Peptide-protein coassembling matrices as a biomimetic 3D model of ovarian cancer

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Bioengineered three-dimensional (3D) matrices expand our experimental repertoire to study tumor growth and progression in a biologically relevant, yet controlled, manner. Here, we used peptide amphiphiles (PAs) to coassemble with and organize extracellular matrix (ECM) proteins producing tunable 3D models of the tumor microenvironment. The matrix was designed to mimic physical and biomolecular features of tumors present in patients. We included specific epitopes, PA nanofibers, and ECM macromolecules for the 3D culture of human ovarian cancer, endothelial, and mesenchymal stem cells. The multicellular constructs supported the formation of tumor spheroids with extensive F-actin networks surrounding the spheroids, enabling cell-cell communication, and comparative cell-matrix interactions and encapsulation response to those observed in Matrigel. We conducted a proof-of-concept study with clinically used chemotherapeutics to validate the functionality of the multicellular constructs. Our study demonstrates that peptide-protein coassembling matrices serve as a defined model of the multicellular tumor microenvironment of primary ovarian tumors.

INTRODUCTION

There is a need for improved three-dimensional (3D) cancer models to study tumor growth and progression as seen in patients and to test responses to new treatments. At present, 90% of successful cancer treatments tested preclinically fail in the early phases of clinical trials, and less than 5% of oncology drugs are successful in clinical trials (1). Preclinical tests rely on a combination of 2D in vitro cell cultures and in vivo models to predict responses to treatment. Conventional 2D cell cultures fail to recapitulate key features of tumor tissues, such as cellular heterogeneity, presence of spheroids, matrix stiffness, cell-matrix interactions, and cross-talk with other cell types (2). On the other hand, murine models allow for cell-matrix and cell-cell interactions, but interspecies differences result in many successful treatments in murine hosts being ineffective in humans. Thus, novel experimental 3D cancer models are needed to better recapitulate the human tumor microenvironment (TME) and incorporate patient-specific differences.

The TME is a rich mixture of malignant and nonmalignant cells, such as fibroblasts, endothelial cells, and immune cells, embedded in an extracellular matrix (ECM) (3). The TME affects tumor growth and undergoes changes in response to cancer progression, such as stiffening of the ECM, shifts in chemical signaling, and tumor angiogenesis (4). Tumor angiogenesis arises when the tumor core undergoes hypoxia and triggers the secretion of proangiogenic proteins and the migration of endothelial cells toward the tumor. The resulting vessels are leaky and disorganized, consequently influencing the components of the TME. Collectively, these changes in the local microenvironment affect both cancer progression and responses to treatments. Thus, modeling the TME is vital and must recapitulate cell-cell and cell-matrix interactions as well as key components of the ECM, such as proteins, growth factors (GFs), and chemical gradients. Furthermore, the model should integrate multiple cell types that enable the growth of tumor spheroids.

Currently, the gold standard for 3D cancer models is the commercially available Matrigel (5), a solubilized basement membrane extracted from mouse sarcoma that consists of an undefined mixture of ECM proteins and GFs. A major reason for Matrigel’s popularity is its capacity to enable cell-matrix interactions, which promote the growth of malignant and nonmalignant cells as well as spheroid formation (6). However, it lacks control in mimicking the TME because of its high batch variability, undefined composition, and murine origin. These features are important limitations to effectively screen and develop new treatments for cancer. To address these drawbacks, natural and synthetic materials are being explored as alternatives to generate 3D cancer models, such as hyaluronic acid hydrogels (7), chitosan/gelatin composites (8), and polyethylene glycol (PEG) (9). Natural materials recapitulate structural aspects of the ECM, but they exhibit limited control over their mechanical properties. While synthetic materials can overcome the limitations in mechanical control, the trade-off is bioactivity. Thus, hybrid systems, such as PEG modified with fibrinogen (10), heparin (11), isoeucine-lysine-valine-alanine-valine (IKVAV) or arginine-glycine-aspartate (RGD) peptides (12), are being studied. However, while these composites greatly improve cellular responses, they do not capture the fibrous ECM nanostructure and heterogeneous composition and tend to require the use of potentially cytotoxic cross-linking components.

Self-assembling peptide-based materials can be programmed to assemble into well-defined nanostructures, such as micelles, nanofibers, or ribbons, while generating porosity and displaying multiple
bioactive epitopes (13). These materials have been optimized to enhance their capacity to recreate the ECM, for example, by enabling alignment (14), tunability of mechanical properties (15), and the incorporation of protein-mimetic sequences to promote angiogenesis (16). Peptide amphiphiles (PAs) are promising self-assembling materials, which are capable of assembling into well-defined nanofibers (17) with the capacity to display cell-constructive motifs that mimic ECM proteins or GFs, such as laminin, collagens, and fibronectin (FN) (18). This approach is increasingly being used to coassemble peptides with a variety of molecules, such as hyaluronic acid (19), heparin (20), and proteins (21, 22), to develop biologically relevant 3D cell cultures.

Here, we report the design and validation of a peptide-protein coassembling hydrogel-based multicellular 3D model for ovarian cancer. We demonstrate the capacity of the system to serve as a complex, yet controllable, alternative to Matrigel. The hydrogel supported the spheroid formation from single ovarian cancer cells reaching comparable sizes to those grown in Matrigel. When cocultured with human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs), the hydrogel enabled cellular interactions between the different cell types. The model demonstrated an increase in spheroid size as a result of increased cell-cell interactions. We also tested the effect of different peptide sequences and proteins within the multicomponent hydrogel, indicating the tunability of the system. In a proof-of-concept study, we determined tumor cell responses to different chemotherapeutics.

