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Advances in biocompatibility and physico-chemical characterization of microspheres for cell encapsulation☆

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ABSTRACT

Cell encapsulation has already shown its high potential and holds the promise for future cell therapies to enter the clinics as a large scale treatment option for various types of diseases. The advancement in cell biology towards this goal has to be complemented with functional biomaterials suitable for cell encapsulation. This cannot be achieved without understanding the close correlation between cell performance and properties of microspheres. The ongoing challenges in the field of cell encapsulation require a critical view on techniques and approaches currently utilized to characterize microspheres. This review deals with both principal subjects of microspheres characterization in the cell encapsulation field: physico-chemical characterization and biocompatibility. The up-to-day knowledge is summarized and discussed with the focus to identify missing knowledge and uncertainties, and to propose the mandatory next steps in characterization of microspheres for cell encapsulation. The primary conclusion of this review is that further success in development of microspheres for cell therapies cannot be accomplished without careful selection of characterization techniques, which are employed in conjunction with biological tests.

Contents

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1. Introduction

Cell microencapsulation is a multidisciplinary research field where polymers, biomaterials, surface chemistry and engineering meet biology, immunology, medicine and surgery. Most researchers entering the field are fascinated by the opportunities for application and, at glance, the simplicity of production of hydrogel microcapsules and microbeads in conjunction with cell encapsulation. However, as the complexity in transplantation of encapsulated cells is associated with a wide variety of factors involving (i) transplanted cells, (ii) biomaterials and (iii) heterogenic recipient properties, over the years it has become apparent that entering the clinics with therapy based on immunoprotected cells by encapsulation is a challenging task.

Research on microencapsulation of pancreatic islets dominates the studies on immune protection of transplanted cells. This is mainly driven by the high number of diabetic patients and benefits of intrahepatic islet transplantation in terms of freedom from endogenous insulin, controlling the blood glucose levels, reducing the diabetic complications and improving quality of life [1]. Immunoisolation of cells by encapsulation, *i.e.*, transplantation of cells without immunosuppression, would be a major breakthrough towards safer and widely applicable cell therapy in diabetes treatment. Recent reviews on islet encapsulation describe the current situation in transplantation of encapsulated islets and provide insight in factors determining successes and failures of transplanted immunoprotected islets [2–4]. Examples of other cell therapies, which would benefit from transplantation of encapsulated cells, include the treatment of neurological and sensory diseases [5], treatment of liver disorders by bioartificial liver devices [6] and cardiac repair [7]. Common for all these therapies is the requirement of safe (in terms of recipient) and functional (in terms of encapsulated cells) microspheres to provide long-term immunoprotection of cells capable of treating the specific disease.

Several types of microspheres have recently been used in clinical trials for diabetes treatment [8–11] as well as for neural and sensory diseases [5]. However, a recipe for perfect and unquestionably clinically applicable microspheres is so far not available. The reason for this situation originates, at least partially, from the inability to specifically point out the factors contributing to success or failure of the transplanted device. This is at least partially due to the high numbers of variations in the way microspheres can be produced. However, in spite of this, the development of successful microspheres for cell therapies has been associated with a number of successful cases. But, up to now, the final set of recommendations with the general validity could not be provided because, similarly as in the case of failures, the reasons for the success could not be completely understood. A few reasons for uncertainties can be provided. Primarily, the polymer characterization, including composition, molecular weight and purity, is not always given for the encapsulation system, as well as the details in protocols for microsphere formation are missing as recently reviewed [12]. Although a terminology for microspheres is regularly given, as e.g. APA for alginate–poly-L-lysine (PLL)–alginate microspheres, the same type of microspheres can have complete different properties. This is caused by variation in alginate composition and molecular weight, gelling ions, non-gelling ions, solubilized or gelled core, PLL molecular weight, size of microspheres, exposure and washing times [13]. Hence, care should be taken in comparing studies and drawing conclusions from these comparisons as different microspheres might be applied. The properties of microspheres are usually determined before implantation and the correlation of performance of encapsulated cells with the properties of microspheres after exposure to the *in vivo* environment is missing. This is seen as an incorrect approach as the properties of microspheres may change after implantation [14]. In addition, the quality and viability of encapsulated cells are important for success. This is especially the case with pancreatic islets, where cell viability varies considerably due to donor variations and differences in efficacy of the enzyme-driven isolation process. Furthermore, the presence of encapsulated cells may influence microsphere properties such as the mechanical stability [15]. These are obviously only a few examples from the number of variables which have to be controlled and documented as a part of the encapsulation and transplantation protocols.

The study of the correlation between microsphere design, the protocol of encapsulation and the *in vitro* and *in vivo* performance requires a series of characterization methods. Many *in vitro* [16,17] and *in vivo* [14,18] approaches have been considered as appropriate to identify the factors determining the functional properties of microspheres. The methods regularly employed for characterization of microspheres are described in a recent monograph [19] and review paper [12]. The aim in applying various methods is to understand the mutual relationship between microsphere characteristics and performance in the presence and absence of cells, and to identify the important contributors to an "optimal window" [20] for the conditions providing reproducible graft function *in vivo*.

The characterization of microspheres with encapsulated cells is carried out on two mutually related platforms: (i) physico-chemical and (ii) biocompatibility. The complexity of parameters involved in microsphere characterization towards safe and functional performance is depicted in Fig. 1. The microsphere is manufactured from a biomaterial which requires characterization of the selected polymers. The mechanical stability, permeability and morphology characteristics of the microcapsules are important for the *in vivo* performance. The microsphere surface properties are highly important for the interaction with the host proteins and cells. The characterization methods are recommended for application in order to get a deeper understanding of the microsphere physico-chemical properties, since these properties are tightly linked to the biocompatibility and the functional performance of the microspheres. The biocompatibility assessments represent the second part of Fig. 1, and include aspects of immune compatibility, cell compatibility as well as characteristics connected to the transplantation site and the recipient.

Also the term "biocompatibility" needs to be considered. "Biocompatibility" has been used as a term within the last 40 years to describe the performance of a material after implantation [21]. The "biocompatibility" definitions are debated [21,22], and may hold different meanings depending on the type of applications and strategy [21,23]. In the field of microspheres, the "biocompatibility" term has been used to describe apparently contradictory outcomes, *i.e.*, microspheres both with no cellular overgrowth and those causing blood vessel formation can be considered as biocompatible. The strategies for functional grafting of

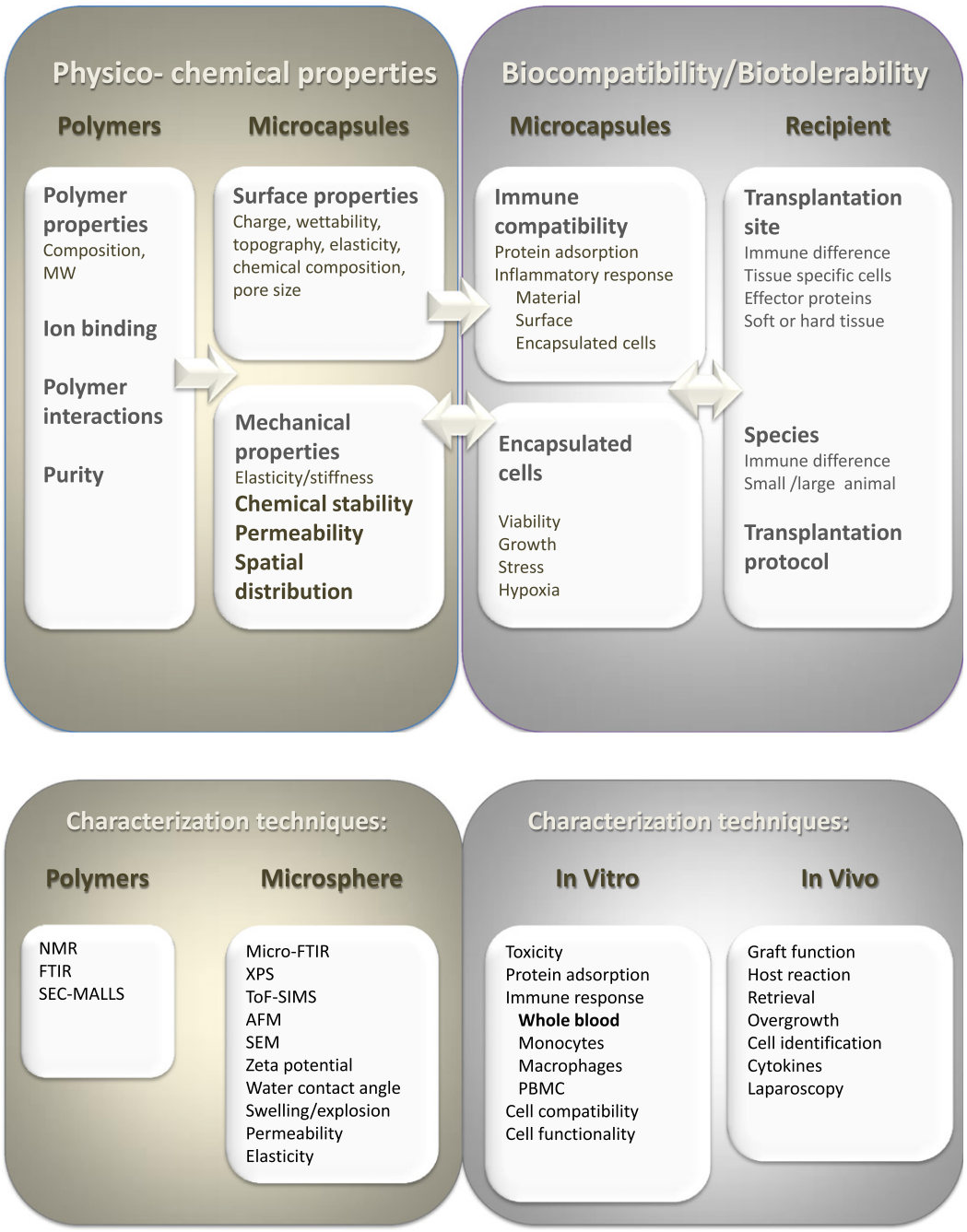

Fig. 1. Determinants of the "optimal window" for functional characterization of microspheres for cell encapsulation. Upper panel: physico-chemical properties and relation to biocompatibility/biotolerability. Lower panel: characterization methods/properties for polymers and microspheres related to physico-chemical characteristics, *in vitro* immune and *in vivo* specific properties related to biocompatibility/biotolerability. Arrows point at the principal mutual dependency of determinants. The abbreviations are explained in the text.

transplanted cells by using microspheres should follow the line to provoke none or, at most, minimal cellular overgrowth to ensure free diffusion of nutrients and oxygen, as well as exchange of the therapeutic proteins. This can be considered as a "biotolerability" strategy, a term recently introduced by Ratner to describe "the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reactions" [21]. To create "biotolerable" microspheres free of cellular overgrowth is one of the most pressing challenges in the field of cell encapsulation. In order to achieve functional and thus biocompatible/biotolerable microspheres, a deeper understanding of the interaction between the physico-chemical properties and the biological responses is needed.

The field of microsphere design and characterization has rapidly developed in recent years. In the subsequent sections we will discuss advances in the understanding of surface characteristics, mechanical properties, permeability, spatial distribution and leakage of microcapsule components. In addition, the biological factors with impact on microsphere biotolerability and functionality are described. The evaluation methods for microsphere biocompatibility are discussed with an emphasis on quantifying inflammation induced by microspheres or enveloped cells. Notably microspheres are immunoprotective and not meant to prevent responses. Immune responses will always occur due to leakage of antigens, breakage of microspheres, protrusion of cells, and due to responses associated with the surgery. This review is prepared with the hope that it will assist in selecting proper approaches for thorough characterization of microspheres, understanding the correlation between microsphere properties and performance to design novel, effective steps towards clinical application of therapies with

microencapsulated cells. Before describing the physico-chemical properties of microcapsule systems used for cell encapsulation in Section 2 and biocompatibility/biotolerability aspects in Section 3, the terminology should be clarified. The terms microspheres, capsules, microcapsules, beads and microbeads have been used interchangeably. To avoid confusion, in this review we use the term *microsphere* as the collective term for all systems used for microencapsulation of transplanted cells, the term *microcapsule* for the encapsulation system consisting of two or more polymers forming the membrane at the microcapsule surface, and the term *microbead* for the encapsulation system typically made of the one-polymer forming a continuous hydrogel of varying degree of homogeneity.

