. 2007).

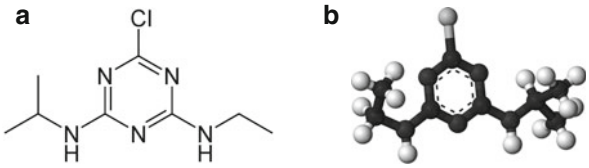

Fig. 3.2 (**a**) Atrazine formula (http://en.wikipedia.org/wiki/File:Atrazin.png) and (**b**) three-dimensional structure (http://en.wikipedia.org/wiki/File:Atrazine-3D-balls.png; courtesy of Benjah-bmm27)

Dry beads enable straightforward handling and distribution, and therefore various dry bead formulations have been assessed and compared to wet bead formats for cell viability. Air-drying had a minor effect on cell viability or survival in beads treated with a supplementary recovery step in nutrient broth media shortly after cell encapsulation (Vancov et al. 2007). The supplementary recovery step enhanced cell survival in both wet and dry beads upon extended storage at 4°C. A number of publications support an additional recovery/growth step for bead-encapsulated cells to promote high bacterial survival rates upon storage and subsequent release

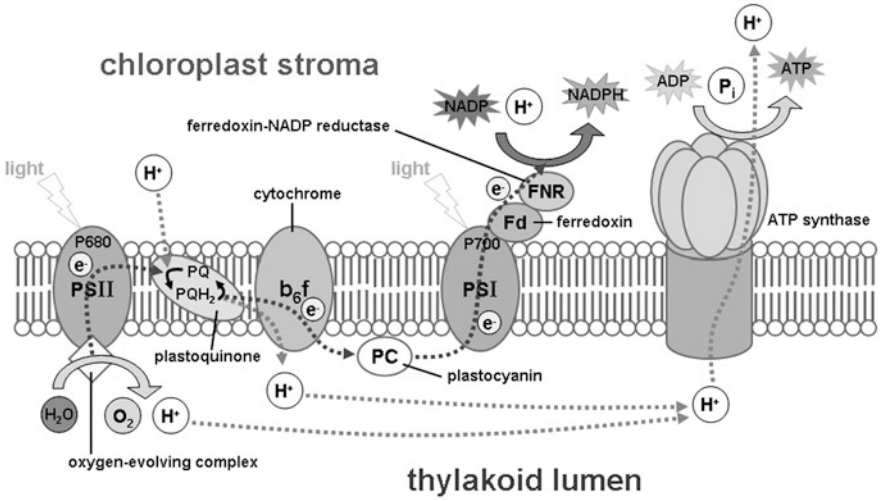

Fig. 3.3 Light-dependent reactions of photosynthesis at the thylakoid membrane (created using Powerpoint, based on Taiz and Zeiger, Plant Physiology, 4th edition, ISBN 0-87893-856-7; http://en.wikipedia.org/wiki/File:Thylakoid_membrane.png; original uploader was Tameeria at en.wikipedia; released into the public domain by the author)

in soils (Bashan 1986; van Elsas et al. 1992; Trevors et al. 1993; Weir et al. 1996). Cell survival in air-dried beads was only 0.35 log units lower than in wet beads on day 0. Cell numbers were comparatively constant over 182 days at 4°C. However, cell survival rates were reduced when air-dried beads were stored at 42°C. Under these conditions, cell viabilities declined by approximately 4.4–6.5 log CFU per bead within 62 days. The air-drying process reduced and altered bead size and shape, from an oval structure with a diameter of 3–4 mm to a granular shape of less than 1 mm in diameter (Vancov et al. 2007). Similarly, dry sodium alginate/hydroxypropylmethylcellulose (HPMC) beads showed a less spherical shape and noticeably reduced size. Addition of HPMC to the formulations had no significant effect on the mean particle size in any of the physical states. Significant differences were observed in some preparations in the reswollen state (Nochos et al. 2008).

Reports on drug release from beads have attempted to elucidate the advantages of particular types of drying. One study compared the release of nifedipine (NP) from alginate gel beads prepared using air- versus freeze-drying methods. NP (Fig. 3.4) is a dihydropyridine calcium-channel blocker. Its main uses are as an antianginal and antihypertensive. Beagle dogs were administered NP via oral ingestion of alginate gel beads that had been air-dried or NP powder alone: significantly less absorption of NP was found in the former case, perhaps due to the limited release of NP from the alginate gel beads in the gastrointestinal tract (Tateshita et al. 1993). On the other hand, when the alginate gel beads were prepared by freeze-drying, NP

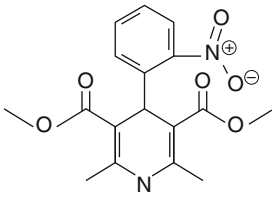

Fig. 3.4 Structure of nifedipine (http://en.wikipedia.org/wiki/File:Nifedipine.svg)

absorption improved, possibly due to increased disintegration of the alginate gel beads with decreased structural strength. However, this method showed poor reproducibility relative to the air-dried alginate gel beads. When PGA (propylene glycol alginate) was used instead of alginate, NP release was highly accelerated, potentially because of the swelling and lower strength of the formed gel (Tateshita et al. 1993). The effect of different drying methods on sustained drug delivery of floatable multiparticulate systems has also been explored. Crosslinked beads were composed of calcium and low-methoxylated pectin (LMP) or calcium, LMP, and sodium alginate. Beads were dried separately in an air-convection type oven at 40°C for 6 h or in a freeze-dryer to evaluate the changes in bead characteristics with process variability. Riboflavin, tetracycline, and methotrexate were used as model drugs for encapsulation. Evaluation of the drying process demonstrated that the freeze-dried beads remain buoyant for over 12 h in United States Pharmacopeia (USP) hydrochloride buffer at pH 1.5, whereas the air-dried beads remained submerged throughout the release study (Talukder and Fassihi 2004). The freeze-dried beads contained air-filled spaces which were responsible for the flotation property of the beads. Nevertheless, the release kinetics from the freeze-dried beads was independent of the hydrodynamic conditions. Calcium pectinate–alginate beads released their contents at much faster rates than did calcium pectinate beads (100% in 10 h versus 50% in 10 h, respectively). It appears that the nature of the crosslinking, the drying method, drug solubility, and the production approach are all important in the potential development of a gastroretentive drug-delivery system (Talukder and Fassihi 2004). In another example which also emphasizes the differences between air-dried and freeze-dried beads, and particularly the additional effect of coating, spherical beads were prepared from a water-soluble chitosan (*N*,*O*-carboxymethyl chitosan, NOCC) and alginate using an ionic gelation method. The swollen calcium–alginate–NOCC beads were then coated with chitosan (Tavakol et al. 2009). To prepare drug-loaded beads, sulfasalazine (SA) was added to the initial aqueous polymer solution. SA (Fig. 3.5) is a sulfa drug used primarily as an anti-inflammatory agent in the treatment of inflammatory bowel disease, as well as rheumatoid arthritis. The effects of the coating and drying procedures on the swelling behavior of unloaded beads and SA release from drug-loaded ones were evaluated in simulated gastrointestinal tract fluid. The rate of swelling and drug release was lower for air-dried and coated beads than for freeze-dried and uncoated ones, respectively (Tavakol et al. 2009).

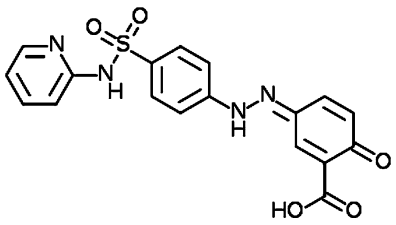

Fig. 3.5 Structure of sulfasalazine (http://en.wikipedia.org/wiki/File:Sulfasalazine.svg)

3.2.3 Fluidized-Bed and Microwave-Assisted Fluidized-Bed Drying

Freeze-drying of hydrocolloid beads produces cellular solids (see Chapter 6). Oven-drying of gum-based beads often ends in their collapse. Fluidized-bed drying at lower temperatures can lead to less structural damage to the bead than regular oven-drying. The design of an industrial-scale fluidized-bed dryer is in somewhat of an art, based more on empirical knowledge than on fundamental research. Fluidized-bed drying permits large-scale continuous operation with easy handling of the feed and product. The dryers do not include any mechanical moving parts and therefore maintenance is not expensive. Moreover, due to the rapid exchange of mass and heat between gas and particles, it is possible to eliminate heating of a heat-sensitive product. Control of the process is reliable due to rapid mixing, and heat transfer rates are high (Hovmand 1987).

Fluidized-bed drying presents the benefits of high heat and mass-transfer coefficients and hence increased drying rates result in shorter drying times (Wang and Chen 2000; Tatemoto et al. 2001). Consequently, fluidized-bed dryers are widely used in the food, chemical, metallurgical, and pharmaceutical industries for the drying of particulate solids (Mujumdar and Devahastin 2000). Drying in a fluidized-bed apparatus generates a monodisperse population of well-separated, spherical, smooth particles. Bead collapse during the drying stage has been shown to be avoidable in the case of chemical crosslinking of chitosan chains (Agnely et al. 2004). A major disadvantage of fluidized beds is the potential for particle size reduction due to attrition and collisions between them. Fluidized-bed drying is a convectional drying method which closely resembles drying under constant external conditions in short beds due to the high degree of mixing in both the solid and gas phases. As a consequence, one can observe the characteristic constant- and falling-rate periods, as well as the mechanisms occurring during the latter (Keey 1972): most of the drying takes place in the falling-rate period (Hlinak and Saleki-Gerhardt 2000; Elbert et al. 2001).

In fluidized-bed drying, layers of beads or other particles cover a grid through which gas is passed upward. When an increase in gas velocity is induced, the pressure drop across the particle layer increases in proportion to the gas velocity until it reaches the equivalent of the weight of the particles in the bed divided by the area of the bed. At that instant, the particles are suspended in the upward-flowing gas, and the frictional force between particles and the gas counterbalances the weight

of the particles (Hovmand 1987). This is the instant of the particles' incipient fluidization. Although the particle layer behaves like a liquid, only moderate mixing takes place. If the velocity of the gas is further increased, the additional gas passes through the particles as bubbles. These small bubbles, which originate from the gas distributor, coalesce rapidly and as they rise they mix with the fluidized particles. At higher velocities, the drag forces are increased such that the particles can be carried from the fluid bed (Hovmand 1987). The particles' size distribution should be fairly narrow and their shape regular. They should be between 20 μm and 10 mm in size—finer particles tend to lump together due to cohesive forces. The lumps break up easily or are retained on the gas distributor and cause defluidization. Finally, the particles must have the strength to withstand abrasion and the product should not be sticky at operational temperatures (Hovmand 1987).

When air passes through a bed of particles (beads) they create resistance to the air flow and reduce the area available for air to flow through the bed (Fellows 2002). When the velocity of the air increases, a point is reached when the weight of the beads is just balanced and the bed becomes fluidized (Fig. 3.6). If the velocity is increased further, the voidage is increased and the bed becomes more open, until eventually the particles are conveyed in the fluid stream. The velocity of the air

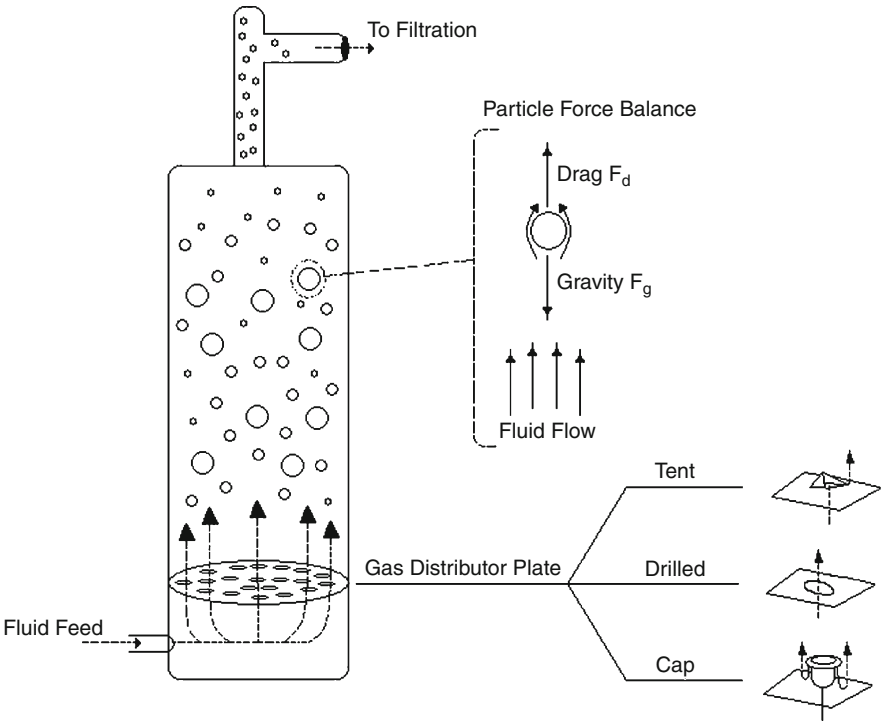

Fig. 3.6 Diagram of a fluidized bed

required to accomplish fluidization of spherical particles is calculated using the formula

$$v_f = \frac{(\rho_s - \rho)g}{\mu} \frac{d^2\varepsilon^3}{180(1-\varepsilon)} \tag{3.4}$$

where v_f (m/s) = fluidizing velocity, ρ_s(kg/m^3) = density of the solid particles, ρ(kg/m^3) = density of the fluid, g(m/s^2) = acceleration due to gravity, μ(N s/m^2) = viscosity of the fluid, d (m) = diameter of the particles, ϵ = the voidage of the bed.

When other than spherical particles are dried, the minimum air velocity needed to convey the particles is

$$v_e = \sqrt{\left[\frac{4d(\rho_s - \rho)}{3C_d\rho}\right]} \tag{3.5}$$

where v_e(m/s) = minimum air velocity and C_d (=0.44 for Re = 500–200,000) = the drag coefficient (Fellows 2002).

At the last stage of drying, Fick's law of diffusion applies:

$$\frac{\partial M}{\partial t} = \nabla(D_{eff}\nabla M) \tag{3.6}$$

where M (kg water/kg of dry solid) is the moisture content, t (s) is time, and D_{eff} (m^2/s) is the effective diffusivity of moisture in both liquid and vapor form. The solution of this equation for a sphere assuming constant moisture diffusivity and no shrinkage is (Crank 1975)

$$\frac{X_t - X^*}{X_0 - X^*} = \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(-n^2\frac{\pi^2 D_{eff}t}{r^2}\right) \tag{3.7}$$

where X_t (kg of water/kg of dry solids) is the moisture content at time t, X^* (kg of water/kg of dry solids) is the equilibrium moisture content, X_0 (kg of water/kg of dry solids) is the initial moisture content, and r (m) is the radius of the particle. When the Fourier number, $D_{eff}t/r^2$, is greater than about 0.1, all terms other than the first one on the right-hand side can be neglected, resulting in (McCabe et al. 1993)

$$\ln\left(\frac{X_t - X^*}{X_0 - X^*}\right) = \ln\left(\frac{6}{\pi^2}\right) - \frac{\pi^2 D_{eff}}{r^2}t \tag{3.8}$$

Plotting $\ln((X_t - X^*)/(X_0 - X^*))$ versus t gives the effective diffusivity from the slope of the linear portion of the curve. The effective diffusivities of some materials follow the Arrhenius-type equation (Treybal 1981):

$$D_{eff} = D_0\exp\left(\frac{-E_a}{RT}\right) \tag{3.9}$$

where E_a is the activation energy (kJ/kg mol), T is temperature (K), R is the gas law constant (kJ/kg mol K), and D_0 is a constant. Equation (3.9) can be rearranged into

$$\ln(D_{\text{eff}}) = \ln(D_0) - \frac{E_a}{RT} \tag{3.10}$$

Microwaves may have an influence on fluidized-bed drying. The mechanism of microwave drying is different from that of fluidized-bed or conventional drying. In this system, as the temperature within the material approaches the boiling point of water, the pressure increase becomes significant. Consequently, this moisture is moved from inside toward the surface in accordance with Darcy's flow (i.e., a simple proportional relationship between the instantaneous discharge rate through a porous medium, the viscosity of the fluid and the pressure drop over a given distance). Generally, this results in a much higher surface moisture level than that from diffusion alone (Ni et al. 1999). The water constituent is selectively heated to achieve substantially higher dielectric properties for the water than for components of the material (Ahmad et al. 2001; Feng et al. 2002). Nevertheless, microwave processing has the disadvantage of non-homogeneous distribution in a microwave cavity, creating setbacks related to non-uniform heating (Drouzas et al. 1999). Fluidized-bed drying with microwave heating compensates for some of the drawbacks of each method: evenness of particle temperature can be provided by good mixing due to fluidization (Feng and Tang 1998), and use of microwave energy can decrease the diffusional period of drying (Jumah and Raghavan 2001; Wang et al. 2002). Microwave-assisted fluidized-bed drying starts by internal heating and liquid water movement from the interior to the surface of the particle (Chen et al. 2001). When the temperature within the material approaches the boiling point of water, the developed pressure pushes the moisture to the surface where it is vaporized into the air. As the drying proceeds, the internal liquid water is not able to maintain the evaporation rate at the surface. The water begins to evaporate inside the particle, at which point Darcy's flow and vapor diffusion are the two major mechanisms for moisture transport in the particle (Goksu et al. 2005). During the final stage, the moisture content near the surface decreases below the critical moisture content. Darcy's flow disappears so that the liquid water has to evaporate and it is then transported to the particle surface only by vapor diffusion. As a result, in the last period of drying, Fick's law of diffusion may be applicable (Goksu et al. 2005).

3.2.4 Freeze-Drying

Freeze-drying (lyophilization) is a process in which the solvent and/or medium in a suspension is frozen at low temperatures and then sublimated from the solid state directly into the vapor phase (Oetjen 1999). Water generally serves as the solvent, and from its phase diagram one can clearly see the area of its transfer from solid to vapor. However, freeze-drying a biological substance containing several components in true solutions or suspensions presents a much more complicated situation. In the drying phase, the ice is transformed into vapor which has a large volume

(Oetjen 1999). The first stage of freeze-drying is to freeze the beads or biological sample in conventional freezing equipment. Rapid freezing will produce small ice crystals and less damage to the cell structure of the object. When the water vapor pressure of the sample is held below 4.58 torr (610.5 Pa) and the water is frozen, upon application of heat the solid ice sublimates to vapor without melting (Fellows 2002). For continuous water vapor removal, the pressure in the freeze dryer is kept below the vapor pressure at the surface of the ice. The vapor can also be removed by vacuum pump and then condensed on refrigeration coils. As the process continues, the sublimation front moves into the frozen object, leaving a partly dried layer behind it. The heat driving the sublimation is passed through the sample or produced by microwaves (Fellows 2002). The sublimated ice creates channels in the sample that correspond with its structure and porosity. The freeze-drying of samples composed basically of water is performed in two stages: sublimation to ~15% and then evaporative drying of the unfrozen water to ~2%, by raising the temperature in the dryer to near-ambient temperature while retaining the low pressure (Fellows 2002). The rate of heat transfer depends on the thickness and thermal conductivity of the ice. Thermal conductivity is the amount of heat conducted per unit time through a unit thickness of a material if a unit temperature gradient exists across that thickness. At 20°C, the thermal conductivities of water, air, and insulating materials are 0.597, 0.0251, and 0.035–0.173 W/m °C, respectively (Singh and Heldman 2001).

In parallel to the progressive drying, the thickness of the ice decreases and the rate of heat transfer increases. The rate of heat transfer to the sublimation front depends on the thickness and the area of the object, as well as on the thermal conductivity of the dry layer and the temperature difference between the surface of the object and the ice front (Fellows 2002). Similar to insulation materials, the dried layer has low thermal conductivity, and it therefore offers high resistance to heat flow. Microwaves are sometimes used to supply heat at the ice front, but this method is less controllable and if water is present as a result of ice thaw, localized overheating is possible (Fellows 2002).

In commercial freeze-dehydration, 1 g of ice converts to 2 m^3 of vapor at 67 Pa, requiring the removal of several hundreds of cubic meters of vapor per second. Factors controlling the water vapor pressure gradient are the pressure in the drying chamber (~13 Pa) in an economical system, the temperature of the vapor condenser, and the temperature of the ice at the sublimation front (~–35°C). One problem which may occur in the freeze-drying process is an unwanted change in the structure of the dried moiety. Such changes can occur when concentrated solutes in the moiety are mobile at temperatures above a critical collapse temperature, causing irreversible collapse of the moiety's structure. Thus mass transfer is controlled by the maximal ice temperature, the minimal condenser temperature, and the minimum chamber pressure (Fellows 2002). When executing the drying operation, the moisture content decreases from an initial high level in the frozen zone to a lower level in the dried layer. The relationship between the pressure at the ice surface and that in the cabinet is

$$P_i = P_s + \frac{k_d}{b\lambda_s}(\theta_s - \theta_i) \tag{3.11}$$

where P_i (Pa) is the partial pressure of water at the sublimation front, P_s (Pa) is the partial pressure of water at the surface, k_d (W/m K) is the thermal conductivity of the dry layer, b (kg/s m) is the permeability of the dry layer, λ_s (J/kg) is the latent heat of sublimation, θ_s (°C) is thesurface temperature, and θ_i (°C) is the temperature at the sublimation front (Fellows 2002).

The drying time depends on many factors. It is proportional to the square of the object thickness:

$$t_d = \frac{x^2 \rho \, (M_1 - M_2) \, \lambda_s}{8 k_d (\theta_s - \theta_i)} \tag{3.12}$$

where t_d (s) is the drying time, x (m) is the thickness of the object, ρ (kg/m^3) is the bulk density of the dry object, and M_1 (kg of water/kg of dry solids) is the initial moisture content and M_2 the final moisture content in the dry layer (Karel 1974; Fellows 2002).

Different types of freeze-dryers are available. In contact freeze-dryers, the heat is transferred via conductance to one side of the object. Thus, the contact is uneven. There are also differences between the drying rates of the object's top and bottom and loss of fine particles of the product with the vapor. Accelerated freeze-dryers involve a different procedure for holding the object that facilitates more rapid heat conductance into the object and thus reduced drying times. Radiation freeze-dryers heat objects in a more uniform way, maintaining constant drying conditions. Heat can also be produced by radio frequency heaters, but microwave and dielectric freeze-dryers are less used commercially due to their lack of controllability (Fellows 2002).

3.2.5 Freeze-Dried Biological Products

Pharmaceutical, biological, and medical products are among the many items that are freeze-dried for a variety of reasons. These products can include ingredients that are not stable in a liquid state, their active ingredient may be present in very small amounts, or they can be designed to reduce microbial contamination (Oetjen 1999). The biological products include, among many others, proteins and hormones, viruses, vaccines, bacteria and yeast, antibiotics, cytostatics, ibuprofen, liposomes, and nanoparticles. Beads containing bacteria and spores are dried to produce carriers for use in water treatment and in the biological control of diseases in soils. When the biological and natural activities of microorganisms need to be retained, cryoprotective agents (CPAs) are added to the material before its freeze-dehydration (Oetjen 1999).

In general, materials which cannot be heated, even to the moderate temperatures of ordinary drying, can be freeze-dried. In many cases, this operation produces a porous, non-shrunken dried product that undergoes rapid and almost complete rehydration when it is reconstituted (Liapis 1987). To produce a satisfactory drying rate, the freeze-drying process requires very low pressure or high vacuum. Most freeze-drying is performed at -10°C or lower at an absolute pressure of ~ 2 mmHg or less,

due to the fact that in most dried moieties, water does not exist in a pure state but in a combined state or as a solution. It is common to assume that if products are dried by this method and stored in the dry state under controlled conditions (i.e., free of oxygen and water vapor and usually in airtight, opaque containers, filled with inert dry gas), most of them can be kept for an almost unlimited period of time while retaining their desired properties (Liapis 1987).

Many studies have provided details on improving microorganism viability after freeze-dehydration. One study observed that trehalose is a very good stabilizer for *Escherichia coli*, even when the freeze-dried suspension was stored at 21°C and 60% relative humidity and/or exposed to visible light (Israeli et al. 1993). To increase the activity and capacity of *Saccharomyces cerevisiae*, addition of 10% skimmed milk saturated with Ar or N_2 was reported (Kabatov et al. 1991). Another study suggested that 0.010 M succinate buffer at pH 4.6 was the best stabilizer for *S. cerevisiae* (Pitombo et al. 1994). Sometimes, instead of immobilizing microorganisms, just the enzymes are immobilized within beads; for example, lipase from *Yarrowia lipolytica* was immobilized using three methods: inclusion, adsorption, and covalent bonding, to study enzyme leaching, storage, and catalytic properties. Sodium alginate and chitosan were the polymers selected to immobilize lipase by inclusion. The beads of each polymer were dried by freeze-drying and fluidization. The results showed that chitosan is better adapted to the inclusion of lipase. Even when freeze-dried, bead activity was low compared to that of fluidized beads. The freeze-drying process appeared to produce suitable beads for storage at 4 and 20°C (Alloue et al. 2008).

An interesting report on the development of porous collagen structures using freeze-dehydration stated that pore sizes are determined by the freezing conditions (Schoof et al. 1999). A homogeneous pore-size distribution was shown to be essential for the implanted collagen to populate living cells. Desirable sizes varied between 20 and 125 μm. The preparation consisted of a suspension with 1.8% collagen, water, and HCl at pH 3.2 followed by the addition of 3.8% acetic acid to pH 2.5, then freezing in a process by which a temperature gradient is applied between the two ends of the sample. The temperatures were further lowered and the samples were freeze-dried. The pore size was influenced by the acetic acid content: a decrease in the acid content from 3.8% (w/w) to 1.5% reduced the size from ~40 to 20 μm (Schoof et al. 1999).

3.3 Drying of Dosage Forms Made of Drug Dispersed in a Polymer

3.3.1 Mathematical Model

The process of drying a polymer or a solid is not simple, as demonstrated in studies carried out with elastomers of various shapes, such as thin sheets (Khatir et al. 1986) and cylinders of finite length (Khatir et al. 1987). Drying is controlled by

diffusion through a solid and evaporation from its surface. The process of drying dosage forms made of drug dispersed in a polymer was modeled (Laghoueg-Derriche and Vergnaud 1991a) using the following assumptions: (i) the process of solvent desorption is controlled by transient diffusion within the solid and evaporation on the surface; (ii) the rate of evaporation is proportional to the difference between the actual concentration of liquid on the surface and the concentration on the surface which is at equilibrium with the surrounding atmosphere, under the operational conditions, the coefficient of proportionality being the rate of evaporation of the pure liquid; (iii) the bead is spherical in shape, and its dimensions do not change during the process; (iv) the diffusivity is constant, as has been found experimentally; (v) the concentration of liquid in the bead is uniform at the beginning of the process (Laghoueg-Derriche and Vergnaud 1991b).

The transfer of liquid within the bead is expressed by Fick's equation for a sphere:

$$\frac{\partial C}{\partial t} = D\left[\frac{\partial^2 C}{\partial r^2} + \frac{2}{r}\frac{\partial C}{\partial r}\right] \tag{3.13}$$

with a constant diffusivity. The rate of evaporation on the surface is defined by the surface condition:

$$-D\left(\frac{\partial C}{\partial r}\right)_R = \frac{F_0}{\rho}(C_S - C_{eq}) \tag{3.14}$$

where C_s (g/cm^3) is the actual concentration of the liquid on the surface, C_{eq} (g/cm^3) is the surface concentration required to maintain equilibrium with the surrounding atmosphere, and F_0(g/cm^2 s) is the rate of evaporation of the pure liquid.

As the sphere is initially at the uniform concentration C_{in}, and the diffusivity is constant, the required solution of the above equation is

$$\frac{C_{rt} - C_{eq}}{C_{in} - C_{eq}} = \frac{2LR}{r}\sum_{n=1}^{\infty}\frac{\sin(\beta_n r/R)}{\sin\beta_n\left[\beta_n^2 + L^2 - L\right]}\exp\left(-\frac{\beta_n^2 Dt}{R^2}\right) \tag{3.15}$$

where C_{rt} is the concentration at position r (position in the bead, between 0 and R) and time t and the β_ns are the roots of

$$\beta_n \cot\beta_n + L - 1 = 0 \tag{3.16}$$

with the dimensionless number L

$$L = \frac{RF_0/\rho}{D} \tag{3.17}$$

The amount of liquid leaving the sphere is given by the expression (Crank 1975)

$$\frac{M_\infty - M_t}{M_\infty} = \sum_{n=1}^{\infty} \frac{6L^2}{\beta_n^2\left(\beta_n^2 + L^2 - L\right)} \exp\left(-\frac{\beta_n^2 Dt}{R^2}\right) \tag{3.18}$$

The process of drying is controlled not only by the rate of evaporation but also by diffusion of the liquid through the solid. The two parameters of interest are thus diffusivity of the liquid through the polymer and the rate of evaporation of the liquid.

The diffusivity of the liquid can be determined by using the experimental kinetics of drying and Equation (3.18). For long times, corresponding with a high value of M_t such as $0.7 < M_t/M_\infty < 1$, the series in Equation (3.18) converges very fast and the first term dominates. By neglecting the other terms, the equation can be written as follows:

$$\ln\left(\frac{M_\infty - M_t}{M_\infty}\right) = -\frac{\beta_1^2 D}{R^2} t + \ln \frac{6L^2}{\beta_1^2\left(\beta_1^2 + L^2 - L\right)} \tag{3.19}$$

By plotting the first term of Equation (3.19) as a function of time, a straight line is obtained. From the slope of this curve, the quantity $\beta_1 D/R^2$ is easily calculated. The value of the diffusivity can thus be obtained by simple iterative calculus, because β_1 is also a function of the diffusivity, as shown in Equation (3.17). The rate of evaporation can be determined in two ways: (i) by evaporating the pure liquid under the same conditions of temperature and pressure used for drying the bead—special attention is given to the shape of the flask, which is flat and full of liquid, as well as to the motion of the air; (ii) by using the initial rate of drying when the concentration of the liquid is uniform and constant, and therefore when the process may be assumed to be controlled by evaporation (Khatir et al. 1986). The validity of the model was tested by comparing the kinetics of drying of various beads obtained either experimentally or from calculation (Laghoueg-Derriche and Vergnaud 1991b).

Eudragit prepared dosage forms using a copolymer of dimethylaminoethyl acrylate and ethylmethacrylate as the polymer, in powdered form: a paste was composed from this polymer and ethanol, followed by pressing into spherical beads of various sizes. These beads were characterized by their weight and radius. Beads had total weights of 697, 352, and 251 mg, radii of 0.49, 0.39, and 0.21 (cm), and alcohol (as fluid) content of 120, 65, and 50 mg, respectively (Laghoueg-Derriche and Vergnaud 1991b). The kinetics of drying of these beads was determined by weighing them at intervals. The beads were dried in the open air at a constant temperature of 20°C. Calculations could be performed using either an analytical expression or a numerical model, the former because the diffusivity was constant. The rate of evaporation of the liquid was determined by following the weight of liquid evaporated under the same conditions in a flat flask of constant area (Laghoueg-Derriche and Vergnaud 1991b).

The following conclusions were drawn from these experiments: (i) the process of drying is very well described by the model that takes into account not only the diffusion of the liquid through the bead but also its evaporation on the surface; (ii)

as the diffusivity is constant during the entire process, the analytical solution gave results that agreed well with the experimental results; (iii) the process is controlled by diffusion, and the rate of drying is very high at the beginning of the process. This rate shows a constant decrease as the process proceeds; (iv) at the end of the process, the rate of drying is very low, and an asymptote is obtained when the drying approaches completion.

Three parameters are of interest: the radius of the sphere, the diffusivity, and the rate of evaporation. The rate of evaporation has an effect on evaporation and diffusion. On the one hand, a smaller radius means a larger external area of unit mass of the solid, inducing faster drying. On the other hand, the dimensionless term found in Equations (3.3) and (3.6), Dt/R^2 shows that the time of diffusion is proportional to the square of the radius. An increase in the radius is thus followed by an increase in the time of diffusion. In general, the diffusivity value depends on the nature of the liquid and the polymer (Vergnaud 1983). It increases with increasing temperature, often following Arrhenius' law with a constant energy of activation (Blandin et al. 1987a). However, it is not possible to calculate the predicted effect of temperature on diffusivity—experiments must be performed. The rate of evaporation is a well-known parameter, as this rate is proportional to the vapor pressure of the liquid, and the vapor pressure is expressed in terms of temperature by the classical Clausius–Clapeyron relation (Fig. 3.7).

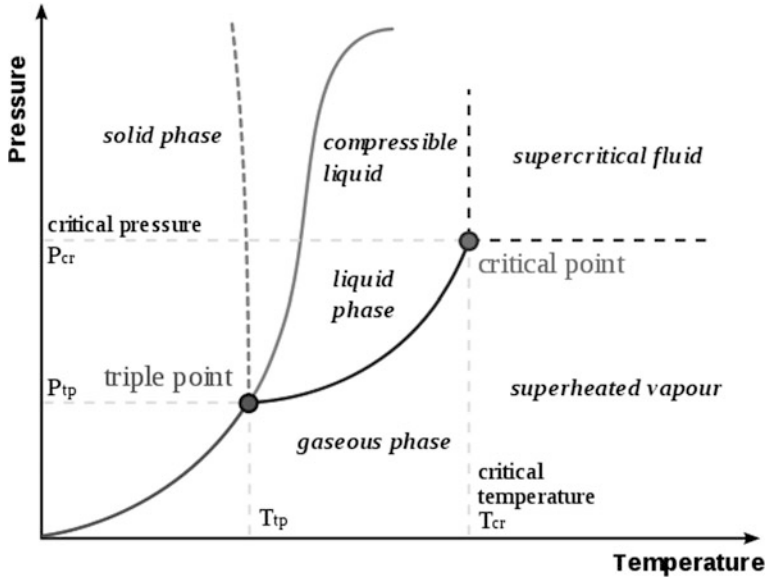

Fig. 3.7 A typical phase diagram. The *dotted line* gives the anomalous behavior of water. The Clausius–Clapeyron relation can be used to (numerically) find the relationships between pressure and temperature for the phase-change boundaries (courtesy of Matthieumarechal, http://en.wikipedia.org/wiki/File:Phase-diag2.svg)

3.3.2 Numerical Model

A numerical model was also constructed to resolve the problem of concentration-dependent diffusivity and a non-uniform initial liquid concentration: a sphere of radius R was divided into N spherical membranes of constant thickness Δr (Fig. 3.8). The mass balance was determined at various positions along a time increment Δt (Laghoueg-Derriche and Vergnaud 1991a). Within the solid (at position r), the new concentration after Δt has elapsed is expressed in terms of the previous concentration by the relation

$$CN_r = C_r + \frac{\Delta t}{r^2(\Delta r)^2}\left[G\left(r - \frac{\Delta r}{2}\right) - G\left(r + \frac{\Delta r}{2}\right)\right] \tag{3.20}$$

where the function G is given by

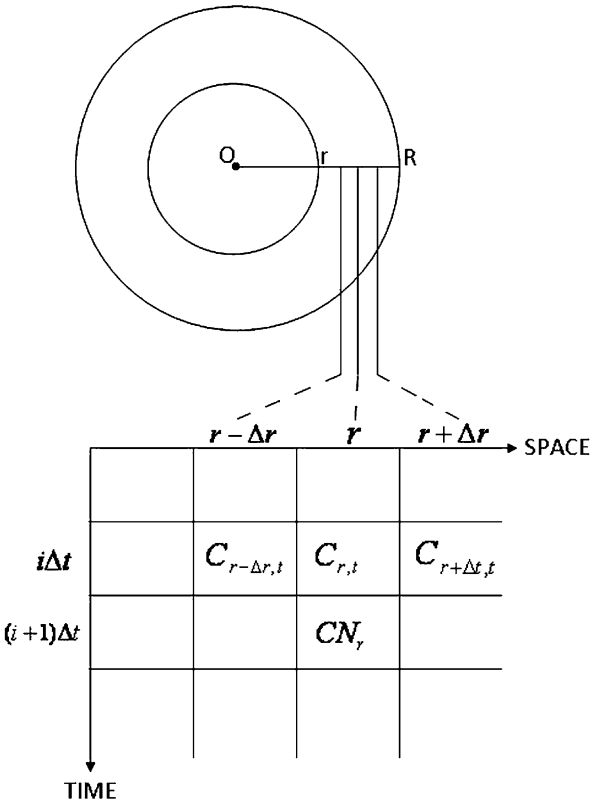

Fig. 3.8 Space–time diagram for numerical analysis for a spherical bead of radius R

$$G\left(r-\frac{\Delta r}{2}\right)=\left(r-\frac{\Delta r}{2}\right)^2\left(C_{r-\Delta r}-C_r\right)D_r-\frac{\Delta r}{2} \tag{3.21}$$

The diffusivity D_T (cm^2/s) is a function of temperature

$$D_T = DA\exp\frac{-E}{RT} \tag{3.22}$$

DA being a constant, E (cal/mole) playing the role of activation energy, and temperature T being expressed in Kelvin.

At the center of the spherical bead ($r = 0$), the following equation is obtained (Laghoueg-Derriche and Vergnaud 1991a):

$$CN_0 = C_0+\frac{24\Delta t}{(\Delta r)^4}G\left(\frac{\Delta r}{2}\right) \tag{3.23}$$

with the function G and diffusivity defined in Equations (3.21) and (3.22).

Surface of the spherical bead (R): The new concentration CN_R on the surface is expressed as a function of the previous concentration C_R after elapsed time Δt by the relation:

$$CN_R = C_R+\frac{2\Delta t}{(R-\Delta r/4)^2(\Delta r)^2}G\left(R-\frac{\Delta r}{2}\right)-\frac{2R^2\Delta t}{(R-\Delta r/4)\,\Delta r}\frac{F_T}{\rho}\left(C_R-C_{eq}\right) \tag{3.24}$$

where the function G has been previously defined.

The diffusivity in Equation (3.21) is a function of temperature (Equation 3.22).

The rate of evaporation also varies with temperature according to the following relation:

$$F_T = FA\exp\frac{-\Delta H}{RT} \tag{3.25}$$

where FA is a constant, ΔH (cal/mol) is the enthalpy of vaporization of the liquid, and the temperature of the bead is a function of time, with the constant heating rate b:

$$T_t = T_{t_0}+b\left(t-t_0\right) \tag{3.26}$$

The kinetics of drying can be determined by recording the weight of the bead, as well as the temperature. The volume of the surrounding atmosphere is so large that the vapor concentration becomes very small and therefore, negligible.

The parameters of interest, such as diffusivity and rate of evaporation, are determined from experiments carried out under isothermal conditions at various temperatures. The diffusivity is calculated from the slope of the straight line obtained by plotting the logarithm of the amount of liquid evaporated as a function of time.

This equation results from the analytical solution of diffusion–evaporation of a liquid out of a sphere (Crank 1975; Laghoueg-Derriche and Vergnaud 1991a, b; Laghoueg-Derriche, Bouzon and Vergnaud 1991). The first term of the series becomes dominant for long times, when $M_t/M_0 > 0.8$. β_1 is also a function of diffusivity, being the first root of Equation (3.16), with the dimensionless number L (see Equation 3.17)

The value of diffusivity is thus obtained by iteration when the rate of evaporation is known. The accuracy of diffusivity is tested by comparing the experimental and calculated kinetics of drying. The rate of evaporation of the liquid from the dosage form is determined from the kinetics of drying at the beginning of the process when the concentration of liquid is uniform (Khatir et al. 1986; Blandin et al. 1987b). The temperature dependence of the diffusivity is obtained by considering an Arrhenius expression while the rate of evaporation is expressed in terms of temperature by the Clausius–Clapeyron equation, the rate of evaporation being proportional to the pressure of vapor at equilibrium with the liquid (Equations 3.5 and 3.8). The values of the pre-exponential term and of E and ΔH can be obtained from the values of the diffusivity and rate of evaporation at various temperatures.

3.3.3 Drying a Polymer Bead with Shrinkage

When a large amount of fluid is absorbed by a polymer, swelling takes place. During the process of desorption, shrinkage occurs through transient diffusion of the liquid within the polymer and its evaporation from the surface. Many mathematical treatments of this phenomenon neglect the change in the bead's dimensions. A numerical method and model have been suggested to take these factors into account (Senoune et al. 1990; Bakhouya et al. 1991; Bouzon and Vergnaud 1991; Mouffok et al. 1991). Beads of ethylene vinyl acetate (EVA) copolymers (David et al. 1988, 1989a, b, c, d), which can be used for the slow release of active agents in agriculture, served as the model for these studies, due to their large absorption capacity. Six assumptions were made in constructing this model: the polymer is isotropic and homogeneous in composition; the bead is spherical and retains its form during drying; the volume of the bead with the included liquid equals the sum of the polymer and liquid volumes; liquid transport is governed by both transient diffusion and evaporation from the surface; the rate of evaporation is proportional to the difference between the actual concentration of liquid at the surface and the concentration necessary to maintain equilibrium with the surrounding atmosphere; and finally, during evaporation, the temperature remains constant (Vergnaud 1992).

Fick's law describes the radial transient diffusion through the polymer:

$$\frac{\partial C}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left[Dr^2\frac{\partial C}{\partial r}\right] \tag{3.27}$$

where C (g/cm^3) is the liquid concentration in the polymer and D (cm^2/s) is the diffusivity, which may or may not be constant. The initial and boundary conditions for the drying stage are

$$t = 0,\ 0 \le r \le R,\ C = C_{\text{in}}(\text{bead}) \tag{3.28}$$

$$t > 0,\ -D\left(\frac{\partial C}{\partial r}\right)_R = \left(\frac{F_0}{\rho}\right)\ \left(C_R - C_{\text{eq}}\right)(\text{surface}) \tag{3.29}$$

where R (cm) is the radius of the bead, C_R(g/cm^3) is the actual concentration of liquid on the surface, C_{eq} (g/cm^3) is the concentration of liquid necessary to maintain equilibrium with the surrounding atmosphere, F_0 (g/cm^{2}s) is the rate of evaporation of the pure liquid, and ρ (g/cm^3) is density (Vergnaud 1992). If the bead is spherical, its radius and the diffusivity are constant and an analytical solution may exist. If the dimensions of the bead are variable, the problem can be solved by numerical procedure.

As stated, beads of EVA copolymers were used as the model for these studies. The EVA polymer has a density of 0.96 g/cm^3 and a melt index of 55, with 40% vinyl acetate used for the creation of beads immersed in *n*-hexane with a density of 0.66 g/cm^3. The beads absorbed the liquid and changes were checked by periodical weighing. They were then dried by slight agitation at constant temperature and exposing their surfaces to large volumes of air relative to their small volume. Kinetic information was collected by periodical weighing. In the case of bead shrinkage, a numerical model was successfully used to describe the experimental results. The diffusivity D was 16×10^{-7} cm^2/s and the rate of evaporation F_0/ρ was 3×10^{-4} cm/s (Vergnaud 1992).

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Chapter 4
Food and Biotechnological Applications for Polymeric Beads and Carriers

4.1 Introduction

Bioreactor systems (i.e., any device or system that supports a biologically active environment) containing immobilized biocatalysts are part of the biotechnology developed in the 1970s. Initially, research focused mainly on the preparation and application of immobilized microbial, plant, and animal cells, not to mention enzymes and cellular organelles. Substantial effort was also invested in improving the design and operation of the bioreactors. Applications for immobilized biocatalysts can be found in many fields, and it was therefore natural that from the laboratory level, a few of the processes would be scaled up to the pilot plant and/or industrial level. The aim of this chapter is not only to include food and biotechnological applications of beads in the laboratory, but also to support the reader with a glimpse of various industrial processes.

4.2 Amino Acid Production

Amino acids are significant constituents of the basic nutrients of living organisms and are employed in feeds, food, and parenteral nutrition. Amino acids and their derivatives are also used in pharmaceutical, cosmetic, agricultural, and other industries (Kirk and Othmer 1991). Nearly all amino acids, including glutamic acid, aspartic acid, lysine, threonine, and leucine, are manufactured by fermentation. These amino acids are obtained in dilute aqueous solutions and have to be separated from excess substrate and by-products. Separation is frequently followed by final purification, concentration, and crystallization. The separation and purification steps may reach up to 50% of production costs (Eyal and Bressler 1993). Amino acids are the building blocks of proteins. The first few amino acids were discovered in the early 1800s. In 1806, a French pharmacist and chemist, Louis-Nicolas Vauquelin, isolated the first amino acid, asparagine, from asparagus (Vauquelin and Robiquet 1806). In 1812, William Hyde Wollaston, an English chemist and physicist who is famous for developing a way to process platinum ore, found a substance in urine that he identified as cystic oxide and which was later named cystine (Wollaston

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1810). In 1819, another French chemist and pharmacist, Henri Miriam Braconnot, published a memoir describing, for the first time, the conversion of cotton, straw, or wood into sugar by sulfuric acid treatment. Using the same acid process, he obtained glycine from gelatin and leucine from muscle fibers (Braconnot 1820). During the digestion of food, proteins are decomposed into amino acids, which are then absorbed into the blood and carried in the bloodstream to take part in the synthesis or formation of new proteins in the body (Nussinovitch 2003). Amino acids that cannot be prepared in the body are called essential amino acids. Except for some infant formulas and parenteral solutions, the use of amino acids as food additives is limited for the most part to the essential ones (Nussinovitch 2003). The amino acid industry started in Japan. A report on extracted basic flavor (Ikeda 1909) from traditional soup stock, which was identified as L-monosodium glutamate (MSG), served for a long time as the only product in this industry. The manufacture of other amino acids followed with the recognition of their value (Yamamoto 1978; Yoshida 1978; Kleemann et al. 1985). Amino acids are used not only as food additives but as components in single drugs, combination drugs, cosmetics, and feeds, as well as for other industrial uses (Kumon and Kawakita 1991). The main commercially produced amino acids are L-glutamic acid, DL-methionine, and L-lysine (hydrochloride). L-Phenylalanine production is on the rise, as the consumption of aspartame increases. Four groups of amino acid production methods exist: chemical synthesis, enzyme reactions, extraction from protein hydrolysates, and fermentation (Kumon and Kawakita 1991). Various bioreactors that utilize membranes for the production of pharmaceuticals have been reviewed (Belfort 1989) and these bioreactors can be applied to the production of amino acids. They include homogeneous (traditional), heterogeneous (immobilized), and multiple layer flat sheet-entrapped cell bioreactors (Belfort 1989).

4.2.1 L-*Aspartic Acid*

L-Aspartic acid [$C_4H_7NO_4$; a white crystalline powder with a melting point of >300°C (decomposes) that is slightly water soluble, stable, combustible, and incompatible with strong oxidizing agents] (Fig. 4.1) can be used as a food additive and in medicines. As a pharmaceutical ingredient, L-aspartic acid is used for cell culture and in manufacturing processes. L-Aspartic acid is widely utilized for mineral supplementation in its salt form. The popular synthetic low-calorie sweetener aspartame is a dipeptide of L-aspartic acid and L-phenylalanine methyl ester and its consumption has increased the demand for L-aspartic acid as a raw material. Since 1958, the

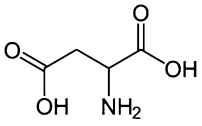

Fig. 4.1 Structure of L-aspartic acid

industrial manufacture of L-aspartic acid has been achieved by enzymatic and fermentative methods from fumaric acid and ammonia, through the action of aspartase (Sato and Tosa 1993). There have been numerous studies on batch and continuous production of L-aspartic acid (Chibata et al. 1974; Tosa et al. 1974; Sato et al. 1979, 1983; Fusee et al. 1981; Chao et al. 2000). In those studies, L-aspartic acid was separated from the reaction mixture by crystallization. Reverse micelle extraction, i.e., a system that consists of nanoaggregates of surfactant molecules containing an inner water core, dispersed in an organic solvent medium, has been used. The polar nanoenvironment inside reverse micelles permits the solubilization of biomolecules while retaining their native structure (Brandani et al. 1996). The process has been used to recover amino acids from fermentation broths. This low-energy-consuming, simple, and flexible procedure also allows recovery and concentration of amino acids from dilute aqueous solutions (Krei and Hustedt 1992; Dovyap et al. 2006). L-Aspartic acid was produced via a continuous process by the Japanese company Tanabe Seiyaku (Sato and Tosa 1993): *Escherichia coli* cells were immobilized in a polyacrylamide gel, which was then cut into cubes of 3×3×3 mm. A suspension of the freshly immobilized preparation at 37°C for 24–28 h increased the activity by ~10-fold, possibly due to increased membrane permeability to substrate and/or product due to cell autolysis within the gel. The immobilized cell column's half-life was 120 days at 37°C. In a column of 1000 l, the theoretical yield was 1915 kg/day, ~60 t/month. The method was observed to be 40% less expensive than the batch reaction method (Sato and Tosa 1993). An attempt to replace the polyacrylamide with κ-carrageenan gel strengthened with 0.3 M KCl solution was very successful. In the polyacrylamide immobilization method, the aspartase activity was 18,850 μmol/h g cells, in comparison to 56,340 μmol/h g cells for carrageenan. When glutaraldehyde or hexamethylenediamine was added to the carrageenan, aspartase activity decreased to 37,460 and 49,400 μmol/h g cells, respectively; however, the respective half-lives at 37°C increased to 240 and 680 days and relative productivity increased by ~4- and ~15-fold, respectively. As a result of this study, the Tanabe Seiyaku Corporation switched to the carrageenan method in 1978: using a 1000 l column, the theoretical yield of L-aspartic acid is 3.4 ton/day and ~100 t/month (Sato and Tosa 1993). In 1982, the corporation began to use *E. coli* strain EAPc-7, derived from appropriate cultivation for higher aspartase activity (Sato and Tosa 1993).

4.2.2 L-*Alanine*

L-Alanine is an amino acid that, due to its attractive flavor, can be used in medicines as well as in foods (Fig. 4.2). Since 1965, it has been produced by Tanabe Seiyaku Corporation from L-aspartic acid. L-Alanine can be produced by co-immobilization of *E. coli* and *Pseudomonas dacunhae* cells. The presence of the two immobilized microorganisms in a single reactor makes it possible to immediately convert L-aspartic acid produced by the aspartase of *E. coli* to L-alanine via L-aspartate β-decarboxylase, without diffusion resistance of L-aspartic acid within the gel

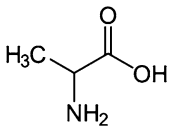

Fig. 4.2 Structure of L-alanine

(Takamatsu and Tosa 1993). The influence of changing the ratio of *E. coli* cells to total cells on the enzymes' activities was studied. At a 30% ratio, aspartase and L-aspartase activities were 9960 and 1310 μmol/h g gel, respectively. These values were lower than those achieved for the single-cell immobilized cell culture. It was assumed that this might be the result of pH inhibition of L-aspartate-β-decarboxylase by fumaric acid (Takamatsu and Tosa 1993). Therefore, optimization of the multireactor system was proposed. Four various bioreactors for the continuous production of L-alanine from ammonium fumarate using two immobilized microbial cells were studied. The most efficient procedure was the one in which pH control was exercised (Takamatsu and Tosa 1993). Other reports have dealt with the industrial manufacture of L-alanine from L-aspartic acid by biotransformation using L-aspartate β-decarboxylase activity of immobilized *P. dacunhae* cells with the benefit of high yield and selectivity (Chibata et al. 1987). Among the different immobilizing agents, i.e., κ-carrageenan, polyurethane, and gelatin, reported for *P. dacunhae* cells producing L-alanine, κ-carrageenan appears to be the most efficient and stable matrix (Yamamoto et al. 1980; Calik et al. 1999). However, contradictory information was reported on the immobilizing and handling conditions in the production of L-alanine from ammonium fumarate by means of a two-step enzyme reaction via L-aspartic acid by aspartase of immobilized *E. coli* cells and L-aspartate β-decarboxylase of immobilized *P. dacunhae* cells (Takamatsu et al. 1986).

4.2.3 L-*Phenylalanine*

L-Phenylalanine is an aromatic amino acid. It has critical medicinal and industrial applications, i.e., as an amino acid infusion in the healthcare industry (Peijing and Zhangcai 1993; Takac et al. 1995) and as a precursor for the production of the low-calorie dipeptide sweetener aspartame in food manufacturing (Chao et al. 1999; Bongaerts et al. 2001). L-Phenylalanine (Fig. 4.3) is produced for the most part by chemical synthesis, fermentation, and enzymatic preparation (Deng and Wang 2007). Nevertheless, the reduced cost of biotransformation utilizing immobilized cells makes it a more attractive alternative (Calik et al. 1999). Cell immobilization technique has several advantages: simple operation, high enzyme activity, the ability to reuse the immobilized cells, utilization of high cell densities, and improved system stability (Chao et al. 2000). Several disadvantages include possible toxicity of the mixed gel to cells and insufficient mechanical strength of the immobilized carriers (Meiyan et al. 2003). The use of a mixed gel for immobilization might lead to a lower gel solidification point, a shorter solidification time, and less enzymatic

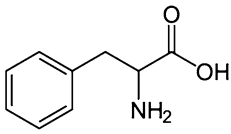

Fig. 4.3 Structure of L-phenylalanine

deactivation (Hu et al. 1995; Hongyu et al. 2003). Calcium alginate–gelatin hydrogels have been used to immobilize α-amylase (Zhu et al. 2004). To prevail over the above-mentioned disadvantages, improve the ratio of reusable enzyme, and reduce the production costs associated with the traditional production route via single-gel immobilization, mixed-gel immobilization for L-phenylalanine production was proposed (Yonghong et al. 2009), consisting of κ-carrageenan and gelatin. The mixed gel containing 87.5% κ-carrageenan and 12.5% gelatin showed the best performance and was selected for further study with *E. coli* strain EP8-10. Optimum pH and temperature were found to be 8.5 and 37°C, respectively. Under the optimal conditions, 98.3% of the phenylpyruvic acid was converted to L-phenylalanine. The activity recovery of the transaminase enzyme in the mixed-gel immobilization was higher than that with immobilization in κ-carrageenan alone (93.6%). The total phenylpyruvic acid conversion rate was over 80% in all 15 batches, suggesting high sustainability in the mixed-gel immobilization (Yonghong et al. 2009).

4.3 Organic Acid Fermentation and Conversion

4.3.1 General

In recent years, cell immobilization procedures have become more and more important and are being effectively applied in industrial processes such as the manufacture of alcohols (ethanol, butanol, and isopropanol), organic acids (including malic, citric, lactic, and gluconic acids) and enzymes (cellulase, amylase, lipase, and others), the biotransformation of steroids for hormone production, wastewater treatment, and food applications (beer, wine, meat, and sugars) (Furusaki and Seki 1992; Kourkoutas et al. 2005a). Organic acids are weak acids that do not dissociate completely in water. A few common examples are lactic, acetic, formic, citric, and oxalic acids. Organic acids with lower molecular weights, such as formic and lactic acids, are miscible in water, whereas higher molecular weight organic acids such as benzoic acid are insoluble in their molecular (neutral) form. Formic or acetic acids are used for oil- and gas-well simulation treatments. The conjugate bases of organic acids such as citrate and lactate are often used in biologically compatible buffer solutions. Oxalic and citric acids are used as rust removers. Organic acids can be useful in food preservation (Brul and Coote 1999): lactic acid and its salts, sodium lactate and potassium lactate, are widely used as antimicrobials in foods such as

ham and sausages. Their mode of action involves penetration of the bacterial cell wall by the non-dissociated organic acids and consequent disruption of the bacteria's physiology, which are not able to tolerate a wide internal and external pH gradient. The dissociation state of the organic acids is extremely important in defining their capacity to inhibit bacterial growth (Dibner and Butin 2002).

