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A 3D-bioprinted collagen *in vitro* model of Ewing sarcoma: Feasibility platform for drug screening

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Abstract

Ewing sarcoma (EwS) is a rare pediatric malignancy which develops in a physiologically stiff bone environment. Conventional *in vitro* models fail to faithfully recapitulate the complexity of this microenvironment, showing limits in translational relevance of preclinical drug screening.

In this study, we present a novel three-dimensional (3D) *in vitro* EwS model using extrusion-bioprinting technology with a collagen-based bioink. Rheological and morphometric analyses were performed to establish a printability framework, optimizing bioink formulations and cell densities for effective biofabrication. Alamar Blue assay was used to identify optimal cell densities for the evaluation of cell viability and growth in short- and long-term studies. The optimized condition (collagen 1:10 ratio; 1.4×10^6 cells/mL TC-71 EwS cell line) enabled reproducible biofabrication of structurally stable constructs with improved viscoelastic properties and shape fidelity. The 3D tumour microenvironment was modulated by hydrogel viscoelasticity, which supported sustained cell growth and remodelling activities as indicated by increased expression of MMP-9 and MMP-13. Under these conditions, viable multicellular spheroids formed within the hydrogel by day 3 and exhibited sustained growth through day 14, enabling both short- and long-term studies. The immunohistochemical analysis confirmed homogenous cell distribution, and together with the gene expression analysis recapitulated key tumor features. As proof of concept, treatment with talazoparib, a potent poly-ADP ribose- polymerase (PARP) inhibitor (PARPi), led to reduced proliferation (Ki-67 labeling) and activation of the DNA damage response (γ H2AX expression) in the 3D EwS model, demonstrating the feasibility of this platform for drug screening. Comparative analysis with a conventional 2D model further illustrates the enhanced physiological relevance of the 3D environment. Notably, the analysis of Ki-67 expression revealed that the 3D model exhibited slower proliferation kinetics compared to the 2D culture, with significantly lower Ki-67 levels at day 3 and comparable levels to the 2D control by day 10. Furthermore, treatment with 20 nM talazoparib in the 3D model significantly reduced Ki-67 expression and increased γ H2AX positivity by day 10, indicating a delayed DNA damage response relative to the rapid accumulation of DNA damage observed in the 2D cultures. Overall, this study provides a reproducible and biologically relevant 3D bioprinted EwS model, supporting its application as a predictive preclinical tool for therapeutic evaluation.

Keywords: Ewing sarcoma; Bioprinting technology; *In vitro* 3D-bioprinted tumor model; Collagen hydrogel; Preclinical drug screening

1. Introduction

Ewing sarcoma (EwS) is a rare and highly aggressive cancer that typically affects bones or soft tissue, predominantly in children and young adults¹. The integration of intensive, multi-agent neoadjuvant chemotherapy and consolidation treatments have substantially boosted prognosis in localized cases, driving long-term survival rates up to approximately 70–75%. However, the prognosis for patients with metastatic or relapsed disease remains poor, highlighting the urgent need for more effective therapeutic advances^{2,3}. Despite extensive preclinical research efforts, EwS has benefited only marginally from the major advances achieved in precision oncology and immunotherapy. One major obstacle is the persistent discrepancy between the high sensitivity to anticancer agents observed *in vitro* and the limited efficacy of these therapies in clinical settings. This discrepancy arises, in part, from the extensive use of long-term cultured cell lines, which have undergone genetic and biological drift, resulting in poor representation of primary human tumors. In addition, conventional experimental systems frequently rely on oversimplified two-dimensional (2D) culture conditions that inadequately recapitulate the biological complexity of EwS cells and the peculiarities of the bone microenvironment. Although 2D cell cultures remain widely used because they are cost-effective, technically accessible and amenable to imaging and high-throughput manipulation, they present well-known limitations, such as a lack of three-dimensional architecture, mechanical properties, extracellular matrix composition, and multicellular interactions. Despite being cost-effective, easy to handle, and ideal for high-throughput manipulation and imaging, traditional 2D cell cultures suffer from well-documented limitations, notably the lack of a three-dimensional structure, proper mechanical cues, extracellular matrix fidelity, and heterotypic cell interactions. These limitations underscore the need for more physiologically relevant preclinical models capable of better reproducing EwS tumor biology and improving translational predictability. To this end, several advanced models have been developed to better mimic tumor architecture, microenvironmental complexity, and drug responses compared with conventional 2D cultures. Among these, patient-derived xenografts have significantly advanced our understanding of cancer biology and facilitated more personalized approaches for evaluating therapeutic efficacy^{4,5}. However, they remain costly, labor-intensive, and unsuitable for high-throughput drug screening. Furthermore, ethical concerns and regulatory constraints related to the use of patient-derived material and animal experimentation limit their applicability in translational research. To address these limitations, a broad spectrum of innovative three-dimensional (3D) culture techniques⁶⁻⁸, including spheroids, microfluidic/organ-on-chip technologies, and organoids, has emerged as promising alternatives⁹⁻¹². In this context, the use of 3D bioprinting technology, thanks to its design flexibility, ensures the replication of complex structural networks through fine control of architecture, mechanical strength, cellular composition, and tissue architecture to achieve cell functional performance¹³. By accurately recreating the native tissue's heterogeneity and the gradients of biochemical and mechanical signals (such as porosity and stiffness), along with the spatial arrangement of cells using various bioinks that mimic key matrix components, this approach provides a valuable tool for studying cancer cell behavior, invasion, and drug responses¹⁴.

The use of natural-origin biomaterials that recapitulate key features of the native biological substrate, particularly the extracellular matrix (ECM) in which tumors develop, has gained

increasing attention in recent years thanks to their capacity to better support cell-cell and cell-matrix interactions^{15,16}. Despite these advantages, the processing and implementation of natural biomaterials remain technically challenging. Their intrinsic properties, including poor mechanical stability, low viscosity, and limited control over structural integrity, often compromise the fabrication of stable structures with adequate shape fidelity^{17,18}. These constraints are especially critical when aiming to generate stable 3D tumor models that should maintain both architectural and functional relevance over time for accurately reproducing tumor progression and therapeutic responses. In this context, extrusion-based 3D bioprinting represents a robust biofabrication strategy, enabling the precise deposition and subsequent polymerization of natural biomolecules, such as collagen type I, thereby partially overcoming the low viscosity and slow gelation kinetics of collagens. Moreover, the integration of cells within ECM-based bioinks via filament deposition makes this technology a valuable tool to model custom-shaped constructs, offering a versatile platform to recapitulate tumor-specific features¹⁹⁻²³.

In this study, we developed a novel collagen-based 3D bioprinted *in vitro* EwS model, capable of supporting EwS cell organization and vitality over time. The 3D model was evaluated in terms of cytocompatibility and functional properties. As proof of concept, we assessed EwS cell sensitivity to talazoparib, a poly-ADP ribose- polymerase (PARP) inhibitor (PARPi)^{24,25}, to check the potential of this biofabricated platform for evaluation of preclinical therapeutic drug responses, comparing the results with those obtained using the standard 2D culture model.

2. Materials and methods

2.1. Materials

Bovine collagen type I bioink (concentration: 3.5 mg/mL, Advanced BioMatrix, Carlsbad, CA, US) was used for 3D bioprinting experiments. The bioink was prepared according to the manufacturer's instructions, with varying component ratios to tune stiffness and achieve optimal stability and reproducibility. The EwS cell line TC-71 was cultured in Iscove's modified Dulbecco's medium (IMDM, EuroClone, #ECB2072L) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, EuroClone, #ECS0180L), 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere, and culture media was changed twice/week. Cells were regularly tested for Mycoplasma contamination (MycoAlert™ Mycoplasma Detection Kit, LT07-418, Lonza) and authenticated by PCR-single-locus-technology using a PowerPlex ESX Fast System kit (CLA service by Eurofins Genomics); the last authentication was performed on 10 July 2023.

2.2. Experimental design

The experimental design of this study aimed to develop a 3D bioprinted model mimicking key architectural features of EwS tumor using 3D extrusion-based printing technology. A commercially available bovine type I collagen-based bioink combined with the TC-71 cell line was first optimized in terms of composition, printability, and cell density to ensure structural

stability, reproducibility and biological functionality (**step 1**). The resulting bioprinted constructs were then evaluated for cytocompatibility using a panel of biological assays, including the assessment of cell viability and proliferation (**step 2**), followed by the characterization for typical markers and spheroid formation over time (**step 3**). Finally, the developed 3D model was employed for drug screening to assess treatment response, thereby assessing its biological relevance for tumor research and preclinical drug screening (**step 4**) (**Figure 1**). Parallel analyses were conducted under 2D culture conditions to assess differences in drug sensitivity between 2D and 3D systems. This comparative approach was intended to evaluate the potential benefits arising from the incorporation of the collagen matrix.



Figure 1: Schematic representation of the experimental workflow for the development of the 3D EwS model. **Step 1-Optimization of the cell bioprinting process:** initial characterization of different bioink composition ratios and printing parameters was performed through rheological analyses. Subsequently, cell-laden constructs with varying cell densities were fabricated using an extrusion-based microvalve system, where the human TC-71 cell line was encapsulated within a collagen-based bioink to find the most suitable formulation; **Step 2- Cytocompatibility evaluation:** The cytocompatibility of the 3D EwS *in vitro* model was evaluated at 3, 7 and 14 days post-fabrication using Alamar blue metabolic assays and Live/ Dead staining; **Step 3-Biological characterization:** The developed 3D EwS constructs were further characterized by histological and immunofluorescence analyses; **Step 4- Drug screening:** The developed 3D EwS model was employed for drug screening to assess treatment response. Created in BioRender. Desando, G. (2026) <https://BioRender.com/h3dh0kp>

2.3. Tuning bioink compositions and cell density for enhanced bioprinting performance and cellular functionality

2.3.1. Rheological characterization of collagen type I-based bioink

The rheological properties of the collagen type I-based bioink were measured by a rotational rheometer (Anton Paar MCR-102, Graz, Austria) with a plate–plate geometry (25 mm diameter) and a fixed gap of 1.0 mm. All measurements were performed at 37°C. A steady rate sweep test was conducted at shear rates ranging from 0.01 to 1000 s⁻¹ to determine the viscosity of the collagen type I-based bioink. By fitting the power-law equation (Equ.1) to the linear region of the shear rate versus viscosity plot, we calculated the shear-thinning coefficients

$$\eta = K \dot{\gamma}^{n-1}$$

(Equ.1)

where η is the apparent viscosity, K is the flow consistency index, $\dot{\gamma}$ is the shear rate, and n is the power-law index. An amplitude sweep test was performed at a constant oscillation frequency of 1 Hz and shear strain ranging from 0.01 to 1000% using a logarithmic ramp profile to obtain a total of 31 data points to study the viscoelastic behavior of the bioink under increasing deformations and identify the linear viscoelastic region (LVE). In this LVE, a temperature ramp test was performed from 4 to 40°C at a constant frequency of 1 Hz and 1% shear strain to measure the storage modulus (G') and loss modulus (G'') as a function of temperature. In addition, a temperature ramp test from 4 to 40°C was performed at a constant shear rate of 10 s⁻¹ to assess changes in viscosity and define the temperature range in which the bioink maintains structural stability post-bioprinting.

