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SHORT RESEARCH ARTICLE

Use of synthetic polymer hydrogels to prepare scaffoldless 3D tissue constructs [version 1; peer review: 3 approved with reservations]

Gerson Florez¹, Eric Williams¹, Minn H Saing¹, Jasvir S Khurana², Solomon Samuel¹

¹Orthopedic Surgery, Einstein Medical Center, Philadelphia, PA, 19141, USA

²Department of Pathology and Laboratory Medicine, Temple University Hospital, Philadelphia, 18140, USA

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Abstract

The use of 3-dimensional tissue cultures is gaining popularity in many fields including drug discovery, toxicity testing and tissue engineering. Currently most of the techniques used to prepare these 3D tissues are time consuming and cannot be reproduced easily. There is an urgent need to optimize the preparation of these 3D tissue cultures. This study evaluated the use of synthetic hydrogel polymers used to manufacture soft contact lenses to guide cells to form multicellular tissue-like structures. It was found that bovine chondrocytes and porcine dental pulp stem cells were able to form 3D tissue structures when placed inside a soft contact lens. Commercially available microarrays, 96 or 384 well plates manufactured using synthetic hydrogel polymers may help overcome many reproducibility issues and simplify the 3D tissue culture process.

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Approval Status **? ? ?**

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1. Wael Kafienah , University of Bristol, Bristol, UK 2. Mikko Lammi , University of Eastern Finland, Kuopio, Finland 3. Carlos E Semino , Ramon Llull University, Barcelona, Spain			
Any reports and responses or comments on the article can be found at the end of the article.			

Corresponding author: Solomon Samuel (samuelsp@einstein.edu)

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Introduction

Three dimensional tissue cultures are gaining popularity because they mimic the natural cell environment^{1,2} and are used in many applications such as drug discovery, toxicity testing and tissue engineering. Scaffoldless 3D tissue cultures may be superior to 3D tissue cultures prepared by immobilizing cells in extracellular matrices such as alginates, agarose or chitosan³. The advantages of scaffoldless tissues include avoiding immune responses to the scaffolds, degradation of scaffolds *in vivo*, the effects of non-physiological mechanical properties of the scaffold and avoiding cross reactivity to scaffolds during *in vitro* testing. There are at least four common methods reported in the literature used to prepare these 3D spherical tissue cultures or multi-cellular tumor spheroids (MCTS) without the need for any scaffolds or matrices. They include (a) the use of centrifugal force to produce cell pellets⁴, (b) the hanging drop technique^{5,6}, (c) culturing cells at high density to enable self assembly, or (d) placing cell suspensions onto hydrophilic surfaces (agarose or alginate based hydrogels) so that electrochemical forces coerce cells to assemble into spherical structures⁷. However, these methods are often time consuming and the results are often poorly reproducible⁸.

The use of synthetic polymer hydrogels may overcome the limitations associated with the previously described 3D tissue culture methods and simplify 3D tissue manufacture. For example, synthetic hydrogel polymers can be used to manufacture microarrays, 96 or 384 well plates and can be made available off-the-shelf. Preparing 3D tissue structures using commercially manufactured hydrogel plates can be as simple as pipetting a known quantity of cells into these plates, incubating the plates for a certain time period (usually 24 h) and then pipetting out the formed 3D cell aggregates for further tests. In fact, Le Gac *et al* (2008) showed that polydimethylsiloxane (PDMS) microwell arrays may overcome some of the limitations (e.g. reproducibility, sterility, and low production yield) associated with conventional 3D tissue manufacture⁹. Synthetic polymer hydrogel materials have been used for a long time in the large scale manufacture of soft contact lenses. Therefore, they may also be ideal hydrogel candidates for large scale manufacture of these microarrays, 96 or 384 well plates, etc. The first logical step is to evaluate whether the synthetic polymer hydrogels used to prepare soft contact lenses have the properties required to guide cells (e.g. chondrocytes) to form 3D tissue-like structures. This property was evaluated by simply placing bovine chondrocytes or porcine dentine pulp cells in commercially available soft contact lens and observing whether they can guide the cells to form 3D structures.

