TITLE: Use of virus-mimicking nanoparticles to investigate early infection events in upper airway 3D models

Running title: Generation of a 3D infection platform

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Abstract

The current coronavirus disease-19 (COVID-19) pandemic, caused by 'severe acute respiratory syndrome coronavirus 2' (SARS-CoV-2), underscores the threat posed by newly emerging viruses. The understanding of the mechanisms driving early infection events, that are crucial for the exponential spread of the disease, is mandatory and can be significantly implemented generating 3D in vitro models as experimental platforms to investigate the infection substrates and how the virus invades and ravages the tissues.

We here describe a protocol for the creation of a synthetic hydrogel-based 3D culture system that mimics in vitro the complex architectures and mechanical cues distinctive of the upper airway epithelia. We then expose the in vitro generated 3D nasal and tracheal epithelia to gold nanoparticles (AuNPs) that display the typical shape and size distinctive of SARS-CoV-2 and of the majority of Coronaviridae presently known.

The infection platform here described provides an efficient and highly physiological in vitro model that reproduces the host-pathogen early interactions, using virus-mimicking nanoparticles, and offers a flexible tool to study virus entry into the cell. At the same time, it reduces the risk of accidental infection/spill-overs for researchers, which represents a crucial aspect when dealing with a virus that is highly contagious, virulent and even deadly.

Key Words: Coronaviridae, Covid-19 pandemic, Hydrogel-based culture system, Gold nanoparticles, SARS-CoV-2, Upper respiratory tract.

1. Introduction

The current coronavirus outbreak caused by 'severe acute respiratory syndrome coronavirus 2' (SARS-CoV-2) is the gravest health crisis the World has seen in a century, with an unprecedent death toll and number of patients in urgent need of hospital treatment ***(1)***. According to the World Health Organization, the situation report update at 08 July 2020 is 11,635,939 of total confirmed cases and 539,026 the deaths, with the United States and Europe as epicenters of the global pandemic, and 6,004,685 and 2,809,848 confirmed cases respectively ***(2)***.

Although, SARS-CoV-2 has a relatively low/medium mortality rate, it is highly contagious, leading to an explosive and exponential spread of the infection ***(3)***. The understanding of the first events controlling the mechanisms that lead to the rapid spread of the disease are therefore crucial for its containment and the early detection. Typical in vitro experiments carried out by virologists use cell lines cultured into 2D culture systems ***(4)***, that are only poorly resemble real tissue behavior, especially in terms of structural and functional proprieties, nor its interactions with SARS-CoV-2. 3D in vitro models have been currently developed to better investigated the in vivo infection substrates and allow for studying how the new coronavirus invades and ravages the body.

We here describe a protocol for the generation of reliable 3D in vitro models of upper respiratory tract, namely nose and trachea. We propose the use of a synthetic hydrogel-based 3D culture system that is able to reproduce in vitro the complex biochemical and mechanical cues deriving from the native extracellular matrix ***(5, 6)*** as well as several aspects of the native cellular microenvironment, mimicking the original conditions of the airway epithelia ***(7)***.

To stimulate the mechanisms active at early stages of infection -prior to viral replication- and allows to study how the virus interacts and invades cells of the upper respiratory tract, we implement a nanomedicine-based approach, exposing the 3D in vitro generated nasal and tracheal epithelia to gold nanoparticles (AuNPs) (Figure 1). In particular, we select AuNPs spherical in shape and with size ranging from 60 to 140 nm, which are distinctive features of SARS-CoV-2 and common to the majority of Coronaviridae presently known ***(8–10)***. In addition, in order to address the different

mechanisms used by Coronaviridae to enter cells, the same protocol can be applied using nanoparticles with surface ad hoc activated or caged with coronavirus envelope spike proteins ***(10, 11)***.

Overall, the bioengineered model of infection here described provides an efficient and highly physiological in vitro platform that mimics the host-pathogen early interaction and offers a powerful insight on the possible strategies to prevent virus entry into the cell (Figure 2). In addition, beside its predictive power and flexibility, it also ensures safety. This latter represents a crucial aspect, since dealing with a virus that is highly contagious, virulent and even deadly, inevitably reduces the manpower and the number of laboratories that have high protection facilities, significantly affecting in a way the intellectual and scientific resources to fight this as well as other future pandemics. Finally, the 3-D cell-based assay here proposed could contribute to reduce the use of experimental animal studies, the uncertainties arising from monolayer cultures and hence the cost of subsequent screening processes.

2. Materials

Prepare all solutions immediately before use (unless indicated otherwise).