4.3.2 Citric Acid

The Krebs cycle is active in higher plant cells in generating a variety of organic acids, including citric, malic, and succinic acids. Citric and malic acids are important constituents of most fruits (Eskin 1990). The utilization of organic acids in foods, e.g., dressings, cakes, pickling solutions, cream soups, custard mixtures, pies, gels, and pastes is on the rise. Examples of immobilization for the production of organic acids from different raw materials can be found in the literature (Gu 1997). Citric acid ($C_6H_8O_7$, MW 192.13, p*K* 3.14) (Fig. 4.4) is generally recognized as safe (GRAS) and is employed in many foods. Typical applications are as an acidulant, a synergist for antioxidants, a plasticizer and emulsifier to provide texture to processed cheese and to enhance melting; it is used to reduce heat-processing requirements by lowering pH and to control acidity in gel formation as a retardant of browning reactions when used with good manufacturing practice (Doores 1990).

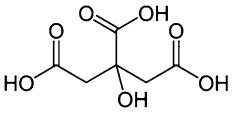

Fig. 4.4 Structure of citric acid

Citric acid can be manufactured by calcium alginate-immobilized *Yarrowia lipolytica* yeast from glucose. By hardening the beads and activating the immobilized biocatalyst in a nutrient solution, a higher produced acid concentration (39 g/l) can be achieved (Rymowicz et al. 1993). Due to the simple manufacture of alginate beads, they were used to produce citric acid by immobilized *Y. lipolytica* yeast in repeat-batch, shaken-flask, and airlift fermentation. In terms of citric acid yields, alginate beads were found preferable to other carriers based on κ-carrageenan, polyurethane gel and foam, or nylon webbing. It was also noted that the smaller the bead size, the greater the productivity (Kautola et al. 1991). Citric acid, oxalic acid, erythritol, and glycerol formation by *Aspergillus niger* immobilized in calcium alginate was also reported. Morphological changes were found to be strain dependent (Hamdy et al. 1992). The well-known gelling agent agarose was also used to produce beads containing *A. niger* to produce citric acid from soy whey. Maximal reported citric acid yields of 21 and 27 g/l with free and immobilized cells, respectively, were reported (Khare et al. 1994).

4.3.3 Malic Acid

Malic acid (E296) was first isolated from apple juice by the German-Swedish pharmaceutical chemist Carl Wilhelm Scheele in 1785. Malic acid [$C_4H_6O_5$; molar mass 134.09 g/mol, density 1.609 g/cm^3, melting point 130°C, solubility in water 558 g/l (at 20°C), pKa_1=3.40, pKa_2=5.13] (Fig. 4.5) contributes to sourness and is a source of extreme tartness. It can be used with or in place of citric acid, and as an acidulant in fruit and vegetable juices, soft drinks, jams, and candies. L-Malic acid salts of basic amino acids are also used in amino acid infusions in the pharmaceutical industry (Takata and Tosa 1993). Industrial immobilization of *Brevibacterium flavum* in κ-carrageenan was achieved by mixing the cell suspension with the gum solution at 45–50°C, where the fumarase activity of the cells is stable. Gel strength was enhanced by soaking the gel in a 0.3 M potassium chloride solution. The gel stabilized the fumarase activity and protected the cells from lysis. The cells' shape and number within the gel matrix remained about the same during 3 months of observation (Takata and Tosa 1993). Addition of polyethyleneimine increased the melting temperature of the formed gel by 20°C. In addition, an interaction between the polyionic polymer and the microorganism occurred. If amines were added to κ-carrageenan, C_2H_5NH, $(C_2H_6)_3N^+$, and NH_2NH contributed to a decrease in gel strength from 910 g/cm^2 (no addition) to 880, 800, and 560 g/cm^2, respectively. Addition of NH_2, $(C_2H_5)_2N$, and $NH_2(CH_2)_6NH$ contributed to an increase in gel strength. The highest fumarase activity (1070 μmol/h ml gel) was detected for $(C_2H_6)_3N^+$ in the modified κ-carrageenan system. On the other hand, the highest operational stability at 37°C (221 days) was observed in a system in which the amine C_2H_5NH was added, in comparison to 160 days for carrageenan without any amine and 172 days for carrageenan with $(C_2H_6)3\ N^+$ (Takata et al. 1982). Addition of 0.1% Chinese gallotannin to the immobilization medium showed its direct interaction with the fumarase protein. The productivity of *B. flavum* in this case was 42.2 kg/h per 1000 l column, three times higher than when no Chinese gallotannin had been added (Takata et al. 1984). Tanabe Seiyaku Corporation has used immobilized *B. flavum* for the industrial production of L-malic acid with ~70% of the theoretical yield (Takata and Tosa 1993).

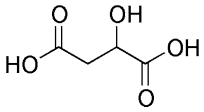

Fig. 4.5 Structure of malic acid

4.3.4 Gluconic Acid

Gluconic acid is an organic compound with the molecular formula $C_6H_{12}O_7$ (i.e., the chemical structure of gluconic acid consists of a six-carbon chain with five hydroxyl groups terminating in a carboxylic acid group) (Fig. 4.6). Gluconic acid

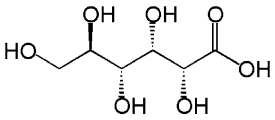

Fig. 4.6 Structure of gluconic acid

occurs naturally in fruits, teas, wines, and honey, among other sources, and is a food additive (E574) which serves as an acidity regulator. In aqueous solution, gluconic acid exists in equilibrium with the cyclic ester glucono-δ-lactone. *Zymomonas mobilis* can convert glucose and fructose to gluconic acid and sorbitol (Rehr et al. 1991). The enzyme, glucose–fructose oxidoreductase, catalyzing the intermolecular oxidation–reduction of glucose and fructose to gluconolactone and sorbitol, was formed in high quantities (1.4 U/mg) when *Z. mobilis* was grown in chemostats with glucose as the sole carbon source under non-carbon-limiting conditions (Rehr et al. 1991). The activity of a gluconolactone-hydrolyzing lactonase was steady at 0.2 U/mg. By means of glucose-grown cells for the conversion of equimolar fructose and glucose mixtures of up to 60% (w/v), a maximum product concentration of only 240 g/l of sorbitol was established (Rehr et al. 1991). The accumulated gluconic acid was further metabolized to ethanol. After permeabilizing the cells using cationic detergents, maximum sorbitol and gluconic acid concentrations of 295 g/l each were attained, and no ethanol was produced (Rehr et al. 1991). Another manuscript described the simultaneous production of gluconic acid and sorbitol from glucose and Jerusalem artichoke using a glucose-fructose oxidoreductase of *Z. mobilis* and inulinase (Kim and Kim 1992). Inulinase was immobilized on chitin by crosslinking with glutaraldehyde. Cells of *Z. mobilis* permeabilized with toluene were co-immobilized with chitin-immobilized inulinase in alginate beads. The optimum amounts of both chitin-immobilized inulinase and permeabilized cells for co-immobilization were determined, and operational conditions were optimized (Kim and Kim 1992). In a constantly stirred tank reactor operation, the maximum productivities for gluconic acid and sorbitol were about 19.2 and 21.3 g/l h, respectively, but operational stability was low because of bead abrasion (Kim and Kim 1992).

4.3.5 Lactic Acid

Lactic acid is a carboxylic acid, also known as milk acid. Lactic acid has the molecular formula $C_3H_6O_3$, a molar mass of 90.08 g/mol, boiling point of 122°C at 12 mmHg, acidity p*K*a of 3.85, and the following melting points for its isomers: L, 53°C; D, 53°C; DL, 16.8°C. Lactic acid was first isolated by the German-Swedish pharmaceutical chemist Carl Wilhelm Scheele (Fig. 4.7). It is miscible in water or ethanol and is hygroscopic. Lactic acid is chiral and has two optical isomers: L-(+)-lactic acid and its mirror image D-(−)-lactic acid, the former being the biologically important isomer. In animals, L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase in a process of fermentation which occurs during

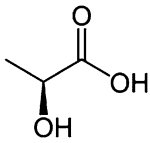

Fig. 4.7 Structure of lactic acid

normal metabolism and exercise. Lactic acid is used extensively for its sensory qualities. It is also used as an antimicrobial agent, pH-control agent, curing and pickling agent, flavor enhancer, flavoring agent and adjuvant, solvent, and vehicle (Doores 1990). Lactic acid has become a large-volume chemical commodity due to its great variety of applications, such as feedstock for biodegradable polymers, oxygenated chemicals, plant growth regulators, environmentally friendly "green" solvents, and specialty chemical intermediates (Tong et al. 2004). Lactic acid production through free cell fermentation provides about 50% of the world supply, but the productivity is very low in conventional batch processes (Datta and Tsai 1995).

Lactic acid was continuously produced from raw starch using a combination of a reversibly soluble, auto-precipitating amylase dependent on pH and *Lactobacillus casei* entrapped in κ-carrageenan (Hoshino et al. 1991). Lactic acid productivity was 3.1 g/l h and this continuous production system, using a novel reactor, may be widely applicable to the production of other useful materials from solid substrates with microorganisms other than lactic acid bacteria for fermentation (Hoshino et al. 1991).

Different beads produced from κ-carrageenan (2.75% w/w)-locust bean gum (LBG) (0.25% w/w) have been used to entrap *L. casei*, and useful diffusion coefficients and equilibrium partition factors for lactose and lactic acid were determined. The use of mathematical models of non-steady-state diffusion into and/or from a sphere and appropriate boundary conditions as a tool for the calculation of the diffusion coefficients has also been described (Arnaud et al. 1992). Production of L-(+)-lactic acid by *Rhizopus oryzae* immobilized in alginate in a tapered-column fluidized-bed batch reactor has been reported (Hamamci and Ryu 1994). Immobilized *Sporolactobacillus inulinus* cells were used to produce L-(−)-lactic acid from topinambour tubers in a stirred reactor. The biocatalyst retained its initial activity level for 400 h of operation at 35°C, providing lactic acid yields of 95–96% (Abelyan and Abelyan 1996). Alginate was also favored for the immobilization of *L. casei* for lactate production under stirred-batch, as well as packed bed conditions (Dong et al. 1991). Stirred-batch experiments, terminated with total glucose utilization, yielded 90–99% lactate and a total productivity of 1.6 g/l h (Dong et al. 1991). Calcium alginate beads were used as a carrier for the immobilization of *L. casei* and *Lactococcus lactis* for the continuous production of lactic acid from deproteinized whey. Average lactic acid productivity and yield and lactose utilization were 24 g/l h, 55%, and 90%, respectively (Roukas and Kotzekidou 1996). A continuous biparticle fluidized-bed reactor was developed for the simultaneous fermentation and recovery of lactic acid. One-week fermentation trials using immobilized *Lactobacillus delbrueckii* with the addition of sorbent demonstrated a volumetric productivity (6.9 g/l h) that was at least 16-fold higher than that of a free

cell batch fermentation with base pH control and identical biomass concentration and medium composition (Kaufman et al. 1996). Regeneration of the loaded sorbent from the fluidized-bed reactor yielded a concentration of lactic acid that was 35-fold the original levels in the fermentation broth (70 versus 2 g/l). Lactic acid concentrations as high as 610 g/l were observed when the loading solution contained 50 g/l lactic acid. Rich medium formulations did not appear to increase the fluidized-bed reactor's performance (Kaufman et al. 1996).

L. casei cells were immobilized on fruit pieces for food-grade lactic acid production (Kourkoutas et al. 2005b). Alginate has been extensively used in various lactic acid production methods (Prasad and Mishra 1995; Yoo et al. 1996; Abdel-Naby, Reyad and Abdel-Fattah 2000; Klinkerberg et al. 2001; Yan et al. 2001; Carvalho et al. 2003; Idris et al. 2003). *L. delbrueckii* was entrapped in an alginate bead matrix through which substrates and products diffused in and out easily. Bead stability is important in maintaining high substrate-to-product conversion ratios. The concentration of sodium alginate and bead diameter were found to have a pronounced effect on the stability of the bead, which in turn affects lactic acid production, and several authors have studied these effects (Abdel-Naby et al. 1992; Rajagopalan Pillutla and Sonal 1992; Goksungur and Guvenc 1997, 1999; Gough et al. 1998).

Grape invertase was reported to improve the production of lactic acid using *L. lactis* and canned pineapple syrup as a substrate (Ueno 2003). The ability to utilize this liquid effluent as an alternative carbon source for useful by-products such as lactic acid could help reduce or eliminate sources of pollution as well as reduce production costs (Idris et al. 2003). Nevertheless, high levels of heavy metals in the medium might inhibit the growth of microorganisms, influence substrate pH, and be involved in the inactivation of enzymes associated with product synthesis (Kotzamnidis et al. 2002). Batch fermentation of liquid pineapple waste to lactic acid was also performed using immobilized *L. delbrueckii* subsp. *delbrueckii* ATCC 9646 under anaerobic conditions for 72 h (Idris and Wahidin 2006). Maximum concentrations of lactic acid were produced after 56 h of fermentation using a sodium alginate concentration of 2.0%. Maximum lactic acid production was also obtained with a bead diameter of 1.0 mm at an initial pH of 6.5 and temperature of 37.8°C (Idris and Wahidin 2006).

Fumaric acid has been used as an antimicrobial agent, in the prevention of malolactic fermentation in wines, and as a means of adding acidity to wines. Malic acid can be used as a flavoring agent, flavor enhancer, adjuvant, and pH control agent (Neufeld et al. 1991). The yeast *Saccharomyces cerevisiae* was used for amplification of the enzyme fumarase by cloning the single nuclear gene downstream of a strong promoter. The overproducing strain converted fumaric acid to L-malic acid at a rate of 65 mM/g h in free cell experiments, and approximately 87% of the fumaric acid was converted to L-malic acid within 45 min (Neufeld et al. 1991). L-Malate was also produced from fumarate by using immobilized *S. cerevisiae* cells entrapped in polyacrylamide. This preparation performed better when pretreated with malonate. Under the experimental conditions, succinate was not detected as a by-product of the reaction, as had been reported for other microorganisms (Figueiredo and Carvalho 1991). Fumarate bioconversion to L-malic acid by *S. cerevisiae* entrapped within polyacrylamide gel beads has been

described (Oliveira et al. 1994). The spherical shape of the beads was improved by the addition of detergent (sodium dodecyl sulfate). Bioconversion was 60 times higher than that observed with free cells (Oliveira et al. 1994). Other reports have dealt with the effects of propionic acid on *Propionibacteria* fermentation (Zhong et al. 1998) and improved organic acid production by calcium alginate-immobilized *Propionibacteria* (Rickert et al. 1998).

4.4 Ethanol, Wine, Vinegar, Sake, and the Like

4.4.1 Vinegar

Vinegar is processed from the oxidation of ethanol in wine, cider, beer, fermented fruit juice, and the like by acetic acid bacteria (Osuga et al. 1985). Fermentation can be fast or slow. Vinegar has been produced for thousands of years. Table vinegar ranges from 4 to 8% acid by volume (typically 5%). Natural vinegars contain small amounts of tartaric, citric, and other acids. The word "vinegar" originates from the old French "vin aigre," meaning "sour wine." Its density is ~0.96 g/ml. Better quality wine vinegars are matured in wood barrels for up to 2 years and exhibit a complex, mellow flavor. Wine vinegar tends to have a lower acidity than that of white or cider vinegars. Vinegar is recognized as an ancient food seasoning.

Freedom in cellular movement can be limited by two principal approaches: adsorption of cells to or physical entrapment of cells within carriers. Insufficient effort has been made to develop these techniques for the vinegar industry. However, several manuscripts have been published describing the use of these techniques for fermentation procedures that involve acetic acid bacteria. In this case, the microorganism is strictly aerobic and it is therefore very important to consider the support's diffusional resistance to oxygen transfer, a property that becomes the main limiting factor in the process (Ghommidh et al. 1982; Osuga et al. 1984). It has been widely reported that immobilization imparts bacterial stability against the negative effects of temperature, pH, ethanol, and acetic acid concentrations and prevents the washing away of cells by the high dilution rates used in the continuous process. Adsorption to surfaces and encapsulation within gels or porous materials have been the most widely studied methods for the immobilization of acetic acid bacteria. Entrapment of acetic acid bacteria in alginate (Saeki 1990; Sun and Furusaki 1990) and κ-carrageenan (Osuga et al. 1984; Mori 1993) gels and carriers of hydrous titanium in a bubble-mixing column (Kennedy et al. 1980), bentonite (Ghommidh et al. 1986), ceramic monolith (Ghommidh et al. 1982; Ghommidh et al. 1982), polypropylene hollow fiber (Nanba et al. 1985), honeycomb ceramic monolith (Kondo et al. 1988; Takada and Hiramitsu 1991), and cotton-like polypropylene fiber (Okuhara 1985), among many others, have been used. These methods represent a particular form of cellular adhesion based on the ability of certain microorganisms to attach themselves to solid surfaces via the secretion of polymucosaccharides (Moonmangmee et al. 2002).

An inclusive report on the use of κ-carrageenan gel beads for the immobilization of *Acetobacter* species K1024 for vinegar production discusses a few interesting points (Osuga et al. 1984; Mori 1993). The immobilized bacteria in the center of the gel beads barely grew, but bacteria on the surface of the beads grew actively for 48–72 h, until a whitish layer with a thickness of up to 200 μm was observed. The formed colonies, about 30 μm in diameter, were concentrated in fig-like clumps in craters that were formed as a result of surface breaks in the beads (Osuga et al. 1984; Mori 1993). Long-term acetic acid production of 180 days using κ-carrageenan gel beads as carriers gave a mean production rate of 5 g/l h (Mori et al. 1989). The authors reported several production problems, such as blockage of the pipe for liquids, coupling of the liquid or gas tubes, and interruption of the liquid or gas supply. Those temporary problems were surmountable and in general, did not change the production rates. However, contamination by wild bacteria can occur and this can terminate production (Mori 1993). Issues that must be considered include oxygen supply, since the immobilized acetic acid bacteria are strongly oxygen dependent, use of closed and pressurized vessels, and recycling of the oxygen to minimize the cost of the operation (Mori 1993). Moreover, the use of κ-carrageenan beads is somewhat limited. They suffer from breakage during operation due to their relative weakness in the face of frequent impact with reactor parts and the powerful shear rate of the gas–liquid flow. Thus, other alternatives for immobilization have been checked. An interesting option involves porous chitosan beads. Their selection was a result of their superior mechanical properties in comparison to κ-carrageenan beads, as well as their smaller size and the easy adhesion of bacteria to their surfaces. This preparation gave a 46% higher production rate than the κ-carrageenan beads as a result of the chitosan beads' hardness (Mori 1993).

Immobilization of *Acetobacter aceti* bacteria by adsorption onto three different solid carriers—Siran (i.e., sintered glass), wood chips, and polyurethane foam—was also studied (de Ory et al. 2004). These carriers were chosen with the idea of scaling-up the procedure for application in industrial vinegar processes. The aim of the study was to analyze the cellular adhesion properties in each case and assess the fermentation properties of the resultant immobilized carriers (de Ory et al. 2004). Of the three carriers assayed, the best for the immobilization of acetic acid bacteria and the subsequent acetification stage with adhered biomass on a laboratory scale was polyurethane foam. It enabled the immobilization of a large number of cells (~10.5 million/mg) in 12.5 days. Moreover, it led to the highest acetification rate of the three assayed carriers (4.74 g/l day), increasing the overall yield. Furthermore, it is an inert material and is very inexpensive, thus making the process potentially suitable for industrial scale-up (de Ory et al. 2004).

4.4.2 Soft Sake

Sake is a traditional alcoholic beverage in Japan produced from rice by the use of parallel fermentation, i.e., hydrolysis of starch and slow fermentation at low temperature, resulting in a high ethanol content of 20% (v/v). The sake brewing

process starts by soaking white polished rice in water and steaming it. The second step is preparation of *moto*-mash, a starter for sake yeast (Nunokawa and Hirotsune 1993). The main fermentation (*moromi*) is carried out by adding steamed rice, rice-*koji*, and water to the *moto*. Rice-*koji* is a culture produced from steamed rice inoculated with *Aspergillus oryzae* for 2 days. It is produced from the saccharification of starch and decomposition of protein contained in the polished rice grain. The volume of *moromi* is increased by further addition of steamed rice, rice-*koji*, and water. Following fermentation at 10–15°C for ~20 days, the insoluble residues are removed by filtration and a clarified sake is obtained (Nunokawa and Hirotsune 1993).

The dense *moromi* slurry cannot be processed in a bioreactor; therefore, the saccharification is separated from the rest of the process, and the clarified sugar solution can be applied to the bioreactor, to which immobilized yeast cells in calcium alginate are added for continuous fermentation. The sake produced in this way has a lower ethanol content and a fruity flavor, making it different from the traditional sake.

The preparation of saccharified rice solution begins with soaking the polished rice in 2.5-fold the amount of water, followed by crushing and addition of 0.14% calcium chloride. Liquefying α-amylase (0.04%) and protease (0.02%) enzymes are then added and the mixture is incubated at 50°C for 4 h followed by heating at 97°C for 0.5 h. To the liquefied mash, rice-*koji* at 20% of the amount of rice and 0.06% glucoamylase are added. Incubation at 57°C for 14 h produces the saccharified mash, which is filtered with a press to obtain the saccharified rice solution which is purified by added activated carbon (Nunokawa and Hirotsune 1993). The immobilized yeast preparation is subjected to the traditional crosslinking reaction of 3% alginate by 4% $CaCl_2$. All media are autoclaved prior to use. The dropping method is used to form 3 mm beads, excess $CaCl_2$ is washed away, and the yeast extract peptone dextrose is diffused for 3 days (renewed every 12 h) into the beads to obtain an approximate 1000-fold multiplication of the initial counts, reaching 2×10^9 cells/ml gel (Nunokawa and Hirotsune 1993).

As mentioned, the sake produced by the reactor method has a lower alcohol content than traditional sake, ~10% compared to 16–20%. The alcohol content can be increased by changing the percent glucose in the medium. However, due to taste considerations, as well as an attempt to emphasize the new sake properties, CO_2, which is not present in traditional sake, was added (Nunokawa and Hirotsune 1993). The second fermentation was carried out with a reduced number of cells per unit gel to eliminate off flavors. In fact, the new sake had a unique ester flavor not observed in ordinary sake due to its possible adsorption to the solid *moromi* mash phase. Since yeast cells leaked from the gel beads and those from a preincubation tank participated in the fermentation process, their contribution to the quality of the sake should be checked. A few bioreactor systems were studied and it was found that free yeast produce more isoamyl alcohol but fewer esters than immobilized yeast. The bioreactor system for the production of sake was validated. It is no surprise that the use of immobilization and bioreactor techniques for many other food applications has been reported. A few examples include alcohol production (Sitton et al. 1980;

Wada et al. 1980; Williams and Munnecke 1981), beer (Onaka et al. 1985), wine (Fukusima 1981), sake (Hirotsune et al. 1987), *miso* (Numata et al. 1990), and soy sauce (Mizunuma 1986).

4.4.3 Malolactic Fermentation

Malolactic fermentation is an important issue for many red wines, particularly those with high acid content produced from grapes grown in chilly weather. The fermentation is performed by lactic acid bacteria of the genera *Lactobacillus*, *Pediococcus*, and *Oenococcus*. It decreases the acidity, influences microbial stability, and affects the sensory attributes of the wine (Jay et al. 2005). Spontaneous malolactic fermentation may occur during the storage of new wines at slightly elevated temperatures over the course of weeks or months (Colagrande et al. 1994). Cell immobilization techniques are applied to induce and control malolactic fermentation in wine, mostly with specific cultures of lactic bacteria. Support for immobilization by entrapment of microorganisms is provided by polyacrylamide, κ-carrageenan, pectin, chemically modified chitosan, and alginate (Naouri et al. 1991). Immobilization by adsorption has also been used for malolactic fermentation of wines (Maicas et al. 2001; Agouridis et al. 2005). Another study demonstrated the promising option of immobilizing *Oenococcus oeni* ATCC 23279 cells on delignified cellulosic material for malolactic fermentation (Agouridis et al. 2008).

4.4.4 Removal of Urea from Sake and Wine by Immobilized Acid Urease

Urea (Fig. 4.8) can be removed from sake and wine by acid urease (Hara et al. 1981; Ough and Trioli 1988). However, batch systems are uneconomical, treatment time is usually long (7–30 days), and continuous treatment is not possible (Matsumoto 1993). A bioreactor with an immobilized acid urease preparation could serve as a good solution if it can support its users with the option of treating sake beyond 150 days at 5–15°C at a high flow rate and can guarantee that urease will not be released from the immobilized enzyme into the effluent (Matsumoto 1993). Four immobilization matrices based on entrapment within calcium alginate or κ-carrageenan or on covalent binding with polyacrylonitrile (PAN) (Fig. 4.9) and chitosan (Fig. 4.10) derivatives were tested. The PAN and chitosan derivatives have specific urease activity and therefore their use as efficient urea removers is of great interest. The procedure with porous PAN fibers (diameter of $20–35 \times 10^{-6}$ m, pore size 400–600 Å) involves several steps. First, the fibers are partially reduced to amino

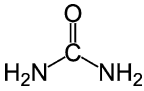

Fig. 4.8 Structure of urea

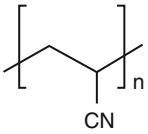

Fig. 4.9 Structure of the repeating unit of polyacrylonitrile

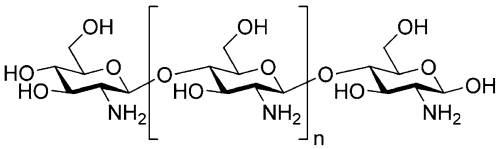

Fig. 4.10 Structure of chitosan: poly(β-1,4-D-glucosamine)

groups by lithium aluminum hydride in ether (Matsumoto 1993). The partially aminated porous PAN fibers are then treated with 3% glutaraldehyde in acetate buffer for 1 h at 25°C with stirring. After thorough washing, the fibers can be added to the urease solution in acetate buffer for 1 h at 25°C with stirring. The enzyme is covalently bound to the fiber and the non-adsorbed enzyme is washed away. The process results in an immobilized enzyme preparation with a high, sufficiently specific activity of 150 U/g wet weight carrier. The preparation is stored in 0.1 M acetate buffer at 4°C for future use (Matsumoto 1993).

Another option for the removal of urea from sake at a higher flow rate is to use chitosan derivative beads as carriers (Chitopearl^{TM}). These commercially available beads are used in the food industry and can immobilize acid urease by absorption crosslinkage or covalent binding. Urea was removed from sake in a bioreactor by passing the sake through the bioreactor with downflow at 10–15°C and a flow rate of 20–100 space velocity (SV—the speed passed through a volume of column per hour). When PAN fibers were used for urea removal from sake by immobilized acid urease, the process was continued for over 100 days and urea concentration decreased from 12 to 37 ppm to below 3 ppm (Matsumoto 1993). It should be mentioned that the removal of urea from alcoholic beverages can be inefficient due to the presence of urease inhibitor and especially fluoride. In this case, slow flow rates are recommended (Matsumoto 1993).

4.4.5 Beer Brewing Using an Immobilized Yeast Bioreactor System

Immobilized microorganisms can be used for the preparation of various alcoholic beverages, such as beer (White and Portno 1978; Godtfredsen et al. 1981; Pardonova et al. 1982), wine (Totsuka and Hara 1981; Gestrelius 1982), vinegar (Osuga et al. 1985), sake (Sato et al. 1983), and shoyu (soy sauce, Akao et al. 1982). Beer-brewing techniques originated thousands of years ago. They are affected by substrate quality and brewing conditions and production is conducted in a batch-wise manner. Important issues related to beer production quality are the fermentation temperature which should be kept below 20°C for better flavors (Nakanishi et al. 1993). There are numerous studies reporting the use of

immobilized yeast in the brewing industry. The mentioned carriers include diatomaceous earth (Narziss and Hellich 1971, 1972; Baker and Kirsop 1973), PVC + diatomaceous earth (kieselguhr) (Navarro et al. 1976), and calcium alginate (White and Portno 1978; Godtfredsen et al. 1981; Polednikova et al. 1981; Pardonova et al. 1982; Masschelein and Francotte 1983; Nakanishi et al. 1985; Onaka et al. 1985).

The immobilized preparation has the advantage of a short fermentation, when the yeast is maintained at high concentrations with minimal loss of fermented wort. However, the disadvantages of the produced beer are a very strong diacetyl flavor (concentration of 3.3 mg/l, which is 11 times the concentration of the observed total diacetyl in beer from conventional fermentation—0.3 mg/l) and a very "unclear" taste, caused by a high residual amino acid concentration in the beer (140 versus 75 mg/l for the conventional fermentation) (Nakanishi et al. 1993). The latter is the result of lack of oxygen. Furthermore, it has been reported that oxygen can only be detected 0.4 mm from the surface of a 3 mm diameter bead because of oxygen consumption by the yeast at the bead's surface (Toda and Sato 1985). While in foods, diacetyl flavor is important, in beer it is not, due to its adverse effect on the beverage's original refreshing taste, popularly referred to as "unripe" or "young" flavor. The threshold value of diacetyl is 0.1 mg/l (Drews et al. 1966). The substrate from which the diacetyl flavor is formed is vicinal diketones. The precursors of vicinal diketones are acetohydroxy acids, which are produced by the yeast through the fermentation. In the immobilized yeast preparation, the acetohydroxy acid concentration reached 3.3–5 times its value in conventional fermentation, the latter amounting to only 0.3 mg/l (Nakanishi et al. 1993). It is possible to age the fermented wort in order to reduce its acetohydroxy acid content to normal levels, but twice the aging (normally 40–50 days) is necessary (Inoue 1981).

A fermentation system containing an immobilized yeast reactor for the rapid production of beer was reported by Nakanishi et al. (1985) and Onaka et al. (1985).

The beer produced by this system was claimed to not differ significantly from conventional beer, but the alginate carrier used in this study suffered from a decrease in strength and swelling during continuous long-term use (Nakanishi et al. 1985; Onaka et al. 1985). Thus a different ceramic bead carrier was chosen as an alternative for further study (Nakanishi et al. 1989). A ceramic carrier has a few "built-in" properties: it is stable to pH changes, chemical corrosion, and high temperatures of several hundreds of degrees Celsius. The bead retained its size, shape, and strength throughout its use and in many cases, the activity of the immobilized preparation remained stable: in beer, the fermentation efficiency was stable for over 6 months (Makishima and Aoki 1984; Nakanishi et al. 1989, 1993). An efficient way to carry out the immobilization is to pass a yeast suspension through a column filled with porous ceramic beads, resulting in $\sim 10^8$–10^9 cells/g bead being immobilized by this method (Nakanishi et al. 1993). The ceramic bead was superior to the calcium alginate bead in terms of its strength and in retaining its fermentation capacity relative to the alginate system. The quality of the beer was good in both cases (Nakanishi et al. 1993).

4.5 Soy Sauce

Soy sauce is a salty, strong-flavored seasoning that enjoys worldwide popularity (Motai et al. 1993). Traditional preparation includes the mixing of cooked soybean and roasted wheat, which are inoculated with spores of *Aspergillus sojae* and/or *A. oryzae* for the preparation of *koji*, which is then mixed with brine to prepare *moromi*. The former is left to sit, with occasional agitation, for over half a year for the purpose of mixing and microbial growth promotion (Motai et al. 1993). The *koji* enzymes, namely proteinases, peptidases, and amylases, hydrolyze most of the protein to amino acids and peptides and convert starch almost completely to simple sugars. These sugars are further fermented by salt-tolerant microorganisms into ethanol, lactic acid, and various aroma compounds (Motai et al. 1993). *Moromi* includes 17% salt. This high salt concentration encourages the growth and lactic acid production of salt-tolerant bacteria, such as *Pediococcus halophilus*, causing a reduction in pH. When the pH is reduced to 5.5 or less, the salt-tolerant yeast *Zygosaccharomyces rouxii* begins fermentating the alcohol. This process yields many aroma components and 2–3% ethanol. In addition to *Z. rouxii*, other types of yeast can be present and active in the *moromi* mash. They include yeasts such as *Candida versatilis* and *Candida etchellsii*, which produce phenolic aroma compounds such as 4-ethylguaiacol and 4-ethylphenol to the product. After fermentation and aging, the *moromi* mash is filtered and the clarified soy sauce is pasteurized. The sauce goes through further clarification by sedimentation and the supernatant serves as the final product (Motai et al. 1993). Since the process can take more than 6 months, an attempt should be made to shorten this period without affecting product quality, and immobilization can be adopted for this purpose.

A system for soy sauce production composed of five stages, hydrolysis of raw material, increasing glutamic acid content, lactic acid fermentation, alcohol fermentation, and finally ethylguaiacol (EG) production, has been described (Motai et al. 1993). Protease can be produced in a continuous culture of *A. oryzae* for the preparation of raw liquid for bioreactors. The main problem is microbial contamination. This can be avoided by inclusion of 10% NaCl, continuation of the culture for over ~30 days, and use of oxygen instead of air. Slow agitation and maintaining a small amount of gas flow prevent both foaming and thicker growth of mycelium, the latter causing plugging hazards (Motai et al. 1993). The cooked soybean and roasted wheat were mixed with the cultured broth of *A. oryzae* for enzymatic hydrolyzation at 45°C for 3 days. The hydrolysate mash was filtered by packing in a press machine and passing through a cloth. NaCl, total nitrogen content, and pH were adjusted to 13, 2, and 6.0%, respectively, before the fluid was transferred to the bioreactor (Motai et al. 1993).

Optimization of continuous fermentation through the use of immobilized cells of *Z. rouxii* was explored in a study of the influence of various parameters, such as aeration, temperature, pH, and NaCl concentration, on the produced ethanol content. High ethanol production through a pH range of 3.5–5.5 was observed. The highest viable cell count in the immobilizing alginate gel was detected at pH 4.5–6.0. Therefore, a pH of 4.5–5.5 was recognized as optimal (Hamada et al. 1989).

The ethanol content was maximal at 25–30°C and the maximum number of viable cells was observed at 18–27.5°C. Ethanol content decreased with an increase in the proportion of nitrogen gas and a supply of air was necessary for considerable alcohol production.

The production of 4-EG is maximal at pH 3.5–4.0 and a temperature of 30–33°C. Air supply contributes to continuous production, since without this the number of viable cells in both the gel carrier and the liquid decreases in parallel to increasing proportions of nitrogen gas (Hamada et al. 1990). The optimum concentration of 4-EG in soy sauce is 1–3 ppm. After 6 months of fermentation, the content of 4-EG in conventional soy sauce is very low; however, by immobilization of *C. versatilis* cells, 20–30 ppm 4-EG can be produced with short residence times (Hamada et al. 1990). During a continuous process, cells are released from the carriers and become free. A higher contribution of free cells of *Z. rouxii* and *C. versatilis* to ethanol production in comparison to immobilized cells was recognized. Free cells showed a lower contribution to 4-EG production for *C. versatilis* (Motai et al. 1993).

Continuous production of soy sauce in a bioreactor system was reported, using immobilized glutaminase and immobilized cells of *P. halophilus*, *Z. rouxii*, and *C. versatilis*. This system included a feed tank, glutaminase reactor, *P. halophilus* reactor, buffer tank, *Z. rouxii* reactor, *C. versatilis* reactor, product tank, feed and air pumps, and means to maintain the feed and product tanks at 5°C. After 100 days of operation, the observed sample compositions for NaCl, total nitrogen, formol nitrogen, lactic acid, residual glucose, and ethanol were 12.90, 2.00, 1.01, 0.92, 1.06, and 2.60%, respectively. Some aroma components of the soy sauce sampled after 100 days of fermentation included isobutyl alcohol, *n*-butyl alcohol, isoamyl alcohol, acetoin, ethyl lactate, furfuryl alcohol, methionol, 2-phenylethanol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 4-EG, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone, and 4-hydroxy-5-methyl-3(2H)-furanone, at concentrations of 17.2, 3.6, 50.4, 28.0, 2.3, 6.6, 1.9, 15.8, 3.1, 1.0, 13.6, and 39.2 mg/l, respectively. Sensory evaluation of soy sauces from the bioreactor system in comparison to conventionally produced soy sauce demonstrated that the former was a bit weaker in aroma and fresh odor; nevertheless, the panel judged the overall qualities to be similar (Motai et al. 1989; Motai et al. 1993).

4.6 The Milk Industry

4.6.1 Immobilization in the Milk Industry

The watery part of the milk that remains after the curd, or casein, precipitates is the whey (Gates 1981). It contains proteins which are not precipitated by either acid or renin, along with nearly all of the water-soluble vitamins, minerals, and lactose. Whey is used chiefly in animal feed, but it is also used in some dairy products, and its use as a liquid for gelatin desserts and salads and in the production of alcohol and beer has been investigated (Gates 1981). The manufacture of lactic acid from deproteinized whey by mixed cultures of free and co-immobilized *L. casei*

and *L. lactis* cells in batch and fed-batch cultures was investigated (Roukas and Kotzekidou 1998). The highest lactic acid concentration (46 g/l) in the fed-batch culture was obtained with both the co-immobilized cells and a free cell mixture at a feeding rate of 250 ml/h and substrate concentration of 100 g/l (Roukas and Kotzekidou 1998). Co-immobilized *L. casei* and *L. lactis* cells gave a higher overall lactic acid concentration compared with the free cell mixture, in repeat fed-batch culture. Calcium alginate beads co-immobilizing *L. casei* and *L. lactis* cells retained their ability to produce lactic acid for 20 days (Roukas and Kotzekidou 1998). Gel beads composed of κ-carrageenan-LBG entrapping *Lactobacillus helveticus* were appropriate for continuous lactic acid fermentation of whey permeate enriched with yeast extract (Norton et al. 1994). Other beads based on κ-carrageenan-LBG gel were employed to immobilize three strains of *Lactococcus*, for the ongoing fermentation of whey ultrafiltration permeate medium. During the process, no strain was eliminated or dominated, and beads retained their integrity and stability (Lamboley et al. 1997). Agarose beads were used to immobilize an isolate from Egyptian Cheddar cheese. The isolate was an effective lactic acid producer (33.4 mg/ml) from salt whey permeates to which yeast extract and minerals had been added (Zayed and Winter 1998). A supplementary manuscript discussed the use of lactic acid production by *L. casei* entrapped within agar gel beads, when salt whey (even at 8%) was used as a substrate. Such a bead maintained its stability and structure for 168 h in the salt medium (Zayed and Zahran 1991).

For practical purposes, fermented milk products consist of hard and soft cheeses, yogurt, and related products (Sutherland et al. 1986). During milk fermentation by immobilized *L. lactis* in calcium alginate beads, some cells were released. Reuse of the beads resulted in 30 times the number of free cells in the medium. Rinsing the beads between fermentations had no influence on the number of free cells in the milk. Even coating the beads with poly-L-lysine did not significantly reduce the release of cells during five successive fermentations (Champagne et al. 1992). Double coating was somewhat more successful, but milk acidification with these beads was slower in comparison to that with non-coated beads. Ethanol treatment and heating of beads killed cells on their peripheral areas and were found to be effective in minimizing release due to cells entrapped near the bead surface (Champagne et al. 1992). Regular and two-layer calcium alginate beads served for entrapment of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* for fermentation of pasteurized cream (31% fat content). The beads allowed an elevated concentration of inoculum and consequently, a reduction in the fermentation time (Prevost and Divies 1992).

4.6.2 Hydrolysis of Lactose in Milk

Lactose (Fig. 4.11) is a disaccharide which constitutes 4.3–4.5% of cow's milk and represents 38–40% of total milk solids. Lactose is not hydrolyzed in the stomach or in the upper section of the small intestine. Due to its low absorption rate, the bulk passes to the next section of the intestine, where β-galactosidase hydrolyzes it to

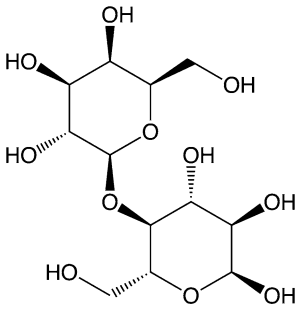

Fig. 4.11 Skeletal structure of lactose

glucose and galactose in the epithelial cells of the mucous membrane (Honda et al. 1993). β-Galactosidase from *A. oryzae* was immobilized on the rugged surface of an amphoteric ion-exchange resin of phenol formaldehyde polymer (28–60 mesh). The hydrolysis ratio of lactose varied between 68 and 73%. Another study reported on entrapping the enzyme from *Kluyveromyces lactis* within the microcavities of fibers made from cellulose triacetate. The ratio of hydrolyzed lactose in this case was reported to be 70.8–81.3% (Honda et al. 1993). Permeabilized *Kluyveromyces marxianus* cells were employed as a source of β-D-galactosidase for the production of lactose-hydrolyzed milk. The yeast cells were entrapped in alginate gel beads for their subsequent use in lactose hydrolysis. Different processing parameters (alginate concentration, bead size, biomass load, temperature, agitation, and incubation time) were monitored to enhance lactose hydrolysis in milk (Panesar et al. 2007). Maximum lactose hydrolysis (87.9%) was observed with yeast cells immobilized in 2% (w/v) alginate with a bead size of 2.90 mm at 30°C under agitation (80 rpm) after 150 min of incubation. The developed system was highly stable and the alginate-entrapped yeast cells could be recycled up to eight times without any marked change in their ability to carry out lactose hydrolysis (Panesar et al. 2007).

4.6.3 Antibiotic Residues in Milk

Food and environmental regulatory agencies have established control programs in response to increasing concern about the possible exposure of consumers to antibiotic residues through foods of animal origin (Bergwerff and Schloesser 2003). Among the different kinds of antibiotics in question, sulfonamides are extensively used, due to their broad spectrum of antibacterial activity for the treatment of infection and preventative control of disease outbreak and to improve feed efficiency and promote growth. A novel electrochemical immunosensing strategy for the detection of sulfonamide antibiotics in milk based on magnetic beads was described (Zacco et al. 2007). Following the performance of immunochemical reactions with sulfonamide antibiotics on the magnetic bead, the modified beads can be easily captured by a magnetosensor made of a graphite-epoxy composite which is also used as the transducer for the electrochemical immunosensing. Electrochemical detection

is thus achieved using a suitable enzyme substrate, such as horseradish peroxidase, and an electrochemical mediator (Zacco et al. 2007).

4.7 Miscellaneous Flavor Materials and Aroma Compounds

4.7.1 Biotransformation from Geraniol to Nerol

Geraniol is an aromatic, pale-yellow liquid alcohol with the molecular formula $C_9H_{17}COH$, derived from the oils of geranium and citronella and utilized in cosmetics and flavorings (Fig. 4.12). Nerol is a colorless fluid obtained from orange blossoms and used in perfumery (Fig. 4.13). Geraniol and nerol are geometric (*cis–trans*) isomers. Geraniol has a waxy, sweet rose scent; nerol also smells of sweet rose, with a citrus tang. The abilities of two grape cell suspensions (Gamay and Monastrell) to biotransform geraniol into nerol in a biphasic system based on culture medium and Miglyol 812 were compared. The Gamay grape-cell suspension transformed a higher concentration of geraniol into nerol than the Monastrell suspension. Immobilization proved to be beneficial in protecting cells from substrate toxicity. In addition, immobilization appeared to have an effect on secondary metabolism: the cells immobilized in polyurethane foam were more efficient at performing the immerization process than either the freely suspended or calcium alginate-immobilized cells (Guardiola et al. 1996).

4.7.2 Limonin

Limonin is a limonoid, a bitter, white crystalline substance found in orange and lemon seeds. It is also known as limonoate D-ring-lactone and limonoic acid di-δ-lactone (Fig. 4.14). Chemically, it is a member of the class of compounds known as furanolactones (http://en.wikipedia.org/wiki/Limonin). Limonin can be successfully degraded by *Rhodococcus fascians* cells for debittering purposes. These bacteria can be entrapped in κ-carrageenan and used in a constantly mixed tank reactor to degrade limonin in a continuous process. The immobilized cells were able to debitter limonin-containing media and the immobilized biomass was quite stable throughout the operational conditions tested (Iborra et al. 1994). The immobilized preparation

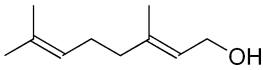

Fig. 4.12 Structure of geraniol

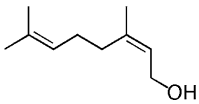

Fig. 4.13 Structure of nerol

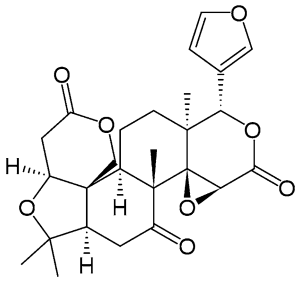

Fig. 4.14 Structure of limonin

was active and stable for more than 2 months of continuous operation and none of the major juice components were noticeably altered during the operation (Iborra et al. 1994).

4.7.3 β-Ionone

β-Ionone, $C_{13}H_{20}O$, is a pale-yellow to yellow oily liquid. It has a particularly intense freesia character, emphasized by a fruity, somewhat raspberry-like background (Fig. 4.15). This product is used in all domains of perfumery and has good substantivity (Nussinovitch 2003). The ionones are a series of very closely related chemical substances and part of a group of compounds known as rose ketones. They are derived from the degradation of carotenoids. Ionones are aroma compounds found in a variety of essential oils, including rose oil (http://en.wikipedia.org/wiki/Ionone). Biotransformation of β-ionone into hydroxy- and oxo-derivatives by *A. niger* IFO 8541 has been reported. Since the fungus *A. niger* develops in the form of pellets when cultivated, its immobilization within alginate beads is a natural way to imitate this feature. In the presence of a carbon source, recovery of ~2.5 g/l aroma compounds after 230 h cultivation with a molar yield of ~100% is feasible (Larroche et al. 1995).

4.7.4 Naringin

Naringin is the major flavonoid glycoside in grapefruit and it gives grapefruit juice its bitter taste. Its molecular formula is $C_{27}H_{32}O_{14}$, and it has a molar mass of 580.5 g/mol and a melting point of 166°C. Naringin (Fig. 4.16) exerts

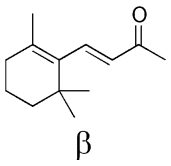

Fig. 4.15 Structure of β-ionone

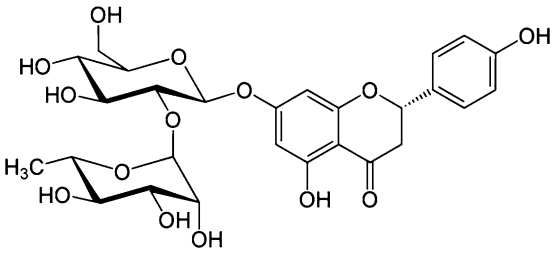

Fig. 4.16 Structure of naringin

a variety of pharmacological effects, such as antioxidant activity, lowering of blood lipids, anticancer activity, and inhibition of selected drug-metabolizing enzymes (http://en.wikipedia.org/wiki/Naringin). Sodium alginate beads were used for immobilization of naringinase to yield naringin hydrolysis. This can be employed for debittering kinnow juice at various pHs and temperatures (Puri et al. 1996). An additional attention-grabbing application is the preparation of a low-oxalate dietary preparation for hyperoxaluric patients: the high oxalate contents of spinach, amaranthus, and the dish *paruppu keerai* can be depleted by banana oxalate oxidase entrapped in alginate (Lathika et al. 1995). *L. lactis* subsp. *lactis* entrapped in alginate beads was used as a biocatalyst in continuous fermentation. Activity inside the beads was limited by the reaction and internal transfer. The higher the quantity of citrate within the medium, the greater its bioconversion into aroma compounds (Cachon, Molin and Divies 1995).

4.7.5 Methyl Ketone (Blue Cheese Flavor) as a Flavor Molecule from Higher Fungi

Conidiospores of *Penicillium roqueforti* as free-spore suspensions or immobilized in calcium alginate were used for the production of 2-heptanone. This ketone and 2-undecanone are flavor molecules in blue-veined cheese (Seitz 1991). Free spores converted sodium octenoate to 2-heptanone in a stirred, aerated fermenter at an efficiency of ~60% (Larroche et al. 1988). Reproducible spores were immobilized in 0.2 mm calcium alginate beads that were coated with a copolymer (Eudragit RL) to improve their mechanical stability. At pH 6.5, and by using chloramphenicol to eliminate bacterial contamination and additional $CaCl_2$ for alginate stabilization, continuous production of the flavor for 30 days was possible (Larroche and Gross 1989).

4.7.6 Capsaicin

Capsaicin, $C_{18}H_{27}NO_3$, is the active component of chili peppers, belonging to the genus *Capsicum*. Its molar mass is 305.41 g/mol, melting point 62–65°C, and boiling point 210–220°C at 0.01 torr. The structure of capsaicin was partly elucidated

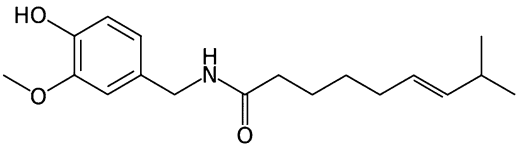

Fig. 4.17 Structure of capsaicin

~90 years ago (Nelson 1919) (Fig. 4.17). It is an irritant for mammals, including humans, and produces a sensation of burning in any tissue with which it comes in contact. It was stated that higher yields of capsaicin can be achieved by immobilized pepper cells of the chili pepper fruit *Capsicum frutescens* Mill. cv. annuum, compared to freely suspended cells (Yeoman et al. 1980; Lindsey et al. 1983; Lindsey and Yeoman 1986). The cells were immobilized within the pores of reticulate polyurethane foam that did not affect cell viability and produced between two and three orders of magnitude higher yields of capsaicin than did freely suspended cells (Mavituna et al. 1987). The production rate of immobilized cells was ~0.1 mg capsaicin/g dry weight per day, in comparison to 0.5 mg/g dry weight per day in ripening pepper fruits (Mavituna et al. 1987). For optimization of the manufactured cell-line selection, depression and alteration of primary metabolism, induction of rate-limiting enzymes, and continuous product removal are necessary (Holden and Yeoman 1987).

4.7.7 Vanillin

Another food-related application is the bioconversion of vanillin (a substance which occurs widely in nature and as a synthetic flavoring with the empirical formula $C_8H_8O_3$, molar mass 152.15 g/mol; it is a white to yellowish crystalline powder with a characteristic strong, vanilla-like odor and very sweet taste) (Fig. 4.18) into vanillic acid. Immobilization of callus cells (ATCC#40354) derived from *Vanilla fragrans* or *Vanilla phaeantha* in polymeric matrices or small-pore filters, alongside the use of polymeric hydrophobic resins or activated charcoal, makes continuous manufacture and extraction of vanilla flavoring possible. The flavoring contained vanillin, vanillyl alcohol, 3,4-dihydroxy benzaldehyde, 4-hydroxy benzyl alcohol, and 4-hydroxy benzaldehyde. Flavor recovery was achieved by using 50% ethanolic solution (Seitz 1991). A suitable medium for vanillin production by non-growing cells should contain a carbon source, calcium and sodium chloride, galactose, mannose, ribose, and xylose (Knuth and Sahai 1989). An additional report claimed that important precursors for the production of vanilla flavor components are dehydroshikimic acid, ferulic acid, phenylalanine, and vanillyl alcohol (Ramagnoli and Knorr 1988). Yet another report dealt with alginate beads that served as carriers for *Pseudomonas*. After 13 h of conversion, transformation rate was only 47%. Nevertheless, optimal yield of higher than 80% has been detected in a continuous immobilized cell reactor during 76 h of operation (Bare et al. 1992).

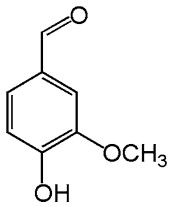

Fig. 4.18 Structure of vanillin

4.7.8 Japanese Seasoning

Seasoning could be prepared from a mash of *koji* wheat that contained 11.5% NaCl and was proteolyzed by the addition of *Bacillus subtilis* and *A. oryzae*. Porous chitosan beads with immobilized glutaminase were loaded on a column and a clarified seasoning passed through them (by centrifugation). The pH was adjusted to between 5 and 6. The enzyme increased the level of glutamic acid by a factor of 2.6 compared to untreated seasoning (Yasuyuki et al. 1989).

4.8 Miscellaneous Applications

4.8.1 Production of Oligosaccharides

Sucrose, maltose, lactose, and other disaccharides are obtained by the elimination of one molecule of water from two molecules of monosaccharide. In the same way, polysaccharides are formed by the elimination of n–1 molecules of water from n molecules of monosaccharides. The boundary between oligosaccharides and polysaccharides is usually taken as somewhere around degree of polymerization (DP) 20. Fructose oligosaccharides (FOSs) can be manufactured by immobilization of a soil-isolated strain of *Aspergillus japonicus* in calcium alginate beads, for fermentation of a medium composed for the most part of sugarcane molasses. Optimum temperature and pH were 55°C and 5.0–5.6, respectively. The microorganisms and the utilized process appear promising for industrial applications (Cruz et al. 1998).

Additional reports discuss the production of high-purity FOSs with co-immobilized *A. niger* and enzyme (Jiang et al. 1996); production of isomalto/branched oligosaccharide syrup by using immobilized neopullulanase and preliminary evaluation of the syrup as a food additive (Kuriki et al. 1997); production of FOSs by immobilized mycelium of *A. japonicus* (Chih et al. 1996). In producing FOS, fructose transformation of sucrose was inhibited by its by-product glucose, making it unfeasible to obtain high-purity FOS: purity was usually 49%. Removal of glucose isomerization was tested by two processes: converting glucose with glucose oxidase or use of immobilized glucose isomerase. Glucose oxidase was crosslinked with 2% glutaraldehyde and 0.1% tannin and co-immobilized with *A. niger* in a calcium alginate gel. Co-immobilized beads were incubated at pH 5 and 50°C for

24 h and FOSs were produced with 71% purity. In the other process, immobilized *A. niger* and immobilized glucose isomerase were co-packed in a column that produced FOSs at 63% purity and 16% fructose. The pH optima were 5–5.5 for cell multiplication and 7 for reaction of immobilized isomerase (Jiang et al. 1996).

4.8.2 Preservatives and Bacteriocins

Preservatives provide a wide variety of functions and may be separated in terms of usefulness into inorganic and organic substances. Inorganic preservatives include, among others, alkalis and alkaline salts, metals, inorganic acids and their salts, halogens, peroxides, and gases. The organic preservatives include organic acids and their salts, formaldehyde, sugars, alcohols, antibiotics, wood smoke, spices, and condiments. Most of the better-known antibiotics have been tested on raw foods, chiefly proteinaceous ones such as meat, poultry, and fish. The antibiotic nisin is employed in Europe to suppress anaerobes in cheese and cheese products. Nisin is a polycyclic antibacterial peptide having the molecular formula $C_{143}H_{230}N_{42}O_{37}S_7$, which is used as a food preservative (Fig. 4.19). It contains the uncommon amino acids lanthionine, methyl lanthionine, didehydroalanine, and didehydroaminobutyric acid. As a food additive, nisin has the E number 234. Nisin is manufactured from the culturing of *L. lactis* on natural substrates, such as dextrose milk or dextrose.

