Supporting Information

Toward Morphologically Relevant Extracellular Matrix: Nanofiber-Hydrogel Composites for Tumor Cell Culture

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Experimental Section

Materials and cells

The PLGA (lactide/glycolide, 50/50; *M*n = 100 000) was purchased from Daigang Biological Engineering Co. (China). Sodium alginate (NaAlg, viscosity of 10g/L dissolved in water at 20 °C ≥ 0.02 Pa·s, Mw 400 kDa) was purchased from Aladdin Reagent Co. (China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were purchased from Gibco (USA). DU145 cell line was purchased from the Chinese Academy of Sciences (Shanghai, China). All reagents were purchased from Sinopharm Chemical Co. if not specifically mentioned.

Fabrication of 2D PLGA fibrous membrane

PLGA molecule dissolved in TCM/DMF blend solvent (v/v = 4/1) and stirring overnight on a magnetic stirrer at room temperature of 25°C with a rotational speed of 500 r/min. Then the prepared solution was transferred into a 10 mL syringe with a 20 G metal needle under the conditions of 18 kV voltage, 4 ml/h feeding rate and collecting distance of 20 cm. The prepared fibrous membrane was then dried at room temperature for 24 hours and then stored, with named as PLGA.

Preparation of NaAlg hydrogel

This work uses a two-step method to prepare a NaAlg hydrogels with uniform structure.

2 g of NaAlg powder was added to 200 mL of deionized water and stirred for 6 hours using a magnetic stirrer at room temperature and 500 r/min to obtain pure NaAlg solution with a concentration of 10 mg mL^{-1}. The NaAlg solution (1 mL) was added to the 12-well plate and subsequently frozen at -80 °C overnight, and then removed and lyophilized in a vacuum freeze dryer for 24 hours. The above samples were then cross-linked overnight in a solution containing 20 mM $CaCl_2$. At the end of cross-linking, the samples were washed 3 times with deionized water, placed in a refrigerator at -80 °C, and frozen overnight. The samples were denoted as NaAlg gel.

Preparation of ECM mimicked 3D tumor cell culture scaffold

ECM mimicked 3D scaffold was prepared by modified the electrospinning process to form fiber and hydrogel homogeneous-type architecture. 4 minutes after PLGA fiber collection, spinning was suspended and NaAlg powder (100 mg or 300 mg) was uniformly sprayed on the fiber membrane, followed by continued collection of PLGA fibers. After repeating the above steps 5 times, the spinning process was completed. Then the obtained samples were impregnated in distilled water for 5 min to swelling the NaAlg and subsequently frozen at -80 °C overnight, and then removed and lyophilized in a vacuum freeze dryer for 24 hours. After that, the samples were cross-linked with $CaCl_2$ (20 mM) overnight, washed three times with deionized water and freeze-dried. The as prepared samples were named as ECMMS-1 and ECMMS-3 according to the quality of sprayed NaAlg powders.

Physico-chemical characterizations of the scaffolds

The surface morphologies were evaluated by the field emission scanning electron microscopy (FESEM, SU8010, Japan). The chemical structures were characterized using Fourier Transform Infrared (FTIR, NEXUS-670, USA). The measurement of pore size was realized by Fiji software (National Institutes of Health, USA). The mechanical properties were evaluated with a YG (B) 026G-500 universal testing system (Wenzhou Darong Textile Instrument Co., Ltd., Wenzhou, Zhejiang, China). The tensile tests were performed at a distance of 30 mm and a tensile speed of 100 mm/min.

The rheological characterization was performed using a Haake Mars 60 rheometer (Thermo Fisher Scientific, USA). G′ (storage modulus) and G″ (loss modulus) were measured at 37°C and at a constant frequency of 1 Hz (in the linear viscoelastic region) in the 0.01 to 10% strain during the amplitude sweep, while the oscillation frequency experiments were carried out at a 1% fixed strain along 0.1 to 100 rad/s.

The compression performance of the sample was tested using unconfined compression[1]. After thorough rehydration of the scaffold in PBS, the compressive stress-strain behavior of the scaffold was measured using a human in vivo biological pipeline compression elasticity tester (LLY-06D, Laizhou Electronic Instruments Co., Ltd., China). A cylindrical indenter with a diameter of 20 mm was used to compress the samples at a rate of 5 mm/min until a strain of 50% was reached. At least 3 specimens of each stent type were tested.