2. Microsphere physico-chemical properties

2.1. Surface characterization

2.1.1. Chemical analysis

Surface characterization of microspheres is important to gain knowledge about the physico-chemical requirements which microspheres have to meet in order to be tolerated by the host. Table 1 contains an overview of methods, which have been used for characterization of microspheres for encapsulation of mammalian cell. These methods will be described in correlation with the microsphere design. It should be mentioned that care should be taken in interpretation of some of the results as most of these methods require drying of the microspheres. The microspheres for cell encapsulation are exclusively made in the form of hydrogels with a high water content, which removal may be associated with artifacts. We will only discuss results where the intactness of microcapsules has been confirmed with microscopy.

The use of X-ray photoelectron spectroscopy (XPS) has brought a new insight to the surface characterization of microspheres [24]. XPS allows for identification of the chemical groups on the surface on an atomic level. The surface is bombarded by X-rays and the number and kinetic energy of ejected electrons are measured. This is proportional to the number of atoms and indicative for the element it originated from. Hence, quantitative information on the elemental composition of the surface can be obtained. Subsequent studies combining XPS with time-of-flight secondary ion mass spectrometry (ToF-SIMS) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) have brought further details regarding the chemical characteristics of microcapsules on a nanometric and micrometric scale, respectively. XPS has a penetration depth of the atomic structure of up to 10 nm and ToF-SIMS up to 1–2 nm. ToF-SIMS is based on sputtering the surface with a beam of primary ions and measuring the mass of secondary ions ejected from the top surface. Quantification is not easily obtained and hence ToF-SIMS is used in combination with other surface characterization techniques. The surface analysis by ATR-FTIR spectroscopy is on a micrometer scale and provides the information on bulk chemistry of materials in a spatially resolved manner [25].

A combination of XPS, ToF-SIMS and ATR-FTIR to characterize the surface of alginate-PLL microcapsules has been performed using slowly dried samples [16,26]. This slow drying avoids artifacts in the membrane. XPS revealed PLL in high amounts at the outermost 1–2 nm layer of the microcapsule surface [26]. ToF-SIMS analysis revealed that PLL was present on the surface in the form of the polyelectrolyte complex with alginate. FTIR was applied to show that the interaction between PLL and alginate at the surface is driven be hydrogen bonds. After binding to the surface of alginate microbeads, calcium is substituted by PLL. The PLL is then present in α-helix, antiparallel β-sheet and random coil conformations [27].

2.1.2. Topography and elasticity

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) can also be used to characterize and visualize the surface structure of microspheres. SEM can give an insight into the pore structure, the permeability and the topography, and in combination with ion beam sputtering can provide 3D images of microspheres. The topography and the morphology of microspheres can be preserved using new microscopy techniques. They do not require drying of the hydrogel samples (Environmental or Wet SEM) or the morphology is preserved by carefully freezing the sample (cryoSEM). The Environmental SEM allows analyses on hydrated materials without fixing, drying, freezing or specimen coating [28]. The application of the so called Wet SEM is to the authors' knowledge technically still limited for microsphere characterizations, although it was recently reported to work for characterization of hollow magnetite-containing chitosan microcapsules crosslinked by glutaraldehyde [29]. The cryoSEM method has been applied for characterization of alginate microbeads, used for encapsulation of bacteria [30] and βTC3 insulin-producing cells, formed by emulsion and internal gelation methods as well as by dripping method [31]. The method-dependent differences were identified in surface topography [31] illustrating the efficacy of cryoSEM for characterization of hydrogel microspheres.

AFM can be used to characterize hydrated surfaces including surface topography and elasticity by nanoindentation performed in a liquid environment [32–34]. A diamond indenter of known dimension is

Table 1
The list of principal methods and equipment used for physico-chemical characterization of microspheres.

Characterization method/ equipment	Obtained information
XPS	Surface chemical composition (sample depth ~10 nm)
ToF-SIMS	Surface chemical composition (sample depth ~2 nm)
ATR-FTIR	Surface chemical composition (sample depth ~a few micrometers)
AFM	Surface local mechanical properties and topography
Zeta potential	Surface charge
WCA	Surface hydrophilicity
Wet SEM	Surface topography
Texture analyzer	Compression resistance
Instron	Compression resistance
Micromanipulator	Compression resistance
Agitation	Resistance to shear stress
Osmotic stress and explosion assay	Resistance against destabilization
Vortex or orbital mixer and chelation	Resistance to shear stress and osmotic destabilization
ISEC	Permeability, molecular weight cut-off and pore size distribution
Ingress/egress	Permeability and rate of diffusion.
CLSM	Polymer spatial distribution and permeability

XPS (X-ray photoelectron spectroscopy), ToF-SIMS (time-of-flight secondary ion mass spectrometry), ATR-FTIR (attenuated total reflectance Fourier transform infrared spectroscopy), AFM (atomic force microscopy), WCA (water contact angle), SEM (scanning electron microscopy), ISEC (inverse size-exclusion chromatography), CLSM (confocal laser scanning microscopy).

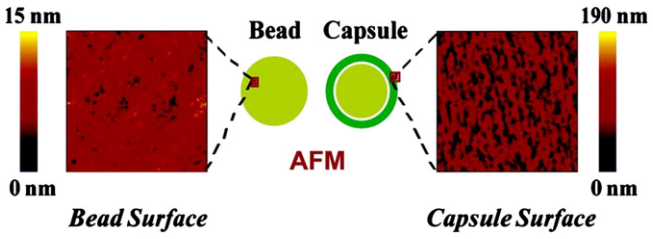

Fig. 2. Topography of microspheres. Selected atomic force microscopy images of alginate microbeads and alginate–cellulose sulfate–poly(methylene-co-guanidine) microcapsules. Reprinted with permission from American Chemical Society, M. Lekka, D. Sainz-Serp, A. J. Kulik, C. Wandrey, Hydrogel microspheres: influence of chemical composition on surface morphology, local elastic properties, and bulk mechanical characteristics, Langmuir 20 (2004) 9968–9977.

pushed into the surface leaving an indent. The depth of penetration, up to a user specified load, is monitored. Lekka et al. characterized the local surface topography of nine different microsphere types by contact mode AFM [32]. The surface roughness of the alginate microbeads and alginate-polycation containing microcapsules varied from 0.9 to 14.4 nm with the lowest surface roughness for alginate microbeads and alginate–PLL–alginate microcapsules and the highest for alginate-cellulose sulfate-poly(methylene-co-guanidine)(PMCG) microcapsules (Fig. 2). The surface elasticity (Young's modulus), was showing a range of 0.4 to 440 kPa and forces at maximal compression in a range of 34 to 455 mN. The highest Young's modulus was observed for alginate–cellulose sulfate–PMCG microcapsules (178–438 kPa) while alginate microbeads and alginate–PLL microcapsules were in the range of 0.4 to 18 kPa. Higher surface stiffness was found for alginate microbeads crosslinked with barium and lower values for microbeads crosslinked with calcium ions. This correlates to the Young's modulus of alginate gels measured by macroscopic compression measurements [35]. Notably, the authors point to a high standard deviation in the measurements resulting from both surface and batch heterogeneity, illustrating possibly a low reproducibility of the microsphere formation process. Hence, AFM can be a powerful tool to determine variability between different batches of microspheres, between different microspheres within one batch and even the variability in the surface characteristics of one microsphere [32].

2.1.3. Hydrophilicity and charge

Other measures of microsphere surface properties include analyses of surface charges that can be measured by the zeta potential and hydrophilicity, *i.e.*, the degree of wettability. The hydrophilic nature of a surface is directly correlated with the water contact angle. A hydrophilic surface exhibits a high wettability and a low water contact angle. The opposite applies to a hydrophobic surface. Hydrophobic surfaces are having a higher adsorption of proteins and denaturation of proteins at the surface. This leads to exposure of new epitopes which are believed to be a cause of immune reactions towards hydrophobic materials [36]. Hydrophilic surfaces are therefore preferred for microspheres aiming on transplantation of encapsulated cells. Hydrophilicity of materials used for formation of microspheres can be determined by measuring the water contact angle on dry films. Tam et al. applied this approach and found similar hydrophilicity of dry alginate films regardless of alginate composition and molecular weight, and type of gelling ions [37]. However, films of alginate–PLL and alginate–poly-L–ornithine (PLO) showed a lower hydrophilicity (*i.e.*, higher water contact angles) than alginate films alone [18]. Thus polycation coating of alginate microbeads increases the hydrophobicity. In addition, an alginate-dependent effect upon the polycation coating was observed [18]. The sample with the lowest hydrophilicity was with an alginate containing 44% guluronic acid (G) gelled with barium and coated with PLO.

The zeta potential is another helpful tool to characterize the surface properties. Preferably the zeta potential should be negative and comparable to cell membranes in the immediate vicinity of the microspheres in the host. Positive charges promote adsorption of proteins, which is the starting point for cellular overgrowth as discussed in Section 3.2. The zeta potential of microspheres is determined by the electrophoretic mobility by forcing an electrolyte through a measuring cell unit containing the microspheres, as the large microsphere size does not allow direct measurements of the zeta potential. A pressure drop depending upon the flow resistance of the sample is detected across the measuring cell. The circulation of electrolyte through the cell results in a flow of ions, and the resulting potential difference is measured. During a measurement the pressure drop and drop in potential are recorded and the zeta potential is calculated. De Vos et al. [14] measured the zeta potential of alginate–PLL–alginate microcapsules of 50% G alginate versus 40% G alginate before implantation and 1 day post implantation. Both types of microcapsules had negative surface charge and similar zeta potential before implantation (-3.6 ± 0.2 mV and -3.5 ± 0.2 mV at pH 7). After explantation the zeta potential for microcapsules prepared using alginate with the G content of 40% was unchanged while those prepared using alginate with the G content of 50% had less negative charges (-0.5 ± 0.7 mV) [14]. For the alginate–chitosan–alginate microcapsules Xie et al. showed zeta potentials in the range from –3.5 to –6.2 mV with variations depending on the alginate used [38]. The zeta potential of Ca-alginate microbeads has been shown to be in the range of –10 mV [39]. It is important to emphasize that the zeta-potential is influenced by the surrounding fluid, therefore the comparison of the zeta potentials of various microspheres can only be applied for comparison when identical conditions are used.

Recently Hook and coworkers [40] proposed a toolbox of techniques consisting of AFM, XPS, Raman or IR microspectroscopy, ToF-SIMS and water contact angle to be applied in a high throughput screening. This type of screening is powerful for new polymers and new combinations of polymers. However, limitations exist when moving from the film format to the format of microspheres. Also it is uncertain whether these laborious techniques can really achieve the stage of high throughput.

2.2. Physico-mechanical characterization

2.2.1. Mechanical resistance and stability

Microspheres applied for long-term immunoprotection of cells must be non-biodegradable, durable and mechanically stable for periods as long as the cells are functional. Mechanical and chemical stability are therefore the most crucial properties for functional microspheres. Mechanical disintegration will lead to loss of immunoprotective function and graft failure. This mechanical disintegration can be caused by many factors. One of them is swelling of the hydrogel network that leads to increased permeability of the gel and impaired immunoprotection as a consequence. Also, the swelling may induce accelerated host responses as leaching of the microsphere components is enforced as well as the leakage of cellular components from the enveloped cells.

