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Review

Multiscale requirements for bioencapsulation in medicine and biotechnology

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ABSTRACT

Bioencapsulation involves the envelopment of tissues or biological active substances in semipermeable membranes. Bioencapsulation has been shown to be efficacious in mimicking the cell's natural environment and thereby improves the efficiency of production of different metabolites and therapeutic agents. The field of application is broad. It is being applied in bioindustry and biomedicine. It is clinically applied for the treatment of a wide variety of endocrine diseases. During the past decades many procedures to fabricate capsules have been described. Unfortunately, most of these procedures lack an adequate documentation of the characterization of the biocapsules. As a result many procedures show an extreme lab-to-lab variation and many results cannot be adequately reproduced. The characterization of capsules can no longer be neglected, especially since new clinical trials with bioencapsulated therapeutic cells have been initiated and the industrial application of bioencapsulation is growing. In the present review we discuss novel Approached to produce and characterize biocapsules in view of clinical and industrial application. A dominant factor in bioencapsulation is selection and characterization of suitable polymers. We present the adequacy of using high-resolution NMR for characterizing polymers. These polymers are applied for producing semipermeable membranes. We present the pitfalls of the currently applied methods and provide recommendations for standardization to avoid lab-to-lab variations. Also, we compare and present methodologies to produce biocompatible biocapsules for specific fields of applications and we demonstrate how physico-chemical technologies such as FT-IR, XPS, and TOF-SIMS contribute to reproducibility and standardization of the bioencapsulation process. During recent years it has become more and more clear that bioencapsulation requires a multidisciplinary approach in which biomedical, physical, and chemical technologies are combined. For adequate reproducibility and for understanding variations in outcome of biocapsules it is advisable if not mandatory to include the characterization processes presented in this review in future studies.

1. Introduction

Bioencapsulation involves the envelopment of tissues or biological active substances in a semipermeable membrane to protect the enclosed biological structures for potential hazardous processes in the direct environment. The field of application of bioencapsulation is broad. In plant cell cultures [1–3], bioencapsulation has been shown to be efficacious in mimicking the cell's natural environment. Thereby bioencapsulation improves the efficiency of production of different metabolites for industrial application. For fermentation [4–8] bioencapsulation is being applied for enlarging the cell density, aroma and capacity of the systems. Additionally during fermentation it avoids washout of the biological catalysts

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from the reactor. Bioencapsulation also has a pertinent application in medicine. It is, for example, applied to protect biological active substances or cells such as probiotica to the deleterious biological environment [9–12] and for delivery in specific sites such as the colon [13,14]. A relatively large group of researchers apply bioencapsulation for the creation of a bioartificial organ [15]. In this application, therapeutic cells are encapsulated in membranes that protect the cells against antibodies and cytotoxic cells of the host immune system. This immunoisolation by encapsulation has a number of important benefits for clinical application of transplantation. First of all, it avoids the use of systemic and permanent immunosuppression. Immunosuppression has serious side effects such as a higher chance for malignancies and frequent infections. Another benefit is that encapsulation allows for successful transplantation of cells from nonhuman origin, i.e. xenografts, which could be a mean of overcoming the obstacle of limited supply of donor tissue [16]. The principal applicability of the technology has been shown for the treatment of a wide variety of endocrine diseases, including anemia [17], dwarfism [18], hemophilia B [19], kidney [20] and liver [21] failure, pituitary [22] and central nervous system insufficiencies [23], and diabetes mellitus [24].

During the past decades many procedures to fabricate capsules have been described. Unfortunately, most of these procedures are dedicated to the technology of the production process but lack an adequate documentation of the characterization of the capsule. As a result many procedures show an extreme lab-to-lab variation and many results cannot be adequately reproduced. The characterization of capsules can no longer be neglected, especially since new clinical trials with bioencapsulated therapeutic cells have been initiated [25] and the industrial application of bioencapsulation is growing. During recent years many technologies have been described to characterize capsule properties. In the present review we discuss these technologies in view of clinical and industrial applications.

2. Polymers for encapsulation

Producing a microcapsule for envelopment and protection of biologically active substances or cells starts with selection of an adequate encapsulation material. The majority of materials used in microcapsules are polymers, either naturally occurring or synthetic. A major pitfall in the field is the absence of guidelines for documentation of the characteristics of the materials applied. It is mandatory that this documentation will be included since it is now widely accepted that the characteristics of the polymer is a dominant factor in determining the capsule properties.

Several characteristics should be taken into account for considering polymers for formation of microcapsules. The polymer selection starts with the description of chemical composition of the monomeric units. The amount and character of functional groups contained in monomer units (one type of monomer unit in case of homopolymers, two and more types of monomer units in case of copolymers) define the primary structure of polymers. The chemical composition of monomer units gives rise to various interactions such as hydrogen bonding, electrostatic interactions, and hydrophobic interactions, which are important for intra- and intermolecular interactions. These interactions are responsible not only for the effects potentially originating from secondary, tertiary, or quaternary structures but also for the interactions that lead to formation of microcapsules. The most common principle used for microcapsule formation by polyelectrolyte and ionotropic complexation represents a typical example. Since the variability in the chemical character of monomer units is virtually infinite, application of well-characterized polymers in terms of chemical composition is critical in order to understand and control the microcapsule properties and performance.

In addition to the chemical composition, the molecular weight characteristics should be part of the conventional documentation. This includes identification of weight number and average molecular weights (M_w and M_n), and polydispersity M_w/M_n. The latter characterizes the molecular weight distribution. The molecular weight averages and molecular weight distribution can be measured by various techniques. Conventionally, static light-scattering, viscometry, and size-exclusion chromatography are used to determine the molecular weight averages. The molecular weight distribution is most typically determined by size-exclusion chromatography, although the mass spectrometry techniques have been advancing to assess the molecular weight distribution of synthetic [31] and natural [32] polymers. Molecular weight characteristics are linked to the viscosity and other rheological properties of the polymer solution, which are important for the process of microcapsule formation. The rheological properties are affected by temperature and concentration of the polymer, and by ionic strength in case of polyelectrolytes, which all should be specified for the materials applied in encapsulation.