Nisin was efficiently incorporated into a calcium alginate matrix and ground into microparticles smaller than 150 μm. Formation of microparticles and incorporation of nisin was confirmed by scanning electron microscopy and by a decrease in the inactivation of nisin activity with proteolytic enzymes. Incorporation efficiency was 87–93% and the nisin in the alginate-incorporated form was 100% active against an

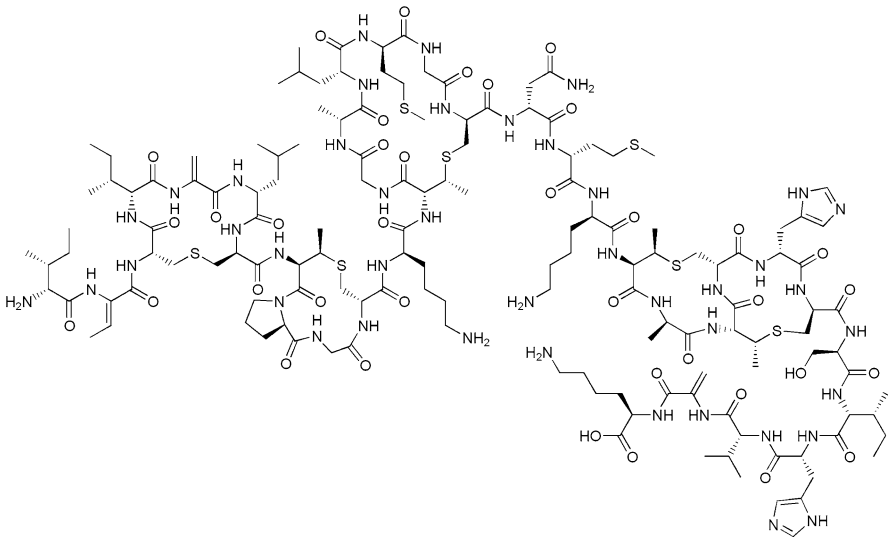

Fig. 4.19 Structure of nisin

indicator culture of *Lactobacillus curvatus* in both MRS broth (i.e., de Man, Rogosa, and Sharpe Medium for *Lactobacilli*) and reconstituted skim milk (Wan et al. 1997). The bacteriocin divercin is active against *Listeria*, which can be produced from cells of *Carnobacterium divergens* V41 by continuous cultivation, by immobilization in calcium alginate beads, or in a membrane bioreactor. Immobilized cells presented the best performance (Bhugaloo Vial et al. 1997). Sterilized, lean, and adipose beef carcass tissues were inoculated with *Brochothrix thermosphacta*, left untreated, or treated with 100 μg/ml nisin, calcium alginate, or 100 μg/ml nisin immobilized in calcium alginate gel. The tissues were ground aseptically and nisin activity and bacterial populations of *B. thermosphacta* were determined for up to 14 days at 4°C. While no effective suppression of *B. thermosphacta* was observed, the nisin application appeared to provide some protection against other undesirable bacteria during grinding (Cutter and Siragusa 1997).

4.8.3 Xylitol Production

Most fruits and berries, as well as vegetables, include xylitol, which is a pentitol. Commercially, it is manufactured by acid hydrolysis from xylan-containing plant material, hydrogenation, and purification. Xylitol (Fig. 4.20) has a caloric content equal to that of sucrose and is a colorless, non-hygroscopic, non-carcinogenic, crystalline sweetener. Xylitol can also be produced by microbiological processes and is used for both dietetic and diabetic foods (Salminen and Hallikainen 1990). Many reports have been written on xylitol production (Dominguez 1998). *Eucalyptus globulus* wood hydrolysates were concentrated by vacuum evaporation to increase their xylose contents. In addition, they were treated with activated charcoal and nutrients and then utilized as culture media for xylitol production by *Debaryomyces hansenii* NRRL Y-7426. The susceptibility of hydrolysates to fermentation depends on the initial cell concentration. Media containing 58–78 g xylose/l were hardly consumed in batch experiments starting with 16 g cells/l, whereas 39–41 g xylitol/l was yielded by fermentations performed with a comparable concentration of carbon source and initial cell concentrations of 50–80 g/l (Parajo et al. 1996).

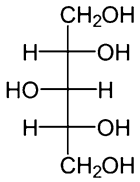

Fig. 4.20 Structure of xylitol

4.8.4 Carotenoids and Leucrose

Alginate entrapment of *Dunaliella salina* followed by fluidized-bed drying of the preparation was used for the manufacture of carotenoid-rich product (Leach et al. 1998). The culture medium was made up of seawater, sea salt, KNO_3, and K_2HPO_4,

and CO_2 was bubbled in to maintain the culture at a pH of 8.0 (Leach, Oliveira, and Morais 1998). Fabrication of leucrose by dextran sucrase has also been described (Buchholz et al. 1998). Leucrose is a new, non-carcinogenic disaccharide and in and of itself is a highly promising sugar replacement for the prevention of cavities. Leucrose possesses excellent nutritional properties with respect to metabolic utilization and is well tolerated (Buchholz et al. 1998).

4.8.5 cis,cis-*Muconic Acid (MA)*

cis,cis-Muconic acid (MA) (Fig. 4.21) is an unsaturated dicarboxylic acid in the degradation pathway of benzoic acid. It can potentially be used as raw material for new functional resins, pharmaceuticals, or agrochemicals or converted to adipic acid for the production of nylon (Yoshikawa et al. 1993). A process for the production of MA from upstream toluene in the benzoate pathway by *Pseudomonas putida* was patented by Celanese Corporation (Maxwell et al. 1986). For continuous manufacture, screening for suitable microorganisms is the first required step, followed by mutation induction by UV irradiation or treatment with nitrosoguanidine. UV mutation of *Arthrobacter* sp. yielded a strain capable of converting 5 g sodium benzoate to more than 4.5 g MA in 24 h of flask culture (Mizuno et al. 1988). Characterization of the mutant strain (Seki et al. 1987) was followed by comparison of gel-entrapping and membrane separation methods. It was concluded that the photo-crosslinked resin ENTG-3800 is superior to polyurethane (Fukushima et al. 1978), κ-carrageenan, alginate, or polyacrylamide (Yoshikawa et al. 1993). In this system, consisting of a membrane separation reactor and downstream processing, *cis*,*cis*-MA can be produced continuously from benzoic acid (Yoshikawa et al. 1993).

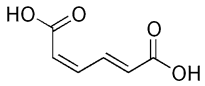

Fig. 4.21 Structure of muconic acid

4.9 Various Industrial Options

4.9.1 Fuel Ethanol Production

Fuel ethanol can be produced using immobilized yeast systems. For the entrapment procedure, both synthetic and natural polysaccharides can be employed. An attractive alternative is to use photo-crosslinkable resin. Such a resin has a main chain of polyethylene glycol (PEG) or a mixture of PEG with polypropylene glycol with photo-crosslinkable ethylenic double bonds at both ends. Polymerization can be induced by UV irradiation of the double bonds. This in turn induces crosslinking,

forming a three-dimensional crosslinked gel structure that entraps the microorganisms. Gels can be produced in bead, sheet, or other forms (Iida 1993). In addition, it is feasible to form beads from a mixture of photo-crosslinkable resin and sodium alginate, followed by crosslinking with calcium chloride and irradiation by actinic light at 300–400 nm. The produced beads have diameters of 2–3 mm and a compressive strength of 15 kg/cm^2 (Iida 1993). To achieve high reaction rates, the ethanol must be removed during the fermentation process, since its accumulation at volumetric concentrations of over 7% reduces the fermentation rate. The continuous process includes packing of the bioreactor volume with 20–30% (v/v) immobilized yeast beads. Then, the sugar solution is continuously applied to the bioreactor, permitted to stand for a few hours, and then broth containing ethanol and by-products is removed. The broth is heated and then introduced into a flash tank, and the ethanol is partially flash vaporized. Part of the resultant low-ethanol broth is cooled to near-fermentation temperature and returned to the bioreactor. The vapor is condensed to contain 30–40% (v/v) ethanol, and part of the condensate is used as an ejector solution after cooling (Iida 1993). In evaluating the continuous process, the yeast concentration in the bioreactor amounts to ∼40 g/l and the final alcohol concentration obtained during the process amounts to 10.6% (v/v) (Iida 1993).

4.9.2 Application of Gels for Separation Matrices

Gels designed for separation can have either spherical or irregular shapes and their sizes range from several micrometers to several millimeters. In characterizing the materials when accurate separation is needed, the requirements of liquid chromatography determine the small particle size, i.e., from several to at most tens of micrometers. For industrial purification of a specific material (or fractionation), slightly larger particles, of several hundreds of micrometers up to ∼1 mm, are chosen (Takayanagi 2001).

The gels used for separation are classified into several groups based on chemical structure, as well as on physical properties such as particle shape, pore structure, network structure, and chemical properties such as pH stability. Inorganic compound gels for separation are porous. Their pore size ranges from several nanometers to several tens of nanometers and their surface area is on the order of several hundred square meters per gram. They are based on silica and alumina as well as compounds that include metallic ions, such as apatite (Takayanagi 2001).

Another group is the synthetic organic polymer gels. A few examples are polystyrene crosslinked with divinyl benzene, poly(acrylic acid), and poly(acrylic acid) derivatives. Synthetic organic polymer gels are synthesized by suspension polymerization or emulsion polymerization and their shape is spherical. Particle sizes range from several micrometers to several millimeters, the choice of size depending on the application (Takayanagi 2001). Other options include natural polymer gels, mainly polysaccharides that are made of agarose, cellulose, and dextrin. They can be used for separation of easily denatured proteins and nucleic acids. In

general, natural polymer gels are soft, deform under pressure, and their structure can undergo degradation or decomposition.

Separation mechanisms are classified into several modes: however, actual separation takes advantage of a combination of modes. In molecular sieving, the separation depends on the solute as well as on the network size. Sieves are tested as part of liquid chromatography. The molecular sieve effect is fundamental to gels and therefore used for separations. When using other modes of separation, an appropriate selection of network size or pores is still required. Another mode of action is adsorption, using mainly porous organic polymer gels. Separation based on adsorption deals mainly with organic compounds of molecular weight $<10^4$ (Takayanagi 2001). Ion-exchange materials can also be used for separations. In general, the solution that contains the target compound is loaded, subjected to ion exchange and adsorbed, and then the target material is desorbed by changing the conditions in the external solution.

4.9.3 Bioartificial Organs

Existing membrane-based bioartificial organs are made up of three basic constituents: a synthetic membrane, cells that exude the product of interest, and encapsulating matrix material. Agarose and alginate have been extensively employed to encapsulate cells for artificial organ purposes. It is important to understand the degree of transport resistance imparted by these matrices in cell encapsulation in order to determine whether adequate nutrient and product fluxes can be obtained (Li et al. 1996). In general, 2–4% agarose gels offer minute transport resistance for solutes of up to 150 kDa, whereas 1.5–3.0% alginate gels offer significant transport resistance for solutes in the range of 44–155 kDa, lowering their diffusion rates by 10- to 100-fold as compared to their diffusion in water. Doubling the alginate concentration has a more significant effect on hindering the diffusion of larger molecular weight species than does doubling the agarose concentration. Average pore diameters were approximately 1.70 and 1.47×10^{-8} m for 0.5 and 3% alginate gels, respectively, and 4.80 and 3.60×10^{-8} m for 2 and 4% agarose gels, respectively. These values were estimated using a semi-empirical correlation based on diffusional transport of different-sized solutes. This technique, developed for measuring diffusion in these gels, is highly reproducible and functional for gels crosslinked in the cylindrical geometry applicable to studying transport throughout matrices employed in cell immobilization in the hollow fiber configuration (Li et al. 1996).

4.9.4 Insect Cell Immobilization

There is increasing interest in insect cell cultivation for possible mass production of viral insecticides for pest control, an environmentally friendly approach relative to the traditional chemical solution. Another reason for this interest is the development

of baculovirus vectors for the expression of many foreign genes whose products are correctly processed post-translationally in the insect host cell (Goosen 1993). The fact that baculoviruses are not pathogenic to vertebrates or plants is also important (Summers and Smith 1985). Baculoviruses are almost exclusively viruses of insects (Kuzio and Faulkner 1993). Transmission is the result of ingestion of occlusion bodies in which the infectious virus particles are embedded. The virus replicates within the insect, causing midgut infection and its systemic spread to other tissues, where progeny occlusion bodies develop. The cycle repeats itself when occlusion bodies from dead insects contaminate potential feeding surfaces (Kuzio and Faulkner 1993). Baculoviruses have been isolated from moths and butterflies (Lepidoptera), bees and wasps (Hymenoptera), flies (Diptera), beetles (Coleoptera), caddisflies (Trichoptera), spiders (Arachnida), and even from shrimp and crab (Decapoda); however, they have never been isolated from vertebrates or plants.

Culture techniques for insect cells are mostly modified from mammalian procedures. Aside from cultivation in suspension, immobilization in an appropriate matrix, adsorption onto surfaces, or entrapment behind polymeric membranes are options (Summers and Smith 1985). The use of microencapsulated animal cells for the production of monoclonal antibodies was first reported ∼17 years ago by Posillico (1986). That report described the cells' preferential appearance on the interior surface of the microcapsule membrane as a possible reason for mass-transfer limitations. When trials of insect cell encapsulation were performed, it was observed that their growth is not influenced by constituents such as $CaCl_2$, KCl, and sodium citrate (Smith et al. 1989). Exposure of cells to low molecular weight poly-L-lysine (22,000), which was used to react with alginate to create an alginate-poly-L-lysine microcapsule system, had no effect on cell viability, while its increase to 102,000 and 270,000 reduced cell viability by ∼75% (King et al. 1987). It was also suggested that growth inhibition depends on alginate concentration and mass-transport limitations.

It is difficult to scale up an insect cell suspension culture (Murhammer and Goochee 1988; Shuler et al. 1990), with agitation and sparging appearing to cause losses in cultured cell viability. Cell damage was associated with bubble entrainment in the fluid due to cavitation or vortexing (Kunas and Papoutsakis 1990; Murhammer and Goochee 1990). Moderate agitation using a marine impeller or flat-blade impeller at 300−500 rpm prevented this problem. In the absence of headspace, cells can grow at higher agitation rates (Kunas and Papoutsakis 1990). Media for insect cell culture are complex and expensive, in part due to inclusion of fetal bovine or calf serum (Grace 1962). Attempts were made to use serum-free medium (Caron et al. 1990), producing high cell-growth rates but decreased recombinant protein production. Another study reported significantly lower product concentration in a serum-free medium (Maiorella et al. 1988).

There are several limitations of the insect cell/baculovirus system. The level of dissolved oxygen can affect the growth rate of insect cells in the stirred bioreactor (Hink and Strauss 1980; Hink 1982). Processing of highly expressed recombinant products by the cell can be incomplete when very late-stage baculovirus promoters are employed (Bishop 1990). Another study reported incomplete glycosylation of

the rabies glycoprotein (Prehaud et al. 1989). After prolonged passage through multiple infection cycles, the virus' ability to infect insect cells also diminishes (Tramper et al. 1990; Van Lier et al. 1990) and the mutant virus particles interfere with the formation of regular virus (Wickham et al. 1991). Despite these and other limitations, insect cell culture has become an important technology for the production of recombinant proteins and biopesticides, via baculovirus expression. The advantages of the baculovirus expression vectors are high levels of foreign gene expression; capacity to produce proteins that are functionally, enzymatically, and immunologically similar to their authentic counterparts; inability to activate dormant oncogenes; and easy manipulation (Agathos 1993).

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Chapter 5
Medicinal Applications of Hydrocolloid Beads

5.1 Introduction

This chapter gathers together information culled from many resources. For example, it describes novel approaches to cell encapsulation for enhanced biocompatibility and immunoisolation. Emphasis is placed on methods using alginate–polylysine alginate for encapsulation and multistep methods of cell encapsulation, and a glimpse is provided of the art and science of encapsulation of living cells and tissues for biomedical purposes. Other topics discussed in this chapter include stem cells in bead environments, charged hydrogel beads as new microcarriers for cell culture, potential support for endothelial cells, vaccine delivery, crosslinked chitosan beads for different medicinal purposes, mucoadhesive beads and their application in eyes and alimentary systems, and polyelectrolyte complexes. For beads as drug carriers, the reader is referred to Chapter 8.

5.2 Encapsulation of Cells in Hydrogels

Bioartificial organs have been fabricated from cells with the requisite functions and hydrogels. Encapsulation methods involve crosslinking of polymer chains after dispersion of living cells within a polymeric solution. Many water-soluble polymers include hydroxyl, carboxyl, and amine groups on their side chains. The reactivity of these groups and the use of crosslinking agents to react with them favor gelation. However, those crosslinking agents that react with side groups can also react with the same groups on proteins and thus cell toxicity is high. Therefore, other approaches have been sought.

The islets of Langerhans (Fig. 5.1) are the pancreatic regions containing endocrine (hormone-producing) cells. The islets of Langerhans constitute approximately 1–2% of the pancreas mass. There are about 1 million islets in a healthy adult human pancreas, which are distributed throughout the organ; their combined mass is 1–1.5 g (http://en.wikipedia.org/wiki/Islets_of_Langerhans). Langerhans cells, which produce insulin, were microencapsulated within alginate gel beads (Zekorn et al. 1992; Lanza et al. 1995). However, when these beads were introduced

A. Nussinovitch, *Polymer Macro- and Micro-Gel Beads: Fundamentals and Applications*, DOI 10.1007/978-1-4419-6618-6_5,

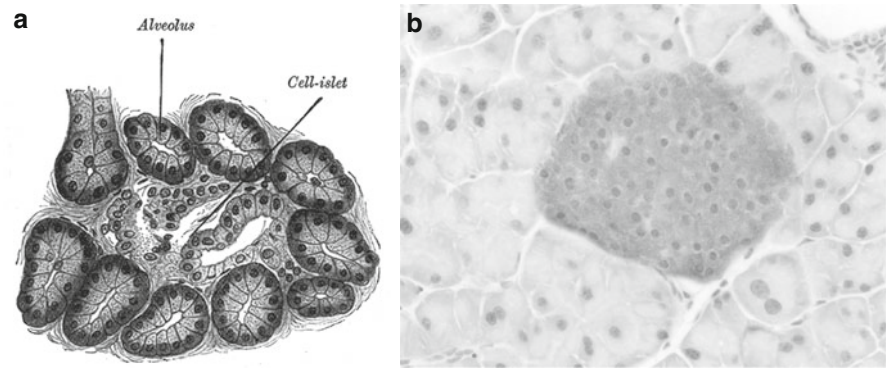


Fig. 5.1 (**a**) Section of dog pancreas (250× magnification) (reproduced from the 20th US edition of Gray's Anatomy of the Human Body). (**b**) Immunohistochemistry of mouse pancreas for insulin (200× magnification) (courtesy of Billyboy, http://en.wikipedia.org/wiki/File:InsulinIHC.jpg)

(transplanted) into the body, the multivalent metallic ions forming the gel could be exchanged with monovalent sodium ions, causing the bead to lose stability and decompose. Moreover, some multivalent ions exhibit cell toxicity (Lim and Sun 1980; Sun, O'Shea and Goosen 1983). Therefore, another approach was used in which gel beads produced of alginate and calcium ions were immersed in poly-L-lysine solution (polycationic) to form a polyion complex at the surface. These altered beads were again reacted with alginic acid to eliminate the possibility of a cationic surface, with rather poor biocompatibility, being present on the outside of the bead. A further step was taken to treat the beads with citric acid, in order to remove the calcium ions and to liquefy the interior of the bead.

Alginate encapsulation technology was applied for the differentiation of embryonic stem cells into insulin-producing cells. The study showed that embryonic stem cells can be efficiently encapsulated within alginate beads, retaining a high level of cell viability. The alginate encapsulation resulted in an approximately 10-fold increase in cell density in the culture relative to the two-dimensional culturing conditions, revealing the potential benefit of this technology for large-scale cell culture applications (Wang et al. 2009). Manipulation of encapsulation conditions, particularly of initial alginate concentration, provided control over both diffusion of the molecules into the alginate matrix (e.g., differentiation factors) and matrix porosity/flexibility, permitting the proliferation and growth of encapsulated embryonic stem aggregates within the bead. The functionality of the encapsulated and differentiated cells was confirmed by their insulin production capacity: upon glucose challenge, insulin production by the cells that had differentiated within the alginate beads was found to be statistically significantly higher than that by cells from a conventional two-dimensional differentiation system (Wang et al. 2009). Although alginate is a convenient option for cell entrapment, another alternative is to encapsulate cells within agarose beads. Separated cells were dispersed in a previously produced low-temperature-gelling agarose solution, to which paraffin fluid was

added. The container was shaken to disperse the agarose solution in the paraffin into suitable particle sizes, then cooled to obtain the formed agarose beads which could then be easily collected by centrifugation (Iwata et al. 1989; Iwata et al. 1992). It was reported that 1500 agarose beads encapsulating islet of Langerhans cells had been produced and implanted in the peritoneum (the membrane lining the abdominal cavity that covers most of the intra-abdominal organs) of diabetic mice with very high levels (500 mg/dl) of blood sugar. After implantation and during the 200 days of the experiment, blood sugar levels returned to normal (~100 mg/dl) and the recipient's immune cells were not able to reach and affect the allogenic (antigenically distinct tissues and particularly cells from the same species; also spelled allogeneic) homograft. In contrast, non-encapsulated cells were rejected by the recipient immune system (Iwata 2001).

The use of a phase change in polymer solutions for cellular encapsulation in polymer film was also reported (Iwata et al. 1990b). Cells can be encapsulated in microcapsules produced from polymers that are insoluble in water, such as poly(2-hydroxyethyl methacrylate). To do so, organic solvents need to be used which are, in many cases, hazardous to cells. Thus to utilize this approach, a relatively low-toxicity solvent—poly(ethylene glycol) (PEG, the most commercially important type of polyether; refers to oligomers and polymers with a molecular mass below 20,000 g/mol, prepared by polymerization of ethylene oxide)—is used and the microencapsulation is performed by a device designed to minimize contact between the cells suspended in solution and the polymer–solvent solution. Despite these efforts, many cells perish as a result of this process (Crooks et al. 1990). Iopamidol (Fig. 5.2) is a non-ionic, low-osmolar iodinated contrast agent, available in various concentrations, from 200 to 370 mg/ml; it is used as a non-ionic water-soluble radiographic contrast medium. Iopamidol blocks x-rays as they pass through the body, thereby allowing body structures not containing iodine to be visualized. The degree of opacity produced by iopamidol is directly proportional to the total amount of the iodinated contrast agent in the path of the x-rays. The visualization of body structures is dependent upon the distribution and elimination of iopamidol. It has been proven to be effective in experiments with encapsulation of insulin-producing cells, but is very expensive (Iwata 2001). Another option for cell encapsulation is the use of photodimerization. A hydrophilic resin comprised of a poly(vinyl alcohol) polymeric backbone and containing styrylpyridinium groups is photosensitive. A photosensitive resin can be prepared by reacting styrylpyridinium salt containing

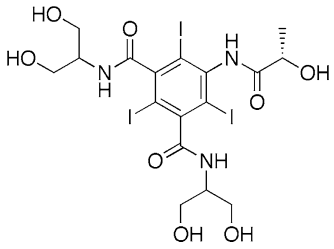


Fig. 5.2 Structure of iopamidol (courtesy of Edgar 181, http://en.wikipedia.org/wiki/File:Iopamidol.png)

a formyl or acetyl group with a poly(vinyl alcohol) compound (Ichimura 1981). Poly(vinyl alcohol) with styrylpyridinium as a side chain can undergo gelation via dimerization of these groups upon irradiation with visible light to encapsulate Langerhans cells (Ichimura and Watanabe 1982; Iwata et al. 1990a,b). Yet another approach to preparing hydrogels for different purposes is by using thiols. Thiol contains a functional group composed of a sulfur–hydrogen bond (–SH). Being the sulfur analogue of an alcohol group (–OH), this functional group is referred to as either a thiol group or a sulfhydryl group. The production of hydrogels for entrapment was reported via thiol formation of disulfide bonds (Hisano et al. 1998). Another study described a new PEG-based copolymer that contains multiple thiol (–SH) groups along the polymer backbone. This copolymer, in the presence of α,ω-divinylsulfone–PEG crosslinking reagent, was capable of forming a polymer hydrogel from aqueous solution in the presence of protein drugs, which are physically but not covalently entrapped. A subcutaneously injected polymer hydrogel depot was formed in situ and could deliver protein drugs for a prolonged period of time, ranging from days to weeks (Qiu et al. 2003).

5.3 Stem Cells in Bead Environments

Bone can lose its natural capacity to respond to injury with age and disease. In such cases, clinical intervention is required to heal larger injuries. The requirement for regenerative bone therapy has been a driving force in the field of bone tissue engineering for the past couple of decades (Salgado et al. 2004). The currently used bone substitutes do not self-renew and have a limited life span. The clinical standard for bone grafting is an autogenous graft harvested from the iliac crest (Fig. 5.3). The graft materials include osteogenic cells and growth factors in a suitable extracellular matrix to support healing at the damaged location (Orban et al. 2002). For patients suffering from diabetes or osteoporosis and for smokers, sufficiently high-quality tissue is not available for grafting (Salgado et al. 2004). Consequently, a current clinical challenge is to stimulate bone formation in cases in which healing is delayed or halted, for the most part in areas where host cell infiltration is prevented or there is a lack of vascular supply, and the rapid generation of high-quality bone is needed to prevent morbidity (Lund et al. 2008). Most efforts to encourage bone formation by human mesenchymal stem cells in vivo make use of osteoinductive (osteogenesis-inducing) scaffolds, with controlled release of growth factor. Osteoinduction is a natural phenomenon that is commonly seen in any type of bone-healing process, which involves the recruitment of immature cells and their stimulation to develop into preosteoblasts. In the case of, for example, a fracture, a major part of the healing depends on osteoinduction (Albrektsson and Johansson 2001). However, a main restriction in using these cells therapeutically is inadequate control over the induction and maintenance of their differentiation. Thus the ability to provide potent and consistent cues is critical to the success of future stem-cell therapies. A number of physiological extracellular matrix proteins have been used as scaffolds for bone tissue engineering, including collagen I (Xiao

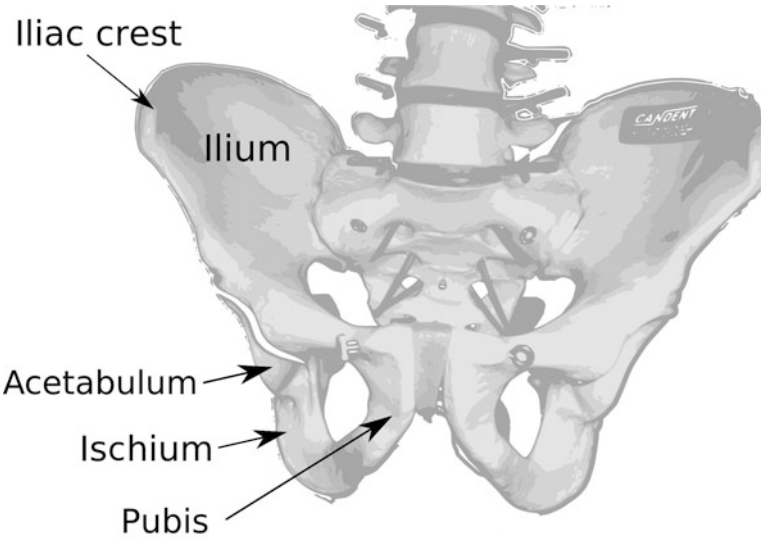
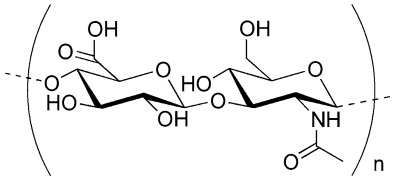


Fig. 5.3 Diagram of the human pelvis (courtesy of Je at uwo, http://en.wikipedia.org/wiki/File:Pelvis_diagram.png)

Fig. 5.4 Structure of hyaluronan (courtesy of Edgar 181, http://en.wikipedia.org/wiki/File:Hyaluronan.png)



et al. 2003), fibrin (Perka et al. 2000), and hyaluronan (Fig. 5.4) (Solchaga et al. 1999). One study described a system to embed cells in three-dimensional hydrogel microbeads consisting of collagen I and agarose (Batorsky et al. 2005). Composite matrices of collagen I and agarose were manufactured by emulsification and concurrent polymerization in the presence of human mesenchymal stem cells to produce 30–150 μm diameter hydrogel "beads." Mesenchymal stem cells are multipotent stem cells that can differentiate into a variety of cell types in vitro or in vivo, including osteoblasts, chondrocytes, and adipocytes (Sell 2004). The proliferation, morphology, osteogenic gene expression, and calcium deposition of human mesenchymal stem cells in bead environments were compared to other two- and three-dimensional culture environments from days 14 to 21 in culture (Lund et al. 2008). Cells embedded in 40% collagen beads exhibited proliferation rates that were equivalent to those in gel disks, but showed upregulated expression of bone sialoprotein (a component of mineralized tissues such as bone, dentin, cementum, and calcified cartilage) and increased calcium deposition relative to two-dimensional

controls (Lund et al. 2008). Osteocalcin gene expression was not changed in the three-dimensional beads and disks, while collagen type I gene expression was down-regulated relative to cells in two-dimensional culture. The hydrogel bead format thus allowed controlled cell differentiation and represented a cell delivery vehicle that may also increase vascular invasion and host incorporation. It was concluded that the use of beads can be applied to promoting the osteogenic phenotype in human mesenchymal stem cells, an important step toward using them in bone repair applications (Lund et al. 2008).

5.4 Charged Hydrogel Beads as New Microcarriers for Cell Culture

Microcarrier systems are more effective in cell culture technology (involving in vitro cellular engineering, particularly the large-scale cultivation of anchorage-dependent cells) than in conventional cell culture plates (Fleming et al. 2000). Because biocompatible substances are considered to be very important in biomedical applications and tissue engineering, the interaction between surfaces and cells has been much investigated (Peter et al. 2000; Gomes et al. 2001). Easier propagation of cells with higher yields in a much lower culture volume is possible when the ratio of surface area to volume in polymeric microcarriers is large relative to the conventional monolayer culture techniques (Barrias et al. 2005). Microcarriers are frequently used for large-scale cell culturing of anchorage-dependent cells and for the manufacture of various cell products, including vaccines, enzymes, and monoclonal antibodies. Dextran-based hydrogel beads are the most frequently used microcarriers for animal cell culturing: CR2 cells were cultured on Cytodex 1 for purification of the virus DISC-HSV (Zecchini and Smith 1999). Mosquito and mammalian cells cultured on Cytodex 1 were used for the production of four serotypes of dengue virus (Fig. 5.5) (Liu and Wu 2004). Cytodex 3 was found suitable for the cultivation of hepatocytes, hepatocarcinoma cells, Vero cells, Fe cells for the production of canine parvovirus, and an NM57 cell line for the production of respiratory syncytial virus (Mittereger et al. 1999; Moran 1999; Sun et al. 2000; Zhang et al. 2001).

Microcarriers of other compositions were also examined, e.g., bioceramic hollow carriers for three-dimensional bone tissue formation in rotating bioreactors (Qui et al. 1999), and modified bioactive glass powders encapsulated in a polylactic acid matrix as a potential substratum for bone tissue formation (Qui et al. 2000). Hollow microcarriers made of degradable poly(lactic acid-*co*-glycolic acid) were used for the cultivation of osteoblast-like cells (Botchwey et al. 2001). Other examples are microcarriers produced from calcium alginate gel beads crosslinked with gelatin for anchorage-dependent mammalian cell culture (Kwon and Peng 2002) and collagen-based microcarriers in a three-dimensional culture of osteoblasts derived from human trabecular bone (Fig. 5.6) (Overstreet et al. 2003). Macroporous gelatin microcarriers were effective for nasal chondrocyte expansion (Malda et al. 2003). Agar-based beads, poly(γ-methyl-L-glutamate) beads, and gelatin–polystyrene magnetic beads were used as microcarriers in mammalian cell

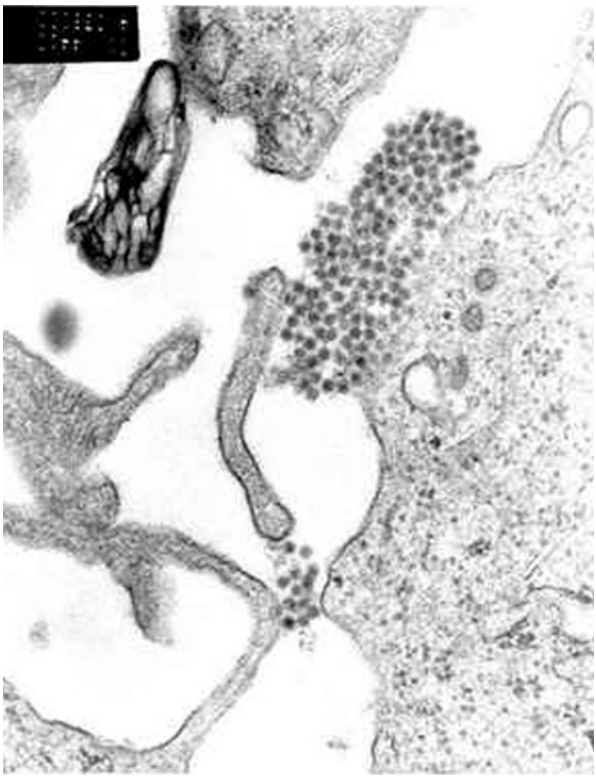


Fig. 5.5 TEM micrograph showing dengue virus virions (the cluster of *dark dots* near the center). This is a work of the United States Federal Government (http://en.wikipedia.org/wiki/File:Dengue.jpg)

culture (Xu et al. 1999; Sakata et al. 2000; Lu et al. 2002). PEG is frequently used to enhance the biocompatibility of the surface of the material interacting with the cells. Amendment of poly(vinyl chloride) with PEG results in an increase in blood compatibility (Balakrishnan et al. 2005). Graded coverage with a surface-immobilized PEG layer was constructed to integrate cell adhesion on a solid surface (Mougin et al. 2005). Incorporation of PEG methacrylate and 2-methacryloyloxyethyl phosphorylcholine onto poly(hydroxyethyl methacrylate)-based hydrogels provided significant improvement in cell viability and proliferation characteristics of human muscle fibroblasts (Abraham et al. 2005). A variety of suspension polymerization procedures were invented for the production of polymeric beads with different functionalities (Kesenci et al. 1996; Tuncel and Piskin 1996; Tuncel et al. 1996; Senel et al. 1998; Tuncel and Cicek 2000).

These beads, crosslinked with ethylene dimethacrylate, were effectively used as carriers in such dissimilar biological applications as enzyme immobilization and isolation, protein separation, and DNA isolation (Bahar and Tuncel 2000; Tuncel 2000; Unsal et al. 2000; Bahar and Tuncel 2004). PEG methacrylate was recognized

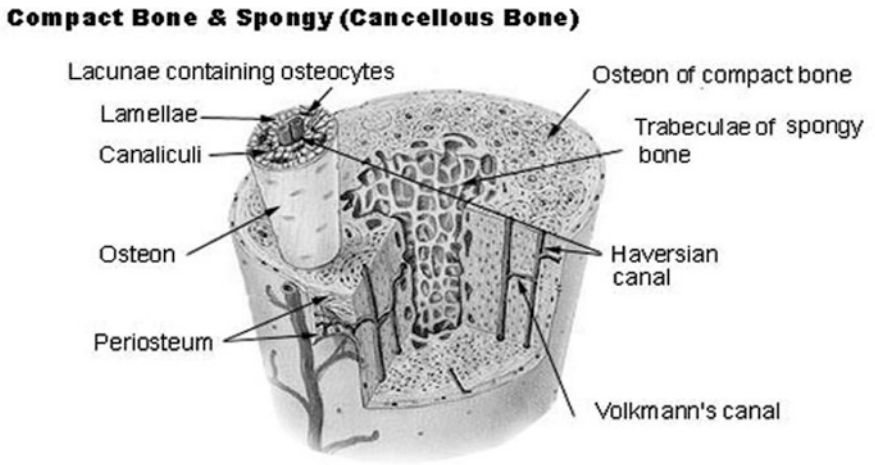


Fig. 5.6 Compact bone and spongy bone (courtesy of the U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program, http://training.seer.cancer.gov/index.html)

as an appropriate candidate monomer for the synthesis of spherical hydrogel beads in the micron size range. The non-toxic and highly biocompatible PEG segments of these microspheres gave this product potential as a microcarrier in cell culturing. PEG methacrylate was selected as the main monomer for synthesis of a new microcarrier suitable for cell culturing (Cer et al. 2007). To achieve cell adhesion onto the microcarrier surface, *N*-[3-(dimethylamino) propyl]methacrylamide was chosen as the comonomer carrying cationically charged functional groups. A suspension copolymerization protocol that includes the use of PEG methacrylate and *N*-[3-(dimethylamino)propyl]methacrylamide was developed for the production of spherical microcarriers with cationic charge. The performance of the developed microcarrier in the cultivation of mouse fibroblast cells was reported (Cer et al. 2007).

5.5 Potential Support for Endothelial Cells

Angiogenesis in mature tissues is a multistep process that engages the creation of new capillaries from pre-existing vessels (sprouting angiogenesis), from the splitting of pre-existing vessels (intussusception), or from the fusion of adjacent blood vessels (anastomosis) (Carmeliet 2000; Bergers and Benjamin 2003; Martineau and Doillon 2007). In vitro models of angiogenesis have been developed to examine the behavior of antiangiogenic and angiogenic compounds. Monolayer cell culture arrangements with subconfluent endothelial cells on extracellular matrix-coated

plates produced cord-like structures; three-dimensional culture systems consisted of growing endothelial cells on top of a gel: upon convergence, the cells sprouted and formed tube-like structures in the gel. Similar angiogenic-like structures are also obtained after mixing endothelial cells in an extracellular matrix (e.g., fibrin, collagen) solution prior to gelling. The latter cell-seeded gel closely imitates the in vivo environment (Folkman and Haudenschild 1980; Haralabopoulos et al. 1997; Davis et al. 2000; Vailhe et al. 2001; Gagnon et al. 2002). Other approaches have consisted of integrating endothelial cell-seeded microbeads in an extracellular matrix gel and introducing mural cells or their equivalents (e.g., fibroblasts) to induce and measure the angiogenic response (Nehls and Drenckhahn 1995; Nakatsu et al. 2003). A tight association of endothelial cells on beads can reduce cell-seeding density, and induction of interconnected angiogenic-like structures does not require a high microbead density. In general, microspheres may represent a potential support for endothelial cells in microvessel networking, with subsequent applications in the prevascularization of bioimplants (Martineau and Doillon 2007).

5.6 Vaccine Delivery

Pasteurella multocida is the most common bacterial pathogen of domestic rabbits (DeLong and Manning 1994). Losses due to *P. multocida*-related disease are a problem since rabbits are used for research and food. Vaccination may serve as a simple economical alternative to therapeutic measures (Broome and Brooks 1991; Suckow et al. 1996a). Potential preparations for immunizations include lipopolysaccharide (Manning 1984), outer membrane proteins (Lu et al. 1988), and potassium thiocyanate extracts (Ringler et al. 1985; Suckow et al. 1996b). In parenteral immunization, injection-site reactions may occur (Hendrick et al. 1994; Doddy et al. 1996) and immunization by vaccine delivery seems to be a promising alternative. A few examples of locally administered vaccines include intranasal administration of the mutant of *Bordetella bronchiseptica* (Fig. 5.7) against the bacterial component of kennel cough (Keil and Fenwick 1998), aerosolization of *P. multocida* and inhalation of the vaccine by chickens as a vaccination against this pathogen, and *Salmonella choleraesuis* var. *kunzendorf* vaccine for disease prevention in swine (Kramer et al. 1992). Microparticles entrapping antigens were observed to induce serum and secretory antibody responses (Mathowitz et al. 1997). Poly-(DL-lactide-co-glycolide) can be used to create those particles, but this involves the use of both organic solvents and high temperatures. The non-toxic alginate can be utilized as a substitute and has already been used for the incorporation of living beta-islets for the experimental treatment of diabetes and for other delivery purposes (Lim and Sun 1980; Bowersock et al. 1996). Alginate microparticles with sizes of 1–50 μm and with 70% of the particles less than 10 μm were used for vaccine delivery (Suckow et al. 1999). Studies in rabbits showed that incorporation of a potassium thiocyanate extract of *P. multocida* results in effective immunization through the drinking water (Suckow et al. 1999).

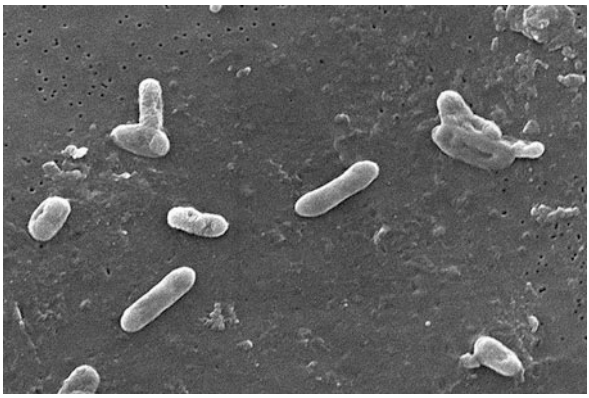


Fig. 5.7 Gram-negative *Bordetella bronchiseptica* coccobacillus bacteria, a common cause of respiratory tract infections in dogs, as well as humans whose immune system has been compromised (photo credit: Janice Carr; Source: The Centers for Disease Control and Prevention's Public Health Image Library)

5.7 Crosslinked Chitosan Beads: Different Medicinal Functions

Chitosan reactivity has been used for the chelation of heavy metal ions (Muzzarelli and Rocchetti 1974), as an ion exchanger (Itoyama and Tokura 1994), and as an antifungal agent (Schlick 1986). To extend the functionality of this biopolymer, its chemical modification was investigated (Kurita 1986). Cyclodextrins (Fig. 5.8) are cyclic carbohydrates that are of interest to both the pharmaceutical and nutriceutical industries (Frank 1975). Inclusion of more than six D-glucopyranose moieties with α-(1→4) glycosidic linkages in a cyclic oligosaccharide has the potential ability to complex various organic compounds in their hydrophobic cavities. This facilitates creation of a chitosan-based supramolecular species through condensation of cyclodextrin and chitosan (Sakairi et al. 1999). Insoluble cyclodextrin-linked chitosan with a highly porous structure was prepared by a coupling method using crosslinked chitosan beads. Two types of cyclodextrin-linked chitosans were prepared, having an amide-containing linker and an amino-containing linker.

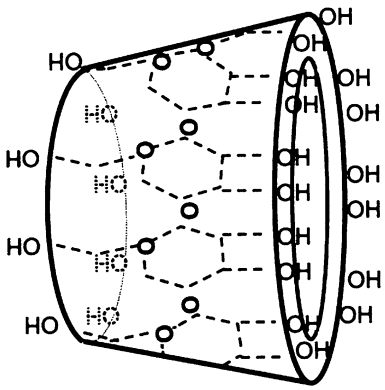


Fig. 5.8 Gamma cyclodextrin cone shape showing arrangement of glucose molecules (courtesy of MDW, http://en.wikipedia.org/wiki/File:Gamma_CD_cone_shape.jpg)

Preliminary experiments were conducted to assess the utility of these polymeric host compounds for reversed-phase chromatography, as adsorbents for controlled-release applications, as a pseudostationary phase in capillary electrophoresis, and as an additive in cosmetics to protect skin from chemical damage (Sakairi et al. 1999).

There is a continual need for biocompatible polymeric materials that can be used for cell or tissue encapsulation under mild conditions. Biocompatible microcapsules were developed from poly(2-hydroxyethyl methacrylate) and poly(methyl methacrylate) with controlled morphology and porosity for selective permeation (Crooks et al. 1990). Alginate-poly-L-lysine was used to encapsulate islet cells (Lim and Sun 1980). Other cells, such as hepatocytes (Matthew et al. 1991; Dixit and Gitnick 1995) and dopamine-secreting cells (Campioni et al. 1998; Aebischer et al. 1991), were encapsulated to normalize metabolic deficiencies or to serve for self-regulating drug delivery. The ongoing need for biocompatible polymeric materials led to the fabrication of a chitosan–Pluronic network (Anderson et al. 1999). Physically interpenetrating chitosan–Pluronic network membranes were fabricated for potential use in microencapsulation of cells and tissues. Human serum albumin was used as a protein permeability marker to examine the permselective nature of the membranes. Scanning electron micrographs showed distinct porous surfaces and cross sections in chitosan–Pluronic membranes. The permeability coefficient of albumin increased from 2.7×10^{-8} cm^2/min in chitosan membranes to 5.5×10^{-7} cm^2/min in chitosan–Pluronic membranes made with 20% (w/w) Pluronic. The results of this study showed that membranes can be designed for selective permeation of microencapsulated products while restricting the entrance of immune-recognition elements (Anderson et al. 1999).

5.8 Mucoadhesive Beads and Their Applications

5.8.1 General

Mucoadhesive polymers have recently become of interest for pharmaceutical scientists as a means of improving drug delivery by promoting the residence time and contact time of the dosage form with the mucous membranes. Mucoadhesion is a process whereby synthetic and natural polymers adhere to mucosal surfaces in the body. If these materials are then incorporated into pharmaceutical formulations, drug absorption by mucosal cells may be enhanced or the drug will be released at the site for an extended period of time (Patil and Sawant 2008). In general, microspheres have the potential to be used for targeted and controlled-release drug delivery; however, the coupling of mucoadhesive properties to microspheres confers additional advantages, such as much more intimate contact with the mucous layer, and efficient absorption and enhanced bioavailability of the drugs due to a high surface-to-volume ratio (Patil and Sawant 2008). In short, mucoadhesive microspheres as a novel carrier system to improve drug delivery by various administration routes, such

as buccal, oral, nasal, ocular, vaginal, and rectal, for either systemic or local effects, have broad potential for use in coming years (Patil and Sawant 2008).

5.8.2 Eyes

A bioadhesive gel is a tissue adhesive gel. The main application area for these gels is the mucosal tissue. The morphology of mucose membranes varies to a great extent depending on their location in the body. Mucoadhesive microspheres or nanospheres can be used to overcome restrictions related to eyedrops and ophthalmic ointments. The residence time of microspheres which were in-labeled using poly(acrylic acid) (Carbopol 907) in rabbit eye was evaluated (Murrani et al. 1995). Microsphere clearance exhibited a bimodal pattern, with rapid partial disappearance at the initial stage followed by complete disappearance. When the microspheres were hydrated at pH 5.0 and administered, clearance was slower than for those hydrated at pH 7.4, in agreement with the in vivo results on adhesion characteristics. Because 25% of the administered material remained after the rapid clearance period, this could be a vehicle for drugs that are not easily absorbed by the eye (Murrani et al. 1995). Microparticles smaller than 10 μm in diameter were fabricated by emulsification with poly(lactic-co-glycolic acid) as a core material and, in some cases, PEG as a mucoadhesion promoter. To examine the effect of particle geometry, microparticles were also cut to have flat surfaces (i.e., microdiscs) and were compared with spherical particles (i.e., microspheres). In vitro mucoadhesion of microparticles was tested on a mucous layer under shear stress, mimicking the human eye blink. The resultant microparticles were also formulated in two dosage forms, an aqueous suspension and a dry tablet, to test the effect of formulation on the retention capacity of microparticles in the preocular space of rabbits in vivo (Choy et al. 2008). Mucoadhesive microdiscs adhered better to the simulated ocular surface than the other types of microparticles. When a dry tablet embedded in mucoadhesive microdiscs was administered in the cul-de-sac of the rabbit eye in vivo, the microdiscs exhibited longer retention than the other formulations tested. More than 40 and 17% of mucoadhesive microdiscs remained on the preocular surface at 10 and 30 min post-administration, respectively. Fluorescence images of the eye surface showed that mucoadhesive microdiscs remain for at least 1 h in the lower fornix. This study demonstrated that mucoadhesive microdiscs formulated in a dry tablet form can achieve prolonged residence time on the preocular surface and thus constitute a promising drug-delivery system for ophthalmic applications (Choy et al. 2008).

5.8.3 Alimentary System

Bioadhesive gels are capable of controlling the rate of movement through the alimentary system (the pathway by which food enters the body and solid wastes are expelled). The alimentary canal includes the mouth, pharynx, esophagus, stomach,

small intestine, large intestine, and anus and is also called the digestive tract. Bioadhesive gels improve localized effectiveness, by restricting the affected area to that which the drugs are designed to affect. Poly(glycerol ester) microspheres coated with Carbopol 934P (CPC-MS) or microspheres in which carbopol was dispersed (CPD-MS) were tested (Akiyama et al. 1995). Mucoadhesive strength was compared to that of microspheres without carbopol. In an in vitro experiment using rat stomach and intestines, CPD-MS showed strong mucoadhesion capability because new carbopol particles were gradually and continually exposed from inside the microsphere. When CPD-MS was administered to a starved rat, it took longer to pass through the alimentary canal than the microspheres without carbopol (Akiyama et al. 1995). Another report dealt with the potential use of mucoadhesive microspheres for gastro-retentive delivery of acyclovir (Fig. 5.9) (a synthetic nucleoside analogue that is active against herpes viruses and is better known by its brand name, Zovirax; De Clercq and Field 2006) by using chitosan, thiolated chitosan, Carbopol 71G, and Methocel K15M. These polymers were chosen for their mucoadhesive abilities. Microsphere formulations were prepared using emulsion–chemical crosslinking technique and evaluated in vitro, ex vivo, and in vivo. Gelatin capsules containing drug powder showed complete dissolution ($90.5 \pm 3.6\%$) in 1 h. Drug release was prolonged to 12 h (78.8 ± 3.9) when incorporated into mucoadhesive microspheres (Dhaliwal et al. 2008). The poor bioavailability of acyclovir is attributed to short retention of its dosage form at the absorption sites (in the upper gastrointestinal tract to the duodenum and jejunum). The results of the mucoadhesion study showed better retention of thiolated chitosan microspheres (8.0 ± 0.8 h) in the duodenal and jejunal regions of the intestine. The results of the qualitative and quantitative gastrointestinal tract distribution study also showed significantly higher retention of mucoadhesive microspheres in the upper gastrointestinal tract. A pharmacokinetics study revealed that administration of mucoadhesive microspheres could maintain a measurable concentration of acyclovir in the plasma for 24 h, as compared to 5 h following its administration in solution form. Thiolated chitosan microspheres were superior to the other formulations. Overall, results indicated prolonged delivery with significant improvement in oral bioavailability of acyclovir from mucoadhesive microspheres due to enhanced retention in the upper gastrointestinal tract (Dhaliwal et al. 2008). Other coating materials, such as a Eudragit–cysteine conjugate, were used to coat the chitosan microspheres for the development of an oral protein drug-delivery system with mucoadhesive and pH-sensitive properties. Bovine serum albumin (BSA) as a model protein

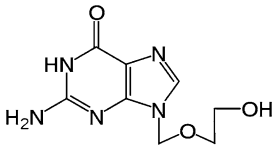


Fig. 5.9 Skeletal formula of acyclovir (aciclovir, acycloguanosine) in nucleoside orientation (courtesy of Fvasconcellos, http://en.wikipedia.org/wiki/File:Aciclovir_standard.svg)

drug was loaded into thiolated Eudragit-coated chitosan microspheres (TECMs) to study the release characteristics of the delivery system. After coating with thiolated Eudragit, the release rate of BSA from BSA-loaded TECMs was visibly suppressed in a pH 2.0 PBS solution, while in a pH 7.4 PBS solution, BSA exhibited sustained release for several hours (Quan et al. 2008). The structural integrity of the BSA released from the BSA-loaded TECMs was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and circular dichroism spectroscopy. The mucoadhesive property of the TECMs was evaluated and compared with those of chitosan microspheres and Eudragit-coated chitosan microspheres. It was confirmed that after coating with thiolated Eudragit, the percentage of TECMs remaining on the isolated porcine intestinal mucosal surface was significantly higher than those of chitosan microspheres or Eudragit-chitosan microspheres. Likewise, gamma camera imaging of Tc-99m-labeled-microsphere distribution in rats after oral administration also suggested that TECMs have comparatively stronger mucoadhesive characteristics. Therefore, results indicated that TECMs have the potential to be an oral carrier of protein drugs (Quan et al. 2008).

5.9 Polyelectrolyte Complexes

A polyelectrolyte complex (PEC) formed from chitosan and an anionic polymer is insoluble in water. Anionic polymers, such as carboxymethyl cellulose, carrageenan, and alginic acid, are frequently used. For instance, a dispersion of potassium chloride with theophylline was added dropwise to a κ-carrageenan solution at pH 9.0 that included chitosan powder, forming a carrageenan capsule. This capsule was treated in a solution of pH 2.1 and the chitosan was charged to form a complex with carrageenan (Tomida et al. 1994). Theophylline is a methylxanthine drug used in therapy for respiratory diseases, such as chronic obstructive pulmonary disease or asthma, under a variety of brand names (Schultze-Werninghaus and Meier-Sydow 1982). The release of theophylline from this capsule was not influenced by ionic strength in a pH range of 1.2–8.0 and it was released at a rate of 10% per hour. Chitosan complex gels do not generally swell in neutral or acidic solutions. However, chitosan–carrageenan complexes swell considerably at pH 10–12 (Sakiyama et al. 1993). Similarly, chitosan–xanthan gum complexes swell greatly at pH 10–11 (Chu et al. 1995). κ-Carrageenan is not the only candidate for creating PECs. The formation of PECs between chitosan of varying degrees of deacetylation and κ-, ι-, or λ-carrageenan was investigated in moderately concentrated solutions, with a focus on the effects of charge density and conformation. Macroscopically electroneutral PECs were formed when chitosan interacted with carrageenan molecules in a coiled or non-aggregated helical conformation. When the κ-carrageenan existed in an aggregated helical conformation, the interaction with chitosan produced PECs with a charge ratio of chitosan to carrageenan that was below unity. Furthermore, the PEC formation process proved to be significantly affected by the presence of low molecular weight polyelectrolyte components (Hugerth et al. 1997).

5.10 Soft Tissue Regeneration

Calcium alginate, calcium alginate/chitosan, calcium alginate/gelatin, and pectin/chitosan microcapsules were prepared for use as innovative injectable scaffolds for soft tissue regeneration by a simple extrusion method from aqueous solutions (Munarin et al. 2010). The prepared microcapsules were spherical and their size was markedly influenced by polymeric composition. When incubated in a simulated physiological environment for up to 30 days, they underwent initial swelling, followed by different rates of weight loss, depending on the microcapsule formulation. Mouse myoblast cells were encapsulated in the calcium alginate, calcium alginate/chitosan, and calcium alginate/gelatin microcapsules. Cells remained viable throughout the encapsulation procedure and were recovered upon mechanical rupture of the capsules. After 7 days, the cells gradually migrated out of the fractured microcapsules (Munarin et al. 2010).

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Chapter 6
Dry Bead Formation, Structure, Properties, and Applications

6.1 Introduction

Dried hydrocolloid gel beads are actually cellular solids. Therefore, formation, structure, and properties of cellular solids in general and cellular dry beads in particular are discussed in this chapter. The word cellular solid originates from the word "cell," derived from the Latin "cella"—a small enclosed space. Unique structures, which can be observed in many natural instances, e.g., wood, cork, sponge (Fig. 6.1), and coral, are cellular solids (Gibson and Ashby 1988). Man-made natural-based or synthetic cellular products include items from disposable coffee cups to crash padding in aircraft cockpits. Foamed polymers, metals, ceramics, and glass are used for insulation, cushioning, and absorption of impact kinetic energy (Nussinovitch 1997). Cellular solids contain different ordered and disordered structures: of major importance is the distinction between open-cell (interconnected) and closed cell (a cell sealed off from its neighbors by membrane-like faces) cellular solids (Gibson and Ashby 1988; Jeronomidis 1988). This chapter describes several methods, such as compression studies, to evaluate the mechanical properties of dried beads. It also includes details of models used to describe stress–strain behavior in cellular solids. A special section is devoted to the elastic properties of dried beads. Bead structure as a result of the production method is also considered. Facts regarding the distribution of entrapped microorganisms within dried cellular solid structures are also revealed. Applications of dried beads (not including drug delivery which is fully described in other chapters), such as for biological control, as a means of preserving microorganisms for long periods, in water treatments, and as a vehicle to protect bacteria against radiation, are illustrated.