Methods

Cell culture

Bovine chondrocytes were harvested from the femoral cartilage of a 2 yr old cow obtained from a local slaughter house and the porcine dental pulp cells were harvested from the dental pulp

of a euthanized 3–4 month old female Yorkshire pig. The cells were isolated from their respective tissues within 4 h of tissue harvest. It should be noted that to avoid yeast contamination, the porcine dental pulp tissue was briefly (< 30 sec) washed with 70% isopropyl alcohol. To isolate the cells, the tissues were cut into very small pieces using a scalpel blade and washed twice using regular Dulbecco's Modified Eagle Medium DMEM, (Invitrogen, Carlsbad, CA) and 5 ml solution of TrypLE™ Express (Invitrogen, Carlsbad, CA) containing 10 mg collagenase type 2 (Worthington, Biochemical Corp., Lakewood, NJ) was added to the washed pieces in a 50 ml centrifuge tube. The centrifuge tube was placed in a shaker water bath for 2 h and maintained at 37°C. The dissociated cells along with the remaining partially dissociated tissue fragments were centrifuged to remove the collagenase and washed twice using regular DMEM media. The washed cells along with the tissue fragments were then placed in T25 cell culture flasks containing high glucose concentration DMEM media supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 µg/ml streptomycin (complete medium). The cell culture flasks were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The cells were allowed to grow in a monolayer for 15 days. Every time (3–5 day interval) the cells reached confluence, they were trypsinized (TrypLE™ Express, Invitrogen, Carlsbad, CA), passaged at 1:1 dilutions and grown in complete medium.

Induction of 3D cell self-assembly using commercially available soft contact lens or 2% agarose hydrogel surfaces

2% agarose hydrogels (control group) were prepared by mixing 2 g of agarose in 100 ml PBS and boiling the mixture in a microwave oven. The clear agarose solution was then poured into six well plates and was allowed to form a hydrogel. For the contact lens group, (chemical composition: galyfilcon A (Acuvue advance™, Johnson & Johnson Vision Care, Inc, Jacksonville, FL), enfilcon (AVAIRA™, Cooper Vision, NY) or Etafilcon A (Acuvue2™, Johnson & Johnson Vision Care, Inc, Jacksonville, FL)), the contact lenses were removed from their sterile packaging and one placed in each well of a 12 well plate. Once placed in the plates, the contact lenses maintained their concave shape and were able to hold up to 200 µl of media without any shape deformation. 100 µl of media containing $6.0 \times 10^5 - 5 \times 10^6$ of the desired cells (counted using the automated cell counter, TC10, Bio-Rad Laboratories, Inc., Hercules, CA) were placed carefully into the contact lens or on top of the 2% agarose hydrogels using a pipette. The cells were allowed to coalesce for 3 hours and then 3 ml of complete medium was added to each well surrounding the contact lens to keep them moist (Figure 1). Within 24 hours, the cells coalesced completely and formed a single 3D tissue like structure. The micromass was removed, placed in fresh 12 well culture plates and the soft contact lens or agarose plates were discarded. The constructs were maintained for 3 weeks and the media was changed regularly every 3–4 days. The experiments

Figure 1 Experimental set up used to evaluate the formation of 3D tissue structures using synthetic polymer hydrogels (in the form of a contact lens, pictured left) and a photograph of a 3D micromass formed using chondrocytes (5×10^6 cells) after 3 weeks (right).

were conducted in triplicates. After 3 weeks, the micromass was then frozen sectioned; hematoxylin and eosin (H&E) stained and observed under a microscope.

Results

Both bovine femur chondrocytes and porcine dental pulp cells self-assembled when placed in contact either with the contact lens or 2% agarose hydrogel surfaces. None of the cells seemed to adhere to the soft contact lens or 2% agarose hydrogel surfaces. The micromass formed was firm after 24 h and did not crumble during handling (e.g. pipetting). This shows that the cells were already producing an extracellular matrix to hold them together. However, these micromasses continue to shrink and by day 10 it reaches a stable size. After 3 weeks, the final

diameter of the micromass depended on the initial number of cells used. The micromass (irregular in shape) was at least 1.5 mm in diameter when 6×10^5 cells were used and 2–3 mm in diameter when 1.2×10^6 cells were used. Visual examination of 3D constructs showed that the type of contact lens used did not seem to make a difference in the size of the micromass at either day 5 (Figure 2) or day 21 (Figure 3). However, further studies are needed to evaluate whether there are any differences in the matrix composition between 3D constructs formed in agarose gels versus different soft contact lens hydrogel surfaces. From a handling point of view thicker contact lenses worked better (e.g. enfilcon (AVAIRATM, Cooper Vision, NY)) and tended to maintain their shape during media placement (Figure 4). Both galyfilcon and etafilcon had similar thickness/

