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Reproductive Tissue Engineering

Alginate: A Versatile Biomaterial to Encapsulate Isolated Ovarian Follicles

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Abstract—*In vitro* culture of ovarian follicles isolated or enclosed in ovarian tissue fragments and grafting of isolated ovarian follicles represent a potential alternative to restore fertility in cancer patients who cannot undergo cryopreservation of embryos or oocytes or transplantation of frozen-thawed ovarian tissue. In this regard, respecting the three-dimensional (3D) architecture of isolated follicles is crucial to maintaining their proper follicular physiology. To this end, alginate hydrogel has been widely investigated using follicles from numerous animal species, yielding promising results. The goal of this review is therefore to provide an overview of alginate applications utilizing the biomaterial as a scaffold for 3D encapsulation of isolated ovarian follicles. Different methods of isolated follicle encapsulation in alginate are discussed in this review, as its use of 3D alginate culture systems as a tool for *in vitro* follicle analysis. Possible improvements of this matrix, namely modification with arginine-glycine-aspartic acid peptide or combination with fibrin, are also summarized. Encouraging results have been obtained in different animal models, and particularly with isolated follicles encapsulated in alginate matrices and grafted to mice. This summary is designed to guide the reader towards development of next-generation alginate scaffolds, with enhanced properties for follicle encapsulation.

Keywords—Alginate, Preantral follicles, Ovary, Cancer patients, Fertility preservation.

ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AMH	Anti-Müllerian hormone
ANGPT-1	Angiopoietin-1
ANGPT-2	Angiopoietin-2
boECM	Bovine decellularized ovarian extracellular matrix
ECM	Extracellular matrix
FSH	Follicle-stimulating hormone
GC	Granulosa cell
LH	Luteinizing hormone
MEF	Mouse embryonic fibroblast
RGD	Arginine-glycine-aspartic acid
VEGF	Vascular endothelial growth factor

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INTRODUCTION

Studies on *in vitro* culture of isolated ovarian follicles or ovarian tissue fragments and transplantation of isolated ovarian follicles have significantly increased over recent years thanks to a growing number of applications. For many decades, these techniques were largely used as a research tool to investigate folliculogenesis,[49,56,81,104] a complex process that is far from being fully understood in all animal species. Now they also represent a potential alternative to restore fertility in cancer patients who cannot undergo other procedures like cryopreservation of embryos or oocytes, or transplantation of frozen-thawed ovarian tissue.[4,32,103] Similarly, they can be used to obtain and exploit female gametes in preservation programs of endangered animal species.[20,42,115] Finally, these techniques have also been applied in toxicological studies evaluating the impact of different chemicals and drugs on female

fertility.[6,24,65,73] However, identifying a system that accurately mimics the natural environment of follicles in order to understand how they develop in the original organ, react to potentially toxic chemical agents, and grow to produce viable oocytes has proved to be very challenging.

Andersen *et al.*[8] stated that "the world around us, including the human body, is constructed in three dimensions", so it is fair to say that the ovarian follicle is, by definition, a three-dimensional (3D) structure. Respecting its 3D architecture is therefore crucial to maintaining proper follicular physiology and obtaining responses resembling the expected behavior of follicles *in vivo*. Although ovarian follicles from rodents have been shown to survive and grow on flat surfaces,[35] follicles from large mammals, including humans, cannot be made to develop normally in two-dimensional (2D) systems.[1,43,82,102] Indeed, such *in vitro* culture conditions cause disruption in follicle architecture due to flattening because of granulosa cell (GC) attachment to the culture dish, which in turn interferes with their interactions among themselves and with the oocyte, leading to uncoordinated growth and differentiation of germ and somatic cells.[75] For these animal species, 3D culture systems are essential, as they prevent dissociation of GCs from the oocyte, ensuring survival and correct development of isolated follicles, and providing follicular responses of biological significance.

A number of studies have reported 3D encapsulation of isolated follicles in various animal models using different scaffold materials, such as agarose,[5,80] alginate,[7,21,112] collagen,[50,54,88,98,135] and hyaluronic acid.[27] To date, the most widely applied biomaterial has been alginate, since it has yielded favorable results with follicles from numerous animal species. The goal of this review is to provide an overview of alginate use as a matrix for 3D encapsulation of isolated ovarian follicles, highlighting the most important results in different animal models. Ways of improving this matrix to optimize its application for *in vitro* culture or transplantation of isolated follicles will also be considered and discussed.

CHARACTERISTICS OF ALGINATE HYDROGELS

Alginate is a collective term for a family of polysaccharides produced by brown algae or bacteria. Chemically, they are copolymers of 1 $\rightarrow$ 4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) arranged in a blockwise pattern along the chain, with homopolymeric regions of M (M-block) and G (G-block) residues interspersed with regions of alternating (MG-block) structures (Fig. 1).[30,45,46]

Alginate has the ability to form stable hydrogels in the presence of millimolar concentrations of calcium or other divalent cations,[47] like barium or strontium, that serve to crosslink G units (Fig. 2). This gelification property allows cell encapsulation under physiological conditions, with uniform distribution of cells throughout the matrix.[60]

The mechanical properties of the alginate beads are modulated by the polymer composition and/or concentration. Ouwerx *et al.*[70] described that Young's modulus of alginate beads formed in the presence of calcium (0.1 M) increased with the square of the alginate concentration: $E = KC^2$ (E = value for Young's modulus; K = alginate specific constant; C = alginate concentration), demonstrating that alginate concentration is the parameter limiting the bead's elasticity. Rheological analysis also showed a positive correlation between guluronic acid concentration and elastic modulus in alginate hydrogels[33] and an influence of the gelling cation type[70] and concentration on the alginate matrix.

Alginate is very easy to handle, as well as being biocompatible *in vivo*, which means its degradation products can be excreted through the urine.[3] Once purified to remove endotoxins, proteins and polyphenol contaminants, it is also non-cytotoxic. For these reasons, alginate has been widely used for tissue engineering applications,[34,64] including follicle encapsulation.

ENCAPSULATION OF ISOLATED FOLLICLES IN ALGINATE BEADS

Encapsulation in alginate has proven to be a rapid, non-toxic and versatile procedure for cells and follicles, performed in two steps. The first involves an internal phase during which the alginate solution containing the biological material is separated into droplets, and the second, solidification of these droplets by gelling.[8] The size of the droplets varies according to the amount of biological material to be encapsulated, the viscosity of the alginate solution, the system used to obtain the droplets, and the alginate flow rate.[8]

For follicle encapsulation, alginate droplets ranging from 5 to 60 μL in size have been investigated.[52,72] Their size was not related to the number of follicles contained inside. For instance, Hornick *et al.*[52] used 5 μL of alginate to encapsulate 1, 5 or 10 mouse primary or secondary follicles, while Amorim *et al.*[7] embedded 4–8 human primordial-primary follicles in 20 μL of alginate.

