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Gelatine methacrylamide-based hydrogels – an alternative 3D cancer cell culture system

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\textbf{Running title:} A gelatine methacrylamide-based 3D cancer model

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Abstract

Modern cancer research requires physiological, three-dimensional (3D) cell culture platforms, wherein the physical and chemical characteristics of the extracellular matrix (ECM) can be modified. In this study, gelatine methacrylamide-based hydrogels (GelMA) were characterised and established as *in vitro* and *in vivo* spheroid-based models for ovarian cancer, reflecting the advanced disease stage of patients, with accumulation of multicellular spheroids in the tumour fluid (ascites).

Polymer concentration (2.5-7% w/v) strongly influenced hydrogel stiffness (0.5 ± 0.2 kPa – 9.0 ± 1.8 kPa) but had little effect on solute diffusion. The diffusion coefficient of 70 kDa FITC-labelled dextran in 7% GelMA-based hydrogels was only 2.3-times slower compared to water. Hydrogels of medium concentration (5% w/v GelMA) and stiffness (3.4 kPa) allowed spheroid formation and high proliferation and metabolic rates. The inhibition of matrix metalloproteinases and consequently ECM degradability reduced spheroid formation and proliferation rates. The incorporation of the ECM components laminin-411 and hyaluronic acid further stimulated spheroid growth within GelMA-based hydrogels. The feasibility of pre-cultured GelMA-based hydrogels as spheroid carriers within an ovarian cancer animal model was proven and led to tumour development and metastasis. These tumours were sensitive to treatment with the anti-cancer drug paclitaxel, but not the integrin antagonist ATN-161. While paclitaxel and its combination with ATN-161 resulted in a treatment response of 33-37.8%, ATN-161 alone had no effect on tumour growth and peritoneal spread. The semi-synthetic biomaterial GelMA combines relevant natural cues with tunable properties providing an alternative, bioengineered 3D cancer cell culture *in vitro* and *in vivo* model system.
1. Introduction

The extracellular matrix (ECM), its composition and physical properties have a high impact on functional behaviours, influencing cell growth, morphology and survival [1–4]. Changes of these properties play a key role in tumour development, progression and metastasis [5,6]. The importance of the ECM is well established, but biomaterials that mimic naturally occurring ECM components and their three-dimensional (3D) architecture are still not routinely used in cancer research. The majority of research groups use cell monolayers grown on tissue culture plastic, which are less complex, less variable and not representative of the physiological, extracellular microenvironment [7]. However, there is increasing evidence that cell-based experiments are performed in more realistic conditions employing different engineered biomaterials [8].

To capture the complexities of the native ECM in a 3D in vitro model, various approaches in material design have been applied. Matrices of natural origin, such as protein-based hydrogels like Matrigel™ [9], collagen [10] and fibrin [11], are used in cancer research [12]. However, the gold standard in mimicking a natural ECM is Matrigel™, a reconstructed basement membrane matrix produced by Engelbreth-Holm-Swarm mouse sarcoma cells [9,13]. The advantage of these naturally-derived materials is the representation of native ECM components, cell binding and cleavage sites, but their limitations rise from high batch-to-batch variations, undefined matrix composition and restricted modification possibilities [14–16]. Their mechanical properties are less controllable due to degradation over time, and their weak mechanical stiffness makes them difficult to handle [16]. To overcome these drawbacks, synthetic biomaterials are engineered and applied as 3D culture systems, creating a link between cell monolayer cultures and in vivo studies [8]. Developing new synthetic systems with a high reproducibility, consistent composition and controllable physical properties for 3D cultures has been challenging in the field of biomaterial sciences [8,16,17].

Engineered technology platforms that are based on self-assembling peptides and nanofibres, undergoing a spontaneous reaction and forming 3D networks due to altered pH or salt concentrations [18], are successfully used for various approaches [18,19]. Other commonly used synthetic materials are based on polyethylene glycol (PEG) and its modifications [16,20]. However, these synthetic materials lack bioactive ECM ligands to stimulate cell surface receptors, which can be overcome by chemical incorporation of protease cleavage sites and integrin cell-binding sites [21]. A cost and fabrication efficient alternative is a semi-
synthetic material design, combining the advantages of native ECM components and tuneable matrix properties, resulting in higher reproducibility, less complexity and better comparability between different groups than traditional cell monolayer approaches.

In this study, gelatine methacrylamide-based hydrogels (GelMA) were used as a 3D cancer cell culture system. GelMA consists of gelatine, an inexpensive component, functionalised with methacrylamide side groups that are cross-linked via a photo-initiated reaction [22]. Gelatine is denatured collagen, mostly collagen type I, and presents integrin cell binding motifs, such as RGD, and matrix metalloproteinases (MMP)-degradable sites [23,24]. Compared to native collagen, gelatine has a lower antigenicity and less batch-to-batch variation due to the denaturation process, in which tertiary protein structures are removed. Cross-linking of the methacrylamide side groups results in hydrogels with stiffness and density that can be controlled by varying the polymer dry mass, degree of functionalisation, photo-initiator concentration, UV intensity and exposure time [22,25,26]. Photo-chemical cross-linking takes place only in the presence of a photo-initiator, resulting in hydrogels which are formed by externally controlled gelation conditions, initiated by UV light [22]. Physical gelation is weaker in gelatine methacrylamide compared to unmodified gelatine because vinyl side groups at gelatine chains interfere with helix formation, and after chemical cross-linking physical gelation is inhibited [25]. This enhances the reproducibility of GelMA preparations. As a starter for the photo-reaction, Irgacure 295 was chosen, which is widely used for cell encapsulation within hydrogels, and its minimal toxicity at the used concentration has independently been proven in several studies [27,28]. Most hydrogels, including GelMA, have the advantage of high transparency permitting microscopic analysis of cells embedded within these matrices.

A validation and quality protocol to measure the physical properties of the hydrogel by unconfined compression tests and Fluorescence Recovery After Photobleaching (FRAP) was designed. The hydrogel porosity has an impact on macro- and microscopic diffusion of particles, including growth factors, nutrients and anti-cancer drugs [29–31]; all features critical for tumour growth and cancer cell survival [32,33]. Growth factors, such as EGF, TGF and VEGF, are key factors in cancer development and progression, with an average molecular weight of 6-45 kDa [34,35]. Hence, a FITC-labelled 70 kDa dextran (FD70) was used to represent these relevant biomolecules. The water-based hydrogel precursor solution has a low viscosity at 37°C and allows mixing GelMA with additional ECM components, such as hyaluronic acid (HA), the ligand for the CD44 receptor, and laminin-411 (LN-411), the binding partner of integrin α6β1. HA influences the proliferation and migration of various
cancer cells, and high levels of this ECM glycosaminoglycan are linked to the ovarian tumour-associated stroma and poor prognosis of ovarian cancer patients [36–38]. The adhesion of ovarian cancer cells onto HA-immobilised surfaces is enhanced and correlates with high CD44 levels, especially for OV-MZ-6 cells, indicating a role of HA in peritoneal tumour spread [38]. CD44 is a key factor in tumour growth and metastasis [39]. LN-411 is a basement membrane protein and promotes upon binding to integrin α6β1 cell adhesion, migration and angiogenesis [40,41]. LN/integrin α6β1 interactions are important for proliferation and adhesion of ovarian cancer cells [42].