2.1 Cell lines

1. Human Nasal Epithelial Cells (HNEpCs).
2. Human Tracheal Epithelial Cells (HTEpCs).

2.2 HNEpC and HTEpC 2D culture

1. Water bath.
2. Culture dish.
3. CO_2 incubator.
4. Inverted microscope.

5. Dulbecco’s phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) and 240 mg of KH_2PO_4 (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
6. Antibiotic/Antimycotic Solution.
7. Trypsin-EDTA solution: dissolve 0.5 gr of porcine trypsin and 0.2 gr of EDTA 4Na in 1 L of HBSS with phenol red.
8. Epithelial cells (EC) culture medium: Dulbecco's Modified Eagle Medium (DMEM) high glucose, 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine solution, 5 ng/mL of recombinant epidermal growth factor (EGF), 30 ug/mL bovine pituitary extract (BPE), 0.05 µM Retinoic Acid, and 1% (v/v) antibiotic-antimycotic solution.

2.3 Hydrogel-based 3D culture system

1. Water bath.
2. 15 mL centrifuge polystyrene tube.
3. Centrifuge.
4. CO_2 incubator.
5. Cell counting chamber.
6. Inverted microscope.
7. Collagen pre-coated hydrogel with Young's modulus ranging from 20 kPa to 30 kPa (see Note 1).
8. Dulbecco’s phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) and 240 mg of KH_2PO_4 (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
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2.4 Cellular uptake of nanoparticles

1. Water bath.
2. 15 mL centrifuge polystyrene tube.
3. CO_2 incubator.
4. Inverted microscope.
5. Polyvinylpyrrolidone coated gold nanoparticle (AuNP-PVP) (see Note 2).
6. Dulbecco's phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) and 240 mg of KH_2PO_4 (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
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3. Methods

All the procedures described below must be performed under sterile conditions. Cell manipulation must be carried out under laminar flow hood and cell cultures have to be maintained at 37 °C during their handling using thermostatically controlled stages.

3.1 HNEpC and HTEpC propagation and maintenance

1. Monitor cells daily.
2. Once cells have reached 80% confluency, carefully aspirate the culture medium (see Note 3).
3. Wash cells 3 time with 4 mL of PBS supplemented with 1% antibiotic antimycotic solution.
4. Add 600 μL of trypsin-EDTA solution (see Note 4) and incubate at 37 °C until cell monolayer begins to detach from the bottom of the tissue culture dish and cells dissociate (See Note 5).
5. Dilute cell suspension with 9 parts of EC culture medium to neutralize trypsin action.
6. Dislodge cells by repeatedly and gently pipetting.
7. Plate cells in a new culture dish and culture at 37 °C in 5% CO2 incubator. Keep the passage ratio between 1:4 and 1:6, depending on growth rate (see Note 6).
8. Change medium every 2-3 days.
9. Maintain cells in culture until they have reached 80% confluency and passage them.

3.2 Establishment of a hydrogel-based 3D culture system

1. Remove the sterile water or buffer used for hydrogel maintenance.
2. Immerse hydrogels in EC culture medium and incubate at room temperature for a minimum of 30 min.
3. Trypsinize cells (see Sect. 3.1, steps 3-6).
4. Collect cell suspension and transfer it in a 15 mL centrifuge polystyrene tube.
5. Count cells using a counting chamber under an optical microscope at room temperature. Calculate the volume of medium needed to re-suspend cells in order to obtain a concentration of 6.5 X 10^4 cells/cm^2 (see Note 7).

6. Centrifuge at 300 g for 5 min.
7. Remove supernatant carefully and resuspend the cell pellet in the previously calculated volume (see step 3) of EC culture medium by pipetting.
8. Plate cells onto hydrogels.
9. Transfer into CO_2 incubator.
10. Incubated overnight for cell attachment.

3.3 Cellular uptake of nanoparticles

1. Prepare PVP-AuNP solution by diluting PVP-AuNPs to 70 μg/mL in EC culture medium (see Note 8).
2. Mix solution by gently pipetting three times.
3. Carefully aspirate the culture medium for culture dish.
4. Wash cells 3 times with PBS supplemented with 1% antibiotic antimycotic solution.
5. Add of PVP-AuNPs solution to cells.
6. Transfer into CO_2 incubator.
7. At the end of the desired culture period, wash cells twice with PBS and analyze PVP-AuNPs absorption of by transmission electron microscopy.

4. Notes

1. Hydrogels must be stored at 4°C and used within six months of the printed manufacture date.
2. PVP-AuNPs must be stored at 4°C. As an alternative AuNPs can be replaced with polyvinylpyrrolidone coated silver NPs, which, however, must be protected from light, beside storing at 4°C.
3. Confluency normally takes between 7-10 days. If cells are not confluent after 10 days, they are not successfully growing.

4. The trypsin volume here reported is necessary for detaching cell cultured in a T25 flask. When working with bigger dish, scale up the volumes accordingly.
5. It usually takes 2-3 min.
6. The recommended seeding density is 10,000 - 15,000 cells per cm^2.
7. The formula to be used depends on the specific type of chamber. Cells/µL = Average number of cells per small grid x chamber multiplication factor x dilution.
8. PVP-AuNPs tend to rapidly clusterize, prepare PVP-AuNP solution immediately prior the use.

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Figure Legend

Figure 1. Schematic representation of the protocol used to generate a 3D infection platform of the upper airways.

Figure 2. An overview of virus-mimicking nanoparticle uptake by target cells.