RESULTS
Rationale of the design
The herein reported coassembling hydrogel is based on our recently developed PA/keratin (KN) bioink (22), which served as a supramolecular platform to incorporate and optimize structural, molecular, and cellular components of the TME of ovarian cancer (Fig. 1A). We synthesized three new PA sequences based on the previously established PA-H (C16VVVAAH2K), which includes a histidine-histidine-lysine (H2K) motif to facilitate coassembly with ECM proteins (22); PA-VH (C16VVVAAVPGIGH2K), which contains an additional elastin-mimetic segment that enhances reinforcement of the ECM and was found to improve hydrogel stability; PA-RGDS (C16VVVAAH2KRGDS), which incorporates an additional arginine-glycine-aspartic acid-serine (RGDS) sequence to promote cell adhesion; and PA-GHK (C16VVVAAH2KGHK), which includes an additional glycine-histidine-lysine (GHK) motif known to promote cell proliferation and to serve as an angiogenic factor (23). PAs were designed to gel by coassembling with proteins through charge screening using the base PA (PA-H) as a control. We included FN, which is up-regulated in the ECM of the ovarian TME (24), and hair KN, which supports cell proliferation and tissue regeneration (25). Specific KNS are involved in the regulation of inflammation and immunity in epithelial layers (26, 27). Furthermore, KN provides structural stability to tissues and is expressed in normal and cancerous ovaries (Fig. 2B) as well as in ovarian cancer cell monolayers (Fig. S2A).

A range of PA/KN hydrogel formulations were used throughout this work (Fig. 1B). In material characterization studies and 3D monocultures, PA-VH was tested using PA-H as a control. In a previous study, we used both PA-H and PA-VK sequences, showing favorable results for PA-VK in terms of hydrogel strength and PA-H for cell interaction. Thus, here, we wanted to build on our previous work by designing the sequence PA-VH and keeping PA-H as control for the influence of “V” (22). The hydrophobic section V (= VGPG) was taken from elastin and has been used as part of biocompatible and biodegradable elastin-like polypeptides (28). We expected V to facilitate self-assembly into fibers and stronger interactions with KN. For 3D tricultures, PA-VH was mixed with PA-RGDS or PA-GHK at 10:1 (vol/vol) ratios before coassembly with the proteins. The rational here was to use the PA’s ability to coassemble between different PA molecules and present epitopes on the fiber surfaces. The PA ratio was selected to optimize the presentation and cellular recognition of the bioactive epitopes of PA-RGDS and PA-GHK. Previous studies have shown that epitope recognition can be maximized by spacing them within PA fibers (29). Last, in addition to the incorporation of multiple proteins and bioactive epitopes, the coassembling hydrogel was designed for encapsulation of three major cell types of the ovarian TME, including epithelial cancer cells, HUVECs, and hMSCs.

Hydrogel formation and characterization at the nano- and macroscale
Hydrogels were formed upon mixing PAs with the ECM proteins by injecting 15 μl of PA solution (10 mg/ml ± cells) into 100 μl of protein solution (10 mg/ml ± cells). PAs were synthesized as previously described (30), and their assembly assessed via circular dichroism (CD) and transmission electron microscopy (TEM). We found that all individual PAs and their mixtures exhibited the characteristic β sheet secondary structure (fig. S3A) and the classical nanofibrous architecture of self-assembled PAs (Fig. 2C and fig. S3C). The control sequence C16VVVAAH2K of all tested PAs dictated that the inter-PA interaction mechanism and the different bioactive epitopes of the PAs did not interfere with the assembly process (fig. S3B). This result was further confirmed by TEM, which revealed that the PA-VH/KN coassembled nanofibers exhibited similar morphologies as those formed by the control PA-H/KN, as previously reported (Fig. 2D and fig. S3, C to E) (22). In addition, upon PA-protein coassembly, hydrogels formed immediately and exhibited a translucent appearance that did not appear to change 30 min after coassembly, at which point they were easily handled (Fig. 2E). However, PA-based hydrogels, like many self-assembling hydrogels, are fragile structures that can be difficult to manipulate without altering their macrosopic shape, despite retaining their inherent nanofibrous architecture (Fig. 2E). The moldability of this nanofibrous network (i.e., ability to expand, contract, and shift) is an important feature for cell encapsulation, creating a porous interactive structure. These characteristics facilitate cell migration, cell-cell communication, and spheroid growth, which are critical to recreate the TME. Nonetheless, the structural integrity of self-assembling hydrogels can be a limiting factor. To improve on this, our PA-protein coassembling strategy is expected to enhance stability over PA hydrogels and enable higher control over their stiffness (e.g., by varying PA sequences, PA-protein affinities, and PA and protein concentrations), as explored in the subsequent two sections.

Hydrogel stiffness characterization
Ovarian cancer stiffness are highly dependent on matrix stiffness (24). While soft hydrogels [storage modulus (G’) ~ 0.5 kPa] lead to loose cell aggregation, stiff hydrogels (G’ ≥ 7 kPa) inhibit cell proliferation, leading to smaller inhomogeneously shaped spheroids with decreased metabolic activity (31). A similar stiffness dependence
is reported for prostate cancer spheroids (32). We tested the hydrogel stiffness by rheometry on acellular PA-VH/KN hydrogels (prepared as described in the “Hydrogel formation and characterization at the nano- and macroscale” section in either cell culture medium or 10 mM HEPES after 24 hours in comparison to the control hydrogel PA-H/KN. Culture medium was used to assess the behavior of the hydrogels during cell cultures, while HEPES served as control.

In HEPES, no significant stiffness differences were observed between PA-VH/KN (\(G' = 1616 \pm 198\) Pa) and the control PA-H/KN (\(G' = 1965 \pm 633\) Pa) hydrogels (Fig. 2F). Comparing PA-VH/KN hydrogels in culture medium and in HEPES, a significant increase in stiffness was observed for hydrogels in medium (\(G' = 2133 \pm 111\) Pa, \(P < 0.05\); fig. S4A). When leaving the PA-VH/KN hydrogels for 15 days in culture medium, a further increase (\(G' = 2633 \pm 633\) Pa) was observed compared to the 24-hour time point, although not statistically significant (fig. S4A). This kind of culture medium–induced increase in hydrogel stiffness has been reported (33). Overall, our findings show that the coassembling hydrogels are within the optimal stiffness range.
(G’ > 0.5 kPa and < 7 kPa) reported to support tumor spheroid formation from single-cell encapsulation (31).

