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PLGA-PEG-PLGA hydrogels induce cytotoxicity in conventional in vitro assays

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1 | INTRODUCTION

PLGA-PEG-PLGA hydrogels formed from poly(lactide-co-glycolide) (PLGA) and poly(ethylene glycol) (PEG) polymers are clinically relevant thermoresponsive biomaterials used to deliver biologicals in long-acting injectable formulations.[1,2] These hydrogels are easily injected as a solution and form a gel in situ at physiological temperature. Despite their successful administration in vivo,[3–5] the in vitro biological response to them is underinvestigated. Cell-based assays are utilized to evaluate cytotoxicity before preclinical studies[6]; however, the compatibility of conventional in vitro assays with PLGA-PEG-PLGA hydrogels is contradictory due to varied experimental procedures.[7–10] Here, we identified that PLGA-PEG-PLGA hydrogels induced significant cytotoxicity in vitro due to the rapid accumulation of degradation products that is not observed in vivo.

2 | MATERIALS AND METHODS

PLGA-PEG-PLGA (molecular composition specified in figure legends) copolymers (PolySciTech) were autoclaved before use, and 20% (w/v) solutions were made in sterile water. Hydrogel solutions were added to 96-well plates (50 µL/well) and incubated at 37°C for 1 h to form a gel. Hydrogels were washed once with warm media (200 µL/well) before experiments. Hydrogel extraction media (EM) was obtained by incubating 200 µL/well of culture medium (or water for polymer release studies) on top of hydrogels at 37°C for 24 h. Where stated, Tris base buffer was added to hydrogel solutions to increase the pH to 6.5–7.5 or incorporated into a culture medium (pH ~8) and added to hydrogels for 30 min (Figure 1D). Tris-media was replaced with a fresh medium to obtain EM. Polymer release was quantified by proton-nuclear magnetic resonance using a calibration sample of known polymer concentration.

Cell medium consisted of RPMI 1640 with 10% (v/v) fetal bovine serum and penicillin/streptomycin (100 U/100 µg/mL) for U937 monocytes or Eagle's Minimum Essential Medium with 10% (v/v) FBS, penicillin/streptomycin (100 U/100 µg/mL), and L-glutamine (2 mM) for human foreskin (BJ) fibroblasts (CRL-2522, ATCC).

Cell viability was measured by WST-1 cell viability/proliferation assay (Roche) and Live/Dead Cell Double Staining kit (Sigma), according to the manufacturer's instructions. U937 monocytes (1×10^5 cells/well) and fibroblasts (4×10^3 cells/well) were seeded in 96-well plates. Cell suspensions were added on top of washed hydrogel surfaces. EM was added to cell monolayers adhered to tissue culture plastic.

In alternative culture formats, fibroblasts (2×10^4 cells/well) were seeded in 24-well plates for 24 h, then Transwell inserts (0.4 µm, polyethylene terephthalate; Falcon) containing hydrogels

(50 μL/insert) were added (Figure 1I). In six-well plates, 50 μL of hydrogel solution was pipetted into the well center to form a dome (Figure 1I). Following gelation, fibroblasts (1×10^5 cells/well) were added and visually monitored daily.

3 | RESULTS

As an initial assessment of cytotoxicity, cells were seeded directly onto PLGA-PEG-PLGA hydrogels. After 48 h of culture <20% of monocytes remained viable compared to control cells ($p < .001$; Figure 1A). To determine whether hydrogels released soluble toxic compounds, monocytes were cultured in hydrogel-EM for 24 h, which caused similar cytotoxicity (Figure 1B) and indicated cell death was caused by reagents released from the hydrogel.

As inflammatory monocytes can destroy their environment, subsequent investigations were performed in fibroblasts. To determine whether toxic products continued to diffuse from hydrogels over time, hydrogels were incubated in a medium for 24 h (EM-1), followed by further 24 h incubation in a fresh medium (EM-2). EM was added to fibroblasts, undiluted or diluted with fresh medium (1:1). Fibroblasts cultured in EM-2 had significantly greater cell viability compared to those cultured in EM-1 ($p < .005$; Figure 1C). Dilution improved cell viability only in the case of EM-1 ($p = .052$; Figure 1C). As hydrogel degradation produces lactic and glycolic acid,[11] Tris buffer was utilized to reduce the EM acidity. Hydrogels were either mixed with Tris buffer before gel formation (Gel 2, Figure 1D) or incubated in a media "wash" containing Tris buffer (Gel 3, Figure 1D). Adding Tris buffer to EM-1 of Gel 3 significantly increased fibroblast viability ($p < .05$); however, fibroblasts cultured in EM-2 of Gel 2 had the greatest improvement in viability ($p < .01$). Nevertheless, all conditions resulted in substantially reduced fibroblast viability compared to control ($p < .001$; Figure 1D). These results indicate that acidic degradation products contribute to the cytotoxicity of PLGA-PEG-PLGA hydrogel. A reduction in the polymer's average molecular weight (Figure S1A) and hydrogel strength (Figure S1B) was observed following autoclaving. However, lactic and glycolic acids were not detected (Figure S1C), and the solution pH of non-autoclaved and autoclaved polymers was 2.73 and 2.83, respectively, which indicates that autoclaving did not substantially increase acidic compounds.