The review articles published in recent years [12,20,41,42] describe the importance of controlling the mechanical properties of hydrogel microspheres and provide details on approaches to analyze and enhance the mechanical stability. In general, the hydrogel materials should have adequate mechanical stiffness (resistance to deformation) and toughness (resistance to fracture) to structurally protect transplanted cells [42] against the forces *in vivo*. Table 2 lists the methods employed for characterization of mechanical properties of microspheres for cell encapsulation. The most commonly applied methods include (i) testing the physical resistance using, most typically, a compression test by texture analyzer or other commercial devices, and such techniques as micromanipulation and atomic force microscopy and (ii) various types of more qualitative experiments such as swelling and explosion assays and exposure to agitation and shear force. The latter assays are important for microcapsules to study undesired swelling. This is especially important for ionically crosslinked hydrogels as the exchange of ions between the hydrogel network and the solvent may lead to influx of water and increased osmotic pressure followed by hydrogel swelling and disintegration. As outlined above this may lead to loss of immunoprotective properties and enhanced host responses. The swelling of microspheres is commonly determined by the increase in size upon exposure to different solutions, e.g. water, saline, chelating agents [35,37,43]. The microsphere bursting can be determined in different solutions and mixtures of water and buffers creating hypotonic solutions of different ionic strength [15,44]. The bursting is usually quantified by counting the number of broken microspheres or by measuring the leakage of immobilized molecules (e.g. dextran-blue, FITC dextran or hemoglobulin) [18,43]. Adding buffers and salt solution to water reduces the osmotic influx of water to the microspheres and thus represents lower osmotic pressure in microspheres than when using water alone.

Table 2
Methods and equipment for characterization of mechanical properties of microspheres applied for cell encapsulation.

Method/equipment	Microsphere type	Reference
Texture analyzer	Alginate–PLL–alginate	[51]
Instron	Alginate–PLO–alginate	[52]
Micromanipulator	Alginate microbeads	[48,51,53]
	Alginate–oligochitosan–alginate	[54]
	Alginate–PLL–alginate	[55]
	Alginate–PLO–alginate	[55]
	Alginate microbeads	[56]
	Alginate–chitosan–alginate	[56]
	Alginate–chitosan–PEG	[56]
	Alginate–chitosan–PEG–alginate	[56]
	Alginate–PLL–alginate	[57]
Atomic force microscopy	Alginate–PLL–alginate	[32,57]
	Alginate	[32]
	Alginate–cellulose sulfate–PMCG	[32]
Agitation in the presence of (hard) beads	Alginate–PLL–alginate	[58–61]
	Alginate–PLO–alginate	[58]
	Alginate–protamine sulfate–alginate	[62]
Osmotic stress and explosion assay	Alginate	[35,37,43]
	Alginate–PLL–alginate	[18]
	Alginate–PLO–alginate	[18]
Vortex or orbital mixer agitation and chelation	Alginate–PLL–alginate	[55,63,64]
	Alginate–PLO–alginate	[55]
	Alginate–protamine sulfate–alginate	[62]
Agitation	Alginate–carrageenan–chitosan	[65]

PLL (poly-L-lysine), PLO (poly-L-ornithine), PMCG (poly(methylene-co-guanidine)), PEG (poly(ethylene glycol)).

Stabilizing the hydrogel network based on physical (Coulombic) interactions is one strategy to enhance mechanical and chemical stability of microspheres. Recent efforts are highlighted below. Alginate microbeads ionotropically gelled by calcium cations are commonly stabilized by barium cations [35,45–48] since barium has a higher affinity for alginate chains with high content of G-blocks than calcium [49]. Thus, high-G alginate (67% G) is stable against extensive exposure to saline solution when gelled with barium or with a mixture of calcium and low concentration of barium [35,50]. Similar results were found for swelling of alginate microbeads in water where both calcium microbeads of intermediate-G (44% G) and high-G (71% G) alginates disintegrated faster compared to gels crosslinked with barium [37]. Increased mechanical stability of alginate microbeads can also be achieved by covalent binding using Staudinger ligation with phosphine-terminated poly(ethylene glycol) chemoselectively reacting with azide-functionalized alginate [66,67]. This covalent crosslinking results in complete resistance of microbeads against osmotic swelling. Beneficial effects on mechanical stability and elasticity were found for the hybrid microbeads formed by interpenetrating networks of alginate and covalently crosslinked multiarm vinyl sulfone-terminated poly(ethylene glycol) [53]. The covalent reinforcement of polyelectrolyte complex has been recently proposed for alginate–PLL microcapsules as well. A crosslinking between the reactive copolymer of poly(methacrylic acid, sodium salt-co-2-[methacryloyloxy]-ethyl acetoacetate) with PLL has been applied that is a versatile system as the strength and permeability of microcapsules can be adjusted by varying the depth of penetration of the reactive copolymer and PLL [63,64]. Recently the covalent crosslinking of PLO by genipin was employed with the goal to enhance the biotolerability of alginate-PLO microcapsules [52]. The series of tests consisted of osmotic pressure test in water, agitation test in the presence of glass beads and a time stability test in buffer. These tests were employed to identify suitable conditions for formation of alginate–protamine sulfate–alginate microcapsules [62,63].

The mechanical resistance of microspheres has to be high enough to resist the mechanical load during manipulation, transplantation and *in vivo* application. Presently the values to achieve that goal and measured by Young's modulus, degree of deformation, mechanical stress, rupture load (burst strength) are not known. Reviewing the literature focused on implantation of microspheres aimed at cell encapsulation revealed an important flaw. Only a few papers report data on mechanical stability of microspheres. Alginate microbeads gelled in 20 mM barium chloride solution, which were used in a clinical trial [10], were tested in compression [48]. The microbeads exhibited a 70% deformation for a compression force of 7 and 16 g/microbead when gelled for 20 and 2 min, respectively. A compression force of 5 to 10 g/microbead was found for 65% deformation for alginate microbeads, gelled in molar ratio of calcium and barium 50: 1, which were tested in mice models [47,68]. A burst strength of 1 to 2 g/microcapsule was reported for DIABECELL® alginate–PLO–alginate microcapsules [52,69], which have been currently tested in clinical trials with encapsulated neonatal pig islets [11]. For microcapsules made of alginate, cellulose sulfate and PMCG implanted intraperitoneally in primates [70] a burst strength value of 10 g/microcapsule was reported [20]. Based on these results it is difficult if not impossible to recommend a value for the mechanical stability of microspheres. More systematic studies designed to quantify these values before implantation and after explantation are urgently required.

It is relevant to mention that in other fields than cell microencapsulation new approaches to measure mechanical properties of microspheres are emerging. Mercade-Prieto and Zhang recently reviewed the mechanical characterization of core-shell melamine-formaldehyde microcapsules [71] and of fluid-filled microcapsules that rupture near the elastic regime [72] for silica-based microcapsules by a micromanipulation technique. This work discusses the elastic regime, the inelastic behavior, the rupture and the time-dependent effects, and provides experimental details of measurements and information on applied models [71]. Atomic force microscopy and compression testing by micromanipulation were highlighted as promising among other novel and system-specific techniques.

2.2.2. Permeability

The permeability is principally important as microspheres are designed to contain a semi-permeable membrane for (i) protecting cells from destruction by the immune system, and (ii) permitting the permeation of oxygen and biological compounds required for viability and normal function of cells. Although this principle is known for many years, the requirements the membrane has to meet to protect the encapsulated cells from the immune system are still not known. The performance of encapsulated cells and permeability of microspheres has been correlated as outlined by reviews in the past [73,74] and more recently [2,12,20,41]. As this correlation depends on a number of factors with unknown impact on the performance of encapsulated cells, it is currently difficult to define the appropriate permeability of microspheres immunoprotecting the transplanted cells [75]. A review dedicated to this subject is included in this journal issue.

The permeability of microspheres is determined by size-based exclusion as well as by the rate of diffusion of macromolecules [12]. The first parameter is the exclusion limit of the semi-permeable membrane commonly defined as the molecular weight cut-off (MWCO). MWCO refers to the minimum molecular weight, respectively minimum size, of a solute that is completely excluded by the microcapsule membrane. Notably however, misinterpretation is a risk in this area. Since the size for solutes of the same molecular weight largely differs between polysaccharides (dextrans, pullulans) and proteins, it is imperative to report on the type of solute applied in each study. It is still insufficiently realized that polysaccharides and proteins cannot be compared with each other [76], since for the same molecular weight, the size of proteins (globular conformation) is significantly lower than the size of polysaccharides (statistical coil). Another pitfall is the neglecting of the rate of solute diffusion through the polymeric network, which is usually characterized only by the MWCO value.

During the booming period of islet encapsulation in 1990s, the encapsulation community was strongly focused on identification of

the adequate semi-permeable properties. It was recommended that immunoglobulins should be excluded with an effective size in the range of 5–15 nm [77] and the pore size should not exceed 13 nm [78]. However, other studies showed that the pore size of even a few tens of nanometers may immunoprotect the transplanted cells. In these cases immunoglobulins and complement proteins may permeate the semi-permeable membrane [79,80]. The difficulties in recommending a permeability value was expressed by Dugas et al. postulating that the principal role of microsphere membranes should be in preventing the immune cells from entering the microcapsule and directly destroying the grafted cells, and that a major limitation to successful transplantation studies lies in the design of immunologically inert polymers [81]. However, this may be debated as illustrated by several laboratories that show that inhibiting the cross-talk between grafted and immune cells via cytokines and chemokines [82,83] may be beneficial for survival of the cells. This large range in permeability characteristics given by these two limiting cases corresponds to a recent statement by O'Sullivan et al. that the requirements for permselectivity are not yet clearly defined [2].

In order to advance the knowledge in controlling the permeability, we need to have a basic understanding of the underlying mechanisms involved in destruction of the encapsulated cells, which have been discussed in [75]. Although it is a complicated topic that is behind the scope of this review, the principal relevant aspects can briefly be summarized as follows: The direct contact between the encapsulated cells and the immune effector cells are of particular importance in allograft rejection, thus, preventing the cell contact by encapsulation may be sufficient for transplantation of allograft. In xenografts, the components leaking from the encapsulated cells will be regarded as foreign antigens, which lead to production of immunoglobulins. The immunoglobulins, in combination with complement, contribute to direct lysis of cells through the membrane-attack complex. This requires the assembly of complement factors on the surface initiated by the large glycoprotein C1q (410 kDa) of the classical pathway, and also involving the C3 (185 kDa), C4 (210 kDa), C5 (190 kDa), C8 (170 kDa) and the smaller fragments of C2, C6, C7, C9 (all in the molecular weight range of 79–128 kDa). In addition to this, cell death may also be induced by cytotoxic cytokines that direct cells to undergo apoptosis. Cytokines in general have a low molecular weight and excluding these cytokines from permeation through the membrane would compromise the encapsulated cells since also important nutritional components may be excluded.

Hence, the permeability of the microspheres most likely needs to be adjusted depending on the specific case of transplantation, *i.e.*, allo- or xenotransplantation, which should lead to understanding of the mechanisms of immunoprotection and the role of permeability properties of microspheres. This emphasizes the requirement to characterize the semi-permeable properties of microspheres by at least determining the MWCO value and the solutes which permeate to the microsphere interior. This is the minimum information that has to accompany all studies related to the microsphere design and *in vitro* and *in vivo* applications. Below the most commonly applied methods employed to determine the semi-permeable properties are listed (Table 3).

The methods can be categorized according to differences in the solute type, the analytical method, the direction of diffusion and the obtained parameters [12,41,73]. Likely the most typical way for determining the semi-permeable properties of microspheres in biological laboratories is tracing the ingress and/or egress of radio- or fluorescently labeled molecules including proteins and polysaccharides. This was done, for example, for alginate microbeads tested by ingress of IgG [35,50,68] and dextrans [84,93], ingress of vitamin B_{12}, IgG and carbonic anhydrase [90] and IgG and bovine serum albumin [89] into alginate–chitosan microcapsules, IgG and bovine serum albumin ingress into alginate–PLO–alginate microcapsules [94], egress of bovine serum albumin from alginate–PLL–alginate and alginate–PLO–alginate microcapsules prepared under different conditions [55], ingress of fluorescently labeled dextrans and bovine serum albumin into alginate–PLL–alginate microcapsules reinforced by covalent bonds [63], and egress of fluorescently labeled dextrans from alginate–PLL–alginate microcapsules prepared using different gelling protocols [86].

