

Cell Encapsulation in Mammal Reproduction

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Abstract: Cell encapsulation is an evolving branch of biotechnology with numerous applications including the enhancing of reproductive performance both in humans and other mammal species. Over the last twenty years male and female gametes and embryos have been encapsulated with or without somatic cells, for different purposes, such as semen controlled release, *in vitro* gametogenesis, embryo culture after *in vitro* fertilization and cell preservation. In this paper the state-of-the-art of this field (leaving aside that involving embryonic stem cells) is reviewed in terms of scientific literature and patent production. The patents and papers underline a widespread use of alginate which is a natural anionic, biocompatible, biodegradable polymer that mimics the extracellular matrix or the basal membrane and supports cell functions and metabolism. Gamete and embryo encapsulation techniques tend to fall into two main groupings: the "classical" three-step method, and the more recent one-step method. However, all of these encapsulation techniques are moving towards new, interesting applications since they can be easily tailor-made to fit a variety of cell lines.

Keywords: Microencapsulation, alginate, spermatozoa, oocyte, gamete, *in vitro* maturation, *in vitro* fertilization, three-dimensional cell culture, Sertoli Cell, Leydig cell, embryo, granulosa.

INTRODUCTION

Cell encapsulation is a strategy whereby a pool of live cells is entrapped within a semipermeable membrane. The first scientific publication describing the principle of bioencapsulation is by Chang [1]. In his two-page article, Chang wrote: "I have found that artificial microcapsules of comparable dimensions and properties can be made simple and in large numbers, and that enzymes and other proteins loaded into these particles retain useful biological activity *in vitro* and *in vivo*". After more than four decades, the huge development of encapsulation in several branches of biotechnology is still based on Chang's work. In the succeeding years, in fact, numerous microencapsulation techniques have been developed in independent labs, following on the footsteps of Chang's original method, consisting in the generation of a controlled-size droplet, followed by the process of stabilizing the interface and creating a membrane around the core.

The breakthrough in applying Chang's principles of bioencapsulation came with the work of Lim and Sun [2]: for the first time, a natural, biocompatible polymer, alginate, was employed for Langerhans islet encapsulation with the aim of preventing the rejection of the transplanted cells in type I diabetes-affected rats. The first application of cell encapsulation in mammal reproduction was made by Nebel *et al.* who devised a technique for bovine sperm encapsulation in calcium alginate and polyamines [3]. Nebel introduced the idea of spermatozoa controlled release and the targeting of the female reproductive system for artificial insemination. Since the works of Nebel *et al.*, the applications of cell encapsulation in reproductive biotechnologies

have been expanded to other fields with different aims, which one briefly sketched out in Table 1.

SPERMATOOZA ENCAPSULATION FOR CONTROLLED RELEASE AND PRESERVATION

Artificial insemination (AI) techniques involve the collection of semen, its preservation and its transference to the fertilization site. This practice is widely diffused in both the animal and human fields, but several difficulties must be overcome. Firstly, to be preserved, sperm must be suspended in a suitable extender that assures the energy supply for spermatozoa, counteracting acidosis deriving from the metabolic processes. This leads to a dilution of seminal plasma, the physiological extender for spermatozoa; seminal plasma contains membrane-stabilizing agents and capacitation-preventing molecules [4,5]: unavoidably, sperm dilution leads to membrane destabilization, capacitation and a precocious acrosomal reaction. Moreover, the antioxidant system connected with plasma-cell factor interactions can be unpaired. All of these phenomena are known as "dilution shock" [4].

In order to increase the shelf life of spermatozoa, they are currently refrigerated or frozen: this induces a cold shock, and the severity of this event is species-dependent. Cold shock involves potential modifications of the phospholipid bilayer fluidity, with phase transitions and structural/functional membrane protein alterations, leading to membrane permeability and functional receptor impairment [6, 7].

Further difficulties in AI practice depend on the female reproductive physiology. In order to obtain the maximum fertilization yield, mature oocyte must be reached by a fertile, capacitated but non-reacted spermatozoon. Therefore, the exact detection of ovulation in terms of time and, in polytocic species, timespan is pivotal, and often difficult to establish.