Although different cell types have been successfully encapsulated in alginate by means of various automated systems, such as coaxial air flow, electrostatic

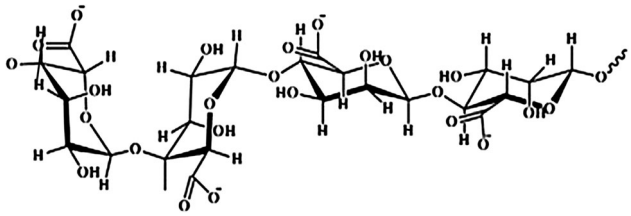

FIGURE 1. Alginate polysaccharide consisting of two guluronic acid and two manuronic acid residues with (1,4) linkages. Reprinted with permission from Manivasagan and Oh, 2016, ©Elsevier (2016).

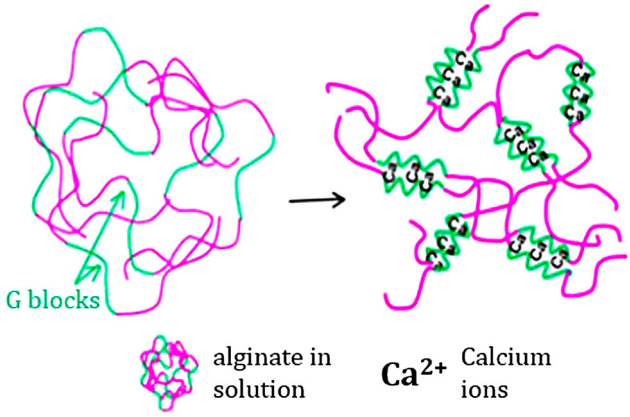

FIGURE 2. Alginate gelation characteristics. Areas with repeating G monomers, termed G-blocks, are cross-linked to form a hydrogel in the presence of divalent calcium ions. Reprinted with permission from Shikanov *et al.*,[90] Journal of Visualized Experiments (2016).

generators, cell electrospinning or jet cutters,[8,10,55,109] procedures described in the literature to embed ovarian follicles do not make use of any instrumentation. Alginate beads are usually made by dropping the alginate solution and follicles from a pipette tip directly into a crosslinking bath consisting of calcium solution,[7,21,112] or immersing a mesh containing the droplets of alginate solution inside the crosslinking bath[72] (Fig. 3). Another technique involves inversing the mesh to suspend the alginate droplets followed by quick shaking or tapping, so that the droplets fall into the crosslinking bath (Fig. 3).[48,52,124,129] All these alternatives are able to successfully entrap isolated follicles in the hydrogel and are gentle on the follicles, not affecting their further survival or development *in vitro*.

Shikanov *et al.*[90] described two different procedures to encapsulate mouse follicles in a fibrin–alginate matrix. In the first, called the drop method, isolated follicles are washed in a rinsing droplet consisting of fibrinogen–alginate solution to remove the medium, before being transferred to the encapsulation droplet, containing the same fibrinogen and alginate concentrations as the initial droplet. Each follicle is individually aspirated in 5 μL of this solution and then expelled into a polymerization bath constituted of thrombin–calcium solution (Fig. 4). In the second, known as the parafilm method, an isolated follicle is first inserted into a 7.5 μL fibrinogen–alginate droplet placed on a parafilm-coated glass slide, to which 7.5 μL of thrombin–calcium solution is then added (Fig. 4).

ALGINATE RIGIDITY

Alginate rigidity, which is modulated by its composition and/or concentration, has been shown to have an impact on follicle survival and development, oocyte maturation and hormone production. Jiao *et al.*[58] reported that the rigidity of an alginate matrix could affect oocyte-specific gene expression levels in oocytes. Moreover, denser matrices could potentially limit access to hormones and other nutrients. Although diffusible signals are generally able to spread throughout the hydrogel, Heise *et al.*[48] found inhibited delivery of follicle-stimulating hormone (FSH) to microencapsulated follicles. Follicle diameters increased with addition of FSH to the hydrogel, but still did not reach the size observed in unencapsulated controls. It is important to bear in mind that in a 3D system, the mechanical force exerted by the matrix on cells around the exterior of the follicle will be transmitted to all cells within the follicle and will influence development and

