Electronic supplementary information

A hydrogel with supramolecular surface functionalization for cancer cell capture and multicellular spheroid growth and release

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Table of Contents

1. Materials and Methods

Materials. β-Cyclodextrin (>98%, β-CD) and methacryloyl chloride were purchased from TCI Co., Ltd (Tokyo, Japan). NHS heterofunctionalized polyethylene glycol (NHS-PEG-SH, MW 5,000) was purchased from Bio basic Pte. Ltd (Singapore), adamantylamine hydrochloride, folic acid (FA), N, N'-dicyclohexylcardodiimide (98%, DCC), 4-dimethylaminopyridine (98%, DMAP), 3-(trimethoxysilyl) propyl methacrylate (acryl-silane), acrylamide (Am), N,N-methylenebisacrylamide (MBA), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), anhydrous triethylamine (TEA), sodium hydroxide were purchased from Sigma-Aldrich. All solvents were purchased from VWR (Singapore).

Synthesis of β-cyclodextrin methacrylate (β-CD-MA). Modification of β-cyclodextrin (β-CD) with methacrylate groups was obtained according to reported protocols[1-3]. Generally, β-CD (3.8 g, 3.4 mmol) was firstly dried at 110 °C overnight. After that, it was dissolved in degassed anhydrous dimethyl formamide (DMF) (30 mL), followed by the addition of anhydrous triethylamine (TEA) (3.4 mL, 24.5 mmol). The mixture was then cooled down to 0 °C using an ice bath. Methacryloyl chloride (2.0 mL, 20.5 mmol) in anhydrous DMF (1.5 mL) was then added dropwise into the mixture with stirring. After stirring for 4 h, triethylamine hydrochloride was filtered, and the solution precipitated in 200 mL cold acetone. White precipitate was obtained and re-dissolved with 5 mL of DMF and precipitated in cold acetone (50 mL) again for purification. The product was further purified by column chromatography using 1-propanol/water/ammonium hydroxide/toluene solvent system (with volume ratio of 6:3:1:1). Pure product, denoted as β-CD-MA was obtained by removal of solvent under vacuum. Yield: 2.4 g, 52%.

Synthesis of adamantyl-thiol-polyethylene glycol (Ad-PEG-SH). 1-Adamantylamine (94 mg, 0.095 mmol) and anhydrous TEA (72 µL, 0.097 mmol) was dissolved in anhydrous dichloromethane (DCM) (1 mL). NHS-PEG-SH (Mw 5000) (500 mg, 0.0019 mmol) dissolved in DCM (4 mL), follow by addition to the mixture and stirred for overnight. After the reaction, the solvent was subsequently removed in vacuo, and deionized water (15 mL) was added to the reaction residue. The solution was transferred into a centrifuge tube and centrifuged at 10,000 rpm for 10 min to remove the unreacted 1-adamantylamine. The solution was dialyzed (molecular weight cut off, 2k) in cold water at 4 °C for 48 h and then freeze dried to obtain the pure product. Yield: 420 mg, 75%.

Synthesis of adamantyl-polyethylene glycol-folate (Ad-PEG-FA). Ad-PEG-FA was synthesized according to our previous reported method[4]. Briefly, folic acid (62.5 mg, 0.142 mmol), DCC (29.2 mg, 0.142 mmol), DMAP (1.73 mg, 0.014 mmol) were dissolved with anhydrous DMSO/pyridine (1:1) mixture (3 mL). Subsequently, Ad-PEG-SH (250 mg, 0.047 mmol) in anhydrous DMSO/pyridine (1:1) mixture (5 mL) was added dropwise to the solution. The reaction was allowed to stir 24 hours at room temperature. After the reaction period, the solution was directly dialyzed (molecular weight cut off, 2k) in DMSO for 3 days to remove unreacted folic acid and deionized water for 5 days. The solution was then filtered to remove precipitants and freeze dried to obtain the pure product. Yield: 270 mg, 80%.

Preparation of hydrogel substrate. Protocol for the functionalization of glass bottom 24 well cell culture plates was adopted from reported literature[5]. The plates were etched for 30 min with 10 mol/L NaOH solution. The etched plates were gently rinsed with distilled water and subsequently covered with 2% (v/v) of 3-(trimethoxysilyl) propyl methacrylate in ethanol for 2 h. After the reaction time, the plates were rinsed with excess ethanol and dried for further use.

