TITLE: Preparation of biological scaffolds and primary intestinal epithelial cells to efficiently 3D model the fish intestinal mucosa

Running head: The artificial intestine: in vitro models

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Abstract

Tissue engineering is an elegant tool to create organs *in vitro*, that can help to obviate the lack of organ donors in transplantation medicine and provides the opportunity of studying complex

biological systems *in vitro*, thereby reducing the need for animal experiments. Artificial intestine models are at the core of Fish-AI, an EU FET-Open research project dedicated to the development of a 3D *in vitro* platform that is intended to enable the aquaculture feed industry to predict the nutritional and health value of alternative feed sources accurately and efficiently.

At present, it is impossible to infer the health and nutrition value through the chemical characterization of any given feed. Therefore, each new feed must be tested through *in vivo* growth trials. The procedure is lengthy, expensive and requires the use of many animals. Furthermore, although this process allows a precise evaluation of the final effect of each feed, it does not improve our basic knowledge of the cellular and molecular mechanisms determining such end-results. In turn, this lack of mechanistic knowledge severely limits the capacity to understand and predict the biological value of a single raw material and of their different combinations.

The protocol described herein allows to develop the two main components essential to produce a functional platform for the efficient and reliable screening of feeds that the feed industry is currently developing for improving their health and nutritional value. It is here applied to the Rainbow Trout, but it can be fruitfully used to many other fish species.

Key words: artificial intestine, *in vitro* model, fish intestine, polymer scaffold, gelatin derivatives, polymer synthesis, primary cell line, Rainbow trout

1. Introduction

The gastrointestinal tract is the organ devoted to digestive and absorptive functions [1]. Specifically, the intestine is composed by an internal lumen and by a wall that in turn is organized in different layers [2]. The predominant layer is the mucosa, typically characterized

by finger-like protrusions [1, 3] aimed to provide a wider absorptive surface [4] and lined by epithelium made up of a heterogeneous cell population [5].

So far, the *in vitro* models of the digestive system have been developed [6, 7] in the context of different applications including toxicology, drug testing and tissue engineering [8, 9]. However, due to the structural and functional complexity of the organ, the challenge to develop an appropriate and predictive *in vitro* model that closely mimics the digestive intestinal physiology and its architecture is still open. Several models are being fabricated at different complexity levels. The most important variables used to generate reliable *in vitro* intestinal models include the option of growing cell lines in mono- or co-culture conditions, the maintenance of the apical and basolateral side of the intestinal epithelium in static or advanced models as well as the option of continuous flow systems [10–12].

Irrespectively of the above, the majority of current *in vitro* models is based on the use of immortalized cell lines [13, 14] which lack a completely differentiated phenotype and therefore fail to reproduce the rich cell heterogeneity found *in vivo* [15]. As an alternative, 3D structures originating from pluripotent stem cells or from intestinal stem cells named organoids and enteroids respectively, are available [16]. These systems are characterized by more differentiated intestinal cells and by their progenitors but are not suitable for functional studies because of their enclosed lumen within a thick mass of hydrogel.

Several studies are trying to overcome this problem by applying well-tailored hydrogel supports [17]. To improve the suitability, their mechanical properties can be varied in a wide range, nonetheless, with increasing number of processing techniques, a structural variety of these hydrogels can be developed in order to achieve an advanced, life-like 3D in vitro intestinal model [18].

However, the keystone of a relevant and suitable tissue model is based on choosing the right combination of scaffold material and cell line. At present, an increasingly amount of data

suggests that the physical and the mechanical properties of the cell culture surface play a crucial role in cell guidance and addressing cell differentiation [19–23] modulating mechano-sensing and mechano-transduction pathways [24].

