

THE ROLE OF METHYLCELLULOSE
IN THE DEVELOPMENT OF PHYSIOLOGICALLY
RELEVANT IN VITRO MODELS FOR CANCER RESEARCH

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Abstract

Three-dimensional (3D) spheroid models are essential in cancer research, offering greater physiological relevance than 2D cultures by mimicking tumour microenvironment features. This study presents the first systematic, concentration-dependent optimization of methylcellulose as a semi-solid scaffold for spheroid formation. We identified 2.5 mg/mL methylcellulose and a seeding density of 30 000 cells/mL as optimal for generating uniform, structurally stable spheroids with high viability. Unlike previous studies using methylcellulose mainly for aggregation, we highlight its dual function in supporting both spheroid integrity and invasion in a concentration- and density-dependent manner. Lower concentrations (1.25 mg/mL) supported formation at low cell densities, while higher concentrations (> 2.5 mg/mL) reduced viability, likely due to limited nutrient diffusion. Moreover, spheroids formed under optimized conditions and embedded in collagen I exhibited strong TGF- β -induced invasion, replicating epithelial-to-mesenchymal transition features. Our findings position methylcellulose as a tunable, reproducible scaffold for producing viable, invasive 3D tumour spheroids, establishing a physiologically relevant platform for investigating cancer progression and therapeutic response.

Key words: spheroids, methylcellulose, invasion, cancer

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Introduction. Advancing cancer research requires in vitro models that replicate the complexity of in vivo tumour biology [1,2]. While 2D cultures have long served as a standard for studying cellular processes and drug responses, they lack critical features such as cell-cell and cell-matrix interactions, limiting their ability to mimic the tumour microenvironment and reducing predictive relevance [3].

In vivo, tumour cells reside in a 3D microenvironment, interacting with the extracellular matrix (ECM) and neighbouring cells, which regulate proliferation, migration, invasion, and treatment response [4,5]. In a 3D setting, gradients of oxygen, nutrients, and signalling molecules further contribute to drug resistance, while ECM mechanics affect cell motility and metastasis [6,7]. These features are challenging to replicate in 2D systems, highlighting the need for advanced 3D culture models.

3D spheroids more accurately represent tumours by replicating in vivo features like proliferative gradients, hypoxia, and ECM-like interactions. These qualities make them valuable for studying invasion, metastasis, and therapeutic response [8,9]. However, current spheroid generation methods such as hanging drop and rotary culture are labour-intensive, require specialized equipment, and often yield inconsistent results [10], creating a need for simpler, scalable, and reproducible techniques.

Methylcellulose, a semi-synthetic polymer, has emerged as a promising alternative. Its non-adhesive, viscous properties support cell aggregation without external scaffolds or complex setups [11]. It is also compatible with high-throughput applications such as drug screening and invasion assays [12]. Additionally, methylcellulose mimics ECM characteristics, enabling studies on cancer cell migration and invasion – key aspects of metastasis [13].

This study presents the first systematic investigation into the concentration-dependent role of methylcellulose in spheroid formation, optimizing both methylcellulose concentration and cell density to improve spheroid viability and invasion potential. Our findings provide novel insights into using methylcellulose for 3D cancer models, with implications for invasion assays and therapeutic testing.

Materials and methods. Methylcellulose preparation. A 50 mg/mL methylcellulose stock solution was prepared by dissolving methylcellulose powder (Sigma-Aldrich, Cat No. 9004-67-5) in sterile distilled water under constant stirring at room temperature. The solution was sterilized by autoclaving and stored at 4°C. For experiments, it was diluted to final concentrations of 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL.

Cell culture. Human MDA-MB-231 cancer cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco™, Cat. No. 11965092) supplemented with 10% fetal bovine serum (FBS; Gibco™, Cat. No. A5256701) and 1% penicillin-streptomycin. Cells were trypsinized, counted, and prepared for spheroid formation.

Spheroid formation. Cells were suspended in mixture of methylcellulose solution (final concentrations: 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL of methylcellulose) and complete medium at densities of 10 000, 30 000, and 50 000 cells/mL. The suspensions (100 μ L/well) were seeded into U-shaped ultra-low attachment 96-well plates (BIOFLOAT™, Cat. No. F202003) and incubated at 37 °C in 5% CO₂. Spheroid formation was monitored daily.

