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REVIEW ARTICLE

Alginate-Based Cell Microencapsulation for Tissue Engineering and Regenerative Medicine

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Abstract: Increasing numbers of requests for transplantable organs and their scarcity has led to a pressing need to find alternative solutions to standard transplantation**.** An appealing but challenging proposal came from the fields of tissue engineering and regenerative medicine, the purpose of which is to build tissues/organs from scratch in the laboratory and use them as either permanent substitutes for direct implantation into the patient's body, or as temporary substitutes to bridge patients until organ regeneration or transplantation. Using bioartificial constructs requires administration of immunosuppressant therapies to prevent rejection by the recipient.

Microencapsulation has been identified as promising technology for immunoisolating biological materials from immune system attacks by the patient. It is based on entrapping cellular material within a spherical semi-permeable polymeric scaffold. This latter defines the boundary between the internal native-like environment and the external "aggressive" one. The scaffold thus acts like a selective filter that makes possible an appropriate supply of nutrients and oxygen to the cellular constructs, while blocking the passage for adverse molecules. Alginate, which is a natural polymer, is the main biomaterial used in this context. Its excellent properties and mild gelation ability provide suitable conditions for supporting viability and preserving the functionalities of the cellular-engineered constructs over long periods. Although much remains to be done before bringing microencapsulated constructs into clinical practice, an increasing number of applications for alginate-based microencapsulation in numerous medical areas confirm the considerable potential for this technology in providing a cure for transplant in patients that excludes immunosuppressive therapies.

Keywords: Tissue engineering, regenerative medicine, encapsulation, alginate beads, transplantation, extracorporeal supply.

1. INTRODUCTION

The growing demand but insufficient availability of transplantable organs for the enormous number of candidates on waiting lists (about 119,712 in the current year according to the data reported by the United Network for Organ Sharing) was the primary motivation for scientists to find alternative permanent or temporary therapeutic treatments in order to guarantee patient survival. In the last thirty years, tissue engineering and regenerative medicine have taken up the cause; these research fields have suggested creating tissue constructs in the laboratory so that these constructs can replace the native organ's failing functions once implanted into the body [1]. Considerable effort has also been made to develop extracorporeal support systems (e.g., bioartificial livers and kidneys) which, thanks to the presence of cells, could bridge patients until organ regeneration or transplantation [1, 2]. Like standard organ transplantation, these strategies involve using life-long immunosuppressant therapies to prevent an immune response in the host body and the subsequent rejection of the bioartificial constructs. Long-term administration of immunosuppressants is, unfortunately, associated with serious side effects, including kidney failure, and, therefore, needs to be decreased or replaced.

In this context, cell microencapsulation has been proposed as effective technology for immunoisolation of biological material to avoid immune suppression and overcome a host immune response in therapeutic applications [3-8]. It consists of enveloping viable and functional cells within a spherical semi-permeable scaffold that provides a suitable inner microenvironment to maintain the cells' physical and functional integrity while protecting them from external attacks. The scaffold is generally made of a polymeric biomaterial, either of natural or synthetic origin, that must have high biocompatibility and biotolerability, and contain no or minimal amounts of endotoxins [9]. Natural polymers are the most suitable choice for this application. This is especially due to their structure, that is similar to the extracellular matrix of many human tissues, and their composition, that is made of macromolecules similarly present in biological environments of the body [10]. Although several biomaterials have been assessed (including agarose, chitosan, hyaluronic acid, collagen, and fibrin [10]), alginate easily satisfies the above-mentioned prerequisites and, consequently, is the most commonly used polymer in this context [9, 10]. In addition, the scaffold must have well-designed physical characteristics (including shape, size, and porosity) that make possible a rapid exchange of nutrients and oxygen between the surroundings and the cells, as well as the transfer of cellular therapeutic products in the opposite direction. A spherical shape, such as beads or capsules, is preferred over other geometries because it offers the best surface-to-volume ratio, responsible for the most efficient mass transfer of the molecules between in and out compartments [11, 12].

This review proposes an analysis of some major areas of application for alginate-bead cell microencapsulation in tissue engineering and regenerative medicine. The subjects are introduced by an overview of alginate material and its features in order to better understand the advantages of using it in this field.

2. ALGINATE: A GOLD-STANDARD BIOMATERIAL FOR CELL MICROENCAPSULATION

Alginate is the most commonly-used biomaterial for cell microencapsulation [10, 13]. It is considered the main encapsulation polymer for clinical applications and is the only encapsulation matrix that has been approved for human use by the U.S. Food and Drug Administration (FDA) [14]. Its excellent physical-chemical

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characteristics inevitably play a part in this success. Alginate has a low gelling capability, is easy to handle and biocompatible, has low toxicity, good *in vivo* performances [10, 13, 15, 16], and, last but not the least, low endotoxin lipopolysaccharide content [9].

Alginate is a naturally occurring unbranched anionic polysaccharide that is generally extracted from brown algae (*Phaeophyta*), but can also be synthesized by bacteria (*Azotobacter* and *Pseudomonas*) [17]. Alginate is formed by linear copolymers containing blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. The G and M residues can arrange themselves in consecutive (GGG and MMM) and alternating (GMGM) order in the blocks. However, the content of M and G residues, the M/G ratio and their arrangement depend particularly on the natural source from which the alginate is obtained. Along with G-block length and molecular weight, these are critical parameters. They not only affect the physical properties of alginate and its resultant hydrogels [18], but also play a role in the body's inflammatory response and fibrotic overgrowth after implantation [19]. In turn, the physical features of alginate hydrogels influence the biochemical responses of the encapsulated cells. For instance, it has been widely proven that alginates, rich in G residues, hinder the growth and metabolic activity of the cells, unlike alginates rich in M residues [20-23].