In this protocol paper, we describe gelatin derivatives as versatile scaffold materials. Gelatin is a collagen derivative, which is the main component of the extracellular matrix (ECM), consisting of various amino acids. The RGD motif (Arginin, Glycin, Aspartate triad) in the amino acid sequence of gelatin ensures the cell interactivity of these materials. In addition, the amino acid sequence of gelatin is highly versatile, with various side groups. These moieties can interact with each other, resulting in triple helix formation below a certain temperature, called the upper critical solution temperature (UCST, 30-35 °C), which leads to a physical gel formulation of gelatin at low temperatures, whilst having a liquid form above the UCST. The side group composition of gelatin gives a unique opportunity to modify these moieties, while introducing chemically cross-linkable side groups onto the macromolecule [25]. To this end, a cell-interactive, both physically and chemically crosslinked, hybrid polymer network is achievable at the utilized temperature (fish's body temperature is 20 °C, which is below the UCST of gelatin). The mechanical properties of the material are tailorable in a wide range via changing the modifying agents, the relative amount of the modifying agent compared to the number of modifiable moieties (degree of substitution, DS), etc.

We describe the synthesis of several gelatin derivatives and the procedure for the reliable derivation of Rainbow trout (*Oncorhynchus mykiss*) intestine primary cell lines. Their combination will lead to a faithful reproduction of the *in vivo* intestinal mucosa.

2. Materials

Prepare all solutions right before the use at room temperature (unless indicated otherwise).

2.1. Hydrogel development

2.1.1. Gelatin-methacrylamide (Gel-MA) development

1. Unmodified gelatin type B.
2. Phosphate buffer, pH 7.8 (17.8 g Na_2HPO_4 and 6.8 g KH_2PO_4 dissolved in 1 l double distilled water).
3. Methacrylic anhydride.
4. Round bottom flask.
5. Mechanical stirrer.
6. Heating plate with water bath at 40 °C.
7. Thermostat.
8. Dialysis bath.
9. Distilled water for dialysis purposes.
10. Dialysis membrane (MWCO: 12-14 kDa).
11. Freeze-dryer.

2.1.2. Gelatin-norbornene (Gel-NB) synthesis

2.1.2.1. Activation

1. Stirring plate.
2. 3-neck flask.
3. Stirring bar.
4. Bunsen burner for flame drying.
5. Teflon sleeves.
6. 2 taps.
7. Stopper.
8. Ar balloon.

9. Vacuum pump.
10. Activation solution (2.21 g EDC, 1.99 g NHS, 1.77 mL 5-norbornene-2-carboxylic acid, dissolved in 75 mL dry DMSO).

2.1.2.2. Reaction:

1. Heating plate with oil bath at 50 °C.
2. 3-neck flask.
3. Stirring bar.
4. Teflon sleeves.
5. 2 taps.
6. Reflux setup.
7. Stopper.
8. Ar balloon.
9. Vacuum pump.
10. Unmodified gelatin type.
11. Activation solution.
12. Dry DMSO.

2.1.2.3. Purification:

2.1.2.3.1. Precipitation

1. Buchner filter.
2. Filter paper (8-12 µm pore size).
3. RT acetone (10-fold excess).
4. Dropping funnel.
5. Distilled water for dialysis purposes.

2.1.2.3.2. Dialysis

1. Dialysis membrane (MWCO: 12-14 kDa).

2. Thermostat.
3. Dialysis bath.
4. Freeze dryer.

2.2 Film casting of gelatin derivatives (Gel-X)

1. Double distilled water.
2. Gelatin solutions (unmodified gelatin type B or in-house synthetized gelatin derivatives, Gel-X, X = MA / NB): 1 g Gel-X in 10 mL double distilled water (sensitivity 18 mΩ at 25°C).
3. Photoinitiator (2,4,6-trimethylbenzoyl) phenylphosphinate, Li-T-POL) solution: 10 mg Li-T-POL in 1 mL double distilled water **(*see* Note 1).**
4. Amber glass vials, 20 mL.
5. Heating plate.
6. Water bath at 40 °C.
7. UV-transparent glass plates.
8. UV-transparent teflon foil.
9. 1 mm thick silicone spacer.
10. Tape, scissors, clamps.
11. 20 mL syringe.
12. Needles.
13. UV-A lamps.