6.2 General Properties of Cellular Solids

Conductivity, density, strength, and Young's modulus are the principal properties of cellular solids. A wide-ranging variety of properties can be attained with dissimilar structures, leading to numerous prospective applications. Low density means light, stiff, large portable structures with flotation ability. Low thermal conductivity leads

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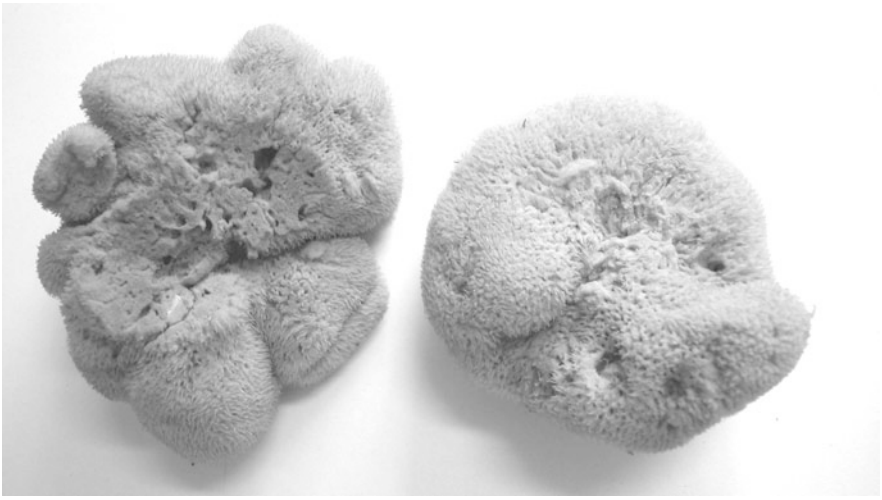

Fig. 6.1 Sponge—a natural cellular solid

to reasonably priced thermal insulation, for use in buildings, cars, trucks, and ships. Glass foams can be used in cases of potential fire hazard or for extending the life span of pipes and roved (i.e., a long and narrow bundle of fiber) products. Packaging with cellular solids absorbs impact energy due to its ability to pass through large strains without producing high stresses. Low density leads to a light package, which in turn reduces shipping costs. Natural-based (such as balsa) or synthetic (cellulose acetate foam) cellular solids can be used in sandwich panels (Gibson and Ashby 1988). A further use of closed cell plastic cellular solids stems from their potential buoyancy. The buoyancy factor (B) is calculated by the simple equation

$$B = \left[\frac{(\rho_{\text{water}} - \rho_{\text{foam}})}{\rho_{\text{water}}} \right] \tag{6.1}$$

where ρ stands for density. Additional applications might be for filtration, where metal is poured through open-cell ceramic foam to achieve high-quality metal castings. Foam sheets can be used as carriers for inks, dyes, lubricants, and enzymes for chemical processing (Gibson and Ashby 1988). Particular applications of dried polymer beads are discussed further on in this chapter.

6.3 Manufacturing Methods for Hydrocolloid Cellular Solids

6.3.1 Drying Bicarbonate-Containing Gels After Acid Diffusion

A cellular solid structure can be formed by drying a hydrocolloid gel. Firm cellular solids have been produced by freeze-drying 2% agar or 1% alginate gels;

however, carrageenan, gellan, curdlan, and many other hydrocolloid gelling agents can also be used for such purposes. The formed cellular solids have distinctive compressive stress–strain curves that can be illustrated by a three-parameter model originally developed for polymeric sponges and edible cellular solids (i.e., baked goods) (see Section 6.5.2 and Equation (6.8)). By immersing bicarbonate-containing gels in an acid bath, which produces entrapped gas bubbles, a considerable loss of mechanical integrity in the dry agar cellular solids but not in those from alginate was observed, possibly due to differences in brittleness (Nussinovitch et al. 1993). The hydrocolloid alginate can be used to form gels filled with manufactured CO_2 gas bubbles: cold-set alginate gels based on alginate, sodium hexametaphosphate, glucono-δ-lactone, and including $CaCO_3$ are immersed in citric acid solutions of various concentrations, the volume of which should be adequate to guarantee excess acid, which then diffuses into the gels. The number of formed gas bubbles can be counted by means of a light microscope. They appear to be trapped within the gel body and protrude from its external surface. The motion of the acid within the gel follows controlled diffusion, as confirmed by the linear relationship between the penetrated distances versus the square root of time. After 2.5 h, ~900 bubbles/cm^3 were counted. This number increased to $\sim 2.5–2.7 \times 10^3$ after 24 or 36 h, depending on the carbonate concentration. As a result of bubble creation, the density of the gels decreased and therefore, they became buoyant. Nevertheless, after a short time, bubbles started leaving the gel, causing some damage to its integrity, and allowing fluid to progressively fill the vacant spaces. As a result, the gels began to sink again. Alginate gels without carbonate immersed for 2.5 h in a 0.5% citric acid solution increased their average stress at failure from 28 to 46 kPa. The Hencky's strain (see Section 6.5.2 and Equation (6.7)) at failure of these gels increased from 0.64 to 0.83, demonstrating that the gels end up being less brittle. An increase in failure stress and strain was found for all tested alginate systems immersed in 0.5–2.0% citric acid solutions. This may have been due to acid-induced crosslinking, which helped the gel retain its mechanical strength, even in the face of the structural disruption caused by bubble formation. The presence of carbonate, nevertheless, had a disruptive effect, evidenced first and foremost by lower stiffness. Thus, gel strength depended on both acid and $CaCO_3$ concentrations (Nussinovitch et al. 1992a, b). Dry cellular solids are obtained upon drying. Because the shape of the final product quite accurately resembles that of the gels before dehydration, products can be designed in every shape and size, by building the desired molds (Fig. 6.2). The structure of the cellular solid is determined by the drying process as much as by parameters of the gel's processing and components.

Several techniques have been suggested for the creation of porous gels: porogenation, freeze-drying, microemulsion formation, and phase separation. Among these techniques, porogenation has the advantage of affording control over pore size and porosity through modification of the porogen's size and amount (Liu et al. 2009). For porous hydrogels, interconnected pores have to be introduced into the gel to provide capillary channels so that water can enter the dry hydrogels by capillary wetting. When the dry gel with its open-pore structure is placed in an aqueous solution, water flows through the open channels via capillary effect,

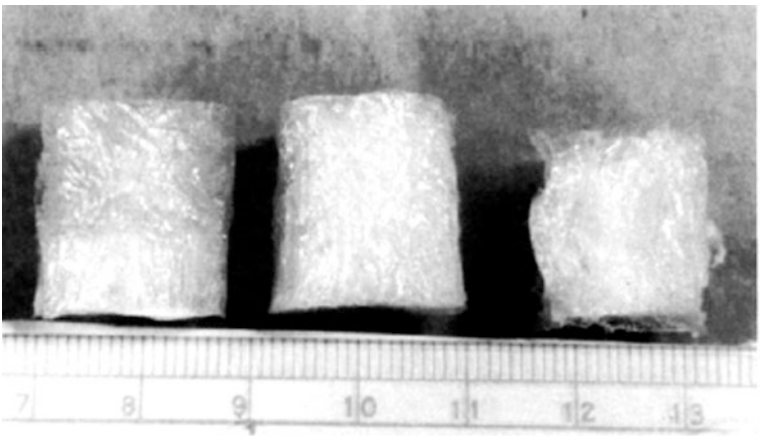

Fig. 6.2 Typical appearance of a freeze-dried gel specimen. The shape of the dried specimen approximates that of the gels before dehydration, thus products can be designed in any shape or size, by building the desired molds

reaching swelling equilibrium in a relatively short period of time (Liu et al. 2009). Porous hydroxypropylmethylcellulose (HPMC) gels were produced in bead form by inverted phase suspension crosslinking with nano-calcium carbonate as the porogen. Interconnected pores were formed in the gel beads at a porogen concentration of 70% (by weight) of the dry HPMC, and the gel's reswelling ability was related to the extent of its porous structure (Liu et al. 2009). In alginate cellular solids, immersing the gels in an acid bath did not result in a drastic loss of mechanical integrity. This appears to be because the disruptive effects of bubble formation were at least somewhat offset by the more extensive crosslinking (Nussinovitch et al. 1992a, b, Nussinovitch 1995).

6.3.2 Cellular Solids Produced by Fermentation

Drying hydrocolloid gels produces dried cellular solids. Gas bubbles can be entrapped in gels to a certain extent as discussed in the previous section and thus influence the structure and mechanical properties of the formed dried cellular solid. Another approach is to immobilize microorganisms that are able to produce gas within a gel under predetermined conditions (composition of culture medium, composition of gel, temperature, pH, diffusion coefficients, and the like) and then to freeze-dry the product to obtain the cellular solid. As an example, yeasts were immobilized in an agar gel: the higher the count of the microorganisms per unit weight, the higher the disturbance to the gel's integrity. In other words, stress at failure and deformability modulus of the gel before drying decreased after immobilization of the yeasts. The brittleness of the gels also increased (their strain at failure decreased). Similar results were achieved for bacterium, yeast, and spore

immobilization in different gels (Nussinovitch et al. 1994). Following yeast immobilization, the gels were immersed in excess 5% sucrose solution. Slow fermentation occurred, possibly because no nitrogen source was added. A longer lag time for yeast growth may also have caused immobilized yeast to react later than their non-entrapped counterparts. As a consequence of the fermentation, CO_2 bubbles and ethanol were produced and the pH decreased. The gas bubbles moved from inside the gel to its surface, causing some mini-cracks and later, influencing the structure of the resultant cellular solid. Longer the fermentation created gels that were less strong and stiff. The gels were freeze-dried to produce cellular solids characterized by a sigmoidal stress–strain curve, which is a manifestation of the three aforementioned deformation mechanisms. By applying Equation (6.8) (see further on) to the stress–strain curves and calculating their constants by non-linear regression, a major difference between cellular solids was observed in the magnitude of C_1: the higher the concentration of the entrapped microorganisms, the smaller the value of this constant, which serves as a scale factor for stress. Yeast cells were distributed in and on the cell walls of the cellular solid and attached to its outer surface. The compression of all cellular solids produced after 3 days of immersion in sucrose solution resulted in stress–strain relationships resembling those of a regular sponge. This was true for all yeast concentrations used in the study (Nussinovitch and Gershon 1997b). After 7 days' immersion in the sucrose solution, a different phenomenon was observed. For a gel containing 10^7 yeast/g gel, a "regular" stress–strain relationship was still observed, whereas at higher initial yeast concentrations, such as 10^8 and 10^9 yeast/g gel, the materials no longer resembled cellular solids in their stress–strain behavior. This can be to some extent explained by noting that an increase in the time of fermentation results in higher biomass (a fivefold increase in the protein content of the gels before freeze-dehydration was observed). In compressed cellular solids with originally high yeast concentrations, after 7 days' immersion in sucrose solution, compaction of a "yeast-hydrocolloid" rather than hydrocolloid network occurred, resulting in different products and properties. Comparing cellular solids prepared from gels without yeast to those prepared from gels with 10^9 entrapped yeast/g showed a decrease in porosity from ~96% in the former to ~92% in the latter. This may be attributable to the increase in cellular solid dry matter content.

6.3.3 Enzymatically Produced Cellular Solids

Another approach to producing cellular solids with some control over their porosity is to embed enzymes within a gel or to immerse a gel that contains a suitable substrate into an enzyme solution that has the ability to decompose the gel or enter via the gel's pores to decompose the embedded substrate. This gel is then dried to obtain a cellular solid. Sponge-like materials with a cellular structure were created by subjecting agar–starch gels to α-amylase activity prior to freeze-dehydration. Various starch concentrations (0.5–1.5%), enzyme concentrations (1000–1500 ppm), and times of exposure to the enzyme were selected to modify the structure and

mechanical properties of the hydrocolloid cellular solid (Nussinovitch et al. 1995). The influence of the extent of enzymatic treatment on the cellular structure of freeze-dried 2% agar–1.5% starch gel specimens (sponges) was studied. The enzyme (1500 ppm of α-amylase) solution was incubated with the agar–1.5% starch gels at 55°C for 24 and 72 h. The enzymes diffused into the gels and began to decompose the substrate: starch hydrolysis caused enlargement of the pores within the gel matrix and consequently within the sponge (Nussinovitch et al. 1998). As a consequence of this process, the dry sponges underwent major structural changes: the longer the exposure to the enzyme, the larger their pores, which in turn may have contributed to changes in the mechanical properties of the sponges. The effect of increasing enzyme concentrations was only slightly detectable: for the highest enzyme concentration (1500 ppm), the stress–strain curve was located only slightly lower than those of the control and 1000 ppm enzyme application (Nussinovitch et al. 1998). The influence of 1500 ppm α-amylase on agar gels containing 1.5% starch incubated for 24 or 72 h was also checked: the longer the immersion time, the greater the starch decomposition, resulting in a weaker cellular solid. Furthermore, the cellular solids became slightly more porous after 24 and 72 h (∼0.93–0.96) than the control cellular solids (∼0.87–0.91). It should be noted that incubation at 55°C for 72 h, even without enzyme activity, led to a decrease in the cellular solid's mechanical properties, as reflected by the stresses measured at a strain of ∼0.2, at the shoulder of the curve. Starch degradation was verified by HPLC analysis. For agar–1.5% starch gels incubated without enzyme for 72 h at 55°C (blank specimen), only traces of the different sugars, i.e., glucose and raffinose, were detected (Gershon and Nussinovitch 1998). Note that because raffinose contains fructose, the very low quantities detected could have simply been reflecting impurities in the starch, rather than an actual degradation product. In comparison, when 1500 ppm enzyme was used to decompose the starch embedded in the agar gels, starch-degradation products (i.e., glucose, raffinose, maltose, and maltotriose) were observed, along with other unidentified oligosaccharides. Thus, the embedded starch can be used, in principle, as an ingredient which can later be degraded; such a process could influence, and maybe even control, the porosity and mechanical properties of the sponge. Under the same experimental conditions (i.e., identical temperature, time, substrate, and enzyme concentrations), enzyme solution decomposed a non-embedded starch solution with greater efficiency than its embedded counterpart, as evidenced by a chromatograph of degradation components (Gershon and Nussinovitch 1998).

6.3.4 Inclusion of Oil in Cellular Solids

Polymeric cellular solids are produced by drying gels. Polysaccharide gels contain a high proportion of water. Upon drying such gels (with no other included constituents), the dried structure's ability to include hydrophobic constituents is small, since no oil was embedded within the cellular solid matrix. To cope with this issue, oil was included within, for example, alginate gels (Nussinovitch and Gershon 1997a). It was observed that the higher the oil content in the gel, the lower

its stress at failure and stiffness, as reflected by the deformability modulus, and the smaller the Hencky's strain at failure; in other words, the gel was more brittle. Two systems of oil gels and sponges were studied: in the first, gels with and without oil were simply freeze-dried directly; in the second, the gels were heat treated at 85°C for 15 min in water, three times in succession. Each time the water and the extracted oil were discarded (Nussinovitch and Gershon 1997a). Oil in the gels and cellular solids was estimated by the Soxhlet method. After heat treatment, 40–50% of the oil had "left" the gel. After freeze-dehydration, the oil percentage within the cellular solid increased. Oil sponges showed ruggedness in their stress–strain curves: the higher the oil content in the sponge, the smoother the curve; in addition, C_3 from Equation (6.8) (see further on) decreased in parallel to the increasing oil content. The higher the bulk density of the cellular solid, the more the stress tended to steepness at smaller deformations. After extraction, the stress–strain curves became more ragged. The heat treatment may have disrupted the gel structure, physically damaging the specimen surface. The porosity of the cellular solids changed dramatically after oil inclusion: porosities of the resultant cellular solids decreased from ~95 (no oil included) to ~80% (40% oil included in the gel before dehydration). In addition, the higher the oil content in the cellular solid, the more closed cells there were within its structure. Moreover, the structure of the cells changed from big openings to rounder, smaller ones. The oil could be detected as mini-drops embedded within the solid wall of the matrix (Nussinovitch and Gershon 1997a).

6.3.5 Porosity Control in Cellular Solids

A method of controlling the porosity of cellular solids and its dependence on solids content was investigated (Rassis et al. 1997). Freeze-dehydration of cold-set alginate gels was used to produce tailor-made porous solids. Gels were composed of sodium alginate, corn starch, oil and other additives necessary to induce gelation. Porosity values were obtained from particle and bulk densities with a helium pycnometer (a laboratory device used for measuring the density, or more accurately the volume of solids, be they regularly shaped, porous or non-porous, monolithic, powdered, granular or in some way comminuted, employing some method of gas displacement and the volume-to-pressure relationship known as Boyle's law) and a volumetric displacement method (Rassis et al. 1997). To achieve a wider range of porosities, the gels were immersed in sucrose solutions of 10–60°Bx. Gel porosity after drying decreased from 0.85 to 0.42 and 0.07 after immersion in 30 and 60°Bx solutions, respectively. Similarly, after immersing gels containing an additional 5% soy oil, porosity decreased to 0.36 and 0.04 after immersion in the 30 and 60°Bx solutions, respectively. Preparation and formulation play a significant role in porosity, and they can both be used to control its value within the range of 0.85−0.04. SEM micrographs revealed changes in the original cellular solid, characterized by numerous large void spaces, to a denser and more uniform appearance of the specimen after immersion in the 60°Bx sucrose solution. The dry gel system with controllable porosity provides a novel tool for the production of tailor-made cellular solid foods.

Edible cellular solids can be produced from alginate–starch gels immersed in a sucrose solution prior to freeze-dehydration. This immersion resulted in an increase in the relative density of the dried samples and a decrease in their porosity. Closed cells were present, particularly at an intermediate time after the diffusion had begun and before termination. The mechanical properties were related to diffusion time (Rassis et al. 1998). Another report discussed the inclusion of oil within such structures by emulsification in the gum solution before gelation occurs. Image analysis clearly showed encapsulated oil droplets of various areas within the produced cellular solid. Gaussian distribution of these areas was found with a maximum of 54% for the $(1–10)\times10^{-12}$ m^2 area range. The properties of the produced cellular solids were also dependent on relative humidity and storage conditions (Rassis et al. 2000).

6.4 Structure of Cellular Solids

Relative density, (ρ^*/ρ_s), is the principal structural characteristic of a cellular solid: ρ^* is the density of the cellular solid divided by ρ_s, the density of the solid from which it is produced. Porosity (P) is the fraction of pore space in the foam, calculated by

$$P = \left[1 - \left(\frac{\rho^*}{\rho_s}\right)\right] \tag{6.2}$$

Cellular solids have relative densities of less than 0.3, most of them much lower. Cell size is less important since most mechanical and thermal properties depend only weakly on this parameter (Gibson and Ashby 1988). Equiaxed cells confer isotropic properties on the cellular solid. Flattened or, to some extent, lengthened cells confer properties that are dependent on direction. Cells can be two-dimensional (cell walls have a common generator) or three-dimensional (randomly oriented in space), within which there is a distinction between open and closed cells in the solid structure. Cell wall number, i.e., number of faces that meet an edge, can be from three to six (Gibson and Ashby 1988). Ordered structures (e.g., bee honeycombs) and disordered three-dimensional networks can be located side by side in the world of cellular solids. Aside from open versus closed cells, structure can be classified in terms of flexible versus rigid (or brittle) cell walls and cell wall distribution, thickness, shape, and uniformity (Gibson and Ashby 1988; Peleg 1997).

6.5 Mechanical Properties of Cellular Solids

6.5.1 Compression of Cellular Solids

Cellular solids compressed to small deformations return to their original shape after removal of the load, demonstrating their elastic properties. Deformability modulus can be calculated from the linear or approximately linear engineering stress–strain relationships (Meincecke and Clark 1973; Phillips and Waterman 1974;

Attenburrow et al. 1989; Warburton et al. 1990). Theoretical investigations of ideal cellular solids have demonstrated a power law relationship between modulus and density (Gibson and Ashby 1988).

For open, interconnected cells

$$\frac{E}{E_s} = k\left(\frac{\rho^*}{\rho_s}\right)^2 \tag{6.3}$$

and for closed cells

$$\frac{E}{E_s} = k'\left(\frac{\rho^*}{\rho_s}\right)^3 \tag{6.4}$$

where E and E_s are the moduli of the cellular solid and cell wall material, respectively, ρ^* and ρ_s are their respective densities, and k and k' are the proportionality constants. Conformity linking the theoretical relationships and the actual behavior of sponge cakes and starch foams was reported by Attenburrow et al. (1989) and Warburton et al. (1990), but the magnitude of the exponent could not be accurately determined due to experimental scatter. Computations provide values that are comparatively close to those derived from theory. In the case of large deformations of cellular spongy materials (with either open or closed cells), compressibility is determined by three mechanisms. At small deformation, the cell's geometry changes only slightly and the structure is more or less elastic (Peleg 1997). At higher deformations and as a result of fracturing or buckling, cell collapse occurs and closed cells can be punctured or burst. At this stage, the force remains more or less steady due to little or no additional resistance. At a certain deformation level, however, the remaining space is filled with fractured and/or collapsed cell wall material and the mechanical resistance of the collapsed structure increases dramatically. At this stage, the compressed material resembles an incompressible solid (Peleg 1997).

6.5.2 Models for Describing Stress–Strain Behavior

Cellular solids can be compressed to high deformations. Their cross-sectional area remains approximately unchanged even after its compression to large deformation, i.e., an engineering stress–strain relationship can justifiably be used. The engineering stress σ_E and strain ϵ_E are defined as

$$\sigma_E = \frac{F}{A_0} \tag{6.5}$$

$$\varepsilon_E = \frac{\Delta H}{H_0} \tag{6.6}$$

where F is the force, A_0 and H_0 the specimen's initial area and height, respectively, and ΔH is the absolute deformation. A characteristic compressive stress–strain curve for a cellular material is composed of three regions (Fig. 6.3). The first is

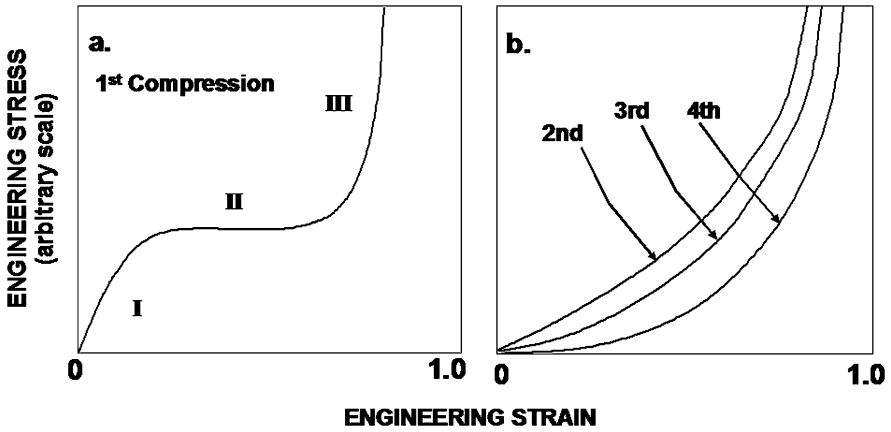

Fig. 6.3 (**a**) Schematic representation of the typical compressive stress–strain relationship of a cellular solid after one compression: (I) deformation of the original matrix; (II) densification; (III) compaction of the collapsed cell wall material. (**b**) Typical stress–strain relationship of a cellular solid after repeated compression cycles

a linear elastic region; the second is a plateau of roughly constant stress, leading to the final (third) region of steeply rising stress. Each region reflects a particular deformation mechanism. While a cellular solid sample is being compressed, the cell walls are bent, giving linear elasticity if the cell wall material is linear elastic. Upon reaching a critical stress, cells begin to crumple and ultimately, at high strains, collapse is sufficient to allow opposing cell walls to touch (or their broken fragments to pack together), and further deformation compresses the cell wall material itself. This gives the final, steeply rising portion of the stress–strain curve, termed densification (Nussinovitch 1997). The representative sigmoidal character of the curve is retained if the strain is presented as Hencky's (natural) strain:

$$\varepsilon_{\mathrm{H}} = \ln\left(\frac{H_0}{H_0 - \Delta H}\right) \tag{6.7}$$

which is a sign of the material's true compressibility (in incompressible materials, the upward concavity of the curve disappears when the cross-sectional area's expansion and the non-linearity of the strain are accounted for) (Peleg 1997).

A variety of empirical mathematical models can be used to describe the compressive stress–strain curve, up to ~80% deformation. Among them (Peleg et al. 1989; Swyngedau et al. 1991a, b)

$$\sigma(\varepsilon) = \left[\frac{C_1\varepsilon}{(1 + C_2\varepsilon)(C_3 - \varepsilon)}\right] \tag{6.8}$$

$$\sigma\ (\varepsilon) = \frac{C'_1}{\left[\varepsilon/(C_3 - \varepsilon)\right]^n} \tag{6.9}$$

$$\sigma(\varepsilon) = -\left(\frac{1}{C''_1}\right)\ln\left[1 - \left(\frac{\varepsilon}{C''_3}\right)^{n''}\right] \tag{6.10}$$

and

$$\sigma(\varepsilon) = C'''_1\varepsilon_1^{n'} + C'''_2\varepsilon_2^{n'} \quad (n_1 < 1,\ n_2 > 1) \tag{6.11}$$

where C and n values are constants. The fit of these models to experimental data collected by compressing two cellular solids (breads) was excellent (Swyngedau et al. 1991a). The calculated constants enable a quantitative comparison of curves from different solid foams, and of curves from the same material subjected to repeated compression–decompression cycles (Peleg et al. 1989), to calculate the compressibility pattern of layered arrays of sponges (Swyngedau et al. 1991b; Peleg 1993). Since constants depend on production conditions, they can serve as a responsive tool to study the influence of processing and storage on the texture of the cellular product.

6.5.3 Elastic Properties of Cellular Materials

A body's ability to return to its original shape after removal of the deforming load is termed elasticity (Peleg 1997). Full (100%) recovery is considered to be 100% elasticity, and 0% recovery is regarded to be 100% plasticity. To estimate the degree of elasticity, the ratio between the areas under the decompression and compression curves needs to be calculated (Kaletunc et al. 1991, 1992). When a cellular solid is compressed, unalterable structural changes can take place due to cell wall rupture and/or opening of closed cells, the extent of which depends on the inflicted strain. The degree of elasticity is dependent on strain as well as on the number of compression–decompression cycles imposed on the specimen (Kaletunc et al. 1992). In edible cellular baked products, the sigmoidal shape of the stress–strain curve disappears after the first compressive cycle. With regard to the previously presented models, the loss of the "shoulder" and transformation of the shape into one with ever-increasing upward concavity is expressed by $C_2 = 0$ in Equation (6.8), and $n_1 > 1$ in Equation (6.11). Comparable performance has been reported for synthetic foams, even though the sigmoidal shape is still detectable in the decompression curve (Peleg et al. 1989).

6.5.4 Layered Cellular Solids and Compressibility of Cellular Particulates

Dissimilar cellular materials can be assembled in layers of the same or different thicknesses. When such arrays are uniaxially compressed, their cross-sectional area

is presumed to be unchanged. In addition the stress along the array is considered to be indistinguishable in the separate layers while the total deformation may be represented as the sum of the deformations of each layer (Peleg 1997). Mathematically this is expressed as

$$\sigma_{\text{total}} = \sigma_i \tag{6.12}$$

where σ_{total} is the array's stress and σ_i is the stress in individual layer i, and

$$\varepsilon_{\text{total}} = \left(\frac{1}{H_{\text{total}}}\right) \sum H_{0i}\varepsilon_i(\sigma) \tag{6.13}$$

where $\varepsilon_{\text{total}}$ is the array's strain, H_{0i} is the individual layer's thickness, and $\varepsilon_i(\sigma)$ is its strain as a function of the stress. The array's initial overall thickness, $H_{0\text{total}}$, is the sum of that of the individual layers, i.e.,

$$H_{0\,\text{total}} = \sum H_{0i} \tag{6.14}$$

Equations (6.8), (6.9), (6.10), and (6.11) are particularly suitable deformability models for cellular solids in that they can be used to express the strain as an explicit algebraic function of the stress, $\varepsilon(\sigma)$. Including $\varepsilon_i(\sigma)$ and the related H_{0i} in Equation (6.11) allows a calculation of the stress–strain relationship of any layered array of cellular solids, given that the assumption of practically unchanging cross-sectional area remains valid (Swyngedau et al. 1991a; Swyngedau et al. 1991b; Swyngedau and Peleg 1992). To avoid problems related to requiring approximations in the nonlinear regression procedure, as well as to learn how to use a polynomial model to eliminate this difficulty, the reader is referred to Peleg (1993). The assumption of the cross-sectional area of the compressed object being uniform and remaining nearly unchanged cannot be made for spherical spongy particles or those having an irregular shape, such as puffed natural or synthetic materials (Nuebel and Peleg 1993, 1994), even though the same three deformation mechanisms control the compressibility patterns. In addition, the specific geometry is a highly important factor. Consequently, the force–deformation curves of individual particles differ from one another and can also lose their sigmoidal shape (Nussinovitch et al. 1991), as in the case of bulk compressibility.

6.5.5 Acoustic Properties of Cellular Solids

The mechanical and acoustic signatures of freeze-dried cellular solids produced from agar, κ-carrageenan, and gellan gels (2%), with and without infused sucrose or included starch, were studied. The presence of sugar, or starch, in the dried cellular solids increased their density in a way that could not be envisaged from the corresponding stoichiometric relations between the hydrocolloid and the additive, demonstrating that the sugars, or starch, are not inert fillers. As a general rule, the

presence of sucrose in the dried gel solid matrix increased the brittleness of the cellular solid. This outcome could be quantified by the increase in the mechanical signature's apparent fractal dimension (Richardson's and Kolmogorov's) (Nussinovitch and Mey-Tal 1998; Nussinovitch et al. 2000). The more brittle cellular solids had a "richer" acoustic signature whose apparent fractal dimension was determined with a "blanket" algorithm. Regardless of the irregular and jagged appearance (Barret et al. 1992) of the stress–strain relationships of all of the cellular solids (i.e., dry gels), they could be described by the same kind of three-parameter empirical model initially derived for soft-baked goods and polymeric foams. Even though the added sugar, or starch, stiffened the cellular solids, the effect could not be quantified unambiguously because of the differences in the density and in the overall shape of the stress–strain curve (Nussinovitch et al. 2000).

6.6 Applications of Cellular Solids

6.6.1 Hydrocolloid Cellular Solids as a Carrier for Vitamins

Vitamin A is an indispensable micronutrient involved in human development, epithelial maintenance, vision, and reproduction (Sommer 2008). Vitamin A deficiency is a prevalent problem worldwide—having the largest impact in developing countries—which carries with it increased risk of morbidity and mortality. Subclinical vitamin A shortage has also been related to high child morbidity and mortality, and vitamin A supplementation has been shown to improve the statistics significantly (Rahmathullah et al. 1990). Hydrocolloid cellular solids containing vitamin A were manufactured by preparing cold-set alginate gels containing soy oil, lecithin (as emulsifier), sodium saccharin (as an artificial sweetener), β-carotene (as a coloring agent), and vitamin A. These components were homogenized and later incorporated into a hydrocolloid solution, to which freshly prepared gluconoδ-lactone solution was added. The manufactured gels were freeze-dried and then kept over silica gel to avoid rehydration prior to testing or packaged in a laminate before clinical testing (Reifen et al. 1998). The aim of the study was to evaluate hydrocolloid cellular solids as carriers for vitamin A. The fortified edible cellular solids were given to 80 children who were screened for vitamin A status. Blood was drawn at the start and after a 3-month period to determine vitamin A levels. Vitamin A levels increased considerably following ingestion of the edible cellular solid, suggesting its usefulness as a carrier of vitamin A for children. In all cases but one, the levels of vitamin A in children who were below the normal range rose to normal following ingestion of the cellular carrier. In one child, the level remained subnormal despite cellular solid carrier ingestion. Of the 80 children assigned to receive the carrier, 3 refused to eat it. The other 77 ate the edible cellular solid all three times during the study period (Reifen et al. 1998). This study demonstrated the usefulness of edible cellular solids as a means of supplementation or fortification for young children. Since such cellular solids are designed for human consumption, coloring, flavoring, and other ingredients can be added (to the slurry before gelation)

to improve their potential acceptance. The success of this study suggests the reasonable possibility that these kinds of tailor-made cellular solids will be utilized in the future as affordable carriers for other food supplements.

6.6.2 Dried Gel Beads as Study Models and for Separation

Drying hydrocolloid gel beads (Fig. 6.4) confers many advantages, including control of some mechanical properties and porosity, in order to produce selected carriers for requested food and biotechnological operations. Drying has also been proven beneficial for the shelf life of beads that include drugs. For example, ceftriaxone–PMMA beads could be made available "off the shelf" for at least 12 months after their preparation (Alonge et al. 2009). In addition, hydrocolloid gel beads serve as study models for drying. For example, agar gel spheres were dried by desiccating air flow (air velocities 0.05–0.32 m/s) at temperatures slightly above and below the freezing point (from –9.3 to 10.7°C). A direct relationship between moisture vapor pressure at the sample surface and drying rates was observed (Suzuki et al. 1989). An additional report discussed the drying rate of agar gel spheres, at a wet-bulb temperature of 25°C and an air velocity of 3480 cm/min. Drying rate equations were developed based on drying shell modes. The use of wet-bulb temperature was preferable to taking the temperature at the center of the sample (Kubota et al. 1977). Another report of gel drying used plate temperatures of 70 and 90°C to dry agar gels using radiation and conductive heat sources. Best results were obtained at 90°C with conductive heat, which gave a more compact product (370% increase in product volume compared with a 450% increase with radiation heating), while reducing the heating time by 20–50% (Maslyukov and Dement'eva 1976). In addition to their many described applications and uses as models for drying studies, dry gel beads can be utilized

Fig. 6.4 Cross section of hydrocolloid-based bead

for separation. Hydrogel [poly (*N*-isopropylacrylamide)] filtration was used to concentrate enzymes. After enzyme production by fermentation and centrifugation (to remove the cells), the supernatant was added to tubes containing the dried gel beads. Separation efficiency depended on temperature and was, at its highest value, 84% at 20°C (Han et al. 1995). Stable gels composed of lard, sodium carboxymethylcellulose, and water were used to examine factors involved in the pro- and antioxidant activities of sodium chloride, other inorganic salts, heme compounds, meat fractions, and other additives. The solid translucent gels, into which additives had been incorporated, were stored frozen or freeze-dried or allowed to oxidize without physical change. When the gel was freeze-dried, a cellular-like spongy structure was obtained which, after an induction period, auto-oxidized rapidly. Freezer-stored gels auto-oxidized at a rate roughly similar to freezer-stored meat (Ellis et al. 1970).

6.6.3 Special Dry Beads for Water Treatment

Water originating from food or agricultural/industrial processes can be contaminated with nitrate. Denitrification methods generally employ immobilized biosystems, i.e., physical or physicochemical bonding of denitrifiers to the surface of insoluble carriers such as sand, plastic, or ceramic particles. Adsorbed microorganisms, immobilized by weak hydrogen bonds or by electrostatic interactions with the carrier (fixed film processes), can be easily washed from the support into the treated water, resulting in microbial pollution (Nussinovitch 2003). Although an alternative method of immobilization, enzyme entrapment, has been used consistently since the 1960s, microorganism containment is a recent approach to wastewater treatment (Power and Schepers 1989; Iza 1991; Metcalf and Eddy, Inc. 1991; Tatsuo et al. 1991). Cellular structures formed as a result of drying gels (composed of several ingredients) serve as matrices for the immobilization of denitrifying isolates. One such isolate (*Pseudomonas* sp.) and starch were included in an alginate gel which was freeze-dried before incubation under denitrifying conditions. The dried beads had physical properties similar to those of porous, sponge-like matrices, with apparent air spaces within and around hydrocolloid-matrix walls. The beads revealed a matrix structure with variously sized pores that enabled gas release without matrix damage. The incorporation of starch granules within the matrix strengthened its structure. The dry matrices sustained their biological activity over a prolonged period, meaning that the drying process did not damage the bacterial activity (Tal et al. 1997). The starch incorporated into freeze-dried alginate beads can serve as a carbon source and filler. Freeze-dried beads containing high concentrations of starch were found to have better mechanical and denitrifying properties than beads containing low concentrations of filler (Tal et al. 1999).

6.6.4 Matrices Entrapping Hydrocolloid Cellular Beads

Composite materials can be manufactured from two or more components, each of which can be prepared from a different material and considered as the matrix or

particulate part of the system. The properties of the composite system are unique and are not usually a simple average of those of the base components. Various examples exist of composite materials in everyday use, for example, fiberglass and different flexible packing materials. Hydrocolloid cellular solids embedded with water-soluble polymer beads are an additional example of a particulate composite system (Fig. 6.5). The physical properties of cellular solids manufactured from a continuous matrix that contained spherical particles (beads) which differed in composition, size, and concentration were studied (Ungar and Nussinovitch 1998; Nussinovitch and Ungar 1999). Two model systems were examined. The first was based on an agar matrix that had embedded alginate beads and the second consisted of a carrageenan matrix with entrapped chitosan beads. The quantity, size, and concentration of the beads embedded in the matrices were varied to study their influence on the physical properties of the composite systems and to determine their ability to control these properties (Nussinovitch and Ungar 1999). In one experiment, 2.5 mm alginate and chitosan beads were introduced into the matrices and only their quantities differed. The maximum percentage of beads that could be introduced into the matrices was 80% in the agar–alginate system and 60% in the carrageenan–chitosan system. In both cases, the higher the percentage of beads introduced, the greater the strength of the dried composite cellular material. In the case of the agar–alginate system, this was because the alginate beads are a tougher material than the agar matrix. The beads were presumed to be the reason for the increasing values of Young's modulus with their increasing percentage in the system. In the carrageenan–chitosan system, results differed because the chitosan beads collapsed during the compression tests (Nussinovitch and Ungar 1999). The effect of alginate and chitosan bead size on the mechanical properties of the agar–alginate and carrageenan–chitosan composite celullar solids was also studied, by increasing the bead diameter from 2 to 5 mm. When the percentage of beads was 20%, the composite cellular solid systems were strengthened by the addition of larger embedded beads. Except for

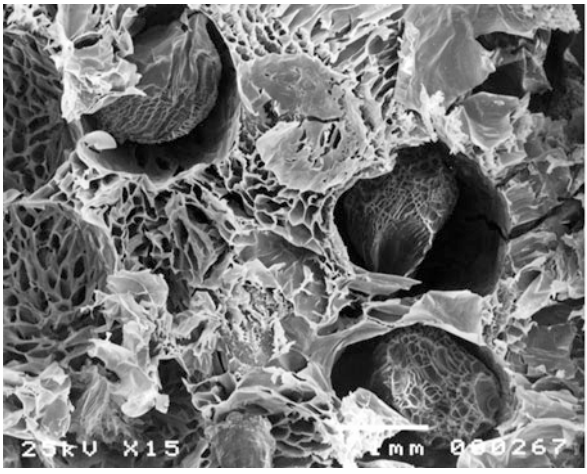

Fig. 6.5 SEM micrograph of dry hydrocolloid cellular matrix, including water-soluble polymer beads embedded within its structure

5 mm alginate beads, the inclusion of 60% alginate or chitosan beads strengthened the cellular composite systems as well. The effect of hydrocolloid concentration in the beads on the cellular solid composite systems was studied. Increasing bead hydrocolloid concentration from 1 to 2% strengthened the cellular solid systems, and the Young's modulus values indicated that the composite systems were more rigid. The apparent strengthening of both systems with a small percentage (20%) as well as a high percentage (60%) of included beads indicated that the addition of dry cellular matter to the dry systems probably causes the higher rigidity in the composite cellular systems. Electron microscopy of the composite cellular solids revealed voids around the beads embedded in the cellular matrix, due to bead shrinkage during the freeze-dehydration process. The size of the voids depended upon the level of bead shrinkage and upon differences in bead type, size, and concentration. The alginate beads shrank to as much as 50% of their initial volume while the chitosan beads maintained their spherical shape and their level of shrinkage was far less (Nussinovitch and Ungar 1999).

A mathematical model was used to describe the dependence of the modulus on the composite system. The model was fitted to a rigid system containing rigid particles in a rigid matrix. E and φ are the modulus and volume fraction of particle p and matrix m, respectively:

$$E_c = x(E_p\varphi_p + E_m\varphi_m) + (1 - x)\frac{(E_pE_m)}{(E_p\varphi_m + E_m\varphi_p)} \tag{6.15}$$

In the sponge specimens, there was no apparent interaction between the particles and the matrix and as a result, x=0 and the expression becomes

$$E_c = \frac{E_pE_m}{E_p\varphi_m + E_m\varphi_p} \tag{6.16}$$

When the percent inclusion was less than 80, there was a correlation between Hirsh's modulus (defined by Equation (6.15)), representing the theoretical model, and Young's modulus, determined experimentally. In addition, a correlation was observed when alginate bead diameter was less than 4 mm. In summary, in this research, use of cellular solids in preparing composite sponge systems with included beads was explored. Bead inclusion enabled control of the mechanical and porosity properties of the new cellular solid. The type of matrix and bead, bead quantity and size, and the interaction between the two components characterized the resultant physical properties. These types of composite systems show promise for use in slow-release applications, as immobilization and packaging materials, and in the production of composite dry foods with new textures (Nussinovitch and Ungar 1999).

6.7 Hydrocolloid Cellular Carriers for Agricultural Uses

6.7.1 General

Yields of vegetable, fruit, flower, and cotton crops are often destroyed by fungal diseases. Because modern chemical disinfectants used to control soil-borne pathogenic fungi have created serious ecological problems, public interest is turning to biocontrol agents. However, there is a wide gap between these agents' potential and their successful field application, due to soil heterogeneity, fluctuations in moisture, temperature, nutrient levels, soil texture and pH, the presence of other microbial species, competition with indigenous microorganisms, and other factors that influence microbial growth, survival, and effectiveness (Weller and Cook 1983; Chet 1987; Chet 1990; Whipps 1997).

6.7.2 Preservation of Biocontrol Agents in a Viable Form by Dry Cellular Bead Carriers

Current advances in biotechnology have led to the isolation of many microorganisms that can improve crop growth and protect plants in agriculture. Cell immobilization technology provides a number of advantages over inoculation with free cells. The appropriate formulation should provide beneficial characteristics for the inoculant, such as long shelf life and suitable survival at its destination, as well as sufficient cell density and performance (Trevors et al. 1992; Bashan 1998). Water-soluble polymeric materials (gums) such as agar, carrageenan, alginate, low-methoxy pectin, gellan, chitosan, and blends of xanthan and locust bean gum, among many others, are commonly used for such applications (Nussinovitch 1997). Each and every one of these materials has been used to manufacture natural-based carriers for the encapsulation of microbial and fungal cells in the food, biotechnology, and agriculture industries (Trevors et al. 1992; Whipps 1997).

By entrapping living cells, the carriers protect the microorganism from various stresses. For their conservation, such inoculated carriers should be dryable. Not as much information can be found on using dried (versus "wet") beads for the addition of encapsulated cells to soils or other functions. Dehydration is carried out by spray-, freeze-, and fluidized-bed drying. The major problem with these techniques, however, lies in the survival prospects of microorganisms during the dehydration process and storage, chiefly with Gram-negative bacteria which are non-spore formers (Fages 1992).

After matrix dehydration, water availability within the polymer-entrapped cell preparation decreases until the cells reach a dormant state during which metabolism slows, sometimes coming to a complete standstill. Final cell survival depends on many parameters, such as the organism being immobilized, the composition of the suspension medium, and the method and conditions of drying. For instance, freeze-drying of a Gram-positive lactic acid bacteria starter suspension reduces the population by 90% (Champagne et al. 1991). Better recovery is usually achieved

when the bacteria are immobilized and cryoprotective agents are added. Various groups of substances have been considered for their possible protective action, including glycerol and other polyols, polysaccharides, disaccharides, amino acids, proteins and their hydrolysates, minerals, salts of organic acids, and vitamin complex media (Kearney et al. 1990; Champagne et al. 2000). In nearly all cases, survival after freeze-drying did not exceed 86 and 3% for Gram-positive and Gram-negative bacteria, respectively (Paul et al. 1993; Kim et al. 1996; Bashan 1998). Furthermore, numerous compounds offer limited protection of microorganisms during the freeze-drying process but do not prevent viability losses during storage or during their application in complex and uncontrolled environments, such as soil (van Elsas and van Overbeek 1993; Bashan 1998). To eliminate these problems, hydrocolloid-based dry carriers with different compositions were tested. The matrix was intended to extend the Gram-negative bacterium's viability during drying and long periods of storage, in order to facilitate further practical applications. The potential Gram-negative biocontrol agent *Pantoae* (*Enterobacter*) *agglomerans* was used as a model microorganism since it produces the antibiotic pyrrolnitrin, secretes a number of chitinolytic enzymes, and has a wide spectrum of antifungal activity (Chernin et al. 1995, 1996). However, the gap between this bacterium's potential and successful field application was wide due to the aforementioned factors influencing microbial growth, survival, and effectiveness. These variables can affect the ecological competence of the inoculum, leading to decreased activity and survival of the added microorganism due to cell stress (Whipps 1997). The chitinolytic *P. agglomerans* strain was used to study the efficacy of freeze-dried alginate-based beads (macrocapsules) as possible carriers for immobilized Gram-negative bacterial cells. These macrocapsules were produced by freeze-dehydration of spherical alginate gel beads, in which different amounts of bacteria, glycerol, and colloidal chitin were entrapped (Fig. 6.6). Subsequent drying produced different unexpected

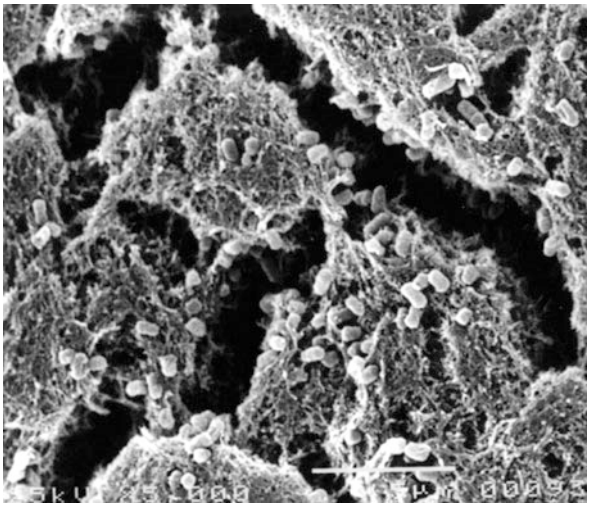

Fig. 6.6 SEM micrograph of a cross section of cellular solid showing microorganisms on and/or entrapped within the solid walls

structures, pore-size distributions, and changes in the outer and inner appearance of the resultant dried cellular solid (Zohar-Perez et al. 2002). With increasing glycerol content, the proportion of larger pores increased. These structures could be related to changes in the slow-release properties of the dried beads. The amount of glycerol in the beads differed from that in the alginate solution as a result of leakage during bead preparation and dehydration. Entrapping 10^9 cells per bead produced from alginate solution containing 30% glycerol and 1% chitin resulted in improved (in comparison to other studies) survival prospects (95%) during freeze-drying. Moreover, immobilization of the bacterium greatly improved its survival in non-sterile irrigated and dry soils compared to bacteria in a water suspension. The results suggested that optimized conservation of Gram-negative bacteria in dry glycerol-containing alginate-based cellular solids is not only possible, but also feasible for a variety of uses (Zohar-Perez et al. 2002).

6.7.3 Dry Carriers' Capacity to Protect Biocontrol Agents Against UV Light

As we have seen, recent progress and requirements in biotechnology have led to the isolation of many microorganisms for the improvement of crop growth and plant protection in agriculture. Nevertheless, success of a microorganism in vitro does not guarantee its success in field applications (Bashan 1998). Soil microorganisms in general, and biocontrol agents in particular, are very sensitive to UV light (Zelle and Hollaender 1954; Cokmus et al. 2000). Furthermore, the direct use of antagonistic antibiotics derived from the biocontrol agents is also limited by their UV sensitivity (Chernin et al. 1996; van Pee and Ligon 2000). Immobilization of microorganisms in dried alginate carriers was proposed as a technique to minimize the effect of this limiting factor (UV radiation) on the efficacy of the microorganism preparations. A controlled natural cellular structure, to serve as a carrier for biocontrol agents or other biological purposes, is obtained by freeze-drying alginate-based gel beads or gels produced from water-soluble polymers (Nussinovitch et al. 1993). Because the effects of UV radiation on the structural properties of cellular solids are of great interest, different types of natural and artificial cellular solids have been examined to determine the penetration depth and transmission properties of UV radiation (Alenius et al. 1995; MacLeod et al. 1995; Day and Demchik 1996; Ruhland and Day 1996; Bornman and Vogelmann 1998; Mitrofanov et al. 1998). For instance, UV radiation penetrates the surface of wood to a depth of approximately 75 mm and causes photodegradation of lignin in the wood's surface (MacLeod et al. 1995); for different leaves, a range of penetration depths of 40–400 mm were found, depending on the leaf type, age, and UV wavelength; in the spectral range of 115–350 nm, the transmission of regular microporous polyethylene terephthalate membranes (an artificial cellular solid) depends mainly on pore size and density (Mitrofanov et al. 1998). While the literature includes thousands of reports dealing with gel beads in general and alginate beads in particular (Nussinovitch 1997, 2003), those on the use of dried beads are less common. Freeze-drying a gel bead

results in a low-density dried cellular product that is composed of many fused open and closed cells. These create the cell wall which contributes to the bead's physical properties and immobilization ability (Tal et al. 1997, 1999). The packaging of biocontrol microorganisms into cellular solids has been developed as a means of reducing loss caused by exposure to environmental UV radiation. The bacterial and fungal biocontrol agents *P. agglomerans* and *Trichoderma harzianum* were immobilized in freeze-dried alginate beads containing fillers and subjected to 254 nm UVC (UV subtype C) radiation. Immobilization of cells in freeze-dried alginate–glycerol beads resulted in greater survival after UVC irradiation than that in a free cell suspension. Adding chitin, bentonite, or kaolin as fillers to the alginate–glycerol formulation significantly increased bacterial survival (Zohar-Perez et al. 2003), with immobilization in alginate–glycerol–kaolin beads resulting in the highest survival levels. The transmissive properties of the dried hydrocolloid cellular solid had a major influence on the amount of protection afforded by the cell carrier. Dried alginate matrix (control) transmitted an average 7.2% of the UV radiation. Filler incorporation into the matrix significantly reduced UV transmission: alginate with kaolin, bentonite, and chitin transmitted an average of 0.15, 0.38, and 3.4% of the radiation, respectively. In addition, filler inclusion had a considerable effect on the bead's average wall thickness, resulting in a 1.5- to 3-fold increase relative to beads based solely on alginate. These results suggested that the degree of protection of entrapped microorganisms against UVC radiation is determined by the UV transmission properties of the dried matrix and the cellular solid's structure. It was concluded that for maximum protection against UVC radiation-induced cell loss, biocontrol microorganisms should be immobilized in alginate–glycerol beads containing kaolin (Zohar-Perez et al. 2003).

6.7.4 Textural Features of Dried Hydrocolloid Beads

Throughout this chapter, we have described various biotechnological uses for dried hydrocolloid beads. These include, but are not limited to, water denitrification (Power and Schepers 1989; Iza 1991; Tatsuo et al. 1991; Tchobanoglous and Burton 1991), matrices for the immobilization of denitrifying isolates (Tal, van Rijn and Nussinovitch 1997), carriers of bacteria or spores for biological control of soilborne root diseases (Zohar-Perez et al. 2002), protectants of spores and bacteria against UV radiation (Zohar-Perez et al. 2003), and carriers of Gram-positive lactic acid bacterial starter cultures in dairy and food fermentation (Champagne et al. 1996, 2000). Freeze-dried alginate beads (i.e., dry carriers) can be reinforced by including starch in the formulation before the beads are manufactured and dried: the starch serves as a carbon source and filler (Tal et al. 1999). After drying, the hydrocolloid beads are stronger than their non-dried gel bead counterparts. In addition, their porosity can be controlled such that produced gases (from fermentation or chemical reaction) can be liberated from their matrix without interfering with its structure. Such beads are essential as carriers for food and biotechnological operations (Nussinovitch 2003). Dried agar gel beads have served as a research

model for drying (Kubota et al. 1977; Suzuki et al. 1989) and dried gel beads in general can also be used for the separation of enzymes (Han et al. 1995).

Scientific data on the shape and surface of dried hydrocolloid beads is limited. Freeze-dried alginate beads after immobilization of *Acinetobacter johnsonii* were described to have some grooves on their external surface (Muyima and Cloete 1995). These authors hypothesized that the "intensity" of those structures may vary according to the chosen dehydration process. An additional manuscript described lyophilized alginate beads as carriers for the slow release of bacteria that affect plant growth. The beads contained concentric layers of dried solidified matrix (Bashan 1986). Freeze-dried alginate beads were employed to entrap probiotic bacteria for survival purposes in frozen, fermented dairy desserts; based on SEM micrographs, the beads were described merely as "spherical" (Shah and Ravula 2000). Coating of alginate gel beads with chitosan at a constant concentration followed by vacuum-drying presented a spherical shape when the alginate concentration was ~3% (Shu and Zhu 2002). While numerous applications of dried hydrocolloid beads have been reviewed (Nussinovitch 1997, 2003), only very limited and generally incomplete information can be located on the regularity of their shapes and surface features. Furthermore, information on how different features are produced on freeze-dried hydrocolloid beads' outer surfaces as a result of technological procedures, and the influence of fillers and other ingredients on their external and internal shape and texture, have not been investigated in detail (Zohar-Perez et al. 2004). These parameters are very important since the surface of the bead is the first part to come into contact with its fluid, solid, or gaseous environment and together with its internal structure will influence, if not determine, the bead's suitability to a predetermined task.

Fillers were found to have an influence on the surface of hydrocolloid beads. Smaller-sized fillers (such as kaolin) gave a smoother surface; a rougher surface was achieved with a larger-diameter filler, such as bentonite. The presence of filler and/or glycerol in the bead increased the number of mid-area "craters" and reduced the number of smaller "craters" formed on the bead's dried surface. Moreover, the inclusion of glycerol had a large influence on the distribution of pores within the beads (Zohar-Perez et al. 2004). Filler inclusion in the dried product reduced its collapse and roundness distortion during the drying process. Thus, the dried bead's components and the method by which it is formed completely determine its weight, volume, shape, and surface features. Taking these parameters into consideration provides the researcher with a useful tool for creating tailor-made dried beads for a predetermined operation, thereby increasing the probability of success, as demonstrated by the improved antifungal activity of entrapped *P. agglomerans* (biocontrol agent) within alginate–glycerol–chitin beads (Zohar-Perez et al. 2004).

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Chapter 7
Liquid-Core Beads and Their Applications in Food, Biotechnology, and Other Fields

7.1 Introduction

Fluid-filled cores are found in many research fields, such as biotechnology, food, and medicine. However, literature and cinematography are also more than a little familiar with them. As children, we were all fascinated by Jules Verne's fantastic voyage to the center of the Earth. A more recent movie, "The Core," directed by Jon Amiel, deals with the issue of our planet's liquid core which has stopped spinning and the heroic crew that is trying to restore Earth's magnetic field by nuclear means. Astronomy is replete with examples of fluid-filled cores. Three years of radio-tracking data from the Mars Global Surveyor spacecraft concluded that Mars, the Red Planet, has a molten liquid-iron core. The core is about one-half the size of the planet, as is the case for Earth and Venus, and contains a significant fraction of a lighter element, similar to sulfur. Mars has not cooled to a completely solid iron core; rather, its interior is made up of either a completely liquid iron core or a liquid outer core with a solid inner core (NASA 2003). NASA's results were published in the March 7, 2003, online issue of the journal *Science*. In another example, analysis of seismic waves generated by the June 1996 earthquake in Indonesia provided proof that inside a liquid core, the very center of the Earth is solid. In 1995, a sea-based Russian rocket was used to carry out the first German microgravity experiment. The experiment, which was funded by the German space agency, simulated thermal convection with respect to the geophysical motion in the Earth's inner liquid core (NASA 2003). Thus, liquid cores are clearly not limited to microscopic phenomena. Another interesting example from the macroscopic world is a string-wrapped, liquid-core golf ball, constructed by mechano-chemical reaction using PVA for the polymer backbone. The ball is composed of a bag containing a dispersion of high-density particles in water. The bag is wrapped with rubber strings for further tactile improvement. Another option is to replace the liquid core with a highly elastic gel. Although this is an interesting idea, such balls are not frequently used due to the significant improvements in the construction and performance of two-piece solid balls and a consequent lack of commercial interest (Tomita 2001). These are but a few examples of the ubiquitous distribution of fluid-core objects and their importance.