2.1. Biocompatibility

Alginate biocompatibility has been well-documented both *in vitro* and *in vivo*. This feature is strictly dependent on purification of the alginate which, accordingly, is an essential step for using the polymer in biomedical applications. Raw alginate, although low in endotoxin lipopolysaccharide content [9], nevertheless has various other impurities, including lipoteichoic acids and proteoglycans [9], which are responsible for activating pro-inflammatory responses and, consequently, compromising the biocompatibility of the construct [9, 13, 16, 24]. In case of implantation, for instance, these processes lead to the failure of the graft with overgrowth of the fibrotic capsule around it, impeding cell nutrition and causing necrosis of the enveloped cells [19].

Several purification procedures have been proposed in different laboratories [25-27]. These are basically based on filtration and charcoal treatment, protein extraction, and precipitation steps. There is evidence that the methods established in various laboratories provide different results [28]. Standardized protocols are therefore required in order to obtain suitable batches of purified alginates with similar characteristics [13, 24]. In 2003, Orive *et al.* expressed the idea of setting up a 'central alginate factory' that could prepare standardized prototypes for use by participating laboratories in their transplant studies [24]. Further strategies have been proposed to improve the biocompatibility of cell-encapsulated alginate microspheres; these include traditional approaches of temporarily administering immunosuppressants [14, 23, 24] and modifying the surface properties by adding chemical groups [25-27], as well as relatively new methods that involve binding long stretched molecules, so-called polymer brushes, with high density on an identical surface area of the microspheres so that they can prevent protein adhesion [28-31].

2.2. Alginate Bead Fabrication and Cell Loading

Alginate has the ability to polymerize. Its gelation process simply occurs at a mild pH, temperature, and salt conditions that slightly affect cell viability. In principle, adjacent alginate chains bind in the presence of divalent cations (e.g. Ca^{2+} or Ba^{2+}), forming ionic interchain bridges [10]. Due to the different affinities of alginate for divalent cations, choosing the latter influences the physical properties of the resultant alginate gel (e.g. mechanical strength) [29]. Cell loading normally occurs prior to gelation; that is, cells are first suspended in a water-based alginate solution and, upon exposure to the cations, are then entrapped within the crosslinked alginate network [30].

Alginate beads are manufactured with respect to the basic principle given above, with the addition of technical procedures which make it possible to build the rigid spherical shape. These methodologies are more often involved in the extrusion of the cell-alginate solution through a syringe needle into a gelling bath. The droplet immediately polymerizes when surrounded by cations, but maintains its spherical shape (Fig. **1**).

Extrusion is the simplest method for producing large-sized alginate beads (millimetric order). To create micron-sized spheres, which are more suitable for cellular applications, external forces (such as electric fields, mechanical vibration, and air flow) must however be applied, so that the extruded alginate can be broken up into small, size-tunable droplets [10, 31]. Microbeads effectively have improved characteristics, such as a higher exchange rate for substances between cells and the external environment, high mechanical resistance, and a milder pericapsular reaction once implanted [10]. More sophisticated technologies have recently been introduced in order to better control the encapsulation parameters (including cellular density, the homogeneity of bead dimensions and reproducibility); these are based on cutting-edge microfluidic platforms [10, 32-34] and bioprinting [10, 35].

2.3. Multilayer and Covalent Cross-Linking Strategies

Although alginate beads are easy to manufacture, tuning their final properties requires several optimization steps in the manufacturing process. Multilayer and covalent cross-linking technologies are commonly used strategies that, of all the others, make it possible to conveniently manipulate the features of alginate microbeads, such as permeability or (mechanical) stability. These latter, together with biocompatibility, are considered important characteristics of the beads and effectively influence the success of the final construct. Modifications − made on the basic structure of the alginate beads − often affect both permeability and (mechanical) stability. However, it is still very challenging to reach perfect tuning of both features by modifying a single physical characteristic of the alginate beads. Ideally, an optimal compromise between mechanical strength and mass transport needs to be found to guarantee the preservation of cell functionalities [8].

The need to adjust the permeability of "nude" alginate beads derives from the limited tuning of their cutoff, which does not make possible total control of the traffic of adverse molecules. In addition to therapeutic molecules, encapsulated cells in fact release antibodies and cytokines. These factors are actively involved in the activation of macrophages and trigger fibrosis processes, which are mainly involved in the failure of the construct. Antibodies are efficiently blocked by the alginate barrier, contrary to cytokines which, being of similar size to beneficial molecules (e.g. insulin), cannot be prevented from crossing the polymeric boundary. The motion is equally unregulated in the opposite direction, and can cause as many undesirable effects. Applying multilayers on top of microbeads resulted in offering the most adequate regulation of substance selection [19]. The strategy also exhibited great potential for improving the surface finish of the beads so as to prevent fibroblast adhesion [36] which can also be an explanation for the fibrosis [37]. Similarly, applying multilayers to the surface of alginate beads prevented cell protrusion; this undesirable phenomenon has been accused of inducing fibrotic reactions and, consequently, determining rejection of the construct [11, 38]. Depositing multilayers on to the surface of alginate beads is commonly made possible by the layer-by-layer technique [39]. This is an easy and cheap method that consists in attaching highly and oppositely charged polyions on to charged surfaces in a self-assembly process [19]. A consistent number of coatings have been assessed so far [40-43]. However, polycations, such as poly-L-lysine and poly-L-ornithine, are the most widely-used in this context. On the contrary, polycationic coatings show a huge variety of drawbacks. Although polycationic coatings determined the reinforcement of alginate beads and de-

