Formation of Embryoid Bodies with Controlled Sizes and Maintained Pluripotency in Three-Dimensional Alginate Inverse Opal Scaffolds

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Statement of Purpose: Embryonic stem (ES) cells hold great potential for therapeutic applications because of their unique capability in terms of unlimited self-renewal and differentiation into a rich variety of cell lineages. During early embryo development, ES cells aggregate into a spherical cluster, in which the cells then gradually migrate and differentiate into different germ layers. Significantly, this feature could be partially conserved and recapitulated *in vitro* by assembling ES cells into embryoid bodies (EBs) prior to the actual fate determination. Although there have been a few methods to produce EBs such as those using non-adhesive substrates, hanging drops or microwell arrays, they suffer from disadvantages including polydispersed sizes of EBs or limited scalability. Recently, three-dimensional (3D) inverse opal scaffolds with uniform and yet precisely controllable pore sizes have been developed for various applications in regenerative medicine. Here we further take the advantage of the uniform structures of these scaffolds and propose that, EBs with uniform, controlled sizes can be obtained in large numbers by seeding and growing ES cells in cross-linked alginate inverse opal scaffolds. The EBs can then be conveniently recovered by disintegration of the alginate scaffolds.

Methods: An alginate inverse opal scaffold was fabricated by templating against a lattice of uniform polystyrene microspheres [1]. Briefly, the lattice was placed in an oven heated at 110 ^{o}C for 2 h to induce the necking between adjacent polystyrene microspheres. The lattice was then treated with plasma and infiltrated with a 4% aqueous solution of alginate. After removing the excess alginate solution with filter paper, the pellet was frozen in a refrigerator (-20 ^{o}C) for 5 h, and then lyophilized in a freeze-dryer overnight. The pellet was immersed in dichloromethane (DCM) for 5 h to completely remove polystyrene. The alginate inverse opal scaffold was afterwards cross-linked in ethanol containing 2% $CaCl_2$ for 1h. The scaffold was finally washed twice with water. A total of 2×10^5 mouse ES cells were used to seed into each scaffold using a spinner flask at 60 rpm for 24 h. The culture was maintained in a non-adhesive plate.

Results: The alginate inverse opal scaffolds had a uniform pore size of 230 μm and windows connecting adjacent pores (Fig. 1A). These windows provided the scaffolds with high interconnectivity which could promote the transport of nutrients and ensure the homogeneous seeding of cells. At day 1 post seeding, small aggregates of ES cells could already form and distribute uniformly throughout a scaffold. These small aggregates could be a result of the proliferation of an anchored cluster of cells during the seeding process, or the aggregation of several anchored single cells (or clusters of cells) in the same pore, which is common in the presence of a surface non-adhesive to cells. The EBs gradually grew in size over time. By the end of culture for 14 days, the size (*ca.* 200 μm) of the EBs had almost reached the size (230 μm) of the pores (Fig. 1B). The physical confinement imposed by the pores limited the EBs from further growth, making it possible to control the maximum size of EBs derived from a scaffold. The EBs could then be efficiently recovered by disintegrating the alginate scaffolds using a Ca^{2+} chelating solution containing 100 mM EDTA and 100 mM K_2HPO_4 (Fig. 1C) [2]. Upon the addition of the solution, the scaffolds could reach nearly complete disintegration in less than 10 min. As a result, we were able to obtain EBs with uniform and controlled sizes from the scaffolds by varying the culture time or the pore size of the scaffold. Further *in vitro* studies demonstrated that the EBs produced using this method conserved high cell viability. More importantly, the EBs maintained their pluripotency, and they were able to differentiate into bone and neural lineages upon proper stimuli.

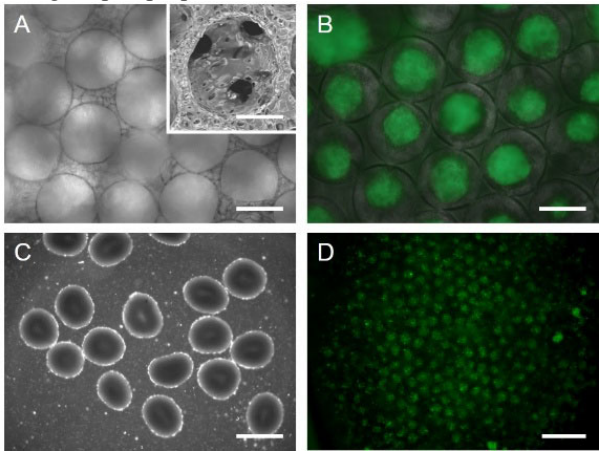

Figure 1. (A) A bright field image showing an alginate inverse opal scaffold. Inset shows an SEM image of a single pore of a freeze-dried sample; scale bar: 100 μm. (B) Superimposed fluorescence and bright field images showing EBs grown in a scaffold after 14 days of culture. (C) A bright field image showing EBs recovered by disintegrating the scaffold. (D) A fluorescence image at lower magnification showing hundreds of EBs formed in the top layer of the pores of a scaffold. Scale bars in (A-C) and (D) are 200 μm and 1 mm, respectively.

Conclusions: We have successfully developed an effective method for producing uniform, spherical EBs using 3D alginate inverse opal scaffolds. It is also noteworthy to mention that, a single scaffold with about 4 layers of pores could generate up to *ca.* 1000 EBs (Fig. 1D). Therefore, we expect our technique for generating large numbers of uniform EBs to be highly useful for regenerative medicine and stem cell research.

References: [1] Zhang Y. Adv. Funct. Mater. 2011;22:121; [2] Li Z. Biomaterials. 2010;31:404.