

Video Article

A Method for Ovarian Follicle Encapsulation and Culture in a Proteolytically Degradable 3 Dimensional System

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Abstract

The ovarian follicle is the functional unit of the ovary that secretes sex hormones and supports oocyte maturation. *in vitro* follicle techniques provide a tool to model follicle development in order to investigate basic biology, and are further being developed as a technique to preserve fertility in the clinic¹⁻⁴. Our *in vitro* culture system employs hydrogels in order to mimic the native ovarian environment by maintaining the 3D follicular architecture, cell-cell interactions and paracrine signaling that direct follicle development⁵. Previously, follicles were successfully cultured in alginate, an inert algae-derived polysaccharide that undergoes gelation with calcium ions⁶⁻⁸. Alginate hydrogels formed at a concentration of 0.25% w/v were the most permissive for follicle culture, and retained the highest developmental competence⁹. Alginate hydrogels are not degradable, thus an increase in the follicle diameter results in a compressive force on the follicle that can impact follicle growth¹⁰. We subsequently developed a culture system based on a fibrin-alginate interpenetrating network (FA-IPN), in which a mixture of fibrin and alginate are gelled simultaneously. This combination provides a dynamic mechanical environment because both components contribute to matrix rigidity initially; however, proteases secreted by the growing follicle degrade fibrin in the matrix leaving only alginate to provide support. With the IPN, the alginate content can be reduced below 0.25%, which is not possible with alginate alone⁵. Thus, as the follicle expands, it will experience a reduced compressive force due to the reduced solids content. Herein, we describe an encapsulation method and an *in vitro* culture system for ovarian follicles within a FA-IPN. The dynamic mechanical environment mimics the natural ovarian environment in which small follicles reside in a rigid cortex and move to a more permissive medulla as they increase in size¹¹. The degradable component may be particularly critical for clinical translation in order to support the greater than 10⁶-fold increase in volume that human follicles normally undergo *in vivo*.

Protocol

1. Follicle Isolation

Experiments on animals were performed in accordance with the guidelines and regulations set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the established Institutional Animal Use and Care protocol at Northwestern University.

For optimal results, all dissections are carried out in L15 media for pH control at ambient levels of CO₂, on 37°C heated stages for temperature control, and on a clean bench to minimize bacterial contamination. The dissection media (DM) is prepared with L15 media supplemented with 50 IU/mL penicillin and 50 µl/mL streptomycin and 1% FBS. The maintenance media (MM) is prepared with αMEM media supplemented with 50 IU/mL penicillin and 50 µl/mL streptomycin and 1% FBS.

1. Transfer freshly dissected ovaries from 16 day old mouse into a new 35 mm Petri dish with 1-2 ml MM containing 0.1% Collagenase and 0.1% DNase. Incubate for 15 minutes inside an incubator at 37°C and 5% CO₂.
2. After incubation, perform several washing steps in DM to remove collagenase, and then transfer to a 35 mm Petri dish with fresh DM. Isolate follicles from the ovary by gently flicking (or cutting) follicles away from the whole ovary by using two 28 5/8 gauge needles attached to disposable syringes. Remove as much stroma as possible without damaging the integrity of follicle. Dissect out 20-40 secondary follicles per ovary (130-150mm). These follicles usually have 2-3 layers of somatic cells.
3. Add 1 ml MM to the central well of a 60 mm IVF (*in vitro* fertilization) Petri dish, and 3 ml MM to the outer ring. Transfer intact follicles to the outer ring of the IVF dish to briefly rinse, and then selectively transfer them into the central well (See 1.4). Store this IVF dish in the incubator.
4. After all the follicles are collected, the selection step is performed under a dissecting microscope with a 5-8x magnification. Healthy follicles have the following morphological characteristics:
 - 2-3 somatic cell layers
 - 130-150 µm
 - No separation between oocyte and somatic cells
 - Intact and round oocytesTransfer the healthy follicles into the center of IVF dishes for encapsulation.