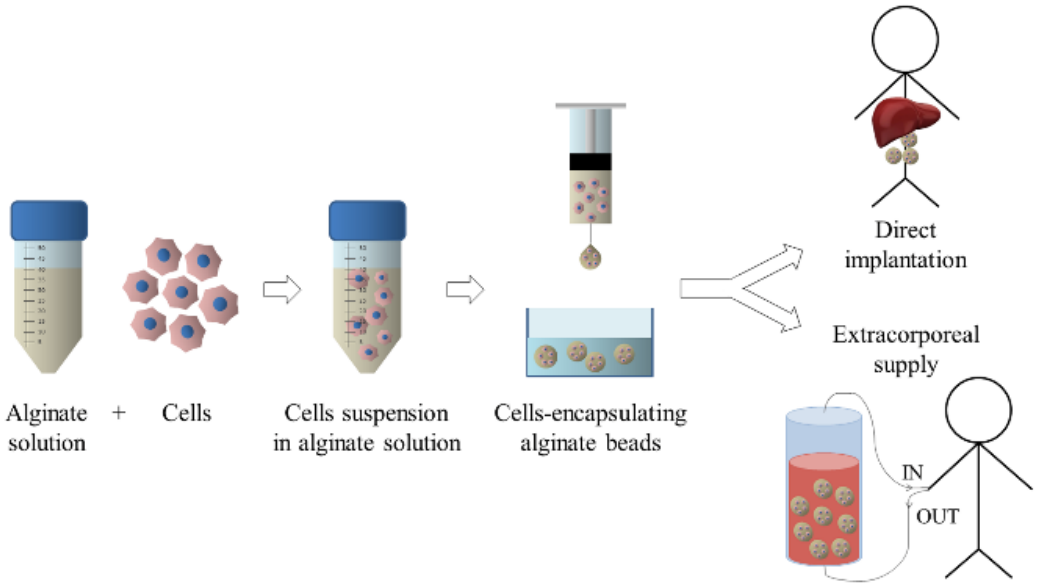

Fig. (1). Representative diagram of the microencapsulation process: from manufacture of the cell-encapsulating alginate beads to their application as direct implants in several body sites or as temporary substitutes in extracorporeal devices.

creased their permeability to the host's immune cells and antibodies [44], they lack sufficient mechanical strength, biocompatibility, and long-term stability *in vivo* [8]. More importantly, they are often a frequent cause of inflammation [45, 46] and fibrosis because they attract macrophages and/or detach from the alginate bead surface [8, 47]. A common solution was to cover the polycation layer with a second alginate coating. However, this risked reducing membrane permeability and correct oxygen and nutrient supply to the cells. Furthermore, the outer alginate layer does not provide efficient results for completely neutralizing the polycation layer, avoiding protein and cellular adhesion and the consequent induction of fibrotic processes [44]. New strategies have recently been proposed as alternatives to the current polycationic coatings. Protamine and cationized potato starch are two examples of new coatings which, in addition to controlling the permselectivity of the beads, demonstrated a promising ability to improve survival [48] and preserve the metabolic activities of the encapsulated cells [49] in comparison with classical polycations.

Most of these strategies do not vary the ionic bridges present between adjacent chains in the internal structure of the alginate beads, as established during their standard polymerization thanks to the inclusion of divalent cations. Ionically-bonded alginate beads nevertheless suffer from very low mechanical and chemical stability in physiological solutions and within the implantation sites of the body; this aspect gives rise to a series of phenomena ending with the rupture of the beads [8, 50]. Covalent cross-linking strategies have thus attracted substantial attention for the manufacture of long-term resistant alginate beads. Photoinitiated polymerization is one of the most commonly-adopted techniques for forming covalently cross-linked gels. In general, polymerization occurs by rapidly exposing the biomaterial and suitable initiators to UV or visible light; the cells and bioactive molecules can already be present in the biomaterial. The final mechanical properties can easily be tuned by changing the degree of photo-curable moiety [8]. Covalent alginate beads can also be obtained by adding phenol moieties into the polymer side chains and establishing alginate-tyramine conjugates. The gelation takes place in the presence of H_2O_2 and horseradish peroxidase and it is almost instantaneous (seconds). The resulting alginate beads manifest high cytocompatibility and mechanical strength [8]. In another study, human hepatocellular carcinoma cells (Huh7) were successfully encapsulated in calcium alginate poly(ethylene glycol) forming hybrid microspheres (Ca-alg-PEG). Grafting some percents of the backbone units of sodium alginate (Na-alg) with □-amine-□-thiol PEG maintained the gelling capacity in the presence of calcium ions, while thiol end groups made it possible to prepare chemically crosslinked hydrogels via spontaneous disulfide bond formation [51]. Additional advanced methods are currently under investigation. An energy dissipating mechanism, for instance, is an appealing alternative to improve the cell microencapsulation field. It consists in promoting the formation of hydrogels with a hybrid backbone, where ionic cross-linked alginate chains are covalently linked to covalent cross-linked chains of polyacrylamide gels [52]. The final construct has excellent mechanical properties.

3. CELL MICROENCAPSULATION FOR APPLICATIONS IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

The concept of cell encapsulation applied in the medical field was introduced in 1933, when Bisceglie *et al.* [53] enveloped tumor cells in amnion tissue-sheets to study the effect of the absence of vascularization on tumors. The construct, when implanted into the abdominal cavity of pigs, maintained cell survival for extended time periods, which was probably a beneficial consequence of the immunoprotection from the host environment provided by the membranes. However, the group did not recognize the potential of this technology, so it was not exploited for the treatment of diseases. It was only in 1950 that the concept of immunoisolation started to acquire importance in the context of the implantation of biological material for therapeutic purposes [54]. Fourteen years later, Chang [55] formalized the need to use ultrathin polymer membrane microcapsules for the immunoprotection of transplanted cells; eventually, this introduced the concept of bioencapsulation. The real breakthrough came with Lim and Sum [56] who were the first to demonstrate the effectiveness of the technology. In their studies, pancreatic islets were encapsulated in polyanionic alginate beads coated with a polycationic poly(L-lysine) layer, and then implanted into chemically-induced diabetic rats. The new microencapsulation procedure actually reduced the problem of immune rejection, making possible long-term maintenance (over 15 weeks) of the cells' morphology and functionalities.