PLGA-PEG-PLGA polymer exhibits an amphiphilic structure, which disrupts cell membrane integrity.[12] We thus investigated whether the release of polymer induced cytotoxicity. Hydrogels released up to 6.3 ± 0.1 mg/mL of free PLGA-PEG-PLGA polymer in EM-1 after 24 h that was reduced to 3.4 ± 0.3 mg/mL in EM-2 (Figure 1E). Fibroblasts cultured in a medium containing up to 10 mg/mL of polymer were viable (Figure 1F), retained normal morphologies (Figure 1G), and showed increased metabolic activity compared to control cells (Figure 1H). These data suggest that whilst fibroblasts detect and respond to PLGA-PEG-PLGA released by the hydrogel, the polymer is not cytotoxic.

Finally, to determine whether the sudden addition of EM to fibroblasts induces cytotoxicity, we indirectly cultured fibroblasts with the hydrogel using Transwell inserts (Figure 1I). Fibroblasts remained viable and proliferative with normal morphologies for up to 7 days. We also seeded fibroblasts in direct contact with a hydrogel dome, which enabled fibroblasts to adhere to the adjacent tissue-culture plastic due to a lack of cell-adhesive sites in the hydrogel (Figure 1I). Fibroblasts appeared viable with normal morphologies for up to 3 days, which corresponds to previous reports of high fibroblast viability in similar direct and indirect contact assays.[13] However, due to weak hydrophobic interactions that govern gel formation[14] (Figure 1J), the volume of media required in 2D cultures disrupted the weak bonds and caused excessive hydrogel dilution that resulted in gel loss within 3 days. This contrasts in vivo studies where hydrogels remain intact for up to 6 weeks.[15] Therefore, controlled exposure of fibroblasts to hydrogel degradation products may improve cell viability, but adapted culture formats are required to retain the integrity of hydrogels that are sensitive to dilution.

Significance statement

We identified that PLGA-PEG-PLGA hydrogels, which have been used in human clinical trials and possess a demonstrable safety profile, induced significant cytotoxicity in conventional in vitro assays. This major contradiction may lead to inconsistent and misleading toxicology due to the limited biological representation of these assays. Cytotoxicity evaluation is a crucial element of screening the biological response to new biomaterials. However, as standard test methods do not recapitulate the in vivo environment, tailored adaptations may be required to reflect the true biological response elicited toward novel biomaterials.

4 | CONCLUSION

PLGA-PEG-PLGA hydrogels exerted cytotoxicity on monocytes and fibroblasts. The high volume of media required in 2D cultures induced rapid hydrogel degradation and accumulation of acidic compounds that are not observed in vivo. To study the cytotoxicity of novel hydrogels in vitro, conventional 2D models may require adaptations to capture a more representative biological response.