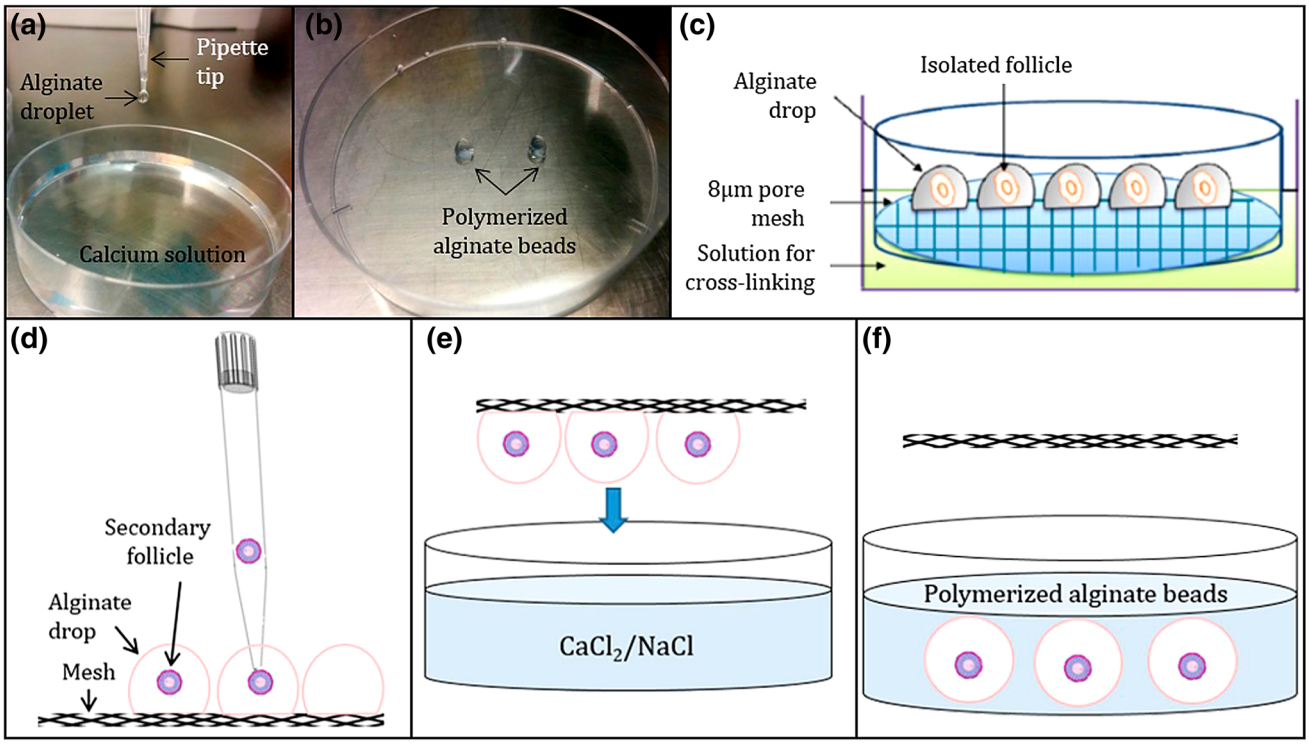

FIGURE 3. Different strategies to prepare alginate beads. Dropping method used by Amorim's group (a, b), where one droplet of 1% alginate solution containing isolated follicles is released into calcium solution (a) to induce polymerization (b). Immersion method developed by Park *et al.*,[72] where an alginate drop containing a single isolated follicle is placed onto a polycarbonate insert that is then plunged into a calcium solution (c). Inversion method applied by Woodruff's team (d–f), where a droplet of alginate solution containing one follicle is first placed onto polypropylene mesh (d), which is then inverted over a dish containing calcium solution (e) and quickly shaken, so the droplets fall into the calcium solution for polymerization (f). ©Reprinted with permission from Park *et al.*,[72] Springer Science + Business Media (2016).

maturation. It may, for instance, affect actin organization, possibly activating one or more mechanoresponsive pathways.

Studies on alginate-encapsulated follicles have demonstrated that hydrogel rigidity needs to be carefully determined, as the scaffold must be sufficiently rigid to maintain the 3D structure of the follicle, yet soft enough to expand and allow oocyte growth, GC proliferation and antrum formation. Clearly, the physical attributes of the 3D matrix selected for *in vitro* follicle culture need to be tailored to meet species-specific requirements and take into account follicle stage. For example, primate secondary follicles embedded in 0.25% alginate beads were able to produce angiogenic factors, notably vascular endothelial growth factor A (VEGF-A), angiopoietin (ANGPT)-1 and ANGPT-2, which were regulated by gonadotropins and oxygen tension.[38] In similar culture conditions, Rodrigues *et al.*[76] showed that survival and growth, as well as steroid and anti-Müllerian hormone (AMH) secretion, could be influenced by direct action of androgens.

Alginate matrix stiffness appears to play a role in the survival and growth of follicles from all animal species. A number of studies have reported that alginate concentrations affect mouse follicle survival,[72] paracrine communication between the cellular compartments of follicles,[121] oocyte development,[133] and antrum formation.[72] Although different alginate concentrations ranging from 0.125 to 3% have been tested for *in vitro* culture of mouse follicles, West-Farrell *et al.*[122] found folliculogenesis in this species to be better supported by lower alginate concentrations.

Brito *et al.*[18] reported that isolated preantral follicles from goats could have undergone alterations to their morphology and hormone and enzyme levels according to concentrations of alginate used for their encapsulation. These authors observed that while higher alginate concentrations (0.5 and 1.0%) improved follicle morphology, a lower dose (0.25%) favored estradiol and progesterone production.[18] In sheep, Sadeghnia *et al.*[84] found that a stiffer alginate matrix (2%) was more suitable for development of primordial follicles.

Songsasen *et al.*[97] showed that a lower alginate concentration (0.5%) enhanced growth of canine preantral and small antral follicles more successfully than a higher concentration (1.5%). Interestingly, smaller follicles from non-human primate species cultured in more rigid alginate scaffolds performed better than

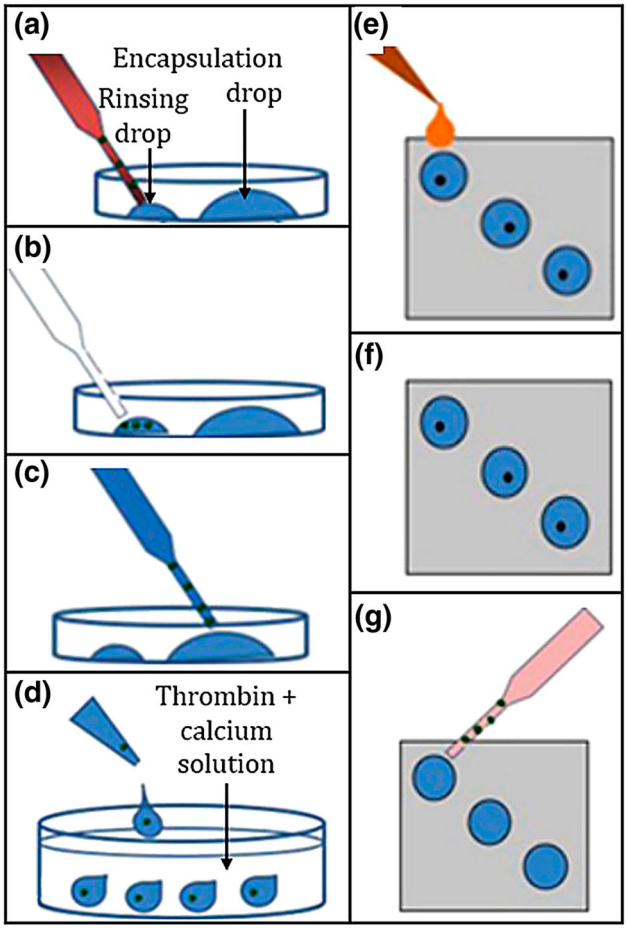

FIGURE 4. Different strategies to prepare alginate–fibrin beads proposed by Shikanov *et al.*[90] Drop method (a–d): Two drops of fibrinogen–alginate solution are prepared on a dish, one for rinsing and the other to encapsulate follicles (a). Up to 5 follicles are washed in the first drop to remove the medium (b), before being transferred to the second drop (c). Each follicle is aspirated in 5 μL of encapsulation solution and then expelled into a polymerization bath containing thrombin–calcium solution (d). Parafilm method (e–g): Fibrinogen–alginate droplets are placed on a parafilm-coated glass slide (e) and, an isolated follicle is inserted into each droplet (f). For polymerization, a droplet of thrombin–calcium solution is added to each fibrinogen–alginate droplet. Reprinted with permission from Shikanov *et al.*,[90] Journal of Visualized Experiments (2016).