Since then, cell microencapsulation technology has obtained considerable attention from the scientific community, extending its application to an increased range of therapeutic areas. The potential of this technique has been demonstrated in different *in vitro* investigations and pre-clinical studies. In some medical fields, it has already been evaluated in preliminary clinical trials. So far, the results of these studies have shown the safety, feasibility, and tolerability of this therapeutic approach [8]. However, there are still many limitations, especially associated with the wide variability in cell survival (from days to years) in the encapsulated environment [8]. The challenge has thus been to further improve the cell microencapsulation procedure in order to define routine clinical practices, different for each therapeutic application, and thus minimize errors.

Of the many of medical fields involving the application of cell microencapsulation, three areas of interest are reviewed below. The idea here is to provide readers with an overview of the current situation, allowing them to understand the growing importance attributed to microencapsulation over the years. Thus, to delineate the time evolution of this technology, our topic selection includes (i) the bioartificial pancreas, where cell microencapsulation found its first application and, accordingly, has developed rapidly; (ii) hepatic substitutes, whose investigation highlights the crucial benefits of using cell microencapsulation in both grafting and bioartificial extracorporeal devices; and (iii) stem cell differentiation, which is a relatively recent and only partially explored application area for microencapsulation.

3.1. The Bioartificial Pancreas

The pancreas is a fundamental organ involved in the regulation of the body's metabolism through the production of a variety of hormones. The release of these molecules is carried out by clusters of four different cell types (alpha and beta, which are respectively 20% and 50% of the total islet cells, delta and gamma cells), called islets of Langerhans. Beta (β) cells are the components responsible for the secretion of insulin; this latter is released in proportional response to actual blood glucose levels and promotes its uptake (mainly in the liver) making it possible to control blood glucose homeostasis. Inefficient pancreatic β cell function thus determines persistent hyperglycemia; the resulting chronic disorder is well-known: diabetes. In this condition, either β-cells are unable to fulfill their tasks because they are completely destroyed by the immune system (type-1 diabetes), or they gradually stop functioning (type-2 diabetes) due to their excessive solicitation as a result of genetic and environmental factors. Besides exogenous insulin injection therapy, β-cell transplantation offers an improved therapeutic solution for type-1 diabetic patients, especially for those whose blood glucose levels are difficult to control despite intensive insulin doses [57], guaranteeing normal stimulus-coupled insulin secretion kinetics. This procedure, however, has certain limitations, including host rejection of the graft in the absence of immunosuppression.

3.1.1. Bead Composition

Alginate microencapsulation of Langerhans islets, which corresponds with the bioengineering approach to design a bioartificial pancreas, has been shown to be a relevant solution to the immunorejection of the graft [58]. As already mentioned, the technique was first introduced by Lim and Sum [56]; then, considering their promising results on transplanted diabetic rats, numerous other studies followed. For about two decades, chemically-induced and spontaneous diabetic animal models (rodents, canines, and primates) made it possible to explore several parameters and criteria for the definition of a system that could maintain its characteristics and functionalities in the long term [59-68]. Great attention has been paid to the manufacture of alginate beads to allow them to offer cells a biocompatible environment, minimize inflammatory processes upon implantation, and manifest enhanced stability over time. Alginate does not support interactions with cells [69]. This aspect is nevertheless crucial in prolonging cell survival within the microbeads after transplantation. Accordingly, the usual inclusion of arginine-glycine-aspartic acid (RGD) sequences in the alginate backbone has been proved to beneficially affect the long-term maintenance of functional and viable grafts in both small and large animals [60, 70]. At a more complex level, a porcine pancreatic extracellular matrix (ECM), obtained from a decellularized organ via ECM lyophilization and liquefaction stages, has recently been crosslinked within poly-L-lysine coated alginate beads [71]. Implantation of the resulting microbeads, encapsulating non-β insulin-secreting cells, in diabetic mice produced a promising outcome; in fact, despite the low density of encapsulated cells, the system promoted increased insulin secretion and good maintenance of glucose levels. An appropriate internal environment can, however be compromised if it is not protected from outside attacks. For certain scientists, outer alginate coatings have become mandatory for permselecting external elements. For this reason, several types of polyamine coatings have been evaluated by several researchers; however, most of these were found to strongly induce the host inflammatory response [45, 72]. In 2006, Ponce *et al.* [73] proposed poly-L-lysine as the optimal coating for reducing host reactions. Previous studies highlighted that selecting an alginate bead crosslinking bath was paramount for permselectivity; the barium bath improved the alginate features compared to calcium [74]. In these circumstances, polyamine coating did not result necessary. However, ionically-bonded alginate hydrogels lack the mechanical stability to support the strains associated with implantation and, therefore, they degrade and rupture easily due to the exchange of cations with sodium ions in the *in vivo* environment [75, 76]. Methods for forming covalent bonds within the alginate beads have thus been developed to enhance their stability. Photo-crosslinking is probably the most common technique used to form covalent bonds in the alginate backbone in the presence of photopolymerizable groups, such as methacrylate groups [77-85]. Although this process gives the alginate beads high stability and increased mechanical strength, it generates free radicals that cause significant cell toxicity. Of the alternative strategies, Hall *et al.* [50] engineered polymers capable of forming spontaneous, covalent linkages. The resulting microbeads, obtained through ionic and covalent interactions between functionalized alginate and poly(ethylene glycol) polymer, presented high levels of stability and high cell compatibility.