2. Follicle Encapsulation, Method 1 - "The Drop Method"

1. Add 1 ml of 50 IU/ml thrombin in TBS with 40 mM CaCl₂ into the middle well of the IVF dish. Thaw and keep fibrinogen stock (50 mg/ml in TBS) on ice. Bring the fibrinogen to room temperature right before use.

2. Prepare the fibrinogen/alginate solution by mixing 1x PBS, 0.5% alginate in PBS solution and 50 mg/ml fibrinogen solution at a 2:1:1 ratio in a 1.7 ml sterile microcentrifuge tube. Avoid introducing bubbles into the solution. Gently vortex and spin-down. The solution appears slightly cloudy and should be prepared immediately before use.
3. Place two droplets of fibrinogen/alginate solution in the outer ring of an IVF dish: a 90 μ l droplet for encapsulation and a 10 μ l droplet for washing. To decrease evaporation, turn off the heating stage on the dissecting microscope. Transfer 10-15 follicles into the 10 μ l droplet with a minimal amount of culture media (< 5 μ l) using a 200 μ m micropipette tip, and quickly mix. Transfer all of the follicles into the 90 μ l droplet with a minimal amount of solution (< 5 μ l) from the first drop, and mix.
4. Simultaneously aspirate one follicle and 5 μ l of fibrinogen/alginate solution using a 10 μ l pipette tip, and release into the thrombin/ Ca^{2+} solution in the IVF dish. Repeat this step until all the follicles are encapsulated.
5. Cross-link the beads for 5-7 minutes in the thrombin/ Ca^{2+} solution. The beads will become darker as fibrin gels. Transfer the beads into a Petri dish containing MM, starting with the darker beads first. Incubate the dish for 15-30 minutes in the incubator to rinse off the remaining thrombin.
6. Transfer the beads into a 96 well culture plate containing 100 μ l of follicle growth media, and image immediately. The growth media (GM) is prepared with α MEM media supplemented with 10 mIU/ml of recombinant FSH, 3 mg/ml of BSA, 1 mg/ml of bovine fetuin, 5 μ g/ml of insulin, 5 μ g/ml of transferrin, and 5 ng/ml of selenium.

3. Follicle Encapsulation, Method 2 - "The Parafilm Method"

1. Prepare the fibrinogen/alginate solution by mixing 0.5% alginate solution and 50 mg/ml fibrinogen solution at a 1:1 ratio in a 1.7 ml sterile microcentrifuge tube.
2. Pipette 7.5 μ l drops of the fibrinogen/alginate mixture onto a parafilm coated glass slide with 3 mm spacers. Transfer 1 follicle into each drop with a minimal amount of media.
3. Add 7.5 μ l of thrombin/ Ca^{2+} solution to each drop. Mixing is unnecessary because the gel forms almost instantaneously.
4. Cover the gels with the second parafilm coated glass slide, put slides in a 100 mm Petri dish that is upside down, and transfer to the incubator for 5 minutes.
5. Transfer the beads into a Petri dish containing MM, and then into the culture well as previously described. If the beads stick together, which is due to cross-linking between the beads, they can be gently separated with forceps.

4. Follicle Imaging and Media Change

1. Every 2 days, the cultured follicles are imaged using a light microscope and the follicle diameter is measured with the software program ImageJ.
2. Every 2 days, half of the growth media (50 μ l) is replaced by fresh, pre-equilibrated growth media.

5. Follicle Recovery from the 3D Matrix and *in vitro* Oocyte Maturation (IVM)

1. After 8 days of culture, the hydrogels appear clear due to complete degradation of the fibrin component, and the follicles expand to a diameter of 300-400 μ m. The remaining alginate is degraded by alginate-lyase, a plant-derived enzyme that specifically degrades alginate and does not affect animal cells.
2. Remove growth media from wells containing the beads and add 100 ml of 10 IU/ml alginate lyase in α MEM. Leave the plate in the incubator for 25-30 minutes.
3. Remove follicles from the dissolved beads with a blunt end tip. Transfer first to the outer ring of an IVF dish containing DM, and then into the center well, also containing DM to wash.
4. After washing, transfer the follicles to the outer, and then inner, ring of an IVF dish containing maturation media. *in vitro* maturation media (IVM) is composed of α MEM, 10% FCS, 1.5 IU/ml of hCG, and 5 ng/ml of epidermal growth factor (EGF)
5. Transfer to CO_2 incubator for 15-16 hours.