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Table 1. Synopsis of Applications of Cell Encapsulation Technology in Mammal Reproduction

Cell type	Aim	Comments	References
Spermatozoa	Controlled release for artificial insemination	Polycation-Alginate beads or barium alginate capsules	[3,14-22,24-26]
Spermatozoa	Preservation	As above	[24,26,27]
Female germ cell lineage	Enhance <i>in vitro</i> maturation yield	Oocyte-granulosa coculture in calcium alginate beads or barium alginate capsules	[30-39]
Male germ cell lineage	As above	Germ-Sertoli-Leydig coculture in calcium alginate beads	[40,41,43-46]
Embryo	Improve <i>in vitro</i> fertilization yield	Zygote-tubal cells coculture in calcium alginate beads	[36,47]

For this reason, different strategies have been proposed: in pigs, for example, the identification of the optimal time of insemination can be tested in terms of standing reflex; ovulation can be detected by ultrasonography [8,9] or hormonal patterns, [10] and/or vaginal mucus conductivity [11]. Another strategy employed to improve AI procedure is hormonal oestrus synchronization [12, 13].

A different approach, proposed by Nebel [3], is the development of controlled release delivery systems by sperm encapsulation: high sperm concentrations were maintained in the bovine uterus during the ovulation, thus, guaranteeing an increase in fertility and prolificacy.

The encapsulation procedure similar to that of Lim and Sun [2] involved three steps: (a) the production of a calcium alginate cell-containing matrix; (b) the formation of a semi-permeable membrane by interfacial polymerization with a multivalent polyamine; and (c) the liquefaction of the semi-solid matrix by chelation of calcium with sodium citrate. The use of a biocompatible polymer as the alginate led to the maintenance of optimal cell viability levels both *in vivo* and *in vitro*. The capsule membrane is permeable to small molecules as glucose and oxygen but impermeable to large molecules such immunoglobulins [2].

Moreover, capsules could protect male gametes from uterine macrophage phagocytosis and promote bioadhesion of the delivery system, preventing semen reflux [3, 14-16]. Microcapsule properties were modulated in order to obtain an optimal release time. Vishwanath *et al.* [17] obtained satisfactory *in vivo* fertilization results in CIDR[®] synchronized heifers: they observed an increase in fertility with encapsulated semen at the optimal time of insemination (48 hours after CIDR[®] removal).

Van Blerkom [18,19] used, for bovine sperm encapsulation, a non-toxic polymer (polyurethane-polyether), which liquefies at body temperature and gels or solidify at storage temperature and transfer. Spermatozoa suspended in the liquid polymer at 37°C were poured into molds and then cooled at a controlled rate until gelling point. When the delivery system reaches the female reproductive apparatus, the polymer matrix liquefies, and motile spermatozoa are released. Polymer concentration appears to be critical for sperm viability and motility, as well as gel cooling rate.

In 1999, Conte *et al.* proposed a one-step reverse technique for encapsulating swine spermatozoa in barium alginate [20]. Briefly, barium chloride is added to the semen and the resulting suspension is dropped into a sodium alginate solution; barium ions contained into the ejaculate diffuse out of the droplets and when they reach the interface, react with the alginate chains, leading to the formation of a barium alginate gel membrane around the semen droplet. The thickness of the gel membrane increased until the diffusion of the barium ions through the semen droplet ends [20].

This method has two advantages: firstly, these capsules consist of a core of highly concentrated spermatozoa surrounded by a polymeric membrane which separate the cells from the extender. The spermatozoa encapsulated with physiological seminal plasma were thus suspended rather than diluted in the storage medium, and a virtual dilution could be obtained after encapsulation [21,22]. Secondly, barium ions were employed as gelling agent in lieu of calcium, as Ba⁺⁺ inhibit a precocious capacitation process that is promoted by Ca⁺⁺ [23]. The latter technique was also successfully applied in other species, as for example the Equidae [24].

Chou and Wang [25] encapsulated boar spermatozoa in calcium alginate beads crosslinked with polycations: the membrane thickness can be modulated to vary the sperm release timespan; the release time and rate can be optimised providing a mixture of capsules of different thickness. Moreover, in order to avoid precocious calcium-induced capacitation spermatozoa, prior to be encapsulated, had to be diluted with a capacitation-preventing extender, and the capsules had to be suspended in a capacitating medium just before AI.

Huang *et al.* [26] recently reported *in vivo* fertility results obtained using encapsulated boar spermatozoa on a small sow sample: they conclude that calcium alginate microencapsulation sustains the fertility of spermatozoa with good farrowing rates and offspring size. Moreover, the findings of this study confirmed that encapsulation could prolong the storage time of boar spermatozoa.

More recently, Herrler *et al.* [27] demonstrated that human semen-containing calcium alginate microcapsules can be cryopreserved, yielding a decreased motility compared to standard protocols, but with a higher vitality of the immotile

spermatozoa. This technique can be useful when small amounts of spermatozoa collected by microsurgical epididymal sperm aspiration, testicular sperm extraction, or from cryptozoospermic men must be preserved in several small samples feasible for a possible Intracytoplasmic sperm injection (ICSI). This avoids repeated freeze-thaw cycles to perform repeated ICSI.