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7.2 General

Encapsulation, on a micro- or macroscale, is a specialized form of edible packaging. In food processes, the approach is to encapsulate only those ingredients which are unstable, volatile, or particularly reactive. Thus, creating an envelope around those ingredients provides the whole product with stability and protection (Daniels 1973). In 1971, Maddox's patent on gelatin capsules was approved. In 1980, Lim and Sun published their seminal manuscript in which microencapsulated islets were used as a bioartificial pancreas (Lim 1984, Lim and Sun 1980). In that manuscript, alginate–polylysine liquid-core capsules were produced and described. In the food area, Sneath's patent (1975) and later our group, among others, contributed to the manufacture and study of liquid-core capsules. This chapter will include a description of such capsules and the procedures used to produce the different types. Methods to include oil within the liquid-core capsules are also described, in parallel to screening their biotechnological and special food applications.

7.3 Soft Gelatin Capsules

Fluids, with or without additional powders, can be conveniently included within a gelatin capsule (Maddox 1971). The capsule is kept in a dry form in a suitable enclosed space until use. Soft gelatin capsules (Fig. 7.1) are frequently used in pharmaceuticals, cosmetics, and food supplements (Moorhouse and Grundon 1994). Gelatin, in many cases a basic capsule shell component, is well suited to encapsulating a wide variety of substances. Gelatin's unique properties are of particular interest in foods since the material acts as a barrier and protects the capsule's liquid contents from the outside environment (Nussinovitch 2003). On the one hand, gelatin operates as a physical barrier to microorganisms and on the other; it offers a low-permeability membrane to gases. The gelatin shell is naturally transparent (but can be artificially colored), it can be manufactured in an extensive range of shapes and sizes (size and shape can be determined by the die-roll), and it melts rapidly in hot water, releasing its encapsulated liquid (Moorhouse and Grundon 1994). The process of encapsulation involves bringing together two continuous strips of

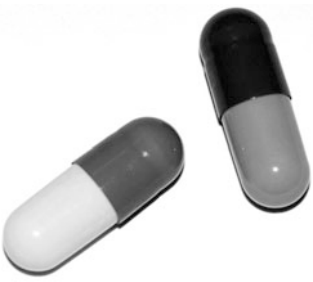

Fig. 7.1
A gelatin capsule for medicine (http://en.wikipedia.org/wiki/File:Kapsel_beredningsform.jpg; courtesy of Ola Rönnerup)

molten gelatin and injecting the fill formulation between them. Machines have been invented to cost-effectively produce seamless gelatin capsules, to achieve excellent shell clarity and to deliver fills to an exacting standard of accuracy. A wide range of materials can be encapsulated within these capsules, such as fish oils, multivitamin suspensions, halibut liver oil, mouthwashes, evening primrose oil, wheat germ oil, flavor oils, oil-soluble vitamins, unsaturated fatty acids, insecticides, perfume oils, inhalation and garlic oils (ITS Machinery Development Ltd 1996). The advantages of encapsulation include portion control, easy use and storage, extended shelf life, improved aesthetic appeal, the variety of available sizes, disposability and edibility, improved product aromatics versus time, and biodegradability (Nussinovitch 2003). A few food applications are "real chicken broth" capsules which retain and deliver flavor more effectively than the powder system, encapsulated lemon oil for meringue pie mix, mint essence capsules for the tinned goods market (Moorhouse and Grundon 1994). Future possibilities could be inclusion of the capsules as part of dry mixes, instant foods, and microwaveable products (see Section 7.7).

7.4 Liquid-Core Capsules

7.4.1 Liquid-Core Hydrocolloid Capsules

Capsules that contain fluids within a spherical polymer membrane are termed liquid-core hydrocolloid capsules (Vergnaud 1992). As already mentioned, a process for manufacturing liquid-core microcapsules to encapsulate pancreatic islets has been described (Lim and Sun 1980; Opara 1999). Production involved suspending cells in a sodium alginate solution, forming minute spherical calcium alginate beads by crosslinking with calcium salt and reacting with polylysine (Fig. 7.2) to create a polylysine–alginate membrane around the bead. The bead's core is composed of calcium alginate gel and it is then solubilized to form a fluid-core capsule that contains cells (Lim and Sun 1980). One of the disadvantages of this procedure is that the cells sometimes end up in the membrane matrix. As a result of this, a later approach suggested entrapping cells in alginate gel microspheres, which were then enclosed within larger beads, resulting in a greater distance between the cells and the surface of the larger alginate bead (Wong and Chang 1991). The exterior (surface) of the larger microsphere was reacted with poly-L-lysine and then alginate to form an outer layer membrane. The contents of the microcapsule were then liquefied with sodium citrate (a sequestering agent) to eliminate the calcium from the array. The cells in the smaller entrapped gel microsphere were released and as a result floated freely

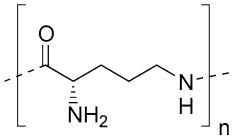

Fig. 7.2 Structure of polylysine (courtesy of Edgar 181, http://en.wikipedia.org/wiki/File:Polylysine.png)

in the fluid core of the resultant beads (Wong and Chang 1991). In these scenarios, the manufacture of the liquid-core hydrocolloid capsules consists of several steps. A one-step method to form and alter the mechanical and slow-release properties of fluid-core hydrocolloid capsules (Fig. 7.3) was described (Nussinovitch 1992, 1994; Nussinovitch et al. 1996). Proteins with molecular masses of 2,500–205,000 Da were included within liquid-core alginate, alginate–chitosan, or alginate–polylysine capsules, and their diffusion into the external fluid in which the capsules were immersed was compared to diffusion from "whole" alginate beads containing the same proteins. Better slow-release properties were achieved when polylysine or chitosan were used to change the permeability of the alginate membrane. These chemical treatments also strengthened the membrane and affected its brittleness (Nussinovitch et al. 1996). Diffusion coefficients for quite a few low molecular weight solutes, such as glucose, fructose, sucrose, lactose, and vitamin B_{12}, have been determined in calcium alginate membrane liquid-core capsules using

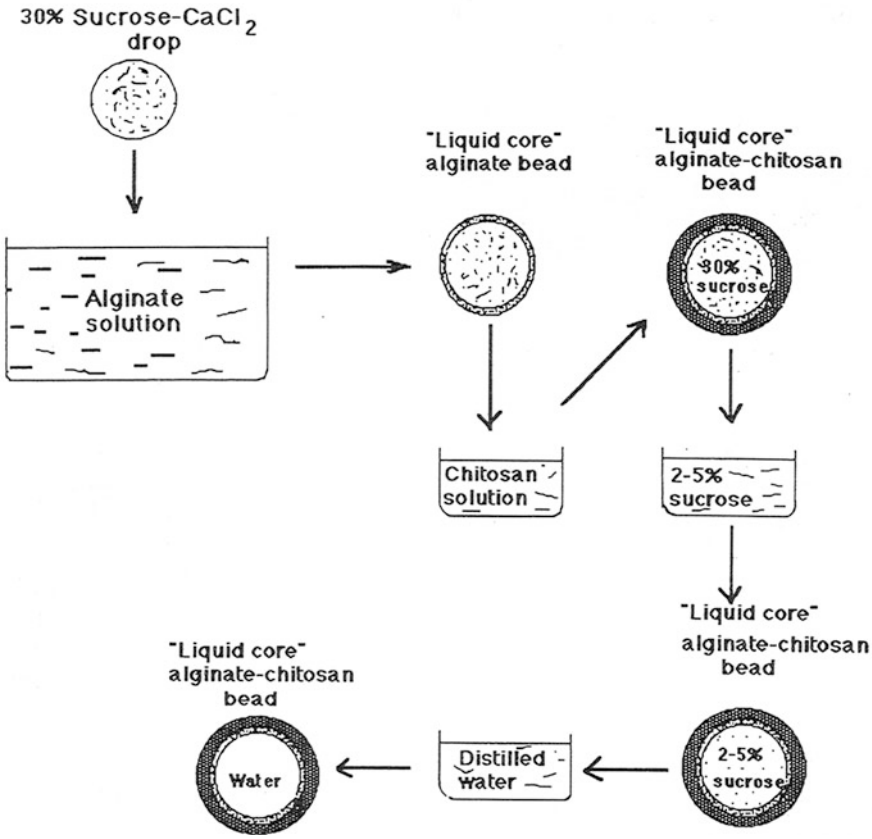

Fig. 7.3 A method for producing various liquid-core capsules (from Nussinovitch, Gershon, and Nussinovitch 1996)

the unsteady-state method following the release of solutes from the capsules to a well-stirred solution of limited volume (Dembczynski and Jankowski 2000a, b). The diffusion coefficients obtained for saccharides were 5–20% lower than the corresponding diffusivity in water while for vitamin B_{12}, it was about 50% that of water. The diffusion coefficients of the investigated capsules were not influenced by a change in alginate concentration in the capsule membrane from 0.5 to 1.0%. Lower diffusivities and higher deviations from the diffusivity in water were obtained for higher molecular weight solutes (Dembczynski and Jankowski 2000b). These same researchers determined the pore diameter and molecular weight cut-off of a hydrogel calcium alginate membrane and hydroxy propylammonium starch liquid-core capsules (Dembczynski and Jankowski 2000b). The idea was that for transplantation purposes, enclosing cells in a semi-permeable biocompatible membrane (e.g., a liquid-core capsule) would protect the cells from attack by the host immune system. To meet the requirements of immunoisolation, the capsule wall had to prevent cellular immune rejection as well as exclude antibodies to the encapsulated cells. Since immune cells are substantially larger than antibodies, the exclusion limit of the membrane was predefined by the size of the immunoglobulin molecules, particularly the IgG antibodies. An exclusion limit (i.e., the largest pore size of the capsules) was determined by means of inverse size-exclusion chromatography (ISEC) with dextran molecular weight standards. In regular ISEC measurements, the examined material constitutes the stationary phase in the chromatographic column and the elution volumes of a series of standard solutes with different molecular sizes are measured (Jerabek et al. 1993). By suitable selection of a mobile phase and standard solute type, specific interactions between the studied material and solutes are eliminated such that the elution volumes only depend on the porous structure of the column filling. A mathematical treatment of elution data can provide detailed information on both the macropores and the microporous structure (Jerabek et al. 1993). The fundamental principle underlying the evaluation of porosimetric information from chromatographic data is the assumption that the authentic porous configuration of an examined specimen can be modeled as a collection of discrete pore fractions, each containing pores of different but uniform size and of simple geometrical shape. The problem, then, is to determine the combination of fraction volumes that will yield the best agreement between computed and experimental values of the elution volumes of standard solutes (Jerabek et al. 1993). It was observed that the exclusion limits of the capsules were influenced by a change in alginate concentration in the membrane from 0.5 to 1.0%, causing the membranes to be less permeable. The diameter of the largest pores in the hydrogel membranes was found to be in the range of 7.2–8.0 nm. Based on the relationship between solute size and molecular weight, the capsules had an approximate exclusion limit of 21–25 kDa for dextran and 78–103 kDa for protein, which is sufficient to block the antibodies penetrating the membrane (Dembczynski and Jankowski 2000b).

Another single-step operation to produce liquid-core spherical alginate–chitosan capsules containing a range of hydrocolloid concentrations in their membrane has been described (Fig. 7.3). This simplified procedure is preferable to other multistage methods of fluid-core capsule production. The contents of the capsule were either

distilled water or sucrose solutions (2.5 and 30%, w/w), although other viscous fluids can be used. Beads with 0, 2, and 5% sucrose were produced by diffusion of sucrose out of liquid-core capsules containing 30% sucrose. The spherical shape (see Chapter 1) of the capsule was maintained after diffusion. Mechanical properties of various capsules were studied after incubation at 25, 37, 45, 55, and 85°C for 5, 30, and 60 min, respectively, while those of the liquid-core water capsules were studied for a further 2 weeks at 25°C (Nussinovitch et al. 1997). Capsules with a higher hydrocolloid concentration within their membrane displayed more stress at failure (strength) and less brittleness than those with lower solid membrane contents. The stress σ, or intensity of the distribution of internal forces, can be obtained by dividing the total tensile or compressive force F by the cross-sectional area A upon which it is acting. Following diffusion, capsules with 2 and 5% sucrose were weak compared to those with 30% sucrose; however, no membrane rupture was observed after incubation. The weakest capsules were those containing water incubated for long times at higher temperatures (Nussinovitch et al. 1997). The temperature-stability relationship of the liquid-core capsules was presented. Projection of three-dimensional curves of mechanical property versus time, temperature, or percent sugar offered a convenient way of examining the desired mechanical properties and their dependence on liquid-core composition and incubation conditions (Nussinovitch et al. 1997).

7.4.2 Synthetic and Additional Liquid-Core Capsules

Due to the many possible applications of liquid-core capsules, it is not surprising that advances for their manufacture from synthetic polymers have been made. Synthetic polymers are industrially produced chemical substances consisting of a number of molecules linked together with covalent bonds. Examples include plastics, synthetic fibers such as nylon, and synthetic rubber. A capsule with a solid shell and a solid (if a molten mass is used for the inner core) or liquid core can be of use to the food, textile, and the building industries. Possible encapsulated materials include aromas, antiallergenic substances, or phase-change materials. The Brace™ double-nozzle processes produce real microcapsules with a solid shell and a liquid or solid core in a single step. The inner core material is chosen from a list of materials that do not weaken the stability of the shell. The shell material, on the other hand, can be a solution, a molten mass or sol, or a combination of these (http://www.brace.de/PagEd-index-topic_id-4-page_id-52.phtml). The encapsulated substances can be released at once (e.g., if pressure is applied to the capsule or the shell material is dissolved) or slowly, the latter required for long-term effects of antiallergenic substances, for instance. If hydrocolloids are used for the production of microcapsules, careful drying can be helpful, especially for the drying of microspheres with an oily core which can start to leak when over-dried. In these driers, the particles are kept in motion by conditioned air flow which minimizes the risk of particle deformation at the drier walls. It is even possible to

dry microcapsules with volatile or sensitive cores to a specified residual moisture (http://www.brace.de/PagEd-index-topic_id-4-page_id-52.phtml).

A method has been described for the formation of microcapsules which contain a liquid composition in the core, surrounded by a polymeric shell, membrane, or coating. The microcapsules were produced by simultaneously extruding the liquid-core material along with a polymerizable liquid through concentrically aligned nozzles to form spherically layered bi-liquid droplets, followed by energy input in the form of heat or light to polymerize the outer layer. The capsules formed by this method are capable of containing a variety of liquid materials having a composition ranging from completely aqueous to completely non-aqueous (Toreki et al. 2002).

Fully synthetic polymers have been used for the preparation of hydrogel beads and capsules (Cellesi et al. 2004). The polymers bear terminal reactive groups that tolerate mild, but efficient chemical crosslinking. The thermal gelation allows for hardening kinetics resembling that of alginate. This process has been optimized for the manufacture of liquid-core microcapsules and monodisperse, highly elastic hydrogel microbeads (Cellesi et al. 2004). The benefits of this practice lie principally in the use of synthetic polymers, which provide immense flexibility in the molecular design. This allows for the precise tailoring of mechanical and transport properties and of bioactivity of the hydrogels, as well as for precise control in material purification (Cellesi et al. 2004). The use of synthetic polymers is illustrated by particles with liquid cores and solid shells that have been prepared by the controlled phase separation of poly(methylmethacrylate) (PMMA) within the droplets of an oil-in-water emulsion (Fig. 7.4). Poly(methyl 2-methylpropenoate) is a thermoplastic and transparent plastic. Chemically, it is the synthetic polymer of methyl methacrylate. The oil phase of the emulsion contained PMMA, a good solvent for the polymer, a poor solvent (hexadecane, decane, octanol, or tetrachloromethane), and in some cases acetone (a water soluble co-solvent) to aid in emulsification (Loxley and Vincent 1998). Core/shell microcapsules were formed when hexadecane or decane was used as the non-solvent, and only when polymeric emulsifiers were employed. All other combinations yielded "acorn"-shaped particles. The thickness of the microcapsule walls was found to be a constant fraction of the overall capsule diameter for all microcapsule sizes and depended, as expected, on the concentration of polymer in the oil phase (Loxley and Vincent 1998). For many applications, particularly in medicine, smaller capsules (between 50 and 300 nm) are of high interest. An important approach for the synthesis

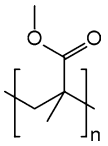

Fig. 7.4 Skeletal formula of the poly(methylmethacrylate) repeating unit $(C_5O_2H_8)_n$ (courtesy of Dr Torsten Henning, http://en.wikipedia.org/wiki/File:PMMA_repeating_unit.svg)

of these nanocapsules is based on the principle of mini-emulsion by interfacial polycondensation. Early on, this methodology was not used for the synthesis of nanocapsules, even though polyester and polyurethane or epoxy nanoparticles had already been prepared by mini-emulsion polyaddition (Tiarks et al. 2001). Later, the formation of polyurethane nanocapsules, composed of an organic liquid core and a polymer shell with mean diameter of 200 nm, was described (Torini et al. 2005). The influence of various parameters, such as the choice of surfactant type (anionic, cationic, or nonionic) and its concentration, and the ratio of surfactant to co-stabilizer were examined (Torini et al. 2005). Another example is the synthesis of hollow polyurea (a type of elastomer derived from the reaction product of an isocyanate component and a synthetic resin blend component through step-growth polymerization), polythiourea, and polyurethane nanocapsules with a hydrophilic liquid core by interfacial polycondensation or crosslinking reactions in an inverse mini-emulsion. The mini-emulsions were built upon emulsification of a solution of amines or alcohols in a polar solvent with cyclohexane as the nonpolar continuous phase. The addition of appropriate hydrophobic diisocyanate or diisothiocyanate monomers to the continuous phase allowed the polycondensation or crosslinking reactions to take place at the interface of the droplets. These syntheses allowed the encapsulation of hydrophilic compounds in the mini-emulsion in a hollow structure (Crespy et al. 2007). The wall thickness of the capsules can be directly controlled by the quantity of the reactants. The nature of the monomers and the continuous phase are the critical factors for formation of the hollow capsules, due to the interfacial properties of the system (Crespy et al. 2007). The resulting polymer nanocapsules can then be dispersed in water. The capsules were found to be spherical when formamide was employed as the liquid core, while elongated capsules were obtained with water. These hollow nanoreactors can be used as a model system for the preparation of silver nanoparticles by reducing silver nitrate solutions encapsulated by the polyurea shell (Crespy et al. 2007).

A suitable approach to preparing liquid-core nanocapsules by crosslinking an amphiphilic copolymer at an oil–water interface has been demonstrated (Ren et al. 2007). The hydrophilic copolymer poly[(ethylene oxide)-*co*-glycidol] was prepared by anionic polymerization of ethylene oxide and ethoxyethyl glycidyl ether, followed by recovery of the hydroxyl groups on the backbone after hydrolysis and in part modified by hydrophobic conjugated linoleic acid (Ren et al. 2007). The copolymer with multiple linoleate pendants was absorbed at an oil–water interface and then crosslinked to form stable nanocapsules. The mean diameter of the nanocapsule was less than 350 nm and the size distribution was relatively narrow (<0.2) at low concentrations of oil in acetone (<10 mg/ml). The particle size could be easily controlled by varying the emulsification conditions. The nanocapsule was stable in water for at least 5 months, and the shell maintained its integrity after solvent removal of the oily core. Pyrene was encapsulated in these nanocapsules and a loading efficiency as high as 94% was measured by UV spectroscopy (Ren et al. 2007).

Microcapsules containing a solvent and reactive epoxy resin are a critical component in the development of cost-effective, low-toxicity, and low-flammability

self-healing materials. The encapsulation method provides protection for a range of oil-soluble solvents and reactive epoxy resins surrounded by a thin, polymeric, urea–formaldehyde shell (Blaiszik et al. 2009). Resin–solvent capsules are produced at high yield with diameters ranging from 10 to 300 μm by controlling agitation rates. These capsules have a continuous inner shell wall and a rough exterior wall that promotes bonding to a polymer matrix. Capsules as small as 300 nm in diameter are achieved through sonication and stabilization procedures (Blaiszik et al. 2009). The presence of both the epoxy resin and the solvent core components is confirmed by differential scanning calorimetry measurements, and the relative amount of epoxy and solvent in the liquid core is determined by thermogravimetric analysis. The capsules are shown to satisfy the requirements for use in self-healing materials including processing survivability, thermal stability, and efficient in situ rupture for delivery of the healing agent (Blaiszik et al. 2009).

7.5 Oil-Core Hydrocolloid Capsules

Oil-core hydrocolloid capsules, 4–5 mm in diameter with a 70–90-μm thick membrane, have been manufactured by a one-step technique (Nussinovitch and Solomon 1998). Three basic types of beads were formed. They contained 40–90% glycerol, 0–50% soybean oil, and 10% of either $CaCl_2$ or $BaCl_2$ (Fig. 7.5). Subsequent to capsule formation, membranes were first reinforced with calcium or barium cations and then immersed in 2% sodium alginate, and this alginate layer was reinforced with either $CaCl_2$ or $BaCl_2$. The higher the inclusion of the oil within the fluid component of the capsule, the lower its liquid medium's density.

Although the *aspect ratio* (i.e., the ratio of a shape's longer dimension to its shorter dimension—this ratio may be applied to two characteristic dimensions of a three-dimensional shape, such as the ratio of the longest to shortest axes, or for symmetrical objects that are described by just two measurements, such as the length and diameter of a rod) of the oil-core hydrocolloid capsules varied from 0.91 to 0.97, most capsules had a value of ~0.95, reflecting their high degree of sphericity (see Chapter 1). The smallest liquid-core volume was recorded for the capsules with the least included oil and for those which underwent further crosslinking and contained 25% included oil (Nussinovitch and Solomon 1998). The maximum projected area (i.e., a two-dimensional area measurement of a three-dimensional object made by projecting its shape onto an arbitrary plane) was smallest with the lowest oil content and barium crosslinking and was a function of time: the longer the time, the smaller the capsule. As a general rule, the higher the oil content within the capsule, the weaker it was. Membrane strengthened with Ba^{2+} was always stronger than that strengthened with Ca^{2+}. With Ba^{2+} capsules, respective stresses at failure were 185, 97, and 64 kPa for 0, 25, and 50% included oil. With Ca^{2+} oil-core beads, strengths were 136, 66, and 40 kPa for 0, 25, and 50% included oil, respectively (Fig. 7.6). None of the capsules were brittle, their engineering strain at failure deviating between 0.79 and 0.78. The Cauchy strain or engineering strain is expressed

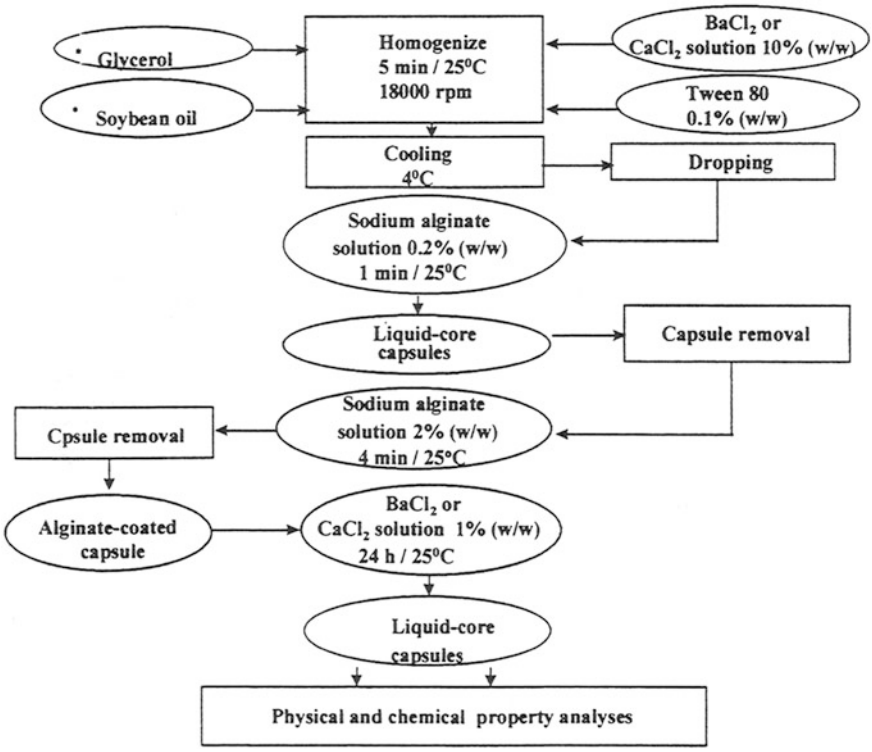

Fig. 7.5 Production of liquid-core hydrocolloid oil capsules (from Nussinovitch and Solomon 1998)

as the ratio of total deformation to the initial dimension of the material body in which the forces are being applied. The engineering normal strain or engineering extensional strain ε of a material line element or axially loaded fiber is expressed as the change in length, ΔL, per unit of the original length L of the line element or fiber. The deformability modulus, calculated at a strain value of about 0.12 (when the highest R^2 of σ versus ϵ was achieved), followed the same trend as strength. In other words, capsules were stiffer when membranes were strengthened with barium versus calcium. Moreover, the higher the oil content within the capsule, the less stiff the membrane and capsule were (Nussinovitch and Solomon 1998). Since the oil is entrapped within the capsule, it is assumed that capsules with even higher oil content can be achieved by changing the crosslinking solution or using other liquids that could further shrink the capsule, such as acetone. Oil-core hydrocolloid capsules can be used for food and non-food purposes, when oil-soluble ingredients are included (Nussinovitch and Solomon 1998). In other studies on microencapsulation by coagulation, soybean oil was used as the core material and gum arabic and gelatin as the shell materials. Conditions for encapsulation were optimized at 1% gum arabic

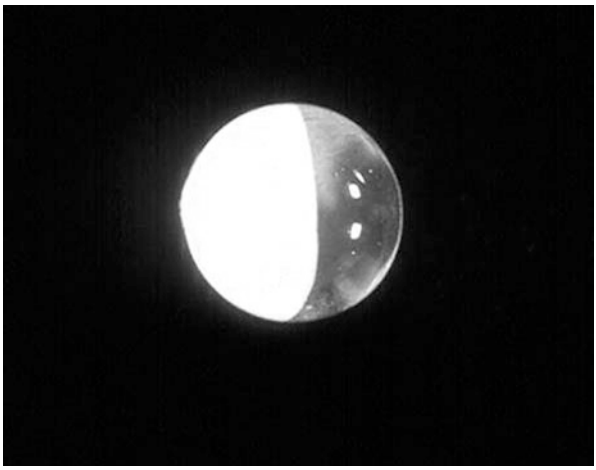

Fig. 7.6 Liquid-core hydrocolloid oil capsule consisting of glycerol and 50% soybean oil before capsule shrinkage. The "white phase" is the oil. This capsule has a 20.7-mm^2 maximum projection area, an average volume of 71.6 mm^3, an equivalent diameter of 5.1 mm, and an aspect ratio of 0.96 reflecting its high degree of sphericity (from Nussinovitch and Solomon 1998)

and gelatin, pH 4.4, 40°C, and a mixing time of 15 min. Under these conditions, inclusion rate was greater than 50%. Microwave dewatering was achieved in 5–6 min in a 500 W microwave oven for a 2-cm thick layer of microcapsules (Wang 1997).

Liquid-core capsules that include up to 50% oil have been described (Nussinovitch and Solomon 1998). Capsules composed of a hydrophobic liquid core surrounded by a crosslinked polyacrylamide/alginate membrane have been also developed (Wyss et al. 2004). Monodisperse spherical liquid-core capsules of between 800 μm and 1.6 mm in diameter, with high mechanical resistance, were prepared by co-extrusion, using the jet breakup technique. Capsules produced from a solution of *N*,*N*-methylenebisacrylamide (MBA)/total monomer (5%) were found to be more elastic and have a higher burst force when exposed to chelating agents such as phosphate or citrate. The mechanical resistance was unaffected by buffer solutions in the pH range of 4–9 or by sterilization at 121°C for 20 min. Capsules having membranes composed of a copolymer of acrylamide and *N*-hydroxymethylacrylamide exhibited even higher mechanical stability toward chelating agents (Wyss et al. 2004). These liquid-core capsules can potentially be used in capsular perstraction for the removal of inhibitory products from bioprocesses and bioconversions. They have the benefit of having a high surface area to promote rapid mass transfer, while separation of the organic core phase from the aqueous environment by the capsule membrane prevents the formation of stable emulsions and potential problems associated with toxicity of the organic phase to microbial cells or enzymes (Wyss et al. 2004).

7.6 Biotechnological Applications of Liquid-Core Capsules

7.6.1 Growth of Microorganisms in Liquid-Core Capsules

Immobilization and entrapment of microorganisms are discussed in detail in Chapter 4. In short, immobilization of cells relates to the prevention of free cell movement, independent of its neighbors, to any fraction of the aqueous phase of the system under study (Bucke 1983). At one extreme, the immobilized cells can be fully capable of division; at the other, they may possess only one sort of enzymatic activity (Mattiasson 1983). Cells are frequently entrapped within a gel matrix. A wide range of characteristics are attributed to gels as an entrapment medium. On the one hand, they consist of macromolecules held together by relatively weak intermolecular forces, such as hydrogen bonding or ionic cross-bonding by divalent or multivalent cations. On the other hand, strong covalent bonding, where the lattice in which the cells are entrapped is regarded as one vast macromolecule, is limited only by the particle size in the immobilized cell preparation (Tampion and Tampion 1987; Nussinovitch et al. 1994, Nussinovitch 1997). Entrapment within gel beads is more common than entrapment within liquid-core capsules. Regardless of the approach used, an awareness of optimization and proper scaling up of a process is of great interest to the manufacturer. For example, a cell-encapsulation technology in alginate has been studied with the aim of confirming the predictability of large-scale production by Good Manufacturing Practice (GMP; Villani et al. 2008). It was observed that it is possible to optimize the performance of the capsules, relating the molecular structure and size of the polymeric membrane to the preferred functional properties (Villani et al. 2008). Furthermore, technological resources are available for large-scale cell encapsulation intended for advanced therapies (gene therapy, somatic cell therapy, and tissue engineering) in a cell factory, following GMP guidelines (Villani et al. 2008).

A few manuscripts have dealt with a comparison of entrapping/growing microorganisms within liquid-core capsules versus gel beads or free-flowing within a fluid. Immobilization of yeasts such as *Saccharomyces cerevisiae* for continuous fermentation or other aims is highly significant. The name *Saccharomyces* derives from the Greek and means "sugar mold" or "sugar fungi": *saccharo*—"of sugar" and *myco*—"of fungi"; *cerevisiae* comes from the Latin and means "of beer." *S. cerevisiae* are round to ovoid cells, reproduced in a division process known as budding, and they are perhaps the most useful yeast, owing to their use since ancient times. This yeast is one of the most intensively studied eukaryotic model organisms in molecular and cell biology. It is the microorganism behind the most common type of fermentation (http://en.wikipedia.org/wiki/Saccharomyces_cerevisiae). In one study, the effects of microcapsule core state (solid or liquid), initial cell density (1.5×10^7, 3×10^6, and 3×10^5 cells/ml of microcapsules), microcapsule diameter (200, 500, 600, and 700 μm), and membrane-formation times (0, 5, 15, and 30 min) on cell growth, including proliferating capacity, metabolic activity, and product secretion of *S. cerevisiae*, cultured in alginate–chitosan–alginate microcapsules, were investigated. No

significant difference was found in the cell growth of microencapsulated cells in solid versus liquid cores (Qi et al. 2005). *S. cerevisiae* cells were also encapsulated in a liquid polyethylene glycol (as a viscosity former) core in an alginate-membrane capsule and cultured (Koyama and Seki 2004). After 24 h of cultivation, the cell concentration in the capsule's liquid core reached 222 $\mu g/mm^3$ on a dry weight basis. This was 1.4 times higher than that in the core of double-layered alginate beads, i.e., alginate-coated alginate gel beads. The diameter increase of the capsule prepared by the proposed method using immobilized cell growth was suppressed compared to those using the double-layer method and simple alginate gel bead entrapment, most likely because of the mobility of the entrapped cells in the capsule (Koyama and Seki 2004). As a means of integrating cell growth and immobilization, recombinant *S. cerevisiae* cells with invertase activity were immobilized in liquid-core alginate capsules and cultured to a high density. *S. cerevisiae* cells of SEY 2102 (MAT alpha ura3-52 leu2-3, 112 his4-519) harboring plasmid pRB58 with the SUC2 gene coding for invertase were grown to 83 g/l of liquid-core volume inside the capsule on a dry weight basis. The cloned invertase was properly expressed in the immobilized cells, exhibiting slightly higher activity than the free cells in batch culture. Invertase in the immobilized cells showed slightly better thermal stability than in the free cells. Storage in a sodium acetate buffer at 4 and 10°C for 1 month resulted in 7 and 8% loss in activity, respectively. The sucrose hydrolysis reaction was stably maintained for 25 repeated batches over 7 days at 30°C. Continuous hydrolysis of 0.3 M sucrose was carried out in a packed bed reactor with conversion of more than 90% at a maximum productivity of 55.5 g glucose/l/h for 7 days. In a continuous stirred tank reactor, maximum productivity of 80.8 g glucose/l/h was achieved at a conversion of 59.1% using 1.0 M sucrose solution, and 0.5 M sucrose solution was hydrolyzed for 1 week with 95% conversion at a productivity of 48.8 g/l/h (Chang et al. 1996). Liquid-core calcium alginate capsules have also been used to immobilize recombinant yeast cells with a plasmid containing the SUC2 gene encoding invertase. Invertase activity in immobilized cells was to some extent higher than that in free cells growing in a batch system. Temperature for optimal enzyme activity was 65°C, in both entrapped and free cultures. However, the immobilized preparation improved thermal activity. Immobilized cells retained more than 90% activity for 7 days at 30°C. Both a stirred tank reactor and a packed bed reactor are options for continuous sucrose hydrolysis (Chang et al. 1996).

Probiotics are dietary supplements of live bacteria or yeast that are considered to provide health benefits to the host organism (Tannock 2005; Ljungh and Wadström 2009). According to the currently adopted definition by FAO/WHO (2001), probiotics are "Live microorganisms which when administered in adequate *amounts confer a health benefit on the host.*" Strains of the genera *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria. Claims have been made that probiotics strengthen the immune system to combat allergies, excessive alcohol intake, stress, exposure to toxic substances, and other diseases (Sanders 2000). Some strains of *Lactobacillus acidophilus* may be considered to have probiotic characteristics (Ljungh and Wadström 2006). These strains are used commercially in many dairy products, such as sweet acidophilus milk and yogurt. *L. acidophilus*

ferments sugars into lactic acid and grows readily below pH 5.0. It has an optimum growth temperature at 30°C. *L. acidophilus* occurs naturally in the human and animal gastrointestinal tract, mouth, and vagina (Forsum et al. 2005). It may provide additional health benefits, including improved gastrointestinal function, a boosted immune system, and a decrease in the frequency of vaginal yeast infections (Anderson and Gilliland 1999; de Roos and Katan 2000). *L. acidophilus* cells were encapsulated in alginate/starch liquid-core capsules and cultured in a whey-based growth medium to enable concentrated biomass production in immobilized form. The population of encapsulated viable cells reached a concentration of 6.5×10^{10} CFU/ml capsule after 30 h of fermentation, while the population of free cells cultured under the same conditions during control fermentation was 7.2×10^{9} CFU/ml growth medium after 45 h. The capsules remained stable and no cell release was observed during the course of cell growth (Dembczynski and Jankowski 2000a). Biocompatible capsules consisting of a liquid starch core with calcium alginate membranes have been developed and their formulation conditions chosen on the basis of membrane strength and diffusivity measurements. Acidification activity of the encapsulated *L. acidophilus* cells was similar to that achieved in free cell fermentation and increased with subsequent reuse (Jankowski et al. 1997).

Lactobacillus rhamnosus is a probiotic bacterium that inhibits the growth of most harmful bacteria in the intestine. It is used as a natural preservative in yogurt and in other dairy products for shelf-life extension. *L. rhamnosus* cells were encapsulated in alginate/starch liquid-core capsules and cultured in a whey-based growth medium supplemented with yeast extract, tryptone, and calcium carbonate to enable concentrated biomass production in an immobilized matrix without centrifugation or filtration. The characteristics of the encapsulated cell culture were compared with those of freely suspended cell fermentation under the same bioreactor conditions. The population of encapsulated viable cells reached a concentration of 4.8×10^{10} CFU/ml of capsule volume, while the population of free cells cultured under the same conditions during control fermentation was 5.7×10^{9} CFU/ml of growth medium (Dembczynski and Jankowski 2002). Despite the higher cell concentration in the capsules, the overall cell productivity of the encapsulated culture was lower than that obtained in free cell fermentation. The effects of lactic acid inhibition on cell growth in the capsules caused by mass-transfer limitations were evidenced by premature growth cessation and earlier uncoupling between growth and acid production as compared to the free cell culture. The capsules remained stable and no cell release was observed during 60 h of fermentation (Dembczynski and Jankowski 2002). *Lactobacillus casei* is found in the human intestine and mouth. *L. casei* is considered to be a probiotic and may be effective in alleviating gastrointestinal diseases due to pathogenic bacteria. Entrapment of *L. casei* cells within alginate liquid- and gel-core capsules was compared. Fluid-core capsules were found to provide more space for cellular growth than gel-core beads. This resulted in a 1.5-fold higher cell concentration in the former; however the calcium alginate structure was unstable throughout repeated batch fermentations for lactic acid manufacture (Yoo et al. 1996). Barium alginate capsules were chemically and physically more stable than calcium alginate capsules in both phosphate and lactate solutions.

Attempts were also made to use a range of hardening agents to stabilize the structure of the barium alginate capsules. Treatment with a mixture of chitosan and $BaCl_2$ solution gave the best results for hardening. In conclusion, stable lactic acid manufacture was achievable with a yield of more than 2.7 g/l per h by *L. casei* cells immobilized in chitosan-coated barium alginate capsules. Cell leakage from the capsules was maintained at a moderately low level during batch fermentations (Yoo et al. 1996).

The mechanical stability of liquid-core alginate–poly-L-lysine capsules used for the encapsulation of hybridoma cells can be largely enhanced by the inclusion of poly(ethylenimine) in a hardening solution containing $CaCl_2$. The poly(ethylenimine) can also strengthen alginate–poly-L-lysine-coated carboxymethylcellulose liquid-core capsules (Hsu and Chu 1992). Murein hybridoma CT04 cells were cultured in these two capsules. Cell concentrations higher than 10^8/ml capsule were obtained, with ~80% of the specific antibody productivity obtained from freely suspended cells. These capsules could withstand severe agitation and aeration in an air-lift reactor over a period of 3 weeks with negligible damage (Hsu and Chu 1992).

7.6.2 Activity of Enzymes Within Liquid-Core Capsules

The activity of penicillin acylase has been studied in aqueous and organic solvents, as a free enzyme and immobilized within the membrane of liquid-core capsules (Wyss et al. 2005). The activity of the enzyme is inhibited by the accumulated product of the hydrolysis reaction, namely phenyl acetic acid. In order to overcome this inhibition, a range of organic solvents was tested for use in in situ product recovery (Wyss et al. 2005). Immobilization of penicillin acylase onto the capsule membranes resulted in increased operational stability of the enzyme and very high enzyme activity. Over 53.3% of the phenyl acetic acid formed could be recovered in the capsule core with a concentration over sevenfold that in the aqueous phase. Higher extraction efficiencies could be obtained by varying the substrate concentration and number of capsules. The capsule-immobilized enzymes could be stored for over 4 months at pH 8 and 4°C with no loss of activity. Over 80% of the initial activity could still be recovered after five repeated batch cycles of the bioconversion process. The importance of capsular perstraction and reactive capsular perstraction was clearly demonstrated (Wyss et al. 2005).

Baicalin was converted to baicalein by β-glucuronidase encapsulated in liquid-core alginate capsules, mimicking the natural enzyme's behavior in lysosomes. Taking both the physical properties of the capsule and the activity of the encapsulated enzyme into account, optimal conditions (1.0% w/v sodium alginate, 0.10 M $CaCl_2$, 30 min gelation time) for β-glucuronidase encapsulation were determined (Jiang et al. 2007). The encapsulated β-glucuronidase retained up to 88% of its free-form activity, with an encapsulation efficiency of 77%. Conversion of baicalin by free and encapsulated β-glucuronidase resulted in baicalein productivities of 80 and 65%, respectively. The encapsulated β-glucuronidase showed no appreciable loss in

activity after four repeated cycles and 90% of its initial activity remained after 26 days of storage at 4°C (Jiang et al. 2007).

Lactate dehydrogenase was entrapped within the liquid core of alginate–chitosan shell capsules and carbon nanotubes were integrated into the alginate or chitosan matrices or both. Encapsulation was carried out to convert pyruvic acid to lactic acid coupled with the oxidation of NADH to NAD^+. The alginate–chitosan capsules doped with carbon nanotubes showed enhanced mechanical strength relative to those without carbon nanotubes. The lactate dehydrogenase loading efficiency of the alginate–chitosan capsules with carbon nanotubes (10 mg/ml) doped in both the shell and the core was 30.7% higher than without carbon nanotubes. The optimal pH value for the bioconversion catalyzed by immobilized lactate dehydrogenase was 7.0, lower than that by free lactate dehydrogenase (7.5), but the optimal temperature for both was 35°C. Operational stability of the immobilized lactate dehydrogenase was greatly improved by including carbon nanotubes in the alginate–chitosan capsules. The results showed that this method is efficient for enzyme encapsulation in biotechnology applications (Ma et al. 2009).

A mathematical model for the analysis and simulation of a heterogeneous bi-enzymatic reaction system was presented. A glucose oxidase–catalase system co-encapsulated within hydrogel membrane liquid-core capsules was chosen as a model for consideration of a non-uniform biocatalyst concentration profile within the support and the deactivation phenomena of the two enzymes. Simulation experiments elucidated the distribution of the two enzymes within the capsules (Blandino et al. 2002). Glucose oxidase appeared to be distributed across the whole of the particle while catalase was confined almost exclusively to the core of the capsule. From the simulated glucose and hydrogen peroxide concentrations within the capsules, it was deduced that the hydrogen peroxide formed in the glucose oxidation reaction led first to deactivation of the catalase, upon which glucose oxidase deactivation accelerated (Blandino et al. 2002).

7.7 Special Food Applications

7.7.1 Jelly-Like Foods

Gums of natural origin are employed in confectionery manufacturing. Gum arabic has been utilized in gumdrops and agar has been used for the fabrication of jellies (candies) and marshmallows. The gum within the recipe serves to shape the "jelly." An additional reason for adding gum is to avoid sugar crystallization and to emulsify fat, keeping it uniformly distributed within the product (Klose and Glicksman 1968). A special ice confection consisting of jelly balls with a liquid core and elastic skin is an example of a product that encloses polysaccharides obtained from *Agrobacterium* or *Alcaligenes* cultures grown on glucose (Takeda Chemical Industries Ltd, Tokyo, Japan) with milk, sucrose, and vanilla flavoring. These fluid-core elastic-skin jelly balls are then frozen (Kimura et al. 1975). These ice confections can include fruit

jellies or milk jellies (Takeda Yakuhin Kogyo KK 1974). The gum powder swells and gels when added to water and heated. Gels that are thermally irreversible and unaffected by further addition of water can be produced over a pH range of 2.0–9.5 in the presence of many food additives. The gels may be used to make novel food products consisting of a jelly-like skin with a liquid core and canned jellies. The concentration of the polysaccharide in water must be greater than 1.5% for gel stability and less than 0.6% for taste acceptability. The gels are freeze–thaw stable and may be used to make an ice confection contained in an elastic gel skin (Anon 1977).

7.7.2 Fruit Products

Fruit or vegetable juices can be extracted by a combination of compression and shearing forces. In several cases, e.g., tomatoes or other soft fruits, they are heated to soften their tissues and pulp is forced through perforations of a pulping device's screen, the size of which determines, to some extent, the textural properties of the product (Fellows 2000). Unique uses of juice, pulp, or puree for the production of soft, viscous, fruit-based liquid-core products were described decades ago. For instance, fruit pulp, puree, or juice containing soluble calcium salt was extruded to form drops which were coated with a thin skin of alginate or pectate sol. The coated drops were exposed to an aqueous setting bath containing a soluble calcium salt (Sneath 1975). Drops of aqueous fruit material were coated with an aqueous alginate or pectate solution and dipped in a solution containing calcium or aluminum ions to gel the surface. Another report deals with food products which simulate the non-uniform structure (soft core and tough skin) of soft fruit, e.g., black and red currants; these are obtained by surrounding droplets of, e.g., fruit pulp or juice containing calcium or aluminum ions with an alginate or pectinate sol, and treating the coated droplets in a gelatinizing bath containing calcium or aluminum ions. Coated droplets are obtained by co-extrusion, the droplets being dripped directly into the coagulating bath (Unilever NV 1974).

7.7.3 Encapsulating Aroma and Health Compounds

Coffee aroma, β-carotene, or any liquid that does not readily dissolve the shell can be included within fluid-core hydrocolloid capsules. A previously described method for encapsulating volatile aroma compounds, which can be incorporated directly into an instant consumer product, involves dissolving an inert gas (such as carbon dioxide) in an aromatized edible liquid, then co-extruding the gas/liquid mixture with a molten carbohydrate material which has a glass transition temperature of 20–80°C (Garwood et al. 1995). The result is a continuous stream of carbohydrate outer shell surrounding an inner core of aromatized, gasified liquid. This stream is extruded into a pressure chamber, at a pressure beyond that of the inert gas in the aromatized liquid core, and the carbohydrate shell is then allowed to cool below

the glass transition temperature and become firm. When these capsules come into contact with hot water, the inert gas in the inner core expands and ruptures the capsule wall, releasing the aromatized liquid. Coffee aroma capsules are prepared from 100% coffee-derived material, in which coffee aroma frost is dissolved in coffee oil and gasified, then encapsulated in the outer shell of hardened amorphous coffee glass (Garwood et al. 1995). A health food containing encapsulated *Dunaliella* algae is manufactured by adding cyclodextrin (chemically and physically stable molecules formed by the enzymatic modification of starch) to *Dunaliella* powder, stirring and blending, adding an antioxidant (vitamin A or E), lubricant (talc, esters of sucrose with a fatty acid) and a binder (a sugar), blending, granulating, and encapsulating in a light-impermeable hard capsule. A variant employing a soft capsule is also described. The food is rich in β-carotene (Tanaka 1990). Microcapsules with a solid, fusible shell and multiple liquid cores (of any liquid, e.g., an aqueous solution, which does not readily dissolve the shell) can be produced by spray-cooling a water-in-oil emulsion. The resultant dry, free-flowing powder can be heated or otherwise processed to release the contents of the microcapsules (Morgan and Blagdon 1993).

Coacervation appears to be a useful method for encapsulating aroma compounds and provides a good barrier against oxidation of sensitive material (Leclercq et al. 2009). Coacervates [liquid (limonene or medium-chain triglycerides) or solid core (menthol)] using gum acacia/gelatin as wall materials were produced. Modified starch was utilized as a carrier material to protect limonene from oxidation. Capsules were crosslinked with glutaraldehyde and freeze-dried. Coacervates were characterized for their structure, shape, size distribution, flavor load, and water-uptake rate and a brief storage study compared coacervate with spray-dried capsules. No detectable increase in limonene oxide could be detected in capsules produced by coacervation over 25 days in storage at 45°C, whereas a significant increase in limonene oxide was detected in spray-dried powder over the same period (Leclercq et al. 2009).

7.7.4 Other Foods

A small number of specific foods that cannot be classified into one unifying category but have liquid-core capsules are listed in this section. One report describes a process for preparing encapsulated foods and drinks filled with a desired edible fluid (Ueda 1985). A core liquid is prepared by adding calcium salt and if necessary, other additives, to a sugar liquid. Membranes, mainly calcium alginate, are formed on the surface of the core liquid by dropping it into an alginic acid salt solution, which surrounds the membranes to form capsules. The core liquid inside the capsules is then exchanged with water or other edible fluid by immersing the capsules in that fluid. Processes are described for the production of alcoholic drinks, encapsulated dressing, fruit juices, liquid sweetening materials, and cut solid foods, e.g., vegetables (Ueda 1985). Production of a roe-like, multilayer (more than two), spherical structure has been described. It involves discharging two edible materials

through a multitube nozzle to form two sol materials having different properties, with the material forming the inner layer being convertible to an aqueous gel that is softer than the outer material layer (Kuwabara and Jyouraku 1983). Another patent describes novel ice-cream products that contain small beads which are solid or hollow with a liquid center. These hollow capsules are based on either alginate or pectate with low-methoxy content (Unilever NV 1979). Inclusion of liquid-core capsules within a food enables the introduction of a blend of textures and tastes in the same bite and is expected to undergo further development once more sophisticated "fun foods" hit the market.

7.8 Agricultural Uses of Liquid-Core Capsules

Plant material can be encapsulated in hollow calcium alginate beads. The technique involves suspending plant cells, tissues, organs, shoot tips, or somatic embryos in a solution containing carboxymethylcellulose and calcium chloride and then dripping it into a stirred sodium alginate solution. This is a classic way of producing a liquid-core capsule (Nussinovitch, Gershon and Nussinovitch 1996, 1997). In initial experiments with *Daucus carota* (carrot), it was found that after 14 days of cultivation, 100% of seeds encapsulated in hollow calcium alginate beads germinated in the liquid core while 13% of these burst the capsules. Embryogenic calli developed within hollow beads and formed somatic embryos while calli in conventional calcium alginate beads became detached from the beads early in development, and no somatic embryogenesis occurred. With *Solanum tuberosum* (potato), callus development was observed in 50% of the hollow beads. Eighty-one percent of shoot tips encapsulated in hollow beads sprouted and grew out of the capsules (Patel et al. 2000). Another interesting use for the production, storage, and regeneration of potato shoot tips in agriculture was reported. Shoot tips of four potato cultivars (Désirée, Genet, Tigoni, and Tomensa), 3–4 mm in length, were precultured for 2 days on Murashige and Skoog (MS) solid medium, then encapsulated in calcium alginate to produce hollow-bead synthetic seed capsules averaging 0.78 cm in diameter. Regeneration and "regrowth" were tested on MS solid medium and on soil in the greenhouse, respectively. The encapsulated shoot tips were stored at 4 and 10°C for up to 390 days. For all cultivars, the encapsulated shoot tips stored at both temperatures for 180 days and at 4°C for 270 days exhibited 100% regeneration on MS solid medium (Nyende et al. 2003). After 360 days in storage at 4°C, 70.8% (Tigoni), 66.7% (Genet), 58.3% (Désirée), and 51.5% (Tomensa) regeneration was recorded on MS medium, decreasing to 15% (Tigoni), 25% (Genet), 10% (Désirée), and 0% (Tomensa) regeneration after 390 days in storage. "Regrowth" of 93–100% was recorded for non-stored encapsulated shoot tips transferred directly to soil in the greenhouse after a 2-week preculture on MS solid medium with an added fungicide (carbendazim) in the encapsulating gel. The "regrown" shoot tips produced plants showing normal development. It was concluded that hollow-bead synthetic seed capsules constitute a feasible alternative propagation method for potato seed production (Nyende et al. 2003).

7.9 Environmental Uses of Liquid-Core Capsules

Chapter 10 deals with environmental uses of beads. The presence of pesticides and herbicides in surface and ground water is becoming a significant environmental problem (Patniak 1997). As a result of groundwater movement and erosion, pesticides utilized in agriculture and local gardens wind up in neighboring streams, making the water unsafe for human consumption and aquatic life. In traditional water-treatment plants, pesticides are not always detoxified and are sent back to the waterways where they can accumulate to high levels. Triazine derivatives, such as atrazine, are the most commonly employed herbicides in agriculture (used, for example, in the manufacture of wheat, citrus fruits, pineapples, and Christmas trees; Liu et al. 1999), and their widespread use results in the contamination of surface and ground water due to their relatively high solubility in water. Organothiophosphorus compounds, e.g., parathions, are frequently used pesticides that have been found in the drinking water and are known to cause neuroses and cancer in humans (Dzyadevych et al. 2002). Substantial research has been reported concerning methods for the detection and removal of pesticides and herbicides. Chemical treatments, such as ozonation to remove atrazine (Beltran et al. 2000), reduction by iron powder (Ghauch et al. 1999), biotreatments using granular activated carbon associated with bacteria to biodegrade low concentrations of xenobiotics (Feakin et al. 1995; Jones et al. 1998), and nanofiltration (Van der Bruggen et al. 2001), have been investigated. Liquid-core microcapsules have already been used as extraction aids in fermentation processes (Stark et al. 2002) and it is therefore not surprising that such an approach has been extended to encapsulating a solvent, suitable for the extraction of atrazine, methyl parathion, ethyl parathion, and 2,4-dichloro-phenoxyacetic acid within a porous hydrogel membrane composed of alginate and polyacrylamide (Wyss et al. 2004). Such microcapsules offer the advantage of a very large interfacial contact area that promotes rapid extraction. In addition, the presence of a hydrogel membrane to physically separate the organic core phase from the water to be treated means that considerably less solvent is required compared to liquid–liquid extraction processes. All of the tested compounds could be rapidly extracted, typically 75% extraction within 10 min using a capsule-to-liquid volume ratio of only 3.5% for ethyl parathion, and the rate of extraction increased with increasing hydrophobicity of the compound being extracted. Higher rates of extraction could be achieved by adjusting the capsule-to-liquid volume ratio. The effects of different liquid-core solvents, capsule sizes, agitation rates, and treatments with complexing agents on the properties of the microcapsules and extraction rate were studied. Capsules of a diameter smaller than 0.800 mm showed little external resistance to mass transfer. The main resistance to mass transfer of the pesticides/herbicides was found to reside in the hydrogel membrane composed of crosslinked alginate/polyacrylamide. Removal of divalent cations from the membrane by the addition of citrate resulted in a 50% increase in the mass-transfer coefficient, probably as a result of solubilization and exo-diffusion of alginate (Wyss et al. 2004).

7.10 Special Applications of Liquid-Core Capsules

7.10.1 Stop-Smoking Aids

Smoking cessation refers to ceasing consumption of a smoked substance, mainly tobacco, but also *Cannabis* and other substances. *Plantago major* is a perennial herb, thought to be of Eurasian origin and now naturalized throughout the world (Fig. 7.7). It is considered a common and noxious weed by some and a miracle plant by others (http://www.altnature.com/gallery/plantain.htm). It also causes a natural aversion to tobacco and is currently being used in stop-smoking preparations (http://www.altnature.com/gallery/plantain.htm). *Piper methysticum* (*Piper* Latin for "pepper," *methysticum* Greek for "intoxicating") is an ancient crop originating in the western Pacific, whose active ingredients are called kavalactones. A composition containing both *Pl. major* and *P. methysticum* is prepared in fluid form and then encapsulated in a soft or hard gelatin shell. The present use of the composition in this form is adapted for oral ingestion, producing a diminished desire for tobacco (i.e., nicotine) without the use of nicotine itself and providing anti-depressive and anti-anxiety effects without sedative or hypnotic effects. Suitable soft gelatin capsules for use with this formulation are soluble in water and in gastrointestinal fluids. Upon swallowing the capsule, the gelatin shell rapidly dissolves or ruptures in the gastrointestinal tract, thereby introducing the *Pl. major* and *P. methysticum* composition from the liquid core into the physiological system (Cody 2000).

Fig. 7.7 *Plantago major*, a perennial herb, thought to be of Eurasian origin and now naturalized throughout the world. Image taken in the outdoor botanical garden of the Technion-Israel Institute of Technology, Haifa Israel (courtesy of Iorsh, http://en.wikipedia.org/wiki/File:Plantago_major.jpg)

7.10.2 The Beauty Industry—Removal of Body Hair

Hair removal describes any method of removing hair, especially from the human body. Reasons for removing hair can be medical, social, cultural, sexual, or religious, for the military or as punishment, etc. Body areas from which hair is often removed include the abdomen, armpits, back, chest, eyebrows, eyelashes, face, legs, head, and pubic area. Temporary hair-removal methods include depilation, i.e., removal of hair down to skin level, which can be achieved by shaving and lasts several hours to several days, depilatories (i.e., creams or shaving powders which chemically dissolve the hair), and rough surfaces that are used to buff away hair. Epilation, or removal of the entire hair shaft, including the root, lasts from several days to several weeks and may be achieved by plucking, waxing, sugaring, threading, burning off with hot wax, or by using turmeric with other ingredients, mechanical epilators, oral medications, or enzymes.