Table 3
Methods used to characterize semi-permeable properties of microspheres applied for cell encapsulation.

Microsphere type	Solute/method	Reference
Alginate	Proteins by CLSM or radiolabeled	[35,50,68]
	Dextrans by fluorescence microscopy	[84]
	ISEC by pullulans	[31,48,68]
Alginate–PLL–alginate	Proteins by UV–vis spectrometry	[55]
	Proteins by CLSM	[85]
	Dextrans and proteins by CLSM	[59,63]
	Dextrans by fluorescence spectroscopy	[86]
	Dextrans by HPLC	[76]
	Dextrans and proteins by ISEC	[87,88]
	Pullulans by ISEC	[76]
Alginate–PLO–alginate	Proteins by CLSM	[89]
	Proteins by UV–vis spectrometry	[55]
Alginate–chitosan	Proteins by UV–vis spectrometry	[89–91]
Alginate–cellulose sulfate–PMCG	Pullulans by ISEC	[92]
Alginate–poly(DADMAC)–PSS	Dextrans and proteins by ISEC	[6]

PLL (poly-L-lysine), PLO (poly-L-ornithine), PMCG (poly(methylene-co-guanidine)), polyDADMAC(poly(diallyl dimethyl ammonium chloride)), PSS (poly(sodium styrene sulfonate)), CLSM (confocal laser scanning microscopy), HPLC (high-performance liquid chromatography), ISEC (inverse size-exclusion chromatography), UV (ultraviolet).

In addition to ingress/egress methods exemplified in the previous paragraph, the inverse size-exclusion chromatography (ISEC) can be used as a powerful method to determine the MWCO values. ISEC also provides the information on pore size distribution of microspheres. This method was established by Brissova et al. for microspheres utilized in cell encapsulation [76]. ISEC is the column separation technique based on the enthalpy-free partitioning of analyzed solutes, typically polysaccharides and proteins, of different molecular weight between mobile and stationary phase. The stationary phase is represented by the microspheres to be analyzed. The ISEC analysis results in determination of a chromatography partition coefficient K_{SEC} value, which is used for evaluation of the calibration curve with the exclusion limit characterizing the MWCO value [76]. The calibration curve can be transformed to integral and differential pore size distributions [92], which overall represent the permeability characteristics for a given batch of microcapsules. ISEC has been used to characterize various types of microcapsules including alginate–PLL–alginate [76,87,88], alginate–cellulose sulfate–PMCG [92], alginate microbeads prepared by dripping [48,68] and by emulsification and internal gelation method [31], and alginate–poly(diallyl dimethyl ammonium chloride)–poly(sodium styrene sulfonate) microcapsules [6]. The MWCO values of 250–350 kDa, related to proteins, determined by ISEC for alginate microbeads [48,68] are in agreement with the conclusions from the ingress/egress methods. Both methods show that the alginate microbeads are permeable to IgG. The increase in alginate concentration in conjunction with the emulsification and internal gelation method [31] was used to control the permeability of alginate microbeads. The ISEC data also confirm previous data that polycation-coated microcapsules exhibit lower permeability than alginate microbeads, which can be tuned by the coating protocol [92].

2.2.3. Polymer spatial distribution

Microspheres are typically formed by gelling the droplets of polymer solutions in the presence of gelling ions and oppositely charged polymers. The gelling conditions influence the spatial distribution of polymers in the microspheres from liquid, almost polymer-free, core and highly concentrated hydrogel membrane to almost homogeneous hydrogel throughout the microspheres. This is an important factor for the long-term performance of microspheres in terms of stability,

permeability, and surface characteristics. Therefore, the information on spatial distribution of polymers in the microsphere volume should be documented along with the information on other properties. Confocal laser scanning microscopy (CLSM) is a method used for characterization of the spatial distribution of polymers in microspheres. The advantage of CLSM is in the ability to visualize samples in the native liquid environment with minimum requirements for sample manipulation and preparation for analysis.

The CLSM images are obtained from the so-called confocal spot, located inside the sample volume, formed by fluorescence or back-scattering light [95]. Most of the light is blocked and only the light from the confocal spot can pass to the photomultiplier. To obtain a full image, the sample is sequentially scanned in axial and lateral directions with the possibility to reconstruct the whole 3D volume image by taking a series of images at successive layers of the sample. Comprehensive information on history, principles, technical issues and applications of CLSM was published in a recent monograph edited by Pawley [96]. Also recently, reviews were published on using CLSM for characterization of colloidal systems [97], in pharmaceutical applications [98] and for characterization of polymeric microspheres [95]. The latter review is highly relevant for this work and describes (i) history, principles and modes of CLSM, (ii) practical considerations in CLSM imaging, (iii) CLSM for characterization of microspheres used for the bioartificial pancreas, (iv) CLSM for characterization of other microspheres and microparticles, (v) CLSM for characterization of hydrogels, and (vi) provides a detailed overview on fluorescently-labeled substances visualized by CLSM.

The multitude opportunities in application of CLSM for characterization of microspheres aimed at cell encapsulation are shown in Fig. 3. They can be categorized into the following groups [95]: (i) determination of the local chemical composition of the membrane as well as of the core, (ii) ingress and egress of various compounds to estimate the diffusion properties of microspheres, (iii) viability and localization of cells, (iv) localization of other immobilized compounds, (v) biocompatibility of membrane, protein attachment and cell overgrowth.

The focus of this part is particularly on application of CLSM for visualization of microspheres for cell encapsulation. Table 4 contains the information on labeled polymers and microspheres, for which CLSM was employed to visualize their spatial distribution of polymers in microspheres. This table demonstrates that CLSM has been utilized for commonly applied microcapsules such as alginate–PLL–alginate, alginate–PLO–alginate, alginate microbeads, as well as for novel microsphere designs and conformal coating. One of the first applications of CLSM in studies on immunoisolating microcapsules was done by Vandenbossche et al. [99], who studied the membrane formation between alginate and PLL using the fluorescently labeled PLL. Lamprecht et al. [100–102] demonstrated the capability of CLSM to localize polymers arabic gum and gelatin [100–102], alginate, acacia and chitosan [100], encapsulated oil [100–102], casein [100,101] and bovine serum albumin [100] in the micrometer-scale microparticles. A detailed CLSM study focusing on alginate–PLL microcapsules was published by Strand et al. [103]. The authors described the spatial distribution of covalently labeled alginate and PLL as a function of selected gelling ions, the conditions used for coating alginate microbeads by PLL and the effects of washing and storing of microcapsules. The authors showed that depending on the gelling conditions the spatial distribution of the alginate chains across the microbead can range from almost homogeneous, *i.e.*, equal concentration of alginate in the core, to highly heterogeneous distribution with up to 10-fold higher concentrations at the microcapsule surface compared to the microcapsule center. The alginate spatial distribution in microbeads using different gelling divalent ions was visualized for covalently labeled alginates [35] showing that the concentration of ions rather than their type of alginate determines the degree of inhomogeneity of microbeads. This degree can be increased by using enzymatically engineered alginates. This results in a denser packing of alginate chains [50]. The reflection CLSM mode was used to visualize 3D distribution of $BaCl_2$ crystals injected in alginate droplets using a crystal gun method. This study suggests that homogeneous distribution of alginate chains throughout the microcapsule can only be obtained with simultaneous internal and external gelation [104,105]. The spatial distribution of polymers in alginate–cellulose sulfate–PMCG microcapsules was determined using both covalently labeled polymers and selected low molecular weight anionic and cationic fluorescent labels [95,106]. The residual positive charge within the membrane (thickness a few tens of micrometers) decreases towards the membrane interior, while the residual negative charge first decreases and then sharply increased at the interface between membrane and core of microcapsule. This information can be correlated with the spatial distribution of fluorescently labeled polymers. Hence,

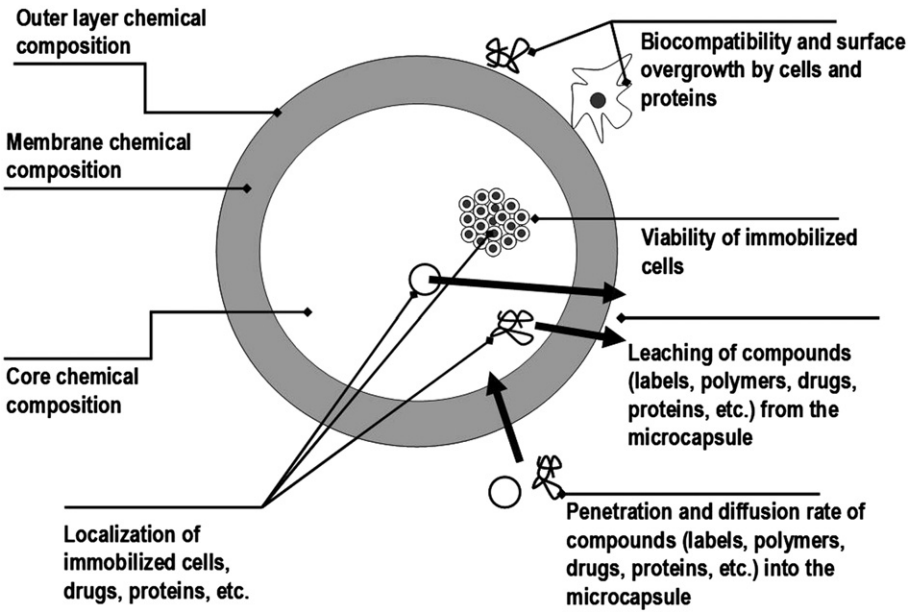

Fig. 3. Opportunities for application of CLSM in characterization of polymeric microcapsules. Reprinted with permission of Transworld Research Network, I. Lacík, D. Chorvát, Jr., Visualisation techniques in the characterization of polymer microcapsules: confocal laser scanning microscopy and atomic force microscopy, in: J. P. Halle, P. de Vos, L. Rosenberg (Eds.), The bioartificial pancreas and other biohybrid therapies; Transworld Research Network; Kerala, 2009, Chapter 8, pp.137–175.

Table 4
Visualization of polymers by CLSM in characterization of microspheres applied for cell encapsulation (in alphabetical order).

Polymer visualized by CLSM	Microsphere type	Reference
Alginate, sodium salt	Alginate–PLL–alginate	[103]
	Alginate–PLO–alginate	[107]
	Alginate	[35,50,103]
Chitosan (via genipin)	Alginate–chitosan	[90,91,108]
Heparin	Conformal coating	[109]
PEGs, and its conjugates	Various types of conformal coating	[110–114]
PLL	Alginate–PLL–alginate	[63,64,86,99,103]
PLO (via genipin)	Alginate–PLO–alginate	[52]
PLO	Alginate–PLO–alginate	[107]
Poly(methacrylic acid, sodium salt-co-2-methacryloyloxy]ethyl acetoacetate)	Alginate–PLL–alginate	[63,64]
Poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate -co-4-vinylphenyl boronic acid)	Poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate -co-4-vinylphenyl boronic acid) combined with poly(vinyl alcohol)	[115]
PMCG	Alginate–cellulose sulfate–PMCG	[95]
Poly(methyl vinyl ether-alt-sodium maleate)	Alginate–PLL–alginate	[116]
Soluble complement receptor 1	Agarose	[117]
Poly(N-vinyl pyrrolidone)	Conformal coating based on poly(N-vinyl pyrrolidone) and tannic acid	[118]
Poly(vinyl alcohol)	Conformal coating	[111]

PLL (poly-L-lysine), PLO (poly-L-ornithine), PMCG (poly(methylene-co-guanidine)), PEG (poly(ethylene glycol).

when low molecular weight fluorescent labels are properly selected they can be used to visualize the residual charge of polyelectrolytes without the need for labeling polymers. In the novel design of reinforced alginate–PLL–alginate microcapsules via covalent cross-linking between reactive copolymer poly(methacrylic acid–sodium salt–co-2-[methacryloyloxy]-ethyl acetoacetate) and PLL of different molecular weights, CLSM was applied to visualize the depth of penetration of PLL, which is critical for determining the degree of reaction between PLL and reactive copolymer [63,64].