3.1.2. Preclinical studies

In all these studies, the overall condition of the animal transplanted with microbeads was positive. Microencapsulated islets were well-protected from the outside environment in the animal's implantation site, unlike the non-encapsulated islets that were rejected a few days after transplantation. Accordingly, cells were healthy and functional in the beads, determining, in most cases, normoglycemia of the diabetic animal for several days (the duration was very variable between the different studies). In cases of severe hypoxia, which is a major contributor to the dramatic drop in β cell viability after transplantation, microencapsulated islets also remained alive and functional when entrapped in alginate beads; it was thus suggested that these latter could prevent islet destruction by playing a protective role in cases of hypoxia [86]. However, the response to the treatment was very dependent on the type of transplanted animal, the source of the islets, and the implantation site. In fact, great success in rodents, dogs, and pigs often corresponded to failures in other large animals [87]. The animal's posture was found to have a significant influence on the final behavior. While this therapy resulted in well-fixed allocation of the microbeads within the intraperitoneal site in quadrupeds [88], the results were remarkably variable in biped non-human primates [3, 88-94]. The results were associated with the strength by which the microbeads adhered to the implantation site, preventing them from falling into the pelvis or not. Implantation site has also been a critical subject of investigation due to its influence on the stability and biocompatibility of microencapsulated islets. Subcutaneous sites have many po-

tential strengths, including their large capacity, the convenience of minimally-invasive implantation procedures, and the simplicity of monitoring the graft. Nonetheless, Kerby *et al.* [95] negatively referred to it. Alginate microencapsulated islets transplanted subcutaneously were not in fact able to reverse the diabetic state of the mice. This limited success was also highlighted elsewhere [96-98]. Several other implantation sites have been assessed in pre-clinical studies (kidney capsule, bone marrow, and omentum); however, intraperitoneal implantation has been considered to be the most adequate as it has made it possible to restore normoglycemia [99, 100]. Accordingly, this is the site used primarily in clinical investigations.

In short, animal pre-clinical studies have mainly demonstrated full safety and the functional performance of alginate microencapsulated allogeneic and xenogeneic islets upon implantation. Preclinical outcomes were, therefore, sufficiently successful to allow some worldwide research centers to obtain authorizations to proceed with phase I/II clinical trials of alginate encapsulated islets implanted into type 1 diabetic patients. The studies are recent, having started just ten years ago.

3.1.3. Clinical Studies

The first records go back to 2005 when Calafiore *et al.* [92, 101] were granted permission to transplant ten non-immunosuppressed patients with long-standing type 1 diabetes and cured with intensive insulin therapy regimens. The patients were grafted intraperitoneally with human allogeneic islets microencapsulated in ultra-purified alginate- poly-L-ornithine -alginate. Two of them received multiple subsequent grafts in better-vascularized sites (i.e. mesentery). Patient follow-up lasted for different time periods and the results are so far available for only some of them [44, 102]. In general, excellent reactions to the transplantation procedure and an absence of immune response were observed in all cases. Significantly, the daily exogenous insulin dose was reduced in all treated patients and suspended for one of them. However, these metabolic improvements were temporary (6 months up to 1 year) and obliged the patients to return to normal insulin injections at the end of 5 years of treatment. In conclusion, alginate-poly-L-ornithine-alginate confirmed its ability to protect allogeneic cells from the recipient's immune system attack and its long-term safety (in patients monitored for 4 years); nevertheless, the encapsulation strategy was not sufficiently optimal to make possible long-term preservation of functional islets.

In 2007, Living Cell Technologies suggested implanting porcine islets encapsulated in alginate-poly-L-lysine-alginate into the intraperitoneal site of a 41-year-old man [103]. Surprisingly, the results were better than expected because the treatment promoted a reduction in exogenous insulin injections and, unlike the previous trial, 10-year maintenance of cell survival. Moreover, the safety of the encapsulation strategy was validated as porcine viral and retroviral infections were not detected. Two years later, the company decided to launch a phase I/IIa study in Moscow. Microencapsulated neonatal insulin-producing porcine pancreatic islet cells were implanted, in a unique or multiple injections, into seven insulin-dependent diabetic patients [102]. The results were promising: the procedure was well-accepted by the patients and their diabetes was better controlled. Phase IIb clinical trials have thus been recently launched in New Zealand and Argentina.

In 2009, another phase I clinical study was carried out by Tuch and his team in Australia [4]. Human islets were encapsulated in barium-alginate microbeads and intraperitoneally implanted into four patients. Transplantation was performed according to different grafting schedules in the four patients: one of them underwent four islet infusions over seven months, another patient had two transplantations ten months apart, and the remaining two were grafted only once. In all cases, anti-inflammatory and anti-oxidant therapy was provided after transplantation. Nonetheless, the microencapsulated islets did not result in substantial improvements. Insulin production was not enough to control the glycemic levels in the patients' blood. The implants were thus removed to figure out the cause of the problem. The beads were found stuck to several tissues in the peritoneal cavity and enveloped in fibrotic capsules. This latter was blamed as the major cause of cell necrosis and loss of graft function.

A recent clinical study ended with similarly negative results [104]. Human islets microencapsulated in barium-calcium alginate were implanted in a 61-year-old female immunosuppressed patient, previously grafted with a whole organ. In this case, it was not possible to reduce daily exogenous insulin therapy even in the three months following transplantation. The detection of diabetes autoantibodies thus obliged the researchers to retrieve the beads, which were found in the presence of inflammatory tissue.

