**Stem Cell-Laden Microspheres as Building Blocks for Large Cardiac Tissue Production Through Secondary Encapsulation in Poly(ethylene glycol)-Fibrinogen**

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**Statement of Purpose:** This work investigates the use of differentiating 3D microspheroidal engineered cardiac tissues (mECTs) as building blocks for future large scale cardiac tissue production (>1 $cm^3$). To produce cardiac tissues of the size required for many regenerative therapies and disease modeling, advances are needed in cell production and tissue assembly. We have shown hydrogel-supported differentiation of encapsulated hiPSCs results in a high degree of hiPSC-derived cardiomyocyte structural maturation, including T-tubule formation, without exogenous electrical or mechanical stimulation[1]. This approach has been applicable in a range of tissue sizes and geometries, including microspheres. These mECTs can be produced in scalable, suspension culture, making them advantageous for use in assembly of large tissues; employing microtissues for production of macroscale cardiac tissue (>1$cm^3$) eliminates concerns about the rapid, uniform distribution of small molecules required to induce cardiac differentiation. However, bringing together differentiating ECTs at earlier timepoints yields better interconnectivity of resulting cardiomyocytes. Therefore, here we investigated the ability to encapsulate mECTs in secondary hydrogel microisland scaffolds and examined the impact of mECT differentiation day (DD) at the time of secondary encapsulation on long-term functional properties of the resulting macroscale ECTs.

**Methods:** Microsphere ECTs (mECTs) were produced using previously established methods[2]; briefly, human induced pluripotent stem cells (hiPSCs) were suspended in poly(ethylene glycol) fibrinogen (PF) polymer precursor solution and microspheres were formed using a custom microfluidic device platform and photocrosslinking using visible light and the photoinitiator EosinY. Microsphere hiPSC-encapsulation occurred on differentiation day (DD) -3. Cardiac differentiation followed previously established protocols[1,3].

Secondary encapsulated tissues were produced during differentiation on DD 3, 5, and 7 through the encapsulation of microspheres into microislands. Microspheres were pretreated with ROCK inhibitor (RI) for one hour before encapsulation. To achieve a high microsphere density, microspheres were centrifuged for removal of excess media and an excess volume of PF was added. The mixture was centrifuged and excess PF removed. The microsphere-PF mixture was crosslinked using UV light and the photoinitiator lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate (LAP) into a PDMS microisland mold; tissues were cultured until DD 40. Phase contrast images were taken every 3 days until DD 20 and contraction analysis and immunostaining (Hoechst, WGA, and αSA) were performed on DD 40.

**Results:** Microspheres on DD 3, 5, and 7 were all successfully encapsulated to form microisland tissues. Centrifugation was found to be important to maximizing microsphere density and removing cell culture media in the larger volumes of microspheres (>1ml) needed for larger cardiac tissue formation. However, centrifugation negatively impacted cell viability. Therefore, pretreating microspheres with RI one hour before secondary encapsulation was tested; pretreatment eliminated negative impacts of centrifugation and resulting tissues showed substantially fewer dead cells than non-pretreated tissues.

For all tested initial secondary encapsulation timepoints (DD 3, 5, and 7), microspheres fused together to form larger composite tissues and microspheres lost their initial microspheroidal shape. Synchronous, full-tissue contraction was observed in the cardiac tissues formed at all secondary encapsulation timepoints (DD40). Contraction analysis showed that contraction frequency and time interval are similar to that of previously studied microisland tissues formed through encapsulation of hiPSCs on DD-3. All tissues showed evidence of αSA; no major differences in tissue formation of secondary encapsulation timepoints 3, 5, and 7 were observed through whole tissue immunostaining.

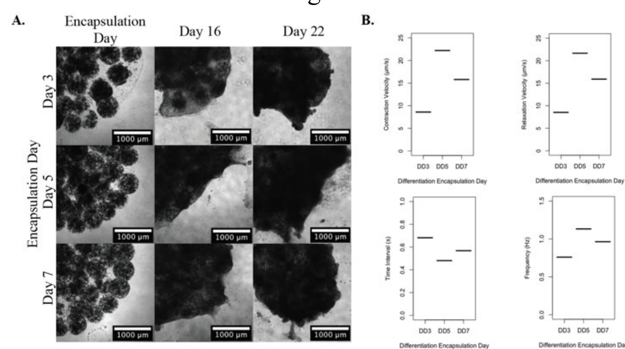


**Figure 1: A.** Phase contrast images of secondary encapsulated microspheres into microislands on their initial secondary encapsulation day (DD 3, 5, and 7) and following further cardiac differentiation on DD 16 and DD 22. **B.** Contraction analysis on DD 40 for each initial secondary encapsulation day (DD 3, 5, 7).

**Conclusions:** The secondary encapsulation of microsphere ECT building blocks into microislands showed that microsphere ECTs from DD 3, 5, and 7 can successfully produce larger tissues and the methods have potential for use in macroscale cardiac tissue production. Microsphere tissues fused together into larger tissues when encapsulated post-initiation of differentiation, resulting in synchronously contracting macroscale cardiac tissues. Further investigation rigorously comparing resulting cardiomyocytes and cardiac tissue functional properties in ongoing.

**References:**

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