To
Dr. Vidhya Iyer,
Review Editor
JoVE

February 15, 2021

Dear Dr. Vidhya Iyer,

Thank you for considering our manuscript, entitled: "In-vitro 3D Cell-Cultured Arterial Models for Studying Vascular Drug Targeting under Flow", Ref. No.: JoVE62279, for publication in *JoVE - the Journal of Visualized Experiments*.

We thank the editor and reviewers for the careful and thorough reading of our manuscript, the positive evaluation of our study and the constructive comments which helped us improve the scientific value of the manuscript. Correspondingly, we have now revised and modified the text as per the editor and reviewers' suggestions. Additionally, we have added new data to the revised manuscript. Please find below a point-by-point response to the editor and reviewers' comments and details of the revisions in the manuscript (the changes are highlighted in the revised manuscript as well). We believe that in its current form the manuscript is suitable for publication in *JoVE - the Journal of Visualized Experiments*.

Sincerely,
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Revision of Manuscript JoVE62279:

"In-vitro 3D Cell-Cultured Arterial Models for Studying Vascular Drug Targeting under Flow"

We are pleased to submit our revised manuscript entitled "In-vitro 3D Cell-Cultured Arterial Models for Studying Vascular Drug Targeting under Flow" to be considered for publication in JoVE - the Journal of Visualized Experiments. We would like to thank the Editor and the Reviewers for their valuable input, which we believe helped improve and strengthen the scope of the paper.

Changes to the text and figure legends are highlighted *in blue font color* in the revised manuscript. A point-to-point response is detailed below:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. E.g. Lines 103, 105, 135: "..at RT/ 60 oC" instead of "..in..", Line 174: "…and imaging" instead of "…and imagine" etc.

Reply: We thank the editor for this comment. The manuscript was re-edited for its English and the grammar issues were corrected.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Reply: The summary was rephrased as follows: "Here, we present a new protocol to study and map the targeted deposition of drug carriers to endothelial cells, in fabricated real-sized 3D human artery models, under physiological flow. The presented method may serve as a new platform for targeting drug carriers within the vascular system".

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Reply: The reference numbers have been changed to subscripts, according to JoVE references style.

4. Use SI units as much as possible and abbreviate all units: L, mL, μL, cm, kg, h, min, s, etc.

E.g. Line 98: use "g" instead or "gr", Line 123, 132, 135, 157: "mL" instead of "ml" etc.

Reply: The units and abbreviates have been changed as requested.

5. Line 78: Please specify the type/grade of plastic used for mold construction. Also use "..Figure 1 D, E" instead of "Figure 1 D +E".

Reply: We thank the editor for this comment. The plastic used for preparing the molds was added in the protocol as follows: "2. Print the geometries using a 3D printer. Note: in our case, the printed molds were made of clear resin v4". Also, the figures were changed as requested.

6. Line 82: Which alcohol? Concentration?

Reply: Isopropyl Alcohol. This was modified in the manuscript as follows: "5. Rinse the sanded model with isopropyl alcohol AR-grade 99.5 % to remove the plastic dust and allow to completely dry in a chemical hood, for 2-3 h".

7. Line 117: For how long should the sterilization be carried out?

Reply: For 20 min. This was changed in the manuscript as follows: "2. Sterilize the model and the connectors by UV irradiating them in a biological hood for 20 min".

8. Line 121: Remove fibronectin from where? Endothelial cells medium composition?

Reply: Prior seeding the cells, fibronectin (in PBS) was added to the model for 2 h in incubator or overnight in 4 °C. Therefore, before adding the cells, the fibronectin needs to be removed from the model. This was rephrased in the manuscript as follows: "3. Coat the models with 4 ml 100 μg/mL fibronectin (in phosphate-buffered saline, X1 (PBS)) for 2 h, at 37 °C, or overnight, at 4 °C. Inject the fibronectin solution to the model through the inlet, using a 5 or 10 ml plastic syringe. 4. Remove the fibronectin through the outlet and wash the model with endothelial cell medium".

9. Line 122: Please specify the unit for the cell concentration. E.g., /mL

Reply: Cells concentration seeded inside the models was 2.5x106 cells/mL. This was added to the manuscript as follows: "5. Suspend 2.5x106 cells/mL human umbilical vein endothelial cells (HUVECs, passage<6), and fill the model with 4 mL suspended cells, using a 5 or 10 mL plastic syringe".

10. Line 132, 134: 1X PBS?

Reply: Yes. This was added to the manuscript: "8. Remove the model from the rotator and wash with X1 PBS using a 10 mL plastic syringe".

11. Line 182: Please specify the step numbers corresponding to "..as explained above..".

Reply: We thank the editor for this comment. This was modified as follows: "6. Connect the inlet and outlets of the carotid as described in steps 3.4-3.6".