2.3 Rheological stiffness characterization of film-casted gelatin films

1. Film-casted gelatin films.
2. 14 mm round puncher.
3. PBS solution.

4. 19 °C incubator.
5. Anton Paar MCR301 Physica rheometer.
6. 15 mm diameter spindle.
7. Torck paper.
8. Tweezer.

2.4 Scaffold preparation from film-casted gelatin films

1. Film casted gelatin films.
2. 7 mm round puncher.
3. 4-well plates.
4. Spatula.
5. Tweezer.

2.5 Sample collection

1. Rainbow trout weighing approximately 500 gr from a fish farming.
2. Tricaine methane-sulfonate solution (MS-222): Dissolve 500 mg of MS-222 in 10 L tap water.
3. Dulbecco's Phosphate Buffered Saline (PBS): Weigh 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) 240 mg of KH_2PO_4 (2 mM) and dissolve in 800 mL of distilled water. Adjust pH to 7.4. Make up to 1 liter adding distilled water Mix and sterilize the solution using autoclave.
4. Ice container.
5. Antibiotic/Antimycotic Solution.
6. 50 mL polypropylene tubes.
7. Surgical scissors.
8. Scalpel and surgical blades.

9. Tweezers.

Prepare all solutions in advance using ultrapure water and store all the reagents at +2-8°C (unless indicated otherwise). Once the reagents are sterilized be sure to use them under a fume hood

2.6 Primary cell culture isolation and maintenance

1. 25-cm^2 culture flasks.
2. 0.1% gelatin derived from pig skin: Weigh 0.1 g of gelatin derived from pig skin and dissolve it in 100 mL of water. Mix and sterilize the solution using autoclave.
3. Dulbecco's Phosphate Buffered Saline (PBS): Weigh 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) 240 mg of KH_2PO_4 (2 mM) and dissolve in 800 mL of distilled water. Adjust pH to 7.4. Make up to 1 liter adding distilled water Mix and sterilize.
4. Refrigerated incubator.
5. Surgical scissors.
6. Scalpel and surgical blades.
7. Tweezers.
8. Glass pasteur pipettes.
9. Micropipettes and tips.
10. Complete cell culture media: Prepare complete Leibovitz's L-15 medium without phenol red, adding 5% (v/v) Fetal Calf Serum (FCS), 1% (v/v) antibiotic antimycotic solution and 200 mM L-Glutamine **(*see* Note 2).**
11. Stereomicroscope.
12. 35 and 60-mm petri dishes.

13. Trypsin-EDTA solution0.5 g/l porcine trypsin, 0.2 g/l EDTA 4Na per liter of Hank's Balanced Salt Solution with phenol red.

2.7 Primary cell culture seeding and maintenance on biological and biocompatible scaffolds

1. 70% ethanol in distilled water.
2. Dulbecco's Phosphate Buffered Saline (PBS): Weigh 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) 240 mg of KH_2PO_4 (2 mM) and dissolve in 800 mL of distilled water. Adjust pH to 7.4. Make up to 1 liter adding distilled water. Mix and sterilize.
3. Complete cell culture media: Prepare complete Leibovitz's L-15 medium without phenol red, adding 5% (v/v) Fetal Calf Serum (FCS), 1% (v/v) antibiotic antimycotic solution and 200 mM L-Glutamine.
4. 4-wells multidish.
5. Refrigerated incubator.
6. 15 mL polystyrene tube.
7. Microscope.
8. Cell counting chamber.
9. Trypsin-EDTA solution: 0.5 g/l porcine trypsin, 0.2 g/l EDTA 4Na per liter of Hank's Balanced Salt Solution with phenol red.

3. Methods

Perform all procedures at room temperature unless otherwise specified.