those in softer alginate structures, suggesting that primate follicles may require stronger physical support.[51,134] Indeed, follicle survival and morphology were optimal when primate primordial follicles were cultured in 2% alginate compared to 0.5% alginate.[51] However, most studies investigating 3D *in vitro* culture of primary–secondary follicles from non-human primate species found low alginate concentrations (0.25%) to work best.[105–107,126,127,131,134]

To humans, like in non-human primates, higher alginate concentrations are generally used for smaller preantral follicles, and lower concentrations for larger follicles. Softer matrices (0.3–0.5% alginate) are able to sustain survival and development of secondary follicles during long periods of *in vitro* culture,[94,124,136] produce oocytes with nuclear maturation competence, demonstrating that this 3D culture system allows maintenance of bidirectional communication between GCs and the oocyte,[13] and create an environment conductive to oocyte growth and proper steroid production.[123,124] On the other hand, Yin *et al.*[136] showed that although follicles from different stages of development are able to survive in such low alginate concentrations, secondary follicles exhibited significantly higher survival rates compared to their primary and primordial counterparts. Moreover, they could develop up to the antral stage and become steroidogenically active.[136]

For smaller human preantral follicles, 1 or 1.5% alginate proved best, as these concentrations were shown to support survival and growth.[7,15,21,112]

ALGINATE-BASED MATRICES

Despite positive results obtained with alginate hydrogels, the biological inertness of alginate has lar-

gely hampered its use in applications where cell adhesion is essential for survival and proliferation.[53] However, extracellular matrix (ECM) molecules can be combined with calcium alginate to construct synthetic ECM matrices for 3D culture without affecting their biocompatibility or stability,[59–62,90,91,125,127,130,137] as detailed below.

For instance, calcium alginate containing the arginine-glycine-aspartic acid (RGD) adhesion peptide sequence showed a positive effect on hormone secretion: increasing RGD concentrations improved progesterone and estradiol secretion.[62] Moreover, RGD peptide and collagen I showed to improve follicle development of secondary follicles and oocyte maturation and meiosis resumption can be promoted when fibronectin, laminin or RGD are added to the alginate matrix.[60]

Alginate can also be combined with fibrin, offering a dynamic mechanical environment, since both polymers contribute to initial matrix rigidity. However, fibrin is rapidly degraded by proteases secreted by growing follicles and matrix support is done exclusively by alginate. Shikanov *et al.* obtained 82% of meiotically competent oocytes after 12 days of culture of isolated secondary murine follicles encapsulated in a fibrin–alginate matrix, which was a significantly higher percentage than in alginate alone.[90,91] Similarly, Jin *et al.*[59] found fibrin–alginate hydrogels to be superior to alginate in terms of murine follicle growth and differentiation, yielding a larger proportion of oocytes competent for fertilization and a greater number of two-cell embryos than alginate alone.

Isolated follicles from goats[19] and dogs[96] were also encapsulated in a fibrin–alginate scaffold. Brito *et al.*[19] reported that oocytes could only mature when groups of isolated secondary follicles were *in vitro* cultured in this matrix, proving that goat follicles can benefit from a component that facilitates cell adhesion. Fibrin was also shown to improve growth of canine secondary follicles.[96] The authors hypothesized that this may be thanks to fibrin's ability to degrade, allowing better follicle expansion than with alginate alone.

Xu *et al.*[130] also reported that an alginate–fibrin matrix increases growth of primary follicles from macaques and *in vitro* production of estradiol, AMH and VEGF. A fibrin–alginate scaffold containing growth factor-reduced Matrigel, which has lower concentrations of several growth factors[28] that participate in follicle development,[36] also appears to be suitable for *in vitro* culture of baboon follicles.[127]

These studies show that *in vitro* culture of isolated preantral follicles in 3D systems can be improved and controlled through the design of ECM structural and biochemical properties. More recently, a fibrin–alginate interpenetrating network was used to evaluate toxic effects on murine ovarian follicles *via* a high-throughput approach, showing that fibrin degradation rate can be correlated to follicle health status: it significantly decreases or stops when follicle is damaged. The changes in proteolytic activity from the proteases secreted by functioning GCs can be measured by the optical fibrin clearance surrounding the follicle.[137] Like this, Zhou *et al.*[137] found that follicles exposed to increasing doses of doxorubicin showed decreasing survival rates, coupled with attenuated fibrin degradation.

Biological scaffolds made of ECM constituents produced by decellularization of mammalian tissues have been widely studied in recent years. Indeed, although they do not retain the 3D ultrastructure of native tissue, they still maintain *in vitro* and *in vivo* biological activity.[16,25,116] Moreover, ECM hydrogels may supply signaling molecules and growth factors.[93,117] Based on encouraging results obtained with alginate and its biological inertness, Chiti *et al.*[22] hypothesized that decellularized bovine ovarian ECM (boECM) hydrogel could be mixed with alginate to provide cell anchorage patterns. A protocol was therefore developed to produce such a decellularized boECM hydrogel and the resulting matrix was used to *in vitro* culture encapsulated isolated murine follicles for 7 days.[22] The findings showed that while boECM hydrogel alone did not promote follicular growth, it did allow follicular survival. However, when 75% of boECM was combined with 25% of 1% SLM alginate, follicle survival and growth were both observed after 7 days of *in vitro* culture.[22]

A very interesting concept for an alginate-based hydrogel for *in vitro* culture of isolated follicles was proposed by He's team,[2,23] whereby a matrix was prepared with a hard shell (made of alginate) and a soft core (made of collagen), mimicking the ovarian cortex and medulla respectively (Fig. 5). Encapsulation of isolated secondary follicles from deer mice in the hydrogel promoted follicle growth up to the antral stage, as well as ovulation,[23] demonstrating that these microtissues can recapitulate the 3D mechanical, physiological, and anatomical ovarian environment.[23]

USING A 3D ALGINATE CULTURE SYSTEM AS A TOOL FOR *IN VITRO* ANALYSIS

The ultimate goal of an alginate system is to promote follicle development in order to obtain healthy oocytes that can be further matured and fertilized to produce embryos for fertility restoration in cancer patients, improve conservation of wild animal species or autochthonous animal breeds, or multiply the potential of genetically superior domestic animals.