12. Line 184: Which particles? Figure 4 mentions use of fluorescent tags – please provide details about these.

Reply: We thank the editor for this comment. This manuscript presents a new method to fabricate real-sized arterial models and how to map deposition of particles inside the model. Carboxylated Polystyrene (PS) particles (2 µm) were used as representative model particles. This was mentioned in the manuscript as follows: "11. At 100 rpm, which equals the maximum flow rate of the physiological waveform of the human carotid artery (~ 400 mL/min), add fluorescent carboxylated polystyrene (PS) particles (2 µm, at a concentration of 1.6 µg/mL) to the 300 ml PBS in the closed-circuit container".

13. Line 192: Please provide details about the imaging E.g. equipment used, recording format (still image/video) etc.

Reply: For imaging we have used our Nikon stereomicroscope and camera, specified details were added to the Table of Materials. This was added as follows: "12. Image the region of interest every 10 s, for 1.5 h (still single images or video as needed)".

14. Line 208: What is the dummy model?

Reply: We thank the editor for this comment. The dummy model is an elastosil 3D carotid model used only for washing, not cultured with cells. This was clarified in the manuscript as follows: "5. Connect a "dummy model", (which is also an elastosil 3D carotid model used only for washing, without cultured cells), instead of the carotid model and rinse the system".

15. Line 214: Designate this as a "note". Remove the bullet point.

Reply: Done. The bullet point was removed.

16. Please use single line spacing between the protocol steps.

Reply: Done.

17. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." E.g. Line 136: "..stain the cells with.." instead of "cells can be stained…", Lines 147-160 etc.

Reply: We thank the editor for this comment. Sentences were rephrased as requested.

18. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Reply: Three pages have been highlighted.

19. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Reply: We thank the editor for this comment. The representative results were modified as follows: "Here we presented a new protocol to map the deposition of particles inside real-sized 3D human artery models, which may provide a new platform for drug delivery research.

Using a 3D printing technique, a model of the human carotid bifurcation artery was fabricated (Figure 1). The model was made of Elastosil and was seeded with human endothelial cells (Figure 2). Importantly, this protocol enabled replication of the physiological conditions, especially with regards to fluid dynamics. A perfusion system was designed to infuse particles to the carotid bifurcation under constant flow, at the magnitude of the physiological waveform characteristic of the carotid. Figure 3 presents the perfusion system which consists of the peristaltic pump, an oscillation damper, the cultured bifurcation model, tubing and fluids containers.

To map the deposition and adhesion of the perfused particles, the arterial model was imaged under a stereomicroscope, at the end of the experiment and after washing (step 5.3). The images were captured using x2 objective and tiled together to form a whole image of the model. Then, the number of adhered particles was calculated using a customized MATLAB code.

To examine the formation of the recirculation pattern at the bifurcation, 10 μm fluorescent glass beads were infused into the model. In Figure 4A, the recirculation was observed, which suggests that the conditions inside the model are physiological.

To map the deposition of particles inside the model, 2 μm fluorescent carboxylated PS particles were infused and their adhesion to the endothelial cells was imaged (Figure 4B, C). These particles adhered differently at various regions along the model, when more adhesion was observed out of the recirculation area, where wall shear stress is high. We have previously elaborated on these results and showed that the adhesion of particles is a function of the model's geometry, particle surface characteristics and shear stress[17].

These deposition maps are relatively simple and may be quickly obtained for screening drug carriers' affinity and targeting under physiological conditions in patient specific models".

20. In the references, please use a period "." between the initials of the last author and the article title instead of a comma ",". Please ensure that the following style is followed: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).]

Reply: The references format was changed to JoVE formatting style.

21. Please sort the Materials Table alphabetically by the name of the material, and do not embed it in the manuscript (Line 251). Also mention the equipment used E.g. microscope, imaging devices etc.

Reply: The table was modified as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The work by M. Khoury et al. introduces a new 3D printing method to create human bifurcated arterial models at anatomical size. They demonstrate the use of such models to study the targeting of vascular cells with various therapeutics. The work is innovative and has the potential to establish a high-throughput robust platform for a wide variety of drug screening assays. There are, however, a number of issues that need to be addressed in order to further improve the submitted manuscript.

Reply: We thank the reviewer for his positive evaluation and comments on the paper.

Major Concerns:

- The authors are generally quite vague about the "particle" used in the study. It is only late in the middle of protocol that they do mention what type of particles they are referring to here, what size/material they are, and what their real significance is. Using polystyrene particles at 2 um size for this study is not making much excitement, as it won't be clinically/biologically relevant. Why not using any of the nanoparticle systems that are actually used for drug delivery purposes (liposome nanocapsules, dendrimers, SPIONs, etc.)? What's the significance of perfusion with PS particles? How does that relate to any specific biological application?

Reply: We thank the reviewer for this important comment. The submitted manuscript presents a new technique to study deposition of particles in real-sized models of human arteries under physiological conditions. In these experiments, carboxylated polystyrene are used as model particles having uniform composition as well as a narrow size distribution, and they adhere to the cells mainly via electrostatic interactions. Indeed, specific targeting and ligand labeled particles, e.g., to anti-ICAM-1, anti-PECAM etc., increase the targeting and delivery of the particles to the site of the disease, i.e., endothelial cells. This also applies to other targeting carriers such as liposome nanocapsules, dendrimers nanoparticles etc.