Preparation of β-cyclodextrin-polyacrylamide hydrogels (β-CD-PAM, Gel_{PAM-CD}) on substrates. Gel_{PAM-CD} were synthesized via copolymerizing monomer acrylamide and crosslinker β-CD-MA via free radical polymerization using ammonium persulfate (APS) and tetramethylene diamine (TEMED) as initiators. Typically, Gel_{PAM-CD} synthesized by mixing monomer solution (24%, w/v) (1.3 mL), DMSO/water (1:1) (0.65 mL) and β-CD-MA. Subsequently, 10% (w/v) APS (50 μL) and TEMED (10 μL) was added to the mixture and vortexed. 212 μL of the above prepolymer solution was immediately pipetted into acrylic silanized glass bottom 24 well cell culture plates. The reaction was allowed for 30 mins and subsequently terminated by submerging the hydrogels on the plates into deionized water. To remove unreacted monomer, cross-linkers and initiators, the hydrogels was soaked in deionized water with 1% penicillin-streptomycin for 5 days. The purified hydrogel on 24 well plates were fully swelled and ready for cell seeding.

Preparation of polyacrylamide (PAM) hydrogel (Gel_{PAM}) on substrates. Polyacrylamide hydrogel (PAM) with no cyclodextrin as control was synthesized by polymerization of N,N'-methylenebisacrylamide (MBA) using the similar procedure as for preparing β-CD-PAM gel.

Polymer and hydrogel characterization. Proton nuclear magnetic resonance (^{1}H NMR) were recorded on a Bruker spectrometer (400 MHz). Fourier transform infrared (FTIR) spectra were measured on a Shimadzu IRPrestige-21 spectrometer in the region of 4000–500 cm^{-1}. Gel_{PAM} and Gel_{PAM-CD} morphology were imaged by scanning electron microscopy (SEM) (Hitachi FlexSEM 1000) at 10 kV. Gel_{PAM} and Gel_{PAM-CD} were lyophilized to remove the water content before SEM observation.

Formation of functional hydrogel via self-assemble of cyclodextrin host and guest substituted polymer. To assemble Gel_{PAM-CD} with different adamantyl-terminated polymers (Ad-PEG and Ad-PEG-FA), Gel_{PAM-CD} was incubated with 500 μL of Ad-PEG or Ad-PEG-FA (5 mg/mL) for 24 h to allow formation of host-guest self-assembly. After incubation, the Gel_{PAM-CD}/PEG and Gel_{PAM-CD}/PEGFA were then roughly washed with deionized water to remove unbound polymers.

Cell lines and cell culture. Hepatocellular carcinoma cell line HepG2, human cervix adenocarcinoma cell line HeLa, human nasopharynx epidermal carcinoma cell line KB and human lung carcinoma cell line A549 were purchased from ATCC (Rockville, MD). HepG2 and HeLa cell lines were separately cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM). KB cell lines were cultured in MEM medium containing 2 mM L-glutamine and 1 mM sodium pyruvate. A549 were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12). All media for cell lines were supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (GE Healthcare). All four cell lines were maintained in 5% CO_2 at 37 ºC.

Determination of α-FR receptor expression level of cell lines. In this study, three folate receptor positive cell lines (HepG2, HeLa and KB) and one folate receptor deficient cell line (A549) were used to evaluate the specificity of our cell capture surface. The α-FR receptor expression level of cell lines was confirmed by detection of immunofluorescence of α-folate receptor using flow cytometry. α-FR specific monoclonal rabbit antibody was purchased from ThermoFisher Scientific. Briefly, cells were trypsinized, centrifuged and re-suspended to a concentration of 1 x 10^7 cells/mL. Cells were block with 2% BSA-PBS solutions on ice for 30 min, incubated with folate receptor alpha (ThermalFisher Scientific) with a dilution of 1:50 for 1 h on ice. After incubation, cells were washed with cold PBS for three times, followed by incubation with FITC-labelled goat-anti-rabbit secondary antibody on ice for 60 minutes. Cells

were then washed and measured by Beckman flow cytometer. Data was analysed by CytExpert.