GERM CELL LINEAGE ENCAPSULATION FOR IVM

a. Female Germ Cell Lineage

During recent decades, several attempts have been made to design oocyte *in vitro* maturation (IVM) and/or *in vitro* fertilization (IVF) culture systems providing optimal yield. Common culture methods are generally based on protein and growth factor-enriched media with the purpose of replacing the biological milieu for the isolated ova, but more recently, as in other fields, alternative oocyte maturation systems have been conceived, based on two simple morphophysiological observations: 1) oocytes are surrounded by a cellular environment (e.g. granulosa cell cumulus), with a well-defined extracellular matrix and 2) *in vivo* cells present a three-dimensional (3D) organization. Thus, a culture system that considers these principles as its keystone more closely approaches the biological maturation system, and forms one of the most recent advances in tissue engineering.

These features have been extensively reviewed by Gilchrist *et al.* [28] and recently demonstrated by Hussein *et al.* [29]; oocyte/cumulus cellular contacts and paracrine factors are the basis for optimal oocyte development and competence. Following these principles, a number of three-dimensional granulosa/oocyte co-culture systems were tested and, in some cases, patented; in this field, encapsulation seems to be a valid technology for obtaining a 3D environment suitable for co-culture. Pangas *et al.* [30] developed an alginate beads 3D culture system designed for cumulus/oocyte complexes (COCs); such technology yielded good results in terms of structural development and meiosis resumption for murine ova. Further successes were achieved in rat oocytes after modifying the cell adhesion properties of the alginate with the use of the tripeptide Arg-Gly-Asp (also known as RGD peptide), a common adhesion sequence for several extracellular matrix proteins [31]. This latter system aims to mimic the extracellular matrix functions more closely. Kreeger *et al.* [32] demonstrated that the alginate-based three-dimensional co-culture system can easily be employed in several murine oocyte development stages when a 3D COCs co-culture system, is supplemented with follicle-stimulating hormone, in a synthetic extracellular matrix with specific components (RGD peptide and laminin). The same research group observed a huge and positive influence of the 3D system not only in the pseudo-tissue architecture, but also in the meiotic competence of mouse oocytes [33,34]; moreover, the offspring obtained from this kind of co-culture technique, is viable and fertile [35]. These strategies, when applied to murine models, could positively affect the researchers attempts of oocyte banking as well as the preservation of fertility in human reproduction.

The 3D approach for COCs/oocyte co-culture in alginate matrix was also employed using barium alginate capsules to mimic a basal membrane. An oocyte and a pool of granulosa

cells, were enclosed in a fluid core and once in the core, the granulosa cells organized themselves into a pseudofollicular structure, morphologically resembling a mature, Graafian follicle, and thus creating a milieu capable of inducing oocyte maturation without exogenous hormones [36,37]. Scanning electron microscopy and the analysis of progesterone/oestradiol synthesis performed on bovine/porcine models show that the barium alginate co-culture maintains a better environmental condition for granulosa cells, when compared with a monolayer culture, with delayed luteinization phenomena [37]. Moreover, porcine granulosa cells cultured in the barium alginate matrix can be successfully transfected by electroporation after 6 days of culture [38]. The membrane-core model was also applied to human oocytes, obtaining better results in terms of maturation yield when compared to a routine method (90.3% vs. 52%, respectively after 48 hours of culture), i.e. microdrop oocyte culture [39]; moreover, neither maturation system contained any hormonal supplements, and oocytes were retrieved from patients after *in vivo* hormonal stimulation.