There are some additional items that should be documented in specific applications, e.g., for medical application it is mandatory to have information on the purity degree of the polymer. At least the endotoxin content, the microbial contamination, and the protein content should be specified and documented.

What the above-mentioned measurements imply for application can be most adequately illustrated with an example. We will do so with alginate which is one of the most dominantly applied polymers in encapsulation. Alginates are natural unbranched binary copolymers of 1 → 4 linked ß-D-mannuronic acid (M) and α-L-guluronic acid (G) (Fig. 1A). The alginate composition and sequential structure together with its molecular weight are essential characteristics in determining the capsule properties and functionality. High-resolution NMR is applied to determine the composition and sequential structure of alginates (Fig. 1B).

The molecular weight is mostly determined from intrinsic viscosity measurements or size-exclusion chromatography using light-scattering detection. As alginates do not have any regular repeating unit, the sequential structure is not only determined by the monomer composition (monad frequencies) alone, but also by measurements of diad and triad frequencies. The four diad (nearest neighbor) frequencies (F_{GG}, F_{GM}, F_{MG} and F_{MM}) and the eight possible triad frequencies (F_{GGG}, F_{GGM}, F_{MGG}, F_{MGM}, F_{MMM}, F_{MMG}, F_{GMM} and F_{GMG}) can be measured by NMR techniques [26–28]. From the frequencies we can estimate the average length of blocks of consecutive G units ($N_{G>1} = F_G - F_{MGM}/F_{MGG}$) and M units ($N_{M>1} = F_M - F_{GMG}/F_{MMG}$). Recent development in the field of polysaccharide sequencing allows for an even more detailed characterization. Nowadays we can assess the true reconstruction of the block structure of alginates. This is done by the use of specific lyases and subsequent analysis of the digest. The digest is fractionated by SEC and the molecular mass and composition of each fraction are then analyzed with NMR and ESI-MS (low molecular weight fractions) or HPAEC-PAD (MALDI-ToF) for the high molecular mass fractions. This latter of course is not yet routinely applied for characterization of alginates for encapsulation.

Since the purity degree of the alginate has been shown to determine the biocompatibility of alginate-based capsules [29,30] it is mandatory to provide details on the purity of this polymer. According to FDA requirements for device implantations, the content of endotoxin must be below 350 EU per patient (below 15 EU per patient for CNS applications). As the chemical properties of endotoxins are very similar to alginates, their removal has been a challenging task but purified alginates with a specified endotoxin

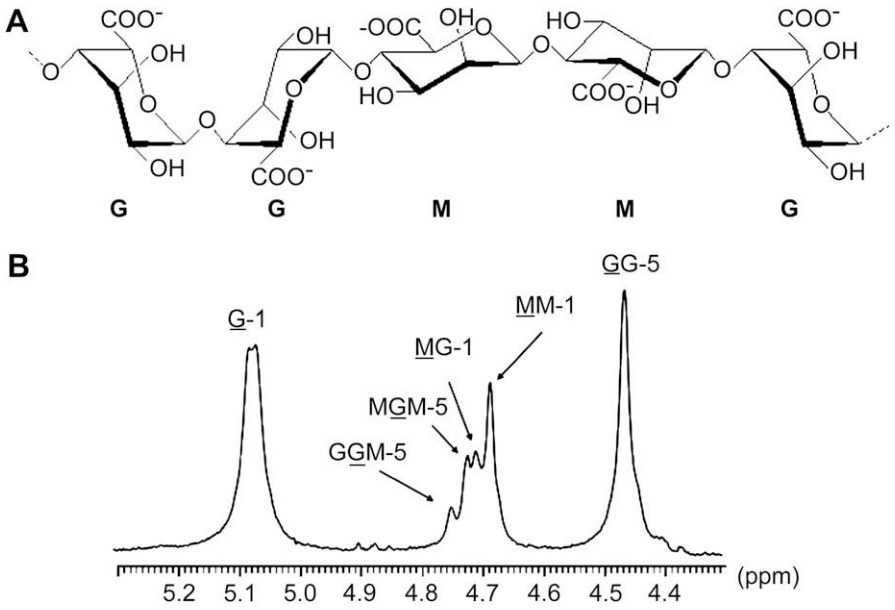

Fig. 1. Structure of alginate. A: β-D-mannuronic acid (M) and α-L-guluronic acid (G), with most probable ring confirmation: M: $^4C_1$ and G: $^1C_4$. B: ^{1}H-NMR spectra of alginate from *Laminaria hyperborea* stipe.

content below 100 EU/g are now commercially available. For Good Medical Practice (GMP), alginates must be characterized by validated methods and every product batch must be characterized and documented with its individual laboratory certificate. Characterization parameters for alginates to be used in biomedical and tissue engineered medical products are now thoroughly described in the ASTM guide F 2064 (American Society for Testing and Materials) of the ASTM Book of Standards.

Other polymers in addition to sodium alginate have been successfully applied in encapsulation research [33]. The polymers involve various polyelectrolytes of anionic (cellulose sulfate, chondroitin sulfate, polyacrylic acid) and cation (poly-L-lysine, poly-L-ornithine, chitosan, poly(methylene-co-guanidine), polyvinylamine) nature as well as non-ionic polymers (polyvinyl alcohol, poly(hydroxyethyl methacrylate-co-methyl methacrylate), polyethylene glycol). It is viewed as a positive trend that researchers recently dedicate much more attention to characterization of these materials used for capsule formation than in the past. Thus, the encapsulation protocols provide at least one of the molecular weight averages, chemical composition in case of copolymers and degree of substitution in case of chemically modified polysaccharides. Often, the lot number is another important parameter to be reported, which can help tracking the properties of the commercial polymers in order to improve the lab-to-lab reproducibility.

3. Permeability properties

Encapsulation is applied to protect the enclosed biological materials for deleterious effects of substances or processes in the immediate vicinity of the capsules. This protection is usually accomplished by restricting the diffusion of deleterious molecules by applying semipermeable membranes. The permeability of the capsules is determined by the desired control over both the size-based exclusion and the rate of diffusion of the molecules, which either have to or must not permeate the membrane. Before discussing the means to measure the permeability properties, it is essential to shortly discuss the factors determining the diffusion characteristics of the capsules since this rationalizes the application of the presented technologies. This will be done with the hydrogel as an example since this is the most commonly applied capsule structure with complicated diffusion characteristics.