Evaluation selective cell capture of $Gel_{PAM\text{-}CD}$/PEGFA. Folate receptor positive cells HepG2, HeLa and KB cells and folate receptor deficient A549 cells were seeded on $Gel_{PAM\text{-}CD}$, $Gel_{PAM\text{-}CD}$/PEG and $Gel_{PAM\text{-}CD}$/PEGFA which were attached to glass bottom 24-well cell culture plate with a density of 100,000 cells/well. Cells were also seeded on bare glass with no gel as control. After incubation for 24 h in 300 μL medium, cells were washed with 1x PBS for three times to remove the unattached cells. Images were captured under bright-field microscope and cell viabilities were tested using cell counting kit-8 (CCK-8). For cell viability testing, 30 μl of CCK-8 solution was added to each well and incubated for 2 h to allow reaction to complete. Absorbance was measured on a microplate reader (TECAN) at 450 nm. Standard curve of cell number vs absorbance was developed by making serial dilutions of 100,000, 50,000, 25,000, …, and 0 cells per well to each well of a 96 well plate using the corresponding cell lines and incubated with CCK-8 for 2 h, followed by measuring absorbance at 450 nm. Cell viability was further tested by Live/Dead Viability/Cytotoxicity Kit (Invitrogen). Cells were incubated with combined Live/Dead assay reagents (200 μL, 2 μM) for 30 min on ice. Following the incubation, cells were washed with D-PBS and observed under fluorescent microscope. Green colour from calcein staining stands for live cells and red colour from ethidium homodimer-1 staining stands for dead cells.

Monitor of cell proliferation of HeLa MCS. HeLa cells were seeded on GelPAM-CD/PEGFA with a density of 2,500 cells/well. Cells were also seeded on bare glass with no gel as control. Cells were imaged by bright-field microscope at 4 h (when all cells settled, Figure S10A), 24 h, 48 h, 72, 96 h, 120 h, and 144 h.

Release of captured cancer cells from $Gel_{PAM\text{-}CD}$/PEGFA. HeLa cells (100,000 cells/well) were seeded on $Gel_{PAM\text{-}CD}$/PEGFA hydrogel which were pre-attached on 24 well plate with glass bottom. HeLa cells were also seeded on bare glass and $Gel_{PAM\text{-}CD}$ as controls. The original capture cell amount was measured and calculated using CCK-8, similar to protocol described above. The captured cells on $Gel_{PAM\text{-}CD}$, $Gel_{PAM\text{-}CD}$/PEGFA and bare glass were incubated with 2.45 mM 2-Hydroxypropyl-β-cyclodextrin (HPβCD) or PBS for 2 h, respectively. After that, cells were washed with PBS for 3 times. Number of cells left over in cell capture surfaces were tested again using CCK-8. Cell released percentage was calculated as [1-(cells left after release / cells attached on platform before release)] × 100%. Experiments were performed in triplicates. Morphology of released MCSs was observed by microscope. HeLa MCSs released from $Gel_{PAM\text{-}CD}$/PEGFA was centrifuged at 200 g for 5 min, resuspended in medium and seeded onto 24 well plate. Image was taken 4 h after seeding, when all the cells settled to the bottom of plate. Images were captured under bright-field microscope.

2. Results on Polymer and Hydrogel Synthesis and Characterization

We first prepared and characterized Gel_{PAM-CD} and all building blocks for the supramolecular hydrogel scaffolds. Crosslinker β-CD-MA was synthesized by modification of β-CD with methacryloyl chloride. Gel_{PAM-CD} was then formed by a free radical polymerization of acrylamide monomer and β-CD-MA (Fig. S1A). Successful modification of β-CD with methacrylate groups was confirmed by ^{1}H NMR (Fig. S1B), from which the average degree of substitution was calculated to be 3. FTIR analysis was performed to further confirm the successful synthesis of b-CD-MA and Gel_{PAM-CD} (Fig. S2). Guest polymers Ad-PEG and Ad-PEG-FA were prepared according to our previously reported method.[6,7] As shown in Fig. S3A, Ad-terminated PEG with a thiol end group (Ad-PEG-SH) was synthesized by conjugating 1-adamantylamine to thiol-PEG-NHS. Ad-PEG-FA was synthesized by conjugating Ad-PEG-NHS to FA using DCC/DMAP coupling chemistry. Successful synthesis of guest polymers was confirmed by ^{1}H NMR (Fig. S3B). The surface and cross-sectional morphologies of freeze-dried Gel_{PAM} and Gel_{PAM-CD} (Fig. S4 and S5) were studied by scanning electron microscopy (SEM). GelPAM was crosslinked by N,N methylenebisacrylamide (MBA) with no cyclodextrin. The freeze-dried GelPAM-CD is more porous with a larger pore size than GelPAM, indicating that b-CD as a crosslinker in the hydrogel helps in the swelling of the hydrogel.