3.1. Hydrogel development

3.1.1. Gelatin-methacrylamide (Gel-MA) development [25].

1. Dissolve 100 g gelatin type B in phosphate buffer (pH 7.8) at 40 °C.
2. Add 14.34 mL methacrylic anhydride to the gelatin solution while vigorously stirring with mechanical stirrer (***see*** **Note 3**).
3. After 1 h reaction time, dilute the reaction mixture with the addition of 1 L double distilled water.
4. Dialyse the Gel-MA against distilled water (MWCO: 12-14 kDa) for 24 hours at 40 °C.
5. Freeze dry the samples (Figure 1).

[Figure 1 near here]

3.1.2. Gelatin-norbornene (Gel-NB) synthesis [26]

3.1.2.1. Activation

1. Dissolve 1.77 mL 5-norbornene-2-carboxylic acid with 1.99 g N-hydroxysuccinimide (NHS) and 2.21 g N-(3Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) in 75 mL dry DMSO at RT in a round bottom flask.
2. Apply Argon atmosphere.
3. The activation step should continue during 25 hours.

3.1.2.2. Reaction:

1. Dissolve 15 g gelatin type B in 450 mL dry DMSO at 50 °C under reflux conditions.
2. After full dissolution, add the activated solution.
3. Let the mixtures react overnight while vigorously stirring.

3.1.2.3. Purification:

1. Precipitate the reaction solution in 10-fold excess of acetone.

2. Dissolve the precipitate in double distilled water.
3. Dialyse the aqueous solution against distilled water (MWCO: 12-14 kDa) for 24 hours at 40 °C.
4. Freeze dry the samples (Figure 2).

[Figure 2 near here]

3.2. Film casting of gelatins (Gel-X)

In order to test the stiffness and cell-interactivity of the applicable scaffold materials, film casting of the gelatin derivatives was performed. Film casting provides the opportunity to create polymer sheets with a well-defined thickness. To this end, identical, flat polymer samples can be fabricated fast and efficiently in a reproducible manner.

1. Dissolve 1 g Gel-X in 10 mL double distilled water (sensitivity 18 mΩ at 25°C) in amber glass vials in a 40 °C water bath.
2. Add 2 mol% photoinitiator (Li-T-POL, from stock solution, with respect to the amount of double bonds, ***see*** **Note 4-7**) to the mixture and homogenize at 40 °C.
3. Prepare the glass plates: attach the teflon foil on the glass plate with transparent tape.
4. Place the 1 mm thick silicone spacer on the top of the teflon foil.
5. Transfer the gelatin solution into the spacer with the help of a syringe and a needle in order to prevent bubbles in the prepared film **(*see* Note 8).**
6. Place the second glass plate on the top of the filled spacer and clamp them together.
7. Place the samples in the fridge for 15 minutes (4 °C, ***see*** **Note 9-10**).

8. Place the glass 2 UV-A lights for irradiation from top and bottom with total intensity of ± 10 mW/cm^2 for 30 minutes (Figure 3).

[Figure 3 near here]

3.3 **Rheological stiffness characterization of film-casted gelatin films**

To study the stiffness of the film-casted Gel-X films, oscillatory rheology was performed on the samples.

1. Punch out round samples from the film-casted polymer sheets with a puncher of 14 mm diameter.
2. Place them in well plates and let them swell for 24 hours in PBS solution at 19 °C in an incubator.
3. The next day, start up the rheometer with a metal bottom plate and a 15 mm diameter spindle **(*see* Note 11).**
4. Apply a frequency sweep program between 0.5-5 Hz, with 0.1% strain applied on the samples **(*see* Note 12).**

3.4 **Scaffold preparation from film-casted gelatin films**

1. Punch out round samples from the film-casted polymer sheets with a puncher of 7 mm diameter.
2. Place them in well plates before sterilization.