2.3.2. Optimizing collagen-based bioink printing parameters for 3D EwS modelling

Three different ratios of collagen type I-based bioink and medium solution (1:2.5; 1:5; 1:10) were carried out to evaluate different parameters, including the extrudability, filament formation and scaffold stability in the culture medium after extrusion-based bioprinting. The desired printing pattern was designed using BioCAD software (RegenHU, Villaz-St-Pierre, CH), subsequently converted into a G-Code, and printed using a 3D Discovery platform (RegenHU, Villaz-St-Pierre, CH) equipped with a high-precision microvalve extrusion-based 3D bioprinting system. The construct design consisted of an 8-layer square (10 x 10 mm with a height of 2 mm) with an internal pattern composed of two parallel lines oriented at relative angles of 0° and 90°. Printing process was performed using a metallic needle (300 µm) and a microvalve system (inner diameter of 300 µm and stroke of 100 µm). The printhead was kept at room temperature (25°C), while the build zone was maintained at 37°C. To optimize bioink extrudability and structure stability for the selected bioink ratios, valve opening time (190 -330 µs), dosing distance (0.03-0.1 mm) and printing speed (8 - 10 mm/s) were systematically varied, while filament thickness (0.18 µm), pressure (0.2 bar) and number of layers (n=8) were kept constant. At the end of this process, an arbitrary classification based on the macroscopic visualization of the printed structures was established, ranging from 0 to 2 (0: no extrudability and no structure stability; 1:

good extrudability and poor structure stability; 2: good extrudability and structural stability). This semi-quantitative scoring system was used as a preliminary screening tool to define the optimal printing parameters for the 3D *in vitro* EwS tumor model. To evaluate the structural evolution of the bioprinted microarchitecture, microscopic analyses were performed on cell-free constructs immediately after printing and on cell-laden constructs at short (day 3) and long-term (day 14) follow-up. Pore size was measured using image analysis software on fluorescence images acquired with the Nikon Eclipse 90i microscope and respective software (Nikon Eclipse 90i, Melville, NY, USA). For each sample, multiple regions of interest (ROIs) within the 3D constructs were carefully selected based on uniform staining and structural integrity. ROIs were defined manually to exclude artefact-prone or edge areas, ensuring consistent sampling across samples. Pore size quantification was performed using image analysis software on fluorescence images acquired with the Nikon Eclipse 90i microscope (Melville, NY, USA). Images were captured following Live/Dead staining, which simultaneously allowed assessment of cellular organization and viability. For each biological replicate, multiple representative regions of interest (ROIs) within the 3D constructs were carefully selected based on uniform staining and structural integrity. Pore diameters were manually measured using the NIS Elements software's measurement tool by identifying and marking the initial and end points across the central region of each pore to capture their physical size. Approximately 6 pores were measured per biological replicate to provide statistically robust data.

2.3.3 Optimization of cell density for long-term functional stability

To effectively monitor tumor cell viability and growth and drug sensitivity in the developed 3D model, we systematically evaluated multiple cell densities to minimize technical variability and ensure reliable long-term functional assessments. Four cell densities were tested (0.7×10^6 cells/mL, 1.4×10^6 cells/mL, 2.8×10^6 cells/mL and 5.6×10^6 cells/mL). This design allowed identification of conditions that balance biological functionality with structural compatibility under extrusion-induced processing constraints.

Optimization was based on two primary parameters: (i) cell viability, and (ii) metabolic activity in relation to spatial organization within the 3D architecture. To this end, Live/Dead and Alamar Blue assays were performed over a 14-day culture period. Constructs were analyzed starting from day 3-4 post-printing, allowing an initial adaptation phase within the hydrogel matrix, followed by a 10-day functional evaluation time. Acceptance criteria were defined a priori to reduce subjectivity and ensure reproducibility. Conditions were considered suitable when: (i) viable multicellular clusters were observed as early as day 3, (ii) Alamar Blue signal showed a progressive increase between days 7 and 14, and (iii) no evidence of metabolic starvation or decline was detected over time. Conditions not meeting these criteria, including those showing signal plateau or minimal change over time, were excluded from further analysis. Overall, optimal conditions were defined as those supporting stable construct architecture, sustained metabolic activity, and spatially organized cell growth over time, enabling reliable assessment of treatment-induced responses while minimizing artifacts related to over-confluence or transport limitations.

2.4. 3D Biofabrication of a structured 3D EwS tumor architecture for in vitro modelling

The 3D *in vitro* EwS tumor model was designed using dedicated CAD software (BioCAD, RegenHU, CH) with a square geometry (10x10 mm, height of 2 mm), and a 0/90° infill pattern to generate a defined internal micro-architecture. The theoretical design parameters included a fiber diameter of 300 µm, a pore size of 1700 µm and a thickness of 8 layers. The bioprinting process was performed using the 3D Discovery platform (RegenHU, CH) integrated into a class II laminar flow hood and equipped with high-precision microvalve-based dispensing technology. During printing, the printhead was maintained at room temperature (25°C), while the build zone and the ultra-low-attachment 6-well plates were kept at 37°C to promote collagen type I-based bioink gelation and ensure accurate fiber deposition. According to the manufacturer's guidelines, collagen type I bioink was prepared by mixing 1 mL of bioink with 100 µl of buffer solution and 400 µl of fetal bovine serum (FBS, #ECS0180L, Euroclone Milan, Italy). TC-71 cells were trypsinized, counted, and resuspended with FBS at the different cell densities, reported in detail above. The cell suspension was placed into a sterile luer-lock syringe and gently mixed with the bioink using a luer-lock coupler (Sarstedt AG & Co.KG, Numbrecht, DE) to obtain a homogeneous cell-laden construct, which was then loaded into sterile bioprinter cartridges. The final cell-laden bioink was printed directly into ultra-low attachment 6-well plates (Corning Inc., New York, NY, USA), placed on a 37°C build plate. A technical control was prepared by depositing approximately 300 µl of TC-71/collagen type I bioink mixture into 6-well plates without printing. Following biofabrication, constructs were cultured in IMDM at 37°C, 95% humidity, and 5% CO₂, with medium changes performed twice weekly. Samples were monitored throughout the experimental timeline for morphological and biological stability.

2.5. Multilevel structural and biological characterization of a 3D bioprinted EwS model

2.5.1 Cytocompatibility of the 3D bioprinted EwS model

As an initial biological evaluation, post-printing cell viability was used to assess the effect of the 3D bioprinting process on the viability of the cell-based system. Cytocompatibility of the 3D bioprinted constructs included evaluations of cell morphology, viability, and metabolic activity over time. Morphological assessment was performed using an inverted microscope (Nikon, Eclipse Ts2, Japan) to evaluate cell distribution, shape, and overall structural organization within the collagen hydrogel. Cell viability was assessed using a Live/Dead assay (# L3224, Invitrogen, Waltham, MA, USA) and then analyzed using an epi-fluorescence microscope (Nikon Eclipse 90i, Melville, NY, USA). Fluorescein isothiocyanate (FITC) and tetramethylrhodamine (TRITC) filters were used to visualize viable (green) and dead (red) cells at post-printing time and at days 3, 7 and 14. Quantitative image analysis was performed to determine the percentage of viable cells and the number of multicellular spheroids formed over time. Cell metabolic activity was assessed at days 3, 7, 10 and 14 by Alamar blue assay (#DAL1100, Invitrogen, Thermo Scientific, Waltham, MA, USA). Alamar BlueTM Cell Viability Reagent was diluted 1:10 in culture medium and incubated with constructs at 37°C with 5 % CO₂ for 4 h. Subsequently, 100µL of supernatant was transferred to a 96-well plate, and fluorescence was measured using a

Tecan Infinite® 200 PRO microplate reader (Tecan Italia S.r.l, Cernusco Sul Naviglio, Italy) with excitation/emission wavelength of 530–560 nm and 590 nm, respectively.

2.5.2 Biological assessment of the 3D bioprinted EwS model

To evaluate the preservation of tumor-specific biological features in the 3D bioprinted EwS model, histological and immunohistochemical analyses were performed at days 3, 7 and 14 post-printing, while gene expression analysis was conducted at day 14 post-printing.

For immunohistochemical assessments, constructs were embedded in Tissue-Tek® optimal cutting temperature compound (#4583, Sakura Finetech, CA, USA), snap frozen in liquid nitrogen, and then sectioned at 12 µm with a cryostat (Leica Biosystems, Nußloch, Germany). Cryosections were fixed in a 4% paraformaldehyde solution (PFA, #P6148, Sigma-Aldrich, St Louis, MO, USA) before staining. For histological evaluation, Gill III Hematoxylin-Eosin (H/E) (#05-M06015, Bio-Optica, Milano, Italy) staining was used to assess cell morphology and spatial distribution within the collagen matrix. For immunofluorescence analysis, non-specific binding sites were blocked using 1% albumin solution (#A4503, Sigma-Aldrich) in Tris-buffered saline (TBS, #T5912, Sigma-Aldrich) for 30 min at room temperature. Sections were incubated overnight at 4°C with primary antibodies against CD99 (clone O13, BioLegend), FLI-1 (C-19, Santa Cruz Biotechnology), MMP-9 (#IM09L, Calbiochem, San Diego, CA, US), and MMP-13 (#Clone 87512, #MAB511, R&D Systems, CA, US). After washing, samples were incubated for 1 h at 4°C with fluorescent secondary antibodies (goat anti-mouse FITC, Thermo Fisher Scientific; anti-rabbit Atto 488, Sigma-Aldrich), followed by nuclear counterstaining with DAPI (#D9542, Sigma-Aldrich). Samples were imaged using an epifluorescence microscope (Nikon Eclipse 90i). Semi-quantitative analysis was performed using standardized image analysis software (NIS Elements), assessing cluster-related parameters including number of clusters, cluster size, and number of cells per cluster. Measurements were performed across multiple representative fields per section to ensure reproducibility.