In this study, GelMA was used as a 3D model to mimic the natural, local microenvironment of a growing tumour in order to investigate the interactions between cancer cells and the surrounding ECM. Bioengineered approaches have already been used as cancer cell delivery vehicles in animals models, including cell-seeded PEG-based hydrogels in an intraperitoneal ovarian cancer mouse model, thereby combining 3D growth and in vivo imaging of cancer growth and treatment responses [7,20,53]. Ovarian cancer was chosen for its unique path of metastasis and the formation of multicellular cancer spheroids that contribute to resistance to currently used chemotherapeutic regimes [21,43–45]. Most patients are diagnosed at an advanced stage of the disease, characterised by low survival rates due to resistance to therapy. The ovarian cancer cell line OV-MZ-6 was derived from a patient with evidence of peritoneal tumour spread and is known to form multicellular spheroids from single cells in a 3D microenvironment [21]. These spheroids showed an increased resistance to paclitaxel compared to cell monolayers [21,46]. The aim of this study was to establish an alternative biomaterial-based platform for ovarian cancer spheroid growth, thereby analysing cell-ECM interactions in vitro and in vivo. Spheroid formation within GelMA-based hydrogels was visualised and proliferation was quantified in relation to varying stiffness of the biomaterial, MMP inhibition, LN-411- and HA-incorporation. To prove the feasibility of these hydrogels as spheroid carriers within an ovarian cancer animal model, tumour growth and metastasis were monitored over 8 weeks. The responsiveness of these spheroid-derived tumours towards paclitaxel [44] and the non-RGD based integrin antagonist ATN-161 [47,48] was determined. Paclitaxel, a mitotic inhibitor, is a widely administered chemotherapeutic, whereas ATN-161 is a recently developed anti-cancer drug that has led to a prolonged stable disease in patients with solid tumours [49].

2. Materials and methods
2.1. 3D cell culture

The human epithelial ovarian cancer cell line OV-MZ-6 was derived from a patient with advanced stage of the disease and cultured as reported [21]. Their potential to form multicellular spheroids in hydrogel-based 3D cultures has been shown previously [21]. For animal experiments, OV-MZ-6 cells were transfected with a lentiviral luciferase expression system (pLenti6/V5-D-TOPO; Invitrogen) using blasticidin-selection [20].

2.2. Preparation of GelMA-based hydrogels

Gelatine methacrylamide (GelMA) was synthesised from gelatine (isolated from porcine skin, type A; Sigma Aldrich, St. Louis, MO, USA) and methacrylic anhydride (Sigma Aldrich; Figure 1A) following a previously published procedure, resulting in GelMA with a high degree of functionalisation with 70-80% of all lysine groups [22,25]. GelMA-based hydrogels were cross-linked by UV irradiation in the presence of 0.5 mg/mL of a water soluble photo-initiator (Irgacure, IC2959; BASF, Germany). GelMA solution was prepared in PBS to a final concentration ranging from 2.5 to 10%, with polymer concentrations in percentages of weight per volume of PBS (w/v). The precursor GelMA solution was transferred into a custom-made, rectangular Teflon mould, covered with a glass slide and cross-linked using a CL-1000 UV cross-linker (UVP; Upland California, USA) with 365 nm wavelength tubes for 10 min (exposed intensity of 2.7 mW/cm² on hydrogel surface). After polymerisation, hydrogels were removed from the mould and cut into 2 x 4 x 5 mm pieces. For cell-laden hydrogels, 2.8 x 10⁵ cells/mL cells were re-suspended in GelMA solution before cross-linking. After cutting, each hydrogel containing 1.12 x 10⁴ cells was cultured at 37°C/5%CO₂ for up to 21 days, with media changes every 3-4 days. The ECM components laminin-411 (LN-411; 20 µg/mL; Biolamina, Stockholm, Sweden) and sodium hyaluronate (HA; 0.1%; Lifecore Biomedical, USA) were incorporated, separately or combined, by mixing into the precursor GelMA solution prior to (addition of cells and) cross-linking.

2.3. Analyses of mechanical properties

Compression tests were performed using a micro-tester 5848 system (Instron, Norwood, MA USA), with a 5 N load cell, immersed in PBS at 37°C. Hydrogels were prepared 1-2 days
before testing and incubated at 37°C/5%CO₂ in PBS. Sample height was derived from the force-displacement data, while cross-sectional area was calculated using the height and weight of the rectangular sample ($\rho = 1.05$ g/mL). The slope of the stress-strain curve from 10-15% strain was taken as the compressive modulus.

2.4. Analyses of diffusion properties

Fluorescence Recovery After Photobleaching (FRAP) measurements were performed using confocal laser scanning microscopy (CLSM; SP5, Leica) to analyse diffusion properties of solutes within the hydrogel. Hydrogels were incubated with fluorescein isothiocyanate (FITC)-labelled 70 kDa dextran (FD70), which was prepared as described previously [50]. Hydrogels were pre-incubated with FD70 solution in the dark on a shaker to ensure uniform distribution within the hydrogel. To allow measurements in the linear concentration range (< 4 mg/mL) of the fluorophore intensity over concentration, a 3 mg/mL FD70/PBS solution was used [51]. Hydrogels remained in FD70 solution throughout the measurements, and FRAP experiments were performed at least 100 µm above the coverslip to ensure measurements within the hydrogel and to avoid artefacts from the glass/hydrogel and solution/hydrogel interfaces. For imaging and bleaching a 488 nm line from an inbuilt Argon-ion laser, a zoom factor of 3 and a scan frequency of 1000 Hz were used. Images were taken using a 10x low aperture objective (NA 0.45) to yield a beam profile with high axial extension, which allows modelling of the fluorescence recovery as two-dimensional diffusion process [51]. Diffusion coefficients and mobile fractions were determined from the FRAP image sequences by an analysis method that includes spatial and temporal intensity changes, rectangular FRAP (rFRAP) [51]. All FRAP analyses were performed with Matlab2011a (Mathworks; Natick, MA, USA). A rectangular bleach area of 50 µm x 50 µm was used throughout all FRAP recordings.