Diffusion and permeability properties of hydrogels are determined by at least four factors. The first is the obstruction effect caused by the presence of impenetrable slowly moving polymer chains that increase the path length for diffusion. The second process is the hydrodynamic drag at the polymer interface due to polymer-solvent and polymer-solute bonds during the solute diffusion. The third is the different extent of heterogeneity of the membrane material with fluctuation of diffusion properties across the membrane material. Finally, residual charges, presence of counter ions, hydrogen bonds, polar and hydrophobic interactions of the membrane material will affect the diffusion of solute exhibiting similar interactive groups. This is especially essential in diffusion of biological molecules.

For assessing and expressing the diffusion and permeability of capsules, two factors are of main interest. The first factor that is relevant for expressing the diffusion and permeability of capsules is the rate of solute diffusion, which is reflected in the mass transfer, permeability, and diffusion coefficients. The second one is represented by the membrane exclusion properties considering minimum size of a solute completely excluded by the capsule membrane. This is usually referred to as the exclusion limit or molecular weight cut-off (MWCO). This is related to the membrane pore size. It is important to emphasize that this parameter is not connected with the solute molar mass. It is determined by the size and shape. The MWCO and the rate of diffusion of solute are obviously connected and are of equal importance for quantification of the permeability properties of a semipermeable membrane. Nevertheless, the vast majority of studies only characterize the MWCO to quantify the diffusion properties. This is not without consequences since it does not adequately predict the diffusion properties when other solutes are applied. This is especially true for the hydrogel-based membranes since these materials show large fluctuations in chemical composition, local viscosity, density, interactions with solutes as well as non-uniformity in pore sizes and their distribution across the membrane [34,35].

3.1. Selection of solute for quantifying permeability

Many experimental techniques to assess the permeability properties of capsules have been described during the past two decades. These techniques are comprehensively compiled in a number of review articles, for example by Schuldt and Hunkeler [36] and Uludag et al. [37]. Which technique is most suitable for a specific application depends on the type of solute that is applied for measuring permeability. The selection of the technique starts with choosing the solute type, which typically involves proteins, dextrans, and pullulans. In the medical field, the permeability of capsules to IgG is considered as the most important criterion since it is assumed to predict the immunoprotective properties of the capsules after implantation in humans. Not surprisingly, different techniques for quantifying IgG permeability have been developed such as diffusion of radiolabeled IgG [38], fluorescently labeled IgG [39] or entrapping radiolabeled IgG or other relevant proteins inside the capsules [40]. Others, however, prefer to quantify diffusion by applying neutral polysaccharides such as dextrans and pullulans since it is fast and reliable. The obtained information from neutral polysaccharides can also be applied to calculate the permeability for specific proteins by applying the universal calibration principle, which allows for mutual recalculating of viscosity radius and molecular weight for respective polysaccharide and protein [41]. It is recommended, however, to verify whether the calibration principle applies for a specific capsule since theoretically an interaction of proteins with the membrane is more likely to occur than with polysaccharides.

During recent years many other relevant solutes have been proposed for quantifying permeability. Mostly these solutes were chosen because they are playing an essential role in the functionality of the capsule. These solutes include glucose, glycerol, Vitamin B12, etc. [42,43]. The solutes may be applied either unlabeled but the preference is for the radio- and fluorescent-labeled solutes to increase sensitivity and specificity of the applied techniques.

3.2. Quantification of permeability

Many different techniques are available to determine the diffusion properties of solutes in semipermeable membranes [36,37]. Before discussing these techniques, it should be mentioned that the overall permeability of a membrane is determined by a number of capsule properties. It has been shown that the rate of solute diffusion is described by the pore size, the pore size distribution and the chemistry of the membrane and solutes [42,44–47]. In addition, the importance of the membrane thickness for the velocity of solute diffusion was recently shown in studies on diffusion properties of a few micrometer thick nanoporous membrane with uniform pores microfabricated of silicon [48,49] and, more recently, alumina [50,51]. It is therefore advisable to document all these factors including the membrane thickness in studies on semipermeable capsules.

The applied technique for measuring permeability as such depends on the methodology used to quantify the chosen solute. The most commonly applied techniques are spectroscopy techniques (fluorescence, UV–VIS), measurement of radioactivity, size-exclusion chromatography with concentration-sensitive detector, and protein assay kits. Determination of solute permeation can be either in diffusion into the capsules (ingress) or from the capsules (egress) [36]. The results of these experiments can be obtained under equilibrium conditions providing the information on MWCO or at different times until reaching the equilibrium, which results in determination of the transport coefficients.

A technique that may be developed into a widely applied methodology for measuring the permeability of microcapsules is the inverse size-exclusion chromatography [40,41]. The advantage of this technique over the others is that it not only provides information on the, MWCO but also on the pore size distribution (Fig. 2). In this technology, microcapsules are used as the column packing of which the calibration curve with slope (pore size distribution) and exclusion limit (MWCO) are determined simultaneously.

From the above follows that diffusion of the chosen solute, the pore size, the pore size distribution, the chemistry of the membrane and solutes, and the thickness of the membrane are essential for describing the permeability properties of a capsule. Unfortunately, these items are only rarely documented. For that reason it is anno 2009 difficult if not impossible to compare permeability results between different laboratories.

4. Mechanical resistance of microcapsules

A capsule should have a sufficient mechanical resistance to withstand the various forces during the whole duration of application. Up to now, mechanical stability did not gain too much attention by the scientific community since it is technically not too advanced to increase the mechanical resistance of microcapsules. Increasing the strength and resistance of the capsules will also increase the durability of the transplant and consequently the drug release time period. However, can this be done without a drawback on other capsule parameters? The answer is no. Increasing the mechanical stability by increasing for instance the membrane thickness of a capsule may influence other important parameters of the capsules such as permeability and intracellular microenvironment [52]. The challenge is therefore not to increase the stability but to determine what mechanical resistance is required for a specific application without changing other relevant capsule characteristics.