For gene expression analyses, total RNA was extracted from 2D and 3D EwS cell constructs using TRIzol Reagent (#15596018; Thermo Fisher Scientific—Life Technologies, Grand Island, NY, USA) at day 14. RNA quality and quantity were measured using a NanoDrop spectrophotometer (NanoDrop ND1000, Thermo Scientific, MA, USA). The total RNA from each sample was reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (#4368813, Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed on a ViiA 7 System (Applied Biosystems) using TaqMan Universal PCR Master Mix (#4304437, Thermo Fisher Scientific-Applied Biosystems) or SYBR® Green PCR Master Mix (#4309155, Applied Biosystem). Predesigned TaqMan assays were used for the following genes: IGFBP3 (Hs00426287_m1), GAPDH (Hs99999905_m1). Alternatively, the forward and reverse primers were used for PCR amplification of the human genes encoding for: LOX (Fw 5'-ACAGGGATTGAGTCCTGGC- 3'; Rv -5' GCTGGGGTTTACACTGACCTT - 3'), NRBO1 (Fw 5'- TGCTCTTTAACCCGGACGTG - 3'; Rv 5'- GCGTCATCCTGGTGTGTTCA - 3') IGF-1 (Fw 5'-TCGCATCTCTTCTACCTGGCGCTGT-3'; Rv 5'-GCAATACATCTCCAGCCTCCTAGA - 3' and for the reference gene GAPDH (Fw 5'-

GAAGGTGAAGGTCGGAGT -3'; Rv 5'- GAAGATGGTGATGGGATTTC -3'). The expression levels of the target genes (RQ) were normalized to that of GAPDH and expressed as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct \text{ target genes} - Ct \text{ GAPDH}$ and $\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ calibrator}$ (mesenchymal cells).

2.6. Proof-of-Concept Evaluation of Drug Response in the 2D system and 3D bioprinted EwS Model

Sensitivity to the PARP inhibitor talazoparib (BMN 673, Selleckchem #S7048) was initially evaluated in EwS cells cultured under conventional 2D conditions. TC-71 cells were seeded into 96-well plates (2,500 cells/well) and treated with a range of talazoparib concentrations (0.003 μM – 1 μM) for 3 days. Stock solutions of the drug were prepared and stored according to the manufacturer's instructions. The cells were also treated with dimethyl sulfoxide (DMSO) (#D2650, Sigma) as a vehicle control. Drug sensitivity was assessed using the TACS® MTT Cell Proliferation Assay kit (#4890-25-K, R&D Systems), according to the manufacturer's instructions. Dose-response curves were generated to determine the half-maximal inhibitory concentration (IC_{50}) and identify a treatment condition that produced a measurable biological effect for subsequent comparative studies.

Following determination of the IC_{50} value in the 2D model, the corresponding concentration was selected as the starting dose for experiments in the 3D bioprinted system. In addition, a higher talazoparib concentration was evaluated to account for the potential impact of the collagen matrix on drug diffusion and cellular drug exposure. This strategy is consistent with previous studies, including our own work in 3D culture systems, which have demonstrated that higher drug concentrations are often required to achieve biological responses comparable to those observed in 2D conditions.

To evaluate the biological relevance of the 3D bioprinted EwS model as a predictive preclinical platform, a proof-of-concept assessment of drug efficacy was performed using the PARPi talazoparib. After 3 days of culture, bioprinted constructs were treated with two concentrations of talazoparib (10nM and 20nM), corresponding to the IC_{50} -derived dose identified in 2D cultures and a higher dose selected to account for the characteristics of the 3D microenvironment. Treatments were administered twice weekly for a total of 10 days in culture medium (**Figure S1**).

Drug response was evaluated after 3 days of treatment in both 2D and 3D systems and, additionally, after 10 days of treatment in the 3D bioprinted model. Treatment efficacy was assessed through live and dead staining, Alamar blue assay, and histological analyses to assess cell viability, metabolic activity, and morphological changes, as reported in detail above. In addition, immunofluorescence analyses were performed in both 2D and 3D models to evaluate cellular proliferation (Ki-67 labelling, primary antibody clone UMAB107, #UM800033; Origene) and DNA damage response (positivity to γH2AX ; primary antibody, clone#2577, 1:100 dilution, Cell Signalling Technology, Massachusetts, US).

Section assessments were conducted using an Eclipse 90i epi-fluorescent microscope (Nikon). A semi-quantitative analysis of immunofluorescence signal intensity for γ H2AX and Ki-67 was performed across six microscopic fields per sample at 20 $\times$ magnification. A semi-quantitative analysis of γ H2AX and Ki-67 immunofluorescence staining was performed on six representative microscopic fields per sample acquired at 20 $\times$ magnification. Images were analyzed using NIS-Elements software (Nikon). The total number of cells was determined by automated counting of DAPI-positive nuclei, while γ H2AX- and Ki-67-positive cells were identified in the FITC channel using a hue, saturation, and intensity (HSI) threshold-based segmentation method. For each field, the number of FITC-positive nuclei was quantified and normalized to the total number of DAPI-positive nuclei, and the results were expressed as the percentage of positive cells. Mean values were subsequently calculated from the six analyzed fields for each sample and experimental condition.

2.6. Statistics

Prism version 7.0 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. Kolmogorov-Smirnov (K-S) and Shapiro-Wilk tests were carried out to test data distribution for the Live/Dead test, Alamar blue assay, and immunohistochemical analyses. Differences among the means were analyzed using Student's t-test or one-way ANOVA when the experimental data included more than two groups, with Dunnett's post-hoc test for multiple comparisons with the control group. Data are reported as mean $\pm$ standard deviation (SD). Data were considered significant with $P < 0.05$.

3. Results and Discussion

3.1. The collagen type-I based bioink exhibits a shear-thinning behavior and ratio-dependent viscoelastic stability

A collagen-based bioink was selected as a proof-of-concept study to mimic the stiffness of the bone marrow to recreate a soft, permissive microenvironment that is conducive to tumor growth and drug testing. This selection was performed considering that EwS arises in bone but tumor cells primarily proliferate within the bone marrow niche, whose viscoelastic properties are considerably lower than those of the mineralized bone matrix, as previously reported²⁶. The rheological properties of type I collagen-based bioinks were systematically investigated to evaluate their suitability for extrusion-based bioprinting and to assess the influence of formulation ratio on flow and viscoelastic response. Three different ratios (1:2.5, 1:5, and 1:10) were analyzed, and the results are presented in **Figure 2 (a-d)**. As shown in **Figure 2 (a)**, all formulations exhibited pronounced shear-thinning behavior, with decreasing viscosity by several orders of magnitude as shear rate increased. This behavior was quantitatively described using the power-law model. The calculated flow consistency index (K) and flow behavior index (n) confirmed the strong dependence on formulation ratio. Specifically, the 1:10 ratio exhibited a significantly higher K value (19.026 Pa*s ^{n}) compared to the 1:2.5 (7.7428 Pa*s ^{n}) and 1:5 (7.4434

$\text{Pa}\cdot\text{s}^n$) ratios, indicating higher intrinsic viscosity and a stronger internal network. The flow behavior index (n) was consistently below 1 for all formulations (0.13 for the 1:2.5 ratio; 0.115 for the 1:5 ratio and 0.147 for the 1:10 ratio), confirming their non-Newtonian shear-thinning behavior. Notably, the higher n value for the 1:10 ratio reflects a more stable viscosity profile under shear, suitable for extrusion-based bioprinting. The viscoelastic properties of collagen-type I bioink were further characterized by oscillatory amplitude sweep tests, as shown in **Figure 2 (b)**. All formulations exhibited a well-defined linear viscoelastic region (LVE) at low strain values (approximately within the range of 0.01 – 1%), where the storage modulus (G') remained constant and higher than the loss modulus (G''), indicating predominantly elastic, solid-like behavior. Beyond this region, a decrease in G' was observed, corresponding to the onset of structural breakdown and transition toward a viscous-dominated regime. The 1:10 ratio showed a larger LVE region, maintaining its elastic plateau up to higher strain values (up to 10%) compared to the 1:5 and 1:2.5 ratios. This behavior can be attributed to the higher collagen concentration, which promotes more resistance to deformation and improves mechanical stability during the extrusion-based bioprinting. Temperature-dependent rheological behavior is shown in **Figure 2 (c-d)**. Across the investigated temperature range (4–40°C), viscosity decreased progressively with increasing temperature for all formulations, while the storage modulus (G') remained relatively stable, ensuring reproducible extrusion behavior and structural integrity after deposition.

