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Notes & Tips

Purification of high-quality RNA from synthetic polyethylene glycol-based hydrogels

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ABSTRACT

Polyethylene glycol (PEG)-based hydrogels, with variable stiffness, are widely used in tissue engineering to investigate substrate stiffness effects on cell properties. Transcriptome analysis is a critical method for understanding cell physiology. However, significant RNA degradation was observed during the process of isolating and purifying RNA from cells encapsulated in the PEG hydrogel, thereby precluding purification of high-quality RNA. Here, we describe a simple protocol that prevents RNA degradation and improves the quality and yield of RNA isolated from cells cultured in PEG hydrogels. This modification produces high-quality total RNA suitable for RNA sequencing and microarray analysis.

Three-dimensional cultures are well-established tools to mimic cell behavior in living organisms more precisely than standard two-dimensional tissue culture plates. They are widely used to model the response of normal and tumor cells to tissue microenvironments [1,2]. One of the most important factors affecting cell behavior, including proliferation and regulation of signaling pathways, is the mechanical properties of the tissue. Polyethylene glycol (PEG)[1] hydrogels are used extensively as a matrix for cell encapsulation because they provide enormous flexibility in designing matrices with tunable mechanical properties for the analysis of matrix-dependent cellular behavior [3]. RNA expression profiling by microarray hybridization and RNA sequencing (RNA-seq) are the most powerful and widely used approaches for global analysis of cellular responses. Both approaches require purification of high-quality RNA from cells or tissues. However, the extraction of RNA from cells encapsulated in PEG gels results in mostly degraded RNA (low RNA integrity number [RIN]) [4,5].

Cells were encapsulated in the hydrogel as in Ref. [2]. Briefly, the hydroxy-terminated PEG was functionalized with acrylate groups by the reaction of acryloyl chloride with PEG hydroxy end groups as described previously [2]. First, 30 mg of the functionalized PEG macromer was dissolved in 270 µl of the initiator solution (0.5% initiator in phosphate-buffered saline [PBS]) by heating to 50 °C and vortexing for 5 min. Next, an MDA-MB-231 cell suspension in 100 µl of PBS was added to the hydrogel precursor solution and mixed gently with a glass rod. The suspension of cells in the precursor solution was degassed and ultraviolet (UV) irradiated with a mercury long-wavelength (365 nm) UV lamp (UVP, Upland, CA, USA) for 10 min as described in Ref. [2]. Initially, the cellular RNA was isolated with a combination of TRIzol reagent (Life Technologies) extraction and column purification as described previously [6] (see Fig. 1 for details). However, this standard procedure failed to provide high-quality RNA with RIN > 7 suitable for microarray analysis or preparation of RNA-seq libraries [5] (Fig. 1). Ribonuclease activity is a common cause of degradation of cellular RNAs. To prevent RNase-driven degradation, the gels were treated with RNAlater solution (Ambion), which is 70% ammonium sulfate and prevents RNA degradation by in-cell precipitation of riboprotein complexes. However, the RNase treatment provided only a minimal effect on RNA integrity (data not shown), suggesting that the gel components, rather than cellular RNases, caused RNA degradation. Therefore, as a control, cell-free hydrogel in an amount equivalent to our gel-embedded cells was added to the TRIzol reagent, and this TRIzol solution was mixed with previously purified high-quality total RNA (RIN > 7). The total RNA purified from the gel-containing TRIzol was significantly degraded, whereas no RNA degradation was observed in the control TRIzol reagent

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[1] *Abbreviations used:* PEG, polyethylene glycol; RNA-seq, RNA sequencing; RIN, RNA integrity number; PBS, phosphate-buffered saline; UV, ultraviolet; tRNA, transfer RNA.

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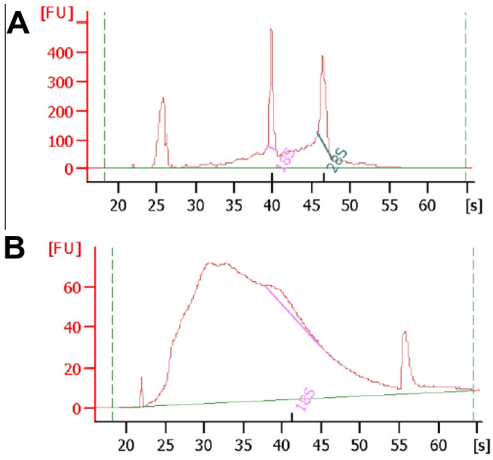

Fig.1. RNA degradation during purification from PEG hydrogels. Total RNA was purified using a combined TRIzol/column purification protocol. (A) MDA-MB-231 cells were embedded in PEG hydrogels (1 million cells/ml). (B) Control MDA-MB-231 cells plated on 60-mm tissue culture plates were lysed with 1 ml of TRIzol (Life Technologies) and homogenized using pellet pestle (Kimble/Kontes, Vineland, NJ, USA) followed by centrifugation. The aqueous phase was collected and total RNA was purified with an RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. RNA integrity was analyzed using an RNA 6000 Pico Kit on an Agilent Bioanalyzer.