A novel idea for easy hair removal has been suggested, consisting of a garment coated with a microencapsulated depilatory agent which effectively and automatically removes unwanted body hair when worn by the user. The invention is suitable for use in women's hosiery, which is typically worn tightly against the skin. The hosiery is coated with a pressure-sensitive microencapsulated depilatory agent. Microencapsulation facilitates deposition of the liquid depilatory on the hosiery in a capsule form without wetting or altering the original dry appearance of the fabric and, in addition, enables dispensing the depilatory only to a localized area adjacent to the hair stubble (Mcgalliard 1979). When the hosiery is worn tightly over the skin, the pressure exerted by the hair stubble against the fabric ruptures the microcapsules, thereby selectively releasing the depilatory agent in the area surrounding the hair stubble. The depilatory agent quickly begins to break down the protein structure of the hair and completely dissolves the hair stubble during normal wearing of the garment (Mcgalliard 1979). The pressure-sensitive depilatory capsules include a depilatory in a liquid core and a surrounding, typically spherical wall. The capsules typically range from a few to several hundred microns in diameter and are deposited upon the hosiery, thereby contacting the hair stubble as well as the user's skin during use. The selected microencapsulation material and thickness should enable rupture in response to either abrasion by the hair stubble or increased pressure at the stubble, but not when simply pressing against the user's skin. The liquid depilatory agent contained within the microcapsules must be inert to the microcapsule wall material and still be able to reduce the disulfide crosslinks of body hair, preferably without causing substantial irritation to the user's skin. Solutions having a moderate concentration of calcium thioglycolate or 1,4 dimercapto-2,3 butane diol have been found to be preferable to sulfate or sulfhydrate solutions due to their inoffensive odor and non-skin-irritating properties (Mcgalliard 1979). The reaction is

$$2\mathrm{SH}-\mathrm{CH_2}-\mathrm{COOH}+\mathrm{R}-\mathrm{S}-\mathrm{S}-\mathrm{R}\rightarrow 2\mathrm{R}-\mathrm{SH}$$

$$+\mathrm{COOH}-\mathrm{CH_2}-\mathrm{SS}-\mathrm{CH_2}-\mathrm{COOH}$$

The calcium thioglycolate breaks down the disulfide bonds in keratin via $Ca(OH)_2$ and weakens the hair so that it is easily scraped off where it emerges from the hair follicle. The resulting combination of alkali and thioglycolic acid is calcium thioglycolate (CaTG). The $Ca(OH)_2$ is present in excess to enable the thioglycolic acid to react with the cysteine present in hair protein.

7.10.3 The Paper Industry

Liquid-core capsules can serve as the origin for the manufacture of hollow particles for the paper industry. A water-dispersant emulsion of hollow particles, having an outside diameter of 0.2–1 μm and a 30–50% internal air pocket, is required. The core of the particle is filled with water. After their production, the particles are dried. During this process, the water contained inside diffuses and passes through the outer shell to be replaced by air, ultimately assuming the form of a hollow capsule. Dried particles function as a white pigment, utilizing the scattering of light generated by the difference in the refractive index at the interface between the outer resin layer and the inner air layer, and consequently provide excellent concealing ability. The gloss, brightness, and printing gloss of coated paper are improved, since particles on the surface are easily crushed during calendering, resulting in a smoother finish. Furthermore, low specific gravity is useful for lighter weight papers. This emulsion is also widely applied to inks and paints (Caruso 2000). In 1997, the Japanese market for such emulsions for paper was ~5000 tons a year. Demand is expected to grow to the levels in Europe and the USA, since the emulsion is applied not only to art papers, high-value-added paper, and high-quality boards, but also to slightly coated paper, following the trend toward lighter weight paper, with annual demand projected to reach four to five times the tonnage in a few years. With lighter weight coated paper, the original paper is thinner, but the demand for low-weight organic pigments with specific gravity like this emulsion increases due to improved concealability and excellent gloss and printability (Caruso 2000).

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Chapter 8
Beads as Drug Carriers

8.1 Introduction

Beads are often used as drug carriers to study both passive and active drug targeting. When discussing drug delivery by beads, topics related to the method of drug incorporation, size and density of the bead, extent and nature of the crosslinking, physicochemical properties of the drug, interactions between the drug and the matrix material, and concentration of the matrix material and release environment, such as the presence of enzymes, are of the utmost importance. Several beads will be described in this chapter including, but not limited to, those that are based on alginate, chitosan, gelatin, gellan, guar, pectin, dextran, and cellulose, and biodegradable hydrogels based on polyesters and polyvinylpyrrolidone (PVP) crosslinked with functionalized albumin.

8.2 Controlled Drug Release

Diffusion through a rate-controlling membrane or matrix, osmosis [i.e., the movement of water across a semi-permeable membrane from an area of high water potential (low solute concentration) to an area of low water potential (high solute concentration)], ion exchange, and matrix degradation are all means of controlling drug release (Heller 1980; Park et al. 1993). Biodegradable drug-delivery systems have a unique advantage in that they do not need to be removed from the site of application once the delivery has been made. In addition, degradation of polymers to low molecular weight fragments that can be eliminated from the body is preferred in many systemic applications (Heller 1980; Park et al. 1993).

Surface (heterogeneous) and bulk (homogeneous throughout) degradation are two different modes of biodegradation based on the site of polymer breakdown (Heller et al. 1978). In bulk polymer degradation, drug diffusion occurs prior to or concurrent with matrix degradation (Chang 1976). In surface polymer degradation, drug release is determined by the relative contributions of drug diffusion and matrix degradation (Heller 1985). In many cases, when biodegradable hydrogels are used, the drugs are in contact with water and their solubility is a main factor in their

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release. If solubility is not a limiting factor, release is rapid and independent of the matrix degradation rate (Park et al. 1993). As a result, most low molecular weight drugs are not transported by hydrogels; instead, these gels are used for proteins and peptides that are entrapped within the gel network until its degradation (Park et al. 1993). Diffusion-controlled and swelling-controlled drug-release mechanisms have been discussed extensively in previous reports (Jost 1960; Baker and Lonsdale 1974; Crank 1975; Baker 1987). Diffusion-controlled systems are divided into reservoir or monolithic devices (Zeoli and Kydonieus 1983; Kost and Langer 1987). In the reservoir system, the drug core is encapsulated by an inert membrane (Park et al. 1993), providing a fairly constant rate of release that depends on drug solubility and excess availability.

8.3 Gels in Drug-Delivery Systems

The shape and size of gels used in drug-delivery systems depend on the way in which they will be administered. Gel particles should be no more than a few nanometers in diameter for injection, and microcapsules, nanocapsules, microgels, coacervates, and microspheres are used for this purpose. Applications of gels in drug-delivery systems include chitosan microspheres manufactured by the emulsion solution dropping method (12 μm), producing mucoadhesive chitosan microspheres of 2.5–50 μm for the alimentary canal, alginate microgel beads (50 μm) for orally delivered drugs, as well as chitosan-coated alginate microbeads or chitosan–chondroitin sulfate-modified alginic acid gel beads (Shi et al. 2007). Alginate–calcium gluconate is used to mask the flavor of bitter drugs (Yonese 2001). Combinations of agar–pectin, agar–alginate, and the like are used in the development of non-gelatinous soft capsules. Pullulan is used to coat theophylline (a methylxanthine drug that is used in therapy for respiratory diseases such as asthma; Fig. 8.1), generating mini-capsules. Dextran has been applied in pH-responsive carboxydextran microspheres, and gelatin and serum albumin have been used for capsule production and as a drug substrate. Semi-synthetic polymers, such as poly(lactic acid) (a biodegradable, thermoplastic, aliphatic polyester derived from renewable resources such as corn starch or sugarcane), have also been used for the sustained release of calcitonin gene-related peptide and poly(lactic acid-*co*-glycocholic acid) has been used for an oral hepatitis type B vaccine using microspheres (Yonese 2001). Control of drug release is governed by phase transition or structural changes in the matrix. Four release-controlling mechanisms are known: differential diffusion coefficient; squeezing of water (squeezing effect); formation of

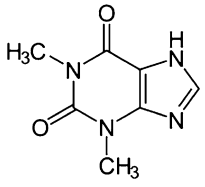

Fig. 8.1 Structure of theophylline (courtesy of NEUROtiker, http://en.wikipedia.org/wiki/File:Theophyllin-Theophylline.svg)

a dense surface layer (skin effect); and coating and squeezing effect (Yonese 2001). In addition, thermoresponsive polymers can be used for drug delivery, such as poly(vinylmethyl ether), poly(*N*-ethylacrylamide), poly(*N*-ethylmethacrylamide), poly(*N*-isopropylacrylamide), poly(*N*-methyl-*N*-isopropylacrylamide), poly (*N* acryloyl piperidine), and acryloyl-L-prolinemethyl ester, with transition temperatures of 38.0, 72.0, 50.0, 30.9, 22.3, 5.5, and 24°C, respectively. Under these phase transition temperatures, the polymers will swell and above them, they will shrink (Yonese 2001).

Drug release can be achieved by adjusting dissolution rate, osmotic pressure, and drug–matrix interactions. For more advanced systems capable of delivering a predetermined amount of drug at the planned time and desired place, gels and liposomes can be used as vehicles. These drug-delivery systems make use of different mechanisms to achieve their target drug storage, controlled-release rate, and activation of release (Yonese 2001). Polymers can be used in drug-delivery systems as bonding, collapsing or masking agents. Gel properties required for drug-delivery systems are the ability to act as a matrix, sol–gel transition, blocking effect toward diffusion, swelling and water-holding, swelling-shrinking phase transition, and mechano-chemical reaction (Yonese 2001). The blocking effect in gel diffusion means that a solute can diffuse through free water in a gel bead. The rate of diffusion is slow. If the volume fraction of the free water, namely the porosity fraction ϵ, is known, then the diffusion coefficient in the gel is given by the Mackie and Meares equation (Mackie and Meares 1955).

$$f = \frac{D}{D_0} = \left[\frac{\varepsilon}{2-\varepsilon}\right]^2 \tag{8.1}$$

where D_0 is the diffusion coefficient in water. When D is 10^{-9} m^2/s, it takes <10 min for a solute to completely diffuse out of a gel sphere that is 0.1 cm in diameter. If $f = 0$ or 0.01, the release time is 100 or 1000 min, respectively. To reach $f < 0.1$, ε has to be less than 0.5 and for $f < 0.01$, $\varepsilon < 0.1$, namely a gel with very small porosity or very small water content must be used. If the diameter of the bead decreases, there is a parallel reduction in release time. To control such systems, it is necessary to make use of the sudden change in swelling degree during the swelling–shrinking phase transition (Yonese 2001). Other important issues are the possible formation of a surface density layer, interaction between the drug and the matrix, and relationship between mesh size and size and shape of the drug (Yonese 2001).

8.4 Dual Drug-Loaded Beads

Sustained release is not always the most favorable option since nearly all bodily functions, including pharmacokinetics and pharmacodynamics in clinical situations, demonstrate significant daily variation and biological rhythms (Giunchedi et al.

1991; Lamberg 1991; Lemmer 1991; Lin et al. 1996). Therefore, a drug-delivery system should be able to deliver the drug not only via controlled release but also in a circadian rhythmic pattern (i.e., a roughly 24 h cycle in the biochemical, physiological, or behavioral processes of living entities, including plants, animals, fungi, and cyanobacteria), so that pharmacotherapy can be optimized by adapting the drug release to therapeutic requirements or biological rhythms (Narisawa et al. 1995; Lin et al. 1996). Pharmaceutical dosage forms that imitate biological rhythms in the body have been extensively studied, including sigmoidal release systems and pulsatile release tablets—a time-controlled explosion system (Giunchedi et al. 1991; Narisawa et al. 1995). Alginate beads have been investigated as drug carriers for the controlled delivery of low molecular weight compunds. The gelling strength and type of release in such beads are highly dependent on the type and concentration of the gel-inducing ions and hydrocolloid grades, gelling time, curing time, release-testing medium, the drug selected, the size of the beads, and formulation compositions (Shin et al. 1996). Dual drug-loaded alginate beads may be used to deliver drugs in a time-dependent manner (Lee et al. 1998). The thickness of such beads, surrounded by an outer layer, typically range from about 57 to 329 μm, with a distinct gap between the inner and outer layers. The release rate of dual drug-loaded alginate beads was found to be stable in gastric fluid for 2 h but largely increased when switched to intestinal fluid. The drug was linearly released for 4 h followed by another linear release thereafter, showing distinct biphasic release characteristics. There was a difference in the release profiles between single-layered and dual drug-loaded alginate beads due to their structural shape (Lee et al. 1998). Since the release profile depends on the formulation compositions of the inner and outer layers and the structural shape of the alginate beads, the current dual drug-loaded alginate beads, which simultaneously contain drugs in their inner and outer layers, may be used to deliver the drugs in a time-dependent manner.

8.5 Drug Release from Beads

8.5.1 Albumin Beads

Albumin generally refers to any water-soluble protein which is moderately soluble in concentrated salt solutions and is subject to protein denaturation. It has a molecular weight of about 65 kDa, consists of 584 amino acids and contains no carbohydrate (http://en.wikipedia.org/wiki/Albumin). The most well-known type of albumin is serum albumin, which is the most common protein in the blood or serum. It is produced in the liver and makes up a large proportion of all plasma proteins. The human version is termed human serum albumin and it normally constitutes ~60% of all human plasma proteins. Serum albumins are important in regulating blood volume and serve as carriers for low water-soluble molecules, thereby isolating their hydrophobic nature (http://en.wikipedia.org/wiki/Albumin).

Albumin was used to create microspheres for drug delivery via either passive or active drug targeting (Morimoto et al. 1980; Burger et al. 1985; Gupta and Hung

1990). Factors that affect drug release from microspheres can be divided into those that are related to the properties of the bead and its constituents, such as size and density of the microsphere and the extent and nature of the polymer crosslinking, and those that are related to the drug, i.e., drug properties, interaction between the drug and the matrix material, and the concentration and position of the drug within the bead (Tomlinson et al. 1984; Gupta et al. 1989). The release of triamcinolone diacetate [a synthetic corticosteroid, anti-inflammatory (steroidal); thought to decrease inflammation mainly by inhibiting activities of mast cells, macrophages, and other mediators of allergic reactions. It also suppresses the immune system by depressing lymphatic activity] from crosslinked human serum albumin nanoparticles was described by the following first-order equation:

$$\ln M = \ln M_0 - kt \tag{8.2}$$

where M_0 is the original amount of drug present in the microspheres, M is the amount of drug remaining in the microspheres, k is the first-order release-rate constant, and t is the time during which the drug release occurs. Another model was used to describe low molecular weight drugs released in vitro from monolithic microspheres:

$$M = Ae^{\alpha t} + Be^{\beta t} \tag{8.3}$$

This model describes biphasic release with an initial burst, followed by a slower release rate (Tomlinson et al. 1984; Gupta et al. 1986; Burgess and Davis 1988, Willmott and Harrison 1988). M is the amount of drug remaining in the microspheres, A and B are constants for zero-time intercepts for the initial and terminal phases, and α and β are the apparent first-order initial and terminal rate constants (Park et al. 1993). When sodium cromoglicate (traditionally described as a mast cell stabilizer and commonly marketed as the sodium salt sodium cromoglicate or cromolyn sodium; this drug prevents the release of inflammatory chemicals such as histamine from mast cells; Fig. 8.2) was released from albumin microspheres stabilized by crosslinking with 5% glutaraldehyde for 18 h into phosphate buffer at pH 7.0, 88% of the drug was released within 10 min, followed by first-order release at a much slower rate. It was claimed that some control of the release can be gained by ultrasonication of the microspheres or changing the crosslinking density (Tomlinson et al. 1984). Rate increases in the terminal phase could be achieved by decreasing the size of the carrier (Tomlinson et al. 1984). A novel method was proposed for the preparation of a free-standing albumin ultrathin, biocompatible nanosheet with heterosurfaces (Okamura et al. 2009). Sheet-shaped carriers, having both obverse and reverse surfaces and thus a large contact area for site-targeting, have several advantages over spherical-shaped carriers, which have an extremely small contact area for site targeting. The nanosheet may be a suitable candidate biomaterial for drug-delivery carriers, hemostatic reagents, wound dressings for burn injuries, and so forth (Okamura et al. 2009).

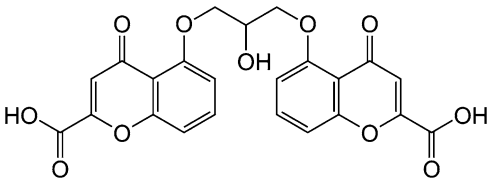

Fig. 8.2 Skeletal formula of cromoglicic acid (from Fvasconcellos; after Triggle et al. (1996); http://en.wikipedia.org/wiki/File:Cromoglicic_acid.svg)

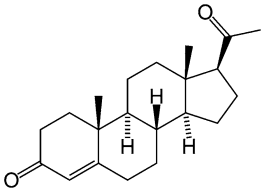

Fig. 8.3 Progesterone: two-dimensional skeletal illustration (http://en.wikipedia.org/wiki/File:Progesterone-2D-skeletal.png)

A good fit for the initial or partial release of L-norgestrel (a synthetic progesterone, which is mainly used as a major ingredient in hormonal contraceptives and is available under numerous brand names) and progesterone (a steroidal hormone involved in the female menstrual cycle, pregnancy, and embryogenesis in humans and other species; Fig. 8.3), from albumin microspheres was achieved by the Higuchi equation [which describes the release of a drug by pure diffusion out of an encapsulating matrix (no erosion occurring)] (Lee et al. 1981; Ishizaka and Koishi 1983; Royer et al. 1983; Willmott et al. 1985; Leucuta et al. 1988). In vitro release of adriamycin (a drug used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas(from albumin microspheres was described by the biphasic zero-order model (Gupta et al. 1986). Special care was taken in washing the drug-loaded microspheres in order to reduce release in the burst phase. The following model describes the release:

$$\frac{\mathrm{d}M}{\mathrm{d}t} = -\frac{ADC_\mathrm{m}}{h} \tag{8.4}$$

where dM/dt is the decrease in the amount of included drug. A is the exposed surface area, D is the drug concentration within the carrier, C_m is the drug concentration inside the microsphere, and h is the thickness of the diffusion layer. When albumin microspheres are used, a bi- or triphasic model is capable of describing the drug release (Gupta et al. 1986; Willmott et al. 1988): there is a distinct difference between the release of a drug associated with the surface and that of one entrapped in the matrix (Willmott and Harrison 1988).

In vivo experiments were conducted to deliver low molecular weight drugs such as antitumor agents (El-Samaligy and Rohdewald 1982; Burger et al. 1985; Willmott et al. 1985; Dau-Mauger et al. 1986; Gupta et al. 1987; Leucuta et al. 1988; Willmott et al. 1988; Gallo et al. 1989, Gupta et al. 1989; Gupta and Hung 1990), steroids (Lee et al. 1981; Gupta et al. 1987; Burgess and Davis 1988), and proteins (Goosen et al.

1982; Royer et al. 1983; Hung et al. 1990; Kwon et al. 1992) from albumin beads. The initial experienced burst effect was accompanied by a more matrix-protected release phase. When progesterone was released from albumin beads strengthened with glutaraldehyde, the initial burst release was observed, followed by drug release at a nearly constant rate for 30 days, as a result of enzymes' proteolytic degradation of the matrix and drug diffusion (Willmott et al. 1985; Burgess and Davis 1988). Hydrogel systems and biodegradable polymers were used to deliver high molecular weight drugs. Inulins are a group of naturally occurring polysaccharides (several simple sugars linked together) produced by many types of plants (Fig. 8.4). Inulin is used by some plants as a means of storing energy and is typically found in the roots or rhizomes. Albumin beads entrapping inulin were used as implants in diabetic rats (Goosen et al. 1982). Royer et al. (1983) improved the system by protecting the amine groups before crosslinking. Another study used crosslinked albumin–heparin microspheres for dextran delivery (Kwon et al. 1992).

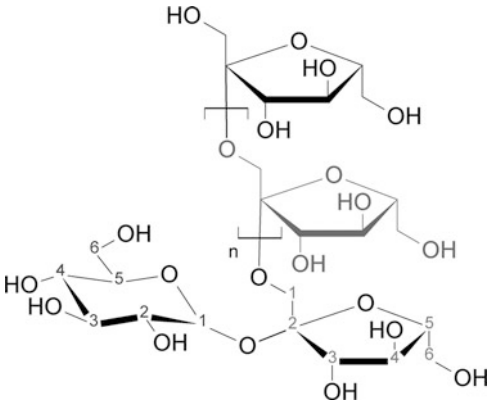

Fig. 8.4 Structural formula of inulin (fructan) (courtesy of Florian Fisch, http://en.wikipedia.org/wiki/File:Inulin_strukturformel.png)

Solid albumin microspheres manufactured by mild chemical crosslinking of serum albumin were introduced as a potential delivery system that could provide sustained release of a drug which targets organs or tissues (Lee et al. 1981). The method of manufacture involves dissolving or dispersing solid drug in an aqueous solution of serum albumin and adding glutaraldehyde as the crosslinking agent. The reaction between the dialdehyde and the ϵ-amino groups of the lysine residues on albumin starts spontaneously but the rate can be controlled by emulsifying the aqueous dispersion in stirred oil before oligomerization has occurred to any considerable extent (Sheu et al. 1986). The product consists of solid particles which can be collected and washed. The size of the produced microspheres is determined by the size of the aqueous droplet in the initially formed emulsion and can be of 3 μm or less, or as large as requested. After drying, powder-like beads entrapping the drug are formed. The microspheres are water-dispersible, biodegradable, and biocompatible, and in vitro-controllable release can be engineered into the system by manipulating formulation and processing variables (Sheu and Sokoloski 1986). The variables that

affect the size of the emulsion droplet include the dimensions of the reaction container and stirrer, the rate of stirring, the density and viscosity of the internal aqueous and external oil phases, and the interfacial tension between them. Other controllable variables affecting release are drug loading and the extent of crosslinking (Sheu and Sokoloski 1991).

8.5.2 Alginate Beads

8.5.2.1 General

The best-studied method of entrapment is sodium alginate gelation by crosslinking via di- or multivalent ions. Such gelation is easy to perform: drug substances or cells are mixed with a sodium alginate solution and added dropwise into a dilute calcium chloride bath. The strength and porosity of the beads can be somewhat controlled by the choice of alginate composition: the higher the L-guluronic acid content, the stronger the gel (Nussinovitch 1997). If alginate with a higher proportion of D-mannuronic acid is chosen for production, a bead with a larger internal pore size is produced. As the concentration of alginate in the beads increases, so does their mechanical strength. If cells are included within the beads, an increase in cell mass has the reverse effect on bead strength (Nussinovitch 1994; Nussinovitch et al. 1994). The interested reader is referred to other sources to read about encapsulation for food and biotechnological purposes (Nussinovitch 2003).

8.5.2.2 Alginate Beads Reinforced by Chitosan

Sodium alginate is a polysaccharide consisting of L-guluronic acid and D-mannuronic acid. It is used as a food additive and its properties have been well studied (Ostberg et al. 1993; Rajaonarivony et al. 1993). In particular, the relationship between drug release and alginate gel bead erosion has been investigated (Murata et al. 1993b). Gel beads reinforced by chitosan, which forms a complex with alginate, erode slowly in phosphate buffer (pH 6.8), and this behavior leads to inhibition of an encapsulated drug's initial release rate (Murata et al. 1993a). Complex formation occurs not only between chitosan and alginate: it can also occur between chitosan and other anionic polysaccharides, e.g., chondroitin sulfate (Fig. 8.5) and chitosan forms a film-like complex (Nakajima and Shinoda 1976). Managing drug release from alginate gel beads by application of a complex formed between chondroitin sulfate and chitosan has also been investigated (Murata et al. 1996). The complex suppressed disintegration of the gel beads and the release pattern of diclofenac incorporated within the beads was obviously changed. Although prolongation of the preparation time gradually decreased the apparent release rate, the pattern of release was not markedly affected. Release of the drug from the gel beads was also influenced by the types of chitosan employed or the properties of the drug itself (Murata et al. 1996). Another report dealt with chitosan-coated alginate beads containing poly(*N*-isopropylacrylamide) (PNIPAAM) (Fig. 8.6), to be

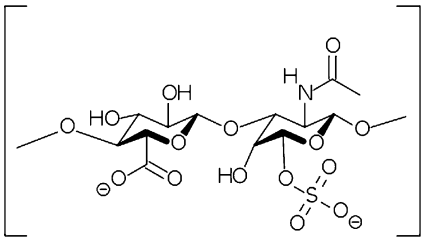

Fig. 8.5 Structure of chondroitin sulfate (http://en.wikipedia.org/wiki/File:Chondroitin_sulfate.svg)

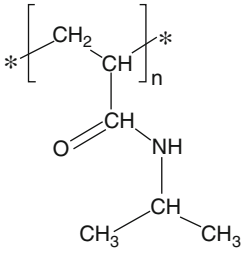

Fig. 8.6 Structure of poly(*N*-isopropylacrylamide) (PNIPAAM) (courtesy of Nickele, http://upload.wikimedia.org/wikipedia/commons/f/f3/Poly_N_isopropylacrylamide.png)

used as a controlled pH/temperature-sensitive drug-delivery system with advanced encapsulation efficiency and delayed release rate (Shi et al. 2006, 2007). The studied beads were characterized by differential scanning calorimetry, scanning electron microscopy, and Fourier transform infrared spectroscopy. Water uptake and release studies using indomethacin as the model drug were also performed. The drug-loading efficiency of the beads with the polyelectrolyte complex coating was considerably higher (84%) than that of the uncoated beads (74%). The equilibrium swelling of the developed materials was found to be pH- and thermoresponsive. Under all conditions, the release profile was found to be slower for the coated beads, indicating that the polyelectrolyte complex coating could effectively slow down the release rate. These results suggest that the studied smart system has the potential to be used as an effective pH/temperature-controlled sustainable delivery system for biomedical applications (Shi et al. 2006).

8.5.2.3 Calcium Alginate/PNIPAAM Beads

Stimulus pH- or temperature-responsive hydrogels are of interest since these two factors can be easily controlled and applied both in vitro and in vivo (Roy and Gupta 2003). Alginate and chitosan are often used as thermosensitive macromolecules in attempts to prepare matrices that offer dual sensitivity to both pH and temperature. PNIPAAM is one of the most extensively studied temperature-sensitive polymers, exhibiting a temperature-dependent volume phase transition at lower critical solution temperature (LCST)—around 32°C (Schild 1992; Kuckling et al. 2003). Alginate is a pH-sensitive and biocompatible natural hydrogel material (Lawrence 1976). Its dissolution and biodegradation under normal physiological

conditions allow it to be used as a matrix for the entrapment and delivery of proteins, drugs, and cells (George and Abraham 2006; Wang et al. 2006). The temperature/pH-responsive drug-release behavior of calcium alginate/PNIPAAM semi-interpenetrating beads has been studied (Shi et al. 2006).

These beads suffer from some limitations, including rapid erosion and a high release rate at neutral pH for dual stimulus-responsive drug delivery. To prevail over these limitations, the alginate gel matrix surface can be modified by ionic interactions with macromolecules through alginate carboxylate ions. Consequently, a coating of macromolecules could cover the alginate gel, with chitosan and poly (L-lysine) being options for typical surface modifications (Anal and Stevens 2005; Ribeiro et al. 2005; Tapia et al. 2005). This amendment method is not so effective for pH- and thermoresponsive alginate beads, possibly because precipitation of PNIPAAM above the LCST can destroy the outer shell constructed by ionic interactions. Inorganic/organic hybrid materials were shown to be of potentially broad significance in the design of biocompatible microcapsules (Carturan et al. 2004; Boissiere et al. 2006) since they repeatedly express complementary properties and, in this regard, synthetic approaches based on mimicking natural processes such as biomineralization could be promising (Green et al. 2006). Biomineralization is in fact a diffusion-controlled deposition of inorganic minerals within porous organic polymeric matrices. Biomineralized polysaccharide capsules can be used for cell growth, release of functional biomolecules, gene delivery, and in general for applications in tissue engineering (Pound et al. 2006). A one-step method to manufacture a biomineralized polysaccharide layer in the preparation of an alginate-based dual stimulus-responsive (pH and temperature) drug-delivery system has been proposed (Shi et al. 2008). It was observed that the mineralized polysaccharide membrane could eliminate the permeability of the encapsulated drug and decrease the drug-release rate effectively when the temperature was lower than the LCST of PNIPAAM. For biomineralized beads prepared with 0.25 M phosphate, the drug-release rate was higher at 37°C (70%) than at 25°C (44%) due to PNIPAAM precipitation and the higher swelling ratio above the LCST. At pH 7.4, the maximum value attained for drug release was 70% (at 37°C), whereas at pH 1.2, the maximum drug release was only ~3%. Based on these results, the studied bioinorganic hybrid materials with dual stimulus-responsive properties have significant importance in a wide range of biomedical applications (Shi et al. 2008).

8.5.3 Chitosan Beads

8.5.3.1 General

Chitosan is unique in its polymeric cationic character and its gelling and film-forming properties and holds potential for pharmaceutical drug-delivery systems (Yao et al. 1995; Illum 1998). Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi (Fig. 8.7). The higher stability of chitosan versus alginate beads has been reported (Stocklein et al. 1983). Chitosan beads

Fig. 8.7 Commercial chitosan is derived from the shells of shrimp and other sea crustaceans, including *Pandalus borealis* pictured here (courtesy of National Oceanic and Atmospheric Administration, http://www.afsc.noaa.gov/race/media/photo_gallery/invert_files/Alaskan_pink_shrimp.htm)

dissolve in the absence of phosphate and swell under acidic conditions but are stable in the presence of chelating agents and monovalent cations (Nussinovitch 2003). Complexation between oppositely charged macromolecules to prepare chitosan beads as a controlled drug-release formulation, particularly for peptide and protein drug delivery, has attracted much attention, because this procedure is uncomplicated and mild (Polk et al. 1994; Liu et al. 1997). The degree of swelling of a chitosan gel is controlled by both pH and ionic strength. Due to the amine group reactivity of chitosan, fixation of drugs is possible and such gels have been used as the matrix in drug-delivery systems (Onishi et al. 1996).

8.5.3.2 Chitosan–Tripolyphosphate Beads

Tripolyphosphate (TPP) is a polyanion (Fig. 8.8) that interacts with the cationic chitosan by electrostatic forces (Kawashima et al. 1985a, b). It was reported that a TPP–chitosan complex can be prepared by dropping chitosan droplets into a TPP solution (Bodmeier et al. 1989) and this possibility was explored for potential pharmaceutical uses (Shirashi et al. 1993; Sezer and Akbuga 1995; Aydin and Akbuga 1996; Calvo et al. 1997; Shu and Zhu 2000). Nevertheless, the mechanical strength of these chitosan beads was poor. To improve their strength and expand their usage in the pharmaceutical industry, TPP–chitosan beads were coated with sodium alginate on the bead surface to form a polyelectrolyte complex film (Aral and Akbuga

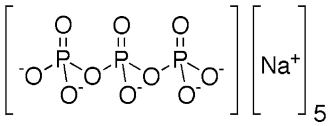

Fig. 8.8 Structure of sodium tripolyphosphate (courtesy of Edgar 181, http://en.wikipedia.org/wiki/File:Sodium_tripolyphosphate.png)

1998). Since the mechanical strength of TPP–chitosan beads still needed to be improved, another method to prepare them with a more homogeneous structure was developed: the beads were prepared under coagulation conditions at 4°C in the presence of gelatin. Cross-sectional analysis indicated that the beads had a homogeneous crosslinked structure and as a result, their mechanical strength increased 10-fold. Furthermore, sodium alginate (a polyanion) can interact with cationic chitosan on the surface of these TPP–chitosan beads to form a polyelectrolyte complex film for the improvement of drug-sustained release performance (Shu and Zhu 2000). This ionic interaction is pH dependent due to the transition of "ladder-loop" complex structures. Chitosan gel beads were cured in a TPP solution with pH less than 6 creating a controlled homogeneous ionic-crosslinking reaction, whereas chitosan gel beads cured in a lower pH polyphosphate solution created a non-homogeneous interpolymer complex reaction due to the mass transfer resistance to diffusion of macromolecular polyphosphate (Mi et al. 1999). Changing the pH value of the curing agent, TPP or polyphosphate from basic to acidic might increase the ionic-crosslinking density or interpolymer linkage of a chitosan–TPP or chitosan–polyphosphate complex. The drug-release patterns of the modified chitosan gel beads in simulated intestinal and gastric juices were sustained for 20 h. These results indicated that the sustained release of anticancer drugs could be achieved by varying the reaction mechanism of chitosan–polyelectrolyte pH-dependent ionic interactions (Mi et al. 1999).

The novel approach improved the strength of TPP–chitosan beads more than 10-fold by harnessing the use of anions such as sulfate and citrate that are able to interact with chitosan by electrostatic force (Shu and Zhu 2000 2001; Shu et al. 2001). Chitosan beads were prepared by adopting a novel chitosan crosslinking method, i.e., chitosan–gelatin droplets coagulated at low temperature and then crosslinked by sulfate, citrate, or TPP (Shu and Zhu 2002). The sulfate–chitosan and citrate–chitosan beads typically had a spherical shape, a smooth surface morphology, and an integral internal structure. Cross-sectional analysis indicated that the crosslinking of sulfate and citrate to chitosan was much faster than that of TPP due to their smaller molecular size. However, once completely crosslinked, TPP–chitosan beads possessed much better mechanical strength and the force required to break the beads was approximately 10 times that of sulfate–chitosan or citrate–chitosan beads (Shu and Zhu 2002). Sulfate- and citrate-crosslinked chitosan beads swelled and even dissociated in simulated gastric fluid and hence, a model drug (riboflavin) (Fig. 8.9) was completely released in 5 h; in simulated intestinal fluid, on the other hand, beads stayed in a shrunken state and released the drug slowly (Shu and Zhu 2002). Chitosan beads, crosslinked by a combination of TPP and citrate (or sulfate) together, had a good shape and improved pH-responsive drug-release properties. Salt weakened the interaction of citrate, and especially sulfate, with chitosan and accelerated bead swelling and hence drug-release rate, but the TPP–chitosan interaction was insensitive to salt. These results indicate that ionically crosslinked chitosan beads may be useful in stomach-specific drug delivery (Shu and Zhu 2002).

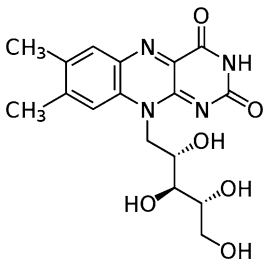

Fig. 8.9 Structure of riboflavin (courtesy of Calvero, http://en.wikipedia.org/wiki/File:Riboflavin.svg)

8.5.3.3 Chitosan Microspheres in Treating Rheumatoid Arthritis

The preparation of chitosan microspheres for intra-articular injection in rheumatoid arthritis (a chronic, systemic inflammatory disorder that attacks the joints, producing inflammatory synovitis that often progresses to destruction of the articular cartilage and ankylosis of the joints) for sustained drug delivery has been studied (Lu et al. 1999). Chitosan can act on the epiphyseal cartilage (the area between the epiphysis and the diaphysis within which bone growth occurs) and enhance wound healing of the articular cartilage. Consequently, it might be useful in healing wounds in the articular cartilage after fulfilling its role as a vehicle for drug delivery. Chitosan was also found to form gel spheres at approximately pH 9 in an amino acid solution, despite usually forming a gel in solutions with pH 12 (Kofuji et al. 1999). Preparations made at lower pH are preferable due to their effect on the solubility or stability of the drug contained in the gel beads and on the tissue into which they are injected. In addition, the release of drug from chitosan gel beads could be controlled by the formation of a complex between chondroitin sulfate and chitosan (Kofuji et al. 2000). Treatment of rheumatoid arthritis might include intra-articular injection of steroids. However, it is difficult to achieve sustained intra-articular drug levels on the basis of drug solubility. During in vivo degradation, drug release is governed by both diffusion and biodegradation of the matrix and it is necessary to clarify the relationship between in vivo biodegradability and drug-release profiles of chitosan gel beads under different conditions (Kofuji et al. 2001). This relationship was studied by implanting novel chitosan gel beads into subcutaneous air pouches prepared on the dorsal surface of mice. No inflammatory response was observed and degradation of the beads in the air pouches increased as their degree of deacetylation decreased. Degradation could be modified by changing the nature of the chitosan or by increasing the chitosan concentration. The release of prednisolone (Fig. 8.10) in vivo from chitosan gel beads was comparable to its release in vitro. When a suspension of prednisolone was injected into the air pouches, it disappeared almost completely within 24 h. Retention of prednisolone in the air pouches was not increased by using a viscous chitosan solution. It was concluded that degradation and drug release of chitosan gel beads can be controlled by changing the structure of the gel matrix, which appears to make these beads a promising biodegradable vehicle for sustained drug delivery (Kofuji et al. 2001).

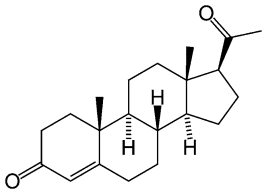

Fig. 8.10 Skeletal formula of prednisolone (courtesy of Fvasconcellos, http://en.wikipedia.org/wiki/File:Prednisolone-2D-skeletal.svg)

8.5.3.4 Carboxymethyl Chitosan Beads

Hydrogels have garnered great interest in the field of controlled-release drug-delivery systems. Crosslinking the hydrogels is important for maintaining their stability in aqueous media. Both chemical and physical crosslinking are frequently used. However, chemical crosslinking has a few disadvantages, the main ones being toxicity of the residual covalent crosslinkers or unwanted side effects with the drugs. To avoid these drawbacks, physical crosslinking is preferred for the synthesis of biocompatible hydrogels, because of the absence of covalent crosslinkers and chemical reactions (Berger et al. 2004). Ionic crosslinking is an effective simple and mild physical method, in which biological activity of the drugs is well retained (Wheatley et al. 1991). Hydrogel networks with ionic crosslinking generally exhibit pH-sensitive and ion-sensitive swelling. These advantages offer immense potential for their application as site-specific or controlled-release drug-delivery systems. Therefore, a few kinds of suitable systems were developed, including Ca^{2+}-crosslinked alginate (Zhang et al. 2004; Lin et al. 2005) and TPP-crosslinked chitosan (Alsarra et al. 2002). Carboxymethyl chitosan has many exceptional properties including no toxicity, biodegradability, biocompatibility, and antibacterial and antifungal bioactivity. This is why carboxymethyl chitosan has gained broad acceptance in biomedical applications (Liu X.F. et al. 2001). Carboxymethyl chitosan should be a good candidate for Ca^{2+}-crosslinked hydrogel beads. Nevertheless, instantaneous precipitation with an irregular shape (rather than bead formation) was observed upon addition of pure aqueous carboxymethyl chitosan into the $CaCl_2$ solution (Lin et al. 2005). Another report discussed the crosslinking of low molecular weight carboxymethyl chitosan with Ca^{2+} to fabricate pH-sensitive hydrogel beads. The swelling, bovine serum albumin (BSA) loading, and release properties of the beads were investigated. Chitosan-coated calcium-carboxymethyl chitosan beads were used to improve BSA entrapment efficiency (Liu et al. 2007). The beads exhibited pH sensitivity. The entrapment efficiency of BSA in the beads was greatly improved (from 44.4 to 73.2%) when the beads were coated with a chitosan–carboxymethyl chitosan polyelectrolyte complex membrane. The polyelectrolyte complex membrane limited BSA release, yet BSA could still be fully released along with the disintegration of the gel beads in pH 7.4 medium. Thus, the chitosan-coated calcium carboxymethyl chitosan hydrogel beads with higher entrapment efficiency and suitable protein-release properties presented a promising protein drug carrier for site-specific release in the intestine (Liu et al. 2007).

8.5.4 Gelatin

8.5.4.1 Gelatin Microspheres

Microparticulate delivery systems are usually manufactured from polymeric materials of synthetic or natural origin (Lawrence 1976). In general, natural polymers are preferred due to their low toxicity and biocompatibility. Gelatin is a good raw material for several reasons: it easily forms films and particles, it is available in a pyrogen-free form and it can be used for the production of delivery systems requiring bioadhesion to mucosal tissues. Bioadhesion is related to adhesion or absorption by the body. Aside from gelatin, some of the materials used today for this purpose are polymer matrices with the ability to absorb water. They include hydroxypropyl cellulose (HPC), cellulose derivatives such as sodium carboxymethyl cellulose (CMC), a carboxyvinyl polymer type which is a poly(acrylic acid), and plant-originated polysaccharides such as tamarine rubber (Machida and Nagai 2001). In this field, spherical or alternatively shaped gel beads are used for the study of adhesion mechanisms based on mucosal tissue location. The adhesion mechanism involves molecular chains that interpenetrate the mucous molecules and contribute to hydrogen bonding (Peppas and Buri 1985). This was demonstrated by introducing urea, which disrupts hydrogen bonding, and measuring the reduction in mucoadhesive force of Carbopol 934P disks that are potentially created by bonds between the polymer and glycoprotein within the mucus (Mortazavi 1995). Hard microspheres of both fumaric and sebacic acid adhered strongly to the mucosa of rat intestine, possibly due to hydrogen bonding (Chickering and Mathiowitz 1995). The adhesive force of disks composed of various mucoadhesive materials was checked and it was observed that they adhere more strongly to poly(vinyl chloride) than to rat intestinal mucosa (Mortazavi and Smart 1995). Water-soluble polymers such as carbopol were used to coat poly(glycerol ester) microspheres or were dispersed within a poly(glycerol ester) bead. The mucoadhesion strength was compared to a microsphere without carbopol. Microspheres in which the carbopol was mixed showed strong mucoadhesion capabilities in vitro (Akiyama et al. 1995).

Gelatin suffers from a few drawbacks, including rapid solubilization in aqueous environments, which results in fast drug-release profiles (Pecosky and Robinsonn 1991). Various factors can influence the rate of gelatin crosslinking (Digenis et al. 1994; Tomihata et al. 1994). These factors are typified by gelatin's molecular weight and isoelectric point and by the reaction temperature and pH. The disadvantage associated with the rapid solubilization of gelatin can in principle be solved by chemical crosslinking procedures (Digenis et al. 1994).

Gelatin is a natural, non-toxic, biodegradable polymer (see Section 2.4.2 for gelatin bead formation, strengthening, and modification). Simple coacervation with gelatin, using sodium sulfate as the coacervating agent and formaldehyde as the hardening agent, helped in preparing a reservoir-type device for in vitro drug release. Release rates were dependent on the hardening time of the microcapsule (Madan et al. 1976). Crosslinking of alginate by formaldehyde or glutaraldehyde is commonly used to form microspheres (Yoshioka et al. 1981; Yan et al. 1991) and

its density influences drug release (Ciao and Price 1989; Tabata and Ikada 1989; Raymond et al. 1990). First-order kinetics governs the release of some drugs from gelatin microspheres (Nixon and Walker 1971; Yoshioka et al. 1981; Goto et al. 1983).

8.5.4.2 Dextran as a Crosslinker of Gelatin Beads

Thermal hardening treatments (Esposito et al. 1996) and crosslinking agents, such as sugars or sugar-derived compounds, have been proposed for the production of gelatin devices to achieve long-term drug release (Ruys et al. 1983). Primarily, oxidized polysaccharides, such as dextran, have been described as crosslinking agents for gelatin beads (Schacht et al. 1993). The influence of dextran in both its native and oxidized forms as a possible crosslinking agent for gelatin microspheres was studied (Cortesi et al. 1999). Gelatin microspheres treated with native or oxidized dextran were produced; in addition, the physicochemical and release characteristics of the antitumor drug TAPP-Br (the bromo-derivative of the *p*-amidino phenoxy neo-pentane), used as a model compound, after dextran crosslinking was evaluated (Cortesi et al. 1999). TAPP-Br (Nastruzzi et al. 1989) belongs to a class of compounds proposed for the experimental and clinical treatment of a large variety of pathologies, including *Pneumocystis carinii* pneumonia in AIDS patients (Smith and Gazzard 1991; Gambari and Nastruzzi 1994) and cancer (Bartolazzi et al. 1989; Nastruzzi et al. 1992). Among aromatic polyamidines, TAPP-Br has been found to be a potent antitumor agent, both in vitro (Nastruzzi et al. 1992) and in vivo (Bartolazzi et al. 1989), in a number of cell lines of different histotype. Both native and oxidized dextran showed the capacity to reduce gelatin dissolution and, more interestingly, to slow down the release of both TAPP-Br and cromoglicate used as model drugs. Results suggested oxidized dextran as an alternative crosslinker of gelatin microspheres. This particular delivery system could potentially be functional for the in vivo administration of antitumor drugs by subcutaneous injection or chemoembolization procedures (Cortesi et al. 1999).

8.5.5 Modified Starch Microspheres

Starch is a polysaccharide carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. It is the most important carbohydrate in the human diet and is found in rice, wheat, maize, potatoes, and cassava. Starch consists of two types of molecules: the linear and helical amylose and the branched amylopectin. Depending on the plant, starch generally contains 20–25% amylose and 75–80% amylopectin (http://en.wikipedia.org/wiki/Starch). Microspheres can be produced from starch that is derivatized, for example, with glycidyl acrylate. These microspheres are biocompatible and enzyme degradable. The degradation is dependent on the degree of derivatization (Artursson et al. 1984; Laakso et al. 1986; Laakso and Sjoholm 1987). Starch-injectable microspheres were used to target lysozyme, human serum albumin, carbonic anhydrase, and immunoglobulin to the

reticuloendothelial system (Artursson et al. 1984; Artursson et al. 1987; Stjarnkvist et al. 1989; Kost and Shefer 1990; Degling et al. 1991; Stjarnkvist et al. 1991a, b). Calcium ions react with the hydroxyl groups on starch and the resultant crosslinking tightens the starch matrix. This affects both the degradation rate and the diffusion of molecules within and from the matrix (Wing et al. 1987). Release of low- and high molecular weight compounds from calcium-crosslinked starch was studied. Myoglobulin and BSA release was affected by amylase activity while release of salicylic acid was not (Kost and Shefer 1990). A triggered delivery system with naltrexone dispersed in a rate-controlling polymer was proposed. This system was composed of four layers from its core to the outside: bioerodible polymer with dispersed naltrexone, a protective coating, antibody-blocked enzyme, and a microporous membrane. The acidic starch coating prevented premature release of the drug, i.e., before activation of the preparation (Heller et al. 1990).

8.5.6 Dextran Beads

Dextrans are glucose polymers which have been used for over 50 years as plasma volume expanders. More recently, dextrans have also been studied for the delivery of drugs, proteins/enzymes, and imaging agents. These water-soluble polymers are available commercially in different molecular weights with a relatively narrow distribution (Mehvar 2000). Dextrans contain a large number of hydroxyl groups which can be easily conjugated to drugs and proteins by either direct attachment or through a linker (Larsen 1989). In terms of pharmacokinetics, the intact polymer is not absorbed to a significant degree after oral administration. Therefore, most of the applications of dextrans as macromolecular carriers are via injection (Mehvar 2000). Nevertheless, a few studies have reported the potential use of dextrans for site (colon)-specific delivery of drugs via the oral route. Subsequent to the systemic administration, the pharmacokinetics of the conjugates of dextran with therapeutic/imaging agents is significantly affected by the kinetics of the dextran carrier (Mehvar 2000).

Drugs were conjugated to dextran by cyanogen bromide activation, direct esterification, or periodate oxidation (Schacht et al. 1987; Schacht et al. 1988; Vansteenkiste et al. 1992). Details on special conjugates can be found elsewhere (Kojima et al. 1980; Takakura et al. 1987). Dextran and its derivatization products are degraded by enzymes present in the liver, intestinal mucosa, colon, spleen, and kidneys (Serry and Hehre 1956; Fisher and Stein 1960; Edman et al. 1980; Larsen 1989; Vercauteren et al. 1990; Crepon et al. 1991). Dextran microspheres were used to entrap several different proteins (Edman et al. 1980). *N*,*N*′-methylenebisacrylamide-crosslinked dextran microspheres were injected into mice: 60–80% of these particles were taken up by cells in the spleen and liver and stored in lysosomal vacuoles (Edman and Sjoholm 1982, 1983; Edman et al. 1983).

Microspheres have the potential to increase drug levels inside tumors by circumventing the tumor vasculature, to decrease normal tissue toxicities by reducing the level of drug in the systemic circulation, to overcome non-cellular

drug-resistance mechanisms, and to act as a depot for the extended release of drugs. Locoregional drug-delivery studies demonstrated that the microsphere system can provide more effective treatment of solid tumors because the drug can be localized to the tumor tissue (Emerich et al. 2000; Goldberg et al. 2002). The anticancer agent mitomycin C (MMC) (Fig. 8.11) is a bifunctional alkylating agent. It is a potent anticancer drug used in the treatment of superficial bladder and breast cancers. MMC delivery is effected by its encapsulation in microspheres using various polymers, including gelatin and ethylcellulose (Davis et al. 1984). Polymers such as poly(lactic-*co*-glycolic acid; PLGA) (Fig. 8.12) are unsuitable for the delivery of acid-labile MMC (Yen et al. 1996) because polymer degradation produces a highly acidic local environment (Fu et al. 2000). Based on successful loading and release studies of cationic drugs using sulfopropyl dextran microspheres (Liu et al. 1999; Liu et al. 2000; Liu Z. et al. 2001, 2003; Liu et al. 2001), and the fact that MMC has the potential to interact with anionic polymers owing to its amino groups, an attempt was made to load MMC into sulfopropyl dextran microspheres. Dextran-based microsphers were employed because dextran has been applied safely in vivo as a blood expander for years and the abundance of hydroxyl groups makes the system easily modifiable for the control of drug loading and release (Cheung et al. 2005). Various biodegradable dextran-based microsphere systems with different hydrophobicities, charges, and crosslinking densities have been investigated for the delivery of MMC. The characteristics of these microsphere systems as carriers of MMC have been evaluated, such as drug-loading capacity, drug-release rate, and drug stability following release. Among the systems studied in this work, oxidized sulfopropyl-dextran microspheres (Ox-MS) possessed superior properties for MMC delivery by offering a higher drug-loading capacity and better sustained drug release while maintaining drug stability. In addition, Ox-MSs physically degraded within a reasonable time frame, allowing for the possibility of repeated administration of

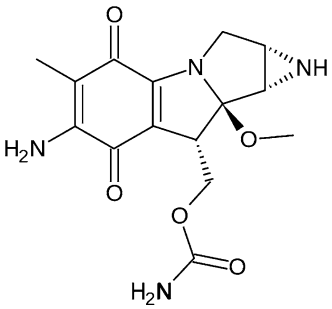

Fig. 8.11 Structure of mitomycin C (courtesy of Fvasconcellos, http://en.wikipedia.org/wiki/File:Mitomycin.svg)

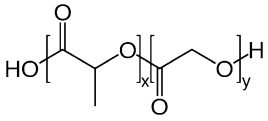

Fig. 8.12 Skeletal formula of poly(lactic-*co*-glycolic acid) (PLGA) (courtesy of Fvasconcellos, http://commons.wikimedia.org/wiki/File:PLGA.svg)

MMC-Ox-MS. Both ionic interactions and covalent bond formation are believed to be predominant mechanisms of MMC loading and release, though hydrophobic interactions play a role as well. The anionic charge of sulfopropyl groups facilitates MMC loading into sulfopropyl dextran microspheres by ionic complexation. Meanwhile, formation of Schiff bases between amino groups of MMC and the aldehyde groups produced by oxidation further enhances the loading capacity of the Ox-MS system. While extracts and degradation products from blank Ox-MSs have been shown to be non-toxic to murine breast cancer cells, the MMC released from Ox-MSs exhibits a cytotoxicity that is similar to fresh MMC. The results of this work suggested that the Ox-MS system has the potential to serve as an effective delivery system for MMC (Cheung et al. 2005).

8.5.7 Cellulose Hydrogels

The reader is referred to Section 2.3.6 to read about cellulose. The hydroxyl groups of cellulose can be partially or fully reacted with various reagents to generate derivatives with useful properties. Cellulose esters and ethers are the most important commercial materials (http://en.wikipedia.org/wiki/Cellulose). Cellulose ethers can be used for microcapsule production as well as for many other formulations (Doelker 1987). Since cellulose is not water soluble, it is necessary to reduce the regularity of the polymer chains and interchain hydrogen bonding. The cellulose derivatives that are used in the pharmaceutical area are methylcellulose (MC), hydroxyethylmethylcellulose (HEMC), hydroxypropylmethylcellulose (HPMC), ethylhydroxyethylcellulose (EHEC), hydroxyethylcellulose (HEC), and hydroxypropylcellulose (HPC). The release of water-soluble drugs from crosslinked HPMC involves both diffusion and matrix dissolution (Huber et al. 1966). The factors that govern drug release are polymer type, its concentration, drug particle size, and type and concentration of different additives (Ford et al. 1985a, b, 1987; Feely and Davis 1988a, b; Hogan 1989). The effect of varying a propranolol–HPMC formulation on the release of 160 mg propranolol hydrochloride into 1 l water at 37°C from tablets containing 57, 71, 95, 140, and 285 mg HPMC was studied. The polymer concentration was shown to control the kinetics of the release with no contribution of matrix erosion (Ford et al. 1985a, b; Feely and Davis 1988a). On the one hand, addition of magnesium stearate, stearic acid, or cetyl alcohol as hydrophobic lubricants did not affect the release of highly water-soluble drugs (Ford et al. 1985b). On the other hand, ionic surfactants reduced the release rate by forming drug–surfactant complexes (Feely and Davis 1988b). Ethylcellulose membranes are useful for polar water-soluble drugs. Release is dependent upon, among many other parameters, membrane topography, porosity, and coacervation manufacturing conditions (Moldenharuer and Nairn 1991) and can be described by first-order release kinetics or the Higuchi model (Jalsenjak et al. 1976; Salib et al. 1976; John et al. 1978; Deasy et al. 1980; Donbrow and Samuelov 1980; Benita and Donbrow 1982a, b).

8.5.8 Gellan Beads

In its natural form, gellan gum is composed of a linear structure of repeating tetrasaccharide units of glucose, glucuronic acid, and rhamnose in a molar ratio of 2:1:1 (Jansson et al. 1983; Nussinovitch 1997). It is partially acetylated with acetyl and L-glyceryl groups located on the glucose residues (Kuo et al. 1986). The presence of acetyl groups interferes with its ion-binding ability. Commercially obtainable gellan gum is deacetylated by alkali treatment (Kang et al. 1982). Because of the presence of free carboxylate groups in gellan gum, it is anionic in nature and, consequently, exhibits the characteristic property of undergoing ionic gelation in the presence of mono- and divalent cations. Nevertheless, its affinity for divalent cations is much stronger than for monovalent cations (Sanderson and Clark 1983). Gellan gum gelation involves the formation of double-helix junction zones followed by aggregation of double-helix segments to form a three-dimensional network by complexation with cations and hydrogen bonding with water (Grasdalen and Smidsroed 1987). Gellan gum beads for sustained release of sulfamethizole were prepared by hot extrusion into chilled ethyl acetate (Quigley and Deasy 1992). Gellan gum beads containing salbutamol sulfate have also been studied for sustained-release applications (El Fattah et al. 1998), and gellan gum beads for the controlled release of propranolol hydrochloride have been produced. In this latter case, drug release was rapid (Kedzierewicz et al. 1999). Gellan gum beads containing cephalexin were prepared by extruding the cephalexin dispersion and gellan gum into a solution containing a mixture of calcium and zinc ions. Beads were spherical in shape, with an average bead size ranging from 925 to 1183 μm (Agnihotri et al. 2006). Cephalexin entrapment at up to 69.24% was achieved. In vitro release studies were performed in phosphate buffer pH 7.4, and cephalexin release for up to 6 h was achieved. The release mechanism deviated slightly from an ideal Fickian trend. The study demonstrated that this manufacturing procedure can produce uniform gellan gum beads with sustained drug-release properties (Agnihotri et al. 2006).