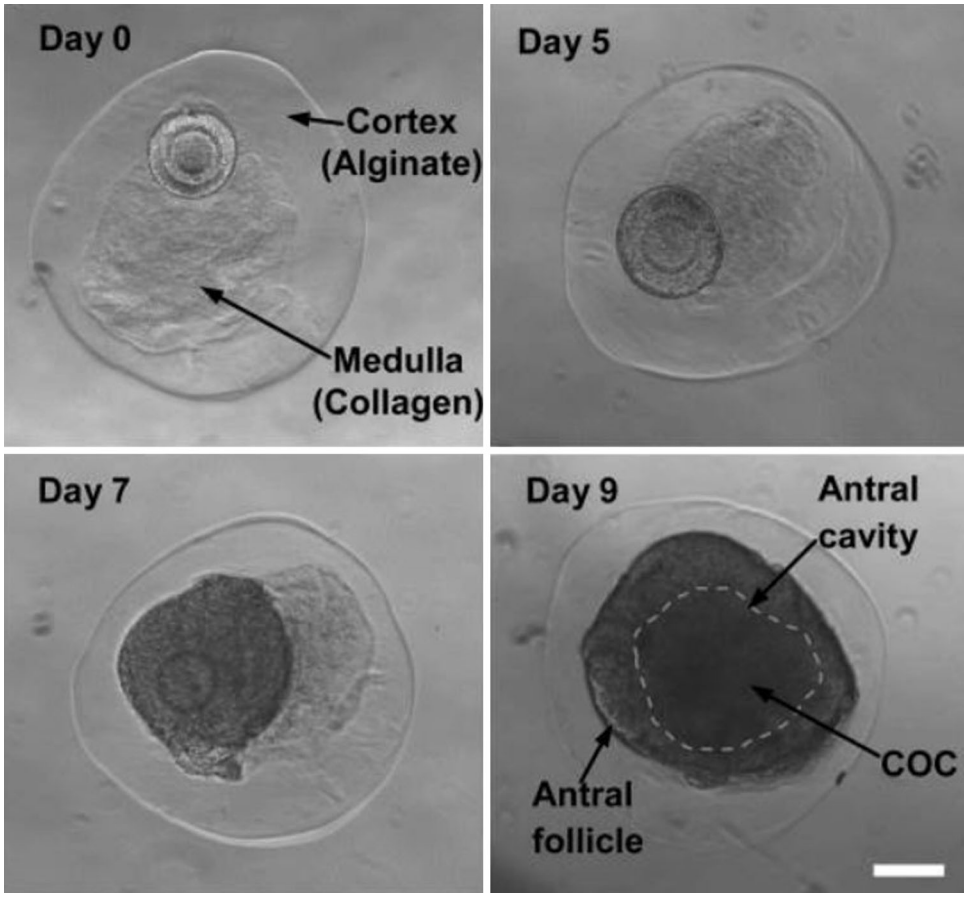

FIGURE 5. Matrix with a hard shell (made of alginate) and a soft core (made of collagen), mimicking the ovarian cortex and medulla, respectively (day 0). Encapsulation of isolated secondary follicles from deer mice in the hydrogels, promoting follicular growth up to the antral stage (day 9). Reprinted with permission from Choi *et al.*,[23] ©Elsevier (2016).

Although positive results have been obtained using this matrix to *in vitro* culture isolated preantral follicles from various animal species, numerous studies have indicated that such outcomes can be significantly improved by optimizing different culture parameters, such as media, co-culture conditions, oxygen tension, and so on. Apart from its main application, this 3D system can also be used as an important tool to investigate follicle biology and evaluate the effect of isolation and cryopreservation protocols on the survival and development of preantral follicles.

Analysis of In Vitro Culture Conditions

Medium Supplementation

FSH has a vital role to play in folliculogenesis, as it stimulates development of antral follicles and ovulation. Because of this, it has been extensively applied in assisted reproduction techniques.[114] Its addition to culture medium has been tested in alginate-encapsulated follicles from different animal species, always yielding positive results. Studies in rodents showed small two-layered murine secondary follicles to be responsive to FSH, demonstrating a positive correlation between FSH dose, follicle growth and steroid secretion,[61] while larger multilayered secondary follicles are FSH-dependent, with follicle survival, growth, steroid secretion, metabolism and oocyte maturation all regulated by FSH. Interestingly, when FSH doses were superior to 25 mIU/mL, follicle survival decreased in the latter follicle class.[61] An enriched culture medium supplemented not only with FSH, but also fetuin, insulin, transferrin and selenium, was found to improve growth of murine primary and early secondary follicles, allowing antrum formation.[99] FSH was also shown to have a positive impact on the growth of rat secondary follicles[48] and, together with insulin, promoted viability and development, as well as estradiol production, in bovine follicles.[77]

Songsasen *et al.*[97] and Nagashima *et al.*[69] reported that FSH supplementation improved development of canine follicles too. Similar results were obtained with cat follicles, which grew faster in the presence of FSH.[95]

In non-human primates, FSH was found to positively influence survival and development of rhesus secondary follicles.[127] Conversely, FSH appeared to have a negative impact on baboon follicles encapsulated in a fibrin–alginate–Matrigel matrix, which exhibited disruption of oocyte integrity and cumulus cell connections. Follicles grown without FSH were able to produce MII-stage oocytes with a normal spindle structure.[131]

Luteinizing hormone (LH) is also an essential constituent during folliculogenesis, as it provides androgen substrate for estrogen synthesis, which in turn contributes to oocyte maturation.[37] It was therefore also tested for *in vitro* culture of alginate-encapsulated follicles from dogs and monkeys. Whether or not associated with FSH, LH was shown to promote development of canine follicles.[69] On the other hand, FSH alone appeared to be more effective for development of secondary follicles in rhesus monkeys compared to combined FSH–LH supplementation.[134]

The influence of vitamins on *in vitro* culture of alginate-encapsulated follicles was also corroborated by two different studies. In order to improve survival and development of mouse primary follicles, Tagler *et al.*[100] supplemented the medium with ascorbic acid, which has been associated with ECM remodeling. The authors reported that follicle structural integrity and survival were significantly enhanced by addition of ascorbic acid and follicles were able to grow up to the antral stage.[100] Xu *et al.*[128] recently investigated the effect of low and high concentrations of vitamin D3 on rhesus secondary follicles encapsulated in alginate beads. They reported that while both doses resulted in antral follicles with a significantly greater oocyte diameter than controls, low vitamin D concentrations improved follicle survival and AMH production, and high doses yielded larger numbers of fast-growing follicles.[128]

