Thermoreversible scaffolds improve transient three-dimensional culture by facilitating rapid cell recovery

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Statement of Purpose: Three-dimensional (3D) scaffolds are important tools for examining biologically relevant cellular behaviors *in vitro*. However, the most common biomaterials used for 3D culture (Matrigel®, PEG, collagen, hyaluronic acid) are chemically crosslinked. Assays that utilize these materials are almost necessarily endpoint, and do not allow for live cells to be easily recovered. Physically crosslinked biomaterials are capable of reversible physical state changes (i.e. soluble to insoluble) in response to environmental stimuli such as temperature without any chemical reaction or byproducts. Our goal is to develop an improved platform for 3D cell culture using a physically crosslinked, thermoreversible hydrogel scaffold to facilitate rapid recovery of live cells under mild conditions. The hydrogel is composed of a poly(N-isopropylacrylamide), or PNIPAAm, copolymer. This material exhibits a lower critical solution temperature (LCST) transition that causes the polymer to undergo a solution-gel phase transition when heated in aqueous solution above the LCST. By adding hydrophilic moieties and functionalizing with an RGD cell-binding motif, this material is ideal for culturing cells in 3D in adherent conditions. We hypothesize that this platform will enable transient culture of human glioblastoma (GBM) cells in 3D while maintaining biologically relevant phenotypes.

Methods: Free radical polymerization of *N*-isopropylacrylamide (NIPAAm), Jeffamine® M-1000 acrylamide (JAAm), and hydroxyethylmethacrylate (HEMA) was used to form temperature-responsive graft copolymers. HEMA side chains were modified with acrylate endgroups through an acyl halide reaction. A cell adhesion peptide (CGRGDS) was conjugated to the copolymer (PNJ-RGD) for culturing cells in adherent conditions. Copolymer composition and molecular weight were characterized with ^{1}H NMR and HPLC. Hydrogel LCST and mechanical properties were analyzed by cloudpoint absorbance and temperature controlled rheology. Cell culture assays were performed using human U118 GBM cells. A live/dead fluorescence assay was used to determine biocompatibility within the gels up to 14 days. Cell recovery from 3D culture in PNJ-RGD was tested by trypsinizing and recollecting cells after up to 7 days in culture. The efficiency of cell recollection was determined by measuring viability of cells remaining in the gel in comparison to recovered cells. Proliferative and invasive behaviors of GFP expressing U118 were analyzed in PNJ-RGD gels by fluorescence confocal microscopy.

Results: Synthesis of PNJ-RGD copolymers was confirmed by ^{1}H NMR and HPLC, indicating a weight average molecular weight of 594 kDa and peptide concentration at 180 µmol/gram copolymer. Rheological measurements of the storage and loss modulus (G' and G'') of PNJ-RGD hydrogels during controlled heating measured the LCST at 29°C. The hydrogels were mechanically tunable, with stiffness (G') ranging from 40 Pa to 3 kPa, depending on total polymer content (Figure 1). PNJ-RGD gels were rapidly reversible when cooled below the LCST (Figure 1), whereas PNIPAAm homopolymer scaffolds did not reverse completely (results not shown). PNJ-RGD hydrogels were found to be biocompatible and maintained a high fraction (>85%) of viable GBM cells over an extended period (14 days). The rapid and complete reversibility of the PNJ-RGD scaffolds facilitated recollection of cells maintained in 3D culture. Cell recovery assays performed up to 7 days after initial culture resulted in a high fraction (>50%) of viable cells being recollected from the scaffolds. Finally, multicellular GFP expressing GBM spheroids cultured on top of PNJ-RGD gels were monitored with fluorescence confocal microscopy and showed signs of both proliferation and invasion into the gels over 6 days.

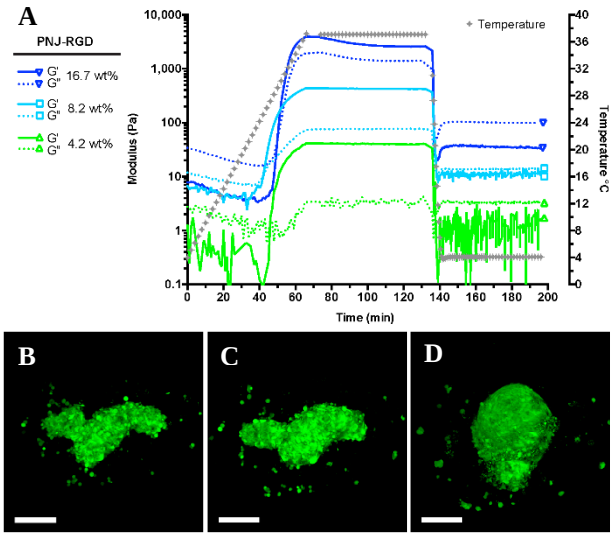

Figure 1: (A) Rheological Measurements of the storage and loss modulus (G' and G'') of PNJ-RGD at various concentrations when subjected to controlled heating and rapid cooling. (B-D) Proliferation and invasion of GFP expressing GBM cells after 1, 2, and 6 days in culture tracked by fluorescence confocal microscopy.

Conclusions: PNJ-RGD copolymers formed viscoelastic, physically crosslinked hydrogels when heated above 29°C, and gels dissolved rapidly upon cooling. The gel stiffness was mechanically tunable over nearly 2 orders of magnitude. These gels are biocompatible, support invasion and proliferation of human glioblastoma cells, and facilitate rapid recollection of viable cells from 3D culture. These attributes make PNJ-RGD a novel platform for performing transient 3D culture of invasive cancer cells.