Conformal coating keeps being one of the emerging strategies for cell encapsulation [111,119]. CLSM plays an important role in visualization of the protective polymer layers, or other protective layers, formed around cells. A number of examples exist, which include alginate and PLO layers [107], heparin–DOPA conjugate [109], various PEG–lipid conjugates [111,112], and other polymers [107,109–111,114,115,118,119]. Other examples involve the combination of conformal coating with cyclosporine, anti-CD4 monoclonal antibody [113], and human soluble complement receptor 1 [120], and the protective layer formed of living cells [121]. Overall, CLSM has demonstrated its applicability for determination of presence, quality and stability of the conformal coating around the cells.

Currently CLSM became a standard visualization technique in the field of cell encapsulation. CLSM utilized for identification of spatial distribution of polymers is a valuable tool for understanding the properties of microspheres. It is applied for the co-localization of various polymers in case of multi-component microspheres, local versus overall concentration of individual polymers, the membrane formation process and the temporal stability in various media. The future applications should be based on individual or combined opportunities of CLSM shown in Fig. 3. Other opportunities of CLSM are in studying the temporal stability of microcapsules under long-term *in vitro* conditions or in identification of the spatial distribution of polymers in microcapsules after exposure to *in vivo* conditions. This may provide insight into the correlation between the chemical microenvironment of immobilized cells (local chemical character of the matrix within the whole microcapsule volume) and their performance.

2.2.4. Leakage of intracapsular components

Leakage of microsphere-forming components (polymers, gelling ions) may cause changes in microsphere properties over time. Furthermore, leaking materials from microspheres may stimulate inflammatory responses by the host. Mannuronic (M) acid has been shown to leak out of Ca-alginate microbeads prepared of high-M alginates [122]. The leaked molecules were analyzed by ^{1}H NMR and SEC-MALLS. The high leakage of molecules correlated with the poor gel forming properties of high-M alginate with low molecular weight. As high-M alginates are shown to induce cytokine production from monocytes [123,124], alginate microbeads should be washed before implantation to reduce leakage and subsequent risk of inflammation *in vivo*. Hydrolysis of alginate at physiological pH occurs at a slow rate and over time the leaked low molecular weight alginate is cleared from the body via secretion by the kidneys [125].

The leakage of PLL from the microcapsules made of high-M alginate was found to be higher than for the high-G gel and is increasing with the exposure to ions of high affinity to alginate [13]. PLL binding to calcium alginate gels is highly dependent on the composition of alginate as high-M (43% G) alginates bind more PLL than high-G (63% G) alginates. Leakage of polycations has been quantified by using radiolabelled polycation [13]. The leakage of barium from Ca/Ba- and Ba-alginate microbeads has been studied as barium ions are known to be toxic [49]. Calcium and barium were quantified by an inductively coupled plasma mass spectrometry in formed microbeads, in different washing solutions as well as in mouse blood and femur bone. Some leakage of barium ions was found in the washing solutions of both high-G (67% G) alginate Ba-microbeads (20 mM $BaCl_2$ in gelling solution) and Ca/Ba-microbeads (1 mM $BaCl_2$ and 50 mM $CaCl_2$ in gelling solution). The barium accumulation in femur bone of mice implanted with microbeads shows dose-dependent deposition and higher barium accumulation in case of microbeads prepared in the presence of high barium (20 mM) concentration than with Ca/Ba microbeads [49]. The leakage data for barium in this study was compared to relevant exposure limits defined by the World Health Organization and found to be under the limits for the Ca/Ba microbeads and slightly over the limits for the Ba microbeads. Leakage of barium should be avoided for safety reasons. Since barium leakage is dependent on the polymer characteristics (e.g. alginate composition), measuring of barium leakage is therefore recommended after implantation of barium-containing microspheres.

3. Biocompatibility/Biotolerability

The terms biocompatibility and biotolerability was introduced and discussed in Section 1. It was also stated that the strategy of making biocompatible microspheres for cell encapsulation therapy should aim at designing systems provoking none or, at most, minimal cellular overgrowth in order to ensure free diffusion of nutrients, oxygen and therapeutic proteins. This could also be defined as a “biotolerability” strategy. In order to achieve this goal, we need to understand the

mechanisms and the processes at the interface between the biomaterial and the host. An important statement regarding these issues has been introduced by D. F. Williams saying: "The mechanisms by which human tissues respond to biomaterials are not unique for a particular application but are merely variations of natural processes between the material and the host" [23]. In view of this statement, in the next sections the current knowledge of acute and chronic inflammatory mechanisms to biomaterials will be reviewed. As shown in Fig. 1, the therapeutic cell encapsulation concept involves layers of factors, which, in addition to the material, include also the encapsulated cells and the transplantation environment. It is beyond the scope of this review to go deeper into these issues, nevertheless, a brief description of factors which are essential for making cell-compatible microspheres will be reviewed. At last the *in vivo* findings in animal and clinical studies with emphasis on various microspheres and their biocompatibility are discussed.

3.1. Host responses to microspheres

The host responses to biomaterials follow a pattern that has been thoroughly described for biomaterials in general [126–129] and for microencapsulation systems [130] in particular. The host responses can be divided into an acute and a chronic inflammatory response followed by a long-lasting granulation tissue phase [126]. Briefly, the early inflammatory responses include adsorption/desorption of proteins and immunoglobulins, activation of effector cascades as complement, coagulation and fibrinolysis, and attraction of granulocytic neutrophils, monocytes/macrophages and lymphocytes. The chronic inflammatory responses are characterized by macrophages and lymphocytes and will disappear during a normal tissue response to injury after around three weeks. The granulation tissue phase is manifested as conditions with variable appearance of fibroblasts and fibrosis, foreign body giant cells (FBGC), macrophages and capillary formation. The host responses are graphically illustrated in Fig. 4. The phenomena of protein adsorption/desorption and the immediate effector cascade reactions are covered in the next section with a focus on microspheres and hydrogel systems. Based on publications describing the host responses to empty microspheres after intraperitoneal implantation in small animals [14,133–135], the cellular responses in the acute inflammatory phase can be described as follows: Granulocytic neutrophils appear early at the implantation site and/or on the implanted microspheres. Granulocytic basophiles are also present, but their presence may be induced by surgical intervention itself rather than as a specific response to microspheres. The granulocytes carry chemotactic cytokines and chemokines important for chemotactic responses. The early appearance of granulocytes is followed by the influx of monocytes/macrophages and lymphocytes within the first days of implantation, and fibroblasts appear on microspheres within the first weeks. Foreign body giant cells have also been identified within this period of the first week, and their presence has been suggested to be a result of surface imperfections [14]. The transition between the acute and chronic phase seems therefore to occur within the first week of transplantation. The description of granulation tissue formation is based on information from clinical studies of islets-containing microspheres transplanted to the peritoneal cavity (Section 3.6). These studies refer to "vascularized fibrous tissue" or "fibrous tissue containing thin-walled capillaries" with specification of mild histiocytic responses as well as scattered macrophages and giant cells with few lymphocytes and polynuclear cells [8,10]. It is important to emphasize that the granulation tissue formation in this case could be a result of the encapsulated cells and their state of performance (described in Section 3.3).

It is important to emphasize that we should not make firm conclusions solely on the studies demonstrating overgrowth. In rodent models, the cellular overgrowth phenotypes against empty microspheres may vary with microspheres compositions [14,135,137]. One example of variations in cellular phenotypes is found in a study comparing alginate–PLL–alginate, alginate–PLL–heparin and alginate–PLL–polyacrylic acid microcapsules [135]. Cells were identified four weeks post-transplantation to peritoneum. The major cell type on alginate–PLL–alginate microcapsules were fibroblasts, whereas also macrophages and endothelial cells were found on alginate–heparin–alginate microcapsules. In contrast, alginate–PLL–polyacrylic acid microcapsules were free of cellular overgrowth. Other studies also report on retrieval of microcapsules free of overgrowth after implantation [47,68,70,137–139].

In order to understand the background for the observed responses and why microspheres also can be devoid of overgrowth, we will focus in the following section on factors that may contribute especially to the early inflammatory stage, since this activation sets the stage for the chronic responses later in time.

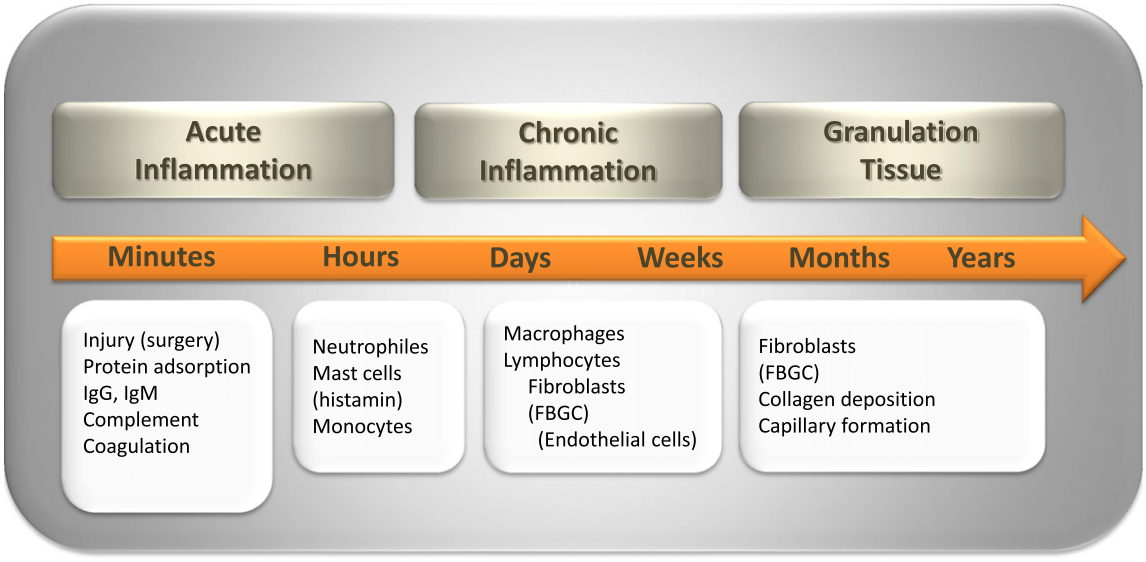

Fig. 4. Overview of the host inflammatory reactions based on earlier descriptions of a normal inflammatory response to biomaterials [126,127]. In addition, responses for the microsphere systems are included. The acute and chronic inflammatory responses are based on information from rodent models evaluating responses of empty microspheres after intraperitoneal transplantation [14,131–136]. The information on granulation tissue responses are based on reported information from human clinical studies upon intraperitoneal transplantation with encapsulated pancreatic islets [8,10]. FBGC; foreign body giant cells.

3.2. Immunocompatibility

3.2.1. Protein adsorption

Protein adsorption is a highly complex phenomenon with a significant impact on the biocompatibility of biomaterials [36]. The amount of adsorbed proteins, the protein types and possible denaturation of proteins determine the further response from the host. In general, hydrophilic surfaces are known to give low adsorption of proteins and a low degree of denaturation, while the opposite is the case for hydrophobic surfaces. Recently, in a comprehensive review by Vogler, important aspects of protein adsorption related to definitions, methods and interpretations of results were discussed [36]. In the following section, we summarize some important findings related to characteristics of biomaterial surfaces and subsequent protein adsorption relevant for immune compatibility before reviewing some recent literature on protein adsorption to microspheres used for cell encapsulation.