2.5. Analyses of cell proliferation

Proliferation assays, AlamarBlue® (Invitrogen) to quantify metabolic activity and CyQuant® (Invitrogen) to measure the DNA content, were conducted as described earlier [21]. Briefly, spheroid growth was detected at several time points (1, 7, 14 and 21 days) after cell encapsulation. To determine the metabolic activity, hydrogels were incubated in media contained 8% (v/v) AlamarBlue® reagent for 2 h at 37°C/5%CO₂. Media without cell-laden
hydrogels with AlamarBlue® reagent served as negative control. The AlamarBlue® solution was transferred into a black 96-well plate (Corning) and fluorescent signals (excitation 544 nm, emission 590 nm) detected using a POLARStar OPTIMA plate reader (BMG LABTECH). The signal of the control media was subtracted from all samples. To determine the DNA content, hydrogels were digested with proteinase K (0.5 mg/mL; Invitrogen) overnight. Then, samples were treated with RNaseA (1.4 U/mL; Invitrogen) for 1 h at room temperature in cell lysis buffer supplemented with NaCl (180 mM) and EDTA (1 mM). CyQuant® dye solution was added and fluorescent signals (excitation 485 nm, emission 520 nm) detected using a plate reader. A λ-DNA standard (10 ng/mL to 2 µg/mL) was used to calculate the change of DNA content for each time point. Each condition was measured in biological triplicates/quadruplicates (unless stated otherwise) in technical replicates.

2.6. MMP inhibition

Inhibition of cell-secreted MMPs and their effect on spheroid morphology and proliferation was examined using media supplemented with a broad-band MMP-inhibitor (GM6001; 20 μM; Chemicon). Cells were encapsulated within hydrogels as described above, and MMP inhibition started on day 1 of 3D culture for up to 21 days. DMSO served as control treatment. The media was replaced every third day. Each condition was measured in biological triplicates.

2.7. Analyses of spheroid morphology

Spheroid morphology over the duration of 3D cultures was assessed with 4’,6-diamidino-2-phenylindole (DAPI) to visualise nuclei and rhodamine415-conjugated phalloidin to stain the cytoskeleton. Hydrogels were washed with PBS, fixed in 4% paraformaldehyde (PFA)/PBS for 20 min and permeabilised in 0.2% triton X-100/PBS for 30 min at room temperature. Samples were washed with 0.1 M glycine/PBS followed by two washing steps in PBS. Hydrogels were stained with 0.8 U/mL rhodamine415-conjugated phalloidin/1% BSA/PBS and 2.5 µg/mL DAPI/PBS and stored in PBS at 4°C until imaged by CLSM. Z-stacks were acquired with a constant slice thickness of 2 µm and a depth of 80-200 µm to reconstruct maximal projections that were processed with Fiji image analysis software [52].

2.8. Analyses of protein expression
For immunohistological analyses, hydrogels were processed as described earlier [53]. Briefly, samples were incubated in TissueTek embedding media (ProSciTech), shock-frozen in liquid nitrogen and stored at -80°C until cryo-sectioned (5-7 µm). Then, samples were permeabilised with 0.2% Triton X-100/PBS for 30 min, washed with PBS and incubated with primary [cytokeratin-8 (1/100; Abcam); integrin α6 (1/50; Chemicon); MMP-9 (1/200; Abcam)] and secondary [Alexa488/568-conjugated anti-mouse/rabbit IgG (1/1,000; Invitrogen)] antibodies in 1% BSA/PBS for 1 h at room temperature. Cytoskeleton and nuclei were stained as above and fluorescence signals detected via CLSM. Hematoxylin and eosin staining was performed using standard procedures [53].

2.9. Analyses of tumour growth and the effect of anti-cancer treatment \textit{in vivo}

The spheroid-based ovarian cancer animal model was conducted as reported before [53]. All animal experiments were performed in compliance with the \textit{Australian Code of Practice for the Care and Use of Animals for Scientific Purposes} and approved by the University Animal Ethics Committee. 3D cultures were prepared as above, and spheroids were grown for 10 days within GelMA-based hydrogels before implantation into 6 weeks old female NOD/SCID mice. Animals received spheroid-containing hydrogel implants adjacent to both ovaries after opening the ventral abdominal wall and the peritoneum (6 animals per group) and were monitored until the predetermined endpoint of 8 weeks was reached. After 4 weeks of tumour growth, animals were treated with paclitaxel (10 mg/kg; twice per week; Sigma-Aldrich) or ATN-161 (10 mg/kg; thrice per week; Peptisynth, Belgium) or a combination of both anti-cancer drugs intraperitoneally over 4 weeks. DMSO and/or water served as control treatment. Tumour growth was assessed fortnightly via bioluminescence imaging (7.5 mg/mL D-luciferin, Caliper Life Sciences, US; \textit{Living Image®} software v.4.3.1, Perkin Elmer). Images were obtained for each group of 3 mice per image with a 13 cm field of view, binning (resolution factor) of 8 (medium), f-stop = 1 and exposure time 0.5-15 sec. Data were analysed using a threshold set at 10% around each bioluminescent source to determine the amount of photons emitted within a given time and presented as radiance (photons/sec/cm$^2$/steridian) from a constant sized region of interest drawn over the murine abdomen. After 8 weeks, animals were sacrificed, and the tumour tissues were removed. Tumours were weighed, and their volume determined according to Tomayko and colleagues [54].
2.10. Statistical analysis

Statistical analyses were carried out using a two-way ANOVA including a Tukey test in the software package ‘PASW Statistics’ (v. 18). Results for all analyses with ‘p’ value less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. The impact of GelMA concentration on hydrogel stiffness and diffusion

To overcome the disadvantage of batch-to-batch variations and inhomogeneous distribution patterns of many 3D cell culture models [8,55] and to ensure reproducible hydrogel properties, the hydrogel preparation was standardised and its properties were tested using a validation protocol. The GelMA polymer was produced after a published protocol [22], and the proportion of gelatine and methacrylamide was kept constant (Figure 1A; step 1). After the polymer was completely dissolved in PBS, ovarian cancer cells were mixed into this precursor solution (Figure 1A; step 2, 3). The solution was cast in customised moulds and UV cross-linked. The closed mould design minimised oxygen inhibition, which is essential for the cross-linking reaction (Figure 1A; step 4). Cutting guides were used to obtain hydrogels of equal size (2 x 4 x 5 mm; Figure 1A; step 5) and cell number (2.8 x 10⁵ cells/mL).

To characterise the hydrogel preparation, two parameters of biological relevance, namely stiffness and solute diffusion within the matrix, were assessed using unconfined compression tests and FRAP measurements. Our previously reported results showed that hydrogel stiffness was a function of GelMA concentration, c, and UV-cross-linking time, t (equation 1) [25].

The dry weight of the GelMA polymer within the hydrogel had only a minor influence on hydrogel swelling [25]. For the used UV source, photo-initiator and mould, the empirical compression modulus (E, kPa) of hydrogels was based on the polymer concentration (c, 5-20% w/v) and UV exposure time (t, min):

\[ E = 7955 \times c^{2.20} \left(1 - e^{\left(\frac{43-t}{11}\right)}\right) \]  

(1)
The compression moduli clearly revealed a positive correlation between polymer concentration and stiffness for the two different batches tested (Figure 1B), which is in line with our reported empirical model [25]. The compression modulus for 2.5% hydrogels was 0.5 ± 0.2 kPa (empirical model: 0.7 kPa), for 5% hydrogels 3.7 ± 0.5 kPa (empirical model: 3.4 kPa) and for 7% hydrogels 8.9 ± 1.8 kPa (empirical model: 7.3 kPa). Variability with respect to metabolic activity (no significant difference; \( p = 0.2 \)), spheroid distribution and size was low, as indicated for 5% hydrogels of different batches for different time points (Figure S1).