In conclusion, these preliminary clinical trials have confirmed the potential ascribed to islet microencapsulation technology and its resulting positive outcome in terms of safety. However, these results could not be effectively exploited because of the lack of reproducibility in the different centers. It is clear that further attention still needs to be given to certain critical technical aspects involved in the production of alginate beads [102]. This would make it possible to establish general optimal criteria for providing ultrapure alginate beads that, whether coated with polyamines or not, could be well-tolerated by recipients. The size and morphology of the beads needs to be carefully designed in order to improve functional performance (smaller size) and minimize the risk of macrophage signaling (smooth surface and absence of cell protrusion). The choice of implantation site perhaps requires additional revision so that the high oxygen requirements of pancreatic islets can be better fulfilled and their functionalities preserved longer.

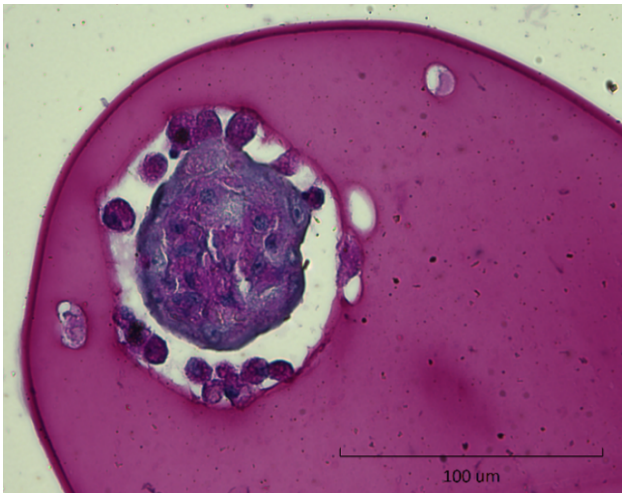

Fig. (2). Hepatic spheroid encapsulated in an alginate bead. The spheroid was formed by the aggregation of hepatocytes and hepatic non-parenchymal cells. The image shows PAS staining that indicates cellular glycogen storage.

3.2. Hepatic Substitutes

Tissue engineered livers are the only alternative treatment to transplantation in cases of severe liver failure. Of the different approaches developed so far, hepatocyte microencapsulation lead to either a short-term treatment, if used in an extracorporeal circuit, or a long-term treatment if implanted.

Different cell sources and materials have been investigated since the pioneering work by Dixit *et al.* (1992). Focusing only on alginate-based materials, the encapsulated cells used are mainly of primary murine [105-120] or porcine [121-125] origin or from hu-

man cell lines [51, 112, 126-138], the most popular being HepG2/C3A. Very few studies have reported the encapsulation of primary human hepatocytes [139-142]. This is likely associated with the inability of the primary human hepatocytes to preserve their phenotypes and biological functions in *in vitro* culture conditions. Co-cultures of hepatocytes with different types of feeder cells (derived or not from the liver, primary or cell lines) have alternatively been proposed as a platform for preserving *in vitro* viability and functionalities of the hepatocytes. However, their encapsulation in alginate beads remains sporadic and investigated little [33, 143, 144]. We recently encapsulated aggregates of primary rat hepatocytes and hepatic sinusoidal non-parenchymal cells (endothelial cells, Kupffer cells, and hepatic stellate cells) in alginate beads (Figure 2). Several primary hepatic activities were analyzed in order to investigate the impact of both non-parenchymal cells and the microenvironment offered by the alginate bead on maintaining *in vitro* hepatic functions.

3.2.1. External Bioartificial Liver (BAL)

In acute liver failure, it is considered that temporary support may be enough to bridge patients until transplantation or to allow the liver to regenerate. An extracorporeal supply with an efficient hepatic biomass thus appears to be the best option, mimicking the treatment proposed by hemodialysis or hemofiltration in kidney replacement. External bioartificial livers generally rely on an extracorporeal circuit, usually composed of a plasmapheresis stage (primary loop) and a secondary loop where additional compounds, such as charcoal or ion exchange column and oxygenator, could be added to the bioreactor hosting the cells [145, 146].

Alginate beads hosting hepatic cells can be considered as the solid fraction of a biphasic compound, the fluid fraction being the patient's plasma. The number of cells requested to fulfill liver functions out of the body is still under question, but a range of 15 to 30 % of a whole liver is generally accepted by the scientific and medical community. This means that about 1L of alginate beads with a cell density of several million per mL of alginate (before the gelling step) should be prepared and then perfused in an adapted bioreactor. In 1999, Doré *et al*. [147] proposed the concept of a fluidized bed to perfuse the beads in a bioreactor. Based on bioreactor design, adequate perfusion conditions led to permanent motion of the beads in a defined volume and promoted interactions and exchanges between solid and liquid phases [148]. Stable bed expansion with homogenous mixing could be obtained by applying well-defined hydrodynamic conditions [127]. The most advanced progress with this technique led to the definition of a whole circuit hosting either HepG2 [149] or potentially other hepatic cells such as primary human cells [138].

3.2.2. Implantation of Microencapsulated Hepatic Cells

As with islet implantation, liver cell microencapsulation was proposed as an alternative to directly injecting hepatocytes into specific sites in the patient's body, enhancing cell functions and ensuring immunoisolation, specifically for xenografts [115, 150]. In addition to the liver, the spleen or intraperitoneal cavity can accommodate a large number of cells. Several *in vivo* studies with Gunn rats (hyperbilirubinemia model) [151, 152] or rats with induced acute liver failure [114, 116, 125] have shown positive results regarding cell functions and animal survival.

Several methods have also been proposed to maintain the specific function and phenotype of the bioencapsulated hepatocytes, such as co-encapsulation with other types of cell. The superiority of encapsulated rat hepatocytes mixed with human fetal liver stromal cells engineered to produce bFGF has recently been demonstrated in the treatment of acute hepatic failure in mice [113]. Liu and Chang *et al*. [153, 154] reported that hepatocyte viability can be maintained longer when encapsulated with mononuclear cells, including stem cells from bone marrow cells. In addition, transplantation of both co-encapsulated cell types improved the ability of the hepatocytes to correct congenital hyperbilirubinemia in Gunn rats during the period of 3 to 10 weeks post-transplantation. These results were confirmed recently by Shi *et al.* with coencapsulated hepatocytes and bone marrow mesenchymal stem cells in a rat model of acute liver failure [118].