Hydrogel stability characterization

Another critical parameter of the ECM is its capacity to degrade and to enable cell and spheroid growth (24). Peptide hydrogels are known to degrade enzymatically through peptide cleavage (34). Conversely, an ideal hydrogel needs to remain sufficiently stable for spheroid formation while enabling ECM secretion and remodeling (35, 36). We tested the hydrogel stability by incubating acellular PA-VH/KN hydrogels and control PA-H/KN hydrogels in cell diluents phosphate-buffered saline (PBS) and HEPES for 3 months at 37°C mimicking cell culture conditions. The hydrogel weight was monitored daily for the first week and subsequently weekly to assess degradation, with the data reported as the weight fraction (Mt) of the weight recorded at the start of the experiment (M0). Similar results were observed for

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**Fig. 2.** PA/KN hydrogel combinations and material analysis. (A) Table of PA sequences and proteins used in this study including the chemical formula, molecular weight (MW), concentration used, and zeta potential at pH 7.5 (*in HEPES (pH 7.5), **in water, ***relative to keratin volume, *theoretical). (B) Immunohistochemical (IHC) staining for keratin type I (KRT32) and type II (KRT82) in normal (healthy) ovary and ovarian cancer tissue samples, with IHC controls in fig. S2. (C) TEM image of self-assembled PA fibers (PA-VH) in HEPES [0.1 mg/ml (pH 7.5)]. (D) TEM image of PA fibers interacting with KN (PA-VH/KN). Pure solutions of PA-VH and KN in HEPES [0.1 mg/ml (pH 7.5)] were mixed in ratio 1:1 before analysis. (E) Photograph of a PA-H/KN hydrogel after 14 days in culture medium at 37°C and a representative scanning electron microscopy image of the internal heterogeneous nanofibrous structure of PA/KN hydrogels. (F) Rheological characterization of the hydrogels, measuring the storage modulus (G’ and G″) of PA-H/KN and PA-VH/KN hydrogels (mean ± SEM). (G) Graphical plot of the hydrogel mass (Mt/M0) against time, indicating the slow degradation of PA-H/KN and PA-VH/KN hydrogels in PBS 1× at 37°C (mean ± SEM). Photo credit: Clara Hedegaard, Queen Mary University of London. ns, not significant.
Cell monocoltures within PA/KN hydrogels generate tumor spheroids

Next, we sought to determine whether the encapsulation of ovarian cancer cells leads to tumor spheroid formation from a single-cell suspension within PA-VH/KN hydrogels compared to cells grown within Matrigel. Spheroid formation occurred over 21 days in PA-VH/KN hydrogels and Matrigel as detected by bright-field and fluorescence microscopy, with a higher spheroid density in Matrigel (Fig. 3, A and B, and fig. S5A) bright-field control of PA/KN. Assaying the metabolic activity as an indicator of cell proliferation, we found that PA-VH/KN hydrogels follow the same trend as Matrigel over 21 days, albeit with slightly lower cell numbers (Fig. 3C). The readout fluorescence count for Matrigel rose to 7600 ± 2037, whereas PA-VH/KN hydrogels showed a high propensity for spheroid formation compared to PA-H/KN (Fig. 3G), which was confirmed by microscopy of both cell-containing PA/VH/KN hydrogels over 21 days, with PA-VH/KN hydrogels supporting comparable spheroid growth and size to Matrigel (Fig. 3 and fig. S5E). These findings demonstrate the feasibility of PA/KN hydrogels in supporting tumor spheroid formation from single cells, with PA-VH/KN being favorable over PA-H/KN. Stable spheroid formation occurred in PA-KN hydrogels over 21 days, representing the key advantage over Matrigel-based 3D cell cultures.

PA/KN hydrogels support 3D monocoltures of HUVECs and hMSCs

The ovarian TME contains not only malignant cells but also nonmalignant cells that support tumor survival and growth through processes like angiogenesis. As tumors grow, their cores become hypoxic, which, in turn, leads to the expression of proangiogenic proteins and GFs, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (40). In previous 3D cocultures of HUVECs and cancer cells, HUVECs migrated toward the tumor spheroids (41). We sought to test the suitability of PA-VH/KN hydrogels to support the formation of capillary-like structures by encapsulating HUVECs and hMSCs. First, we explored the compatibility of PA/KN hydrogels with each cell type. Encapsulation of either HUVECs or hMSCs in PA-VH/KN hydrogels showed a high viability of 85 and 75%, respectively, over 7 days (Fig. 5A and B). Thus, PA/KN hydrogels are suitable for multicellular cultures of malignant with nonmalignant cells.