The required mechanical resistance for in vivo application of encapsulated therapeutic cells has been studied in more detail than for other fields of application. The required mechanical stability for encapsulated therapeutic cells depends on the proposed site of implantation [53]. It has been shown with alginate-based

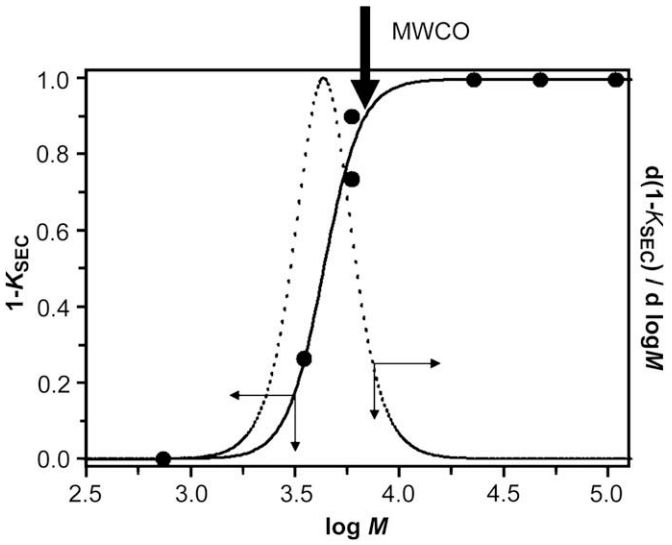

Fig. 2. A classical example of inverse size-exclusion chromatography. Filled circles represent measured data points of chromatography partition coefficient K_{SEC} as a function of molecular weight of testing standards (pullulans) used to test for permeation to the microcapsules forming the column packing. Full line is the Boltzmann fit to the experimental data, of which the first derivative (dashed line) represents the pore size distribution. The exclusion limit of the column packing corresponds to the MWCO value of microcapsules.

microcapsules that they should have a higher mechanical stability in the peritoneal cavity than in the stratium or subcutaneous space. Other important factors that influence the required mechanical stability is the type of cells applied [54–56]. At the moment, there are no guidelines for the mechanical resistance capsules should have in the various applications. Lacik reported that the rupture load from a few grams to tens of grams per capsule for intraperitoneal application [33] should be in the sufficient range. This, however, holds for empty capsules and not for cell containing capsules.

Up to now, broken capsules in explants from recipients are used as a rough indication that the mechanical stability of the capsules should be increased. The approach to accomplish such increase without interfering with other important capsule parameters depends on the following factors: the type of biomaterials used for the elaboration of the polymer matrix and membrane [54–56], the type of gelling ion [39], the type of cell, and the selected encapsulation technology. These factors have a mutual influence on the resistance as will be illustrated with alginate-based capsules as an example. The mechanical resistance of an alginate-based capsule depends on the ionic linkages between the gelling ion (usually calcium) and alginate block structure. However, in physiological solutions and in vivo conditions, the calcium-alginate beads are sensitive for chelating and non-chelating agents such as phosphate, sodium and potassium ions, which provoke the osmotic swelling of the beads and their final rupture [57]. To increase the stability of calcium-alginate beads, a polycation is often added [24]. The latter will increase the stability of the encapsulation system due to the polyelectrolyte complex between the alginate and the polycation. Some report mechanical stability problems with this approach. The electrostatically linked complex might still compete with other charged molecules in the environment, limiting the long-term stability of the microcapsule [58]. A possible approach to overcome such a problem is to improve the mechanical characteristics of the alginates [59,60] or to design novel covalently reinforced and photo-crosslinked capsules [61,62].

The foregoing should not be interpreted as a suggestion that hydrogels made of alginate are not mechanically stable enough to allow application for long periods. In general, hydrogels used for cell encapsulation purposes are likely to contain the desired mechanical rigidity (resistance to deformation) and toughness (resistance to fracture by being pliable) to structurally protect enclosed cells. The mechanical properties of the gels are controlled by both the polymer concentration and the molar ratio between polymers and crosslinking molecules [63]. In fact, reducing the distance between the crosslinks and increasing the polymer concentration led to an increase of the mechanical rigidity in hydrogels [64]. Interestingly, unlike other hydrogels formed from covalent crosslinking, calcium crosslinked alginate hydrogels permit increases in both the rigidity and the toughness with higher crosslink density.

A major pitfall in the studies aiming on improving the mechanical resistance of capsules is the lack of standardization of technologies to quantify the durability of capsules. Many are the disputes about laboratory variations in stability of capsules. At present most groups apply home-made procedures for quantifying mechanical resistance in which specific details such as incubation solutions, compressive force, and shaking speed are rarely documented.

One of the most commonly applied assays to quantify mechanical resistance is the osmotic pressure test in which capsules are exposed to various deleterious solutions with the aim to quantify the swelling of capsules. Swelling of the capsules leads to capsule heterogeneity and to a gradual increase in undesired capsule pore size and permeability. Different types of reagents have been used as swelling solutions including water [55], saline, citrate [56], dilutions of serum free media [65], serum [66], glycine buffer [61], and hepatozyme culture medium [67]. The advantage of this technique is that it is nonlaborious and readily available in all laboratories. However, in order to allow reproducibility it is imperative to standardize the solution reagents.

Another assay that is nowadays more commonly applied is evaluation of the physical integrity of the capsules by using a surface texture analyzer [53,68]. With this technique a specific force is placed on the capsules. The quantity of deformation or rupture of capsules is applied as a measure for the mechanical stability of the capsules. Although very reproducible the technology requires consensus about the speed of the compressive force and the extent of such compression in order to allow comparisons between laboratories. The texture analyzer can be combined by shaking the capsules by means of an orbital shaker to compare the stability between different types of microcapsules [69]. Also, there are new approaches which are not yet generally applied in the field of encapsulation. A promising approach is the probing technique to mechanically characterize small-scale structures ($<$100 μm). This can be done by optical tweezers [70], micropipette aspiration [71], atomic force microscopy (AFM) [72], and magnetic bead measurement techniques [73]. Recently, a new force feedback microelectromechanical (MEMS) microgripper has been reported (Fig. 3). This MEMS combines the capacity to manipulate micrometer-sized biomaterials and hydrogel particles while simultaneously quantifying their mechanical properties [74]. This new MEMS microgripper integrates two-axis force feedback to protect the fragile microgripper by detecting contact between the particle and the microgripper. By that it provides gripping force feedback for achieving secure grasping without applying excessive gripping forces. Using this approach, Kim et al. [74] were the first to apply this on capsules and successfully measured Young's modulus and viscoelastic parameters of 15–25 μm-sized chitosan coated alginate microparticles. These advances may help to establish a range of predictable techniques which will provide comprehensive and comparable data without too much lab-to-lab variations about the strength of the polymer microcapsules.