Insulin and androgens have also been found to play a positive role in the survival and growth of rhesus secondary follicles, enhancing steroid production and oocyte quality.[76,126] Similarly, basic fibroblast growth factor was reported to improve survival and development of human preantral follicles.[119]

Co-culture Systems

Since ovarian cells support folliculogenesis through secretion of key factors required for follicle development, Tingen *et al.*[108] decided to evaluate *in vitro* co-culture of mouse primary and small secondary follicles encapsulated in alginate beads and investigate these cells further. They found that the ovarian stromal cell feeder layer did indeed have a positive impact on follicles, significantly increasing their survival and growth, but isolation of these cells resulted in a heterogeneous population that eventually changed over the course of culture. Tagler *et al.*[99] therefore replaced ovarian cells with mouse embryonic fibroblasts (MEFs), hypothesizing that these cells could provide essential paracrine factors for follicle survival and development. After two weeks, follicles co-cultured with MEF showed higher survival rates than controls. Moreover, they maintained an intact structure, while follicles without MEFs showed extruded oocytes.[101]

Culture Environment

Apart from maintenance of their 3D structure and medium supplementation with hormones, growth factors and antioxidants, other important requirements for *in vitro* survival and growth of isolated follicles concern the culture environment, including aspects like oxygen tension. Although optimal oxygen concentrations have not yet been established for ovarian follicles, studies have indicated that oxygen has a significant impact on folliculogenesis and that oxygen consumption by oocytes varies during follicular development.[41,44]

Makanji *et al.*[67] reported that alginate-encapsulated mouse secondary follicles showed a significant increase in survival and growth rates when *in vitro* cultured in lower oxygen concentrations (2.5%). They believe that such findings were due to hypoxia-mediated pyruvate production for oxidative energy metabolism, and suggest that oxygen tension levels should be lower (2–5%) during the preantral stage. In the antral phase, oxygen concentrations should increase to 15–20%, then fall again during the gonadotropin peak and ovulation.[67] Xu *et al.*[127] also found lower oxygen concentrations (5%) to deliver higher growth rates in rhesus monkey secondary follicles than did higher concentrations (20%). Using 20% oxygen, Yin *et al.*[136] *in vitro* cultured alginate-encapsulated preantral follicles isolated from human ovarian medulla. While secondary follicles grew, primordial and primary follicles exhibited poor survival and did not develop to the antral stage. These results corroborate the theory of Makanji *et al.*,[67] who speculated that higher hypoxia-mediated glycolysis may be necessary for preantral follicles.

Analysis of Isolation or Cryopreservation Procedures

To assess follicle survival and morphology, histological, immunohistochemical and ultrastructural analysis soon after isolation or thawing/warming is an important tool to calculate success rates of isolation and cryopreservation protocols. However, it is not enough to simply ascertain if a follicle will survive and

resume its normal development. For in-depth ultrastructure evaluation, it is necessary to incubate follicles, allowing time for them to reveal changes in their compartments and organelles that may have been caused by isolation or cryopreservation procedures. *In vitro* culture therefore represents a valuable means of investigating these techniques, and 3D alginate systems have been largely applied to this end.

Aiming to develop an isolation procedure for human preantral follicles that could be used in a clinical setting, Vanacker *et al.*[110] showed that follicles were able to successfully survive and grow after isolation with Liberase DH blendzyme and 7 days of *in vitro* culture. Yin *et al.*[136] recently optimized an isolation protocol for preantral follicles enclosed in ovarian medullary tissue. To assess their survival and development, these authors used the alginate system for *in vitro* culture and found that while secondary follicles could survive and grow, most primordial and primary follicles died. Dong *et al.*[31] compared two different protocols (mechanical and mechano-enzymatic) to isolate human preantral follicles. They reported that growth rates and estradiol levels were higher in mechanically isolated follicles than in follicles subjected to a combination of mechanical and enzymatic isolation.

Development of an optimal cryopreservation procedure has been the goal of numerous studies, as it is a crucial aspect of fertility restoration and preservation of genetic material. In mice, Asgari *et al.*[12] used an alginate culture system to compare slow freezing and vitrification protocols. Their results suggest that vitrification is superior for cryopreservation of mouse ovaries, as follicles exhibited better antrum formation than follicles from frozen-thawed ovarian tissue. On the other hand, Sadeghnia *et al.*[84] reported that viability and growth of follicles isolated from vitrified-warmed sheep ovarian tissue were significantly inferior to fresh follicles.

Zelinski's group conducted a number of studies to assess the impact of cryopreservation on the survival and development of preantral follicles from rhesus monkeys. In their first paper, they compared slow-freezing and vitrification, demonstrating that after isolation and *in vitro* culture in alginate beads, follicles from vitrified-warmed tissue had greater capacity to form an antrum than follicles from frozen-thawed tissue.[106] Looking to optimize their vitrification protocol, they conducted further investigations, showing that shorter periods of incubation of ovarian tissue in a vitrification solution containing synthetic polymers resulted in follicle survival rates similar to controls.[107] Finally, the team assessed the ability of ovarian tissue to be successfully vitrified in a closed system.[105] They found that although their protocol preserved tissue and follicle morphology, it had a negative effect on follicle development *in vitro* and antrum formation rates.[105]

In vitro culture in a 3D alginate system by our team showed that isolation of human preantral follicles before or after cryopreservation had a similar impact on follicle survival and growth.[112] Interestingly, some authors were able to successfully cryopreserve isolated follicles embedded in alginate beads.[15,21,112,118] According to Ji *et al.*,[57] hydrogel encapsulation offers the best protection for isolated cells with low cryotolerance. Although primordial follicles have been showed to be cryoresistant,[89] alginate encapsulation was still beneficial, as it maintained their 3D structure, thus preserving connections between GCs and the oocyte during freezing and thawing procedures.[21] Moreover, it may decrease exposure to cryoprotectant and prevent damage due to ice formation,[57,87] protecting follicles during cryopreservation. Indeed, once polymerized, the alginate barrier organizes the extracellular fluid, limiting deleterious ice crystal formation during cooling and warming.[14] These studies show that alginate encapsulation is not only a precious tool for *in vitro* culture of isolated follicles, but also for their cryopreservation.

ALGINATE FOR *IN VITRO* CULTURE OF FOLLICLES FROM DIFFERENT ANIMAL SPECIES

As previously mentioned, alginate matrices have been successfully used to *in vitro* culture isolated follicles from a number of animal species. Figure 6 summarizes studies from the literature on alginate-encapsulated follicles from rodents, ruminants, carnivores, and primates.