Next, the propensity of HUVECs to form endothelial networks was explored by comparing 3D monocoltures of HUVECs in PA-VH/KN hydrogels to Matrigel. At day 2, limited cell spreading and networks were observed in cells grown within PA-VH/KN hydrogels. HUVECs in Matrigel seemed to form networks at the edges; however, these networks disappeared over time and were dissolved after day 5 (Fig. S6C). Conversely, when HUVECs were instead seeded on top of PA-VH/PA-RGDS/KN hydrogels and Matrigel, they adhered (Fig. 4A) and interacted with both matrices (Fig. S6D). It was not possible to process Matrigel samples for immunostaining because of the gels disintegrating when immersed in paraformaldehyde (PFA), a well-documented issue (Fig. 4B) (6). Using PA-VH/PA-RGDS/KN hydrogels, endothelial cell networks were formed 5 days after seeding (Fig. 4, A and B) and CD31 was detected on day 9 in proximity to tight junctions between the cells [Fig. 4, C (control) and D (sample)]. In comparison to the monolayer control, the CD31...
staining of the hydrogels was relatively weak. Nonetheless, the network was confirmed by scanning electron microscopy, which depicted complete HUVEC coverage on top of the PA-VH/PA-RGDS/KN hydrogels (Fig. 4E). As with the encapsulation, networks did not remain intact and started to disappear with no CD31 staining evident after day 9. These results are in line with other studies reporting the formation of a HUVEC monolayer on collagen/chitosan hydrogels (42). Comparatively, cells grown on Matrigel aligned directionally to form tubular polygonal networks, which remained present for 8 days (Fig. 4A and fig. S6E). The network was not verified beyond day 8, as previous studies have shown this timeline to be sufficient (12). Because of the difficulties in fixing Matrigel with PFA (5), we were unable to use these samples for immunofluorescent (IF) analysis. Thus, while neither matrix supported cell spreading in 3D, both matrices promoted adhesion and proliferation when cells were seeded on top.

Overall, our results demonstrate that, while PA/KN hydrogels support encapsulation of HUVECs, additional factors are needed to promote endothelial network structures within these hydrogels. Studies have indicated a role of hMSCs and ECM proteins, such as...
FN, in promoting capillary-like network formation (43). Thus, the inclusion of bioactive peptides (PA-RGDS or PA-GHK) and proteins in combination with 3D cocultures including hMSCs may further support HUVEC behavior.

3D cocultures of cancer cells with stromal cells in PA/KN hydrogels promote intercellular network formation

The TME mediates important interactions between tumor and endothelial cells (12). To investigate the potential of PA-VH/KN hydrogels to support cell-cell interactions within a controlled microenvironment, we conducted 3D tricultures of ovarian cancer cells together with HUVECs and hMSCs. Cell spreading was observed in PA-VH/KN hydrogels, which was not the case in 3D monocultures of HUVECs or hMSCs, suggesting a synergistic effect between the three cell types in 3D tricultures. Before in-depth analysis of the cell behavior in 3D tricultures, we first assessed the effect of the three different peptides PA-VH/PA-RGDS (10:1), PA-VH/PA-GHK (10:1), and PA-VH (1:0) and compared the extent of the F-actin networks. Therefore, all three cell types were encapsulated in PA-VH/KN, PA-VH/PA-RGDS/KN, and PA-VH/PA-GHK/KN hydrogels and F-actin stained using rhodamine-conjugated phalloidin (Figs. 1B and 5A). The immunofluorescent (IF) images were analyzed using AngioTool to calculate the area covered by the F-actin network (fig. S7A). A significant increase in F-actin network coverage was observed for PA-VH/PA-RGDS/KN (38 ± 7%) compared to PA-VH/KN (18 ± 6%) hydrogels (P < 0.05). On the other hand, PA-VH/PA-GHK/KN hydrogels did not yield a significant difference in F-actin network formation compared to PA-VH/KN or PA-VH/PA-RGDS/KN hydrogels due to high sample variability as a result of hydrogel instability and small sample range (n ≤ 3). Conversely, consistent results with smaller variability were obtained for PA-VH/PA-RGDS/KN with and without FN to evaluate cell behaviors in comparison to Matrigel (Fig. 1B).
First, we determined the cell proliferation in 3D tricultures in PA-VH/PA-RGDS/KN(/FN) hydrogels compared to Matrigel. On the basis of the metabolic activity, a similar cell proliferation was observed in both matrices (Fig. 5B; readout fluorescence values in fig. S7B). There was an initial decrease in metabolic activity on day 4, most likely as a result of minor HUVEC and hMSC death, followed by a steady increase in metabolic activity. While cells in the PA-VH/PA-RGDS/KN(/FN) hydrogels showed an initial lower metabolic...
activity compared to Matrigel, no difference was observed from days 8 to 14. Whereas the cell monocultures in Matrigel were prone to gel fragmentation, the tricultures in Matrigel contracted in size over the 14 days. We speculate that this contractile behavior was due to the relatively high cell density in these cultures, increasing the number of cell-cell interactions.

Next, we compared spheroid formation in the 3D tricultures in PA-VH/PA-RGDS/KN(FN) hydrogels and Matrigel to the spheroid data from the 3D monocultures (see the “Cell monocultures within PA/KN hydrogels generate tumor spheroids” section). In both matrices, we observed an immediate increase in spheroid formation and size compared to the monoculture counterparts, as detected by IF microscopy (Fig. 5, C and D; Live/Dead assay and bright-field images in fig. S7C). Comparing 3D mono- and tricultures within PA/KN hydrogels on day 14, we detected an increase in spheroid size in the tricultures (Fig. 5C versus Fig. 3B). Spheroid sizes observed in 3D monocultures in PA-VH/KN on day 21 (discussed in the “Hydrogel formation and characterization at the nano- and macroscale” section) were now observed by day 14 in the tricultures using PA-VH/PA-RGDS/KN(FN) hydrogels. This accelerated growth may be the result of enhanced intercellular interactions between the different cell types, which have been associated with spheroid growth (45). No significant difference was detected between PA-VH/PA-RGDS/KN and PA-VH/PA-RGDS/KN(FN) hydrogels on the basis of the fluorescent micrographs and proliferation data. We hypothesize that this may be a reflection of keratin-containing adhesion sequences that mask the effect of FN. However, to determine the influence of FN in our system, a more detailed analysis of the cell expression is required. Thus, on the basis of the comparative results without FN, our subsequent work focused on PA-VH/PA-RGDS/KN. Overall, we conclude that not only 3D tricultures in PA-VH/PA-RGDS/KN exhibit enhanced spheroid size and growth compared to 3D monocultures but also they are comparable to Matrigel.