Examine the follicles for cumulus cells expansion. To remove the cumulus cells from the oocyte, add hyaluronidase solution to final concentration 0.1 mg/ml and incubate in the incubator for 2-3 minutes. Use a 75 μ m micropipette tip to shear the cumulus cells from the oocyte by pipetting up and down several times. Determine maturation stage of the oocyte under a light or a dissecting microscope. The possible stages, from least to most mature, are:

- Degenerated. The oocyte is fragmented into two or more pieces.
- Germinal vesicle (GV) stage. The oocyte did not resume meiosis in response to hCG exposure, which is evident by the continued presence of the nucleus of the oocyte (GV).
- Germinal vesicle breakdown (GVBD). The nuclear membrane is absent from the oocyte, but there is no polar body present. Therefore, the oocyte is still in meiosis-I.
- Metaphase-II arrested oocyte (MII). The oocyte resumed meiosis, and is now arrested at metaphase-II. A polar body should be visible on a light or a dissecting microscope. The oocyte will remain at MII unless fertilized in later experiments.

6. Representative Results:

We described a novel encapsulation method of ovarian follicles in a FA-IPN for *in vitro* culture (Figure 1). Ovarian follicles consist of an oocyte surrounded by several layers of somatic cells. Communication between the multiple cellular compartments is essential for healthy follicle development and oocyte maturation. Follicle encapsulation in a 3D hydrogel supports follicle expansion while maintaining the architecture of the follicle^{9,11,12}. As follicles develop, their volume expands exponentially and the encapsulating biomaterial should allow this expansion without the development of an inhibiting compressive force. FA-IPN is a network built of two natural materials, where fibrin is proteolytically degradable by plasmin activated by the follicle and alginate is biologically inert¹³(Figure 2). During follicle growth, fibrin degradation begins locally near the follicle, and continues until the fibrin is cleared from the hydrogel. The non-degradable alginate component, which remains intact throughout the culture period, supports the 3D structure of the hydrogel.

Follicles were isolated at the secondary stage of development (150-180 μ m diameter) and expanded to 400 μ m at the antral stage of development in the FA-IPN gels. With hCG stimulation, the cultured follicles can undergo cumulus cell expansion, and oocytes can resume meiosis and progress to metaphase II for fertilization. These results suggest that the encapsulation method and the encapsulating material allowed follicle culture and successful maturation *in vitro* (Figure 3).

Fibrin degradation around the encapsulated follicles starts on the first day of culture and is completed by day 6. Aprotinin, a soluble plasmin inhibitor, can be used to alter fibrin degradation and to extend mechanical gradient in the encapsulating material (Figure 4). If follicles are cultured with 0.01 TIU/mL aprotinin, the fibrin is degraded slower for the first 4 days. Nonetheless, the follicles can still develop to the antral stage and oocytes remain competent to resume meiosis to metaphase II after aprotinin is removed. A high concentration of aprotinin (0.1 TIU/mL) significantly inhibits fibrin degradation, matrix stiffness prevents follicle expansion and somatic cell invasion into the matrix is observed.