Isolated follicles from rodent species have been shown to develop in 2D systems, but they have also been found to survive and grow in alginate scaffolds. Pangas *et al.*[71] were the first to apply this system to *in vitro* culture of isolated follicles from 12-day-old mice. Follicles were embedded in alginate beads, and morphological and ultrastructural studies suggested that alginate does not interfere with oocyte or GC development, as there was no evidence of this over 10 days of *in vitro* culture.[71] Mouse secondary follicles were also able to develop up to the antral stage,[67,72] producing oocytes capable of reaching the MII stage after *in vitro* maturation,[12,94,101,123] or even ovulating.[94]

The reason why high-quality cultured oocytes can be obtained from mice is probably thanks to maintenance of vital corridors of communication between the two cellular compartments of the alginate matrix. In-

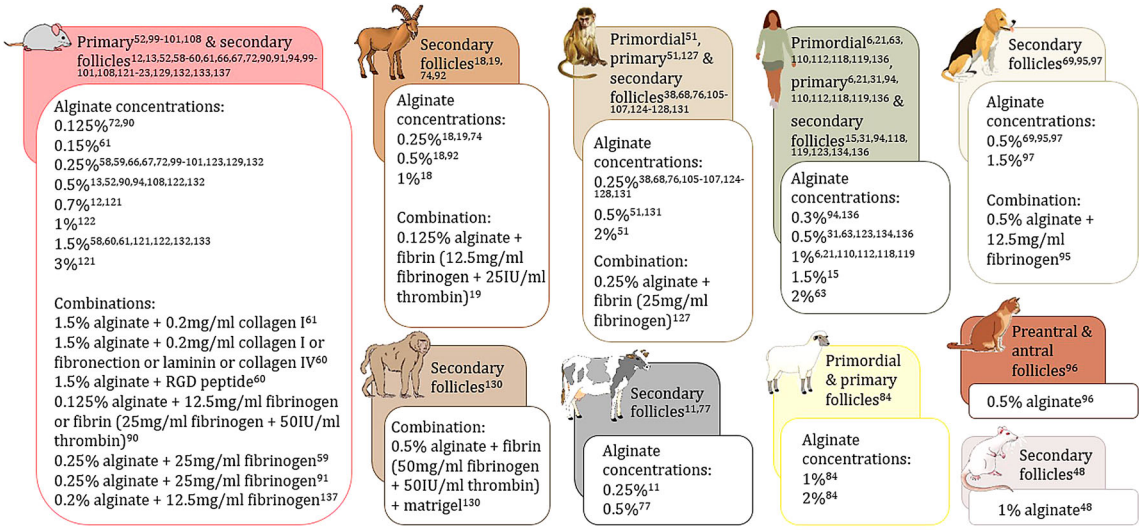

FIGURE 6. Studies on *in vitro* culture of isolated follicles from different animal species encapsulated in alginate beads or alginate-based matrices.

deed, using a similar model, Heise *et al.*[48] demonstrated the presence of connections between GCs and oocytes in isolated rat preantral follicles cultured in alginate, which may have been due to preservation of their unique morphology supported by the alginate matrix.[85] Moreover, Mainigi *et al.*[66] reported that murine oocytes developing *in vitro* in an alginate-based culture system undergo normal transition to transcriptional quiescence, and the transcriptome of these oocytes is very similar (99.5%) to that of oocytes that develop *in vivo*. However, they also revealed that the developmental competence of these oocytes is compromised, as evidenced by the very low rate of fertilization following *in vitro* maturation and insemination, due to an increased incidence of abnormal spindle formation and chromosome alignment, as well as abnormal cortical granule biogenesis and failure to emit the first polar body. Nevertheless, Xu *et al.*[129] obtained viable offspring after encapsulation and *in vitro* culture of isolated mouse follicles.

Caprine follicles exhibited a greater follicle diameter and better oocyte integrity when encapsulated in alginate beads compared to 2D *in vitro* culture.[74] Silva *et al.*[92] showed that 0.5% alginate allowed survival and development of isolated caprine preantral follicles for up to 12 days, producing MII oocytes. Follicles from other ruminant species have been also isolated and encapsulated in alginate beads for further *in vitro* culture. Araújo *et al.*[11] demonstrated that isolated cow secondary follicles were able to grow up to the antral stage in 0.25% alginate, and their estradiol production increased when growth hormone was added to the culture medium.

Looking to improve conservation of wild canids, Songsasen's group showed that alginate was capable of supporting *in vitro* growth of isolated preantral and antral follicles from dogs and cats. This team used 0.5% alginate and FSH to *in vitro* culture isolated cat preantral and antral follicles. Their results revealed that follicles maintained their 3D structure in alginate beads and their growth was significantly superior when the culture medium was supplemented with FSH.[95]

Non-human primate ovarian follicles have also been successfully cultured in calcium alginate beads. Apart from one study on baboon secondary follicles,[127] all have essentially focused on rhesus monkey follicles at different stages of development.[51,68,106,127,130] Other have used alginate-encapsulated follicles from non-human primates to test different cryopreservation protocols for ovarian tissue,[105–107] *in vitro* culture medium supplements,[76,125,127,131] or the effects of diet,[132] proving that these animals can successfully serve as a model for humans. Oocytes retrieved from *in vitro*-grown primate follicles were also able to reinitiate meiosis for fertilization, and hence early embryonic cleavage.[127]

In vitro development of isolated human preantral follicles is an alternative to use cryopreserved ovarian tissue instead of transplantation. Human preantral follicles have indeed been effectively cultured in calcium alginate hydrogels by numerous teams (Fig. 6).[7,21,63,112,118,119,124,136] Although Laronda *et al.*[63] and Yin *et al.*[136] reported that their isolated human primordial follicles died after a few days of *in vitro* culture, Amorim's group showed that these follicles are not only able to survive *in vitro*, but actually grow (Fig. 7).[7,21,112] Such a discrepancy in the

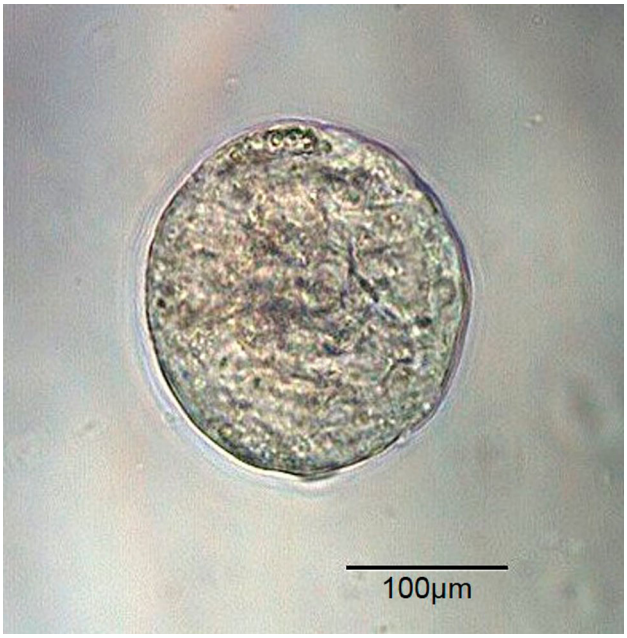

FIGURE 7. Isolated human preantral follicle encapsulated in 1% alginate after 7 days of *in vitro* culture.

results may have been due to the alginate concentration used to encapsulate the follicles, or the protocol applied for their isolation.