Cell viability. Viability was assessed using a dual-staining method with propidium iodide (PI) and Calcein-AM. A dye mixture of 200 μ g/mL PI and 2 μ g/mL Calcein-AM was added to the spheroids, and the plates were incubated for 1 h at 37 °C in 5% CO₂. Spheroids were visualized under a Zeiss AxioVert 200M microscope. Calcein-AM (green) labelled live cells, and PI (red) labelled dead cells. Images were captured to assess viability and structural integrity.

Invasion assay. Mature spheroids (72 h post-formation) were embedded in a 2 mg/mL collagen I matrix (Corning® Collagen I, Cat. No. 354236) and incubated for 72 h. Invasive behaviour and cell morphology were observed using light microscopy.

Statistical analysis. The data are presented as the mean values $\pm$ SEM of two independent experiments. One-way ANOVA (Graph Pad Prism8, San Diego, CA, USA) was used to compare the means between groups. *P*-values less than 0.05 were considered to indicate statistically significant results.

Results. Effect of methylcellulose concentration on spheroid formation across cell densities. To assess the effect of methylcellulose on spheroid formation, we tested varying cell densities (10 000, 30 000, and 50 000 cells/mL) and methylcellulose concentrations (1.25, 2.5, and 5 mg/mL). In the absence of methylcellulose, spheroid formation was density-dependent: no uniform spheroids formed at 10 000 cells/mL after 72 h, while uniform spheroids formed at 30 000 cells/mL by 72 h and at 50 000 cells/mL within 24 h (Fig. 1A). At 1.25 mg/mL methylcellulose, spheroid formation was enhanced, with uniform spheroids forming at 10 000 cells/mL by 72 h, and at 30 000 and 50 000 cells/mL within 24 h (Fig. 1B). Increasing methylcellulose concentration to 2.5 mg/mL further accelerated spheroid formation, with uniform spheroids forming at all cell densities within 24 h and maintaining structural integrity for 72 h (Fig. 1C). No further improvements were observed at 5 mg/mL, suggesting 2.5 mg/mL is the optimal concentration for spheroid formation (Fig. 1D).

Cell viability assessment of spheroids across varying cell densities and methylcellulose concentrations. Cell viability analysis revealed distinct patterns across conditions. In low-density (LD; 10 000 cells/mL) spheroids, 1.25 mg/mL methylcellulose favoured viable cells, while 2.5 mg/mL and 5 mg/mL conditions showed a higher proportion of dead cells. In medium-density (MD; 30 000 cells/mL) spheroids, 1.25 mg/mL methylcellulose resulted in a balanced live-to-dead ratio, while 2.5 mg/mL favoured a higher proportion of live cells. For high-density (HD; 50 000 cells/mL) spheroids, the live-to-dead ratio was sim-