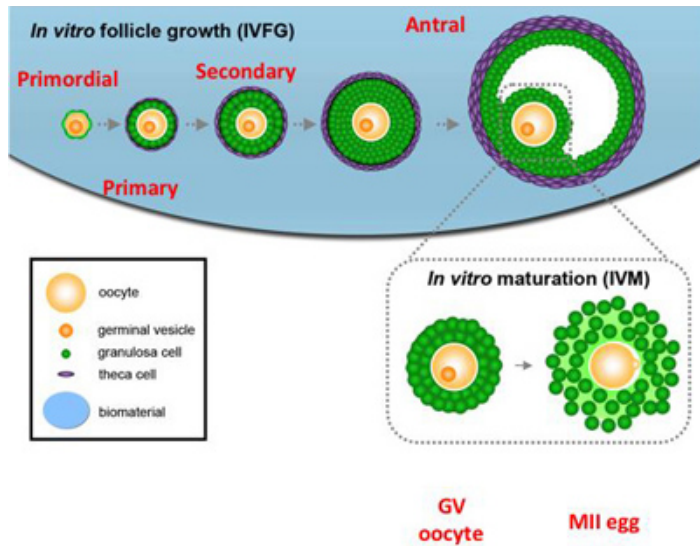


Figure 1. Flowchart of follicle development. The ovarian follicle consists of a centrally located oocyte surrounded by one or more layers of somatic cells, which support oocyte development. As follicles develop from secondary to the preovulatory antral stage, the somatic cells surrounding the oocyte proliferate and differentiate, and the oocyte increases in size. *in vitro* maturation (IVM) is the final step for the follicle culture, when the somatic cells adjacent to the oocyte, termed cumulus cells, expand after hCG stimulation, and the oocyte resumes meiosis and progresses to a metaphase II (MII) stage.

Alginate gelation

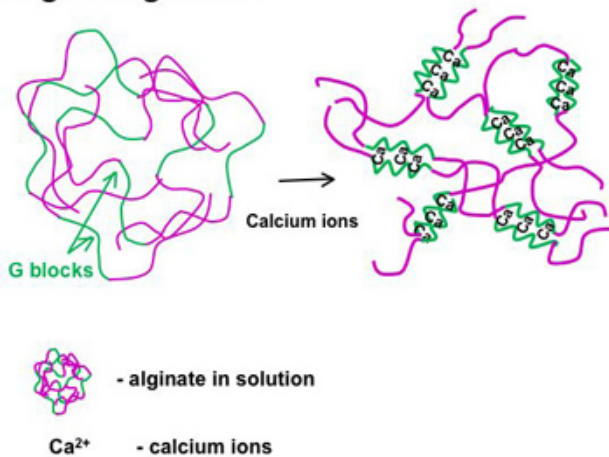


Figure 2a. Alginate and fibrin-alginate for 3D follicle culture *in vitro*. (a) Alginate is a natural biomaterial that is suitable for *in vitro* follicle culture due to its gentle gelation and biochemical characteristics, such as mesh size, controllable rigidity and biological inertness. Alginate is a linear polysaccharide copolymer of α -L-guluronic acid (G) and β -D-mannuronic acid (M). Areas with repeating G monomers, termed "G-blocks", are cross-linked to form a hydrogel in the presence of divalent calcium ions.

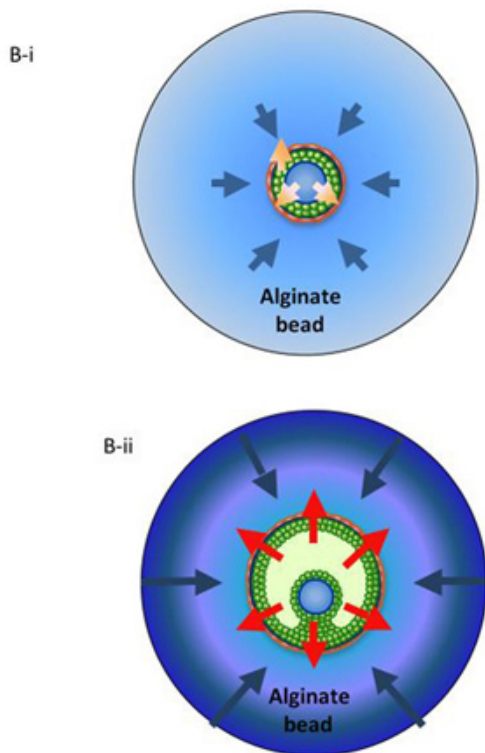


Figure 2b. (b-i) Small encapsulated follicles experience low compressive force in alginate at the beginning of the culture. However, as the follicle expands the displaced volume in the bead is increasing, which results in greater compressive force in the opposite direction of follicle expansion (b-ii).