The wettability was measured with contact angle system (OCA15EC, Dataphysics, Germany). The swelling ratio was determined as before[1]. Briefly, the samples were placed in centrifuge tubes with 15 mL of deionized water per well and kept at room temperature for a specified time (2, 4, 6, 8, 10, 12, 24 h), and then the water was gently wiped off the surface of the samples with filter paper, weighed, and then the swelling ratio of each sample was calculated based on the weight change. Degradability was measured by the rate of mass loss of the sterilized sample inside sterile PBS solution. Briefly, the sample (diameter: 1 cm) of known weight were placed in 50 mL of sterile PBS, the medium was changed every other day. After that, the samples were freeze-dried on days 1, 3, 5, 7, 14, and 28, and the mass was weighed to calculate the weight loss rate. Three individual specimens were tested for each experiment.

Cell proliferation and infiltration

The NaAlg gel, PLGA, ECMMS-1 and ECMMS-3 scaffolds were washed three times and preconditioned with DMEM in 24-well culture plates. 1×10^5 DU145 cells in 50 μL was seeded onto per scaffold after removing DMEM. The cell-loaded scaffolds then were placed in cell culture incubator (37 °C, 5% CO_2) for 2 h allowing cells to attach to scaffolds. After that, 1 mL culture medium was added and changed every other day.

Cell proliferation was analyzed on days 1, 4, 7, and 14 using Cell Counting Kit-8 (CCK-8) assay (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, the CCK-8 reagent was diluted 10-fold and added to a 24-well plate in equal

amounts, and the absorbance at 450 nm of the supernatant was read after incubation for 3 hours. At days 7 and 14, cells were stained using FITC (green, staining the cytoskeleton, Invitrogen, China) and 4,6-diamidino-2-phenylindole (DAPI, blue, staining the nucleus, Beyotime, China) to analyze cell morphology by laser scanning confocal microscope (LSCM, LSM 510, ZEISS, Germany). The depth of cell infiltration within the scaffold was observed using the Z-stack function of the confocal microscope.

Chemosensitivity assay

To assess chemosensitivity, 3D and 2D samples were treated with anti-tumor drugs enzalutamide (10, 20, 50, 100 and 200 μg/mL), etoposide (15, 75, 150, 200 and 300 μg/mL), paclitaxel (5, 20, 50, 100 and 200 μg/mL), and axitinib (5, 20, 50, 100 and 200 μg/mL). After 7 days of proliferation, samples were cultured in medium supplemented with the drug for another 48 h. Almar Blue assay (Yeasen, Shanghai, China) was used to assess cell viability according to the manufacturer's instructions. In brief, after 48 h of drug treatment, samples were washed three times with PBS, and then drug-free medium and Almar Blue were added to each sample at a 9:1 volume ratio. After incubation for 3 h, the reaction solution (100 μL) was transferred to the wells of a 96-well plate and the medium was assayed for fluorescence under excitation at an excitation wavelength of 570 and 600 nm. As controls, medium changes were performed without treatment. Each experiment including three control samples.

RNA isolation and quantitative real time-polymerase chain reaction (qPCR)

DU145 cells were collected after 7 days of culture for RNA isolation and quantitative real time-polymerase chain reaction (qPCR) analysis as previously described[2]. In brief, total RNA was isolated from cells using TRIzol reagent (Invitrogen) and the amount and purity of RNA was determined using a spectrophotometer (Nanodrop 2000, Thermofish, USA). The primer sequences of specific genes are shown in Table S1. Calculation of relative gene expression levels was conducted using the $2^{-\Delta\Delta CT}$ method, and β-actin was used as the reference.

Table S1. Primer sequences.

Primer	Forward (3'-5')	Reverse (5'-3')
β-actin	CTCCATCCTGGCCTCGCTGT	GCTGTCACCTTCACCGTTCC
CD133	CCGGAGGAAGTGCTTAGCCATC	CTTCTGGGTCCTTTGGATCCGAGT
CDH1	ACCTTCCTGGTGCCGGAGA	ATGACGCCATCTGTAGACACTTTGA
CDH2	ATGGGTCTGTTCCAGAGGGATCA	CCTGGGAGAGGATCCTGTACCTC
EpCAM	TGGACATAGCTGATGTGGCTTACT	CGAGGAGCTCCCCGTTCAC
MMP2	AATATGGCTTCTGTCCCCACGA	GTACGGCCCTCGGTGGTACA
MMP9	TGCATGAAGACGACATAAAAGGCAT	GGAGGTGCAGTGGGACACAT
VEGF	GCTGCACCCACGACAGAAGG	TCATCGGGGTACTCCTGGAAGAT