The surface properties of a biomaterial seem to be the most important factor for protein adsorption, as shown in proteomics studies of blood perfusion devices [140,141]. Also, the patient's characteristics were secondary to the biomaterial surface properties as shown in a prospective cross-over study of chronic hemodialysis patients [141]. Conformational changes of the proteins may occur during adsorption [142], a phenomenon more frequently observed for larger proteins [143]. The conformational changes and proteins adsorbed may actuate the activation of host effector mechanisms involving reactive protein cascades as complement, immunoglobulins, and immune cells. The surface chemical characteristics [144–148] as well as the pore sizes [149] are factors that could influence the ability of complement C3 to be activated. The alternative complement activating pathway can be triggered by surface mediated activation of C3 [150], and the classical pathway can be mediated through an initial binding of IgG or IgM on biomaterial surfaces [144,151,152]. In a study using protein films of either IgG, human serum albumin or fibrinogen employing a quartz crystal microbalance dissipation for analysis, it was demonstrated that complement C3 was activated by IgG or human serum albumin, while activation was avoided on a layer of fibrinogen [153].

Fibrinogen can still have a potential role in cellular reactions to biomaterials [154] through mechanisms involving the binding to the integrin receptor CD11b [155]. Xu et al. published interesting data related to the adsorption of fibrinogen on surfaces with variable hydrophilicity [156]. Fibrinogen bound stronger to poorly hydrophilic surfaces through formation of multiple energy barriers, whereas highly hydrophilic surfaces formed single energy barriers leading to reduced binding. The lower energy barriers on hydrophilic surfaces also reduced the protein unfolding [156]. By increasing the loading force and rates between fibrinogen and surface, the protein unfolding profile rapidly changed to the patterns seen for poorly hydrophilic surfaces [156]. These data indicate that force, mostly absent in the *in vitro* model systems, may be an important factor in protein adsorption. Transmitted to animal models, "force" or gravity may be a factor that is contributing to differences between small and large animal models by directly impacting fibrinogen adsorption, as illustrated in the present *in vitro* model.

Microspheres used for cell encapsulation are almost exclusively made of hydrogels, and in general possess high hydrophilicity due to their high water content. Biologically relevant proteins such as immunoglobulin, serum albumin, fibrinogen and coagulation Factor XII are shown to bind less to experimental surfaces exhibiting high hydrophilicity [143,156,157]. Also the conformational changes of proteins are reduced on surfaces with higher hydrophilicity [157]. In spite of this generally high hydrophilicity of the alginate-based microspheres, common plasma proteins from complement, coagulation and fibrinolysis cascades were identified on alginate–PLL–alginate microcapsules implanted intraperitoneally in dogs [131]. As discussed in 2.1.3, different microcapsule formation protocols result in differences in hydrophilicity [18,37], which may be of importance for adsorption of proteins. Tam et al. detected IgG, IgM and IgA on the surface of the alginate–PLL–alginate microcapsules, but not on alginate microbeads after exposure to human serum or human peritoneal fluid [132]. Another study demonstrated that various chitosan-containing microcapsules adsorbed IgG, while coating with a layer of methoxy PEG could reduce the adsorption [158]. Rokstad et al. found complement factor C3 on the surface of alginate–PLL–alginate and alginate–cellulose sulfate–PMCG microcapsules after incubation in whole blood [17]. By incubating in plasma, an accumulation of C3 was demonstrated on the surface of alginate–PLL–alginate microcapsules but not on the surface of the alginate microbeads [159]. These studies suggest there are variable protein deposition patterns and activation states possibly depending on the characteristics of the microspheres. This can explain the varying degrees of success of the different systems.

Alginate hydrogels seem to resist collagen deposition. This was shown in a study aiming at facilitating fibroblast growth in an alginate hydrogel. It was shown that despite matrix proteins production only small amounts of collagen proteins were deposited within the hydrogel [160]. Protein adsorption seems to be a key step for alginate to allow collagen deposition as it was reported for alginate microbeads exposed to human peritoneal fluid [16].

3.2.2. Reactive protein cascades

As discussed in Section 3.2.1, surface adsorption may lead to conformational changes of the proteins. This can lead to activation of the effectors of complement, coagulation and fibrinolysis cascades. Proteins from these effector cascades were found on surfaces of microspheres after intraperitoneal transplantation [131,136]. In addition to this, the coagulation and complement cascades are present in inflammatory and fibroproliferative responses [161] and in cellular overgrowth [162]. Complement components are constituents of the peritoneal fluids [16,163], present in fat tissue [164] and produced by macrophages [165,166]. Hence, complement activation may be a part of the inflammatory responses in the peritoneal cavity and sites normally not in contact with blood. The coagulation and effector cascades involvement in inflammation are therefore briefly summarized below.

The complement effector proteins contribute to inflammatory events by generating "danger signals" recognized by leukocytes and other cells involved in inflammation. An excellent overview of the complement system and its involvement in homeostasis and inflammation is given by Ricklin et al. [167]. The coagulation system is a tightly regulated system for maintenance of blood homeostasis. However, the system is also contributing to inflammatory events [161,168,169]. Accumulating evidence suggests that also the fibrinolysis system is involved in inflammation [170]. The complement and coagulation systems are highly interconnected through several interaction points as demonstrated in original articles [171–173] and in reviews [169,174]. Since these systems are easily activated, they are tightly regulated by proteins present on the surface of host cells or in solution [167]. Foreign surfaces, as bacteria and biomaterials, lack these regulators thus the effector systems are easily activated upon contact.

Of the three complement activation pathways, only two seem to be involved in the biomaterial-induced complement activation. The activation of the alternative pathway is generally believed to be the most important for biomaterials [175]. In the activation stage, C3 splits to C3b and C3a, a process that is probably accelerated by interaction with a surface [176]. This leads to a sequence of thoroughly described steps [150,177] that lead to the formation of C3 convertase (C3bBb) and C5 convertase (C3BbC3b). The split products C3a and C5a are strong chemo attractants for leucocytes. Particularly C5a is involved in a multitude of inflammatory events [178]. The activation also leads to opsonization of the biomaterial membrane by C3b and iC3b (inactivated C3b), which are ligands for the leukocyte receptors CR1 (CD35) and CR3 (CD11b/18). The classical pathway of complement activation may also be involved through activation via immunoglobulin surface adsorption [144,151,152]. The binding of antigen–immunoglobulin complex

normally induces conformational changes in the Fc portion of the immunoglobulin exposing a binding site for the C1 components (C1q, C1r, C1s) of the classical pathway. For proper binding, a clustering of the IgG molecules is needed. The adsorbed IgG on the biomaterial surfaces likely also needs to be present in clusters and subsequently undergoes conformational changes in order to activate the classical pathway.

The coagulation pathway is divided into intrinsic and extrinsic pathways. The extrinsic pathway is activated by Tissue factor (TF) inducibly expressed by blood monocytes, and constitutively expressed on extravascular cells as fibroblast [179,180]. The complement C5a is among the factors that may induce TF. The intrinsic pathway is activated by binding of factor XII to negatively charged surfaces with the involvement of prekallikrein–kallikrein system. Both activation pathways activate factor X to factor Xa. Subsequently this leads to a downstream activation ending in clot formation through conversion of fibrinogen to fibrin.

3.2.3. Whole blood model

Whole blood contains central effector proteins and immune cells involved in the early stages of inflammation. These systems consist of complement, coagulation and fibrinolysis cascades, as well as of leukocytes and platelets. The complement and coagulation systems are closely interacting with leukocytes; therefore whole blood can be used to measure the inflammatory potential of microbeads and microcapsules. A prerequisite for an efficient whole blood model is that the functional effector systems can be activated and interact with each other. The used anticoagulant in the whole blood assay will determine which effectors are still able to be activated. One of the most physiological models is based on the coagulation inhibition through blocking thrombin using lepirudin [181]. Within this model, all of the complement components and most of the effectors for coagulation (with exception of thrombin) are able to interact. Details regarding the model and various anti-coagulants have recently been described [182]. The model has been used for mechanistic studies of bacterial blood infections [181,183–187], blood perfusion biomaterials [188–190] and later also for glucose sensors [191,192]. Studies on microspheres have recently been performed [17,159] and results are summarized below.

Various microspheres based on alginate with a high ratio of guluronic acid (≥60%) have been evaluated. The studies were performed with Ca/Ba microbeads, Ba microbeads, alginate–PLL–alginate (APA), alginate–PLL (AP) microcapsules and alginate–cellulose sulfate–PMCG microcapsule ("PMCG" is used as an abbreviation for this microcapsule) [17]. The PLL-containing microcapsules provoked complement and the inflammatory mediators TNF, IL-1b, IL-6, IL-8, MCP-1, MIP-1a, and VEGF [159]. A direct connection between complement activation and the ability to trigger inflammatory cytokines was confirmed. In contrast, alginate microbeads did not provoke complement activation or inflammatory cytokines. The PMCG microcapsule provoked complement activation and cytokines but, in general, it was less responsive than the PLL-containing microcapsules. C3 has been repeatedly found to accumulate on the surface of PLL-containing microcapsules but not on alginate microbeads, as shown in [17] and in Fig. 5. The deposition of C3 is not homogeneous but variably on the surface of PLL-containing microcapsules as shown in Fig. 5. These results indicate that active C3 and C5 convertases (C3bBb and C3bBbC3b) are formed on the surface of PLL-containing microcapsules. The comparison of PLL-containing microcapsules and alginate microbeads illustrates that the PLL has to be held responsible for the complement activation. This is also supported by testing the microcapsule individual components, where PLL does and alginate does not activate complement [17]. When using PLL-containing microcapsules, one usually incubates the Ca-alginate microbeads first with PLL (forming AP microcapsules), and thereafter with alginate (forming APA microcapsules). A comparison between APA and AP microcapsules showed that they both activate complement and cytokines with a slightly higher activation by the AP microcapsules [159]. In this respect, a study by Tam et al. showed that PLL is located in the outermost surface

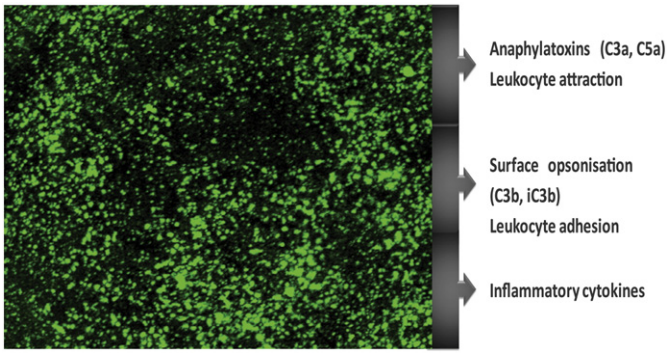

Fig. 5. The deposition of complement C3 on the surface of an alginate–PLL–alginate microcapsule made of high-G alginate. The spotted pattern of complement C3 indicates that complement has been activated and complement C3 and C5 convertases (C3Bb and C3BbC3b) have been formed on the surface. Activation of C3 on the surface will lead to inflammation as a consequence of released anaphylatoxins (strong chemoattractants), opsonization of the surface with C3b and iC3b resulting in leukocyte-attachment, and subsequently secretion of cytokine [17,159]. The larger spots indicate early activation and formation of convertases by a positive loop effect (from the alternative complement pathway). The smaller spots indicate the formation of convertases at a later stage. Detailed description of the formation of the C3 and C5 convertases can be found in [150,177]. The picture was taken after incubation of microcapsules with human lepirudin-plasma for 6 h, staining with FITC-marked antibody (anti-C3c). Image was taken by sectioning through the microcapsule by CLSM and converted to 3D using Zeiss software (LSM510) at the CMIC core facility at NTNU.

of APA microcapsules complexed with alginate in various states forming superhelical coil formations and beta-sheets [26]. This confirms older data that alginate reduces the immunogenicity of surfaces by interaction with PLL, and that the complexation with PLL is influenced by the flexibility, composition and molecular weight of the alginate chains. Inappropriate neutralization of PLL could lead to the complement activation by C3. The C3 cleavage products C3b and iC3b function as the surface opsonins. Leukocytes bind to the inactivated C3b (iC3b) through CD11b integrin receptors. This might explain our findings that parallel on C3 deposition cell adhesion was found on the PLL-containing microcapsules, but not on the alginate microbeads [17].