Fibrin Alginate Interpenetrating Network Formation

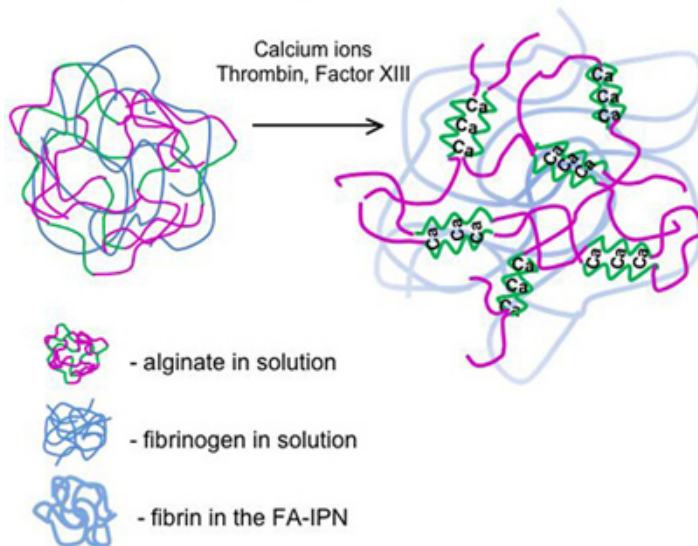


Figure 2c. The chains of individual polymers are completely entangled in the fibrin-alginate solution prior to cross-linking. Both components of FA-IPN start to cross-link immediately as they are exposed to the mixture of thrombin and calcium.

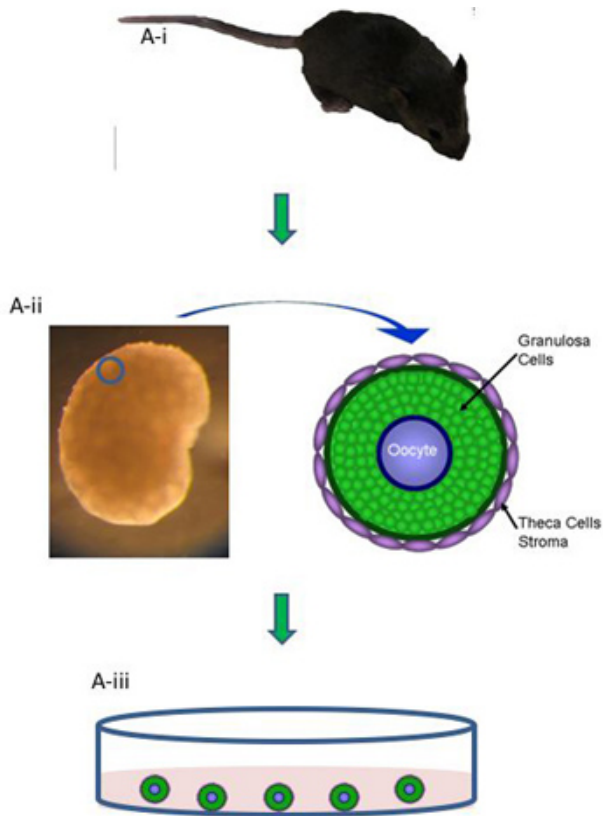


Figure 3a. Flowchart for follicle isolation and encapsulation in a FA-IPN. Secondary follicles are isolated from a 16-day old mouse (A-i). The reproductive organs are dissected (A-ii), and isolated follicles are transferred to a dish with maintenance media (A-iii).