ALGINATE TO TRANSPLANT FOLLICLES

In recent years, the possibility of transplanting isolated preantral follicles inside an artificial ovary has been investigated[6] with the aim of restoring endocrine activity and fertility in cancer patients. A key challenge for construction of such an artificial ovary is identifying an optimal matrix to support these grafted follicles. To this end, the growing field of tissue engineering, which exploits and combines cells, materials, and biological signals, could offer indispensable help. All engineered tissue has different requirements related to metabolism, growth factors, the ECM, and mechanical characteristics, and novel techniques have been developed to meet these needs.[120] Nevertheless, creating the right microenvironment for an artificial ovary to support isolated ovarian follicle development *in vivo* is a task that presents unique challenges, while offering countless opportunities.[120]

To graft isolated follicles and ovarian cells, an artificial ovary must provide structural support for the cells and follicles until complete restructuration of the stromal ovarian tissue matrix. The main goal of the artificial ovary is to offer an environment that allows follicle survival and development, mimicking conditions encountered in a natural ovary. Furthermore, as human ovarian follicles are exceptional in that they can grow to around 600 times their size during folliculogenesis, the artificial ovary, unlike other artificial organs, should also be biodegradable. Because of these requirements and the favorable results obtained with *in vitro* culture of isolated follicles encapsulated in alginate, this polymer looks to be a promising candidate for the artificial ovary matrix.

Vanacker *et al.*[113] conducted a first study conceived as a starting point for construction of a transplantable artificial ovary. They set out to develop a biodegradable artificial scaffold that offers an environment in which follicles and OCs are able to survive and grow. First, they autografted isolated mouse OCs encapsulated in a biodegradable matrix made of alginate and matrigel to a pocket created in the internal part of the mouse peritoneum. After one week of grafting, they demonstrated that an alginate-based matrix was able to degrade, allowed vascularization, and supported cell survival and proliferation. Moreover, it elicited a low inflammatory response.

Encouraged by these results, the authors performed a second study, this time to graft not only OCs, but also isolated preantral follicles. Based on tests showing that 1% SLM alginate completely degrades after 4 weeks of grafting (Vanacker *et al.*, unpublished results), this material was selected to encapsulate the follicles and cells. In this new study, Vanacker *et al.*[111] autografted isolated mouse preantral follicles and OCs enclosed in a 1% SLM alginate matrix to a peritoneal pocket. After one week, they reported a follicle recovery rate of 22%, with isolated follicles able to reach the antral stage. Moreover, grafted OCs survived and proliferated, and vessels were formed. As observed in the previous study,[113] the inflammatory response was low. These findings indicate that an alginate-based artificial ovary may well be capable of restoring endocrine function and fertility in patients suffering from cancer that carries a high risk of ovarian involvement.

CONCLUSIONS AND FUTURE PERSPECTIVES

Since the first study on encapsulation of isolated ovarian follicles in alginate,[71] a considerable amount of research has been conducted to test and optimize not only the matrix itself, but also different variables (culture media, stage of follicle development, cryopreservation protocols, *etc.*). In this review, we have summarized all studies performed in different animal species, underscoring our knowledge and understanding of use of such an artificial scaffold for ovarian follicles. These studies provide important insights into how alginate-based matrices help follicles to survive and grow *in vitro* and *in vivo*, and can thus make a

great impact in the fields of reproductive medicine, biotechnology, and animal conservation.

However, although alginate appears to be a promising biomaterial for follicle encapsulation, it may be not sufficient by itself. Indeed, the biological inertness of alginate has largely hampered its use in applications where cell adhesion is essential for survival and proliferation,[53] which is why it should be combined with other molecules. For instance, to induce cell interaction, bioactive peptide sequences known to bind to receptors on cell surfaces can be covalently linked to alginate[39,78,79] without affecting its biocompatibility.[60–62,90,91,125,137] These bioactive peptides mimic both the adhesive properties of ECM proteins containing these sequences and stimulation of cellular responses like differentiation and proliferation. A variety of such peptides have been identified and used in cell attachment studies, particularly the RGD tripeptide sequence because of its abundance in adhesion proteins (e.g. fibronectin, laminin, fibrinogen, vitronectin) and its ability to bind to a wide variety of integrins.[83]

Alginate modifications with bioactive peptides are traditionally achieved by carbodiimide chemistry and have been shown to yield around 0.1–1.0% mol of peptide per mol of uronate monomer.[40,79,86] Although peptide densities in this concentration range are known to induce attachment of myoblasts,[79,86] olfactory ensheathing cells,[86] mesenchymal stem cells[39] and endothelial cells,[17] greater peptide densities influence the impact on cell attachment and differentiation.[29,79] Increasing the degree of peptide substitution is relevant for use of peptide–alginate combined with non-substituted alginates to form bioactive hydrogels.[9] Carbodiimide chemistry also contributes to the destruction of gelling properties without the addition of bioactive compounds.[86]

An alternative method has recently been described for high-efficiency coupling of bioactive peptides to alginates by means of periodate oxidation followed by reductive amination.[26] Alginates with covalently attached fibronectin-derived Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide were used to demonstrate the activity of the material and impact of high peptide densities on attachment of mouse skeletal myoblasts and human dental stem cells to peptide–alginate hydrogels.[26] The authors showed that enhanced functionalization of alginate was crucial, as dental stem cells only adhered to alginate gels containing the highest concentrations of peptide, in contrast to myoblasts.[26]

Taken together, the most recent advances in alginate modifications have yielded promising results in different cell types. These studies should facilitate the development of next-generation alginate scaffolds specifically tailored for follicle encapsulation. Determining and engineering the structural and biochemical aspects of the alginate matrix for each stage of follicle development will therefore represent some of the future challenges and opportunities in this field.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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