8.5.9 Guar Beads

Hypothetically, any anionic polymer can be crosslinked to form particles. In the past, attention has been directed to alginate and to a lesser extent pectinate. Carboxymethyl guar gum (CMGG) has the ability to form microparticles for the gastrointestinal delivery of sensitive drugs such as proteins. Guar gum (Fig. 8.13) is a polygalactomannan derived from the seeds of the Leguminacea plant *Cyamopsis tetragonolobus*. Its anionic carboxymethyl derivative can be used for the production of high-viscosity solutions and can also be prepared in several degrees of derivatization (Whistler 1959, Nussinovitch 1997). Such derivatives have found application as inexpensive thickeners in the textile and paper industries and have also been studied for food and drug-delivery purposes. Beads of this material, containing indomethacin (Fig. 8.14), were made by dropping it into either very cold aqueous solution or a non-solvent (Nagori and Mathur 1996). Further study described

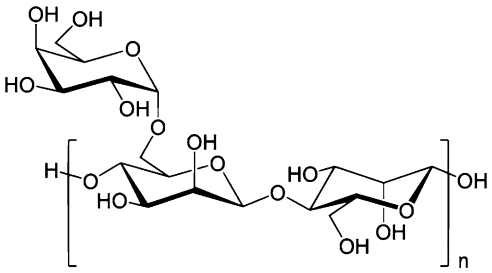

Fig. 8.13 Structural formula of a guaran unit (courtesy of Yikrazuul, http://en.wikipedia.org/wiki/File:Guaran.svg)

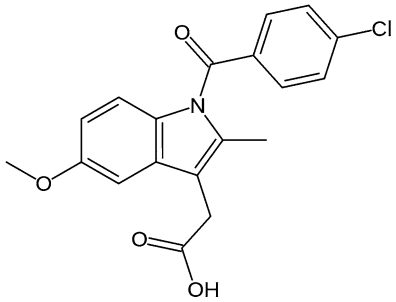

Fig. 8.14 Skeletal formula of indometacin (INN), also known as indomethacin (USAN) (courtesy of Fvasconcellos, http://en.wikipedia.org/wiki/File:Indometacin_skeletal.svg)

the preparation of CMGG, capable of forming spherical crosslinked beads when exposed to $Ca^{2+}$and Ba^{2+} (Thimma and Tammishetti 2001). As a model proteinaceous drug, BSA has been encapsulated in barium chloride-crosslinked CMGG beads and its in vitro release in simulated gastric and intestinal buffers was investigated. It was found that barium chloride-crosslinked beads protect the protein from the low pH conditions and deliver it completely in simulated intestinal fluid (Thimma and Tammishetti 2001).

8.5.10 Pectin

8.5.10.1 General

Pectin is a natural water-soluble polysaccharide. It is composed of D-galacturonic acid and a methyl ester. Pectin is consumed as part of the human diet and serves as a food additive, i.e., as a viscosity and gel former. The polysaccharide is not digested by enzymes released within the human gastrointestinal tract; rather, it is digested by the intestinal flora (Vargo et al. 1985). Pectin's ability to form a gel by complexing with metal ions is dependent on both its molecular weight and degree of esterification (Lofgren et al. 2002). Low-methoxy pectin forms a gel matrix with Ca^{2+} and can complex with chitosan (Durand et al. 1990).

8.5.10.2 Pectin Beads

Pectin has been studied as a potential medium for drug delivery since it transiently forms gel beads in solution by ionotropic gelation. In addition, the matrices of these beads are capable of incorporating drugs under mild conditions (Wakerly et al. 1997; Munjeri et al. 1998; Wong et al. 2002). Micrometer-sized calcium pectinate gel (CPG) beads containing insulin, as a model amphoteric protein, were prepared by ionotropic gelation technique together with an air compressor. Phosphate buffer, pH, and calcium and pectin concentrations of the crosslinking solution greatly affected the entrapment efficiencies and release profiles of the microbeads. The pH of the working solutions was at or close to the isoelectric point of the protein loaded during the preparation process. Consequently, CPG microbeads of perfect spherical shape, uniform size, enhanced mechanical strength, good entrapment efficiencies, and delayed release profiles were prepared for a load of amphoteric protein and peptide drugs, without any use of organic solvents or harsh ingredients. Therefore, CPG microbeads could be a promising carrier for oral controlled-release systems containing amphoteric protein and peptide drugs (Si et al. 2009). Pectin beads allow controlled drug release in the gastrointestinal tract when administered orally (Sriamornsak 1998; Musabayane et al. 2000; Ei-Gibaly 2002). A number of attempts have been made to control drug-release rates from pectin beads. When hardening agents such as glutaraldehyde were used to prepare rigid pectin gel matrices, safe drug delivery required the removal of such agents from the matrix (Sriamornsak 1999). Since pectin beads appear to disintegrate after oral administration when the crosslinked calcium ions within their matrices are replaced with other ions in the gastrointestinal tract, further control of their erosion is needed (Murata et al. 2004). An attempt to prepare spherical gel matrices, capable of containing drugs, without the use of a hardening agent was reported (Murata et al. 2004). In that study, pectin hydrolysate was used. The drug-release profiles of the pectin beads were affected by the rates of erosion of their gel matrices, which could be controlled by the preparation conditions. Therefore, pectin beads show promise with regard to the development of controlled-release drug formulations (Murata et al. 2004).

8.5.10.3 Pectin Beads for Colon-Specific Drug Delivery

Natural polysaccharides, such as pectin, chitosan, cyclodextrin, and dextran, have been used to a great extent for the development of solid dosage forms for drug delivery to the colon. The underlying principle is the colonic microflora's ability to degrade various types of polysaccharides that escape digestion in the small bowel (Vandamme et al. 2002). Pectins are broken down by a range of microbial sources including human colonic bacteria and may potentially be employed as colonic delivery systems if their solubility is reduced (Liu et al. 2003). Consequently, major effort has been focused on the quest for pectin derivatives, which are more water resistant while still enzymatically degradable (Liu et al. 2003). Colonic drug delivery is intended for the local treatment of ulcerative colitis and irritable bowel syndrome,

and can potentially be used for colon cancer or the systemic administration of drugs that are adversely affected by the upper gastrointestinal tract (Yang et al. 2002). Local treatment has a few advantages, among them diminished occurrence of systemic side effects, administration of lower drug doses, and maintenance of the drug in its intact form as close as possible to the target site (Lee and Mukkerjee 2002). An appealing approach is the use of calcium salts of pectins because calcium binding diminishes the solubility and induces non-covalent associations of carbohydrate chains through "egg-box" complexes (Sriamornsak 1998). Pectinate gel beads were manufactured by a crosslinking reaction between amidated low-methoxy pectin with ketoprofen and different solutions of crosslinking agents (calcium or zinc ions). Calcium pectinate and zinc pectinate beads were characterized by their morphological aspect and their drug content. The beads were then tested in vitro under dissolution conditions mimicking the gastric colon transit in order to check their efficacy at targeting the colon (Chambin et al. 2006). Zinc counterions created pectin beads with a stronger network matrix than that with calcium counterions. Acidification of the counterion solution increased the strength of the pectinate gel via two mechanisms: intermolecular crosslinking following the "egg-box" model and non-ionic hydrogen bonding or hydrophobic interactions (Chambin et al. 2006). The formed pectinate beads were evaluated for drug release under conditions mimicking the overall gastrointestinal tract. The strongest matrix, obtained with 10% zinc counterions at pH 1.6, reached the colonic medium with nearly the entire loaded drug dose and was attacked gradually by enzymes with subsequent drug release (Chambin et al. 2006).

8.5.10.4 Pectin–Chitosan Beads

The application of pectin as a carrier material for small-molecule drugs requires the adoption of a new formulation approach. Generally, the pectinate matrix is coacervated with chitosan on its external surface to further retard drug release (Munjeri et al. 1997; Takka and Acarturk 1999a, b). Loading of chitosan into the core of the pectinate matrix could successfully slow drug release without the need to process the beads with microwaves, which were needed to retard the release of drug from pectinate beads when the chitosan was introduced onto the matrix by means of coacervation (Wong and Nurjaya 2008). In response to the influence of microwaves, the drug-release property of beads made of pectin and chitosan was mostly modulated via the C–H, O–H, and N–H moieties of the polymers and the drug, with the C–H moiety assumed to retard while O–H and N–H functional groups were purported to enhance the extent of drug released from the matrix (Wong and Nurjaya 2008).

8.5.11 Modified Poly(Vinyl Alcohol) Microspheres

DC Bead^{TM} is a product for the treatment of hypervascular tumors and arteriovenous malformations (Lewis et al. 2007). The beads consist of poly(vinyl alcohol)

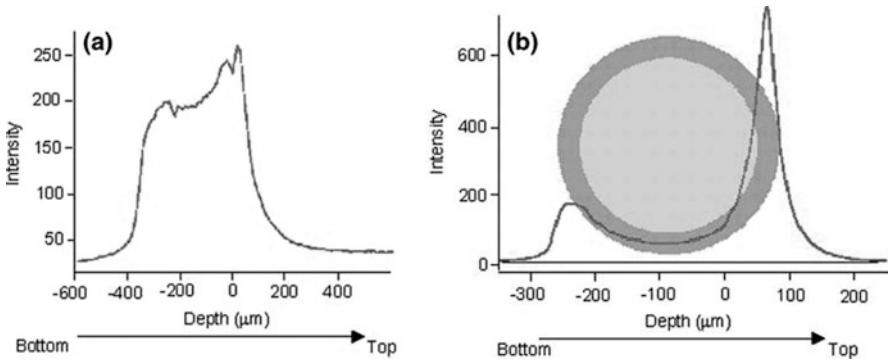

Fig. 8.15 Raman fluorescent microscopy depth profile of (**a**) an unloaded bead and (**b**) a doxorubicin hydrochloride-loaded bead (25 mg/ml 500–700 μm beads) (adapted from Lewis et al. 2007)

microspheres modified with sulfonic acid groups and are available in different sizes, ranging from 100 to 900 μm in diameter. The beads were shown to actively sequester doxorubicin hydrochloride (Dox) (Fig. 8.15) from solution, in a length of time that depended upon drug dose and bead size (Lewis et al. 2007).

Adriamycin® (Dox) is a cytotoxic, anthracycline antibiotic used in antimytotic chemotherapy. It is infused intravenously to treat neoplastic diseases such as acute lymphoblastic leukemia; Wilm's tumor; soft tissue and osteogenic sarcomas; Hodgkin's disease; non-Hodgkin's lymphomas; Ewing's sarcoma; and bronchogenic, genitourinary, breast, and thyroid carcinomas. Drug uptake was through an ion-exchange mechanism (Gonzalez et al. 2008), and in the absence of additional ions in solution, the beads could load a maximum of approx. 40 mg Dox/ml hydrated beads, with >99% of the drug being sequestered from the solution. A loading of 25 mg Dox/ml beads was recommended as a realistic therapeutic dose with optimum handling characteristics. There was a reduction in the equilibrium water content of the beads with increasing Dox loading, which resulted in a decrease in the average diameter of the beads and an increase in the compressive modulus (Lewis et al. 2007). The deliverability properties, however, were not affected after drug loading. Using a variety of microscopic methods, the drug was shown to be distributed throughout the bead structure, but concentrated in the outer 20-μm surface layer, a feature related to the method of synthesis (Lewis et al. 2007).

8.5.12 Biodegradable Hydrogels Based on Polyesters

Polyesters constitute a category of polymers which include the ester functional group in their main chain. Polyesters include naturally occurring chemicals, such as in the cutin of plant cuticles, and synthetics created by step-growth polymerization, for instance, polycarbonate and polybutyrate. Natural polyesters and a few synthetic ones are biodegradable, but most synthetic ones are not (Rosato and Rosato 2004).

Polyester prepolymers were crosslinked with PVP chains to create hydrogels. BSA release from the hydrogel was regulated by varying the amount of PVP (Heller et al. 1983). Since BSA is not capable of diffusing out, its release was dependent on polyester degradation. At 37°C and pH 7.4, less than 40% of the BSA was released in 40 days. This release could be substantially increased by using polyesters containing dicarboxylic acids. Another hydrogel was prepared from water-soluble polyesters containing itaconic or allylmalonic acid (Heller et al. 1983). In this case, no comonomers were necessary for the crosslinking. BSA release from the gels depended on the chemical structure and concentration of the unsaturated ester in the solution prior to crosslinking (Heller et al. 1983).

8.5.13 Hydrogels with Degradable Crosslinking Agents

8.5.13.1 Hydrogels Crosslinked with Small Molecules

Polymerized acrylamide in the presence of *N*,*N*′-methylenebisacrylamide and dissolved insulin formed hydrogels that were injected into alloxan (Fig. 8.16), inducing diabetic rats (Davis 1982). Diabetic rats implanted with 40% acrylamide maintained their normal growth rate for a few weeks. If a smaller concentration of acrylamide (25% by weight) was used, the implanted rats gained no weight due to the rapid release of insulin from the implant (Davis 1982). PVP hydrogels were crosslinked with *N*,*N*′-methylenebisacrylamide and chymotrypsin was included within the matrix. The hydrogels degraded slowly via hydrolysis of the crosslinker. This was dependent on the concentration of *N*,*N*′-methylene bisacrylamide: the lower it was, the more porous the structure of the gels and the more rapid the release of the drug (Torchilin et al. 1977).

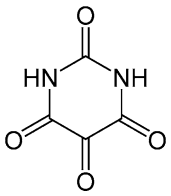

Fig. 8.16 Structure of alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone) (courtesy of Yikrazuul, http://en.wikipedia.org/wiki/File:Alloxan.png)

8.5.13.2 Hydrogels Crosslinked with Azo Reagents

Vasopressin was loaded into gelatin capsules and insulin was fabricated into pellets. The cores were coated with a film composed of poly(hydroxyethyl methacrylate-*co*-styrene) and crosslinked with divinylazobenzene. The coating protected the drugs from the conditions in the stomach and from digestion by enzymes in the intestines. The film was degraded by microorganisms in the colon that break down the azo bonds, causing release of the drug (Saffran et al. 1986).

8.5.13.3 Hydrogels Crosslinked with Albumin

PVP hydrogels can be crosslinked with albumin for long-term oral delivery of flavin mononucleotide (FMN; Fig. 8.17) (Shalaby et al. 1992). The half-life of FMN is ~70 min and its absorption is restricted to the upper small intestine (Juskor and Levy 1967). In vitro release of FMN in simulated gastric fluid occurred for over 400 h. The initial burst was accompanied by a zero-order release up to 300 h (Juskor and Levy 1967).

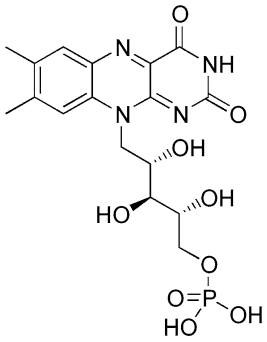

Fig. 8.17 Structure of flavin mononucleotide (courtesy of Edgar 181, http://en.wikipedia.org/wiki/File:Flavin_mononucleotide.png)

8.5.13.4 Hydrogels with Biodegradable Pendant Chains

Release of drug molecules that are bound to the polymer backbone is possible by enzymatic degradation of the labile bond attaching the drug to the polymer (Dittert et al. 1968; Kopecek et al. 1981; Rejmanova et al. 1981; Ulbrich et al. 1981; Ulbrich et al. 1982; Kopecek 1984; Bennett et al. 1991). The enzyme-induced release of *p*-nitroaniline from poly[*N*-(2-hydroxypropyl) methacrylamide] was studied. The rate of hydrolysis of the oligopeptides was dependent on their structure (Duncan et al. 1983; Rejmanova et al. 1983, 1985; Rihova and Kopecek 1985; Rihova et al. 1989). *p*-Nitroaniline release was linear for the first 30 min in all cases, with release slowing after a few hours (Subr et al. 1990).

8.5.14 Floating Beads

Multiparticulate systems obtained by ionotropic crosslinking of pectin, chitosan, carrageenans, sodium alginate, gellan gum, and similar natural biodegradable polysaccharides have been used to develop floating drug delivery. A variety of approaches to induce buoyancy in crosslinked beads exist, among them freeze-drying, entrapment of gas or gas-forming agents, and use of entrapped oils (Iannuccelli et al. 1998; Whithead et al. 2000; Sriamornsak et al. 2004). These approaches have several limitations, e.g., coalescence of oil droplets within the oil-containing beads yielding beads of wider particle size distribution, volatilization, or leaching of the oil (Murata et al. 2000). When sodium bicarbonate is used as

a buoyancy-imparting agent, evolution of carbon dioxide occurs when the bead is in contact with an acidic environment followed by the ability of the matrix to entrap the gas and decrease its density below one. Nevertheless, violent gas generation, disintegration of the dosage form, burst release, dose dumping, and alkaline microenvironment are limitations of these dosage forms (Stockwell and Davis 1986). Another approach is to generate carbondioxide gas within a polymeric bead followed by freeze-drying to improve its porosity (Choi et al. 2002). A floatable multiparticulate system was manufactured by crosslinking low-methoxylated pectin and sodium alginate. The beads obtained by freeze-drying remained buoyant for more than 12 h, while the air-dried beads stayed submerged. The study revealed the occurrence of air-filled hollow spaces inside the freeze-dried beads, which accounted for their flotation property (Talukder and Fassihi 2004). Floating calcium pectinate beads can be prepared by emulsion–gelation method, an alternative that overcomes the limitations of sodium bicarbonate-containing floating drug-delivery systems (Sriamornsak et al. 2005). Hollow calcium pectinate beads containing diclofenac sodium (Fig. 8.18) were manufactured by straightforward technique with in situ action of buoyancy-imparting agents throughout formation. In general, the buoyant beads demonstrated a lag phase during gastroretention followed by pulsatile drug release, which is likely to be valuable for chronotherapy of rheumatoid arthritis and osteoarthritis (Badve et al. 2007). The floating pulsatile concept was applied to increase the gastric residence of the dosage form having a lag phase followed by burst release. The resultant floating beads were porous (34% porosity), hollow with a bulk density <1, and had $F_{t50\%}$ (i.e., the time required to sink 50% of beads assuming linear approach of sinking) of 14–24 h (Badve et al. 2007). In vivo studies of rabbits by gamma scintigraphy showed gastroretention of beads for up to 5 h. The floating beads provided the expected two-phase release pattern with an initial lag time during flotation in acidic medium followed by rapid pulse release in phosphate buffer. This approach suggested the use of hollow calcium pectinate microparticles as a promising floating-pulsatile drug-delivery system for site- and time-specific release of drugs in disease chronotherapeutics (Badve et al. 2007).

8.5.15 Xyloglucan Beads

Materials that can pass through a thermally reversible sol–gel transition are of interest in the development of sustained-release vehicles with in situ gelation properties

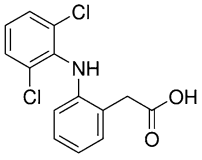

Fig. 8.18 Two-dimensional structure of NSAID-class drug diclofenac (courtesy of Harbin, http://en.wikipedia.org/wiki/File:Diclofenac.svg)

(Kawasaki et al. 1999). Characteristically, poloxamer, a triblock copolymer composed of poly(ethylene oxide) and poly(propylene oxide), transforms into a gel upon warming to body temperature by undergoing a sol–gel transition (Schmolka 1972). Nevertheless, the use of poloxamer as a carrier for drug delivery is limited due to the high concentration (20–30%) necessary for gelation (Schmolka 1972) and potential toxicity at several sites. These difficulties may be avoided through the use of natural polymers (Pitt 1990).

A good candidate is the polysaccharide xyloglucan, which also exhibits sol-to-gel transition in the required temperature range, and has the additional benefits of recognized non-toxicity and a much lower gelation concentration than poloxamer (Yuguchi et al. 1997). The xyloglucan derived from seeds of tamarind is composed of a (1-4)-β-D-glucan backbone chain, which has (1-6)-α-D-xylose branches that are partially substituted by (1-2)-β-D-galactoxylose. While xyloglucan is partially degraded by β-galactoxylose, the resultant product demonstrates a thermally reversible transition from sol to gel between 22 and 27°C in a concentration range of 1–2% (Miyazaki et al. 1998; Shirakawa et al. 1998).

To utilize xyloglucan for oral drug delivery, dosage forms that are convenient for administration need to be developed. Xyloglucan gel beads have quite a few advantages, for instance, the capacity for drug loading, decreased risk of dose dumping, and ease of dose adjustment (Bodmeier 1997; Bulgarelli et al. 2000), in addition to convenient administration. Nevertheless, the stability of xyloglucan beads is lower in the gastrointestinal tract. A special coating process can improve their stability as well as enable the colon-specific release of drugs that are known to irritate the gastric mucosa. Specific acrylic polymers, Eudragit, have been developed for oral dosage forms with stepwise release of the active ingredient in the digestive tract (Breitkreutz 2000; Fan et al. 2001; Cole et al. 2002). Drug-loaded xyloglucan beads were prepared by dropwise extrusion of an indomethacin dispersion in an aqueous xyloglucan solution through a syringe into corn oil. Enteric coating of the xyloglucan bead was performed using Eudragit to improve its stability in the gastrointestinal tract and to achieve gastroresistant drug release. The release behavior of indomethacin from xyloglucan beads was investigated in vitro as a function of loaded drug content, pH of the release medium, and concentration of the coating agent. The adhesive force of xyloglucan was also measured by tensile test (Yoo et al. 2005). Uniform-sized spherical beads with particle diameters ranging from 692 ± 30 to 819 ± 50 μm were obtained. The effect of drug content on the release of indomethacin from xyloglucan beads depended on the medium pH. Release of indomethacin from xyloglucan beads was retarded by coating with Eudragit and increased rapidly with a change in medium pH from 1.2 to 7.4. Adhesive force of xyloglucan was stronger than that of carbopol, a well-known commercial mucoadhesive polymer, in the wet state. Results indicate that enterically coated xyloglucan beads may be suitable as carriers for oral drug delivery of gastric-irritant drugs through the stomach (Yoo et al. 2005).

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Chapter 9
Beads and Special Applications of Polymers for Agricultural Uses

9.1 Introduction

Beads can be used to encapsulate microorganisms, agricultural chemicals, genes, exogenous DNA, seeds, and other agents for use in agriculture. The appeal of this concept lies in the fact that immobilization products are easy to produce, store, and handle during industrial operation. This chapter describes a few of the goals of encapsulation in agriculture: immobilization of plant cell suspensions and single seeds, carriers for the slow release of bacteria that affect plant growth, inoculation of seedlings and plants with bead-encapsulated fungal inoculum, joint immobilization of plant growth-promoting bacteria and green microalgae, and cryopreservation by encapsulation/dehydration technique. In addition to the controlled release of agricultural chemicals, some biotechnological applications, such as gene delivery by beads, bioactive beads for obtaining transgenic plants, and synthetic seed technology, will also be described. Finally, the chapter provides a very brief description of the unique applications of polymers in agriculture, such as in cases where superabsorbency is required or when seed coatings may be beneficial.

9.2 Immobilization of Plant Cell Suspensions and Single Seeds

Cells isolated from higher plants can be cultivated in submerged cultures. However, the plant cell is much larger than a microbial cell, and these large cells are held together by only a fragile wall; as such, they are susceptible to shear forces in culture. Plant cells in suspension culture grow in aggregates containing from a few up to hundreds of cells, making their large-scale cultivation technically more difficult (Brodelius and Nilsson 1980; Brodelius and Mosbach 1982; Brodelius 1983, 1985). In addition, the slow growth of plant cells poses some special problems for culturing in large volumes, since it may take up to 2 weeks from inoculation to the stationary phase; furthermore, a plant cell culture cannot be diluted too much because the cells condition their own medium. Thus, the time needed for growth from a small culture (100 ml) to a technical-scale culture (1000 l) may be as long as 2 months, much longer than the time required for large-scale cultivation of

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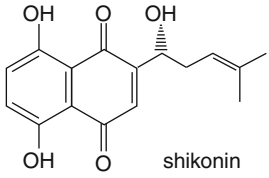

Fig. 9.1 Structure of shikonin, a naturally occurring dye found on the outer surface of the roots of many traditional medicinal plants

microorganisms (Brodelius 1985). The earliest chemical compound obtained from plant tissue culture was shikonin (Fig. 9.1), a dye and pharmaceutical used in Japan for its antibacterial and anti-inflammatory properties. It was produced by cultures of *Lithospermum erythrorhizon* developed in a two-stage batch process by Mitsui Petrochemical Industries Ltd (Tokyo, Japan). The cells were cultured for 3 weeks and the product yield was estimated at a few kilograms per batch. Mitsui's development can be considered a major advance in plant tissue culture technology, since it demonstrated that a secondary metabolite can be produced by plant tissue culture techniques on a commercial basis (Brodelius 1985). Many studies have reported the immobilization of plant cell suspensions (Brodelius 1985; Suvarnalatha et al. 1993; Vanek et al. 1994; Dornenburg and Knorr 1995; Aburjal et al. 1997; Seki et al. 1997; Verpoorte et al. 1999; Gilleta et al. 2000). Plant cells have been immobilized within a variety of matrices, e.g., alginate, carrageenan, and different synthetic supports. Nevertheless, the effects of variations in immobilization parameters toward enhancing the excretion and production of secondary metabolites have been only infrequently evaluated (Vanek et al. 1999). An interesting example is the immobilization of *Nicotiana tabacum* cells within alginate beads. *N. tabacum*, or cultivated tobacco (Fig. 9.2), is a perennial herbaceous plant. It is found only in cultivation, where it is the most commonly grown of all plants in the genus *Nicotiana*, and its leaves are commercially grown in many countries to be processed into tobacco (Ren and Timko 2001). Such entrapment strongly affects the physiology of *N. tabacum* cells and substantially enhances the production and removal of scopolin. Therefore, the immobilization of *N. tabacum* cells under optimum conditions is of major interest for scale-up and use as a novel bioreactor (Gilleta et al. 2000).

Immobilization can be performed on a small or large scale. It is perhaps not surprising that several techniques for the immobilization of single seeds have been invented. For example, a technique for mechanically encapsulating hybrid tomato seeds singly in sodium alginate capsules was studied (Garrett et al. 1989, 1991). In this technique, tomato seeds climb spiral paths in a vibrating bowl. Air jets and seed sensors send one seed at a time to annular nozzles where alginate, pumped in discrete drops, forms a meniscus which catches the seed. Air inflates the meniscus slightly as a pulse of alginate material traps the seed (Garrett et al. 1991). Drops, falling into a hardening bath, are inspected by a digital camera. An air jet blows capsules without seeds into a reject receptacle; seed-containing capsules fall into a bath of calcium chloride and are hardened. The developed system was capable of producing 100-μl gel capsules at a maximum rate of one capsule every 1.2 s per nozzle. However, capsules of 80 μl could not be produced and a production rate of

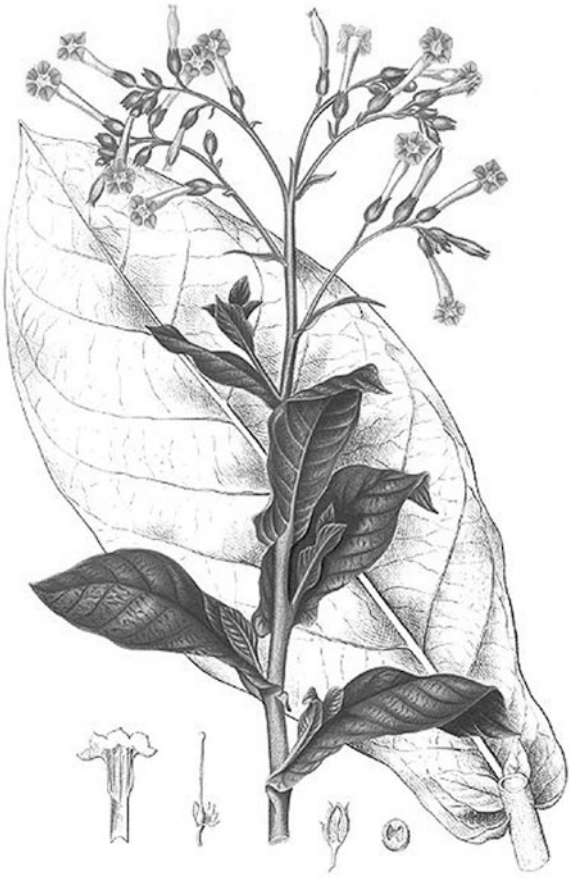

Fig. 9.2 Illustration of *Nicotiana tabacum* (from Franz Eugen Köhler, in *Köhler's Medizinal Pflanzen,* published in 1887)

5×10^5 capsules/day was not possible (Garrett et al. 1991). Another method for encapsulating seeds consists of a loop of wire, dipped into a solution of sodium alginate, which captures a meniscus of gel (Garrett et al. 1994). A seed, placed on top of the meniscus, is driven inside the drop and the drop detaches from the loop during impact. The detached drop of gel, with the seed inside, falls into a solution of calcium chloride where a skin of insoluble calcium alginate forms around the drop. Wire and loop diameters and gel concentration all affected the volume of gel in the formed capsules. Wire loops ranging from 3.2 to 11.1 mm in diameter were fabricated from high-strength wire of diameter 0.56–1.04 mm. Gels in concentrations ranging from 1.25 to 2.50% by weight were used. Capsules ranging in volume from 2.5 to 240 ml were formed from the gel at temperatures ranging from 5 to 35°C. A device with 16 loops, complete with a vacuum-powered seed-singling unit, was capable of producing at least 160 capsules per minute (Garrett et al. 1994).

9.3 Carriers for Slow Release of Bacteria that Affect Plant Growth

In the last decades, increasing attention has been paid to beads from a range of materials and dimensions suitable for the immobilization of microorganisms, enzymes, antibodies, etc. The main rationale for using such beads was the ability to immobilize organisms or proteins for a long period and then collect the products of the entrapped living material. Liberation of the immobilized living material was considered a drawback in such systems (Hackel et al. 1975; Chibata and Tosa 1977; Cottrell and Kovacs 1977; Brodelius and Nilsson 1980; Birnbaum et al. 1981; Fukui and Tanaka 1982). Reports suggested the use of several different polymer gels for entrapping fungi and bacteria for use as agricultural inoculants (Dommergues et al. 1979; Jung et al. 1982; Fravel et al. 1985; Muguier and Jung 1985). In an alginate-based formulation, the populations of two immobilized plant growth-promoting bacteria, *Bacillus subtilis* and *Pseudomonas corrugata*, were on the order of 10^8 CFU/g. The plant-based bioassay indicated that the plant growth-promoting ability of both bacterial isolates was equal to that of fresh broth-based formulations. The bacterial isolates retained root colonization and antifungal and enzyme activities during storage (Trivedi and Pandey 2008). The direct immobilization of soluble peroxidase isolated and partially purified from shoots of rice seedlings in calcium alginate beads and in calcium-agarose gel was carried out. Peroxidase was assayed for guaiacol oxidation products in the presence of hydrogen peroxide (Nahakpam et al. 2008). The presence of calcium ions helped immobilize the peroxidase and mediated direct binding of the enzyme to the agarose gel. Agarose appeared to be a better immobilization matrix for the peroxidase than sodium alginate (Nahakpam et al. 2008).

Inoculation of plants with non-symbiotic rhizospheric bacteria for crop improvement is a complicated task. Most studies have produced variable and inconsistent results (Albrecht et al. 1981; Kapulnik et al. 1981; Reynders and Vlassak 1982; Millet and Feldman 1984; Sarig et al. 1984; Smith et al. 1984). In addition, the modes of action of these bacteria need to be further studied (Okon 1985). Inoculation of legumes with their symbiotic rhizobia (Thompson 1980), a widely and commonly used practice, is carried out by two main methods: (i) direct inoculation with bacterial culture and (ii) use of a variety of solid-phase bacterial inoculants anchored in organic materials (mainly peat variants), including organic granular particles. Both means are reasonably priced, straightforward to use, and have simple formulation preparations. Nevertheless, commercial formulations of peat-based bacterial inoculants used to coat seeds or pellets for sowing in furrows have quite a few severe drawbacks because of the nature of peat and its unavailability in many countries (Kenney 1997).

Carriers for the slow release of uniform bacterial inoculant (beads) were developed to replace the organic inoculants (Bashan 1986). These beads, manufactured from sodium alginate and skim milk, enclose a large reservoir of bacterial culture, releasing their enclosed bacteria at a slow and constant rate. The beads are biodegradable and pose no ecological hazard, and their mechanical properties, rate of bacterial release, and time of survival in the soil are controlled (Bashan 1986).

Use of the final product, the lyophilized dry beads (see Chapter 6), is straightforward and they are applied to the seeds concomitantly with sowing. The released bacteria are instantly available for root colonization at seed germination (Bashan 1986). Furthermore, dry beads containing bacteria can be stored at ambient temperatures for long periods without loss of bacterial content; storage requires little space, and quality control of the bacteria in the bead is simple (Bashan 1986). Another report dealt with an optimized process for manufacturing a crop inoculant with the strain *Azospirillum lipoferum*. This process also involved the immobilization of living cells in alginate beads and dehydration. The influence of several parameters—alginate concentration, the stage at which adjuvants were added, dilution of culture broth, water activity, and dehydration method—on bacterial survival was studied (Fages 1989). Maximum survival was achieved by adding skim milk and using controlled air dehydration of the alginate beads. The end product was a powdered inoculant, containing more than 10^{10} cells/g, which was easy to store and handle and which could be used in the field as microgranules. Furthermore, its biodegradability ensured environmental friendliness (Fages 1989).

As stated, the most common experimental formulation for bacterial inoculants is macrobeads with a diameter of 1–4 mm, for either agricultural or environmental use (Gonzalez and Bashan 2000). Nonetheless, their relatively large size might have disadvantages for agricultural uses. Two possibilities exist to produce smaller beads: (i) mechanically crush large beads or solid alginate sheets and then sieve the powder to the desired size and (ii) produce microbeads directly using a suitable technology (Bashan et al. 2002). Alginate microbeads were produced by low-pressure spraying of an alginate solution mixed with liquid bacterial culture suspended in a very rich medium through a small nozzle, resulting in small-diameter droplets. These droplets, when sprayed into a slowly stirred solution of calcium chloride, immediately hardened into microbeads with diameters ranging between 100 and 200 μm (Bashan et al. 2002). Dried inoculant was prepared using dry air at 38°C, which created a powdery substance loaded with beads containing >10^9 CFU/g. Alternatively, dried microbeads were produced using a standard freeze-drying procedure (Bashan et al. 2002). This dry preparation was easily attached to dry seed surfaces with the addition of 1% alcohol-diluted lecithin or 0.5% synthetic adhesive. The bacteria were slowly released from the microbeads in amounts ranging from 10^4 to10^6 CFU/g depending on the bead type (wet or dry, with or without skim milk) and time of incubation (the longer the incubation period, the smaller the amount of bacteria released with time). The wet and dry inoculants enhanced the development of wheat and tomato seedlings growing in unfertile soil and biodegraded within 15 days in moist soil (Bashan et al. 2002).

9.4 Inoculation of Seedlings and Plants with Beads Containing Fungal Inoculum

Different types of carriers of fungal inocula, for uses such as biological control, fungal spawn, introduction of mycorrhizal fungi, and bioaugmentation, are of great interest. For instance, carriers of fungal inocula for biological control consist of

an alginate hydrogel, with or without an additional nutrient source (Walker and Connick 1983) and vermiculite (Walker 1981). Vermiculite is a natural bronze-yellow mineral, with the chemical formula $(MgFe,Al)_3(Al,Si)_4O_{10}(OH)_2 \cdot 4H_2O$, which is formed by hydration of certain basaltic minerals and expands with the application of heat (Fig. 9.3). Examples of carriers for mycorrhizal fungal inocula include an alginate hydrogel, with or without a supplementary nutrient source, and vermiculite and peat (Le Tacon et al. 1985). Examples of carriers of fungal inocula for fungal spawn include grain, sawdust–grain mixtures, vermiculite saturated with nutrient broth (Stamets 2000), and alginate hydrogel (Romaine and Schlagnhaufer 1992). Ectomycorrhizal (ECM) fungi form a symbiotic relationship with the plant by forming a sheath around the plant's root tip. The fungus then forms a hartig net (an inward growth of hyphae—a fungal cell growth form—which penetrate the plant root structure). There are actually seven types of mycorrhizae and 90% of plants form mycorrhizal associations with fungi, but ECM refers only to the sheath-forming type of mycorrhizae (http://www.nifg.org.uk/ecto.htm). In a mycorrhizal association, the fungus may colonize the roots of a host plant either intracellularly or extracellularly. Such associations are an important part of soil life (Kirk et al. 2001). In mycorrhizal symbiosis, in return for carbohydrates, a fungal partner provides the host plant with several advantages. Trees planted in soils that are deficient in ECM fungi and even on routine reforestation sites have been shown to benefit from mycorrhizal associations, although the positive effect of mycorrhizae on seedlings is not consistent. ECM associations are characteristically formed between the roots of ~10% of plant families, mostly woody plants including the birch, dipterocarp, eucalyptus, oak, pine, and rose families, and fungi belonging to the *Basidiomycota*, *Ascomycota*, and *Zygomycota* (Wang and Qiu 2006).

Commercially produced seedlings are often grown in greenhouses before being transferred to the open sales outlets, usually with an unfavorable impact on

Fig. 9.3 Vermiculite (the image was featured in the *Minerals and Materials Photo Gallery* on the web site of the U.S. House Subcommittee on Energy and Natural Resources)

mycorrhizal formation. One option for nursery managers in the production of ECM planting stock is to artificially inoculate the substrate used for producing seedlings (Kropp and Langlois 1990). Along with the use of spores and various carriers containing mycelium (Honig et al. 2000), alginate bead inoculum was developed and recommended (Kropacek and Cudlin 1989). The mycelium entrapped in alginate beads is better protected, survives longer, and has a longer term outcome than that grown on a vermiculite-peat mixture (Mortier, Le Tacon and Garbaye 1988). Substrate conditions significantly affect seedling emergence (Oleskog et al. 2000), growth (Tammi et al. 2001), ECM abundance (Van der Heijden and Kuyper 2001; Rincon et al. 2005), and composition (Jones et al. 2003).

Peat (Fig. 9.4) is the principal growth medium employed for the production of tree seedlings in several parts of North America and Europe. The physical properties of peat are comparatively poor for plant growth (Heiskanen 1993), as well as for ECM formation (Tammi et al. 2001). If there are insufficient natural sources of peat and/or it comes at a high cost, a supplementary medium such as bark might be tested. Bark material is rather coarse, and it is therefore repeatedly composted in order to enhance water retention and mechanical strength and to diminish the harmful effects of toxic components (Repac 2007). Growth substrate is evaluated by quantifying the growth response of seedlings or by comparing the conditions in the substrate to those recommended for plants. Norway spruce (*Picea abies* (L.) Karst.) is a coniferous tree species used for reforestation (Fig. 9.5). Its seedlings are mostly produced as bareroot nursery stock in a 3- to 4-year rotation. Since almost all Norway spruce roots in natural forest stands are in ECM associations (Ostonen and Lohmus 2003), inoculation in the nursery may favor subsequent seedling establishment in the field. Numerous species of ECM fungi that colonize *P. abies* have been identified (Mihal 1999), many of which may hold considerable potential for

Fig. 9.4 Milled peat production in the Irish Midlands

Fig. 9.5 Norway spruce (from *Köhler's Medizinal Pflanzen*, 1887)

artificial inoculation of seedlings. The effects of selected fungal species and growth substrates on the formation of ECM associations and growth of 1-year-old bare-root Norway spruce seedlings inoculated at sowing with alginate beads containing fungal inoculum and grown under standard operational conditions in a greenhouse without the addition of fertilizer were studied. The fungal inoculum-containing bead was prepared by immobilizing the fungal mycelium in alginate gel and forming beads in calcium chloride solution. Details on the production of the inoculum are reported in Repac (2007). Alginate beads, applied at a dose of 125 ml per vessel (500 ml/m^2), contained ~5 g/m^2 of mycelium (dry weight) from each of the fungi. Several authors had reported increased emergence, ECM formation, or growth of forest tree seedlings inoculated with fungal mycelium encapsulated in alginate beads (Mortier et al. 1988; Ingleby et al. 1994; Parlade et al. 1999). However, with Norway spruce, inoculation of the substrates with vegetative alginate beads containing inoculum of three ECM fungi was not efficient. It appeared that indigenous fungi play a crucial role in mycorrhizal formation with Norway spruce seedlings (Repac 2007). Furthermore, it was concluded that further studies contributing to the analysis of factors affecting the belowground dynamics of mycorrhizal fungi and

the development of seedlings are needed to define practical recommendations for seedling cultivation (Repac 2007).

Early inoculation of mycorrhizae throughout the weaning phase of micropropagated plantlets is advantageous for quite a few tropical species, such as papaya, avocado, pineapple, and banana (Jaizme-Vega et al. 2002). A variety of sources of pot, hydroponic, and aeroponic inoculum, i.e., spore, colonized roots, and rhizospheric soil, respectively, have been used effectively. The use of an in vitro root-organ culture (ROC) technique (Fortin et al. 2002) for the production of arbuscular mycorrhizal fungus (AMF) inoculum is another option compatible with plant micropropagation (Declerck et al. 2002). An overview of the potential techniques for in vitro propagation of AMF has been provided (Moutoglis and Béland 2001) and a bioreactor-based production technique using ROC has also been proposed (Jolicoeur et al. 1999). The effect of ROC-produced AMF, i.e., *Glomus proliferum*, *Glomus versiforme* and *Glomus intraradices*, entrapped in calcium alginate beads on the first developmental stages of micropropagated bananas (*Musa* spp. cv. Grande Naine) was investigated (Jaizme-Vega et al. 2003). Banana plants were inoculated with one of the three AMFs or one of two controls—empty alginate beads and no beads. Calcium alginate-entrapped ROC-produced AMF spores were able to colonize the root system of the micropropagated banana cultivar under nursery conditions, increase plant phosphorus nutrition and biomass, and proliferate in the commercial nursery substrate, consequently increasing the fungal inoculum biomass. The entrapment of ROC-propagated spores, adaptable to a wide range of Glomeromycetes, thus represents a promising alternative pathogen-free inoculum (Jaizme-Vega et al. 2003).

9.5 Joint Immobilization of Plant Growth-Promoting Bacteria and Green Microalgae

The *Chlorella* genome is the smallest characterized genome of all eukaryotic, photosynthetic microorganisms (Higashiyama and Yamada 1991), which makes it a desirable alternative to higher plants with their large genomes in interactions with plant growth-promoting bacteria (de-Bashan and Bashan 2008). *Chlorella* spp. (Chlorophyceae) are simple, non-motile, unicellular, aquatic green microalgae. They were one of the first algae to be isolated as a pure culture (Oh-Hama and Miyachi 1992). *Chlorella* has been employed in studies of photosynthesis and respiration (Ilangovan et al. 1998). A great deal of the knowledge on the synthesis of carbohydrates in microalgae was acquired with this species (Hosono et al. 1994; Ramazanov and Ramazanov 2006). Since the late 1940s, attention has been drawn to the prospective mass cultivation of this microalga for the manufacture of high-value, low-volume compounds, such as pigments for the food industry, including the health food market in developed countries (Richmond 1990), and its application in wastewater treatment (Oswald 1992). A simple, quantitative experimental model offering a convenient and basic approach to studying plant–bacterium interactions has

been proposed. This involves immobilizing a unicellular, freshwater microalga—a *Chlorella* species—serving as the plant, with a plant growth-promoting bacterium—an *Azospirillum* species—in alginate beads to enable their close interaction while avoiding external interference from bacterial contaminants (de-Bashan and Bashan 2008).

Depending on the microalga–bacterium combination and with time (6–48 h), both microorganisms are found in the same cavity within the bead (Lebsky et al. 2001). The bacteria excrete indole-3-acetic acid (IAA) (Fig. 9.6) and other undefined signal molecules, possibly reaching the nearby microalgal cells. At this stage, the activities of the microalgal enzymes glutamine synthetase and glutamate dehydrogenase are not enhanced. However, in the next phase of interaction, beginning at 48 h, glutamate synthetase and glutamate dehydrogenase activities, photosynthetic pigment production, and nitrogen and phosphorus uptake into microalgal organelles are enhanced (Gonzalez and Bashan 2000). The jointly immobilized system liberates oxygen produced by the *Chlorella* sp. as a by-product of photosynthesis and the population of microalgae increases two- to threefold over that of microalgae immobilized alone (Gonzalez and Bashan 2000). It was concluded that such a model would be an appropriate experimental tool to study events related to growth and nitrogen and phytohormone cycles in eukaryotes (de-Bashan and Bashan 2008).

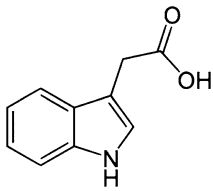

Fig. 9.6 Structure of indole-3-acetic acid

9.6 Cryopreservation by Encapsulation/Dehydration Technique

The production of callus and in vitro conservation of haploid cells might be considered an option for extending the period during which haploid culture can be used. Nevertheless, subcultured cells undergo various chromosomal and ploidy (i.e., the number of complete sets of chromosomes in a biological cell) changes that may result in genetic changes in the cultures (Towill and Walters 2000). In view of the significance of haploid cultures in mutation studies, biochemical genetics, and haploid breeding programs, use of isolated anthers may be very important. Freeze preservation may be relevant to anther storage and needs to be expanded. Storage of cryopreserved anthers would encourage an even distribution of labor, time, and space in the production of homozygous lines of each genotype. Physiological and biochemical studies also require anther storage without change over time. Some attempts have been made to cryopreserve pollen (Towill and Walters 2000), pollen embryos, and segments of anthers in *Triticum* spp. (Bajaj 1990a), *Arachis* spp. (Bajaj 1990b), and isolated microspores of *Brassica* spp. (Chen and Beversdorf 1992).

Cryopreservation has been studied in rice embryogenic suspension cells (Zhang et al. 2001), with callus for the characterization of cryopreserved plants (Moukadiri et al. 1999) and with callus to assess the surviving cells' capacity to yield competent protoplasts for genetic transformation (Cornejo-Martin et al. 1995). On the other hand, cryopreservation of rice anthers showed limited success (Coulibaly and Demarly 1979; Bajaj and Sala 1991) in that only minimal callus was formed and only albino plants were regenerated. The encapsulation/dehydration method for cryopreservation was detailed for shoot tips of a *Solanum* sp. (Fabre and Dereuddre 1990), which were encapsulated in alginate beads, precultured in liquid medium with a high sucrose concentration, and partially desiccated before cooling in liquid nitrogen. This encapsulation/dehydration procedure has been effectively applied to shoot tips of more than a few plant species, including several genotypes of pear, apple, sugarcane, and potato (Engelmann 1997). Anther-derived rice plants were obtained after cryopreservation by an encapsulation/dehydration technique. A distinct characteristic of the rice flower is its six anthers, rather than the customary three in other grasses. Spikelets have a single floret, the lemma and palea completely enclose the caryopsis or fruit, which may be yellow, red, brown, or black (http://www.answers.com/topic/rice). Immature anthers, removed from spikelets pretreated at 88°C for 8 days, were encapsulated in calcium alginate beads (Marassi et al. 2006). The beads were cultured on medium with naphthaleneacetic acid and furfurylaminopurine. Fifteen percent of the encapsulated anthers formed calluses when pretreated with sucrose for 3 days in liquid medium, desiccated on silica gel, slowly cooled to –30°C, immersed in liquid nitrogen, thawed, and recultured. The cryopreserved encapsulated anthers produced 1.67 shoots per callus, in contrast to the control (non-cooled encapsulated anthers), which produced 6 shoots per callus. After transfer to the greenhouse, 80% of the plantlets developed into normal plants. Histological observations showed that the origin of the plants was not modified by the cryopreservation process (Marassi et al. 2006).

9.7 Controlled Release of Agricultural Chemicals

Polymers can be used for the controlled release of agricultural chemicals (i.e., fungicides, germicides, growth regulators, herbicides, insect diets, insecticides). Many considerations are involved in fine-tuning a polymer for a specific system, including: price, seasonal conditions, desired release rate, duration, simplicity of formulation, and application. Other issues include the nature of the polymer, its thermostability or thermoplasticity, melting and glass transition temperatures, its compatibility with bioactive agents, stability of the combination, processing conditions, and desired shape and size of the final product (Paul 1976). These polymers control the rate of delivery, mobility, and the length of time for which the constituent will be regarded as effectual. The ultimate benefit of controlled-release formulations is that less chemical is used for a given time interval, thus lowering its impact on non-target species and limiting leaching, volatilization, and degradation (Nussinovitch 1997). Controlled-release systems are divided into two

categories: in the first, the active agent is dissolved, dispersed, or encapsulated by coating or a polymer matrix and in the second, polymers enclose the active agent as part of a macromolecular backbone or pendent side chain. Numerous natural and synthetic polymers and synthetic elastomers are utilized for controlled-release purposes. Natural polymers include carboxymethyl cellulose, gelatin, gum arabic, starch, and arabinogalactan. Synthetic elastomers include polybutadiene, polyisoprene, neoprene, polysiloxane, styrene–butadiene rubber and silicone rubber. Many synthetic polymers exist including: poly(vinyl alcohol), polysterene, polyacrylamide, poly(vinyl chloride) (Nussinovitch 1997). Physical systems that integrate agricultural chemicals include microencapsulation, physical blends, dispersion in plastics or rubbers, laminates, hollow fibers, and membranes. Models for the release of active materials have been developed and processing techniques are discussed in Harris (1975) and Kydonieus (1980). Natural polymers are used for controlled-release purposes because they are abundant, comparatively inexpensive, and biodegradable. They can participate in derivatization and formulation, whereas their inadequacy lies in their insolubility in some solvents that are suitable for encapsulation, dispersion, reaction, and formulation. In some instances, this could limit the amount of bioactive agent per unit weight of polymer. This problem has been solved in some cases by in situ encapsulation, which is achieved by crosslinking gelatinized starch containing the material of interest by either calcium chloride or boric acid, or by xanthation followed by oxidation. The outcome of such a procedure is entrapment of the material of interest within a granular formed particle. Using this technique, many herbicides—vernolate, butylate, EPTC, cycloate, surpass, pebulate, metribuzin, trifluralin, chloramben, picloram, dicamba and thiocarbonates, the nematicide DBCP and the insecticides dimilin, diazinon, parathion, malathion, captan and zineb—have been formulated (Harris 1975; Allan et al, 1980; Kydonieus 1980; Mark et al. 1985).

9.8 Biotechnological Applications

9.8.1 General

As its name implies, biotechnology is technology based on biology, agriculture, food science, and medicine. Modern use of the term usually refers to genetic engineering as well as cell- and tissue culture technologies (http://en.wikipedia.org/wiki/Biotechnology). Biotechnology can also be regarded as "any technological application that uses biological systems, dead organisms, or derivatives thereof, to make or modify products or processes for specific use. Biotechnology draws on the pure biological sciences (genetics, microbiology, animal cell culture, molecular biology, biochemistry, embryology, cell biology) and in many instances is also dependent on knowledge and methods from chemical engineering, bioprocess engineering, information technology, and biorobotics" (http://en.wikipedia.org/wiki/Biotechnology).

9.8.2 Gene-Delivery Systems Using Beads

Several kinds of drug-delivery systems have been developed to transfer mRNA and DNA molecules, as well as proteins, into fungi and animal cells. Most of these systems make use of liposome (Rodicio and Chater 1982; Rizzo et al. 1983) or organic polymer beads that entrap the molecules of interest, for instance DNA molecules for a cell's genetic transformation (Suryakusuma and Jun 1984). Oil/water (O/W)- and water/oil (W/O)-type emulsions are used to produce small, spherical particles. O/W emulsions are used to manufacture particles that will carry hydrophobic molecules, while W/O emulsions are used to create carriers for hydrophilic molecules. By carefully controlling the reaction and concentration of the solidified materials, the mean size and size variation of the particles can be regulated. Some researchers have utilized a copolymer of 2-hydroxyethyl methacrylate and acrylamide as the core and matrix ethyl cellulose as the barrier membrane (Suryakusuma and Jun 1984). Other studies have reported that polyethyleneimine is a suitable material to obtain high transfection efficiency in cultured human cells (Boussif et al. 1995; Godbey et al. 1999). Chitosan, a cationic polymer of deacetylated chitin, is also useful for high-efficiency transformation in mammalian cells (Borchard 2001) and alginate is a kind of hydrophilic polysaccharide that undergoes gelation in the presence of appropriate crosslinking agents (see Chapter 2). Alginate is non-toxic to both animal and plant cells and has been used as a material to immobilize bacteria in bioreactors and to encapsulate plant somatic embryos as artificial seeds (Kersulec et al. 1993). Micrometer-sized calcium alginate beads encapsulating plasmid DNA molecules carrying a reporter gene were manufactured and checked for their potential gene delivery abilities. To evaluate the efficiency of these "bio-beads" in mediating genetic transfection, protoplasts isolated from cultured tobacco cells (BY-2) were transfected with bio-beads containing a plasmid that carries the modified green fluorescent protein (GFP) gene *CaMV3.WsGFP*. Treatment with the bio-beads generated approximately 10-fold higher GFP expression after 24 h incubation compared to that with the conventional method using a naked plasmid solution. Transfection was up to 0.22% efficient. These results indicate the potential of bio-beads for efficient transformation in plants (Sone et al. 2002).

9.8.3 Bioactive Bead Method for Obtaining Transgenic Plants

Genetic transformation of plants is an essential step in gene manipulation. Several transformation methods are currently available for delivering exogenous DNA into animal and plant cells: *Agrobacterium*-mediated transformation, particle bombardment, and electroporation are regularly used (Rakoczy-Trojanowaka 2002). Some alternative systems, such as infiltration and silicon carbide-mediated transformation (Kaeppler et al. 1990), are also frequently used. Nevertheless, every one of these systems has definite shortcomings, such as low transformation efficiency, plant species limitations, difficulty acquiring regenerated plants, and unprofitable results because of the presence of multiple copies of introduced genes (Liu et al. 2004). Therefore,

there is interest in developing new transformation methods for plant genetic manipulation research. An efficient gene delivery method for plants using calcium alginate bioactive beads to immobilize high-density DNA molecules in combination with polyethylene glycol (PEG) treatment has been reported. The efficiency of transient expression with bioactive beads was reported to be 5- to 10-fold higher than PEG treatment with naked plasmids (Sone et al. 2002). Using this method, chromosomal DNA of up to 450 kb has been successfully transformed into yeast cells (Mizukami et al. 2003). Transformation of tobacco SR-1 by bioactive beads showed more efficient transient expression than that by PEG treatment alone. The transformation efficiency of the method was higher than that obtained using the naked DNA and PEG treatment methods. Only one flowering transgenic plant was obtained with both the *GFP* and neomycin phosphotransferase II (*NPTII*) genes although a high degree of transient GFP expression was detected (Liu et al. 2004).

9.8.4 Synthetic Seed Technology

Encapsulation technology has garnered a great deal of attention in recent years because of its wide use in tissue conservation and delivery. Production of synthetic seeds by encapsulating somatic embryos, shoot buds, or any other meristematic tissue minimizes the cost of micropropagated plantlets for commercial uses as well as their delivery. In most fruit crops, seed propagation is unsuccessful because of seed heterozygosity, minute seed size, presence of reduced endosperm, low germination rate, and the existence of seedless varieties ((Manoj et al. 2009). Many species have moderately desiccation-sensitive or recalcitrant seeds which can be stored for only a few weeks or months. Thus for these fruit crops, encapsulation technology for propagation and conservation could be advantageous. Fruit plants are studied worldwide for breeding, genetic engineering, propagation, and pharmaceutical purposes. In this context, synthetic seeds would be more amenable to the exchange of elite and axenic plant material among laboratories and extension centers due to small bead size and their resistance to handling. Thus, interest in using encapsulation technology for fruit species is on the rise (Manoj et al. 2009).