Having evaluated the changes in spheroid formation between 3D tri- and monocultures, next we assessed the F-actin network formation in our PA/KN hydrogels. As discussed, 3D tricultures in PA-VH/PA-RGDS/KN led to an extensive F-actin network formation, which was absent in our stromal cell monocultures (see the “Cell monocultures within PA/KN hydrogels generate tumor spheroids” section). Using IF images, we performed a detailed analysis on our 3D tricultures to determine the origin of the F-actin networks within PA-VH/PA-RGDS/KN hydrogels. The presence of hMSCs was confirmed by staining for α-smooth muscle actin (α-SMA), which was aligned with the F-actin network (fig. S8A). We hypothesize that the presence of the F-actin network corresponds to the beginning of angiogenesis. To test this, we investigated the position of HUVECs within the hydrogels. Cells grown within PA-VH/PA-RGDS/KN hydrogels and Matrigel were stained with CD31, phalloidin/F-actin and 4′,6-diamidino-2-phenylindole (DAPI)/nuclei (Fig. 5E and fig. S8B). CD31 was stained positive in both matrices; however, unspecific staining was observed in the tumor spheroids. Thus, from the immunostaining alone, the F-actin network cannot be interpreted as the beginning of angiogenesis from HUVEC spreading. However, from the IF images, we created pseudo-volume filled images (Fig. 5F and fig. S8C). These images revealed that F-actin networks were integrated between adjacent spheroids, confirming that intracellular interactions between stromal and cancer cells play a role in the increased spheroid size observed in 3D tricultures compared to monocultures.

In a second attempt to elucidate the HUVEC behavior within the PA/KN hydrogel, we used a cell tracker dye to label HUVECs, allowing cell position to be tracked in relation to cell spreading over 14 days (Fig. 5G and fig. S8D). The image sequence confirmed that the F-actin network formation took place between days 2 and 7 (Fig. 5G), with a decreasing network by day 14, when instead large spheroids were visible (fig. S8E). Analyzing the cell tracker staining, some of the HUVECs coincide with the F-actin network. However, the majority was outside the F-actin network, suggesting that it did not originate from HUVECs. Thus, further optimization of the hydrogel formulation is required to promote tumor angiogenesis within PA/KN hydrogels. However, the images demonstrate the continuous cell spreading as evident by the buildup of F-actin networks. In summary, our 3D tricultures promote intercellular interactions in PA/KN hydrogels, while highlighting the positive impact of stromal cells on spheroid size and growth rate.

**Responses to treatment in spheroid-containing PA/KN hydrogels**

We have demonstrated the consistent tumor spheroid formation in our PA/KN hydrogels, as well as the benefits and added accuracy in modeling the TME by incorporating different cell populations. We have shown the versatility of the PA/KN hydrogels by changing PA sequences and protein composition to tailor the model’s applicability to the ovarian TME. To assess the functionality of our hydrogels, we tested the responses of tumor spheroids grown in PA/KN hydrogels to three different treatments: the matrix metalloproteinase inhibitor and antiangiogenic agent GM6001 and the first-line chemotherapeutics paclitaxel (TXL) and carboplatin (PLT) (Fig. 6A) (9, 31). The dosages used in this study for GM6001 (20 μM), TXL (100 μM), and PLT (100 μM) are commonly used for in vitro drug testing (6, 9, 31) and thus allow comparison with previous studies. For the treatment studies, the fold change in metabolic activity was normalized to day 1 to allow comparison between the different treatments. The readout values are in fig. S10.

Ovarian cancer cells were encapsulated in PA-VH/KN hydrogels and Matrigel and treated 1 day after encapsulation for 14 days, with treatment changes every 2 to 3 days (control hydrogels at day 1; fig. S9A). While GM6001 did not affect spheroid size and metabolic activity (fig. S9B), as reported previously (9), TXL and PLT prevented spheroid formation as detected by bright-field and fluorescence microscopy (Fig. 6, B and C). A small number of cancer cells were still present after 14 days of treatment, although with lower metabolic activity compared to controls on day 1 (TXL, 0.70 ± 0.05; PLT, 0.61 ± 0.09; control, 1.70 ± 0.18; Fig. 6D). There was no difference in the response to both chemotherapeutics. When comparing the response in spheroid-containing Matrigel (Fig. 6E), TXL and PLT treatment appeared more effective than in PA-VH/KN hydrogels, with a very low metabolic activity observed at day 14 compared to day 1 (TXL, 0.38 ± 0.02; PLT, 0.40 ± 0.02; control, 2.60 ± 0.05). However, previous studies have shown that drug uptake within Matrigel is unrealistically high, resulting in uptake values similar to those in 2D cell cultures (38). Thus, we conclude that cellular responses in PA/KN hydrogels are more physiological than Matrigel.

To further explore the effect of treatment, we conducted a brief study using PA/KN tricultures. Our preliminary results indicated a similar behavior to that of 3D monocultures, with reduced metabolic activity upon TXL and PLT treatment, however, with minor effects of GM6001 (Fig. 6E and fig. S9D). Interference from GM6001 may
suggest inhibition of the network structures formed by HUVECs and hMSCs as previously reported (46). This aspect may be explored in future experiments. A follow-up study may also include the separation of multiple cell types after 3D culture for separate analysis to measure the effect of treatment. Within the scope of this study, the experiments with chemotherapeutics have shown the anticipated response in ovarian cancer cells.

DISCUSSION

There is a need to advance 3D cancer models to capture the key components of the TME and to screen improved or personalized therapeutics. Supramolecular hydrogels offer an opportunity to replicate the nanofibrous features of the extracellular microenvironment as well as include desired signaling epitopes. These self-assembling systems can also be engineered to interact with and incorporate key ECM components. Here, we demonstrate that PA/protein coassembling bioinks can model the TME of ovarian cancer to a level that is similar to, yet more controlled than, that provided by Matrigel.