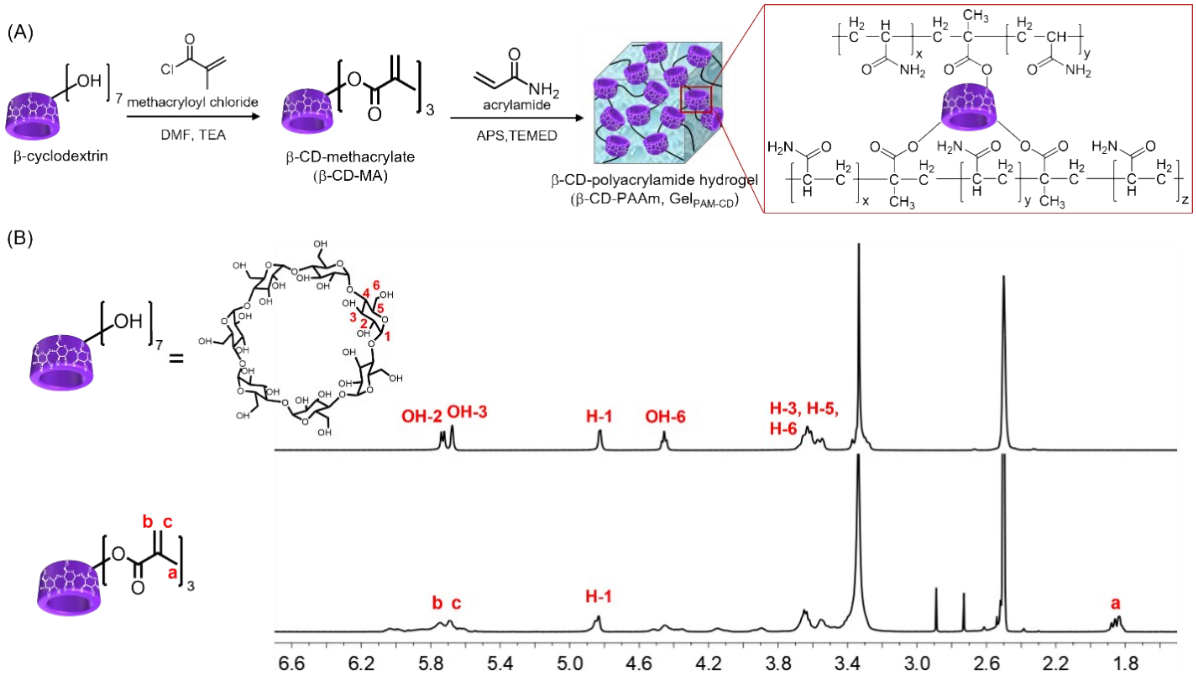

Figure S1. (A) Synthesis scheme of Gel_{PAM-CD}. (B) ^{1}H NMR of β-CD and β-CD-MA (d_6-DMSO).

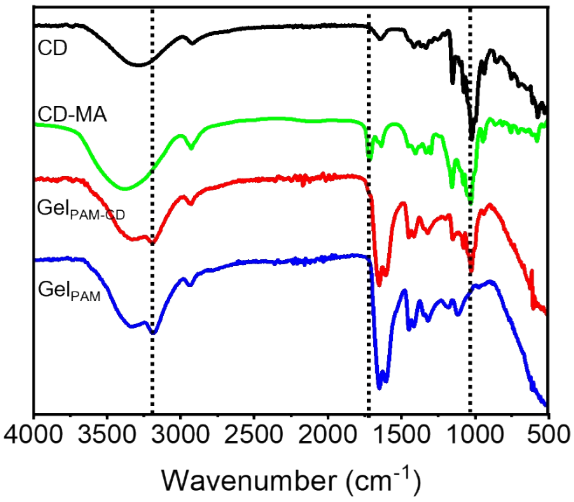

Figure S2. FTIR spectra of β-CD, β-CD-MA, lyophilized Gel$_{PAM-CD}$ and lyophilized Gel$_{PAM}$. FTIR spectrum of β-CD-MA showed an appearance of new peak at 1715 cm^{-1} (C=O stretching of ester groups), and an absorbance increase of peak at 1637 cm^{-1} (C=C double bond stretching). For dried Gel$_{PAM-CD}$, there was a peak at 3340 cm^{-1} (N-H stretching), which was responsible for poly(acrylamide) (PAM) component of hydrogel, and the peak at around 1017 cm^{-1} was assigned to the ether groups of β-CD.