Statistical Analysis

Quantitative results for all data were obtained from at least three independent experiments, and data are expressed as mean ± SD. Statistical analysis was performed using Tukey's post hoc analysis for parametric data. All experimental groups were analyzed in technical triplicates unless otherwise noted. For all tests, the statistical significance level was represented by ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

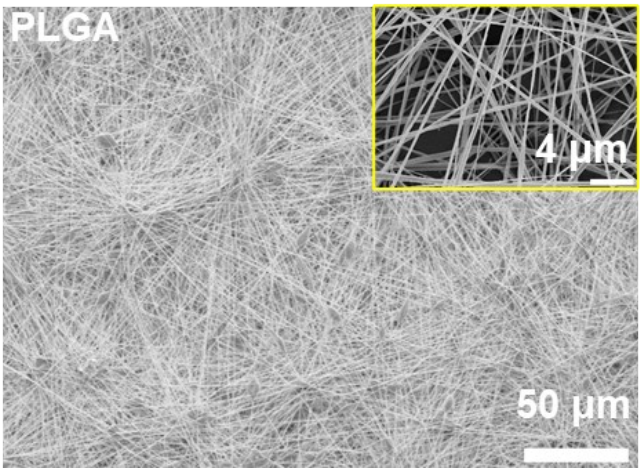

Figure S1. Typical SEM photo of PLGA nanofiber mesh.

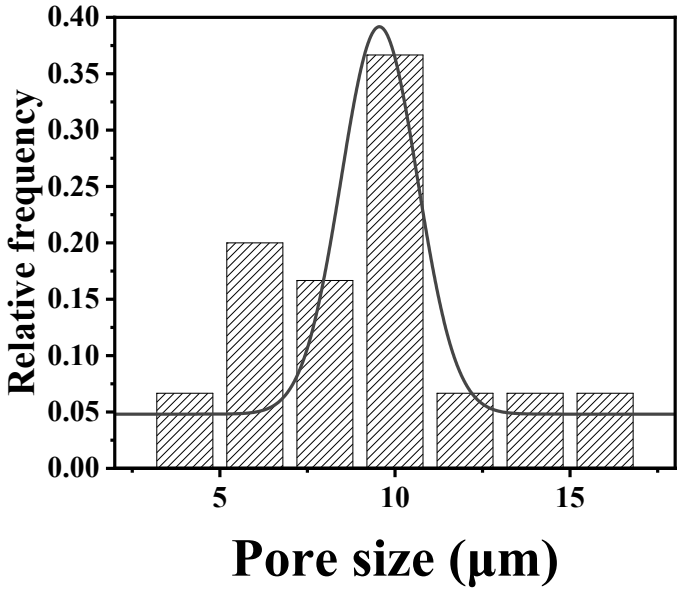

Figure S2. Pore size distribution of PLGA mesh.

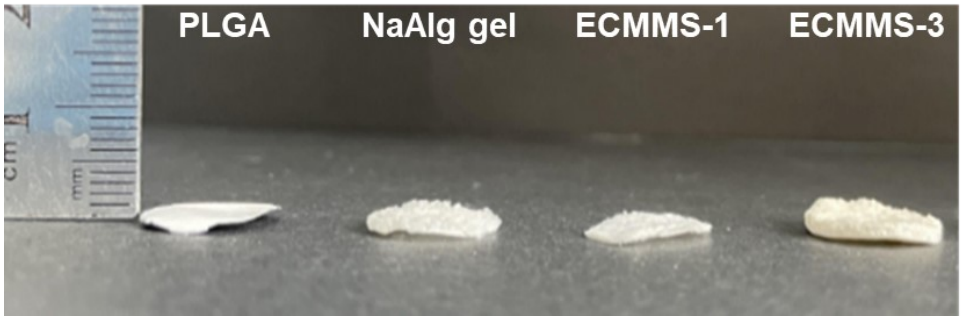

Figure S3. Typical picture of PLGA, NaAlg gel, ECMMS-1 and ECMMS-3.