Ovarian cancer spheroids were grown over 21 days and reached a similar cross-sectional size compared to previous studies (9). We did not observe a hypoxic core found in many spheroids or tumors. However, this phenomenon is only observed when the spheroids or tumor mass reaches a diameter of >200 μm, at which point diffusion is inhibited, eventually leading to a hypoxic core (47). We have shown the PA/KN hydrogel’s compatibility with multiple cell types and ability to selectively modify the peptide sequences to optimize cellular responses. In 3D tricultures, tumor spheroids interacted with HUVECs and hMSCs as well as the hydrogel matrix and their proliferation were enhanced. F-actin networks were formed throughout the course of the experiment.
the hydrogel and intercalated between adjacent spheroids. The extent of network formation was enhanced by using the bioactive sequences RGDS and GHK. Many other sequences, for example, IKVAV and GFOGER (12), have been shown to specifically promote angiogenesis, which may be used instead of GHK. In addition, GFs, such as VEGF or FGF, may be entrapped within the fibrous network to allow slow release. We validated the PA/KN hydrogels using cytotoxic drugs, with cellular responses observed for all tested treatments.

In this study, we primarily assess the performance of the PA/KN hydrogel while comparing it with Matrigel, showing similar results in most of the performed experiments. In spheroid monocultures, Matrigel led to an increased proliferation compared to most of the performed experiments. In spheroid monocultures, hydrogel while comparing it with Matrigel, showing similar results or FGF, may be entrapped within the fibrous network to allow slow GFOGER (RGDS and GHK. (et al.

Many other sequences, for example, IKVAV and both a structural and a signaling role. This emulates the way these macromolecules function in the native ECM. Overall, these hybrid hydrogels enable the design of 3D matrices with controlled of molecular composition, nanofibrous architecture, and the capacity to present both specific bioactive epitopes and macromolecules. Some of the limitations of PA-based hydrogels are cost of material and scalability. However, peptide-based self-assembling hydrogels offer attractive opportunities for in vitro applications, which is evidenced by a number of recent commercially available materials. Examples include Biogelx, which is currently being used in 3D breast cancer models for screening of new drugs; PeptiGels from Manchester Biogel, which have been used for postendoscopic treatment; and PuraStat from 3D Matrix, which has been developed for hemostasis. While these systems demonstrate that peptide-based self-assembling hydrogels are viable products for 3D culture applications, Matrigel remains the material of choice. Our study demonstrates that self-assembling materials can be rationally coassembled with ECM macromolecules to improve molecular and structural complexity while maintaining a high degree of control. Hence, it is possible to easily explore more complex hydrogels in a systematic manner, enabling the design of 3D microenvironments with high molecular diversity and reproducibility.

It is important to mention that the PA/KN coassembling system can be easily integrated with 3D bioprinting to provide macroscale precision and reproducibility (22). Bioprinting has already been explored to build 3D cancer models (48, 49). However, the nanofibrous network and the selective presentation of bioactive epitopes are unique properties to self-assembling materials and are known to directly affect cell behavior. While 3D bioprinting was not used in this study, the potential of the system to be used as a bioink offers additional opportunities for building more complex, yet controlled and reproducible, 3D cancer models (50). Nonetheless, in this study, we have demonstrated the possibility to use our PA/KN system to engineer an innovative 3D ovarian cancer model with a high degree of molecular versatility and tunability, enabling more effective testing of anticancer therapeutics.

METHODS

PAs (synthesis and purification)

PA-H (purity, 97.8%), PA-VH (purity, 98.9%), PA-GHK (purity, 99.5%), and PA-RGDS (purity, 99.2%) were synthesized in-house (fig. S1) as previously described (22). Additional PA-H (purity, 98.1%) was purchased from Biomatik (ON, Canada). In-house synthesis was carried out using an automatic microwave assisted solid-phase peptide synthesizer (SPPS) (CEM Liberty Blue) using 4-methylbenzhydrylamine Rink amide resin (0.52 mmol/g, Novabiochem Corporation, UK) with the tail coupled subsequently. Purification of all peptides was carried out through reverse-phase high-performance liquid chromatography (HPLC) using a 2545 Binary Gradient Preparative HPLC (Waters 2767, Waters, USA), a C18 column (Atlantis Prep OBD T3 Column, Waters, USA) and a gradient of 2 to 100% acetonitrile in water [0.1% (v/v) trifluoroacetic acid] over 40 min. Detection was carried out concomitantly with 2489 UV/Vis and an electrospary ionization mass spectrometer (ESI-MS) detector (Waters, USA). Peptide purity was determined by analytical HPLC as well as ESI-MS (Thermo LXQ, Thermo Fisher Scientific, USA). Purity ≥95% was accepted suitable for usage. Peptides were freeze-dried to yield
lyophilized powder. Peptide solutions were prepared using 10 mM HEPES, (pH 7.5).

**Matrigel**

Matrigel (Coming Matrigel Growth Factor Reduced Basement Membrane Matrix, LDEV-Free) was purchased from Scientific Laboratory Supplies (catalog no. 356230). To minimize the potential influence of batch-to-batch variations of Matrigel, only one batch of Matrigel was used for this study (LOT 7202001).

**Proteins**

Keratin was extracted from human hair and freeze-dried using a modified protocol (51), which yields mainly in intact hair keratin. Bovine-derived FN was purchased from R&D Systems (1030–FN) provided as 1 mg/ml in a 400 mM urea, 500 mM NaCl, and 50 mM tris-HCl solution (pH 7.5). Peptide solutions were prepared using 10 mM HEPES, (pH 7.5).

**Zeta potential**

Samples (n = 3 per condition) were prepared at a concentration of 1 mg/ml in 10 mM HEPES and filtered before measuring on a Malvern Nano ZS series Zetasizer, with each measurement repeated three times.