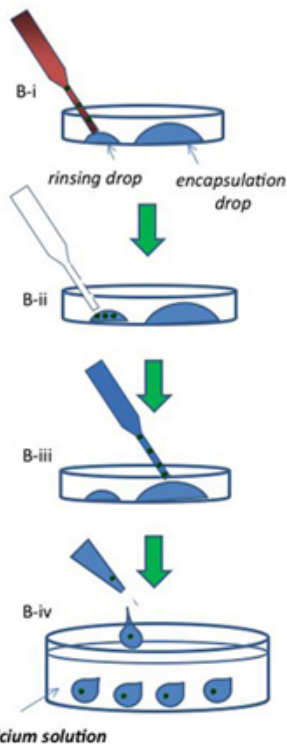


Figure 3b. The drop method for follicle encapsulation in fibrinogen alginate solution (B:i-iv). Two drops of fibrinogen-alginate solution, the rinsing drop (10 μ L) and the encapsulation drop (90 μ L) are pipetted into the dish (B-i). Next 3-5 follicles are transferred to a rinsing drop (B-ii). After rinsing and media removal, follicles are transferred to the encapsulation drop (B-iii). Each follicle is aspirated with 5 μ L fibrinogen-alginate solution with a 10 μ L pipette tip and then expelled into thrombin/calcium solution (B-iv).

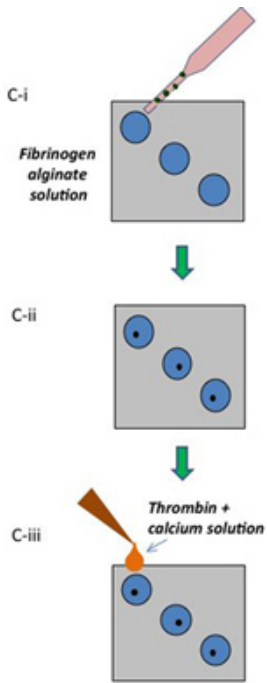


Figure 3c. The bead crosslinks for 5 minutes. The parafilm method for follicle encapsulation in fibrinogen alginate solution (C:i-iii). Fibrinogen-alginate solution (7.5 μ L) is pipetted on the parafilm coated glass slide and follicles are transferred individually to each drop after rinsing (C-i, ii). Thrombin solution (7.5 μ L) is added to each drop (C-iii).

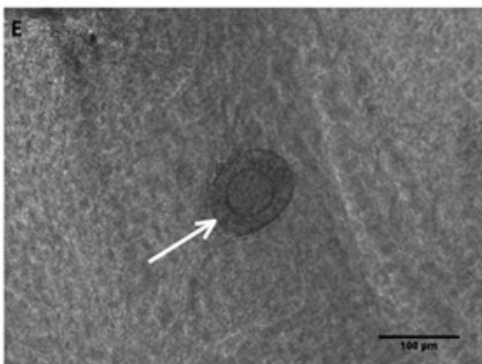
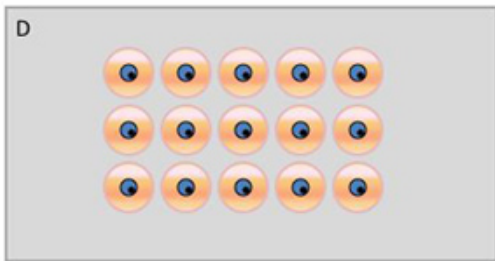


Figure 3d - e. The drops are covered with a second parafilm coated glass slide and the FA-IPN is crosslinked in the incubator for 5 minutes. (D) The encapsulated follicles are transferred to growth media in a 96-well plate. (E) An image of an encapsulated follicle in the FA-IPN (white arrow).