5. Surface properties of capsules

The surface properties of capsules determine the functional performance of the capsule. It is the site that is responsible for the biocompatibility and it determines the diffusion properties. Surprisingly until a few years ago the surface of the capsule only received minor attention. This has recently changed after the introduction of new physico-chemical technologies to the field [75–80]. To illustrate the importance of the surface analysis in the field, we will discuss a few findings with alginate-based microcapsules for encapsulation of mammalian cells.

In order to provide more insight in the structure of alginate-PLL capsules a physico-chemical analysis of the capsules has been performed by applying X-ray photoelectron spectroscopy [81]. This technique allows for identification of the chemical groups on the surface of the capsule on an atomic level. Up to now the capsule was assumed to be composed of a core of calcium-alginate which is enveloped by a membrane composed of two layers, i.e. an inner layer of alginate-PLL and an outer layer of calcium-alginate. The data, which have lead to this model, were almost exclusively obtained by studying the chemical interactions of PLL with solved, non-calcium bound and often individual components of alginate (i.e. G and M monomers) and not by studying the chemical structure of the capsules as such. In subsequent studies on true capsules, Fourier transform infrared spectroscopy, X-ray photoelectron

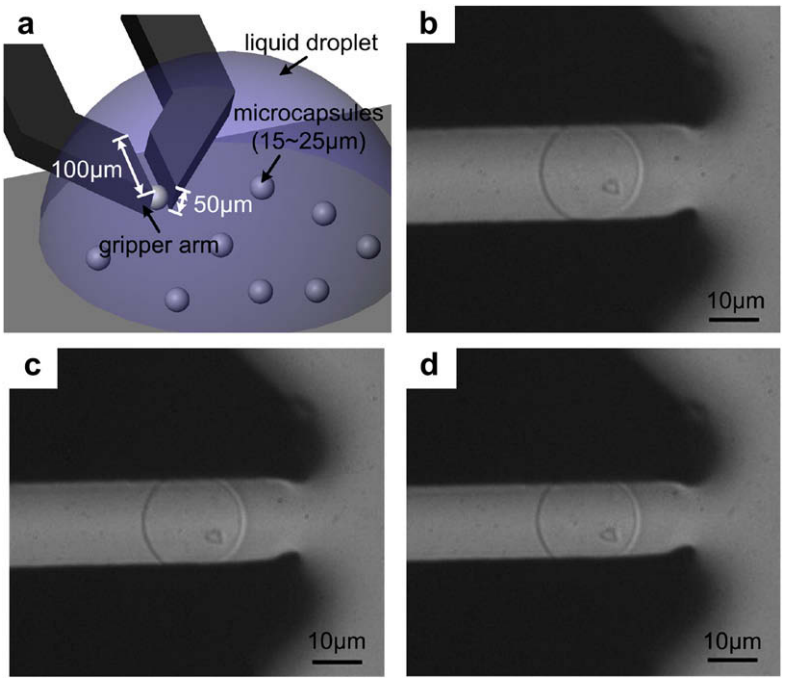

Fig. 3. (a) Schematic illustration of an experimental situation in which a microcapsule coated with 2% chitosan is analyzed; (b) the capsule is compressed; (c) 10% deformation of the capsule and (d) 20% deformation at 962 nN. Printed with permission from Kim K, Park H, Kwon KH, Park JY, Baek JY, Lee TS et al. A cell culturing system that integrates the cell loading function on a single platform and evaluation of the pulsatile pumping effect on cells. Biomed Microdev 2008;10:11-20.

spectroscopy (XPS), and confocal microscopy were applied to study the structure of the alginate-PLL capsule membrane [75–80]. From confocal images and from electron microscopy pictures it was visualized that the PLL penetrates the alginate core, forming an alginate-PLL complex of about 30 μm, depending on the exposure time to PLL. It was found that the capsules were not composed of a generally considered three-layer system of alginate-polycation, and an outer alginate layer but only of an alginate-core surrounded by an alginate-polycation shell. This was recently confirmed by Tam et al. by applying ToF-SIMS imaging [80]. Fig. 4 shows the actual structure of alginate-PLL capsules.

6. Biocompatibility of microcapsules

The design of a standard technology for measuring biocompatibility of microcapsules is a very complex and difficult task mainly due to the complicated interactions between biological systems and microcapsules. Biocompatibility issues of microcapsules are often connected with the ability of a material to perform with an appropriate host response in a specific application [82]. For encapsulation this "specific application" is dependent on the field of application. Roughly, we can distinguish two specific applications in which optimal biocompatibility is essential. The first one is the application of encapsulation in the field of medicine and pharmacy. In this field, the capsules are applied to encapsulate cells for transplantation in recipients [83–85], and for controlled release of drugs [86–90]. The second application is the encapsulation of cells in biotechnology. Usually in this field encapsulated microbial cells are applied as biocatalysts for the production of valuable substances. Production processes are often performed under non-physiological conditions. The cells themselves either use substances with a sequestering effect on immobilization matrices [91] or produce compounds that have inhibitory impact on cells [92]. Therefore, it is desirable to recognize and standardize proper encapsulation methods, which provide mild and physiological conditions to cells during encapsulation and post-encapsulation procedures.

Traditionally the medicine and pharmacy field were focused on the host response to the capsule materials while the biotechnology was concentrated on the compatibility of the materials with the cells in the capsules. Nowadays, it has become more and more recognized that also in the field of medicine the materials should allow adequate function of the cells in the capsules. Therefore, in the present review we discuss both the field of medicine and pharmacy and the biotechnology since it is our expectation that this will contribute to the exchange and introduction of new technologies in the different fields.