(without gel) (see Supplemental Figs. S1A and S1B in online supplementary material). Because guanidine thiocyanate in concentrations used in TRIzol solution effectively inhibits any RNase activity, the results provide further support for the effect of gel components on RNA degradation. Moreover, incubation of purified RNA with hydrogel components (PEGDA macromer and photoinitiator) caused significant RNA degradation in a concentration-dependent manner (Supplemental Figs. S1C–G). Therefore, the results suggest that components of the PEG gel affected RNA stability in TRIzol. The mechanism of RNA degradation by the gel components in TRIzol reagent is unclear; however, recent studies suggest that the acidic conditions in TRIzol (∼ pH 4.5) [7] can accelerate degradation of acrylate-functionalized PEG gels [8].

To overcome the effect of gel components on RNA stability, RNA exposure to the gel-containing TRIzol was limited by grinding the gel in liquid nitrogen, followed by immediate lysis in TRIzol and column purification. As a result, RNA quality improved significantly. Whereas total RNA purified from the gels with a high concentration of encapsulated cells (4 million/ml) was of good quality (RIN > 7), RNAs purified from gels with a low concentration of cells (<0.5 million/ml) showed significant degradation (Fig. 2A and B). These results suggest that the large amounts of rRNAs (ribosomal RNAs) and tRNAs (transfer RNAs) in the gel samples with a high concentration of encapsulated cells acted as competitive inhibitors for the gel's RNA degradation activity. Therefore, the addition of heterologous tRNAs may protect the cellular RNA extracted from the cells encapsulated in the gel. As an external RNA, commercially available *Saccharomyces cerevisiae* tRNA (Sigma–Aldrich, cat. no. R8508) was added. Short (70–90 nucleotide) tRNAs are mainly washed out during column purification steps (RNAs < 200 nucleotides are excluded with a Qiagen RNeasy Kit [9]), and the remaining amount can be eliminated during steps in the RNA-seq library construction. Indeed, the addition of *S. cerevisiae* tRNA drastically improved the quality of RNAs purified from cells encapsulated in PEG hydrogels (Fig. 2C).

Based on our results, the following procedure was established for high-quality RNA purification from cells encapsulated in PEG hydrogels: (i) flash-freeze the cross-linked gels with encapsulated cells (prepared from ⩽ 0.5 ml of gel solution) using liquid nitrogen;

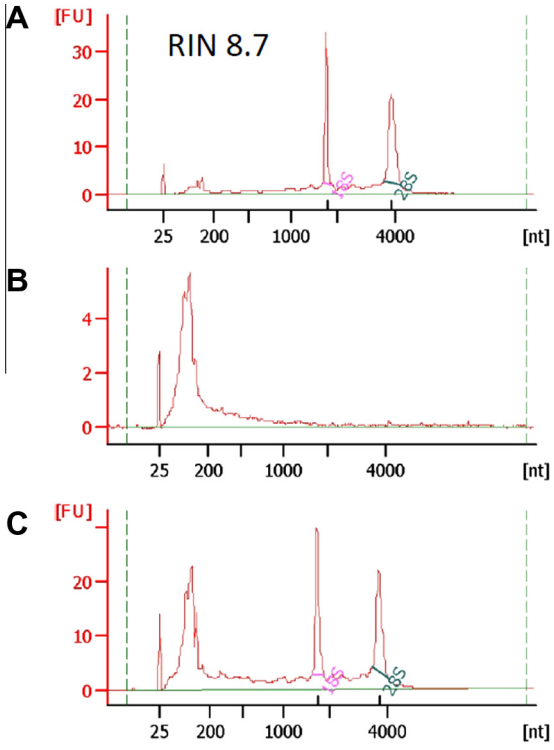

Fig.2. The addition of heterologous tRNA protects cellular RNA from degradation. PEG hydrogels (0.25 ml) with encapsulated cells (A: 2 million/ml; B and C: 0.5 million/ml) were homogenized in liquid nitrogen and lysed in TRIzol (A and B) or TRIzol with 5 mg/ml *Escherichia coli* tRNA (Sigma–Aldrich) (C). RNA integrity was analyzed using an RNA 6000 Pico Kit on an Agilent Bioanalyzer.

(ii) grind the gel (using mortar and pestle) in liquid nitrogen; (iii) quickly transfer the ground gel to a Dounce homogenizer by a liquid nitrogen-chilled spatula with 1 ml of TRIzol containing *S. cerevisiae* tRNA (5 μg/ml) and mix for five or six cycles; (iv) Incubate for 5 min at ambient conditions followed by centrifugation (10,000*g*, 10 min, 2–4 °C); (v) continue purification using the TRIzol/RNeasy hybrid protocol [6].

The procedure was used for purification of total RNAs from gels encapsulating MDA-MB-232, PC3, and DU145 cell lines. The purified RNAs were successfully used for the preparation of RNA-seq libraries. *S. cerevisiae* tRNAs are removed on multiple steps of library construction due to short size (<100 bp) and lack of polyadenylation. In addition, traces of yeast tRNA sequences are not aligned to the genome of mammalian cells; therefore, they will not affect RNA-seq results. Our modification of the total RNA purification procedure from cells encapsulated in PEG hydrogels significantly improved gene expression profiling of tissue microenvironments modeled with PEG hydrogels.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.05.002.

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