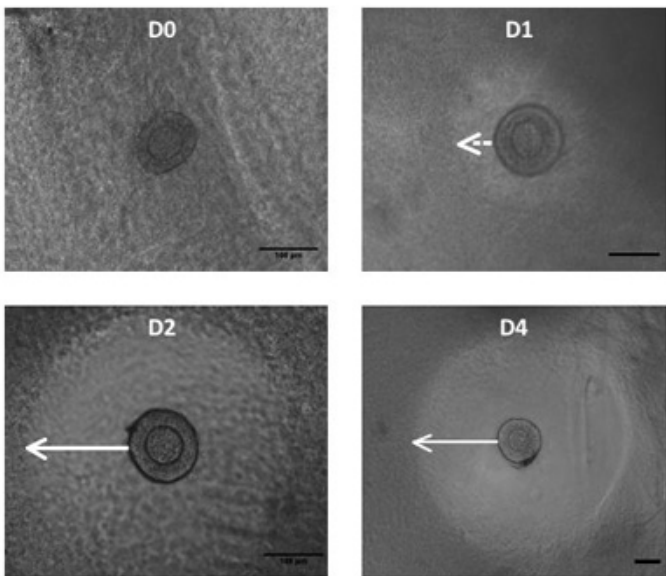


Figure 4. Fibrin degradation by the growing follicle. Follicles degrade the fibrin component of the FA-IPN during the culture period, which is demonstrated by a clear circle around the follicle. The follicle's 3D architecture is supported by the remaining alginate. On day 0 (D0) the fibrin is still intact and the matrix around the follicle is cloudy; after 1 day in culture (D1) the clear ring around the follicle appears (white arrow) and the fibrin degradation front continues to radially expand on day 2 (D2) and day 4 (D4) of culture.

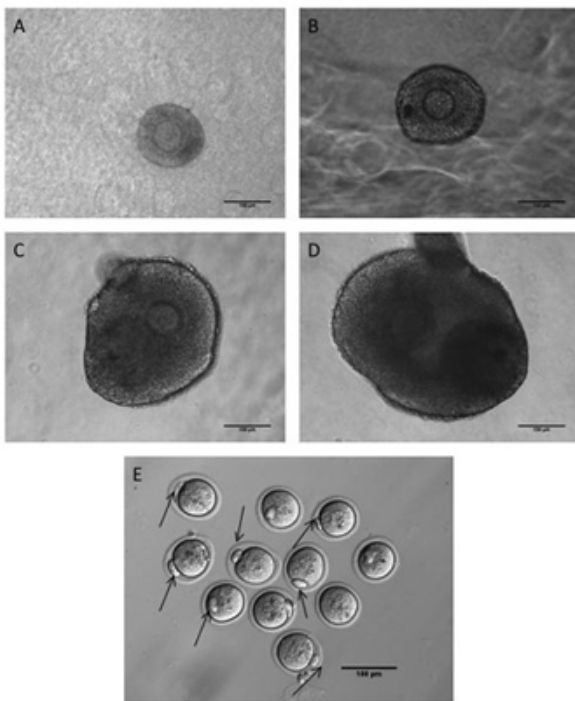


Figure 5. Images of follicle growth. Representative images of secondary follicle growth in the FA-IPN on day 0 (A), 4 (B), 6 (C) and 8 (D) of culture. After 8 days, antral follicles were matured *in vitro*, and the resulting MII stage oocytes are shown (E, the polar body is shown with black arrow).