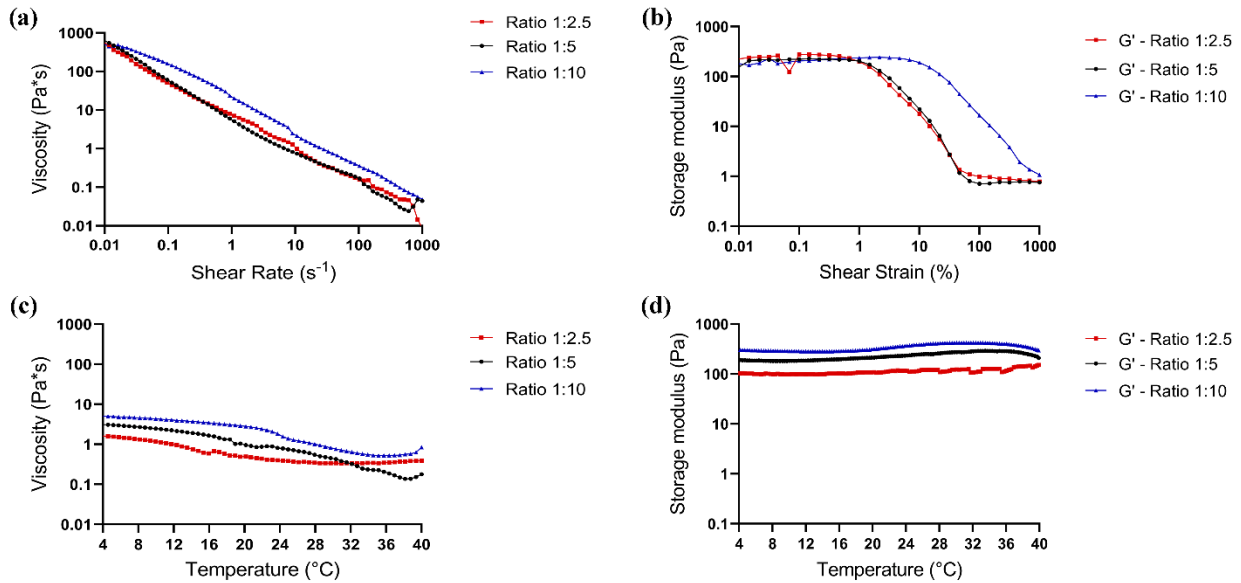


Figure 2. Rheological characterization of collagen type I-based bioink at varying mixing ratios (1:2.5, 1:5, and 1:10). (a) Flow curves of the shear rate sweep test of the collagen type I-based bioinks as a function of shear rate (0.01 – 1000 s^{-1}); (b) Amplitude sweep test displaying the storage modulus (G') against the shear strain (0.01 – 1000%); (c) Viscosity properties of collagen type-I based bioink under the temperature sweep testing (4–40°C); (d) Storage modulus of collagen type-I based bioink under the temperature sweep testing (4–40°C). Data are expressed as the mean of three independent replicates for each ratio.

3.2. Optimized Collagen-Based Bioprinting Enables a Reproducible 3D *In Vitro* EwS Model

To obtain reproducible 3D *in vitro* EwS constructs, a systematic optimization of the printing parameters of collagen type I-based bioink was performed by evaluating the combined effect of ratio, valve opening time, dosing distance, and printing speed on extrudability and structural stability using a high-precision microvalve-based bioprinting technology. As shown in **Figure 3**, macroscopic analysis showed a significant interdependence between bioink formulation and the extrusion process window. At a low valve opening time of 190 μs , the energy delivered to the microvalve was insufficient to induce consistent material flow, resulting in discontinuous extrusion and failure to form a stable 3D porous structure across all tested conditions. Incremental increases in valve opening time to 210 μs and 330 μs significantly enhanced extrudability, allowing the formation of continuous filaments. Dosing distance also played a key role in determining scaffold fidelity. Larger distances (0.06–0.1 mm) enabled filament deposition but often led to poor layer stacking and structural instability. Conversely, a shorter dosing distance (0.03 mm) promoted accurate filament stacking and enhanced structural fidelity. Bioink composition ratios influenced the printability across the tested bioprinting parameters. No extrusion was observed for the 1:2.5, 1.5 and 1:10 ratios using a low valve opening time (190 μs) at different dosing distances. This indicated that a high printing speed of 10 mm/s combined with larger dosing distances (0.06–0.1 mm) led to filament displacement and reduced layer-by-layer adhesion, ultimately compromising structural fidelity. A maximum printability score of 2, defined by both extrudability and structural stability, was obtained within a specific parameter window: valve opening time of 330 μs , dosing distance of 0.03 mm, and printing speed of 8 mm/s. These conditions enabled the fabrication of well-defined and reproducible constructs suitable for downstream biological applications. Morphological characterization by bright-field microscopy revealed a homogeneous architecture with a fiber diameter of $340.00 \pm 18.10 \mu\text{m}$ and a pore size of $1320.55 \pm 124.6 \mu\text{m}$. The constructs exhibited an interplay of regular fiber diameters and large, interconnected pores, creating an ideal environment for cell migration and growth, while supporting optimal nutrient and oxygen exchange throughout the construct.

Building on these structural features, our study introduces a novel 3D bioprinted collagen hydrogel designed to replicate some properties of the ECM, leveraging the hydrated polymer network and intrinsic bioactivity of collagen²⁷. Furthermore, the use of collagen bioink adds significant biological value, as collagen fibers can self-assemble into 3D networks that recapitulate ECM characteristics—supporting cell adhesion, migration, and growth, while guiding biodegradation and providing essential biochemical cues for cellular homeostasis^{21,28}.

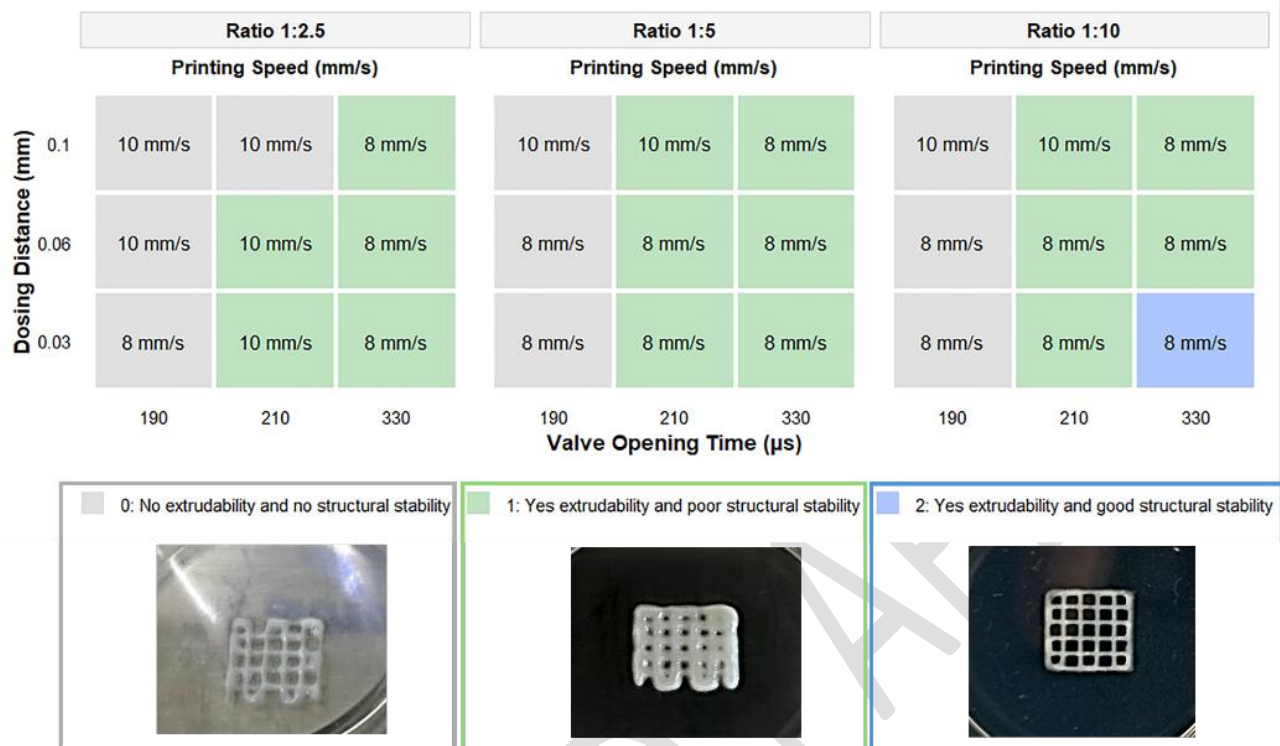


Figure 3. Parametric optimization of high-precision microvalve-based bioprinting process. Heatmap showing the influence of collagen type I-based bioink ratios, valve opening time (μ s), and dosing distance (mm) on printability. Printing performance was categorized into three levels, as defined by the corresponding macroscopic images shown in the panel below: (0) No good extrudability and no structural stability; (1) successful extrudability with poor structural stability; (2) successful extrudability with good structural stability. Representative macroscopic images for each category are provided to visually support the scoring criteria. Data are expressed as the mean of three independent replicates for each ratio.

3.3. Optimized cell density enables short- and long-term studies in the 3D EwS model

Initial evaluations employed a Live/Dead assay immediately following the post-printing phase to assess cell viability under the optimized bioprinting parameters. As a control, a technical sample involving the simple mixing of cells and collagen without printing confirmed the absence of cytotoxicity. During the 3D extrusion cell printing process, data showed a modest decrease in cell viability of approximately 10% at hour 0, reflecting the mechanical shear stress associated with extrusion, typically linked to cellular damage and reduced survival rates²⁸. Importantly, this initial reduction was followed by a recovery phase, with viability improving markedly by day 3, reaching levels consistent with healthy, viable constructs. (**Figure S2**).

These findings confirm that, despite the mechanical challenges intrinsic to extrusion bioprinting, the process parameters are well calibrated to preserve cell integrity and cytocompatibility, supporting the robustness of our 3D bioprinted model. to create cell-rich constructs with minimal constraints on ink viscosity and cell concentration compared to alternative techniques²⁹.

Utilizing cell encapsulation within a collagen type I-based bioink, rather than post-printing seeding, enabled precise spatial control of cell distribution through microvalve extrusion bioprinting, promoting uniform patterning and early cell–matrix interactions essential for maintaining physiological relevance in long-term culture. Cell density was optimized to ensure reliable longitudinal assessment of drug response over a 14-day culture period. Constructs seeded at 0.72×10^6 , 1.4×10^6 , 2.8×10^6 and 5.6×10^6 cells/mL maintained high cell viability throughout the study and exhibited a progressive increase in metabolic activity over time, as indicated by mitochondrial dehydrogenase activity (**Figure 4 a-b**). Notably, the lower cell densities (0.72×10^6 and 1.4×10^6 cells/mL) showed the most pronounced increase in metabolic activity between day 3 (short-term) and day 14 (long-term) ($P < 0.0001$), consistent with sustained cell proliferation. A comparable, albeit less marked trend was observed at 2.8×10^6 cells/mL. In contrast, constructs seeded at the highest cell density (5.6×10^6 cells/mL) exhibited a decline in metabolic activity starting from day 7.