Solute diffusion was measured using FD70 representing relevant biomolecules, such as growth factors. The diffusion coefficients were determined by rFRAP, a method which allows quantitatively correct diffusion values in 3D samples [51]. In addition to a rectangular bleach area (red box), a rectangular fitting region (green box) and background region (blue box) was defined (Figure 1C). Within the fitting area, the spatial intensity distribution was mathematically described for each time point of the recovery sequence (Figure 1D). In addition to the intensity, this spatial information of the bleach profile was used to obtain the recovery curve (Figure 1E). In the last analysis step, a fit to the recovery curve yielded in the diffusion coefficient, which is a measure of the mobility of the diffusing components and the mobile fraction of the diffusing molecule, including molecules that contribute to the recovery within the FRAP ROI (Figure 1E). The mobile fraction was quantified by the percentage of total fluorescence recovery (plateau of the fitted recovery curve) of initial fluorescence intensity in images taken prior to bleaching. As a control for the accuracy of the FRAP method, FD70 was measured in water, as the expected diffusion coefficient for this sample was calculated using the Stokes-Einstein Equation:

\[
D = \frac{kT}{6\pi\eta r_H}.
\]

Equation 2 gives a hydrodynamic radius for FD70 of 5.55 nm. The shaded blue area denoted the range of possible diffusion coefficients that resulted from the molecular weight distribution of FD70. According to the manufacturer, the mean molecular weight of FD70 was 63-77 kDa as measured by size exclusion chromatography. This yielded hydrodynamic radii (\( r_H \)) were calculated according to the empirical formula for dextran [56]:

\[
r_H = 0.015 M_0^{0.59} \pm 0.05.
\]

Equation 3 gives a hydrodynamic radius for FD70 of 5.55 nm. The shaded blue area denoted the range of possible diffusion coefficients that resulted from the molecular weight distribution of FD70. According to the manufacturer, the mean molecular weight of FD70 was 63-77 kDa as measured by size exclusion chromatography. This yielded hydrodynamic
radii for FD70 ranging from 5.25-5.83 nm. Assuming a 10% error in the hydrodynamic radius for FD70, a range of expected diffusion coefficients in the FRAP calibration experiment across a range of temperatures were plotted (Figure 1F). The mobile fraction of FD70 in water was 1. For hydrogels, values were close to 1, indicating that the majority of FD70 was mobile within the hydrogel. For the concentration range from 0% (water calibration) to 7% GelMA a linear decrease in the diffusion coefficient was observed. However, diffusion into hydrogels was a fast process, and FD70 did not interact with GelMA structures, indicated by mobile fractions close to 1 in all samples tested (Figure 1G). These findings suggest that the polymer concentration was linked to the hydrogel stiffness but only marginally to the diffusive properties.

3.2. The influence of cell-laden hydrogels on diffusion properties

Local microenvironmental changes play a key role in tumour growth and are limiting factors in 3D cell culture systems [6,57]. Hence, FRAP measurements within cell-laden hydrogels at various time points were performed to determine potential cell-related changes to the diffusive properties of the hydrogels. Therefore, ovarian cancer cells were embedded within GelMA-based hydrogels and FRAP measurements using FD70 were performed on day 1, 7, 14 and 21 of 3D culture. Cell-free hydrogels incubated for the same time in culture media served as controls. As spheroids were formed out of single cells after 7 days, the bleaching ROI was placed next to a single cell on day 1 day and next to a spheroid on day 7, 14, and 21 of 3D culture (Figures 2A-B). Cell-free hydrogels showed a slightly increase in diffusion coefficients over time. Higher diffusion coefficients were detected in cell-laden hydrogels compared to cell-free hydrogels, in particular on day 7. However, a comparison between diffusion coefficients over all time points showed diffusion values for FD70 without significant difference comparing cell-laden with cell-free hydrogels ($p = 0.8$) and each day ($p = 0.3$). The measured average of the diffusion coefficient for cell-free hydrogels was $27.1 \pm 4.8$ and for cell-laden hydrogels $30.2 \pm 4.6$; the values scattered around the mutual mean of $28.5 \pm 4.9 \mu m^2/s$ in all conditions tested (Figures 2C-D). These findings suggest that diffusion was not influenced by the culture time or presence of cells or spheroids indicating that the structural hydrogel properties were not altered outside the spheroid volumes.

3.3. The dependence of spheroid behaviour on GelMA concentration
Our previous study demonstrated that the biomaterial stiffness has a high impact on cell behaviour; spheroid morphology was dependent on matrix stiffness, and proliferation decreased with increasing hydrogel stiffness [21]. The stiffness of GelMA-based hydrogels was a function of polymer concentration as shown above, which allows the modification of the stiffness by changing the amount of polymer dry weight. Hence, hydrogels with 2.5, 5, 7 and 10% GelMA concentration, with - according to our empirical model [25] (equation 1) - stiffnesses of 0.7, 3.4, 7.3 and 16.5 kPa were examined. Maximal projections of CLSM images showed a single cell distribution on day 1 for all conditions tested (Figure 3A, top panel). Already at early time points, differences of cell morphology in dependency of the hydrogel stiffness were observed. On day 7 of 3D culture, low stiffness (2.5%, 0.7 kPa) resulted in loose cell aggregation and cell migration out of these aggregates, while in stiff (7%, 7.3 kPa; 10%, 16.5 kPa) hydrogels smaller and inhomogeneous-shaped spheroids were formed. In contrast, in medium stiff (5%, 3.4 kPa) hydrogels, distinctive round and compact spheroids were formed (Figure 3A, middle panel). The spheroid size increased from day 7 to day 14 of 3D culture (Figure 3A, bottom panel), which was also observed using bright field microscopy (Figure 3B).

An increase in metabolic activity, indicative of cell proliferation, from day 1 to day 7 was observed across all conditions tested. While the metabolic activity from day 7 to day 14 was constant or increased in soft and medium stiff (2.5%, 0.7 kPa; 5%, 3.4 kPa) hydrogels, stiff (7%, 7.3 kPa; 10%, 16.5 kPa) hydrogels resulted in a constant or decreased metabolic activity (Figure 3C). The same effect was observed for the DNA content, indicative of cell proliferation, in 3D cultures up to 21 days (Figure 3D).