**Circular dichroism**

The experiments were run at concentration 0.1 mg/ml in 10 mM HEPES buffer for both PA and keratin. Measurements were carried out at 25°C using a 0.1-cm–path length and 300-μl-volume cuvette (Chirascan, Applied Photophysics, UK). Three scans were run per sample and at least three independent repeats per sample condition (n ≥ 3). The data were normalized to the baseline (blank control; 10 mM HEPES buffer) and the averaged trace smoothed to reduce noise without causing spectrum distortion. The data manipulation was carried out using the Chirascan trace manipulation software.

**Transmission electron microscopy**

Samples were prepared at a concentration of 0.01 mg/ml in 10 mM HEPES. Carbon copper grids (Agar Scientific, Stansted, UK) were used to mount the samples. Excess was removed using filter paper before incubation with 2% filtered uranyl acetate solution for 30 s. Grids were then washed with ultrapure water for 30 s, air-dried for 24 hours at room temperature (RT) and imaged using a JEOL 1230 transmission electron microscope operated at an acceleration voltage of 80 kV. All the images were recorded by a Morada charge-coupled device camera (Image Systems). At least 10 images were taken in random locations per condition.

**Degradation study**

Hydrogels were formed using PA-VH and PA-H by injecting 30 μl of PA into 70 μl of KN, incubated over 4 hours before being transferred into vials filled with PBS or 10 mM HEPES. Hydrogels were kept in excess buffer (PBS or HEPES) to prevent false negatives as a result of dehydration. At predefined time points (daily in the first week, then biweekly; PBS, n = 6 per time point; 10 mM HEPES, n = 4 per time point), the solution was removed from the vial and the vial with hydrogel weighed. Liquid removal was removed with a pipette and a small cotton swab. After 3 months, the final vial with hydrogel weight was recorded, and the vial was cleaned and weighed. Mass loss was calculated as “M0 – Mtime point/M0,” where M0 is the weight at day zero minus the weight of the vial.

**Rheology**

Hydrogels were prepared as 30 μl of PA in 70 μl of KN and formed for 4 hours at RT before being transferred to a 10 mM HEPES (n ≥ 6) solution or medium [Dulbecco’s Modified Eagle’s Medium (DMEM); n ≥ 3] and subsequently stored overnight before measurement. Samples were analyzed using a Discovery Hybrid Rheometer (DHR-3, TA Instruments, USA) equipped with an 8-mm-diameter parallel plate geometry. Rheological characteristics were monitored by amplitude sweep and frequency sweep. G’ (storage modulus) and G” (loss modulus) were measured at 25°C and at a constant frequency of 1 Hz in the 0.01 to 10% strain during the amplitude sweep, while the oscillation frequency experiments were carried out at a 0.1% fixed strain along 0.1 to 100 Hz.

**Scanning electron microscopy**

Samples were fixed using 4% PFA solution for 2 hours at RT. Then, the samples were dehydrated from a 100% water solution to a 100% ethanol solution in serial steps and dried using a critical point drier, K850 (Quorum Technologies), exchanging the ethanol for carbon dioxide. Carbon black tape was used to adhere the samples to metal stubs. Before imaging, samples were cut open to reveal the inside of the hydrogel, gold coated for 30 to 45 s, and imaged on a FEI Inspect F.

**Cell culture**

Human epithelial ovarian cancer cells (NIH:OVCAR-4) and hMSCs (PromoCell C-12974 hMSC-BM-c) were maintained in DMEM (4.5 g/liter D-glucose) with GlutaMAX, supplemented with 1% penicillin-streptomycin (P/S) and 10% fetal bovine serum (FBS). HUVECs were purchased from PromoCell (pooled donors, C-12203) and grown in endothelial cell growth medium (C-22010; PromoCell). HUVECs and hMSCs were used at passage 2 (P2) to passage 7 (P7) and NIH:OvCar-4 at < P20. Monocultures were placed in DMEM supplemented with 1% P/S and 10% FBS. Tricultures were placed in endothelial cell growth medium, supplemented with VEGF (2 ng/ml). Treatments were carried out using DMEM without P/S and FBS.

**2D cell cultures**

For cell seeding experiments with HUVECs, a density 10,000 cells per gel was used. The cells were suspended in cell media and added to a well plate with preformed hydrogels (Matrigel or PA/KN hydrogels). In the case of PA/KN gels, HUVECs were suspended in a KN solution (10 mg/ml), and 70 μl of the KN/cell solution (~140 cells/μl) was added per well in a 96-well plate. Subsequently, 15 μl of PA was injected into the KN/cell solution and incubated for 30 min at 37°C on a shaker. One-day post- seeding hydrogels were transferred into a 48-well plate. In the case of Matrigel, the HUVECs were suspended in media and added to the preformed gels (10,000 cells per gel) and incubated for 30 min at 37°C on a shaker. One-day post-seeding hydrogels were transferred into a 48-well plate.

**3D cell cultures**

For cell encapsulation, lyophilized PA and proteins were treated with ultraviolet light for 30 min before dissolving in 10 mM HEPES at a concentration of 10 mg/ml. Cells were suspended in PA solution or Matrigel using the following cell densities:

- Stromal cell viability: 1000 HUVECs/μl and 600 hMSCs/μl, respectively.
All 3D cell culture experiments were performed in biological replicates at 37°C and transferred into a 48-well plate with culture medium. Peptide sequences were used as PA-H or PA-VH (10 mg/ml) or in combination 1:1 with either PA-GHK or PA-RGDS (10 mg/ml), i.e., 10 μl of PA-RGDS or PA-GHK (10 mg/ml) per 100 μl of PA-VH (10 mg/ml). KN was used at a concentration of 10 mg/ml either alone or with addition of FN (1 mg/ml) at 100 μl FN per 900 μl of KN. As controls, undiluted Matrigel (GF reduced; Corning) was mixed with cells, and 15 μl was pipetted onto each PDMS square. Samples were incubated for 30 min at 37°C and transferred into a 48-well plate with culture medium. All 3D cell culture experiments were performed in biological replicates with at least triplicate samples.