The long-term maintenance and efficacy of microencapsulated hepatocytes depend on the graft site, the potential neovascularization of the implant and the type of cell to be immobilized, and the cytokines and antigens that are secreted. Attempts have been made to prevent an initial host immune-inflammatory response and favor the establishment of tolerance with time-released immunosuppressive drugs or antibody treatment [155]. Another way of further improving implant biocompatibility and nutrition of the encapsulated hepatic cells would be to promote vascularization around the encapsulated cells [156]. This is not straightforward because alginate is inert and, as already stated, does not promote cell adhesion.

Very recently, and for the first time, human hepatocytes have been administered in a clinical study with encouraging safety [141]. Their efficiency was demonstrated in a rat model [142]. These findings suggest the potential of encapsulated hepatocyte transplantation in treating liver failure and, particularly, using stem cell microencapsulation as a new alternative to hepatocytes.

3.3. Stem-cell Differentiation

The shortage of transplantable organs also coincides with the issue of low availability of suitable cells, which are often not enough to engineer cellular substitutes capable of recovering the functionalities of a damaged organ. The need to find alternative cell sources has, therefore, become primordial and stringent. The opportunity arose from the high availability of stem cells. These latter, which are unspecialized cells, present the ability to self-renew and to differentiate into specialized cell types through specific development pathways [157, 158]. Stem cells are categorized according to their origin in embryonic or postnatal/somatic/adult [159] that, in addition, show different differentiation potentiality. Embryonic stem cells are, in fact, totipotent and, therefore, able to generate all types of cells. Instead, postnatal/somatic/adult stem cells can be pluripotent (able to generate all types of cells except cells of the embryonic membrane) or multipotent (able to differentiate into more than one mature cell) [159, 160]. Over the last decade, it has been firmly demonstrated that the extraordinary features of pluripotency can be induced in adult cells using four embryonic transcription factors, creating so-called induced-pluripotent stem cells [161, 162]. Accordingly, the paramount role of both stem cells (SCs) and induced-pluripotent stem cells (iPSCs) has been corroborated in the field of the regenerative medicine. It is clear that SCs and iPSCs have different cellular portfolios; however, the potential associated with these cells is huge. They may, in fact, be a real breakthrough in the current situation by representing an unlimited *in vitro* source of cells for *in* or *ex vivo* applications. However, there are still many limitations associated with the different types (from the lack of reproducible differentiation protocols to ethical problems).

Increasing understanding of SC behavior has highlighted the fundamental importance of designing a biologically-inspired *in vitro* cellular microenvironment to guide their growth, differentiation, and functional assembly [163]. Biomaterial scaffolds and bioreactors are paramount tools in this engineering process [164]. As already mentioned, the scaffold generally supports cell survival and function by means of a plethora of chemical and biophysical cues. Cell-cell and cell-matrix interactions, which are most easily established within cell-embedded three-dimensional structures, are mainly involved in SC differentiation [165, 166]. The alginate beads have been referred to as simple bioreactors; they in fact favor the assembly of a scalable number of undifferentiated SC bodies [167] and make them ready to differentiate into specific cellular lineages [168]. The alginate beads thus identified a method for synchronizing the SCs: these SCs were then able to expand within the

beads without differentiating, and, when desired, started the differentiation process under specific conditions [169]. Thanks to their egg-box-like microstructure, alginate beads not only offer SCs a niche-like microenvironment [170], which can recover the phenotype of a specific cell lineage [171], but also act as a shield and protection for the SCs against native immunological responses and external mechanical forces and frictions (especially due to *in vitro* pre-differentiation steps in bioreactors) [172]. However, the advantages of using alginate beads in this context were not only associated with the aspects described above, but also with the possibility of easily scaling up SC production and recovering the differentiated cells, when necessary, by means of simple depolymerization techniques [169, 173].

3.3.1. In vitro Cell Differentiation

Microencapsulation of SCs has mostly been performed *in vitro* so far [174-184]. The investigations have shared similar research plans, that is, direct SC differentiation in alginate beads under chemical (culture medium supplements) and/or physical (culture in bioreactor) stimuli, providing a tissue-mimetic microenvironment, without prior monoculture differentiation steps [172]. In 2010, for the first time, Jing *et al.* [185] presented a culture platform for inducing cardiogenic differentiation of both mouse and human embryonic stem cells (ESCs) encapsulated in PLL-coated alginate beads with a liquefied core. This latter morphological feature was indicated as being responsible for enhanced size control of the ESC aggregates, formed within the beads. Moreover, the core was also claimed to have made possible more efficient circulation of paracrine factors inside the beads. This aspect had a beneficial impact on the differentiation of the ESCs and massive production of cardiomyocytes. The configuration and physical/chemical characteristics (e.g. G/M ratio) of the alginate beads thus have a significant influence on the expansion rate of the SCs, as well as on their differentiation and acquisition of a selected phenotype [186, 187]. Stiff alginate beads, presenting high G residue content, delayed SC growth and inhibited loss of pluripotency; SCs thus remained in an undifferentiated state for longer time periods. Instead, flexible alginate beads, with a high M residue content, had an impact on the selection of the differentiation path promoting SC phenotypical changes toward the endodermal lineage [188].