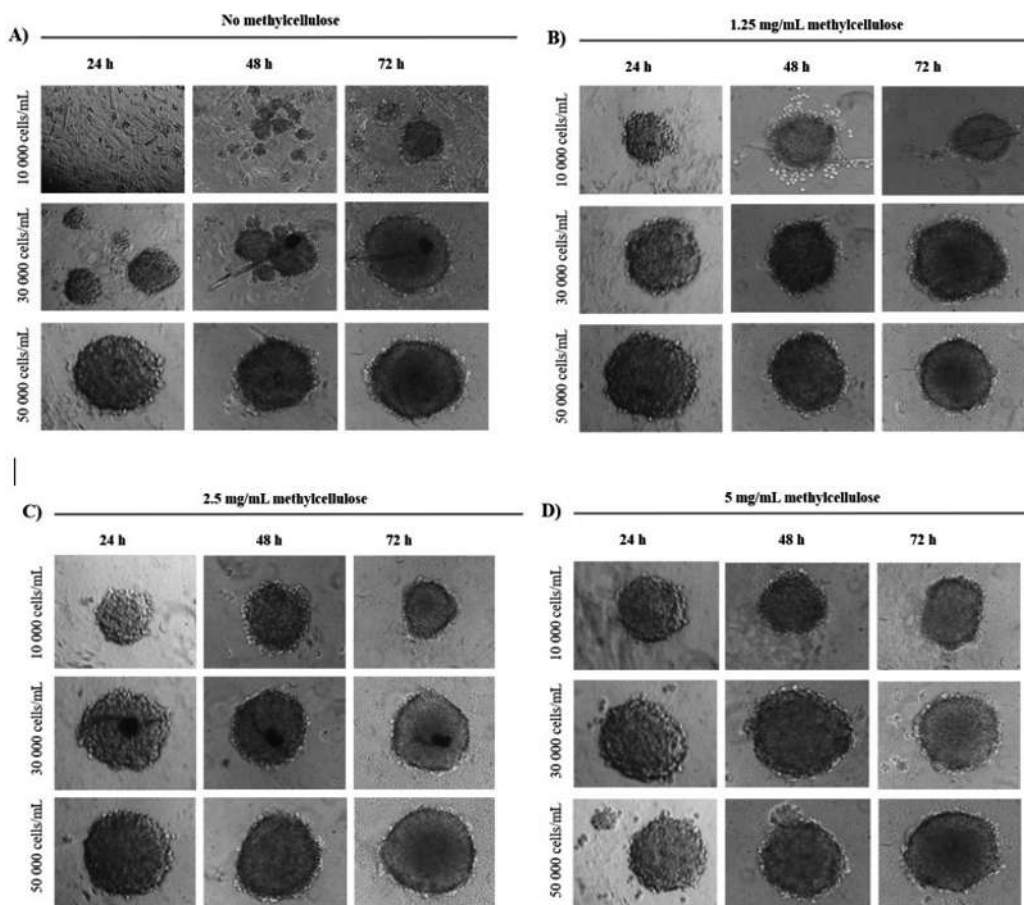


Fig. 1. Effect of methylcellulose concentration on spheroid formation across cell densities and time points. (A) Representative images of spheroids formed with 0 mg/mL methylcellulose at 10 000, 30 000, and 50 000 cells/mL after 24, 48, and 72 h, showing initial aggregation at lower cell densities and compact spheroids at higher densities. (B) Spheroids formed with 1.25 mg/mL methylcellulose under the same conditions, demonstrating increased compactness and uniformity with higher methylcellulose concentrations. (C) Spheroids formed with 2.5 mg/mL methylcellulose, showing enhanced compaction and circularity, particularly at higher cell densities. (D) Spheroids formed with 5 mg/mL methylcellulose, displaying denser and more compact spheroids at all cell densities, though smaller spheroids are observed at 10 000 cells/mL

ilar across all methylcellulose concentrations, with no significant differences. The 30 000 cells/mL and 2.5 mg/mL methylcellulose condition was chosen for further studies due to its optimal balance of viability and spheroid formation (Fig. 2). This combination produced the most uniform spheroids with high viability and robust invasive capabilities, emphasizing the importance of parameter optimization to achieve reliable models that accurately mimic *in vivo* tumour biology.

TGF- β -induced spheroid invasion. Spheroids formed with 30 000 cells/mL and 2.5 mg/mL methylcellulose exhibited significant invasion when embedded in a