In replicate experiments, a high variation of the metabolic activity and DNA content, and the formation of cell monolayers on the surface of the plastic culture dishes used for 3D cultures, was noticed, in particular for hydrogels cultured over 14 and 21 days. However, the highest reproducibility over time in metabolic activity, DNA content and morphology was detected in 5% hydrogels. Consequently, for all further experiments, a 5% GelMA concentration was chosen as it promoted a consistent spheroid growth and reproducible cell behaviours (Figure S1). Overall, these findings suggest that a high polymer concentration inhibited spheroid growth, while low concentrations stimulated migration showing a high dependence of cell proliferation and spheroid formation on the mechanical properties of the extracellular microenvironment.

3.4. The effect of MMP inhibition on spheroid behaviour
Changes of the local microenvironment mediated by cell-secreted proteases, such as MMPs, play a key function in cell growth and matrix remodelling [21]. Therefore, spheroid-containing hydrogels (5%, 3.4 kPa) were treated with culture media supplemented with a broad-band MMP-inhibitor (20 µM). MMP inhibition resulted in smaller spheroids compared to controls as indicated by maximal projections of CLSM images and H/E staining (Figure 4A). Immunostaining of cytokeratin-8, an epithelial marker, MMP-9 and integrin α6 revealed the expression of these proteins in the presence and absence of the MMP-inhibitor (Figure 4B). Inhibition of MMPs resulted not only in smaller spheroid size, but also decreased proliferation rates significantly indicated as metabolic activity (p < 0.001; Figure 4C) and DNA content (p < 0.001; Figure 4D). These findings suggest that cell growth and spheroid size were a function of biomaterial degradability.

3.5. The relevance of native ECM components for spheroid behaviour

The biomaterial GelMA offers the opportunity to modify its cell functional features by additional cell binding sites by mixing natural ECM components to the polymer solution. Here, the effects of LN-411 (20 µg/mL) and/or HA (0.1%) on hydrogel stiffness, diffusion and spheroid proliferation were examined. Maximal projections of CLSM images of spheroids growth within hydrogels containing additional components, separately or in combination, showed a single cell distribution on day 1 and spheroid growth by day 14 of 3D culture (Figure 5A). Brightfield images indicated a different hydrogel structure by addition of HA to GelMA, resulting in larger macro-pores and a heterogeneous polymer-network within the hydrogels (Figure S2). Compression moduli of hydrogels containing LN-411, HA or the combination of both were increased compared to controls (5%, 4.0 ± 0.5 kPa). The incorporation of HA increased the hydrogel stiffness by 77% (7.1 ± 0.7 kPa) compared to controls, while the combination of HA and LN-411 by 58% (6.4 ± 0.5 kPa), and LN-411 alone by 31% (5.3 ± 0.7 kPa; Figure 5B). The metabolic activity increased from day 1 to day 7 in all conditions tested. The metabolic activity changed over time and a significant difference between 5% GelMA controls compared to LN-411- (p = 0.02), HA- (p < 0.001)) and LN-411/HA-containing hydrogels (p < 0.001) was observed. However, after 21 days of 3D culture, the metabolic activity was reduced in LN-411- and HA-containing hydrogels. Control hydrogels showed an increase in metabolic activity from day 1 to day 21 of 3D culture, but with an overall decreased activity compared to hydrogels containing LN-411 and
HA (Figure 5C). The same effect was visible in the DNA content, which was increased in hydrogels with additional components after 14 days of 3D culture compared to controls. An increase in the DNA content was observed in all conditions from day 1 to day 7. The comparison over time showed a significant difference in DNA content between 5% GelMa controls and hydrogels with the additional components HA (p = 0.001) and LN-411/HA (p = 0.003). There was no difference between controls and LN-411-containing hydrogels (p = 0.49). However, there was a decreased DNA content after 21 days of 3D culture in LN-411- and HA-containing hydrogels compared to controls (Figure 5D). The positive effect of the addition of ECM components on spheroid proliferation was more pronounced upon HA incorporation compared to LN-411 (Figures 5C-D). Diffusion properties were not influenced by the addition of these ECM components (Figure 5E). These findings suggest that spheroid growth is a function of the ECM components LN-411 and HA, with HA displaying a greater spheroid growth-promoting effect.

3.6. The effect of GelMA-grown spheroids on tumour growth and anti-cancer treatment in vivo

To investigate whether spheroid-containing hydrogels can induce tumour formation and growth in vivo, spheroids were grown for 2 weeks within GelMA-based hydrogels (5%, 3.4 kPa) prior to implantation into mice, adjacent to each ovary (Figure 6A). Spheroid-containing hydrogels induced tumour formation and peritoneal spread over 8 weeks detected by bioluminescence imaging (Figure S3).

To study the responsiveness of tumour-bearing mice towards paclitaxel, ATN-161 and the combination of both, treatment started 4 weeks after implantation of the hydrogels. DMSO and/or water served as control treatment. Animals of the control group developed tumours on the right and left implantation site next to the ovary, with evidence of tumour fluid (ascites) and metastatic spread to the omentum, liver and colon (Figures 6B-D, S3). Paclitaxel treatment decreased tumour growth by 33% and prevented the development of tumour fluid (ascites) and metastatic outgrowth (Figures 6B, 6D). The combined treatment of paclitaxel and ATN-161 reduced tumour growth by 37.8% compared to controls, while ATN-161 alone had no effect on tumour growth (Figures 6C-D). These findings suggest that intraperitoneal implantation of spheroid-containing hydrogels promoted tumour formation, growth and metastasis, which were reduced upon paclitaxel but not ATN-161 treatment.
4. Discussion

In this study, we presented an alternative biomaterial platform for the *in vitro* and *in vivo* analysis of ovarian cancer spheroid growth. Our optimisation and validation protocol resulted in hydrogels with reproducibly tailorable properties. Increasing polymer concentrations resulted in stiffer hydrogels and indicated that spheroid formation, size and shape was highly dependent on hydrogel stiffness. The degradable features of the hydrogels were also linked to spheroid growth. The modification of hydrogels by addition of the ECM components LN-411 and HA enhanced spheroid growth. *In vivo* experiments showed tumour formation and metastasis induced by spheroid-containing hydrogels, which were sensitive to paclitaxel, but not to ATN-161. Hence, the semi-synthetic biomaterial GelMA combines relevant natural cues with tunable properties providing an alternative 3D cancer model.

3D cell culture techniques are a promising tool for cancer research as they more closely mimic the *in vivo* microenvironment. However, 3D models are still not routinely used [7]. A potential reason for the slow acceptance in comparison to cell monolayer cultures might be the demand for a complex ECM with defined structure and composition, as this introduces challenges regarding the reproducibility and complexity of such 3D systems. While the use of non-synthetic matrices is associated with a high variability due to their natural origin, synthetic biomaterials generally fail to provide a replica of the natural microenvironment, as cell binding and cleavage sites are missing or composed incorrectly [57]. To overcome these limitations, we approached this problem by employing a semi-synthetic hydrogel. Its naturally-derived component, gelatine, provides cell binding and cleavage sites, while its chemical modification via introduction of methacrylamide side groups allows the control of its mechanical properties through a chemical cross-linking reaction, in contrast to a physical polymerisation which cannot be precisely controlled and modified [22]. GelMA harbours optimal properties in representing natural ECM components combined with mechanical robustness as *in vitro* 3D culture model for ovarian cancer by allowing long-term cultures and as cancer cell delivery vehicle for *in vivo* applications.