Further analyses on pore size revealed a density-dependent reduction in scaffold porosity following cell encapsulation, as shown in **Figure 4 (c)**. This effect became more pronounced over time, particularly at higher cell densities after 14 days, likely due to cell proliferation, matrix remodeling, and hydrogel compaction. Such changes in microarchitecture may hinder nutrient and oxygen diffusion, potentially contributing to the reduced metabolic activity observed especially at the highest density and highlighting the interplay between cell loading and mass transport limitations. The observed decline in metabolic activity despite sustained cell viability can be attributed to several factors. First, diffusion limitations of oxygen and nutrients within the 3D construct likely establish spatial gradients, whereby cells located in the inner regions experience reduced availability of essential substrates. This condition, often associated with hypoxia, can drive cells toward a low-energy, non-proliferative state³⁰. Second, cells in densely populated 3D environments may undergo metabolic reprogramming and transition toward cellular quiescence³¹. In this state, cells preserve membrane integrity and viability while down-regulating ATP production and overall biosynthetic activity, resulting in reduced metabolic readouts. Despite its strong proliferative capacity, the lowest cell density presented an immature structural organization for short-term studies, characterized by sparse spheroid formation and predominance of isolated cells. This lack of stable 3D multicellular architecture may compromise the physiological relevance of drug response. Considering both early structural maturity and sustained metabolic activity, 1.4×10^6 cells/mL was identified as the optimal condition for long-term studies, including drug screening.

After establishing the optimal cell density for the EwS model, rheological analyses were also performed to investigate changes in the viscoelastic behavior of the bioink upon cell encapsulation within the 3D construct. Amplitude sweeps and steady rate sweep tests enabled us to assess how cellular incorporation influenced the mechanical properties of the hydrogel network after their loading. The incorporation of 1.4×10^6 cells/mL into the bioink resulted in a slight decrease in viscosity without altering the shear-thinning behavior of the material (**Figure 4 d**). As shown in **Figure 4 (e)**, the viscoelastic properties of the type I collagen-based bioink were

significantly influenced by the presence of embedded cells. In particular, the cell-laden bioink exhibited higher storage modulus (G') at low shear strain values, compared to the control (Col1), indicating an increase in elastic behavior. This suggests that the incorporation of cells contributes to reinforcing the collagen network. Moreover, the crossover points between the storage modulus (G') and the loss modulus (G'') occurred at slightly lower values in the cell-laden condition, indicating an earlier transition from viscous to a more elastic response, suggesting that the presence of cells promotes faster structural organization, likely through cell-matrix interactions and remodeling of the surrounding hydrogel network.

Collectively, these findings highlight how the 3D microenvironment can decouple metabolic activity from cell viability, emphasizing the need to carefully interpret metabolic assays in 3D systems. Overall, they underscore the importance of developing 3D bioprinted *in vitro* model that balances cell density, structural organization, and transport properties to obtain both reproducible and physiologically relevant conditions for drug screening applications.

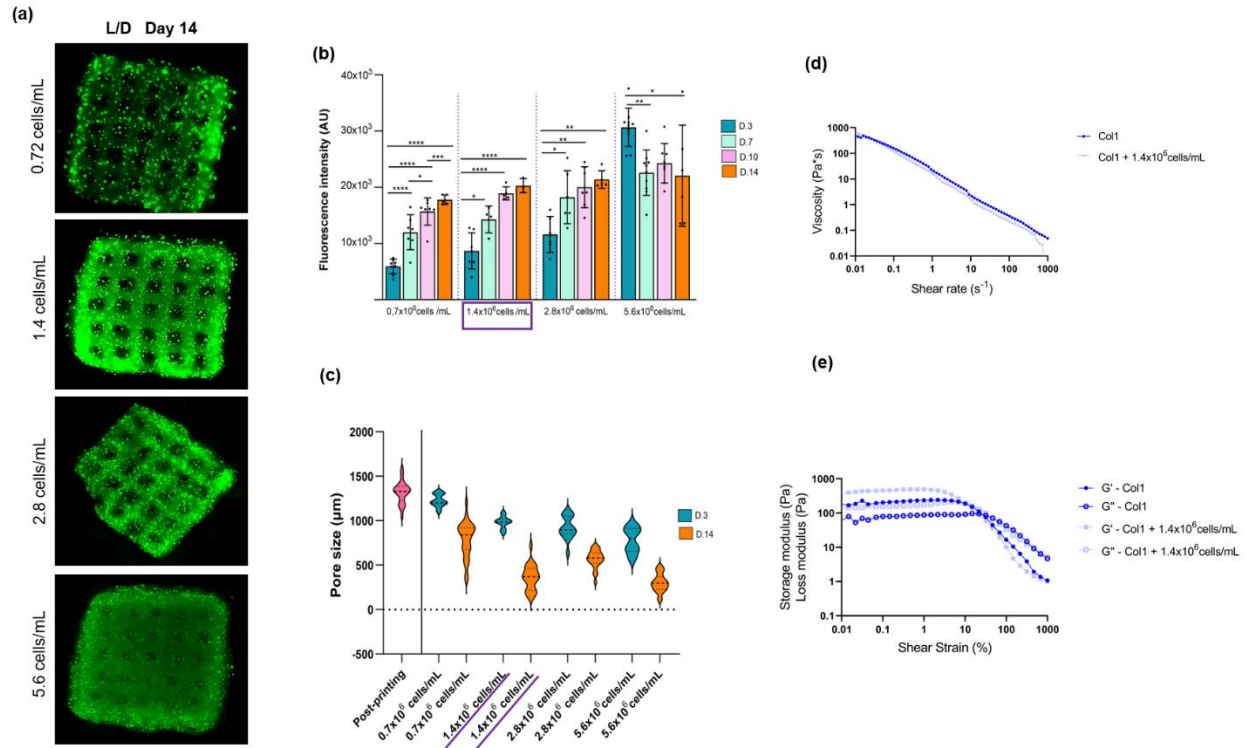


Figure 4. Cell density optimization for drug sensitivity studies in a 3D bioprinted model. **(a)** Representative merged fluorescence micrographs from the Live and Dead assay at day 14, showing four cell densities: 0.72x10⁶ cells/mL; 1.4x10⁶ cells/mL; 2.8x10⁶ cells/mL; 5.6x10⁶ cells/mL; scale bar: 50 μm. **(b)** Quantification of metabolic activity across different cell densities from day 3 to day 14; one-way ANOVA test followed by multiple comparison post-hoc test for each experimental group was performed. **(c)** Quantification of pore size (μm) in the construct at the post-printing phase, free from cells and embedded with different cell densities at 3 and 14 days. **(d-e)** Rheological assessments of viscosity and viscoelastic moduli for the optimal bioink formulation (1:10) alone and combined with the most effective cell density (1.4x10⁶ cells/mL). The dark blue lines represent the values obtained from the collagen bioink formulation (1:10) depicted with filled circles, for storage modulus and empty circles for loss

modulus. Conversely, the light blue lines correspond to the combination of collagen bioink and TC-71 EwS cells, represented by filled squares for storage modulus and empty squares for loss modulus. This comprehensive graphical representation provides insight into the mechanical properties of the bioink formulations and their interactions with cell density.

3.4 Extrusion-Based Approach Effectively Preserves Typical Hallmarks of EwS

Based on the optimization analysis, subsequent experiments were conducted using a cell density of 1.4×10^6 cells/mL, which provided the optimal balance between structural integrity, metabolic activity, and long-term viability.

Consistent with the Live/Dead results, EwS cells maintained high viability over time, indicating that the bioprinting process does not adversely affect cell survival. Immunofluorescence analysis showed a spatially heterogeneous distribution of Ki-67 expression, with higher proliferative activity at the spheroid periphery, as shown in **Figure 5 (a)**. This pattern could be likely due to enhanced access to nutrients and oxygen in outer regions, while the reduced signal observed in the spheroid core suggests the presence of quiescent or less metabolically active cells, likely due to diffusion limitations.

We can speculate that such spatial organization could reflect the establishment of physiologically relevant gradients within the construct, partially recapitulating the tumor microenvironment. These gradients are known to regulate cell behavior and intercellular signaling, representing a key advantage of 3D bioprinted systems over conventional 2D cultures^{32,33}.

Histological analysis further supported these observations. H&E staining revealed a homogeneous colonization of the collagen matrix by TC-71 cells, which retained the characteristic small, round cell morphology typical of EwS. Cells were distributed throughout both peripheral and inner regions of the construct and progressively organized into multicellular spheroids, as shown in **Figure 5 (b)**. Quantitative image analysis demonstrated a significant increase in spheroid numbers from day 7 (30.33 ± 5.4) to day 14 (73.67 ± 13.13), corresponding to a 2.5-fold increase ($P < 0.01$). Similarly, spheroid size increased by approximately 2.1-fold over the same period ($P < 0.0001$). Beyond the presence of multiple spheroids distributed throughout the construct, variability in chromatic staining intensity, well visible at higher magnification, was observed among individual cells, suggesting the emergence of a heterogeneous cell population. In H&E-stained sections, such differences may reflect variations in nuclear density, chromatin organization, and cytoplasmic composition, commonly associated with distinct functional states, including proliferative, quiescent, or early apoptotic phenotypes. The coexistence of densely stained nuclei and more lightly stained cells within the same construct may indicate differential cell cycle activity or metabolic states. Such histological heterogeneity represents an important strength of the bioprinted model, as it more closely mimics the complexity of native tumor tissue compared to uniform 2D cultures. Overall, these findings indicate that the bioprinted 3D collagen model supports not only cell viability but also promotes tumor-relevant architectural features, such as spheroid formation. While this enhances physiological relevance, the presence of diffusion-driven heterogeneity likely highlights a potential inherent trade-off between biomimicry and experimental uniformity. Therefore, further

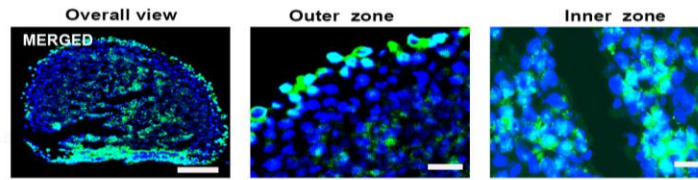
investigations are warranted to explore such effects, aiming to elucidate how such conditions impact tumor architecture and response to therapies.

Crucially, within the context of EwS, this biomimetic environment serves as a functional template for progressive spheroid assembly. This multi-step process is dynamically driven by the orchestration of cell-cell adhesion molecules, such as E-/N-cadherins, often aberrantly expressed in mesenchymal-derived tumors like EwS³⁴, and specific collagen-binding integrins (primarily $\alpha1\beta1$ and $\alpha2\beta1$), which mediate bidirectional mechanotransduction³⁵. Working in tandem with active matrix remodeling, cells exert traction forces while upregulating matrix metalloproteinases, specifically MMP-2 and MMP-9, to cleave collagen fibers and overcome steric barriers³⁶, ultimately guiding the transition from loose cellular clusters into dense, highly organized 3D tumor aggregates. Consistent with this mechanism, our 3D model exhibited a progressive increase in the expression of MMP-9 and MMP-13 between day 7 and day 14 ($P<0.001$), accompanied by a parallel increase in the number of cell clones. These findings highlight the dynamic evolution of the model over time and support its ability to recapitulate key aspects of EwS tumor progression (**Figure S3**).