Figure X – near here punched out samples

3.5 Sample collection

1. Sacrifice the fish immersing it in the tank containing tricaine methane-sulfonate solution **(*see* Note 13).**

2. Immediately after euthanasia perform a longitudinal incision along the fish ventral line and gently remove the whole gastrointestinal tract using surgical scissors and tweezers.
3. Identify starting and ending point of proximal and distal intestine and collect them into 50 mL tube stored in ice and containing freshly prepared PBS supplemented with 1% antibiotic antimycotic solution.

3.6 Primary cell culture isolation and maintenance

Perform all procedures at room temperature and under sterile conditions unless otherwise specified.

1. Coat the 25-cm^2 culture flasks surface using 1.5 mL of 0.1% gelatin derived from pig skin and incubate for at least 20 minutes.
2. Place proximal and distal intestine into two different 60 mm petri dishes.
3. Carefully remove the intestinal content by slightly pressing the intestine from a side to another to let them get out using tweezers.
4. Gently, open the intestinal lumen longitudinally under the stereomicroscope using forceps and tweezers to expose the internal mucosa.
5. Move intestinal segment into two 35 mm petri dishes containing 2 mL PBS.
6. Vigorously, wash samples using a glass Pasteur pipette with fresh PBS to remove completely the mucus layer lining the mucosa **(*see* Note 14).**
7. Transfer tissues devoid of mucus into two new 35 mm petri dishes containing 2 mL PBS.
8. Cut samples into small diced (1 mm^2) using scalpel and tweezers.
9. Carefully, take each intestinal diced one by one and insert it into in 25-cm^2 culture flasks (3-4 pieces/flask) precoated with 0.1% gelatin using tweezers.

10. Very gently, add the sufficient complete L-15 medium to cover all explants (approximately 1.5 mL) making it percolate from a side of 25-cm^2 culture flasks.
11. Maintain cells in complete L-15 medium at 20 C° in incubator under ambient atmosphere (Figure 4).

[Figure 4 near here]

12. Refresh the medium once a week.
13. After 5-10 days culture, cells will start to grow out from intestinal explants and will form colonies.
14. Maintain cells in 1.5 mL complete L-15 medium at 20 C° in incubator under ambient atmosphere at least for 3 months (Figure 5) **(*see* Note 15).**

[Figure 5 near here]

15. Once cells reach their confluence state, carefully remove the intestinal fragments from flasks surface gently aspirating them using a micropipette tip.
16. Maintain cells in 6 mL complete L-15 medium at 20 C° in incubator under ambient atmosphere.
17. Propagate cells refreshing medium once a week, splitting and passing adherent cells using a trypsin/EDTA solution.

3.7 Primary cell culture seeding and maintenance on biological and biocompatible scaffolds

1. Gently, move freshly synthesized gelatins discs (previously punched out) into 4-wells multidish.
2. Incubate gelatins in 70% ethanol solution for 2 hours to sterilize them.

3. Let ethanol evaporate for at least 20 minutes **(*see* Note 16).**
4. Take out from the incubator 25-cm^2 culture flasks and remove the maintenance medium.
5. Gently, wash 3 times adherent cells using around 2 mL PBS supplemented with 1% antibiotic antimycotic solution.
6. Add 600 µl of trypsin/EDTA solution and let cells detach from the culture surface **(*see* Note 17).**
7. Add 5.4 mL of complete L-15 medium and vigorously mix to resuspend cells.
8. Collect the cell suspension solution in a 15 mL polystyrene tube.
9. Count cells within cell suspension under optical microscope using a cell counting chamber.
10. Based on the cell counting results, define the required cell suspension volume in order to seed each gelatine disc 10^7 cells in a 20 µl complete culture media drop.
11. Culture cells in a humidified chamber at 20 °C normal atmosphere.
12. Add 10 µl of fresh complete medium 5 hours after seeding.
13. The day after, add 10 µl of fresh complete medium to the drop.
14. The following day, add 40 µl of fresh complete medium to the drop **(*see* Note 18).**
15. After 24 hours, wash once using complete fresh medium and cover gelatins completely adding 500 µl of fresh complete culture media.
16. Culture cells in complete L-15 medium at 20 °C in the incubator under ambient atmosphere **(*see* Note 19).**

4 Notes

1. Synthesis of the Li-T-POL in brief: Dissolve 9.45 g LiBr in 150 mL butanone at 65°C and apply stirring. Add 8.6 g TPO-L to the solution and stir the reaction

mixture for 24h at 65°C. Collect the precipitate by suction filtration and wash the precipitate with 400 mL petroleum ether. Dry the precipitate under vacuum at room temperature. Perform the reaction and drying step shielded from UV light.