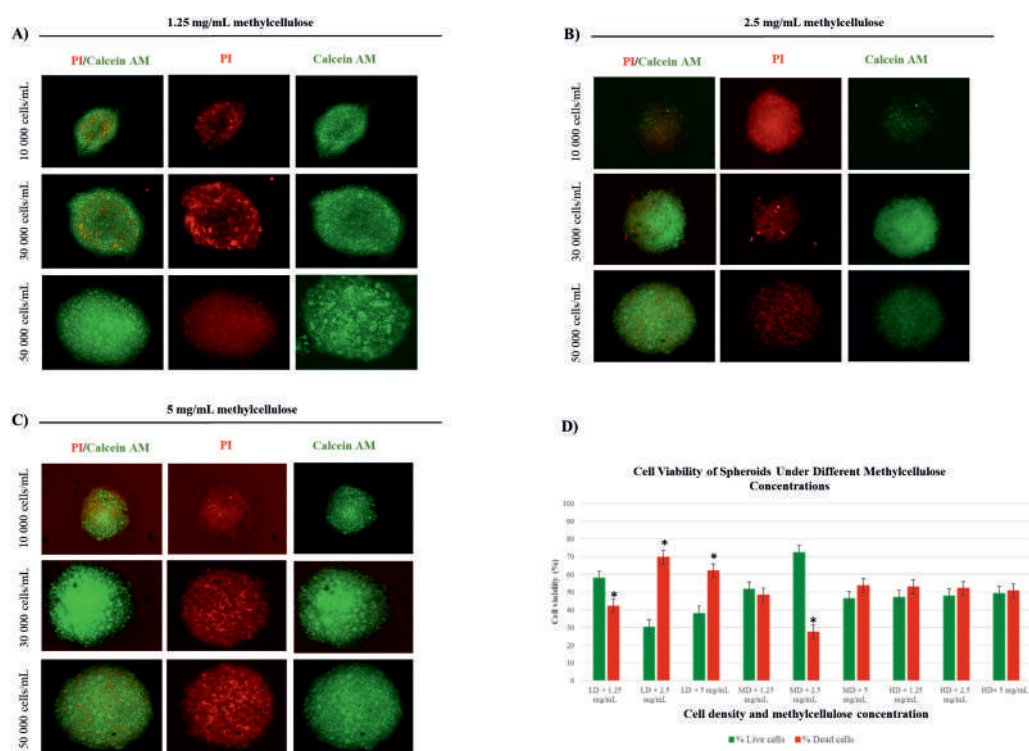
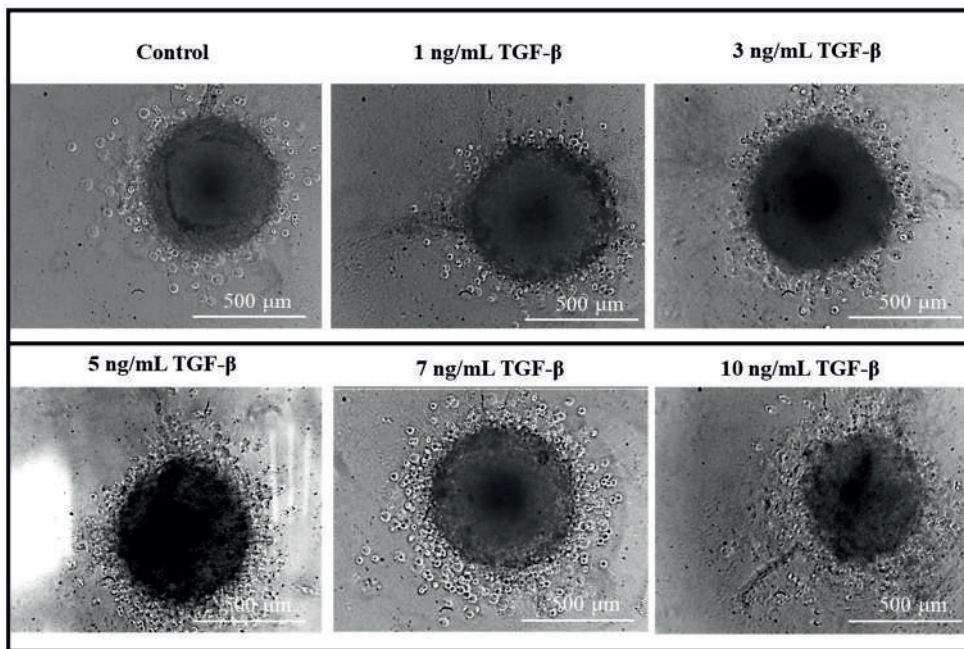


Fig. 2. Live/dead staining of spheroids after 72 h of maturation. (A) Representative images of low-density (LD; 10 000 cells/mL), medium-density (MD; 30 000 cells/mL), and high-density (HD; 50 000 cells/mL) spheroids formed with 1.25 mg/mL methylcellulose. (B) Representative images of LD, MD, and HD spheroids formed with 2.5 mg/mL methylcellulose. (C) Representative images of LD, MD, and HD spheroids formed with 5 mg/mL methylcellulose. (D) Graphical representation of the percentage of live vs. dead cells in spheroids, showing cell viability for the different cell densities and methylcellulose concentrations. Data represent the mean $\pm$ standard error of the mean (SEM) from two independent experiments, ANOVA (Graph Pad Prism8, San Diego, CA, USA) was used to compare the means between groups; p -values less than 0.05

2 mg/mL collagen I matrix and treated with varying TGF- β concentrations. After 72 h, radial invasion increased with TGF- β concentration, peaking at 7 ng/mL (Fig. 3), indicating its potent effect on spheroid invasion. This system effectively supports invasion assessments, demonstrating the utility of methylcellulose-based spheroids for evaluating invasive phenotypes in a 3D tumour-like model.