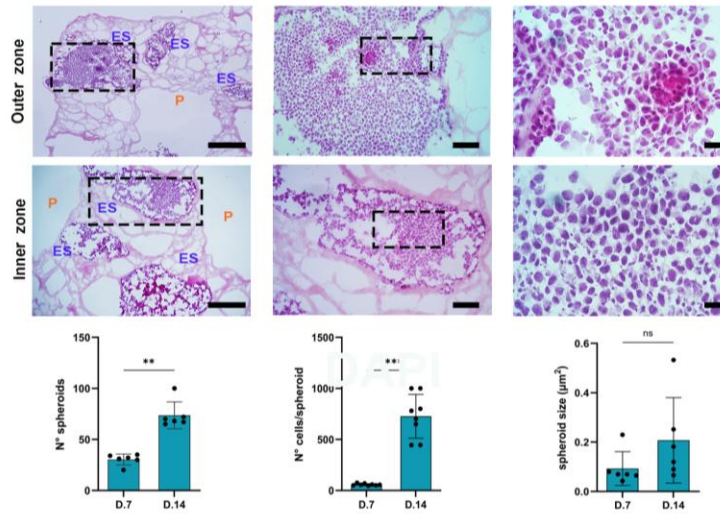
Further in-depth characterization was performed by immunofluorescence to assess two hallmarks of EwS cells: the cell surface molecule CD99 and the oncogenic fusion protein EWS::FLI1. Expression and localization were confirmed in TC-71 spheroids grown on the 3D collagen hydrogel, as shown in **Figure 5 (c-d)**. CD99 was uniformly distributed along the extracellular membrane of EwS cells, with marked enrichment at the interface between the spheroids and the surrounding collagen matrix (**Figure 5 c**). In contrast, FLI1 immunoreactivity was predominantly nuclear, consistent with its role as a transcriptional regulator (**Figure 5 c**). To confirm whether the expression of EWS::FLI1 detected via immunofluorescence directly reflected the preservation of its oncogenic program, we evaluated the expression profile of well-established downstream target genes. This evaluation was conducted in both 2D and 3D models, confirming a strong correspondence between the two conditions. Specifically, we analyzed both positively regulated (*NROB1* and *IGF1*) and negatively regulated (*LOX* and *IGFBP3*) EWS::FLI1 targets. Consistent with our immunofluorescence findings, the transcriptional modulation of these key target genes was fully preserved (**Figure 5 d**), thereby providing robust molecular evidence of a functional and active EWS::FLI1 signaling pathway in our model.

Overall, these findings demonstrate that EwS cells cultured in collagen-based 3D bioprinted scaffolds retain key phenotypic and molecular characteristics, supporting the suitability of this model for recapitulating EwS biology in a physiologically relevant 3D microenvironment.

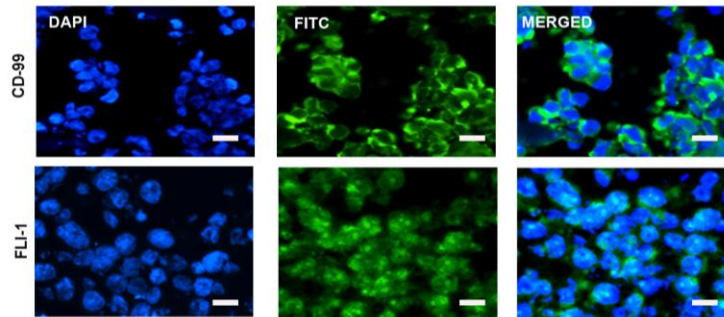
(a) Immunofluorescence analysis- ki-67 marker F.up= D.14



(b) Hematoxylin and Eosin analysis F.up= D.14



(c) Immunofluorescence analyses F.up= D.14



(d) Gene expression analyses F.up= D.14

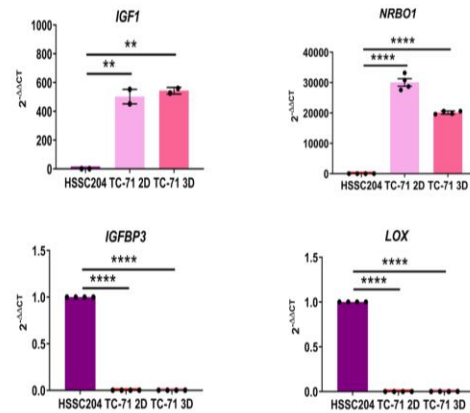


Figure 5. Characterization of the 3D bioprinted EwS model. (a) Representative immunofluorescence images of the 3D bioprinted TC-71 model at day 14 showing Ki-67 expression. Images are presented at different magnifications (scale bar: 200 μm ; 50 μm and 20 μm). Ki-67-positive cells are shown in green, and nuclei are counterstained with DAPI (blue). Panel (b) Representative hematoxylin and eosin staining of TC-71 embedded in collagen hydrogels at day 14. Images are shown at increasing magnifications (scale bars: 200 μm ; 100 μm , 20 μm) to highlight both global architecture and cellular morphology in inner and outer regions. Quantitative analysis of spheroid number, number of cells per spheroid, and spheroid size (μm^2) was performed using NIS-Elements software with Hue Saturation Intensity (HIS) analysis. Data are expressed as mean $\pm$ standard deviation. Statistical significance was determined using Student's t-test: ** $P < 0.01$ N° spheroids day 7 vs day 14; **** $P < 0.0001$ (N° cells/spheroid and spheroid size, day 7 vs day 14). Data are presented as mean $\pm$ standard error. (c) Representative fluorescence micrographs of TC-71 spheroids cultured within the 3D collagen-based bioprinted scaffold, showing expression of CD99 and FLI1 at day 14. Images are presented at high (20 μm) magnification to illustrate single-cell resolution. CD99 and FLI1 immunoreactivity is shown in green (FITC channel), while cell nuclei are counterstained with DAPI (blue), enabling visualization of cellular distribution and morphology within the 3D microenvironment. (d) Relative mRNA expression analysis of *IGF1*, *NROB1*, *IGFBP3* and *LOX* in the 2D or 3D culture conditions by qPCR at day 14. The data are shown as mean $\pm$ SE. Relative quantification calculated as $2^{-\Delta\Delta C_t}$ (** $p < 0.01$, **** $p < 0.0001$, one-way ANOVA test).

3.5. Evaluation of drug response in a 3D Bioprinted Ewing Sarcoma Model

Miniaturized bioprinted scaffold systems enable rapid and scalable drug testing, allowing pharmacologic assessment within three days post-bioprinting due to the formation of structurally homogeneous, viable and proliferative spheroids. Here, we tested the robustness and translational relevance of our collagen-based 3D bioprinted model by evaluating the response of EwS cells to PARPi under defined initial culture conditions optimized to enhance physiological relevance and reproducibility. EwS has been identified as a BRCA-deficient-like tumor, as the abnormal EWS::FLI1 fusion product disrupts the homologous recombination (HR) system by trapping BRCA1³⁷. Although preclinical studies showed promising therapeutic efficacy with PARPi, their clinical efficacy, either as a monotherapy or in combination with cytotoxic chemotherapy, has remained limited^{38,39}. These discrepancies highlight the need for more predictive and physiologically relevant preclinical platforms to evaluate PARPi sensitivity. Among clinically relevant PARPi, talazoparib is one of the most interesting compounds because it exerts its anticancer effects through two complementary mechanisms: on one side, it inhibits the catalytic activity of PARP1/2, preventing PARylation; on the other side, it traps PARP1/2 enzymes on DNA at sites of damage, leading to a highly cytotoxic complex that blocks replication fork progression and impedes DNA repair machinery⁴⁰. Notably, talazoparib is particularly potent in this “trapping” effect—much stronger than other PARPi like olaparib or rucaparib. Here, we assessed the efficacy of talazoparib in TC-71 cells cultured within collagen-based 3D bioprinted scaffolds after 3, 7 and 10 days of treatment.

As illustrated in **Figure 6 (a-b)** and **Figure 7**, cell exposure to talazoparib not only disrupted spheroid architecture but also induced a dose-dependent decrease in spheroid size and viability, with the most pronounced effects observed at 20 nM ($P < 0.0001$) in the 3D model.

Quantitative analysis using NIS-Elements software confirmed a significant reduction in cell viability post-talazoparib treatment (**Figure 7**), aligning with the diminished metabolic activity observed by the Alamar Blue assay from day 7. Notably, the impact of the drug was more pronounced in the inner regions of the spheroids, while viable cells persisted predominantly at

the periphery (**Figure 7**). This spatial distribution suggests the presence of either heterogeneous drug penetration or microenvironment-dependent resistance mechanisms.

Assessment of cell proliferation by Ki-67 immunostaining revealed different proliferation kinetics between the 2D and 3D culture systems (**Figure 8**). Although both models exhibited a similar overall trend over time, Ki-67 expression in the 3D untreated control was significantly lower than in the corresponding 2D culture at day 3. Notably, the level of Ki-67 expression observed in the 3D control at day 10 was comparable to that measured in the 2D control at day 3, indicating a delayed proliferative progression in the 3D model.

Treatment with 10 nM talazoparib did not significantly reduce Ki-67 expression compared with the corresponding untreated controls at day 3 in either the 2D or 3D model, although a slight downward trend was observed in both conditions. In contrast, treatment with 20 nM talazoparib exerted a significant anti-proliferative effect in the 3D model, resulting in a marked reduction in Ki-67 expression relative to the untreated control at day 10 ($P < 0.05$). These findings suggest that the 3D model exhibits slower proliferation kinetics than conventional 2D cultures and that the anti-proliferative activity of talazoparib becomes evident only after prolonged exposure at day 10 and at the higher concentration tested, likely due to the presence of the collagen matrix.