**alamarBlue and Live/Dead assays**
alamarBlue reagent was used at a concentration of 700 μl in 10-ml medium (7%). Therefore, culture medium was removed, and 300 μl of alamarBlue solution was added and incubated for 6 hours including three control samples. Subsequently, fluorescent signals of 100-μl samples were measured in triplicate on a FLUOstar OPTIMA (BMG Laboratory) plate reader (excitation, 530 nm; emission, 590 nm) using a black 96-well plate. Media without cell-laden hydrogels with alamarBlue reagent served as negative control for background reading. The signal of pure media (no alamarBlue) was subtracted from all samples. Subsequently, the readout values were divided by the background reading (Media + alamarBlue + no cells), following the simplified calculation from Thermo Fisher Scientific. The corrected fluorescent signals were normalized to day 1. The uncorrected values can be found in the Supplementary Materials (figs. S5C, S7B, and S10). For Live/Dead staining, 2-μl ethidium homodimer-1 (2 mM) and 1-μl calcein AM (4 mM) were added to 1 ml of culture medium, and samples were imaged after 15 min using a confocal microscope. A monolayer was used as a control to check the Live/Dead staining.

**Immunohistochemistry**
Slides of normal and cancerous ovarian tissue were prepared as serial formalin-fixed paraffin-embedded tissue sections (5 μm). Sections were deparaffinized in xylene and rehydrated in dilutions of ethanol and water. Antigen retrieval was performed using 1× citrate-based solution (pH 6; H-3300; Vector Laboratories) at 95°C for 10 min. Then, slides of normal and cancerous ovarian tissue sections (5 μm) were deparaffinized in xylene and rehydrated in dilutions of ethanol and water. Antigen retrieval was performed using 1× citrate-based solution (pH 6; H-3300; Vector Laboratories) at 95°C for 10 min. Then, sections were blocked with 5% bovine serum albumin (BSA)/PBS for 1 hour. At RT and treated with 3% H2O2. Antibodies (KRT32 ABIN517410 and KRT82 ABIN561240; Antibodies-online) were diluted 1:50 in 5% BSA/ PBS and incubated overnight at 4°C, followed by biotinylated goat anti-mouse immunoglobulin G (BA-9200; Vector Laboratories) at 1:200 in 5% BSA/PBS for 45 min. Samples were washed with 1× tris-buffered saline (TBS-T), blocked with 5% bovine serum albumin (BSA)/PBS for 1 hour. At RT and treated with 3% H2O2. Antibodies (KRT32 ABIN517410 and KRT82 ABIN561240; Antibodies-online) were diluted 1:50 in 5% BSA/PBS and incubated overnight at 4°C, followed by biotinylated goat anti-mouse immunoglobulin G (BA-9200; Vector Laboratories) at 1:200 in 5% BSA/PBS for 45 min. Samples were washed with 1× TBS-T and stained using an ABC HRP Elite kit (PK-6100-NB; Vectastain) and hematoxylin (GHS116; Sigma-Aldrich), mounted, and imaged using a NanoZoomer S210 (Hamamatsu). Staining controls without the primary antibody, as well as without primary and secondary, were included for all experiments. Examples of the controls for no primary are in fig. S2.

**IF staining and confocal microscopy**
DAPI (D1306) and rhodamine phalloidin (R415) were purchased from Sigma-Aldrich. Primary (CD31 ab32457, α-SMA ab7817, KRT32 ABIN517410, and KRT82 ABIN561240) and secondary (Alexa Fluor 488/555/568/594) antibodies were purchased from Abcam, Antibodies-online, and Life Technologies, respectively. Samples were fixed in 4% PFA/PBS for 2 hours, permeabilized in 0.2% Triton X-100/PBS for 2 hours and blocked in 2% BSA/PBS overnight at RT. Primary antibodies were diluted 1:100 in 2% BSA/PBS and incubated for 48 hours at 4°C. Secondary antibodies were diluted 1:1000 in 2% BSA/PBS and incubated overnight at 4°C. An Alexa Fluor 488–conjugated antibody (303110, BioLegend) was used at 1:100 in 2% BSA/PBS overnight at 4°C. DAPI and rhodamine phallloidin were diluted 1:500 and 1:250, respectively, in PBS and incubated for 2 hours at RT. Samples were washed with PBS between each step. A green cell tracker dye (CMFDA C2925; Life Technologies) was also used to label HUVECs by adding 4 mM to the cell suspension for 30 min before cell encapsulation into the hydrogels. Fluorescent images were acquired using Leica TCS SP2 and Zeiss LSM710 confocal microscopes. Samples were imaged acquiring z-stacks ranging from 5 to 20 μm depending on the sample size. The PA/KN hydrogels were checked for autofluorescence before staining. In addition, bright-field images were taken of PA hydrogels without cells after 21 days of incubation.

**Image analysis**
ImageJ (version 1.32i) was used to compile z-stacks and adjust colors. AngioTool (version 0.5a) was used the measure the density of F-actin networks (fig. S7A). Imaris (version x64 9.2.1) was used to create 3D videos of the z-stacks and volume images. Spheroid cross-sectional area was calculated using ImageJ, with cutoff points for days 1 and 4 at 100 μm² and for days 7 and 14 at 300 μm².

**Treatment with cytotoxic drugs and inhibitor**
3D cell monolucres were treated on day 1 using GM6001 (20 μM), TXL (100 μM), and PLT (100 μM). Treatment solutions were prepared in DMEM without supplements and exchanged every 2 to 3 days. As controls, medium changes were performed without treatment.

**Statistical analysis**
Statistical analysis was performed using GraphPad Prism 5.0 (version 8.0.2). One-way analysis of variance for multiple comparisons was used. Nonparametric statistics were used when the samples did not present a normal distribution (Mann-Whitney test). Statistical significance was set to P < 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001).

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/40/eabb3298/DC1

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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials (figs. S1 to S10). Additional data related to this paper may be requested from the authors.

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