6.1. Biocompatibility tests in medicine and pharmacy

Biocompatibility of microcapsules in medicine and pharmacy has been the subject of intensive research, as summarized in several review articles [55,83,85,86]. Table 1 lists the most important technologies and approaches to the measurement of biocompatibility of microcapsules in the mentioned fields. Mostly the studies are dedicated to the host response against the capsule's surface. The biocompatibility was usually evaluated from multiple points of view, and assessed by a combination of techniques. The most commonly applied approach is the correlation of biological responses to capsules with their chemistry [81] and correlation of tissue reactions against capsules with the structure of the capsule's surface [75]. Many have been the efforts to identify and quantify the key markers of biocompatibility of microcapsules. However, divergence of technologies for measuring biocompatibility markers may lead to the never-ending development of experimental protocols that lack standardization. Additionally, the non-consistency of biocompatibility markers can be illustrated by a number of approaches used to evaluate the same marker as shown in the first column of Table 1. The presented description of discrepancies in the biocompatibility

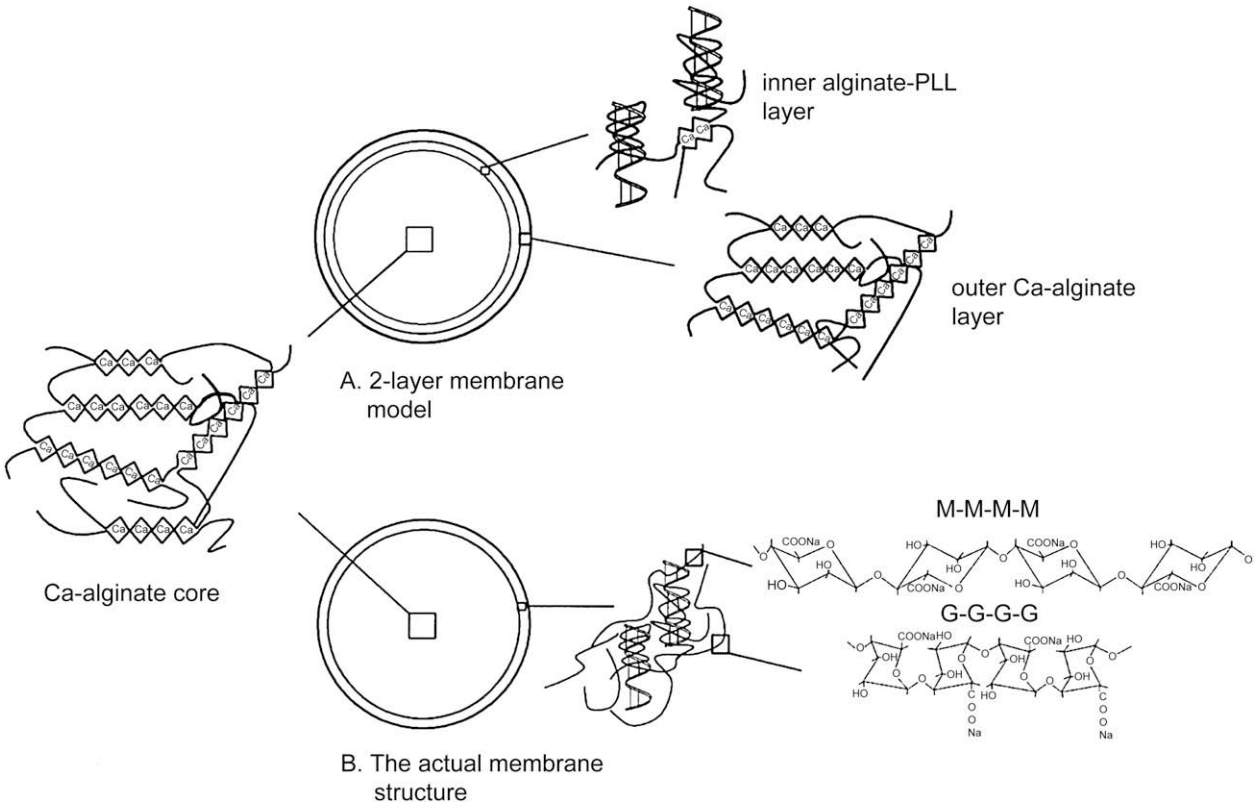

Fig. 4. The considered and the actual structure of alginate-PLL capsules. The capsule is not composed of three layers as generally assumed but of two layers.

evaluation of microcapsules can be considered to be an important milestone of standardization activities in the encapsulation community.

6.2. Biocompatibility tests in biotechnology

Basic studies of the physiological behavior of immobilized microbial cells have gained minor attention in spite of the broad application and rapid development of biotechnological processes based on immobilized cells since the early 1980s [93]. Though the range of more fundamental investigations has been limited in this field [93], research activities have resulted in the identification of key parameters regarding the physiology of immobilized microbial cells which may be considered as biocompatibility markers (Table 2). Importantly, the viability of cells in the capsules is a mutual attribute of the presented biocompatibility markers. Therefore, development and standardization of methods for rapid determination of viable (active) biomass are necessary in order to fully understand the physiology of microbial cells in the immobilized state, and to fine-tune a given biosystem. It has been shown that bioluminometry is a useful tool for determining the concentration of viable microbial cells encapsulated or entrapped in different matrices [92,94,95]. Since this technology is expected to play a major role in measuring biocompatibility of microbial cells, it will be explained in more detail below. An additional reason is that we also expect that this technology will be applied to the medicine and pharmacy field to quantify the survival of cells in immobilizing matrices.