Discussion. Methylcellulose is an effective, tunable scaffold for 3D spheroid culture, supporting cancer research by modelling tumour growth, invasion, and therapeutic response. This study demonstrates that methylcellulose enables rapid, uniform spheroid formation across various cell densities, with optimal viability at 2.5 mg/mL, consistent with prior reports on semi-solid ECM-mimicking scaffolds [14]. By optimizing on both concentration and cell density to enhance

A)



B)

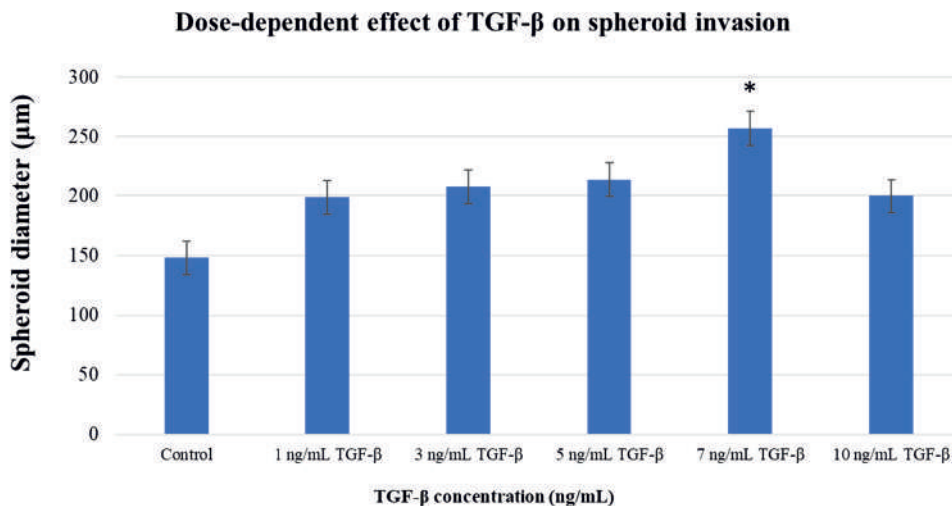


Fig. 3. TGF- β -induced cell invasion in spheroids after 72 h. (A) Representative images of spheroids at 72 h, showing distinct invasion patterns in response to TGF- β treatment. (B) Graphical representation of the measured invasion distances (diameter in μ m) for spheroids treated with different TGF- β concentrations, highlighting the correlation between TGF- β concentration and invasion distance. Data represent the mean $\pm$ standard error of the mean (SEM) from two independent experiments, ANOVA (Graph Pad Prism8, San Diego, CA, USA) was used to compare the means between groups; p -values less than 0.05

spheroid viability and invasion, our work expands on earlier studies that solely used methylcellulose for aggregation [11].

Notably, our study is the first to show a concentration-dependent role of methylcellulose in 3D culture. Lower concentrations (1.25 mg/mL) support viability at low seeding densities, while higher levels (> 2.5 mg/mL) reduce viability, potentially due to increased viscosity limiting nutrient diffusion or imposing mechanical stress – consistent with observations in hydrogel-based systems [15]. Our optimized model showed that at 2.5 mg/mL and 30 000 cells/mL, spheroids showed ideal structural integrity and matrix porosity for nutrient diffusion. This emphasizes the importance of optimizing scaffold properties for 3D culture systems.

This model's physiological relevance was validated by TGF- β -induced invasion. Optimized spheroids embedded in collagen exhibited dose-dependent invasion, with the strongest response at 7 ng/mL, reflecting EMT-related cytoskeletal and enzymatic changes. Compared to 2D cultures, this 3D model better captures tumour microenvironment features such as gradients and matrix interactions that affect drug response and invasion [16].

In summary, our findings support methylcellulose as a robust and tunable scaffold for generating viable, invasive 3D cancer spheroids. The optimized conditions provide a reproducible and physiologically relevant model for studying tumour behaviour and drug responses. Future studies should investigate the broader applicability of this system across different cancer cell lines and its potential integration with high-throughput screening platforms to enhance translational cancer research.

Conclusion. In conclusion, methylcellulose offers a simple, cost-effective, and reproducible platform for generating 3D spheroids that accurately model cancer invasion and tumour behaviour. By optimizing the concentration (2.5 mg/mL) and cell density (30 000 cells/mL), high-quality, viable, and invasive spheroids can be produced, mimicking key features of the tumour microenvironment. This approach is advantageous for cancer research, drug screening, metastasis modelling, and therapeutic development. Furthermore, the system holds potential for broader applications, including co-culture systems to better replicate tumour heterogeneity and evaluate new therapies.

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