Figure 3 Micromass at day 21. On visual inspection of the micromass it was concluded that the type of contact lens used did not seem to make a difference. The micromass (left to right) was formed in galyfilcon A (Acuvue advance™, Johnson & Johnson Vision Care, Inc, Jacksonville, FL), enfilcon (AVAIRATM, Cooper Vision, NY), and etafilcon A (Acuvue2™, Johnson & Johnson Vision Care, Inc, Jacksonville, FL) respectively.

Figure 2 Chondrocytes grown in (top to bottom) enfilcon (AVAIRATM, Cooper Vision, NY), galyfilcon A (Acuvue advance™, Johnson & Johnson Vision Care, Inc, Jacksonville, FL), and etafilcon A (Acuvue2™, Johnson & Johnson Vision Care, Inc, Jacksonville, FL) at 5 days.

Figure 4 Thinner contact lenses (bottom) tended to fold and release the cell contents during initial cell placement.

Figure 5a & b Frozen H&E section (400X) of 3 week old 3D tissue structure formed using bovine femur chondrocytes **(a)** and porcine dental pulp cells **(b)**. The microscopy slide shows viable cells embedded in a hyalinized background (hydrogel material). The cells show rounded slightly eccentric nuclei with a clear space around them.

handling properties (they both tended to fold easily) and were thinner compared to the enficon. It should be noted that the ease of handling is based purely on thickness of the lens, rather than the materials themselves. An example of the frozen section of micromass tissue formed with either bovine femur chondrocytes or porcine dental pulp cells is shown in Figure 5a and 5b respectively. Hematoxylin and eosin (H&E) stain was used to visualize the cells and the presence of extracellular matrix produced by the cells.

Micromass dimensions of dentin pulp and chondrocyte cells after 21 days

<http://dx.doi.org/10.6084/m9.figshare.106813>

Discussion

Hydrogels such as agarose, alginate or PDMS have been used before to guide or coax cells to form 3D multicellular tissues^{3,9}. This study evaluated the use of synthetic polymer hydrogels used to prepare soft contact lenses to do the same. The results

showed that both chondrocytes and dentine pulp cells reproducibly formed 3D tissue structures when placed inside these soft contact lenses. Therefore, the study demonstrated that the synthetic polymer hydrogels (e.g. galyfilcon A, enficon or etafilcon A or even poly HEMA¹⁰) may provide an alternative to agarose, alginate or PDMS based hydrogel surfaces.

It is well known that soft contact lens can be manufactured in large scale without much variability within batches and stored for a long time in sterile solutions. The same technology can theoretically be used to manufacture and store 3D cell aggregation devices in the form of microarrays, 96 or 384 well plates using these synthetic polymer hydrogels. A commercially available 3D cell aggregation device manufactured using synthetic polymer hydrogels has the potential to simplify and help large scale manufacture of reproducible 3D cell cultures for various applications including *in vitro* chemical toxicity testing and preparation of small scaffold-less tissue engineered constructs.

Author contributions

SS conceived the study. SS and GF designed the experiments and carried out the research. EW and MS contributed to the design of experiments and the preparation of manuscript. JK helped with the histology sections. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

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Version 1

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Carlos E Semino

Department of Bioengineering, Ramon Llull University, Barcelona, Spain

The authors described an interesting technology consisting of seeding cells on an hydrogel made out of synthetic polymer used to manufacture contact lenses. They used bovine chondrocytes and porcine dental pulp cells and placed them on top of soft contact lenses. After a few days they observed the formation of 3D-cellular structures. Then, they cultured the constructs for three weeks.

It is important to mention that the use of commercial polymers benefits the experimental setup, in terms of better reproducibility and potential production. The idea of turning this into a 96- and 384-well plate platform looks very attractive since future technologies and platforms are required in order to produce easy 3D-constructs for pharmacology, toxicology, stem cell and cancer research. I believe this is a excellent technical contribution, which is simple and feasible to be adapted to a HTS (high-throughput screening).