6.3. Bioluminometry for determining biocompatibility properties

Viable biomass measurements are often complicated by the fact that some immobilization materials (e.g. hydrogels and polymers) may be rather difficult to dissolve in order to facilitate the biomass release followed by a gravimetric assay. Additionally, the results of typical gravimetric methods do not reflect the amount of active (viable) biomass present in the sample, and non-biocompatible immobilization materials can negatively affect the viability of cells over time. Bioluminometry offers the ability to determine the active biomass content by measuring the ATP concentration extracted from cells immobilized in hydrogel beads [94,95] and polyelectrolyte complex microcapsules [92]. Because ATP is rapidly degraded after cell death, its concentration is a good indicator of the cell viability and can be used to determine the concentration of the active biomass. The released ATP reacts with luciferin (LH_2) in a reaction catalyzed by luciferase, accompanied by the emission of bioluminescent light:

$$ATP + LH_2 + ½O_2 \xrightarrow{\text{luciferase}} LAMP + P - P + H_2O + h\nu$$

The efficient extraction of intracellular ATP is possible mainly due to the fact that 90% of the active biomass is located in a 140-μm thick outer layer of gel beads [96], which allows for easy release and diffusion of ATP out of the immobilized cells (Fig. 5a). Subsequent addition of the ATP monitoring reagent is followed by measuring the bioluminescence response (Fig. 5b). The light output expressed as RLU (relative light units) corresponds to the concentration of active biomass in the beads. The comparison shown in Fig. 3c exhibits a high degree of correlation ($r^2 = 0.9998$), making the bioluminometric method a rapid and accurate alternative to the well-established gravimetric assay.

7. Storage conditions for microcapsules

Storage of encapsulated cells for transport or in the time period between manufactory and application is mandatory for almost all fields of encapsulation. Determination of suitable conditions for

Table 1
The most common technologies for measuring biocompatibility properties of microcapsules in biomedicine and pharmacy.

Biocompatibility marker	Technologies of measurement	References
Pericapsular cell overgrowth		
Score of cell overgrowth	Light microscopy evaluation	[76,87,89,114–119]
Fibrosis score	Analysis of digitized images	[119–121]
Percentage of clean capsules	Cell adhering test	[122]
Area of capsular fibrosis	Histological analysis	[81,88–90,115,120,123–131]
Percentage of capsular overgrowth	Immunohistochemical evaluation	[114,116]
Degree of capsular overgrowth	SEM[a] analysis	[90,118]
Frequency of overgrowth	Fluorophotometry	[129,132]
Cellular composition of overgrowth	Liquid scintillation counting	[129,132]
Cell adhering ratio		
DNA content		
Glucose oxidation rate		
Viability of encapsulated cells		
Cell vitality	Fluorescent live/dead staining by CLSM[b]	[120]
Cell viability		
In-bead survival rate		[130]
Proliferation of cells	Life-Dead-Assay examined by fluorescent microscopy	[65]
Cellular mortality		
Cell death	Alamar Blue assay by fluorometry	[123,131]
	Propidium iodide staining	[133]
	MTT[c] assay	[133–136]
	Trypan blue exclusion assay	[130]
	Optical density measurement	[131,136]
Response of implantation site to microcapsules		
Inflammation at the implantation site	MTS[d] colorimetric assay	[87,131]
Tissue damage	Optical microscopy evaluation	[137]
Pro-inflammatory response	Quantitative autoradiography	[138]
Immune/immunological response	Quantification of tumor necrosis factor alpha	[126]
Immunoreactions	Mitogenic activity assay	[75,88,90,127,130]
Host reaction to microcapsules	Histological analysis	[139]
Tissue reactions/responses	Absorbance to detect antibodies in serum	[134,140]
	RT-PCR[e] measurement of cytokine mRNA expression in macrophages	[65,76,81,116,117,120,127–129,132]
Recovery rate of microcapsules		
Retrieval/recovery rate	Volumetry	[138]
Microcapsule recovery	Toxilight assay	[135]
Cytotoxicity	Measurement of lactate dehydrogenase activity, MTS colorimetric assay	[136]
Floating cells in peritoneal cavity	Hemocytometry	[115]
Secretion of proteins	Bradford dye-binding procedure	[135]

[a] Scanning electron microscopy.
[b] Confocal laser scanning microscopy.
[c] 3-(4,5-Dimethyl-2-yl)-2,5-diphenyltetrazolium bromide.
[d] 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt.
[e] Semiquantitative reverse transcriptase-polymerase chain reaction.

storage of microcapsules, however, plays an underestimated role in microencapsulation research. This is rather surprising since it is broadly accepted that microcapsule characteristics and functionality are often very sensitive to environmental parameters such as temperature, humidity, osmotic pressure, storage solution, or solvent [97–100]. In this review we have decided to separately discuss storage requirements since it is predictable that the large variations in outcome of encapsulation systems can partly be attributed to differences in storage of the encapsulated cells before application. It is mandatory to choose the storage conditions as such that it ensures maintenance of optimal performance.

The adequacy of a storage condition depends on the field of application and on which capsule characteristic should be maintained. It also involves the measurement of characteristics over time preferably focused on the variation of only one environmental parameter such as type of storage solution or temperature. Thereby, analysis can focus on changes in physical or chemical microcapsule properties such as diffusivity, mechanical strength or swelling [101–103], or on changes in properties of encapsulated cells such as catalytic activity [104,105], or performance as analyst. Time course, choice of parameter, and focus of the investigation strongly depend on the exact composition and application of the microcapsules.

For characterization of changes in essential physical and chemical microcapsule characteristics many techniques as already described in the previous sections of this article can be applied. It is essential to monitor the microstructures of capsules. Commonly applied technologies up to now are phase transition, retention. Integrity is usually assessed by imaging techniques such as optical microscopy, scanning electron microscopy (SEM), electron spin resonance spectroscopy (ESR), nuclear magnetic resonance spectroscopy (NMR), radioactive tracers, or fluorescence quenching [101], respectively. An indirect, but particularly realistic measure with regard to application comprises the determination of rupture time by an in situ observation of the release of active compounds such as drugs from microcapsule carriers [102,106]. Such an investigation requires a rather different set of analytical methods involving standard techniques for analysis of structure and concentration of dissolved compounds, such as NMR, mass spectrometry, GC, or HPLC. These are also useful for the determination of changes in properties of the encapsulated material. If functionality of this material mainly depends on chemical stability, as for drugs, cosmetics, or food ingredients [100], complete extraction of the active compounds from the microcapsules must usually precede the measurement [107–109], as methods for direct quantification of

Table 2
The most common technologies for measuring biocompatibility properties of microcapsules and beads in biotechnology.