Seeds are in essence the delivery system for agricultural biotechnology. High-quality seed leads to excellent seedling performance in the field. Seeds are the crucial foundation for success in companies that breed crop plants for seed production. Synthetic seed technology has been successfully developed for the use of somatic embryos and/or other micropropagules as seed analogues in the field or greenhouse and for their mechanical planting at a commercial level (Ara et al. 2000). The technology provides methods for the preparation of seed analogues, called synthetic seeds or artificial seeds, from micropropagules such as somatic embryos, axillary shoot buds, apical shoot tips, embryogenic calli, and protocorm or protocorm-like bodies (Ara et al. 2000). A propagule is any plant material used for the purpose of plant propagation. In asexual reproduction, a propagule may be a woody, semi-hardwood, or softwood cutting, a leaf section, or any number of other plant parts. In sexual reproduction, the propagule is the seed or spore. In micropropagation, a type of asexual reproduction, any part of the plant may be used,

though the highly meristematic tissues are best (i.e., meristematic tissues are cells or groups of cells that have the ability to divide; these tissues in a plant consist of small, densely packed cells that can keep dividing to form new cells), such as root and stem ends or buds (http://en.wikipedia.org/wiki/Propagule).

Encapsulation of vegetative propagules is a potentially cost-effective clonal propagation system that can be used as an alternative to synthetic seeds derived from somatic embryos (Adriani et al. 2000; Chand and Singh 2004). These encapsulated vegetative propagules can also be used for germplasm conservation of elite plant species and for the exchange of axenic plant materials between research institutions (Maruyama et al. 1997). Encapsulation of shoot tips in calcium alginate presents a space-saving alternative for storage at low around 0°C temperatures (Lisek and Olikowska 2004). Regardless of these benefits, there are only a handful of reports on the encapsulation of vegetative propagules (Pattnaik and Chand 2000; Brischia et al. 2002; Danso and Ford-Lloyd 2003; Chand and Singh 2004; Lisek and Olikowska 2004; Singh et al. 2006b). *Phyllanthus amarus* is an important medicinal plant used in traditional medicine for the treatment of jaundice, asthma, hepatitis, tuberculosis, ulcer, and urinary diseases (Calixto et al. 1998). It also possesses antibacterial, antifungal, antiviral, diuretic, hypoglycemic, antihypertensive, and insecticidal properties (Verpoorte and Dihal 1987; Bhattacharya and Bhattacharya 2001; Ghanti et al. 2004). Encapsulation of *P. amarus* shoot tips in calcium alginate beads can be used for mass clonal propagation as well as for germplasm conservation (Singh et al. 2006a). Shoot tips excised from in vitro-proliferated shoots were encapsulated in calcium alginate beads: the best gel complexation was achieved using 3% sodium alginate and 75 mM $CaCl_2 \cdot 2H_2O$. The maximum response for conversion of encapsulated shoot tips into plantlets was 90% after 5 weeks of culture on Murashige and Skoog (MS) medium without plant growth regulators. The regrowth ability of encapsulated shoot tips was affected by the concentration of sodium alginate, storage duration, and the presence or absence of MS nutrients in the calcium alginate beads (Singh et al. 2006a). Such research could prove useful for germplasm preservation, the disease-free micropropagation of plant species, and the production of synthetic seeds in which somatic embryogenesis has not been reported or in which somatic embryos fail to germinate into complete plantlets (Singh et al. 2006a).

9.9 Unique Applications of Polymers

9.9.1 Superabsorbent Polymers

Peat moss, perlite, and superabsorbent polymers (SAPs) are all used to improve water-holding capacity. The water uptake of peat moss and perlite amounts to 10 kg per 1 kg of dry product. The water is easily released upon pressurization since it is held by capillary effect. The water uptake of SAPs, on the other hand, is several hundred kilograms per kilogram of dry product and the water is not easily released by pressure application. The potential use of these polymers for agriculture appears promising; however, current use is low due to their high cost relative

to that of the resultant products and to other factors related to the environment such as soil condition, climate, and growing methods (Ouchi 2001). HSPAN (hydrolyzed starch–polyacrylonitrile) has a unique water-absorbing capacity of over 800 times its weight in distilled water. Other water-soluble polymers have found use in other applications in planting and growth development. To eliminate drying of the root zone in seedlings, gel coatings have been used. Advantages of such techniques are prevention of root drying, reduced wilting and transplant shock, and improved plant survival via the incorporation of fungicides, pesticides, or nutrients in the hydrogel slurries (Mark et al. 1985). Poly(ethyleneoxide) gel can absorb ~100 times its weight in water. The absorbed water is also readily desorbed by drying these hydrogels. Therefore, they can be used as soil amendments. Mixing with soil at a wt % ratio of 2×10^{-4}:1 reduces evaporative loss of water from the soil, resulting in less frequent watering in addition to full development of plants grown in such treated soil (Herrett and King 1967).

Commercially available superabsorbent preparations include one of the following polymers as their main component: poly(acrylic acid)-type, starch-graft copolymer-type, poly(vinyl alcohol) (PVA)-type, PVA-poly(acrylic acid)-type, polyacrylamide-type, isobutylene-maleic acid copolymer and cellulose-type polymer. These main components are used for a large variety of superabsorbent products that are manufactured by various companies, not necessarily for agricultural purposes (Ouchi 2001). Particle sizes of 0.15–0.25 mm and a bulk density of 0.85 g/cm^3 were reported for SIG (a SAP manufactured by Sumitomo Chemicals, tradename IgetagelP). SIG is produced from vinyl alcohol and sodium acrylate as its main components. It has a water absorption capacity of 500 kg/kg for pure water and ~100 kg/kg for water from a river in Iran, one of the locations in which it was field tested. The discrepancy in these values may be due to the fact that 75% of the contained cations were divalent, since the electrical conductivity of this river is 1.2–1.4 mS/cm. With different irrigation water and soil compositions, a range of 100–200 kg/kg was observed (Ouchi 2001). Most of the water (98–99%) held by SIG is free water and as such, it is available to plants. Semi-bound and bound water accounts for 1–1.5 kg/kg dry SAP. SIG is applied by mixing with soil, and its particles are thus dispersed among the soil particles. Upon water absorption, the volume of SIG increases due to swelling. Upon re-drying, SIG shrinks, but does not return to its original volume. As a result of SIG shrinkage, the gas phase also increases, along with soil aeration. Other properties that are changed as a result of SIG inclusion within a soil are a small increase in the permeation coefficient of water at saturation, smaller variations in soil temperature relative to a reference soil, a decrease in the elimination ratio of positively charged cations (mainly due to the negative charge of SIG), and a higher absorption of elements (nutrients) than in a reference area (Ouchi 2001).

9.9.2 Seed Coating

Pea seeds were treated to reduce root rot. To prepare the desired coating, the fungus *Trichoderma harzianum* isolate ThzID1 was grown in liquid culture, formulated

with alginate and PEG 8000, and milled into fine granules (average diam. 500 μm). Granules contained *T. harzianum* chlamydospores, conidia, and hyphal fragments. Viability of the encapsulated fungus remained high for at least 6 months when stored at 5°C (>90% of the granules produced hyphal growth when incubated on agar), but viability was significantly reduced when granules were stored at 22°C. Application of the granular formulation to pea seeds reduced root rot caused by *Aphanomyces euteiches* in growth-chamber experiments and also increased plant top weights compared with non-coated seeds. The results were a potentially improved formulation methodology for coating seeds with biocontrol organisms and a method for evaluating the compatibility of fungal and bacterial biocontrol agents applied to seeds (Dandurand and Knudsen 1993). In another study, two methods of applying a biological control agent (*T. harzianum* T581 from soil) directly to asparagus seeds were evaluated. Seed coating was achieved using talcum and sodium alginate as dispersant and thickening agent, respectively, for the conidial suspension, with or without the addition of a food-based compound (chitin). Encapsulation of *Trichoderma* was then obtained by dropping the coated seeds into a 0.1 M calcium gluconate solution. It was concluded that seed treatment with *T. harzianum* enhances asparagus seed germination (Nipoti et al. 1990).

Encapsulation was also useful in the preparation of artificial seeds by encapsulating somatic embryos, obtained on MS medium containing 3.0 mg 2,4-D and 0.5 mg kinetin per liter, in calcium alginate beads. Germination frequencies of 96 and 45% were achieved in vitro and in the soil, respectively. The in vivo plantlet conversion frequency was increased to 56% following an additional coating of mineral oil on the alginate beads. Germinated artificial seeds could be raised into plantlets (Mukunthakumar and Mathur 1992). Most of the reports on seed coating deal with alginate coatings; however, reports on other gels can also be found. In field trials, sorghum seeds were left to dry, soaked in water for 4 h (primed) and dried, or pregerminated by soaking for 18 h until radicle extrusion and then sown in a 1.4% Laponite gel suspension into a dry seedbed of self-mulching Mywybilla clay soil and given different amounts of irrigation. Emergence increased with irrigation and was higher with dry seed than with primed or pregerminated seed. At intermediate soil contents (0.2 g/g), gel-treated seeds showed enhanced emergence relative to dry sowing (Ferraris 1989). The authors found that at 9 ml 1.2% Laponite gel/seed, the gel application overcame the limitations of a dry soil and seedling emergence rate improved. Priming or pregermination of sorghum seeds did not afford any significant advantage in either field or laboratory studies (Ferraris 1989).

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Chapter 10
Beads for Environmental Applications

10.1 Introduction

In our industrialized civilization, a large variety of contaminants are released to the environment on a daily basis, from residential, commercial, and industrial sources. Significant release of a contaminant/hazardous substance has the potential to impact humans' physical condition and the environment. Environmental pollution is contamination of the environment which causes discomfort, instability, or disorder and has a harmful impact on physical systems and living organisms (Fig. 10.1). Pollution can take the form of chemical substances or any waste in a solid, semisolid, liquid, or gaseous form. Pollutants are considered contaminants when they exceed natural levels. Ecology is all about how the environment is maintained, degraded, or destroyed by human beings and the various harmful effects associated with the ecological imbalance. There are many types of pollution: air pollution, water pollution, soil pollution, and radioactive contamination. Note that this chapter does not deal with noise pollution, light pollution, visual pollution, or thermal pollution (http://dnr.wi.gov/org/aw/rr/archives/pubs/rr674.pdf; http://www.energy-environment.net/environmental_pollution.html).

10.2 Water Treatments

10.2.1 General

Water pollution is the contamination of water bodies such as lakes, rivers, oceans, and groundwater caused by human activities, which can be harmful to organisms, including plants, which live in these water bodies (Fig. 10.2). Water pollution has many causes and characteristics. The most important sources of water pollution are usually assembled into two categories based on their point of origin: point source pollution refers to contaminants that enter a waterway through a discrete "point source"; non-point source pollution refers to contamination that, as its name suggests, does not originate from a single discrete source (http://www.energy-environment.net/environmental_pollution.html).

A. Nussinovitch, *Polymer Macro- and Micro-Gel Beads: Fundamentals and Applications*, DOI 10.1007/978-1-4419-6618-6_10,

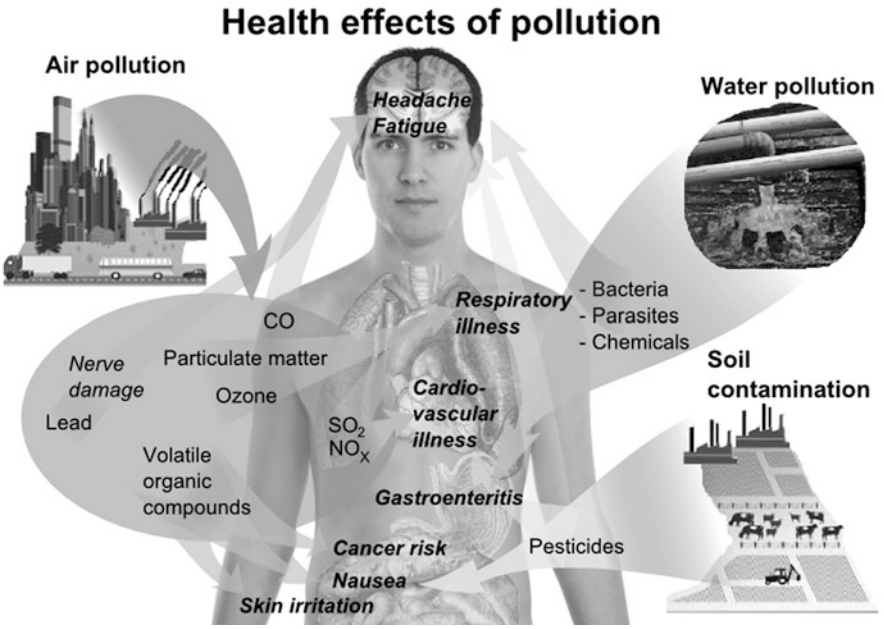

Fig. 10.1 Overview of the main health effects on humans of some common types of pollution (source: Mikael Häggström—http://en.wikipedia.org/wiki/Pollution)

10.2.2 Wastewater Treatment by Anaerobic Fixed Bed Reactor

Wastewater is any water whose quality has been adversely affected by anthropogenic influences. Anthropogenic sources include industry, agriculture, botany, mining, transportation, construction, habitations, and deforestation. Wastewater comprises liquid waste discharged by domestic residences, commercial properties, industry, and/or agriculture and can encompass a wide range of potential contaminants at various concentrations. In its most common usage, it refers to the municipal wastewater that contains a broad spectrum of contaminants resulting from the mixing of wastewater from different sources (http://en.wikipedia.org/wiki/Wastewater).

Often, wastewater from food-processing plants exhibits high biological oxygen demand (BOD) and may be held at high temperatures. This concentrated wastewater can be diluted with other, lower strength wastewater and aerobically treated by an activated sludge method, but this method consumes a great deal of energy, not to mention suffering from reactor efficiency that could be much improved (Kawase 1993). Anaerobic treatment has potential merit but conventional anaerobic treatments are not comparable to aerobic treatments because of instability and low treatment speed. Effort has therefore been invested in improving anaerobic treatments. A ceramic-filled anaerobic fixed-bed reactor can stably treat high-strength organic wastewater in a short time using thermophilic methanogen (Kawase 1993). Carriers based on ceramics are not new (Fig. 10.3). Ceramics as

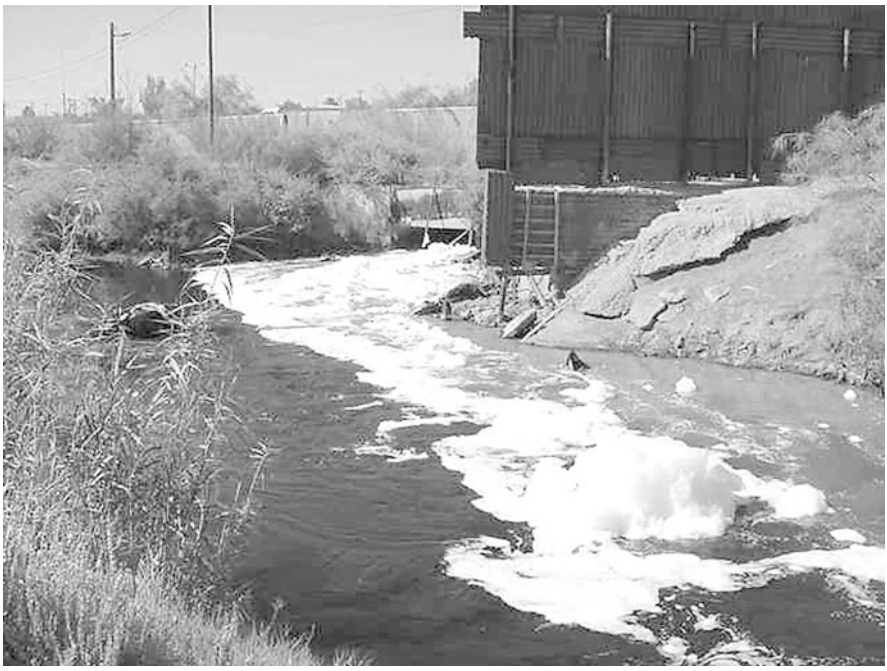

Fig. 10.2 Raw sewage and industrial waste flows into the US from Mexico as the New River passes from Mexicali in Baja California to Calexico, California (source: Calexico New River Committee—CNRC)

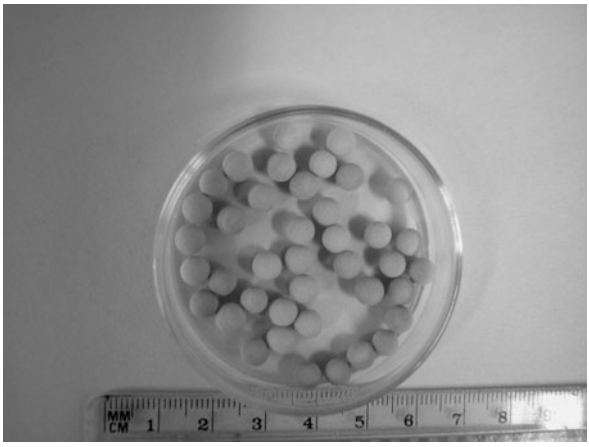

Fig. 10.3 A ceramic carrier

support materials for stationary fixed film reactors (Van der Berg and Kennedy 1981) and porous ceramic carriers for methanogen immobilization have been reported (Majima et al. 1984). A porous ceramic-filled anaerobic fixed-bed reactor is filled with methanogen-immobilizing ceramic as the carrier. The mean pore size diameter

is 15 μm, suitable for microbial growth, and the 70% porosity facilitates the immobilization of large quantities of anaerobes per unit carrier volume (Kawase 1993). The porous ceramic carrier unit consists of a ceramic carrier and resin frame (300 × 300 × 500 mm), the former having narrow ditches (5–10 mm wide × 40–60 mm deep) that create vertical passageways after the units are filled.

Experiments with a laboratory-scale porous ceramic-filled anaerobic fixed-bed reactor were conducted on wastewater from the miso, *natto* (fermented tofu), food (boiled adzuki beans and soybean whey), and beer industries. BOD ranged from 6600 mg/l (soybean whey) to up to 80,000 mg/l (beer). The BOD loading rate was 7 kg/m^3 per day (adzuki beans) up to 70 kg/m^3 per day (beer). BOD removal rate was 71% (minimal) for the *natto* process and maximal at 90% for the beer process. In general, the treatment capacity was observed to be related to the overall surface area of the filled carrier. Further pilot plant experiments with boiled soybean wastewater (BOD of ~4000 mg/l) demonstrated a high removal rate (>80%). Even higher rates are possible if the biomass is removed by other methods. The system has been successfully introduced into the soybean industry and its use is being promoted for the beer industry (Kawase 1993).

10.2.3 Wastewater Treatment Using Immobilized Microorganisms

A comparison of the features of fixed biofilm and immobilization methods revealed that in the former, immobilization depends on natural adhesion of the microorganism, growth occurs on the carrier surface at a rate greater than that of autolysis, and enzyme production is low. Immobilized microorganisms, on the other hand, can be stored selectively, growth occurs on the interior of the carrier, bacterial growth and autolysis are balanced, and enzyme production is considerable (Sumino et al. 1993). Organic substances (BOD) and nitrogen or phosphorus from wastewater can diffuse into the gel carrier and be consumed by the entrapped microorganism. Moreover, excess sludge production appears to be lower than that in conventional methods of biological treatment, since the leakage of bacteria to the gel exterior is limited, sludge aging is prolonged and propagated bacteria can easily undergo autolysis. Another issue is that the gel interior differs from a regular propagation environment: ATP yield for cell generation is reduced and therefore the volume of bacterial cells generated decreases (Sumino et al. 1993).

BOD treatment requires large-scale equipment and long treatment times. Efficiency can be improved by enhancing the bacteria's natural adhesion to specially designed plates or fine particles of sand. Immobilization in pellets is also a good option. The synthetic acrylamide (Fig. 10.4) was found superior to the natural polysaccharides agar, alginate, and carrageenan due to its better stability over time (Sumino et al. 1993). The acrylamide concentration was fixed at 18% to decrease the escape of bacteria that occurs in the looser gel structure at lower acrylamide concentrations. In addition, the activated sludge was protected from the inhibitory effects of the acrylamide polymer by a macromolecular coagulant (Praestol 444 K). The respiration rate of the immobilized microorganism was 3–15 times that in the

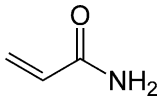

Fig. 10.4 Structure of acrylamide

traditional method. The BOD of the treated water using this system stabilized 2 days into the experiment and remained as low as 20 mg BOD/l or less until the load reached 8.6 kg BOD/m^3 per day (Sumino et al. 1993).

10.2.4 Arsenic Removal from Water

Arsenic has been classified as one of the most toxic and carcinogenic chemical elements (Fig. 10.5). In numerous countries, especially Argentina, Bangladesh, India, Mexico, Mongolia, Thailand, and Taiwan, a large proportion of the groundwater is polluted with arsenic at levels from 100 to over 2000 μg/l (Smedley and Kinniburgh 2002). The existence of arsenic in groundwater as a consequence of anthropogenic pollution has become a major public health concern (Pokhrel and Viraraghavan 2006; Dinesh et al. 2007). An additional difficulty facing many countries is the occurrence of naturally high incident levels of arsenic in the groundwater (Pokhrel and Viraraghavan 2006). Consequently, the removal of arsenic from industrial effluents, groundwater, and even drinking water systems has become an essential aim. A maximum concentration of arsenic of 10 μg/l in the drinking water has been recommended by the WHO (World Health Organization 1993).

Long-term exposure to arsenic in the drinking water can lead to both cancerous and non-cancerous effects and disorders (Chen et al. 1997). In addition to its

Fig. 10.5 Arsenic production based on the US Mineral Commodity Summaries Report 2007 (source: BlankMap-World,_compact.svg)

tumorigenic potential, arsenic has been recognized as genotoxic (Rudel et al. 1996) and the US Environmental Protection Agency (EPA) has lowered its drinking water limit to the recommended 10 μg/l (Schnoor 1996). Arsenic can be removed from the water by a variety of treatment technologies. Widespread methods include co-precipitation using $Fe_2(SO_4)_3$ or $FeCl_3$ or coagulation with ferric or aluminum salts such as $Al_2(SO_4)\cdot 18H_2O$ as coagulants (Hering et al. 1997; Meng et al. 2002), ion exchange (Vagliasindi and Benjamin 1998), use of adsorption media (Singh and Pant 2004; Vaughan and Reed 2005), use of zerovalent iron (Su and Puls 2001), and reverse osmosis and electrodialysis (Waypa et al. 1997). Comparatively speaking, adsorption is considered to be less costly than membrane filtration, easier and safer to handle than precipitation with its production of contaminated sludge, and more adaptable than ion exchange (Schnoor 1996).

The adsorption of arsenic, both as As(III) and as total arsenic, has been studied using a range of natural solids, including clay minerals (Manning and Goldberg 1997), manganese dioxide (Ouvrard et al. 2002), sand (Lo et al. 1997), zeolite (Bonnin and Tampa 2000), and red mud (Fuhrman et al. 2004). Exclusion of As(III) and As(V) has been performed by means of activated alumina (Singh and Pant 2004) and activated carbon (Pattanayak et al. 2000), as well as lanthanum and zirconium compounds (Suzuki et al. 2000), oxides and iron hydroxides (Appelo et al. 2002), open-celled cellulose sponges (Munoz et al. 2002), and biomass materials (Loukidoua et al. 2003). Cellulose beads can serve as potential adsorbents due to their hydrophilic properties, porosity, and high surface area. Cellulose and cellulose-derivative beads are widely used as ion exchangers, filler materials for chromatography, adsorbents for heavy metal ions and proteins, cosmetic ingredients, and carriers for the immobilization of biocatalysts (Stamberg and Peska 1983; Boeden et al. 1991; Wolf 1997). Therefore their capacity to remove arsenic from the groundwater appears promising. An adsorbent cellulose bead loaded with iron oxyhydroxide (BCF) was prepared and applied for the adsorption and removal of arsenate and arsenite from aqueous systems (Guo and Chen 2005). Akaganeite (β-FeOOH), the reactive core of BCF, exhibited high sensitivity to arsenite as well as arsenate, with an adsorption capacity of 99.6 and 33.2 mg/g BCF, respectively, at pH 7.0 with a Fe content of 220 mg/ml. Kinetics data fitted the pseudo-second-order reaction model well. Arsenate elimination was favored at acidic pH, whereas the adsorption of arsenite by BCF was found to be effective in a wide pH range, from 5 to 11. Furthermore, BCF sustained its high removal efficiency of arsenic following regeneration (Guo and Chen 2005).

The focal point of arsenic pollution remediation has shifted from treating groundwater to treating industrial wastewater, predominantly effluent from the manufacture of gallium arsenide (GaAs) supports. These effluents include comparatively high As(V) and trace As(III) concentrations compared to groundwater polluted with arsenic compounds. The compound GaAs is composed of two elements, gallium and arsenic. It is an important semiconductor and is used to make devices such as microwave-frequency integrated circuits, infrared light-emitting diodes, laser diodes, and solar cells (Coleman and Monzyk 1988). Numerous arsenic-removal technologies and their combinations have been developed. Aluminum

and iron salts are functional coagulants in removing arsenic compounds via the coagulation-precipitation method (Han et al. 2002). Nevertheless, the production of large amounts of toxic sludge has limited the use of such systems (Dinesh and Charles 2007). As a result, addition of, e.g., chlorine (as an extra oxidizing agent) to the treated water is typically needed to convert As(III) to As(V). Regrettably, such agents frequently damage the reactor membranes, substantially shortening their lifetime. The adsorption technique is considered promising as it has been proven effective in a variety of schemes (Xu et al. 2002). Many systems, such as activated alumina, chromated copper arsenate-treated wood, polymeric hybrids, natural solids, and iron oxide-loaded slag, have been applied to the elimination of arsenic from water (Elizalde-González et al. 2001; Demarco et al. 2003; Singh and Pant 2004; Deschamps et al. 2005; Kartal and Imamura 2005; Zhang and Itoh 2005; Murugesana et al. 2006). In such systems, an efficient, inexpensive, and environmentally friendly adsorbent based on chitosan can be advantageous. The free amino groups of chitosan provide sites for the coordination binding of metal ions such as Cd(II), Cu(II), Cr(III), Pb(II), Zn(II), Ni(II), Hg(II), As(III), and As(V) (Onsoyen and Skaugrud 1990). Arsenic is more frequently found in natural waters in inorganic form as oxyanions of trivalent arsenite As(III) or as pentavalent arsenate As(V). Membrane technology is more effective at removing As(V) than As(III) (Ning 2002; Xu et al. 2002). In addition, a number of chemical modifications of chitosan have also been carried out to introduce novel functions for this biopolymer (Chen et al. 2007). For instance, chitosan impregnated with molybdate has been used to advance the absorption of metals and organic contaminants (Chen and Chung 2007). Chitosan has a strong affinity for molybdate and molybdate ions are known for their ability to form complexes with metal ions. The focal point of some studies was arsenic sorption mechanisms, but they were evaluated under limited operating conditions, or were deficient in continuous environmental data (Dambies et al. 2000; Dambies et al. 2002). The removal of As(III) and As(V) from water by molybdate-impregnated chitosan beads (MICBs) was studied in both batch and continuous operations (Chen et al. 2008). The results indicated that MICBs favor the adsorption of both As(V) and As(III). The optimal pH value for As(III) and As(V) removal was 5. The adsorption of arsenic onto the MICB is most likely an exothermic reaction (Chen et al. 2008). In the continuous-operation tests, the MICB-packed column exhibited excellent arsenic removal from wastewater without any pretreatment. These results strongly support the potential of MICBs for arsenic removal from industrial wastewaters (Chen et al. 2008).

10.2.5 Chitosan and Removal of Heavy Metal Ions

Waste streams from mining operations, metal-plating facilities, and electronic device manufacturing operations frequently include very dilute concentrations of heavy metal ions (Rorrer et al. 1993). In addition, the groundwater near many nuclear fuel-processing facilities and army bases is contaminated with low levels

of heavy metal ions such as chromium and cadmium. Such ions must be eliminated from the contaminated water in order to meet the increasingly severe environmental quality standards and this requires novel separation technologies (King 1987). Chitosan (Fig. 10.6) selectively binds to almost all group III transition metal ions at low concentrations but does not bind to group I and II alkali and alkaline earth metal ions (Muzzarelli 1977). The amine groups on the chitosan chain provide a chelation site for transition metal ions and the β-1,4 glycosidic linkages joining the glucosamine units resist both chemical and biological degradation. The adsorption isotherms and heat of adsorption for Cu^{2+}, Hg^{2+}, Ni^{2+}, and Zn^{2+} on chitosan powder as a function of particle size and temperature (25–60°C) at near-neutral pH were measured (McKay et al. 1989). Additional isotherm data for Cu, Zn, Cd, Cr(III), and Pb under similar conditions can also be found elsewhere (Yang and Zall 1984). Adsorption isotherms and adsorption kinetics for Cd^{2+} were also measured (Jha et al. 1988). It was reported that 90% of the Cd adsorbed on chitosan powder could be released back to the liquid phase within 8 h using 0.01 N HC1 at pH 2 (Jha et al. 1988).

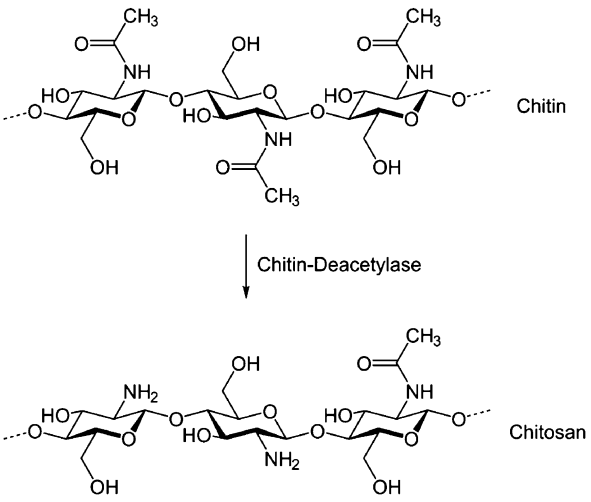

Fig. 10.6 Schematic drawing of the enzymatic synthesis of chitosan (source: NEUROtiker)

The advance of non-crosslinked, porous chitosan beads for biomedical applications was first reported ~20 years ago (Bodmeier et al. 1989). It was suggested that freeze-drying of gelled chitosan particles produces a highly porous matrix that easily dissolves in acid solution. For metal ion adsorption, the high internal surface area of the porous beads could boost the metal-binding capacity and also increase the transport rate of metal ions into the particle. Chemical crosslinking of linear chitosan chains with glutaraldehyde can cause chitosan to be insoluble in acidic media and improve resistance to chemical and biological degradation. Following crosslinking with glutaraldehyde, gelled beads were freeze-dried, and 1 mm diameter beads possessed surface areas exceeding 150 m^2/g and mean pore sizes of 560 Å, and

were insoluble in acid media at pH 2 (Rorrer et al. 1993). Well-mixed batch adsorption experiments revealed that both metal and hydronium ions compete for available adsorption sites via a chelation mechanism. Adsorption isotherms at 25°C and pH 6.5 over the concentration range of 1–1690 mg Cd^{2+}/l exhibited a stepwise shape, which was explained by a pore blockage mechanism. Maximum adsorption capacities for the 1 and 3 mm beads were 518 and 188 mg Cd/g bead, respectively (Rorrer et al. 1993).

Many microbial cells are known to sorb metals to their cell walls (Mendil et al. 2008) and some microbial cells with low viability accumulate metal ions (Vijayaraghaghavan and Yun 2008). The mechanisms of heavy metal removal by microorganisms can be classified as extracellular accumulation/precipitation, cell surface sorption/precipitation, or intracellular accumulation (Quintelas et al. 2008). Lead, copper, and cadmium recovery by aged calcium alginate beads containing a cell suspension from the waste of beer fermentation broth was reported (Choi and Park 2009). The beads were prepared by adding sodium alginate to the cell suspension and dropping the mixture into calcium alginate solution and they were then stored in a calcium chloride solution for 1 year (Choi and Park 2009). The specific metal uptake of the aged cell suspension-immobilized beads was 312 mg Pb^{2+}, 158 mg Cu^{2+}, and 112 mg Cd^{2+} per gram bead (dry weight) at a metal solution pH of 7.5. The specific cadmium uptake capacity of aged cell suspension-immobilized beads was within the range of the specific cadmium uptake capacities of the commercial beads Duolite GT-73 and Amberite IRA-400, and higher than those of fresh *Saccharomyces cerevisiae* ATCC 834- and *S. cerevisiae* ATCC 24858-immobilized beads (Choi and Park 2009).

10.2.6 Water Denitrification

Environmental nitrate contamination has become a global concern. Wastewater from the food and agricultural industries could be one of its major sources in our growing society. Being relatively reasonably priced and consistent, bacterial denitrification is the main general method of biological nitrate removal. Denitrification methods regularly utilize immobilized biosystems. Even though enzyme entrapment has been used consistently since the 1960s, microorganism containment is a recent approach to wastewater treatment. Denitrification and its connection to immobilization have been reviewed elsewhere (Power and Schepers 1989; Iza 1991; Metcalf & Eddy, Inc. 1991; Tatsuo et al. 1991). The denitrifier *Pseudomonas stutzeri*, entrapped in chitosan beads, was incubated under denitrifying conditions in a column receiving a permanent supply of full-strength growth medium (Nussinovitch et al. 1996). Biochemical, structural, and mechanical properties of the beads were studied during the first 11 days of incubation; nitrate removal was followed for 45 days. Under these conditions, the beads contracted and became lighter with time. The mechanical compression properties of the beads strengthened for the first 9 days and then weakened. Concomitantly, structural changes were observed in the beads: denitrification

and nitrite accumulation were lower in entrapped versus free cells (Nussinovitch et al. 1996). Alginate-based beads including a denitrifying bacterium (*Pseudomonas* sp.) were freeze-dehydrated for better performance. Freeze-dried beads containing 40% granular starch (Fig. 10.7) had better mechanical and denitrifying properties than beads containing lower concentrations of this carbon source and filler. The biological activity, i.e., nitrate removal, was correlated with the starch content (Tal et al. 1999). The stronger, freeze-dried beads sustained denitrifying activity over a longer period than the regular beads (Tal et al. 1997). Another study reported the removal of nitrate-N and organic pollutants from dairy industry wastewater by denitrification (Zayed and Winter 1995). Artificially prepared wastewater, containing 250 mg/l nitrate-N and 1.5 g/l whey powder, was completely denitrified with the removal of 90–93% of the chemical oxygen demand (COD) of the whey powder by suspended or immobilized mixed cultures and by a suspended or immobilized pure culture that was isolated from the mixed culture inoculum (Zayed and Winter 1995).

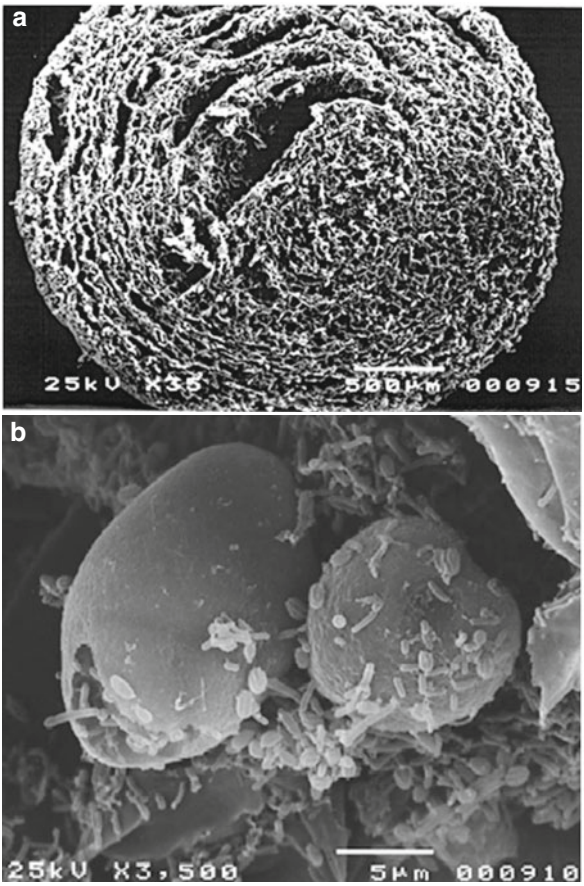

Fig. 10.7 (**a**) Cross section of a bead filled with starch. (**b**) Bacterial colonization on the starch granules (courtesy of Dr. Y. Tal)

10.2.7 Anaerobic Ammonium Oxidation

The anammox (anaerobic ammonium oxidation) process is a promising alternative for the treatment of ammonium-rich wastewater. In this process, ammonium is converted to nitrogen gas using nitrite as the electron acceptor. Compared with a conventional nitrogen-removal system, less oxygen is required, the addition of organic matter is not necessary, and a lower amount of surplus sludge is produced, all of which lead to reduced operational costs (Jetten et al. 2001). Several carriers were prepared to immobilize anammox sludge and nitrogen removal by these beads was evaluated. The removal ratios of ammonium and nitrite by anammox-immobilizing carboxymethylcellulose (CMC) beads were 100 and 95.3%, respectively, in a period of 48 h. The removal efficiencies of ammonium and nitrite by sodium alginate, PVA–sodium alginate, and anammox-immobilizing PVA beads were lower than that of the CMC beads (Zhu et al. 2009). The physical properties of the beads were then studied. PVA–sodium alginate was found to be the best support material in an overall comparison of immobilization procedure, nitrogen-removal efficiencies, and cost of the materials. PVA–sodium alginate gel entrapment was optimized by an orthogonal experiment. Scanning electron micrographs displayed a loose and finely porous surface structure for the PVA–sodium alginate-immobilized beads, facilitating nitrogen diffusion. Micrographs also clearly showed the presence of anammox bacteria in these gel beads. Taken together, results proved that immobilizing anammox sludge in gel carriers is feasible and exhibits good performance (Zhu et al. 2009).

10.3 Soil Treatments

10.3.1 General

Soil contamination characteristically occurs from the rupture of underground storage tanks, application of pesticides, and percolation of contaminated surface water to subsurface strata, oil and fuel dumping, leaching of wastes from landfills, or direct discharge of industrial wastes to the soil. There is a very large set of health consequences from exposure to soil contamination depending on pollutant type, pathway of attack, and vulnerability of the exposed population (http://www.energy-environment.net/environmental_pollution.html).

10.3.2 Agrochemicals

Agrochemicals (fungicides, pesticides, herbicides, etc.) have a significant input in contemporary agricultural know-how and have become indispensable for crop protection and pest control (Singh et al. 2009a). They are dispersed in the soil, atmosphere, and water to enhance agricultural production. Different formulations of agrochemicals can be applied via dusts, sprays, wettable powders, and emulsifiable concentrates, among many other preparations, in order to make them safer and

easier to handle, with more precise release (Singh et al. 2009a). A good formulation is one that limits the available quantity to that which is sufficient for pest control and leaves minimal amounts of residue on the crops and in the environment (Wing and Otey 1983; Trimnell et al. 1985). This can be accomplished by encapsulating the chemicals in a polymeric matrix. Encapsulated formulations extend activity, reduce evaporative and degradative losses, reduce leaching, and decrease dermal toxicity (Singh et al. 2009b). Different types of alginate-based controlled-release formulations of herbicides have been developed for application in freshwater systems (Bahadir 1987). Release from calcium alginate formulations lasted up to 2 weeks, in comparison to release from wettable powder formulations which was completed within 1 day (Pfister et al. 1986). When sorbents such as bentonite, anthracite, and activated carbon were incorporated into a basic alginate formulation, they further reduced the release rate of the herbicides and thus decreased the risk of groundwater pollution (Perez et al. 2001; Cespedes et al. 2007). The release rate of metribuzin (a herbicide) was also controlled by the inclusion of linseed oil, kaolin, or lignin in the formulation. Important factors in controlling release rate were the ratio of oil to metribuzin in the formulation, the temperature at which the formulation was dried, and the aging of the dried formulation (Pepperman and Kuan 1993, 1995). Thiram is an extensively used dithiocarbamate fungicide. It controls soil fungi (Worthing 1987) and protects harvests such as cereals, seeds, fruits, and vegetables from deterioration during storage or transport. The disulfide group present in thiram is cytotoxic due to the oxidation of –SH groups of cellular proteins, peptides, and cofactors (Cereser et al. 2001). Thiram induces sensitivity to contact dermatitis (Korhonen et al. 1982; Bjorge et al. 1996; Mishra et al. 1998) and has damaging effects on the hepatic system (Dalvi and Deoras 1986), the reproductive system (Hemavathi and Rahiman 1993), and on developmental processes. In view of its wide usage and adverse effects on the environment and human health, controlled and sustained-delivery devices are required for thiram's release. Therefore, starch–alginate–Ca^{2+} beads were produced as a controlled-release delivery device for this fungicide. The release of thiram from these beads occurred in a very controlled and sustained manner for secure handling of the pesticide, reducing its toxic effects and resulting in better delivery (Singh et al. 2009b).

10.3.3 Controlled Release of Pesticides into Soils

The danger posed to the environment by existing pesticide practices can be decreased by using advanced pest control practices, including a decrease in the quantity of active constituent needed to obtain adequate pest control (Gerstl et al. 1998). To guarantee satisfactory pest control for a sufficiently long period, pesticides are used at concentrations far higher than that required for control of the target organism. Thus, a great deal of the quantity of soil-applied pesticides is wasted owing to losses resulting from physical, chemical, and biological processes (Gerstl 1991; Triegel and Guo 1994). The excessive quantities added augment the probability of runoff or leaching and, consequently, contamination of surface or groundwater (Guyot 1994). In fact, groundwater pollution by pesticides is quite

common (Gish et al. 1994; Isensee and Sadeghi 1995; Williams et al. 1995; Cohen 1996). The use of controlled-release formulations can, in many cases, make the active component available at the required rate, consequently reducing the quantity of compound needed for pest control and decreasing the risk to the environment (Gerstl et al. 1998). Controlled release of pesticides often permits more efficient, safer, and more economical crop protection. The role played by controlled-release formulations in crop protection is limited, however, to those cases for which other, less expensive alternatives do not function, for example, compounds that are too rapidly degraded in soils or those that, while effective, are too readily leached to water sources or lost by volatilization (Gerstl et al. 1998). Clay minerals are extensively used in pesticide formulations. Clays, especially smectites, are typified by a large surface area, considerable ion-exchange capacity, and colloidal properties in suspension that make them very useful in many industrial applications (Gerstl et al. 1998). The smectite group includes monoclinic clay-like minerals (chemical formula $A_{0.3}D_{2-3}[T_4O_{10}]Z_{2.}nH_2O$). Positive identification of minerals in the smectite group may require data from differential thermal analysis (DTA) curves, dehydration curves, and X-ray (diffraction) patterns of the powder before and after treatment by heating and with organic liquids. The most common members are montmorillonite, nontronite, and saponite (http://www.mindat.org/min-11119.html). Smectites can be modified into pillared clays by a straightforward method. These pillared clays are smectites in which the exchangeable cations (usually Ca^{2+} or Na^{+}), flanked by the clay layers, have been replaced with large polyoxocations of multivalent metals such as Al, Si, Cr, Zr, or Ti (Lahav et al. 1978; Tsou and Pinnavaia 1988; Fetter et al. 1994). The product is a zeolite-like structure in which the clay platelets are separated from each other by hydroxyoxide pillars, exposing a large interlayer surface area (Gerstl et al. 1998). The spacing between the clay platelets is 3 nm and the pores are ~0.7–2 nm. Pillared clays have a large surface area, a large network of micropores (0.15 cm^3/g), both Lewis and Brønsted acidity (Poncelet and Schutz 1986), and thermal stability (Occelli and Laster 1985). Consequently, they have found use as molecular sieves and catalysts (Lussier et al. 1980). The use of clay and clay–polymer (i.e., alginate or pectin) combinations for the preparation of controlled-release formulations was also studied (Gerstl et al. 1998). The release of the pesticide alachlor [Fig. 10.8; 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide] from Al-pillared interlayered clay in soil column studies showed that these clays can be used for the controlled release of herbicides. Nevertheless, much more work is needed to fully understand the effects of the many factors involved in the release of the active ingredient from pillared interlayered clays (Gerstl et al. 1998).

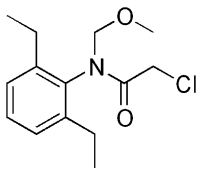

Fig. 10.8 Structure of alachlor (courtesy of Edgar181)

10.3.4 Sustained Release of a Fungicide

Conventional pesticide formulations might be the source of ecological hazards due to their dispersal in the soil, atmosphere, and water (Mogul et al. 1996). These chemicals have a variety of adverse effects, including acute or chronic toxicity, neurotoxicity, reproductive toxicity, carcinogenicity, and teratogenicity (Singh et al. 2009a). Controlled-release formulations of such chemicals should afford the possibility of decreasing the hazards connected with their use and handling (Tsuji 2001). Controlled-release formulations based on alginate have been found to decrease insecticide leaching compared to technical-grade formulations (Gonzalez-Paradas et al. 1999). Herbicides from conventional formulations are completely released within 1 day, while release was sustained for 2 weeks with a calcium alginate formulation and for quite a few months with ethylene–vinyl acetate formulations (Bahadir 1987). Natural polymers, i.e., starch, lignin, cellulose, alginate, gelatin, guar gum, have gained substantial acceptance over synthetic polymers. They are comparatively cheaper, biodegradable, and leave no polymer residues in the atmosphere (Cao et al. 2005; Kim et al. 2005; Sanli and Isiklan 2006; Isiklan 2007; Singh et al. 2007). Addition of starch during bead preparation improved the size, shape, and swelling of the alginate beads (Kim et al. 2005). The formation of alginate beads with different crosslinking agents changed the release rate of insecticides, possibly due to the different structures formed. For example, insecticide release from nickel alginate beads is faster than that from calcium alginate beads (Isiklan 2007). In another study, slower release of carbaryl was observed in barium alginate beads as compared to copper alginate beads (Sanli and Isiklan 2006). Another study concluded that formulation composition affects bead swelling and the release pattern of fungicides from starch and alginate beads. These polymeric beads may therefore be utilized for the safe handling of pesticides, reducing their toxic effects and improving their delivery (Singh et al. 2009a).

10.4 Air Pollution

10.4.1 General

Air pollution is the introduction into the atmosphere of chemicals, particulate matter, or biological materials that cause harm or discomfort to humans or other living organisms, or damage to the environment. Sources of air pollution can be natural (i.e., erupting volcanoes, forest and prairie fires, dust storms), anthropogenic (i.e., industrial, utilities, personal sources), combustive, stationary (i.e., emissions from chemical processes, resins and plastics, varnish and paints, acid manufacturers, soaps and detergents, phosphate fertilizers, other inorganic chemicals, petroleum and coal, primary metals industry, stone and clay products, forest products, and noxious trades), mobile, or emission inventory (i.e., an accounting of the amount of pollutants discharged into the atmosphere). The air pollution problems of the future are predicated on the use of more and more fossil and nuclear fuels as the world

population increases and/or the development of some world economies accelerates. It is important to note that there is not one air pollution problem but rather several distinct problems, each with its own characteristics. These problems may be distinguished by their category and timescale, i.e., local category and temporal scale of hours, urban and days, regional and months, continental and years, and global and decades, respectively.

10.4.2 Sampling Air

Porous polymer beads can be used as packing substances for the sampling of air contaminants (Cropper and Kaminsky 1963; Novak et al. 1965). This method is convenient when sampling in the field at ambient temperature, as a good concentration can be achieved. Porous polymers are very useful materials for the sampling of low-polarity air contaminants. Due to their low concentrations (which must be concentrated in most cases, though larger concentrations may be determined directly in air), the requisite large air samples may be drawn through absorption columns without breakthrough, yielding a high concentration factor, and a more sensitive subsequent gas chromatography determination (Jeltes 1969).

10.4.3 Determination of Trace Contaminants in Air by Concentration on Porous Polymer Beads

The development and use of selective detectors has become crucial in gas chromatography with the increasing need for determinations of trace contaminants in complex mixtures (Cremer 1967). A selective detector eliminates superfluous peaks from the chromatograms and thus greatly facilitates the qualitative and quantitative analyses of complex mixtures. Nevertheless, the best detector for a particular analysis frequently cannot be used due to deficient sensitivity. Various concentration techniques, most of which use cryogenic trapping, have been detailed in the literature and are reviewed in Altshuller (1963). A method was developed to concentrate gas samples at room temperature on porous polymer beads, which eliminated the use of cold traps or cold columns and provided the possibility of on-column injection (i.e., the gas sample is concentrated in the same column that is subsequently used for the gas chromatographic analysis) (Williams and Umstead 1968).

10.5 Miscellaneous

10.5.1 Biodegradation

Immobilization of microorganisms has more than a few benefits relative to freely suspended cells when used in bioreactor systems. These include straightforward partitioning of carriers from the reaction medium, reuse of immobilized cells, and

avoidance of cell washout during continuous fermentation at high dilution rates (Willaert and Baron 1996). Increased volumetric reaction rates are achieved as a result of the higher local cell concentration made possible by the use of immobilized cells (Lee et al. 1994; Rhee et al. 1996; Manohar et al. 2001). Immobilized cells can have improved tolerance to toxic compounds compared to purified enzymes or other processed biological materials (Westmeier and Rehm 1985; Dwyer et al. 1986; Keweloh et al. 1989; Lee et al. 1994).

Coumaphos (an organophosphate insecticide) is used by the US Department of Agriculture Animal and Plant Health Inspection Services in its tick eradication program along the USA–Mexico border. Imported cattle are dipped in vats that include ~15 m^3 of coumaphos solution, at a concentration of about 1500 mg/l (Karns et al. 1995). This method produces ~500 m^3/year of concentrated waste, which must be removed, treated, and discarded. Coumaphos can be hydrolyzed to chlorferon and diethylthiophosphate by organophosphate hydrolase enzyme. Another study described hydrolysis of coumaphos using recombinant *Escherichia coli* as freely suspended cells or immobilized in polyvinyl acetate cryogel. The immobilized preparation functioned twice as well as the free cells (Kim et al. 2002). Recently, calcium alginate-immobilized cell systems were developed for the detoxification and biodegradation of coumaphos (Ha et al. 2009). Optimum bead loading for bioreactor operation was found to be 200 g beads/l for chlorferon degradation and 300 g beads/l for diethylthiophosphate degradation. Using waste solution from the cattle dip as the substrate, the degradation rate for an immobilized consortium of chlorferon-degrading bacteria was five times higher than that for freely suspended cells (Ha et al. 2009).

10.5.2 Carbon Nanotubes

Removal of chemical pollutants from a contaminated environment is one of the most significant steps in environmental remediation (Kaiser 2000). Polychlorinated dibenzo-*p*-dioxins and related compounds make up a group of very toxic, stable aromatic pollutants. They accumulate in the food chain and end up in the human body upon consumption of animal and/or fish products (Kaiser 2000). Activated carbons are extensively used as traditional adsorbents in Japan and Europe for the removal of dioxins from the gaseous emissions of waste incinerators (Yang et al. 1999). It has been reported that multi-walled carbon nanotubes (MWCNTs), a new class of carbon materials, offer significantly higher dioxin elimination efficiencies than can be achieved with traditional activated carbons (Long and Yang 2001). Carbon nanotubes (Fig. 10.9) were discovered in 1991. They are made up of one or several concentric walls in which carbon atoms are arranged in a hexagonal pattern and they measure several tens of microns in length and less than a few nanometers in diameter. A MWCNT-based adsorbent column was obtained by simply packing a stainless steel tube with MWCNTs. However, its potential for adsorptive applications appeared to be restricted to the purification of polluted gas streams (Long and Yang 2001). Another study described the encapsulation of MWCNTs

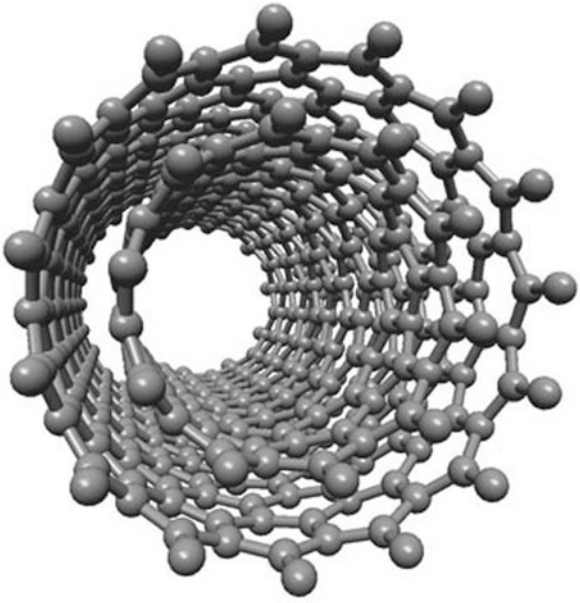

Fig. 10.9 Carbon nanotubes

in Ba^{2+}−alginate to form coated microbeads. Water containing the model compounds dibenzo-*p*-dioxin, dibenzofuran, and biphenyl was pumped into an open-top glass column, which was packed with MWCNT/Ba^{2+}−alginate composite beads. Quantitative determination of contaminant concentrations in the treated water and in the adsorbents using GC-MS demonstrated that the model compounds were readily taken up by the MWCNT/Ba^{2+}−alginate composite microbeads (Fugetsu et al. 2004). High-affinity binding of the target compounds to MWCNTs can be attributed to the latter's unique structure and electronic properties. The hexagonal arrays of the carbon atoms in the graphene sheets of MWCNTs interact strongly with the aromatic bonds of the model compounds, probably due to van der Waals interactions. As a result of these interactions, the target species dibenzo-*p*-dioxin, dibenzofuran, and biphenyl were efficiently eliminated by the MWCNTs of the MWCNTs/Ba^{2+}−alginate composite adsorbents. In addition, the MWCNTs/Ba^{2+}−alginate composite adsorbents could be reused by regenerating the beads with hexane or methanol (Fugetsu et al. 2004).

10.5.3 Removal by Microalgae

Chlorella vulgaris is a unicellular freshwater microalga. It is employed in tertiary wastewater treatment for the most part to remove nitrogen and phosphorus compounds and heavy metals (Aksu et al. 1992; Oh-Hama and Miyachi 1992;

Gonzalez et al. 1997; Tam et al. 1998). Interactions among microalgae and other microorganisms during wastewater treatment have not been well studied. The two obligate aerobes *Pseudomonas diminuta* and *P. vesicularis* were found to stimulate the growth of the microalgae *Scenedesmus bicellularis* and *Chlorella* sp., without releasing any growth-promoting substances (Mouget et al. 1995). Co-immobilization of *C. vulgaris* with the plant growth-promoting bacterium *Azospirillum brasilense* was also reported to significantly increase growth and pigment production of the microalgae (Bashan and Holguin 1998; Gonzalez and Bashan 2000; Lebsky et al. 2001). Co-immobilization of the microalga with its associated bacterium from the wastewater treatment pond, *Phyllobacterium myrsinacearum*, changed the metabolism of the microalga, but did not enhance its growth (Gonzalez-Bashan et al. 2000). Co-immobilization of *C. vulgaris* with *A. brasilense* in alginate beads under semi-continuous synthetic wastewater culture conditions considerably increased the exclusion of ammonium and soluble phosphorus ions compared to immobilization of the microalga alone (de-Bashan et al. 2002). A similar trend was observed in continuous- or batch culture removal of these ions. It was suggested that co-immobilization of a microalga with microalga growth-promoting bacteria can serve as a means for devising original wastewater treatments (de-Bashan et al. 2002).

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