In my opinion the work needs some more characterization at the cellular/construct level with relatively simple tests:

1. I believe it will be important to monitor, as the previous referee mentioned, some live/dead analyses to see how the cells perform inside the construct over time.
2. In addition, constructs could be stained with phalloidin/DAPI to assess cellular configuration by fluorescent microscopy.
3. Also, a general morphological analysis of the construct could be performed, to see if they also form a necrotic core like the classical spheroids, as this would be important.
4. Finally, since the authors used bovine chondrocytes I agree with the previous referee that it would be interesting to test for GAG (glucosaminoglycan) production by simple staining with Toluidine blue.

In the discussion section, the authors should further explore the potential use of this technology,

especially if they suggest translating it into 96- and 384-well format for potential HTS.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 16 August 2013

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Mikko Lammi

Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland

The report by Florez *et al.* describes the use of contact lenses as a material to culture chondrocytes and dental pulp cells. The cells formed scaffold-free cell aggregates of variable sizes, which depended on the original amount of cells seeded. The cultures were apparently biocompatible, although the viability was not checked after 3 weeks of cultivation. This would be easy to confirm with Live/Dead fluorescent markers.

Centrifugation is often used to form cell pellets of chondrocytes (or mesenchymal stem cells in chondrogenesis assays), so in a way this study appears to aim to replace such technology. Therefore, it would be advisable to compare the results of this technology to pellets made by centrifuging, since centrifuging as such is practically costless. Also, looking at Figure 5A, the H&E staining locates mainly to cells in bovine chondrocytes, which is rather surprising. I would have expected that staining would be more like the one shown for dental pulp cells in Figure 5B. The stainings would benefit from immunostaining for type II collagen, and maybe from additional staining for GAGs by stains such as toluidine blue or Safranin O (even though hematoxylin should do the same in H&E staining).

The irregular shape of the aggregates makes me wonder whether the number of cells differs in parallel samples. Therefore, DNA analysis would be useful to clarify this issue. The irregularity is problematic for tissue engineering purposes (even for small constructs), but as commented in the discussion, might be useful for chemical toxicity testing.

As another referee commented, the discussion in its present form is very minimal. In particular, what is required is the reasoning for why this technology is needed, and how it can overcome the present methods.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 05 February 2013

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Wael Kafienah

School of Cellular and Molecular Medicine, University of Bristol, Bristol, UK

This brief report described a method for aggregating matrix producing cells such as chondrocytes using synthetic hydrogel polymers. The polymers were used merely as non-adherent 'containers' to aggregate the cells. Indeed, many labs coat plastic wells with agarose to prevent cells seeded onto other biomaterials from sticking to the plastic well during the tissue engineering process.

It is important to note that the proposed study does not prove in any way if using the investigated polymers in this fashion is any better than say centrifuged pellets or using ultra-low adherence plates that can come in all kinds and shapes. The study however proves that the tested commercial polymers are biocompatible and permissive to cell aggregation. Hydrogels are inert material by nature unless they are functionalised or modified chemically. Indeed, the authors demonstrate no difference in the gross appearance of the aggregates between the different polymers used.

Furthermore, it is not clear what outcome is expected using either cell type in the study. What was the expected outcome for culturing chondrocytes or pulp cells using this method? The results do not determine whether the polymers merely supported cell aggregation or driven their differentiation. To this end, staining of specific matrix proteins such as aggrecan or type II collagen in the case of chondrocytes for example is critical to answer this question.

Other issues that the authors should address include:

- 1) show photos for histological analysis of all samples (including control). This applies for all kinds of staining (H&E, type II collagen, aggrecan, markers of dental pulp cells?).
- 2) dental pulp cells are uncharacterised. What are they?! How are they expected to behave on plastic or in other similar culture systems?
- 2) state the number of animals used in each case (n number). Anything less than 3 makes the results questionable.
- 3) higher magnification photos are needed for figures 1 to 4.

4) rehash the discussion which is not structured and minimal. The authors are advised to read this article:<http://www.bmj.com/content/318/7193/1224>

If the authors fulfill all of the above then this short report can be approved for publication.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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