According to our reported empirical model [25], hydrogel stiffness increased proportionally with polymer concentration in a quantitatively predictable manner. A high variability was only observed for the softest hydrogels with a low polymer concentration (2.5% GelMA, 0.5 ± 0.2 kPA), and these preparations had the highest discrepancy to the empirical model. As the compression testing was performed using a micro-tester system equipped with a 5N load cell,
the sensitivity of the measurements decreased towards low biomaterial stiffness. Despite this systematic error, our compression moduli were robust throughout all hydrogel conditions tested, within one batch and also between two different batches. Hence, GelMA-based hydrogel preparations allow the stiffness to be precisely controlled over the physiological range of most tissues [58].

Next to the molecular composition and stiffness of a matrix, its diffusive properties play an important role in the effort to reproduce a physiological 3D microenvironment. Diffusion into and within a hydrogel directly influences the local cell microenvironment that supplies cells with nutrients, oxygen, growth factors, as well as the effectiveness of drug treatment [29–31,33,59]. This is mainly determined by the pore size and affinity of the diffusing species to the matrix of the hydrogel polymer. In order to assess the diffusive properties of GelMA-based hydrogel preparations, FD70 was used as a tracer molecule, and its diffusion quantified using a robust FRAP protocol [51]. A negative linear relationship between diffusion coefficients of FD70 and polymer concentrations was observed. This is caused by a decreasing mesh size as a result of the higher crosslink density, as well as increased obstruction by the higher volume fraction of polymer chains [60]. Nevertheless diffusion of FD70 into GelMA-based hydrogels was fast in all tested conditions, and diffusion properties were only slightly affected by polymer concentration. Diffusion of FD70 in 7% GelMA-based hydrogels was only retarded by a factor of 2.3 compared to water, indicating that large molecules can still diffuse freely and relatively rapidly through the densest GelMA-based hydrogels. For smaller molecules, the retardation factor will be smaller, and thus, diffusion rates of oxygen, most nutrients and metabolites will be close to those in media in the absence of the hydrogel [61]. Long term assessment of diffusion properties in medium concentrated hydrogels (5% GelMA, 3.7 ± 0.5 kPa) showed robust values over 21 days, indicating a stable polymer structure. The direct comparison of cell-free and cell-laden hydrogels showed nearly constant diffusion coefficients over the complete sample period of 21 days in both conditions, indicating that the overall hydrogel properties are minimally affected by both hydrolytic and cell-mediated degradation or compression, and thus, modification of the hydrogel structure is spatially limited to the pericellular microenvironment, enabling cell proliferation and spheroid growth. It can be assumed that due to cellular matrix modification, local structural changes result in a changing diffusion coefficient. However, it was difficult to detect these because the variability of the diffusion coefficient in cell-free hydrogels is increasing over time. Furthermore, FD70 is a very small molecule (radius of 5.25-5.83 nm), so its diffusion
coefficient might be less affected by cellular compression or degradation, compared to those of larger molecules. Altogether, even in hydrogels with the highest concentration, diffusion of essential components within the hydrogel is possible, and occurs at acceptable rates. Hence, GelMA-based hydrogels provide promising properties as a platform for long term 3D cultures.

Stiffness of the local microenvironment directly affects cell behaviours [21,58]. The variation of the hydrogel stiffness changed spheroid morphology and size: low stiffness caused loose cell aggregation, medium stiffness led to distinctive, round-shaped spheroids and high stiffness resulted in smaller spheroids. Spheroid morphology and size formed in medium stiff (3.7 ± 0.5 kPa) GelMA-based hydrogels were similar to spheroids formed in PEG-based hydrogels without integrin binding sites [21]. An increase in metabolic activity and proliferation rate were determined in low (0.5 ± 0.2 kPA) and medium stiff (3.7 ± 0.5 kPa) hydrogels compared to stiff (8.9 ± 1.8 kPa) hydrogels. Distinctive spheroid formation, similar to those formed in the tumour fluid (ascites) that accumulates in the peritoneal cavity of patients with ovarian cancer at the advanced stage of this disease [43], was observed exclusively in medium stiff (3.7 ± 0.5 kPa) GelMA-based hydrogels. Notably, a high variation in metabolic activity and DNA content was observed, in particular after 14 and 21 days of 3D culture. This phenomenon might be the result of an infrequent observation that cell monolayers were formed on the surface of the plastic culture dishes that were used for 3D cultures upon cell migration out of the hydrogels. As OV-MZ-6 cell monolayers have a higher proliferation rate than cells cultured in 3D over 5 days [21], this effect might influence the measurements of spheroid growth.

Enzymatic degradation of the local microenvironment through MMPs plays a key role in spheroid formation, growth and migration [21,62]. MMPs are frequently expressed in ovarian cancer and contribute to disease progression by degradation and remodelling of the ECM [63]. MMP-2, MMP-9, and MMP-14 are upregulated in advanced ovarian cancer, tumour fluid (ascites) and abdominal metastasis and associated with shorter survival times [64,65]. There is a complex crosstalk between MMPs, growth factors and growth factor receptors [64–67]. MMP-9 levels are linked to a loss of E-cadherin and adherens junctions, thereby promoting a more invasive phenotype [66]. The ovarian cancer spheroids used in this study express MMP-9 but not E-cadherin [21]. We have previously reported that spheroid growth was reduced by 73% using MMP-sensitive PEG-based hydrogels [21]. Using the same MMP-inhibitor, spheroid proliferation within GelMA-based hydrogels was reduced, further
indicating a regulatory role of MMPs in spheroid formation and growth and underlining the importance of the biodegradability of the 3D culture model used. Immunostaining of MMP-9, one of the up-regulated MMPs in ovarian cancer [68], confirmed its expression with and without MMP inhibition over 14 days. Immunostaining of cytokeratin-8 and integrin α6 confirmed that the spheroid morphology and cell adhesive properties were not altered upon MMP inhibition.

Bioengineered, semi-synthetic matrices with close to nature properties become more and more important tools for investigation of cells in approximately native conditions [8]. GelMA offers the possibility of including native ECM components into the precursor hydrogel solution. It was shown that a progressive tumour growth of ovarian cancer occurs along with increased levels of stromal hyaluronan, and that HA promotes metastasis and invasiveness of tumours [37,38,69]. Increased levels of laminin were also found in the tumour fluid (ascites) of ovarian cancer patients [70]. Therefore, LN-411 and HA were used as additional ECM components within GelMA-based hydrogels. An increased stiffness in hydrogels, but similar diffusive properties, that contained LN-411, HA or a combination of both was observed. Interestingly, there was a positive correlation of these additional ECM components on spheroid growth in GelMA-based 3D cultures, despite their stiffness being higher compared to 5% control hydrogels. It was shown that within PEG-based hydrogels, the elastic modulus was slightly enhanced compared to hydrogels containing low levels of HA [71]. The incorporation of HA resulted in only small changes regarding the physical and chemical properties of the hydrogel, but increasing fibroblast proliferation activity and cell spreading. Due to the high molecular weight of HA leaching from the hydrogel is unlikely [71]. Microscopic phase separation of HA and PEG is a more probable cause, as this might result in a modified microstructure, facilitating cell activity and proliferation. This effect is further increased by the fast degradation of HA, both hydrolytically and through enzymes, such as hyaluronase [71]. Similar effects were observed in the GelMA-based hydrogels containing HA-methacrylate [72]. The incorporation of HA caused heterogeneous hydrogel formation, containing larger macro-pores and pockets that might support spheroid formation.