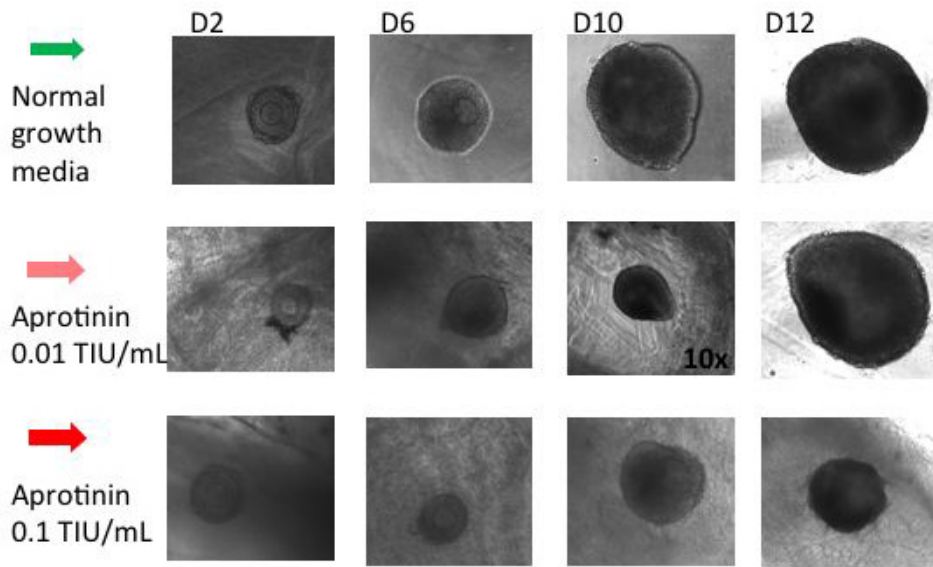


Figure 6. Fibrin degradation was inhibited by aprotinin. Growing follicles on day 2, 4, 10 and 12 are shown in the first row. Aprotinin at concentrations 0.01 TIU/mL (second row) and 0.1 TIU/mL was added to the culture media on days 0, 2, and 4. Only follicle cultures with 0.01 TIU/ml aprotinin degraded the fibrin after aprotinin removal and reached antral stage. Follicles cultured in 0.1 TIU/ml aprotinin did not grow in the FA-IPN.

Abbreviation	Full name
CO ₂ incubator	37°C incubator with 5% CO ₂
COC	Cumulus oocyte complex
3D	3 dimensional
DM	Dissection media
EGF	Epidermal growth factor
FA-IPN	Fibrin-alginate interpenetrating network
FBS	Fetal Bovine Serum
GM	Growth media
GV	Germinal vesicle
hCG	Human chorionic gonadotropin
ITS	Insulin Transferrin Selenium supplement
IVF dish	Center well 60 mm <i>in vitro</i> fertilization dish
MM	Maintenance media
MII stage oocyte	Metaphase II stage oocyte
rFSH	Recombinant Follicular Stimulating Hormone
TBS	Tris buffered saline
TIU	Trypsin inhibitory units

Table 1. Abbreviations

Disclosures

No conflicts of interest declared.

Discussion

The presented ovarian follicle encapsulation method in a FA-IPN allows follicle culture in a 3D environment *in vitro*. A FA-IPN is a dynamic, cell-responsive matrix in which the initial mechanical properties are determined by the combination of both fibrin and alginate. During the culture, the encapsulated follicle activates proteases that degrade only one component of the IPN, the fibrin, which results in a gradually decreasing gel rigidity that is contributed solely by the remaining alginate at the end of the culture. The dynamic mechanical properties obtained with a FA-IPN have been proposed to be consistent with the natural environment of the developing follicles and contributed to the improved rate of oocyte meiotic maturation comparing to alginate alone.

The FA-IPN demonstrates mild and fast gelation, with each component of the system cross-linking by an independent mechanism. We have described elsewhere⁵ that the speed of the gel formation can be controlled by fibrinogen and thrombin concentrations. The degradation rate can be adjusted by aprotinin inhibition of fibrin proteolysis. Murine secondary follicles are usually cultured for 8-12 days and follicles from other species require longer culture periods. Thus, delayed fibrin degradation by aprotinin could potentially provide an extended dynamic environment for longer cultures.

The described encapsulation methods can be applied to other systems, such as the encapsulation and culture of micro-tissues or embryoid bodies, in which cell-cell contact can be retained yet the aggregate can partially degrade the matrix and create a space for expansion. In conclusion, the FA-IPN encapsulation method presents a sterile hydrogel culture system with dynamic, cell-responsive mechanical properties and a controllable degradation rate.

Acknowledgements

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