The sensitivity of the whole blood model makes it particularly suited for revealing the inflammatory potential of various microspheres and materials used for their formation. It may be useful for determining the immunogenicity of various alginate and polycation combinations as well as for studying the immunogenicity of microspheres produced by various protocols. At present it is possible to compare up to 20 types of microspheres in one whole blood assay, and to study the impact of minor differences in the microsphere-formation protocols on initiating the inflammatory response. This so far could not be achieved by any other technique. The human whole blood assay can thus be an efficient tool to correlate the design of microspheres with the inflammatory potentials in order to design low-inflammatory encapsulation devices for the human recipient.

3.2.4. In vitro studies

Biomaterials and their constituents have also been tested in various *in vitro* cultures of primary cells from blood or with macrophage cell-lines. These systems are useful to elucidate the direct effects of microspheres and particularly their constituents on immune cell function; however, due to the use of serum instead of plasma and heat inactivation they exclude the reactive protein cascades important for studies of surface reactivity as discussed in Sections 3.2.2 and 3.2.3. Despite of this, other phenomena related to cell adhesion, activation and fusion with relevance to overgrowth and foreign body reactions can be studied.

The effect of the charge of hydrogel surfaces on macrophage spreading was investigated by Smetana et al. [193,194] in the early 1990s. Hydrogels rich in –OH, –CO–NH–, and $(CH_3)_2N$-groups induced spreading of macrophages, whereas spreading of macrophages was inhibited on materials containing $-SO_3H$ and –COOH groups [193]. The fusion of

macrophages was inhibited on the surface of materials containing acidic groups (–SO_3H, –COOH), while the fusion increased on hydrogels containing 30 mol% of alkaline $(CH_3)_2N$-groups. Albumin adsorption was found to decrease by increasing the amount of –COOH groups, which also coincided with lower macrophage binding and spreading [194]. Human monocyte culture system (autologous serum) was used to determine whether adherent monocytes/macrophages cytokine production was influenced by hydrophobic, hydrophilic, anionic and cationic surfaces using polyethylene terephthalate as a substrate [195]. The hydrophilic and anionic surfaces promoted the anti-inflammatory type of response by inducing more IL-10 and less IL-8 from adherent monocytes/macrophages. Monocyte adhesion and IL-4-mediated macrophage fusion was also inhibited by these surfaces. Hydrophilic and anionic surfaces have also been shown to increase the ability of macrophages to undergo apoptosis [196].

Lymphocytes can be found on the surface of biomaterials, but their role in foreign body reactions has been poorly understood. Brodbeck et al. demonstrated that human lymphocytes contribute to foreign body reactions by increasing the monocyte adhesion (autologous cells and serum) [197]. Co-culturing of macrophages and fibroblasts has also been efficient for increased responses related to the foreign body reactions [198].

The effect of hydrogel stiffness on macrophage (RAW 264.7) revealed that stiffer gels induced more cell spreading and increased integrin staining. The stiffer hydrogels also induced higher levels of cytokines compared to softer hydrogels [199]. The impact of biomaterial topography was studied using polytetrafluoroethylene materials with variation in intranodal distance from 1.2 μm to 4.4 μm [200]. The cytokine expression and fibrous capsule formation was increased by larger distance of nodes implying that a rougher surface contributed to more inflammation and overgrowth.

To the authors' knowledge, only a small number of studies on microspheres have been published using primary immune cells or cell lines. The ability of microcapsules and alginate microbeads to produce cytokines was evaluated in a rat alveolar cell line using heat inactivated fetal calf serum [201]. The PLL-containing microcapsules induced more TNF, IL-1b and IL-6 mRNA than alginate microbeads. In contrast, after exposure to peritoneal fluid and subsequent addition to human PBMC, alginate microbeads, but not PLL-containing microcapsules, induced TNF secretion [16].

A few more studies have been undertaken to explore the immunostimulatory properties of the microspheres constituents. The exposure of monocytes to soluble PLL showed induction of TNF at low concentrations of PLL and cell necrosis at higher concentrations [202]. The effect of alginate compositions was extensively studied 20 years ago using human monocytes [124,203,204]. High-M alginates (85–95% M) stimulated inflammatory cytokines (IL-1, IL-6, TNF) by mechanisms requiring CD14 [204]. Short alginate G-blocks inhibited the effect of the high-M alginate demonstrating the sequence specific stimulation [203]. The follow-up studies demonstrated the importance of the way of exposure of polymers to monocytes [205,206]. The stimulatory properties of high-M alginates was shown to depend on the pattern recognition receptors toll-like receptors 2 (TLR2) and 4 (TLR4) [123].

The effect of alginate on stimulation of dendritic cells has recently been studied [207,208]. In one of the studies, alginate films stimulated dendritic cells to increased MHCII, CD86 and CD80 and to increased expression of IL-1β, IL-6, TNF, IL-10 and IL-12 [208]. The technical grade alginate was used in this study, which was purified before use, but no specification on M/G content was provided, nor were there any measures to judge the final purity of the alginate. Another study used alginate with a mannuronic acid content higher than 50% [207]. This high-M alginate was shown to increase CD83 and CD86, TNF and IL-6, as well as the ability to stimulate allo-responses. These studies suggest that some alginate-components may have the potential to stimulate dendritic cells. It remains to be elucidated whether there are differences related to these properties for alginates with different chemical composition.

3.3. Cell compatibility

The encapsulated cells are encountering a new type of environment upon encapsulation, which can directly impact their functional performance. The matrix affects cellular growth and survival. Previous studies with cells with a high proliferative capacity have demonstrated that alginate matrices with higher hydrogel stiffness and stability negatively impact cell survival and secretion of therapeutic proteins [15,209–213]. In addition, the survival patterns vary between different cell types, thus optimization of the hydrogel matrix should be considered for various cell systems. Some cell types need to grow in clusters/spheroids to survive. These cell types benefit from solubilizing the core of microspheres since more space is available for growth [214]. Also in other systems it is demonstrated that the matrices have impact on the cell fate [215]. Another issue to be considered is the stability of the microspheres that are challenged by cellular growth [15]. Nutritional and immunological challenges are additional factors related to cell proliferation. Overcrowding within an enclosed compartment may lead to competition for nutrients and massive cell death [210]. In microspheres, the dying cells are not removed by the normal clearing mechanisms involving phagocytes taking care of apoptotic cells, but rather become necrotic and release their cellular content. The cellular components normally hidden from the host immune system contain potent inflammatory mediators [216], collectively designated as damage-associated molecular patterns (DAMPS) or alarmins [217]. The alarmins are detected by the host immune system through the toll-like receptors [217]. Cellular overcrowding in mild form will also reduce the availability of oxygen for encapsulated cells. Hypoxia might also lead to secretion of mediators involved in inflammation and angiogenesis as has been demonstrated for islets [218]. Islets are susceptible to produce pro-inflammatory cytokines [219] and, thus, the post-encapsulation conditions could also have impact on the islet function.

Transcriptome analyses has been used to evaluate pancreatic islets performance after encapsulation and showed no negative effects of encapsulation on the gene expression of islets [220]. Positive effects of the encapsulation to islets function using Ca/Ba-alginate microbeads of about 500 μm diameter have also been described [8]. Supporting cells as mesenchymal stem cell [45,221,222] or protein film networks [223] have been proposed to improve the graft function. Although functional survival of an intraperitoneally implanted graft has been shown for more than 450 days in mice [224] a major challenge remains to further enhance survival by stimulating oxygenation and access to nutrients for the encapsulated graft in vivo [225].

3.4. Vascularization

Insufficient oxygenation due to the absence of vascularization has been considered to be the Achilles heel of cell-encapsulation. For therapeutic application of pancreatic islets, a high number of cells are required in a device that cannot be much larger than a few centimeters in size. This requires a high oxygen tension to supply sufficient amounts of oxygen to these metabolic and oxygen-consuming cells. A strategy for increased oxygenation of cells in microspheres is through addition of chemicals such as perfluorocarbon [225,226]. This is the subject of another review in this issue. The alternative strategy is through vascularization.

Vascularization processes seem to be influenced by the physical structure of the biomaterial. Different reports have shown that the biomaterial pore size in the micrometer range allows cells to organize within the micropore structures improving vascularization [227–230]. The pore sizes also impact the relationship between vascularization and fibrotic tissue formation [227]. The impact of micropore structures on vascularization, macrophage polarization, and fibrosis formation has recently been reviewed [231]. The ideal pore size for promoting vascularization is within the narrow micrometer range between 20 and 60 μm [231]. Within this pore range, HEMA-based sphere templates induce more vascularization and lower fibrotic response in various

implantation sites [231]. The pore sizes of the various alginate based microspheres are within the nanometer range: alginate–PLL–alginate microcapsules between 7 and 15 nm [76,87] and Ca/Ba microbeads approximately 15 nm [68]. Therefore, the microcapsules surface does not represent a design to allow for the vascular ingrowth.

Another argument against vascularization of microspheres is that vascularization is a process that is tightly coupled with inflammation [232,233]. Inflammation in the vicinity of microspheres leads to cytokine and chemokine release which is associated with cell death. In a whole blood model evaluating the inflammatory properties of empty microspheres it was found that microspheres causing inflammatory cytokine release from leukocytes also caused the release of the vascular growth factor VEGF [159]. Also, cellular hypoxia and oxidative stress may induce angiogenesis through inflammatory mechanisms [234,235]. Since encapsulated cells may suffer from hypoxia, consequently factors that promote angiogenesis may be secreted and subsequently trigger vascularization that is related to inflammatory reaction. It should be understood that this type of vascularization resulting from inflammation is an uncontrolled response of the host towards the graft and, hence, it is not desirable and should be avoided.

3.5. Biocompatibility in animal studies

Cells adhesion has been used as an indicator for biocompatibility analyzed by various scoring systems [37,236]. Another parameter has been the quantitative infiltration of leukocytes at the transplantation site [237]. Other methods include the microsphere retrieval rates, scaling of the overgrowth and identification of host cells on the surface [14,202,238]. DNA measurements and oxygen consumption are alternative quantitative methods [239]. The peritoneal fluid phenotyping of cellular infiltrates [224] as well as the cytokine profiles have also been evaluated [133]. Recently, microspheres and polymers were injected subcutaneously to estimate the biocompatibility in connection with the reactive oxygen species using a luminescence probe as a reporter for inflammatory reactions [240,241]. The advantage of this method is that it allows for comparison of different materials within the same animal with real-time measurements. The method is, however, limited by the detection of luminescence. As a consequence it can only be applied in the subcutaneous site and not in other relevant sites such as the peritoneal cavity.