In ovarian cancer patients, the primary tumour releases multicellular spheroids into the abdomen which then accumulate in the tumour fluid (ascites) mediating secondary tumour outgrowth and metastasis. This leads to significant morbidity and is a characteristic of the end stage of the disease. Metastatic spread is therefore seen mainly in adjacent organs or tissues,
such as the omentum, liver and colon [43]. Implantation of our spheroid-containing hydrogels into NOD/SCID mice showed that this model is able to exactly replicate the tumour growth pattern and route of metastatic spread as it is seen in the clinic. This study provided proof of the application of GelMA-based hydrogels as an ovarian cancer cell carrier. In all mice, tumour formation, growth and metastasis were detected, which was in accordance with the clinical sequence of this disease. Tumour-bearing mice showed sensitivity to paclitaxel treatment, a clinically used anti-cancer drug [44] resulting in reduced tumour growth and a lack of metastasis. Previous studies showed promising effects of the anti-cancer drug ATN-161, a non-RGD-based integrin antagonist on breast and prostate cancer progression and metastasis in vivo [47]. A phase I trial evaluating patients with advanced solid tumours, including prostate cancer, showed a prolonged stable disease upon ATN-161 administration [49]. However, ATN-161 had no effect on tumour growth and establishment of metastatic lesions and did not enhance the effect of paclitaxel in our in vivo study. So far, anti-tumour effects of ATN-161 treatment have been shown for tumours that metastasise via the bloodstream [47,49]. However, ovarian cancer is predominantly confined within the abdominal cavity and, unlike breast or lung cancer, rarely metastasises hematogenously. In the reported animal studies, ATN-161 was injected intravascularly [47]. In order to compare the anti-cancer effect of ATN-161 to paclitaxel treatment, both drugs were applied intraperitoneally. This might have impacted on the treatment outcome because drugs reach vascularised metastatic outgrowth faster via the vascular system than intraperitoneally applied drugs that are distributed within the peritoneal fluid.

5. Conclusions

GelMA-based hydrogels provide a bioengineered, semi-synthetic 3D platform for spheroid growth of ovarian cancer cells in vitro and in vivo. Employing a controlled preparation and validation protocol, hydrogels of equal size, diffusion and physical properties were generated. The semi-synthetic origin of GelMA combines the features of naturally-derived matrices, including RGD-binding and MMP-cleavage sites, with high stability and reproducibly tailorable characteristics, thereby allowing binding to cell adhesion receptors and degradation by cell-secreted proteases. Spheroid formation is a function of hydrogel stiffness. Only in medium stiff hydrogels (3.7 ± 0.5 kPa) round-shaped spheroids of distinctive size were formed, resembling those present in the tumour fluid (ascites) within the abdominal cavity of patients with this disease. The incorporation of the native ECM components LN-411 and HA
resulted in a more pronounced spheroid formation and growth, in particular upon addition of HA. GelMA-based, spheroid-containing hydrogels implanted into NOD/SCID mice caused local tumour growth and a metastatic spread as observed in ovarian cancer patients. In conclusion, GelMA-based hydrogels offer a low cost, reproducible and tailorable matrix for 3D cancer cell cultures, thereby representing an alternative, close to nature 3D approach to improve our understanding of disease progression on a cellular level and to screen anti-cancer drugs.

Acknowledgements

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Figures

Figure 1. GelMA-based hydrogel preparation and characterisation of the physical properties. A. Critical steps of GelMA-based hydrogel preparation are depicted. Gelatine (1) and methacrylic anhydride (ratio 1:0.6 w/w) reacted to gelatine methacrylamide (GelMA; 2). The GelMA polymer is dissolved in PBS at 37°C and mixed with the photo-initiator, cells, and optionally with other ECM components (3). The presence of photo-initiator allowed hydrogel formation during a radical cross-linking reaction induced by UV light (4). Polymerisation within the Teflon mould produced a hydrogel that was cut into smaller units (5). B. Compressive moduli indicated that increasing polymer concentrations resulted in stiffer hydrogels; tested in two independent batches (n = 5-8 hydrogels for each batch, error bars denote SD). The measured compressive moduli were in accordance with our reported empirical model [25]. C-F. Diffusion coefficients were determined by FRAP measurements on fluorescent dextran (FD70) absorbed in GelMA-based hydrogels. Within a rectangular ROI (red box), FD70 was bleached, and the recovery of fluorescent intensity was monitored in an additional fitting (green box) and background (blue box) region. Scale bar, 100 µm (C). The spatial intensity distribution within the fitting area was determined for each time point of the recovery sequence (D). In addition to the intensity, the spatial information of the bleach profile was used to obtain the recovery curve (E), which yielded in the diffusion coefficient and mobile fraction of the diffusing biomolecules. The method was validated against measurements of FD70 in water. Calculations following equations 2 and 3 predict the theoretical diffusion coefficient of FD70 in water (straight line) and upper and lower boundaries for an error in the hydrodynamic radius ($r_H$) of 10% (F). G. Diffusion coefficients and mobile fractions indicated a decreasing diffusion rate upon increasing polymer concentrations, while the mobile fraction was unchanged and close to 1 for all experiments.

Figure 2. Diffusion properties are not influenced by growing spheroids. A. A representative image of a cell-laden hydrogel at day 1 is shown (left panel). Diffusion coefficients were measured, as described earlier, within the rectangular bleached ROI next to a single cell (red box), the data were analysed within the fitting area (green box) and the determined background area (blue box; middle panel), and a recovery curve was obtained (right panel). Scale bar, 100 µm. B. A representative image of a FRAP measurement at day 14 of spheroid formation is shown. The bleached area was chosen next to a growing spheroid (red box). All measurements were performed at several time points (day 1, 7, 14 and 21) of
3D culture. Scale bar, 100 µm. C. The diffusion coefficients were determined for cell-free and cell-laden hydrogels that were prepared on the same day and cultured at the same conditions. Diffusion coefficients were relatively constant over the incubation period of 21 days. D. A comparison between diffusion coefficients showed no significant difference between cell-laden and cell-free hydrogels ($p = 0.8$) at each time point ($p = 0.3$). The mobile fraction was close to 1 for all experiments.