Biocompatibility marker	Technologies of measurement	References
Viability of immobilized cells	Bioluminometry and gravimetry	[91,94,95]
	Sample staining with Live/Dead BacLight viability kit examined by epifluorescence microscopy and CLSM	[136]
	Viable cell counting	[141,142]
	Determination of CFU[a]	[143,144]
	Radiometry for measurement of protein and nucleic acid synthesis through ^{14}C amino acids and ^{14}C nucleic acids incorporated into cell proteins and DNA	[143]
Growth rate of immobilized cells	Optical density measurement	[141,145,146]
	Chlorophyll absorbance assay	[147]
	Counting of cells by hemocytometry	[148]
	Colony counting on agar plates	[142,149,150]
	On-line microscopy analysis	[151]
	Assessment of dry cell weight	[150,152]
	Cell protein content by Lowry method	[150]
	^{31}P and ^{13}C NMR studies	[153]
Biocatalytic efficiency and enzyme expression of immobilized cells	HPLC analysis	[154,155]
	Estimation of enzymatic activity by spectrophotometry	[142,145,156]
	Measurement of induced leakage of UV-absorbing substances from immobilized cells by spectrophotometry	[157]
Stress resistance of immobilized cells	Scintillation counting and ion release	[158]
	Spectrophotometry	[159]
Variations in protein spot densities observed on protein maps	Principal component analysis of spot quantity variations on electrophoretogram obtained by 2-D electrophoresis	[149,160]
Plasmid stability of immobilized recombinant cells	Screening of cell colonies for antibiotic resistance by culturing on agar plates	[161–164]
Respiratory activity	$^{14}CO_2$ measurement	[143]

[a] Colony-forming units.

chemical compounds within capsules are still rare. In contrast, performance of catalytically active compounds, like whole cell biocatalysts or enzymes, is usually approached while the catalysts remain in the capsules. The activity is then monitored via the reaction kinetics which can either be derived from the concentration of reactants allowed to diffuse in and out of the capsules [105,110], or more elegantly from the heat release during reaction. The latter uses flow calorimetry (FC) as a thermal biosensor, and has been successfully applied to the characterization of various types of encapsulated biocatalysts [111–113].

8. Concluding remarks

In spite of the tremendous growth of the industrial and clinical application of encapsulation in the past decade, it is still difficult if not impossible to define the requirement capsules have to meet in order to provide long-term functionality of the enveloped cells or bioactive components. For a further development of the technology and an exchange of technologies it is mandatory to standardize and define technologies that measure specific characteristics. The present review is the direct results of a common effort of

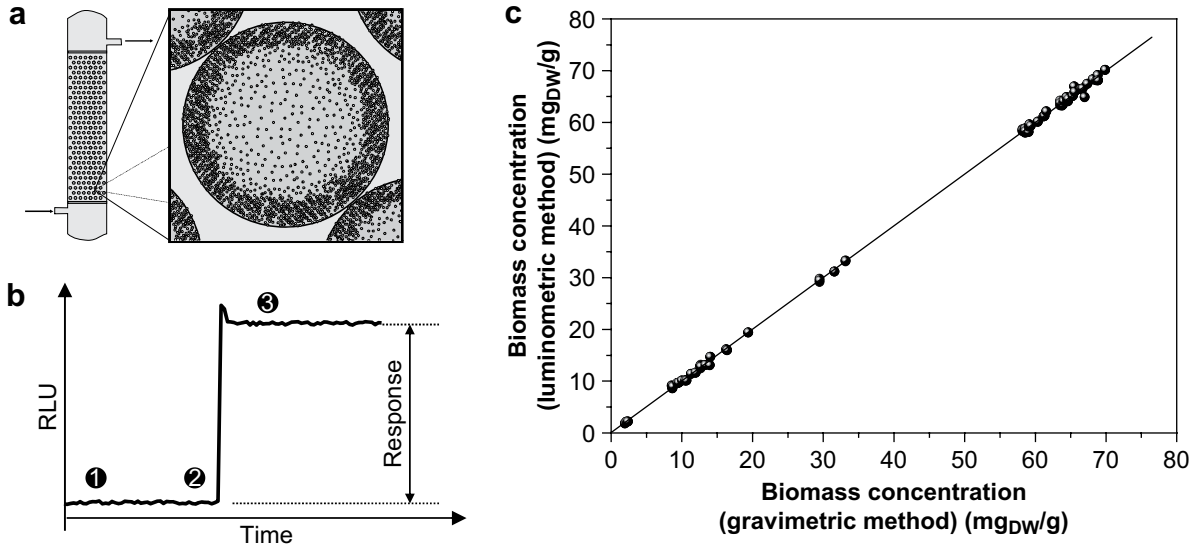

Fig. 5. Measurement of the active biomass concentration in hydrogels. (a) 90% of the active biomass is located in a 140-μm layer of a gel bead. (b) Upon addition of ATP release buffer ❶ and ATP monitoring reagent ❷ to immobilized cells, bioluminometric response ❸ proportional to the intracellular ATP content is generated and detected. (c) Comparison of the biomass concentration as determined by the luminometric (Y-axis) and gravimetric (X-axis) methods. All data points represent an average of at least 5 consecutive measurements.

researchers combined in the COST865 – action to define standardized protocols for characterization and standardization of microcapsule properties for a given application. The studies involve not only inventarisation and comparison of technologies on the basis of own experiences and published results but also on the basis of exchange of capsules and technologies in order to understand lab-to-lab variations and identification of technical details that require further standardization. The efforts are summarized on http://impascience.eu/COST865/index101.html.

Up to now five types of characterizations have been identified as mandatory for adequate description of a capsule. These are the characterization of the polymers applied, the permeability, the surface properties, the biocompatibility, but also the storage conditions. All these factors have a mutual influence on the functional properties of the final capsule.

Obviously, the authors are aware that the identification of mandatory characterizations will not immediately lead to a full implication of this assessment in studies in the near future. The application requires a multidisciplinary approach in which biomedical, physical, and chemical technologies are combined. However, for adequate reproducibility and for understanding variations in outcome of capsules it is advisable if not mandatory to include the characterization in future studies. The described technologies in the present review may be helpful in accomplishing these goals.

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