However, different type of particles and the use of specific ligands have their specific attributes that require a study of their own – which is beyond the scope of our study, that focuses on the general methodology. We believe that our method would serve as a valuable platform to study targeting of other carriers under blood flow, as detailed by the reviewer (e.g., liposome nanocapsules, dendrimers), however these require a comprehensive study in which the specific drug carrier and their targeting approach and characteristics need to be considered and examined (e.g., magnetic targeting). This was added to the manuscript discussion as follows: "It is important to note that in this paper, carboxylated polystyrene particles, which have a uniform composition and a narrow size distribution were used. Moreover, these particles adhere to the cells primarily through electrostatic interactions. However, other drug nanocarriers can be used and, specific targeting, with ligand-labeled particles, e.g., to anti-ICAM-1, anti-PECAM etc., which increase particle accumulation to ECs at the site interest, should be examined as well.".

Moreover, we are currently performing experiments, in collaboration with other groups, where we use the presented protocol and system to study targeting of deformable particles with different moieties (e.g., anti-ICAM1, Sial-Lewis-A) to inflamed EC in our model.

- It is not clear why authors fixed the cell prior to perfusion. The main application for these types of systems would be to do live culture of cells in the models and perfusing them various molecules/particles. If fixed, how could particle targeting be of relevance? A live culture/perfusion and targeting assay would be needed here.

Reply: We thank the reviewer for this comment. In our perfusion experiments we use fixed cells, because we focus on the initial adhesion step of the particles, especially large ones, upon reaching the vessel wall, as previously described by other groups in past studies (see for example: PMID: 19721193). In this work, it was shown that particles, in the first stage of adhesion, bind to live and fixed cells similarly. However, internalization of particles was observed only by live cells in following stages. Therefore, we understand the reviewer's comment and the need to perform such experiments in live-cell models since full replication of the physiological conditions should include targeting to live ECs. 3D models with live cells have been used by other groups, however, they were used for biological studies (see for example: PMID: 24841070) and therefore, we believe our system can be used for targeting experiments with live cells, but this was not the focus of our work. This note was referred in

the discussion by added the following: "In addition, in the presented protocol, ECs were fixed prior to connecting the models to the perfusion system and injection of the particles. Particles' adhesion to fixed cells represents the first stage in the binding process and therefore, experiments with live cells need to be performed, where internalization of particles may occur during later stages of the adhesion process".

- Figure 4 is not clear and properly presented. Did authors use two different particle types with different fluorescence? This needs to be clarified in the caption and with proper labeling in the figure. Further, there is too much background (auto)fluorescence in panel B which makes it really difficult to conclude any rigid observation from this image. A control picture of untreated sample should be presented to compare the background signal. Also, higher magnification images should be provided to show clearly the cells, the particle accumulation, etc..

Reply: We thank the reviewer for this comment, and Figure 4 was updated accordingly. Prior to studying targeting of particles in cell cultured model, we have conducted experiments with 10 µm glass particles to visualize whether the formation of the recirculation occurs at the bifurcation sinus. Figure 4A confirms that using our system, physiological flow structures are obtained as expected. Afterwards, we performed experiments with 2 µm fluorescent carboxylated PS particles (in red) to ECs cultured inside the models (in blue) and tracked their deposition and adhesion (Figure 4B, 4C). Figure 4 legend was changed to: Figure 4: Perfusing and mapping the adhesion of particles. (A) Streak-line image of streamlines and recirculation (dashed rectangle) generated upon perfusion of 10 µm fluorescent glass particles, at a constant flow of 400 mL/min, through the model. (B) Deposition map of the 2 µm fluorescent carboxylated PS particles (in red) inside the 3D cultured model. (C) Adhesion of the particles (in red) to the cultured ECs (in blue – DAPI) inside the model at a 10X magnification."

Regarding the control untreated sample, we include an image of the cultured ECs inside the models without the particles, which is shown in Figure 2D. It is important to notice that Figure 4B shows the deposition of the red fluorescent 2 µm particles with high accumulation at one side of the bifurcation sinus which is characterized with a high shear regime – and it is not an autofluorescence effect.

Minor Concerns:

- Add proper labeling in Figure 3 to introduce all components of the perfusion system in the figure.

Reply: Done.

Reviewer #2:

Manuscript Summary:

The manuscript is well-written and the contents are of interest of a broad readership especially in 3D printing in medical research. The video presentation of the work would be a very interesting one and beneficial.

Reply: We thank the reviewer for his positive evaluation on the paper's contribution to the field.

Major Concerns:

Nil.

Minor Concerns:

The manuscript needs further copyediting for its English.

Reply: We thank the reviewer for this comment. English copyediting was done, and the manuscript was re-edited as requested.