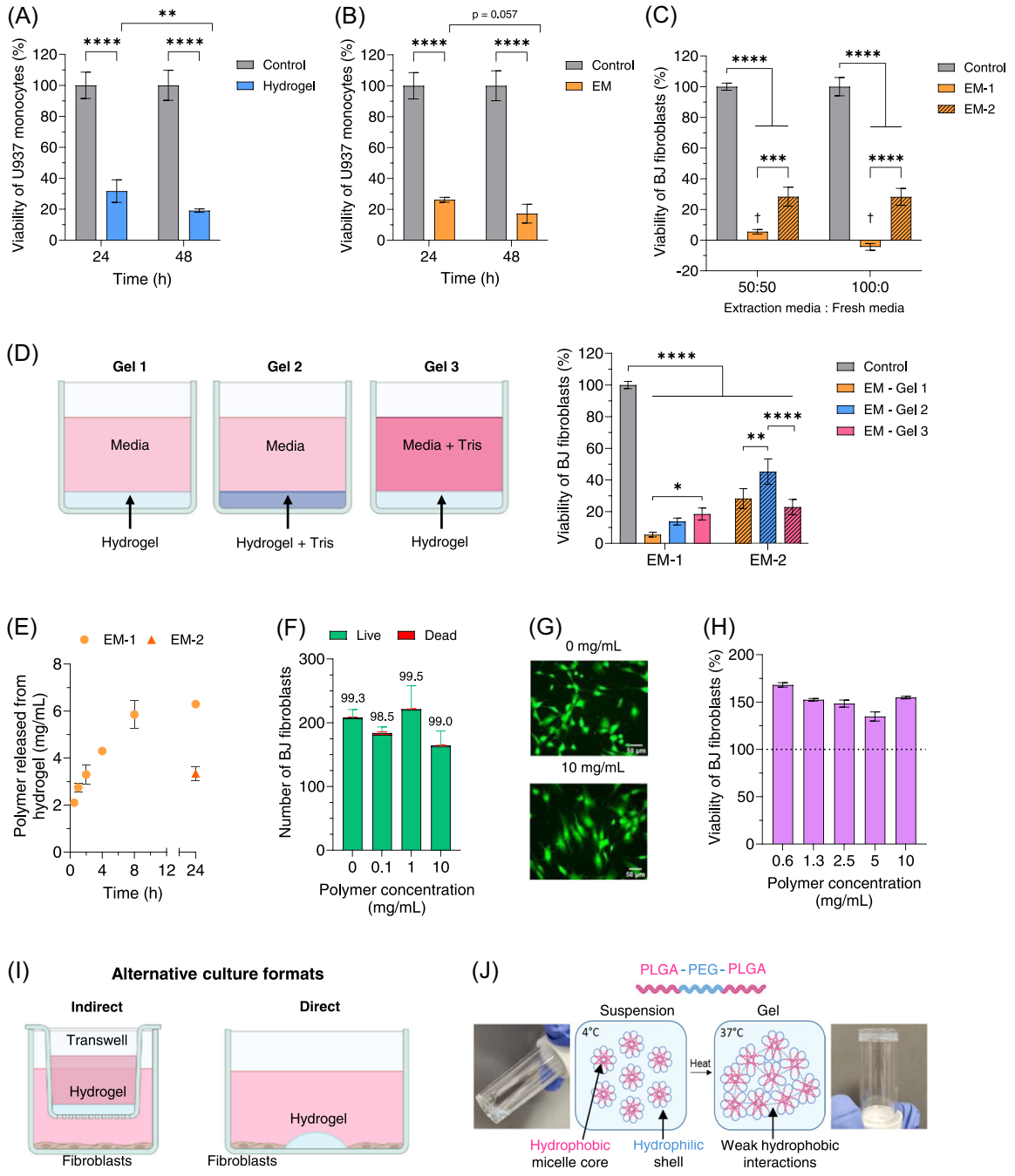

FIGURE 1 (A) Viability of monocytes cultured on hydrogels ($n = 4–6$). (B) Monocyte viability in hydrogel-extraction medium (EM) ($n = 4–5$). (C) Viability of fibroblasts cultured in EM-1 and EM-2 either undiluted or diluted with equal parts of fresh medium ($n = 3$). (D) Viability of fibroblasts cultured in diluted (1:1 with fresh medium) EM-1 and EM-2 obtained from Gels 1, 2, and 3 ($n = 3$). (E) Amount of PLGA-PEG-PLGA released in EM-1 and EM-2 when the hydrogel is incubated in water at 37°C. (F) Number of live and dead fibroblasts in polymer-spiked media (0.1–10 mg/mL) ($n = 3$); the percentage of viability is expressed above each bar. (G) Images of live fibroblasts in polymer-spiked media (10 mg/mL) and stained with calcein-AM. (H) Viability of fibroblasts in polymer-spiked media (0.6–10 mg/mL) expressed as a percentage of control cells. (I) Schematic of indirect culture with hydrogel placed into a Transwell insert and direct culture of fibroblasts with hydrogel. (J) Schematic depicting PLGA-PEG-PLGA polymer structure and hydrogel formation at 37°C by weak hydrophobic interactions between aggregated polymer micelles. Error bars represent standard deviation. (A–D) Two-way ANOVA with (A, B) Fisher's LSD and (C, D) Sidak's post hoc testing (†$p = .052$, *$p < .05$, **$p < .01$, ***$p < .005$, ****$p < .001$). PLGA-PEG-PLGA copolymer consisted of M_n 1410-1500-1410 g mol^{-1}, lactide:glycolide molar ratio 13.5:1, PLGA:PEG weight ratio 1.90. PEG, poly(ethylene glycol); PLGA, poly(lactide-co-glycolide).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data generated in this study are available within the article and its Supplementary Information files or from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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