In parallel, the increase in γ H2AX positivity following talazoparib treatment indicates that its anti-proliferative effects are accompanied by the accumulation of DNA double-strand breaks and increased genotoxic stress, ultimately leading to a reduction in the viable tumor cell population. However, the magnitude and kinetics of this response differed markedly between the 2D and 3D culture systems (**Figure 8**). In the 3D bioprinted model, treatment with 10 nM talazoparib for 10 days induced only a modest reduction in Ki-67 expression together with a slight increase in γ H2AX positivity, neither of which reached statistical significance. In contrast, exposure to 20 nM talazoparib resulted in a significant increase in γ H2AX staining ($P < 0.05$), accompanied by a significant reduction in cell proliferation, indicating that a higher drug concentration was required to elicit a robust DNA damage response within the 3D microenvironment, as previously evidenced in Ki-67 analysis. These findings differ from those obtained under conventional 2D culture conditions, where treatment of TC-71 cells with 10 nM talazoparib for only 3 days was sufficient to induce a significant increase in γ H2AX, reflecting rapid accumulation of DNA damage. The attenuated and delayed γ H2AX response observed in the 3D model suggests that the collagen-based microenvironment confers a greater degree of protection against PARP inhibition, likely through cell–cell and cell–matrix interactions that influence drug penetration, DNA damage signaling, and cellular stress responses.

In summary, these results provide compelling evidence of talazoparib's efficacy in targeting tumor spheroids, positing a robust model for investigating the mechanisms of drug action. From a methodological perspective, these findings underscore the advantage of utilizing the 3D bioprinted EwS model to effectively capture treatment-induced alterations in tissue-like architecture, rather than solely relying on single-cell viability readouts. The ability to quantify changes in spheroid number and morphology provides additional insight into drug-induced effects on tumor-like organization and growth dynamics.

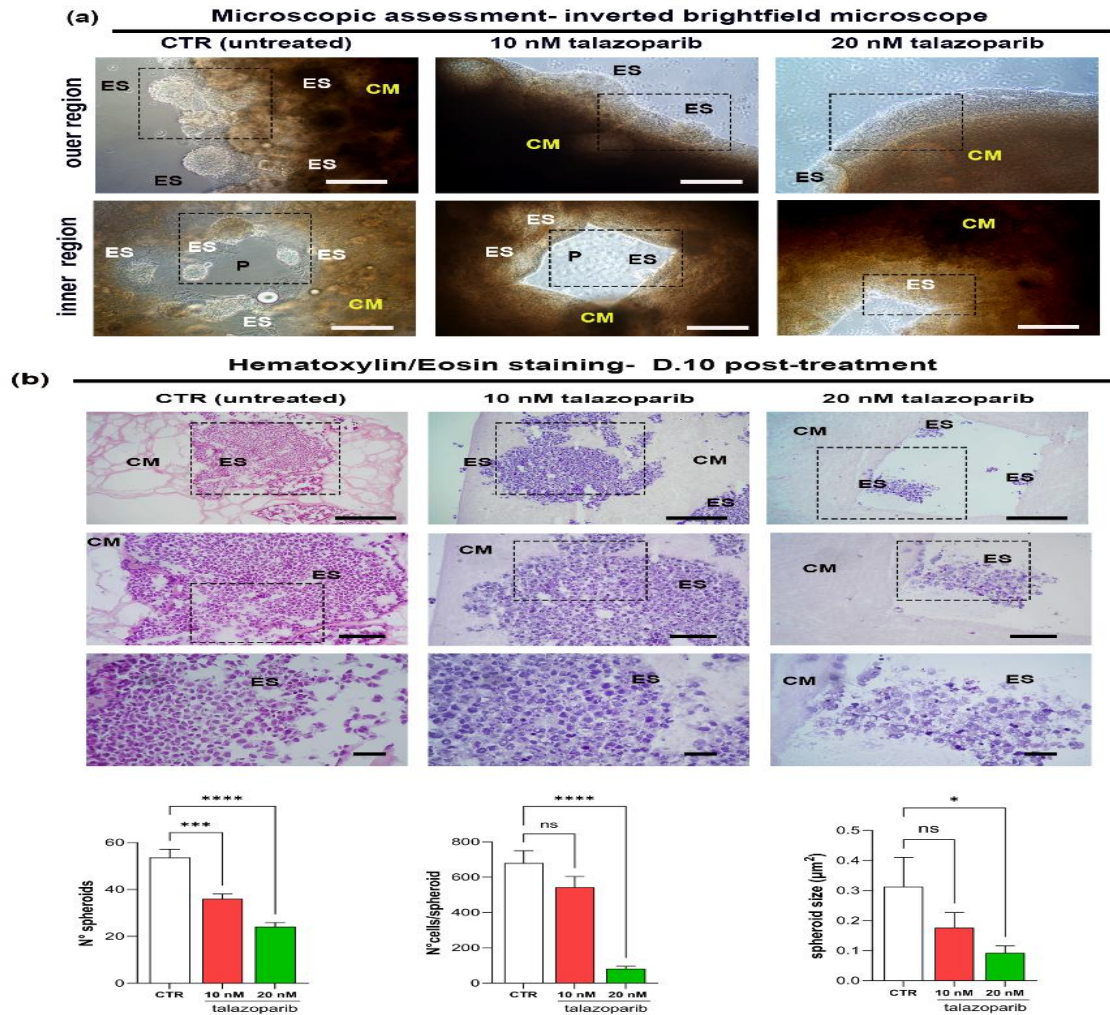


Figure 6. Microscopic evaluation of the 3D bioprinted EwS model following drug treatment. (a) Representative bright-field images acquired using inverted and optical microscopy of TC-71 cells embedded in collagen-based constructs, shown under untreated conditions and following treatment with talazoparib (tala) (10 and 20 nM) at day-10 of culture. Images depict both outer and inner regions of the constructs at low (10 x) and high (20 x) magnification. Details with dashed rectangles indicate the high-magnification images. (b) Representative microscopic images, stained with haematoxylin and eosin, showing the morphology and spatial distribution of TC-71 cells embedded in collagen-based constructs, under control (untreated) and following treatment with talazoparib at 10 and 20 nM. Images are presented at low (10x and 20x) and high (40x) magnification with details, with regions of interest highlighted by dashed rectangles corresponding to the higher-magnification images. Graphs with quantitative data of the number of cell spheroids, the number of cells per spheroid, and spheroid size (μm^2), in both control and treated groups. Differences over time among groups were evaluated using one-way ANOVA. Results demonstrated a significant reduction in the number of cell spheroids in the 10 nM talazoparib group compared to control ($***P < 0.001$), with an even more pronounced decrease in the 20 nM group ($****P < 0.0001$). A significant reduction in the number of cells per spheroid was also observed in the 20 nM group compared to control ($***P < 0.001$). In addition, spheroid size was significantly reduced following treatment with 20 nM talazoparib ($*P < 0.05$), while no statistically significant change was observed at 10 nM.

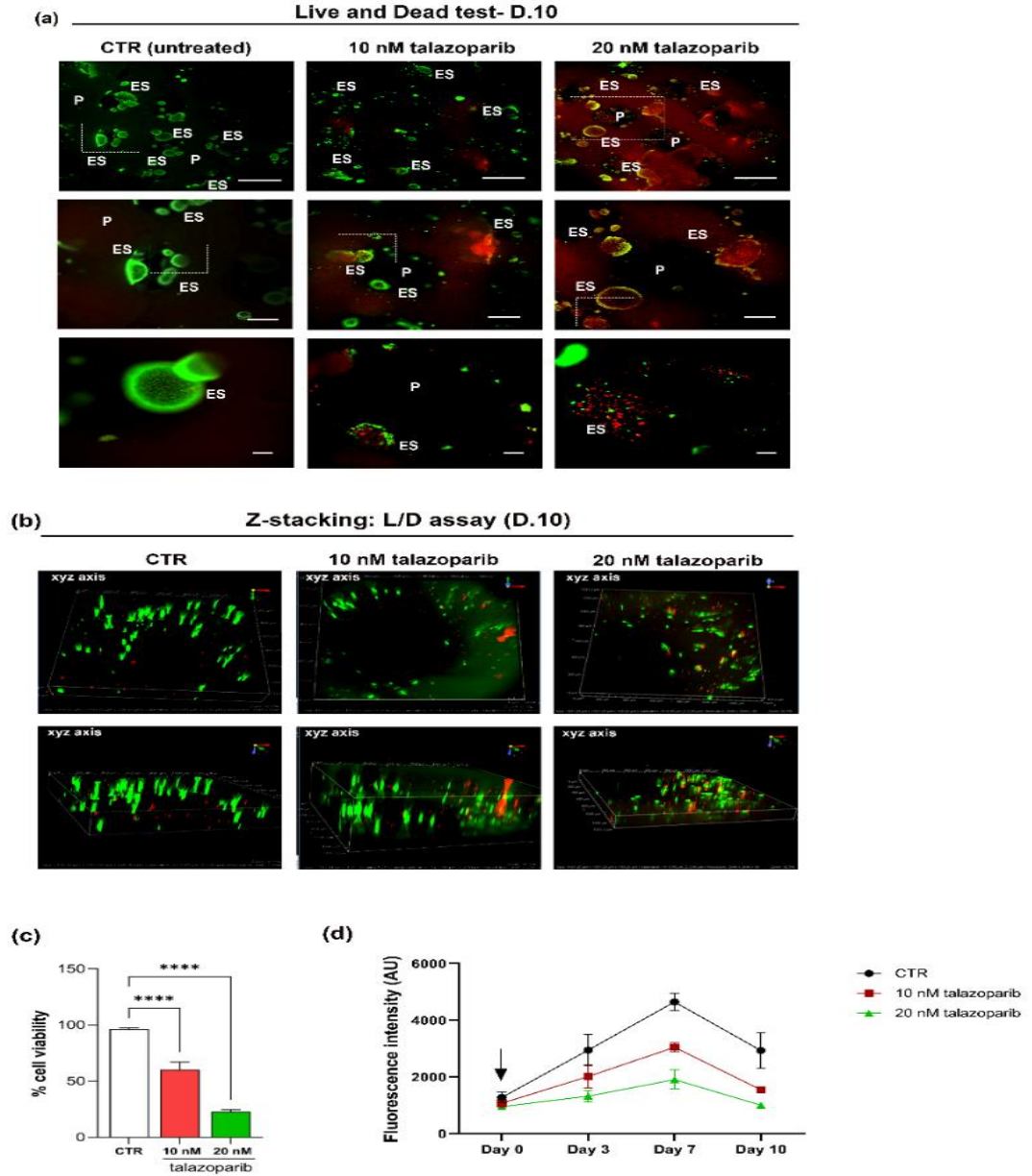


Figure 7. Evaluation of cytocompatibility of the 3D bioprinted EwS model following drug treatment. (a) Representative merged fluorescence images showing live (green) and dead (red) TC-71 cells embedded in collagen-based constructs, under untreated (CTR) conditions and following treatment with talazoparib (10 and 20 nM), as assessed by Live and Dead test. Scale bar: 100 μ m. (b) Representative Z-stack fluorescence images of Live/Dead-stained constructs from CTR and treated groups (10 and 20 nM talazoparib). Z-stacking acquisition provides 3D information across multiple focal planes (x-y-z axis), enabling spatial assessment of cell viability within the construct. (c) Quantitative analysis of cell viability (%) for control and treated groups following talazoparib exposure at 10 and 20 nM at day 10. One-way ANOVA was used to compare differences with the control group over time. **** $P < 0.0001$ (d) Quantification of metabolic activity assessed by Alamar blue assay in control and treated groups (10 and 20 nM talazoparib). Data are expressed as mean $\pm$ standard deviation.