Nevertheless, directed differentiation trajectories can be more efficiently modulated by incorporating specific cues into the alginate bead matrix [188]. Depending on the final application, the alginate bead matrix can be functionalized with additional compounds that improve SC adhesion and environment recognition. Hence, theoretically, a single alginate microencapsulation culture system may be enough to induce differentiation of large numbers of distinct differentiated cell lineages, using specific compounds (i.e. growth factors, extracellular matrix proteins, etc.) in the alginate bead matrix. Focaroli *et al.*[189], for instance, defined a new platform for stimulating chondrogenic differentiation of human adipose-derived mesenchymal stem cells (ADMSCs) within calcium/cobalt alginate beads. The rationale of the study was to chemically reproduce, by adding cobalt, the native hypoxic environment of articular cartilage. The synergic action of cobalt and alginate was evaluated and nevertheless highlighted the favorable impact of the resulting hypoxia-mimicked environment on ADMSC differentiation in cartilage-producing chondrocytes. Song *et al.* [170] used bone powder, sourced from natural bone and containing hydroxyapatite (HA) and bone morphogenetic proteins, as a signal for promoting osteoinduction and osteogenesis of human ADMSCs encapsulated in alginate beads. Dynamic culture, conducted via spinner flask, was adopted to further enrich the niche-like environment during the osteogenic induction period. This condition made possible accelerated expansion of the SCs, which then readily differentiated into osteoblasts with extensive mineralized nodules forming bone aggregates. The hybrid alginate beads thus presented characteristics that can be considered as promising for tissue-engineered bone substitutes for treating skeletal injuries in future applications.

Overall, incorporated compounds showed great potential in leading SC differentiation, although the role of the alginate beads must be recognized in these circumstances. The alginate beads, in fact, supported SC differentiation without interfering or inhibiting the action undertaken by the incorporated compounds. This aspect was stressed in a recent study. Ba^{2+} cross-linked alginate beads were prepared with the addition of synthetic octacalcium phosphate (OCP) and used to induce osteoblastic differentiation of encapsulated mouse BMSCs. OCP is a highly osteoconductive material which tends to convert into HA under physiological conditions [190, 191]. This process enhances bone regeneration [192]. In the study, alginate beads did not inhibit the thermodynamic conversion of the OCP, which could act unconditionally and, thus, it increased the osteoblastic differentiation of the SCs as compared to alginate beads without OCP.

Including peculiar compounds in the alginate bead matrix has also been used to provide the final construct with specific features[193]. Interestingly, Zhang *et al.* [194] presented an innovative strategy for creating constructs intended for bone regeneration in contaminated sites. Alginate beads were manufactured with dopamine and silver nanoparticles, and encapsulated with mouse bone marrow mesenchymal stem cells (BMSCs). Dopamine (a derivate of DOPA) was incorporated to improve cell adhesion and viability, whereas silver nanoparticles were added to provide the alginate beads with anti-bacterial potential, which is beneficial in case of transplantation into contaminated sites. The results suggested that (i) the anti-bacterial effect was well accomplished, (ii) living BMSCs increased in number, and (iii) underwent osteogenic differentiation, showing increased gene expression and protein production of mineralization.

3.3.2. In situ Cell Differentiation

A minority of studies designed the microencapsulation of SCs in alginate beads for *in vivo* regenerative purposes [172, 195-200]. Most of these studies focused on chondrogenic and osteogenic differentiation of SCs to repair fractures in small-sized animal models. Wang *et al*. [201], for instance, introduced HA into the structure of the alginate beads for osteoblastic differentiation of human MSCs. The construct was maintained in *in vitro* culture for about 21 days in order to monitor MSC differentiation in both dynamic and static conditions. In both situations, the inclusion of HA was extremely important for initiating transmission of differentiation signals to the cells. HA inclusion entailed shape changes for the alginate beads and increased their stiffness; these modifications had a beneficial effect on osteoblastic differentiation. The *in vitro* preformed construct was then implanted into rat femoral condyle defects; this implantation site was chosen as a non-load bearing site as the goal here was to investigate the construct's capacity for osteointegration. Tissue formation in the defect site and recognition of the implant by native cells was made possible thanks to the presence of newly-formed *in vitro* cell-laid matrix from the differentiated MSCs within the alginate beads. This probably closely mimicked the native bone. The authors thus speculated that the construct may be used as an accurate bone template in critically-sized defects.

The increasing number of studies proves the potential associated with this technology. Alginate beads may thus be an effective alternative tool for the optimal expansion and differentiation of SCs. Moreover, alginate beads may offer the differentiated cells a ready-implantable structure. This platform may therefore be a way of overcoming the shortage of cells needed to engineer bioartificial substitutes for damaged organs, which is the biggest challenge for their clinical applications. There is still a long way to go, however, and there remain many obstacles. A real barrier is put by the lack of defined differentiation protocols. Consequently, the differentiated cells often present low purity and unwanted cells, which could rise

to tumors; these drawbacks make the differentiated cells inadequate for the actual transplantation [202]. Therefore, the *in vitro* differentiation pathways should be further investigated and clearly defined in order to obtain specific lines of adult cells with less contamination. Moreover, well-designed induction protocols may be beneficial to solve the problem of low frequency of iPCs. In this manner, iPCs may be directly reprogrammed from the patient's own cells overcoming, thus, the issues of immunorejection and ethics associated with the use of SCs [202].

CONCLUSION

The aim of the present review was to point out the crucial role of cell microencapsulation technology in the fields of tissue engineering and regenerative medicine as a strategy for treating different diseases, such as diabetes or liver failure. Alginate-based microencapsulation covers a broad spectrum of medical applications beyond those reviewed here. However, the growing number of studies also suggests that supplemental basic analysis (e.g. optimization of alginate parameters in relation to each specific cell type) is needed to concretize its actual contribution in clinical cases. These studies carried out worldwide by different research groups are promising and allow us to believe that real progress will be made in the near future.

CONFLICT OF INTEREST

None.

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