2. All the medium should be at room temperature before starting the procedure.
3. In order to achieve different degree of substitutions (DS) for gelatin type B (to tailor the stiffness of the material), vary the amount of methacrylic anhydride added. Add 2.87 mL of methacrylic anhydride to achieve the DS of 30% (Gel-MA30), 5.74 mL methacrylic anhydride for the DS of 60% (Gel-MA60), 14.34 mL methacrylic anhydride for the DS of 90% (Gel-MA90).
4. No Li-T-POL was added to the unmodified Gelatin Type B solution.
5. A 10 mg/mL stock solution was prepared from Li-T-POL, in order to overcome difficulties associated with the precise measurement of the Li-T-POL powder to the solutions, as only a small amount (some mgs) of it is needed to initiate the free radical polymerization.
6. Add the photoinitiator to the solution in the dark, and keep the solution shielded from UV light until use.
7. Bubbles on the surface of the polymer film are functioning as inhomogeneities, they have a weakening effect on the film.
8. According to the UCST behavior of gelatin, cooling the samples before UV crosslinking promotes the formulation of a physically crosslinked polymer network, and has a strengthening effect on the samples.
9. In case of the gelatin Type B solution, no UV crosslinking is needed, the cooling of the sample is sufficient to maintain a stable, physically crosslinked network.

10. Wipe off the excess PBS from the surface of the samples before placing in the rheometer. If the samples are significantly swollen (diameter>15mm), re-punch them with the 14 mm puncher.
11. In order to prevent the detachment of the spindle from the sample, if the sample would dry out during the process, apply a normal force on the sample (0.1-1 N).
12. Be sure to perform the procedure within 1-1.5 hour after the sacrifice of the rainbow trout.
13. Be sure to completely remove the mucos layer during the cleaning procedure.
14. Three months represent the time frame required to obtain epithelial-like colonies able to be propagated.
15. Be sure to let ethanol completely evaporate.
16. Verify under the microscope cells detachment in order to avoid to let cells adherent on the plastic surface.
17. At this point, cells should be attached to gelatines.
18. Perform Hoechst staining and DNA quantification to evaluate cells growth and biocompatibility.

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Figure 1: Synthesis setup of Gelatin-methacrylamide (Gel-MA).

Figure 2: Synthesis setup of Gelatin-norbornene (Gel-NB).

Figure 3: Film casting and sterilization of Gel-X discs. Prepare a 10 w/v% Gel-X solution with photoinitiator (Li-T-POL) in an amber vial at 40 °C. Transfer the solution into a syringe for film casting. The film casting setup consists of a) 2 UV-transparent glass plates, covered with teflon foil, b) a 1 mm thick spacer, c) an inlet for the Gel-X solution. After transferring the Gel-X solution in the film casting setup, 30 minutes of UV-A light exposure is required. Punch out 7 mm diameter discs of the crosslinked Gel-X films and sterilize them in 70% ethanol.

Figure 4: Images showing selected regions for the samples collection (A: proximal; B:distal) and describing the main steps of primary cell culture isolation; C: distal intestine devoid of mucus after several vigorously wash in PBS, D: small intestinal pieces diced; E: explants inserted in a 25-cm^2 culture flask precoated with of 0.1% gelatine derived from pig skin.

Figure 5: Representative pictures showing different primary cells culture morphology isolated from intestinal explants.