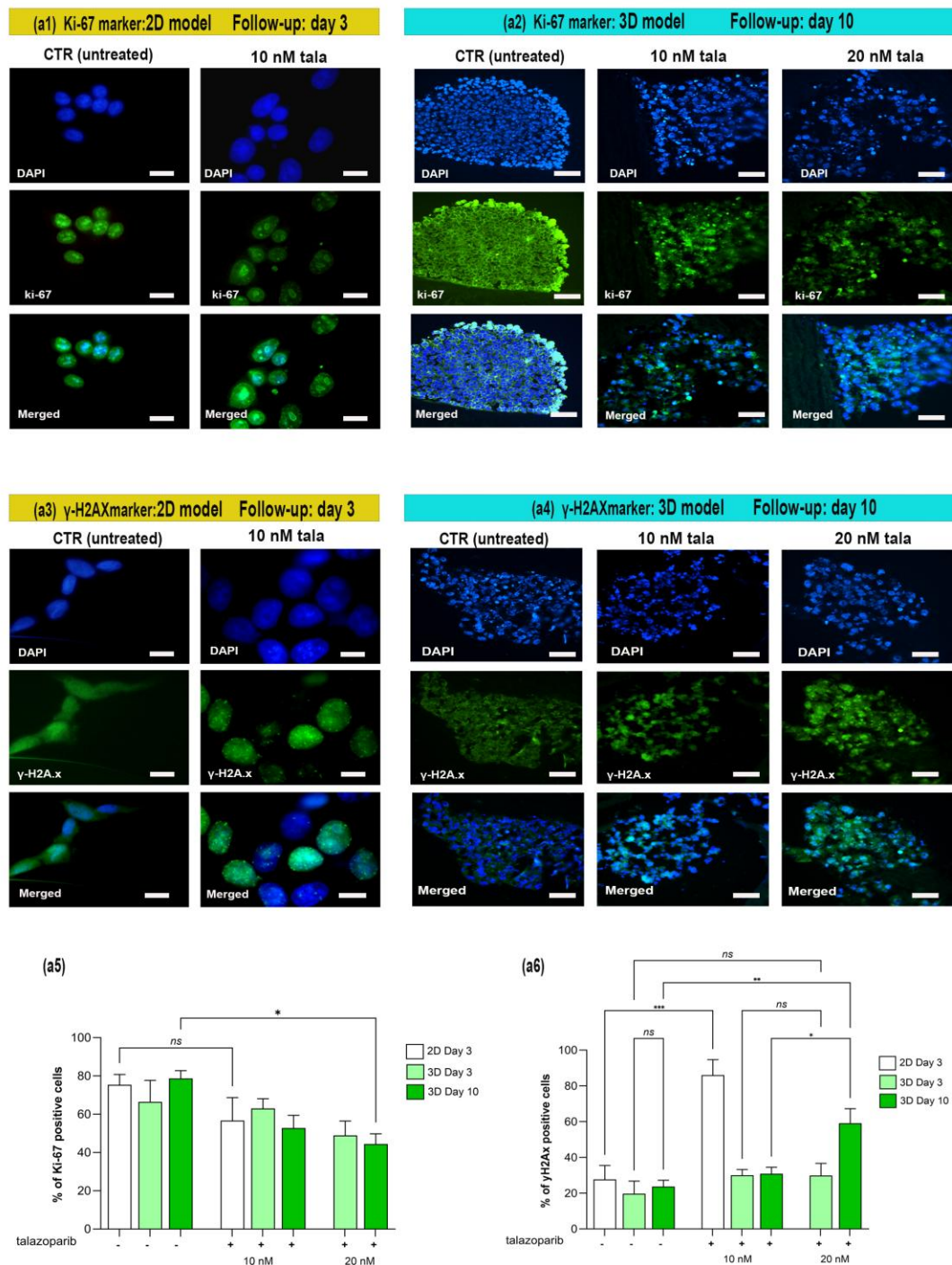


Figure 8. Immunofluorescence staining of Ki-67 and γ H2AX in 2D and 3D EwS model following talazoparib treatment. (a1-a2) Representative micrographs showing Ki-67 expression in control and experimental groups treated with talazoparib at concentrations of 10 nM in 2D condition at day 3 (*left panel*) and 10 and 20 nM in 3D condition at day 10 (*right panel*). Merged images display DAPI (blue) and FITC (green) staining (scale bar:40 μ m). (a5-a6) Graphical representation of semi-quantitative analysis of % expression of Ki-67. Data are presented as mean

± standard deviation. Results from one-way ANOVA to test differences in the control group over time are reported. **P<0.01; ns, not significant. (b) Representative micrographs depicting γ H2AX expression in control and experimental groups treated with talazoparib at concentrations of 10nM in 2D condition at day 3 (*left*) and 10 and 20 nM in 3D condition at day 10 (*right*). Merged images show DAPI and FITC staining (scale bar:40 μ m). Graphical representation of semi-quantitative analysis of the percentage expression of γ H2AX, presented as mean ± standard deviation. One-way ANOVA was used to compare differences with the control group over time. *P<0.05; **p<0.01; *** p<0.001; ns, not significant.

4. Conclusion

The development of a 3D bioprinted EwS model represents a significant advancement in cancer research, specifically in the study of tumor biology and therapeutic responses. To our knowledge, this is the first cell-laden 3D bioprinted model specifically designed for EwS, addressing the limitations of conventional approaches that predominantly rely on static seeding methods. Existing approaches often utilize synthetic biomaterials, which may fall short in terms of biocompatibility and the ability to replicate the intricate dynamics of the natural tumor ECM⁴¹. By employing a collagen bioink⁴², we successfully created a scaffold that closely mimics the structural and microenvironmental complexity inherent to EwS tumor, facilitating a more physiologically relevant platform for drug screening. This model demonstrated the capability to promote homogeneous cell dispersion and defined architectural formations, overcoming challenges associated with conventional static seeding methods.

The gap between high *in vitro* drug sensitivity and limited clinical efficacy reinforces the necessity for innovative models that more accurately predict treatment outcomes. While EwS cell lines often show high sensitivity to therapeutic agents, including PARPi such as olaparib, niraparib, and talazoparib, in traditional 2D cultures, these promising responses have not translated into substantial clinical benefits (e.g., NCT01583543, NCT01858168, NCT03233204 clinical trials). Our evaluations of EwS cell sensitivity to talazoparib in a 3D bioprinted model revealed a dual effect: the drug markedly reduced cell viability and proliferation, particularly in the outer spheroid regions, while enhancing cytotoxic responses in inner regions. This spatially dependent reduction in viability suggests that drug delivery and penetration play critical roles in determining therapeutic outcomes, highlighting the need to consider tumor heterogeneity and inner microenvironments when designing cancer treatments. Notably, we observed pronounced cytotoxic effects in the inner regions of the bioengineered tumors, suggesting that these cells, typically more isolated from pharmacological interventions, can be sensitized under appropriate conditions. Furthermore, the differential responses observed between the inner and outer spheroid regions advocate for combinatorial treatment approaches that may more effectively target both rapidly proliferating peripheral cells and more resistant core cells.

While the proposed model provides a robust platform for evaluating drug responses in EwS, several limitations should be acknowledged. First, the study relied on a tumor monoculture and does not include key components of the bone tumor microenvironment, such as osteoblasts, osteoclasts, endothelial cells, or immune populations (myeloid and lymphoid cells), all of which are known to influence tumor progression and therapeutic response.

A further limitation is the use of a static culture system, which may lead to the formation of gradients in oxygen, nutrients, and drug distribution, particularly in longer-term or more complex multicellular constructs. To overcome these limitations, future studies should adopt a stepwise strategy to increase model complexity. Osteoblasts and osteoclasts could first be incorporated to recapitulate bone remodeling dynamics, followed by the addition of endothelial cells to promote vascular-like network formation and improve mass transport. Subsequently, immune components could be integrated to model tumor–immune interactions and evaluate immunomodulatory responses.

Moreover, considering that the storage modulus of trabecular bone is strongly influenced by the bone marrow compartment, with values reported to range between approximately 0.1 and 10.9 kPa, depending on marrow composition and trabecular architecture²⁶, the development of collagen-hydroxyapatite composite bioinks could better recreate the biochemical and mechanical properties of this microenvironment. Together with dynamic culture conditions, these improvements may provide a more physiologically relevant platform for studying tumor biology, cell–cell interactions, and therapeutic responses. Recently, it has been shown, mainly in hematological malignancies^{43–45}, that 3D tumor models consider not only the environment and ECM mechanics created by the hydrogel, but using an organ-on-a-chip platform, they analyze the environment combined with the mechanical effects. All these aspects will represent important evolution for obtaining 3D models that have the advantage of microfluidic perfusion, helping to guarantee sustained nutrient delivery.

Therefore, despite its limitations, this platform represents a valuable starting point for the progressive development of more complex models that better recapitulate tumour–microenvironment interactions. Future refinements incorporating multicellular components and dynamic perfusion are expected to further enhance its physiological relevance, ultimately improving the predictive power of preclinical drug screening.

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Conflict of interest

Brunella Grigolo is an Editorial Board Member of this journal, but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Data are available from the corresponding author upon reasonable request.

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