Different implantation studies with empty microspheres resulted in variable outcomes related to overgrowth. This may be due to variations in the microspheres composition resulting in different physicochemical properties as well as in biological variations by using different animal models [239] and different implantation sites [239,242]. The purity of the used materials [243] could be another variable. However, some general conclusions can be drawn for alginate-based microspheres in rodent models after implantation in the peritoneal cavity. Microbeads of high-G alginate crosslinked with calcium provoked less overgrowth than alginate–PLL–alginate microcapsules with a high-G alginate core [18,202,239,244]. For alginate–PLL–alginate microcapsules, less overgrowth is observed when a core of intermediate G (*i.e.*, high-M) alginate is used and compared to a high-G core [14,18,236,245]. For alginate microbeads, less fibrotic overgrowth was observed for microbeads of intermediate-G alginate than for high-G alginate [37,246]. These differences caused debate about using high-G versus intermediate-G alginates and their impact on overgrowth of microbeads. In a recent study, Tam et al. directly compared the overgrowth of microbeads of high-G and intermediate-G alginates reporting more host cells on the surface of the high-G alginate microbeads [18,37]. These authors also confirmed the reduced overgrowth on alginate–PLL–alginate microcapsules for intermediate-G compared to high-G alginate and showed a further reduction in attachment of cells by the use of PLO compared to PLL. Most interestingly, the direct comparison between the high-G alginate microbeads and the alginate–PLL–alginate microcapsules of intermediate-G shows the same score for host cells. Tam et al. concluded that the lowest overall host response was towards the microbeads of intermediate-G alginate. However, the stability measured as resistance against osmotic swelling is lower in microbeads of intermediate-G regardless of gelling ions [35], thus the long-term *in vivo* stability may be compromised for these microbeads. Although the studies were conducted with a high level of characterization regarding microsphere surface and stability, conclusions could not be drawn regarding the underlying reasons for the observed difference in overgrowth [18,37].

Limited numbers of reports are available on empty microspheres transplanted to large animals. Most of the studies have been performed with microspheres with encapsulated cells. Dufraine et al. showed that porcine islets transplanted subcutaneously to primates using a high-M alginate microbeads crosslinked with calcium were intact (86% of microbeads) and completely free of cellular overgrowth or fibrosis [138]. The islets in microbeads explanted after 135 and 180 days showed a residual insulin content and responded to glucose challenge. Similarly, Elliot et al. reported 95% recovery of empty alginate–PLO–alginate microcapsules, which were free of overgrowth in five out of six primates [139]. No free microcapsules could be retrieved from primates by peritoneal lavage using the same microcapsules containing neonatal porcine islets. Microcapsules were found broken accompanied with a slight local inflammation. In another study by Elliot et al., neonatal porcine islets encapsulated in the alginate–PLO–alginate microcapsules and transplanted to cynomolgus monkey. These grafts survived for at least 8 weeks and seemed to be free of cellular overgrowth [247]. For the primates (as well as humans), the upright position may lead to sedimentation of microspheres in the peritoneal cavity. Qi et al. recently reported that alginate–cellulose sulfate–PMCG microcapsules were free of overgrowth in a primate model, where a careful distribution of microcapsules was performed using a laparoscopic procedure to avoid the sedimentation of microcapsules in the lower part of the peritoneal cavity [70].

The majority of *in vivo* studies related to biocompatibility of microspheres have been performed in rodents due to lower ethical concerns, cost considerations, accessibility and easier experimental set up. In our hands, empty alginate microbeads of high-G alginate crosslinked with calcium and barium and mixtures of these ions have repeatedly shown absence of overgrowth in Balb/c mice, Lewis and Wistar rats. The C57Bl/6J mice are highly responsive with cellular reactions towards empty microspheres [239,248]. Tam et al. recently used male C57Bl/6J mice to compare overgrowth of empty microcapsules of different composition 2 days after intraperitoneal implantation [18,24]. Comparable results to previous data [239,242] and good internal reproducibility seem promising for the use of C57Bl/6J mice for comparing overgrowth of microspheres *in vivo*. The C57Bl/6J mice could therefore be recommended as a challenge model. Also, since various gene deletion models (knock out) exist, the C57Bl/6J mice could serve as a valuable tool to study the role of specific factors in the overgrowth response.

In summary, the animal studies obviously keep being significant in the design of microspheres for cell encapsulation with the vision of clinical trials. The current level of knowledge indicates that (1) studies in rodent models such as less (nude mice) and more stringent (C57BL6 and/or NOD) ones, (2) the studies in large animal models, preferably in NHP because of the upright position compared to dogs or pigs, and (3) the tests like human whole blood assay should be performed in parallel to combine the options for feasibility, preclinical and human-safety related studies, respectively. This approach may likely lead to identification of the candidate microspheres for the clinical studies.

3.6. Biocompatibility in clinical studies

The first human allo-transplantation of encapsulated islets to a type 1 diabetic immune suppressed recipient [249] reported insulin independence for nine months post-transplantation. Since then, more clinical studies on intraperitoneal transplantation of encapsulated allografts

have been published. These studies were intended as safety assessments and used lower islet numbers compared to the initial study [249]. The insulin independence reported in this study was not repeated either for immune suppressed [8] or non-immune suppressed recipients [9,10]. Calafiore et al. also reported reduced need for exogenous insulin post-transplantation in four patients, transient insulin independence in one patient [9] with the continuation study reported recently [250]. Graft function seen as increase in C-peptide was reported from one to three months by Tuch et al. [10] and Jacobs-Tulleneers-Thevissen et al. [8] respectively, and for up to 3 years in the study by Basta et al. [250]. To the author's knowledge, the presence of porcine C-peptide has not been reported in clinical neonatal porcine islet transplantation using alginate–PLO–alginate microcapsules under the trademark DIABECELL®, although the number of hypoglycemic unawareness events has been significantly reduced [11].

All these studies showed that encapsulated islet grafts can be applied without significant interference with the well-being of the host. Both Calafiore et al. [9] and Jacobs-Tulleneers-Thevissen et al. [8] found no induction of HLA class I or class II antibodies upon transplantation. Basta et al. stated that the graft is bioinvisible according to U.S. Food and Drug Administration criteria [250] which means it does not cause complications and is safe. Further, no changes in diabetes autoantibody status were noticed over a three months follow-up [8]. In contrast, Tuch et al. [10] detected cytotoxic antibodies in three out of four recipients. In the first clinical trial in 1994 [249], antibodies towards alginate were detected about 20 days post-transplantation with a further decline [251]. Also a peak in TNF and IFN-γ, together with TNF receptors was detected two weeks post-transplantation, indicating an acute and transient inflammatory response to the graft [251]. Espevik et al. concluded that such a transient response would not affect the graft function to a large extent, although one cannot exclude the possibility that it contributes to the overgrowth of the microcapsule surface [251].

The reasons for graft failure in the clinical studies are not clear. Graft volume, the quality of the islet preparations and status of the transplanted cells may impact the outcome. Tuch et al. reported positive C-peptide 2.5 years post transplantation in a patient with three infusions of encapsulated islets [10]. Both alginate–polycation–alginate microcapsules and alginate microbeads were used in the studies: alginate–PLL–alginate microcapsules by Soon-Shiong et al. [249], alginate–PLO–alginate microcapsules by Calafiore et al. [9], Basta et al. [250] and DIABECELL® [11], Ba-crosslinked alginate microbeads by Tuch et al. [10], and Ca/Ba-crosslinked alginate microbeads by Jacobs-Tulleneers-Thevissen et al. [8]. As the published studies demonstrated the presence of C-peptide post-transplantation, it may be concluded that the microspheres provided sufficient immunoprotection against the early rejection. Again it should be emphasized that microspheres are not meant to prevent a response but merely to protect against such a response. Leaking antigens, protruding cells, cellular stress, and microcapsule breaking due to *in vivo* shear forces are phenomena that could be responsible for pro-inflammatory responses.

Jacobs-Tulleneers-Thevissen et al. reported numerous single microbeads loosely attached to the peritoneum easily retrievable by lavage [8]. Retrieved encapsulated islets were functional as assayed by insulin secretion upon static glucose response. Although many microbeads were easily washed out, the vast majority was clustered and contained debris, but also contained a small number of living cells located at the surface of clusters. The clusters were attached to the peritoneum or to the greater omentum. The microbeads were embedded in vascularized fibrous tissue containing scattered macrophages and giant cells with few lymphocytes and polynuclear cells [8]. Tuch et al. [10] performed laparoscopy on a recipient of encapsulated human islets 16 months after infusion and found intact microbeads containing necrotic islets. Further, a large number of microbeads were found scattered throughout the peritoneal cavity in clusters attached to the parietal peritoneum spleen, omentum, and kidney. A biopsy demonstrated that microbeads remained intact but were surrounded by fibrous tissue containing thin-walled capillaries with a mild histiocytic response [10]. Finally, Basta et al. [250] reported the explantation of alginate–PLO–alginate microcapsules 5 years after implantation due to the patient's abdominal discomfort. Via ultrasound a hyperechoic cyst-like formation was identified, which was surgically removed. It consisted of a fibrotic clump containing mostly intact microcapsules with no viable islets. The authors commented that this clump originated from the microcapsules incorrectly injected beneath the muscle fascia.

Hence, from the perspective of clinical trials, there seem to be problems with aggregation of microspheres and with host cells growing on a portion of the microsphere surface, both reducing the amount of oxygen and nutrients to the transplanted graft and, possibly, resulting in loss of graft function. This is in contrast to allo- as well as xenotransplantation in various animal models showing insulin independence and graft function for extended periods of time for similar microspheres as those used so far in clinical trials. Human islets are shown to function in Ca/Ba-alginate microbeads in immune deficient mice for more than 300 days [47] as well as in immunocompetent mice for 70–200 days [68]. Thus, human islets are able to function for a prolonged period in transplanted microspheres without surface overgrowth. Since empty microspheres have not been transplanted to humans, it is difficult to conclude from the clinical studies how the microspheres themselves contribute to the overgrowth, *i.e.*, in the absence of encapsulated islets, or any cell types. As discussed in Section 3.3, inflammation might follow hypoxia [218] and cell death [216,217], and the release of inflammatory cytokines from islets may be difficult to avoid [219]. The microspheres are challenged both from the outside by the host factors including proteins and cells as well as from the inside by the encapsulated cells releasing inflammatory mediators further provoking the host immune system. Even though the concept has been proven in small animal models, it has been more challenging in larger animals as well as in humans. Could, for instance, the differences in size of recipient impact the protein adsorption patterns due to force differences? Could this be a part of the explanation for differences between small and large recipients including humans? What about differences in hydrophilicity, matrix stiffness and surface reactivity? This could be some of the many factors that might contribute to the variability between the microspheres. These questions along with others remain to be fully elucidated.

4. Concluding remarks

A multitude of disciplines including chemistry, material science, protein science, immunology, cell biology, endocrinology and transplantation are involved in the field of microencapsulated cell therapies. Apparently this field is more challenging than initially assumed after formulating the basic principles and seeing the first proofs of concept a few decades ago. For moving this field forward, a principal understanding of the multidisciplinary requirements is mandatory. In this review we focus on the physico-chemical properties *per se* and summarize the currently available relevant knowledge regarding the characterization of microspheres designed for cell therapies. We also highlight biological processes that can be activated by these physico-chemical properties. The host responses will occur against the encapsulated cells, but these responses are to be protected by the microspheres. The microspheres need to be designed in a way to avoiding overgrowth, thus the surface characteristics are of essential importance for a functional system. We conclude that for cell encapsulation using microspheres the term biocompatibility may be substituted by the term biotolerability, since the long-lasting function by the grafted cells requires a surface free of overgrowth.

The methods for physico-chemical characterization of surface chemical composition, charge, hydrophilicity, mechanical stability, chemical stability, permeability and spatial distribution of polymers should be combined and more commonly applied in the field to understand the factors leading to success and failure of the encapsulated cells. This is a principal premise for moving forward in this field. Although such an

approach is logical and highly required, so far this has not been done. It is hard if not impossible to create a learning curve from studies with only a partial characterization of microspheres, *i.e.*, a detailed physicochemical characterization without corresponding biological characterization. The mandatory set of characterization methods should provide information on at least (i) mechanical and chemical stability, (ii) characteristics of microspheres regarding the surface topography and chemical composition, permeability and morphology, (iii) the inflammatory potential of microspheres and their individual components, (iv) the compatibility of the microspheres with the encapsulated cells. Only this can lead to identification of the factors that have been responsible for the function of encapsulated cells after transplantation. Finally, since there has been significant lab-to-lab variations in results determining identical characteristics of microspheres, the properties of microspheres should likely be benchmarked, *i.e.*, the same microspheres should be analyzed in different laboratories using both the same techniques and various techniques to determine a given property.

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