Figure 3. Spheroid morphology, metabolic activity and proliferation depend on GelMA concentration. A. Representative maximal projections of CLSM images of cells embedded within hydrogels of various polymer concentrations (2.5%, 0.7 kPa; 5%, 3.4 kPa; 7%, 7.3 kPa and 10%, 16.5 kPa) are shown, with nuclei stained in blue and actin cytoskeleton in red. A single cell distribution was present on day 1 in all conditions of 3D culture (top panel). A low GelMA concentration (2.5%, 0.7 kPa) resulted in loose cell aggregates, whereas high GelMA concentrations (7%, 7.3 kPa; 10%, 16.5 kPa) caused smaller spheroids after 7 days (middle panel) and 14 days (bottom panel). The medium GelMA concentration (5%, 3.4 kPa) led to well defined and round-shaped spheroids. Scale bars, 100 µm. B. Representative bright field images of cells embedded within hydrogels of various polymer concentrations (as above) are shown. Scale bars, 100 µm. C. An increase in metabolic activity, indicative of spheroid proliferation, from day 1 to day 7 was observed in all conditions tested. An enhanced metabolic activity was detected in soft hydrogels compared to stiff hydrogels ($n = 4$ for day 1-14; $n = 2$ for day 21). D. The DNA content increased from day 1 to day 7. From day 7 to day 21 of 3D culture, a higher DNA content was detected in soft hydrogels compared to stiff hydrogels. Shown are mean values, normalised to day 1 ($n = 4$ for day 1-14; $n = 2$ for day 21).

Figure 4. MMP inhibition resulted in smaller spheroids, reduced metabolic activity and proliferation of spheroids. A. Maximal projections of CLSM images, with nuclei stained in blue and actin cytoskeleton in red, and H/E staining of hydrogels are shown on day 7 and day 14 with and without MMP inhibition. MMP inhibition resulted in smaller spheroids compared to non-treated controls. Scale bars, 100 µm, H/E staining 10 µm. B. Immunostaining of cytokeratin-8, MMP-9 and integrin α6 is shown with and without MMP inhibition; proteins stained in green or red and nuclei in blue. Protein expression was detected independent of MMP inhibition. Scale bars, 10 µm. C. The metabolic activity upon MMP inhibition was reduced compared to controls after 21 days of 3D culture ($n = 3$, $p < 0.001$).
D. MMP inhibition resulted in a reduced DNA content on day 7 up to day 21 compared to controls. Shown are mean values, normalised to day 1 (n = 3, p < 0.001).

Figure 5. LN-411 and HA increased hydrogel stiffness, metabolic activity and proliferation of spheroids. A. Maximal projections of CLSM images indicated spheroid formation upon LN-411 and HA incorporation into hydrogels on day 1 (top panel) and day 14 (bottom panel); nuclei stained in blue and actin cytoskeleton in red. Scale bars, 100 μm. B. The addition of the ECM components LN-411 and HA into hydrogels increased stiffness compared to 5% GelMA controls (4.0 ± 0.5 kPa). C. The metabolic activity was significantly different in hydrogels containing LN-411 (p = 0.02), HA (p < 0.001) and LN-411/HA (p < 0.001) over time compared to controls. D. The incorporation of LN-411 and HA into hydrogels increased the DNA content compared to controls. Shown are mean values, normalised to day 1 (n = 3). The DNA content was significantly different in hydrogels containing HA (p = 0.001) and LN-411/HA (p = 0.003) over time compared to controls. There was no significant difference between LN-411-containing and control hydrogels (p = 0.49). E. The diffusion properties were not influenced by addition of LN-411 and HA and were in the range of solute diffusion of FD70 measured in 5% GelMA controls.

Figure 6. Spheroid-based implants resulted in tumour formation and peritoneal spread that were reduced by paclitaxel treatment. A. The surgical procedure was carried out by a left incision to implant the rectangular-shaped, spheroid-containing hydrogel adjacent to the ovary (a). After implantation, the abdominal wall and skin were closed (b). Then, a right incision was made to implant a second hydrogel (c), and the abdominal wall and skin sutured (d). B. Animals were sacrificed 8 weeks after implantation and tumours developed on the right (e) and left (f) implantation site. Evidence of tumour fluid (ascites; arrow) and metastatic spread (arrow) was visible without paclitaxel treatment (g). Upon paclitaxel treatment, neither tumour fluid (ascites) nor metastatic outgrowth was detected (h). C. Tumour growth, indicated as radiance, was reduced upon paclitaxel and combined paclitaxel/ATN-161 treatment starting in week 4 after implantation compared to ATN-161 treatment alone and controls (DMSO and/or water). ATN-161 had no effect on tumour growth compared to controls. A significant reduction in tumour growth was observed upon paclitaxel alone (p = 0.003) and combined paclitaxel/ATN-161 (p = 0.002) treatment compared to non-treated controls. There was no significant difference in tumour growth upon ATN-161 treatment alone compared to non-treated controls (p = 0.95) and between paclitaxel
alone and its combination treatment with ATN-161 ($p = 0.99$). **D.** Luciferase-transfected ovarian cancer spheroids (bioluminescence $9.1 \times 10^5 \pm 1.6 \times 10^5$) were implanted into mice and caused formation of tumours over the monitored time frame. Paclitaxel and ATN-161 responsiveness was calculated as percentage of change in tumour volume compared to non-treated conditions. While paclitaxel and its combination with ATN-161 resulted in a treatment response of 33-37.8%, ATN-161 alone had no effect on tumour growth and peritoneal spread.

**Figure S1.** Highly reproducible GelMA properties resulted in similar cell behaviour within hydrogels of different batches. **A.** Cell-laden hydrogels were prepared from one cell suspension and mixed into precursor solutions of two different GelMA batches. The metabolic activity was increased from day 1 over 14 days of 3D culture in both batches tested, indicating highly reproducible results of the two different batches tested ($n = 3$; $p$ values not significant). **B.** Spheroid formation out of single cells (day 1) was observed after 7 and 14 days respectively in both batches tested. Scale bars, 100 μm.

**Figure S2.** HA addition to GelMA-based hydrogels resulted in heterogenous polymer-network with larger macro-pores. Brightfield mages indicated spheroid formation upon LN-411 and HA incorporation into hydrogels on day 1 (top panel) and day 14 (bottom panel). Addition of HA resulted in hydrogels with larger macro-pores and pockets. However, LN-411 alone did not affect the hydrogel structure. Scale bars, 100 μm.

**Figure S3.** Paclitaxel reduced tumour growth in vivo. Spheroid-containing GelMA-based hydrogels were implanted into mice adjacent to each ovary and caused tumour formation and growth over 8 weeks as indicated by bioluminescence imaging. While paclitaxel treatment reduced tumour mass and peritoneal spread, treatment with ATN-161 did not affect tumour growth. The combination treatment of paclitaxel with ATN-161 showed the same effect as paclitaxel treatment alone.

**References:**


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