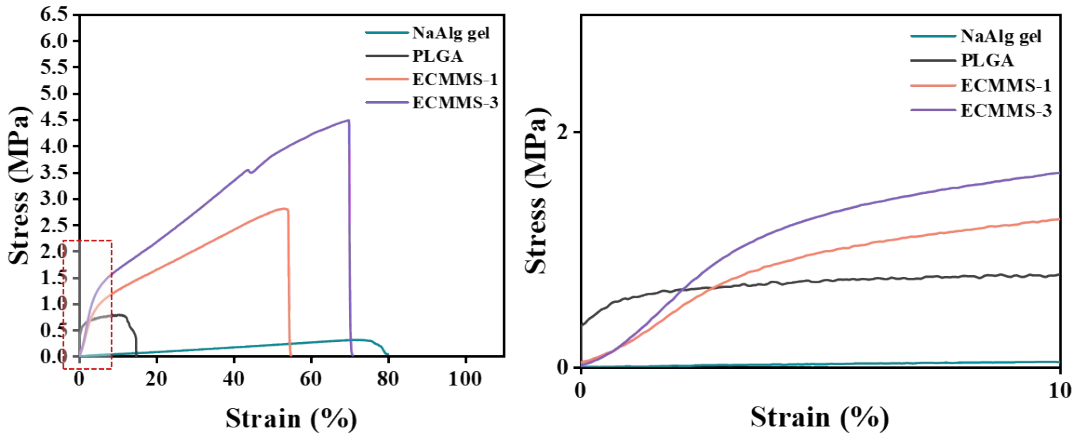

Figure S4. Typical stress-strain curves of NaAlg gel, PLGA, ECMMS-1, and ECMMS-3. Typical stress-strain (0-10%) curves of NaAlg gel, PLGA, ECMMS-1, and ECMMS-3.

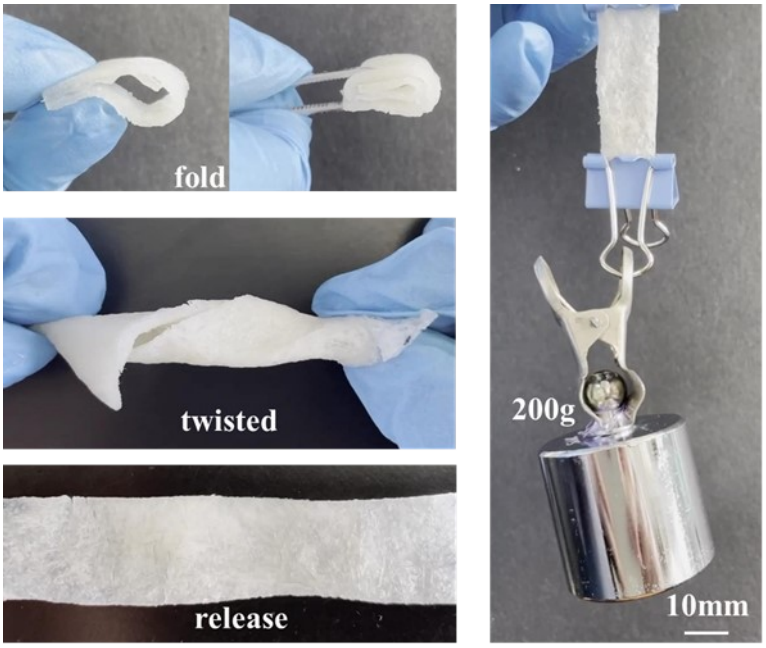

Figure S5. Excellent maneuverability and mechanical properties of the ECMMS-1.

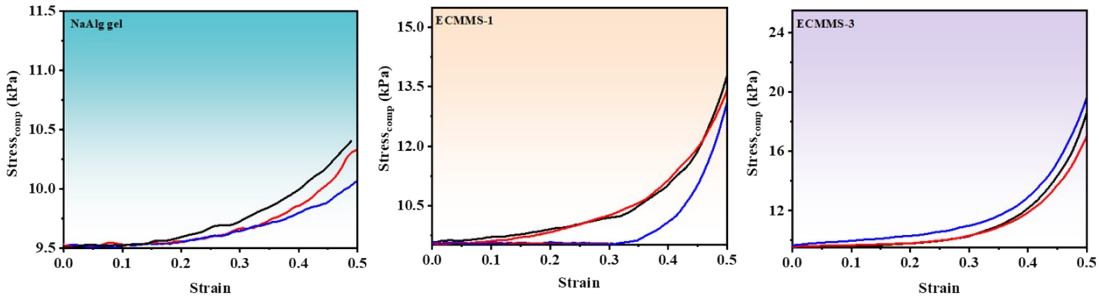

Figure S6. Compressive stress-strain curves of the NaAlg gel, ECMMS-1, and ECMMS-3.