b. Male germ Cell Lineage

In recent years the “reproductive counterpart” for oogenesis, spermatogenesis, has also received the attention of several research groups. *In vitro* spermatogenesis can be a valid tool for facilitating and improving the treatment of male infertility for a better clarification of the sperm differentiation mechanisms. *In vitro* spermatogenesis techniques are somewhat difficult to apply, since the complex architecture of seminiferous tubules constitutes a biological obstacle to a harmonic and temporally-organized gamete development. Initially, the successful attempt to reconstitute sperm cell lines was made by Lee *et al.* [40]: testis tissue from young bulls was dissociated in order to recover Sertoli cells and germ cells, and after harvesting, isolated cells were reaggregated and encapsulated in calcium alginate. After 10 weeks, several spherical cells (7-10 μm) appeared at the margin of the cell culture. Polymerase chain reaction analyses for protamine-2 and transition protein 1 (two molecules present in adult bull testes) conducted on *in vitro* developed germ cells confirmed the presence of presumptive spermatids [40]. Parks *et al.* [41] reviewed these fundamental findings underlining that alternative *in vitro* spermatogenesis techniques, included encapsulation, can be tailor-made to notably improve the culture conditions. Progress in the improvement of the germ cell culture has been confirmed by the work from Lee *et al.* [42]: testis tissues from human patients with non-obstructive azoospermia (NOA) were dissociated, and then isolated male germ stem cell-like cells were encapsulated in calcium alginate and cultured for up to 6 weeks. Presumptive spermatides were able to induce cleavage of mature oocytes. In view of this, the authors conclude that encapsulation might be useful for confirming the existence of germ cells and for the subsequent treatment of NOA patients. As reported by Kubota and Brinster [43], spermatogonial stem cell expansion could lead to a significant number of applications in the near future, with regard to human infertility and perhaps gene therapy as well. These culture techniques for germ line stem cells have been recently patented by Kim *et al.* [44] and Lee *et al.* [45]. Briefly, the isolated germ-line stem cells from azoospermic patients have

been encapsulated in alginate and maintained into an hormone-enriched medium; meanwhile, testicular cells were added to the encapsulated culture as feeder, and the system was maintained in coculture. The coculture system allowed the development of an acrosome and the expression of *c-kit* protein as a marker of spermatogonia and spermatocytes better than other culture systems.

EMBRYO ENCAPSULATION TO IMPROVE IVF YIELD

As well as gamete preservation, a number of efforts have been made to find a valid method for the amelioration of *in vitro* conditions for the preservation and the early development of *in vitro*-obtained embryos. From the very beginning, encapsulation has proved to be a promising technique in this field: a work by Krentz *et al.* [46] reported that encapsulated mouse morulae (either in alginate or poly-L-lysine) gave results similar to unencapsulated morulae, reaching 87.5% of pregnancies for both encapsulation methods, and 71.4% for non-encapsulated morulae. However, there was a smaller percentage of viable foetuses, perhaps, as the authors noted, because "mortality occurred *in vivo* due to an asynchronous condition between the uterine environment and the embryos". Yaniz *et al.* [47] proposed a bovine model, merging the calcium alginate semisolid 3D matrix and the coculture with oviduct cells; a large quantity of blastocysts was reached by day 7 of culture, with results matching the unencapsulated morulae but with a significant reduction in the ability to hatch; such result could be ascribed to the encapsulation process. Vigo *et al.* [24] propose fluid core alginate capsules as an alternative method for facilitating embryo development, since the virtually hollow capsule permits a better self-organization of the cellular systems.

CURRENT & FUTURE DEVELOPMENTS

Nowadays encapsulation is a widespread technique for cell storage and controlled release in AI protocols, as well as in the 3D culture of mono/multicellular systems. Among the advantages of this technique are:

- 1) encapsulation enhances the *in vitro* storage of spermatozoa, allowing for *in vivo* semen controlled release;
- 2) IVM/IVF yield in human and veterinary fields are improved, since the possible self-organization of multicellular systems express a huge number of cell biological potentials. In such a way it is therefore possible just to employ autologous cells, limiting or avoiding the use of exogenous molecules (e.g. growth factors and hormones);
- 3) capsules can be made in a simple way, in large number and with homogeneous characteristics (as permeability, size, mechanical properties), tailored for the type of treated cells and the aims required; polymers are available at different degrees of purity and with different physicochemical properties (though at variable costs): these can be chosen depending on the policy and the aims of the application; encapsulated cells can easily be handled without direct contact, resulting in reduced risks of contamination.

These findings are supported by the abundant literature published over the last 40 years: despite this, patent applications are still relatively scarce.

One reason for this might be the difficulties involved in standardizing culture methods: the classical fluid culture media are fixed in their composition, while the cocultures include cellular systems, often of primary cultures, endowed with intrinsic variability. However, the application of the novel, sophisticated cell-sorting methods can overcome this obstacle, allowing for the selection of the desired cell population. Notwithstanding standardization difficulties, system versatility appears to be immense, given that a wide number of polymers are currently available, either native, native/elaborated or synthetic.

New cell encapsulation technologies are going to be patented over the coming years: these will include standardized procedures for large scale capsule production for livestock artificial insemination as well as easy-to-use kits for "homemade" capsules, which could be routinely used in the therapy of human reproductive disorders. In conclusion, the experimental evidences herein reviewed provide the groundwork for a full technological development leading to large scale production and marketing.

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