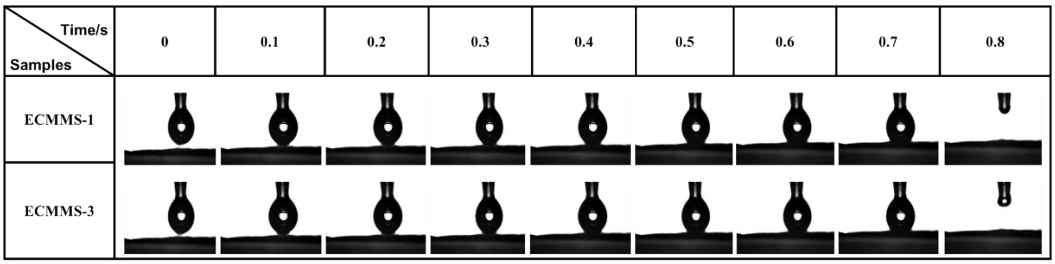

Figure S7. Photograph of contact angle at different time points.

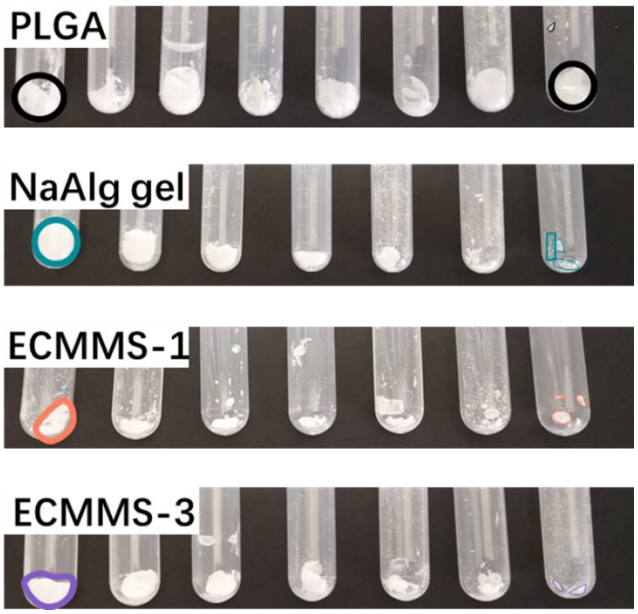

Figure S8. Photographs of the PLGA, NaAlg gel, ECMMS-1 and ECMMS-3 at different degradation time point.

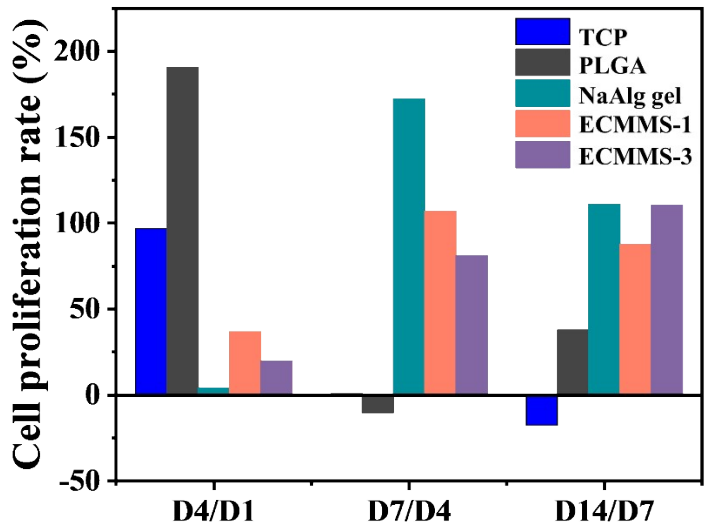

Figure S9. Comparison of the metabolic activity of prostate cancer cells over time in TCP, NaAlg gel, PLGA, ECMMS-1, and ECMMS-3 using an alamarBlue assay. The plot shows the values normalized to day 1, day 4, and day 14 against time, respectively.

References

[1] X. Liu, S. Fu, Y. Jiao, M. Hu, C. Li, F. Wang, L. Wang, A loofah-inspired scaffold with enhanced mimicking mechanics and tumor cells distribution for in vitro tumor cell culture platform, Biomater. Adv. 135 (2022) 112672. https://doi.org/10.1016/j.msec.2022.112672.

[2] S. Mao, J. He, Y. Zhao, T. Liu, F. Xie, H. Yang, Y. Mao, Y. Pang, W. Sun, Bioprinting of patient-derived *in vitro* intrahepatic cholangiocarcinoma tumor model: establishment, evaluation and anti-cancer drug testing, Biofabrication. 12 (2020) 045014. https://doi.org/10.1088/1758-5090/aba0c3.