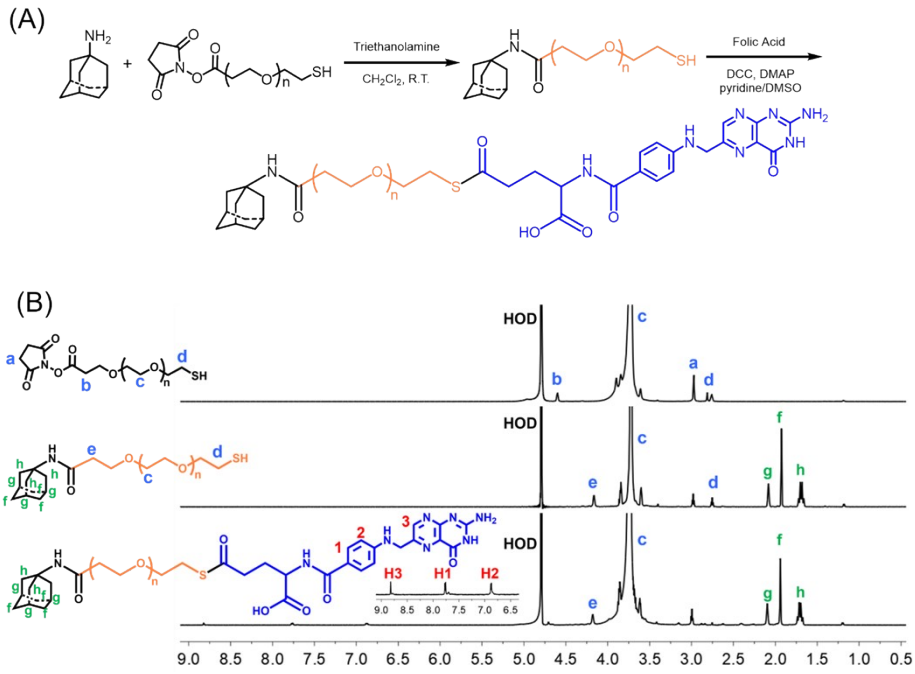

Figure S3. (A) Synthesis scheme of Ad-PEG-FA. (B) ^{1}H NMR of NHS-PEG-SH, Ad-PEG-SH and Ad-PEG-FA in D_2O. First, conjugation of adamantyl group to PEG was confirmed by peak e (4.18 ppm, 2H, -CH_2-CONH-), peak c (3.68 ppm, 454H, -CH_2CH_2O-), and peak f-h, which correspond to signals of adamantyl group. The number of adamantyl group conjugated to PEG was calculated to be ~1, based on integration of peak e of PEG and peak f-h of adamantyl group. Next, FA conjugating to Ad-PEG-SH was confirmed by the appearance of peak H1, H2, and H3 at 7.78 ppm, 6.88 ppm, and 8.81 ppm. The number of FAs conjugated to Ad-PEG was calculated to be approximately 1, by comparison of the integration of peak e of PEG and H1-H3 of FA.

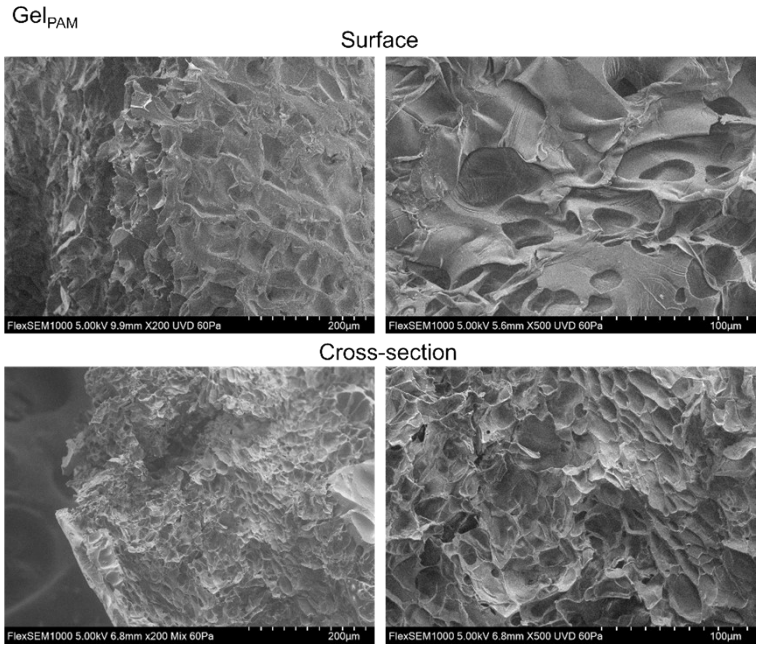

Figure S4. SEM images of surface (scale bar = 200 μm and scale bar = 100 μm) and cross-sectional (scale bar = 200 μm and scale bar = 100 μm) morphologies of freeze-dried Gel$_{PAM}$.

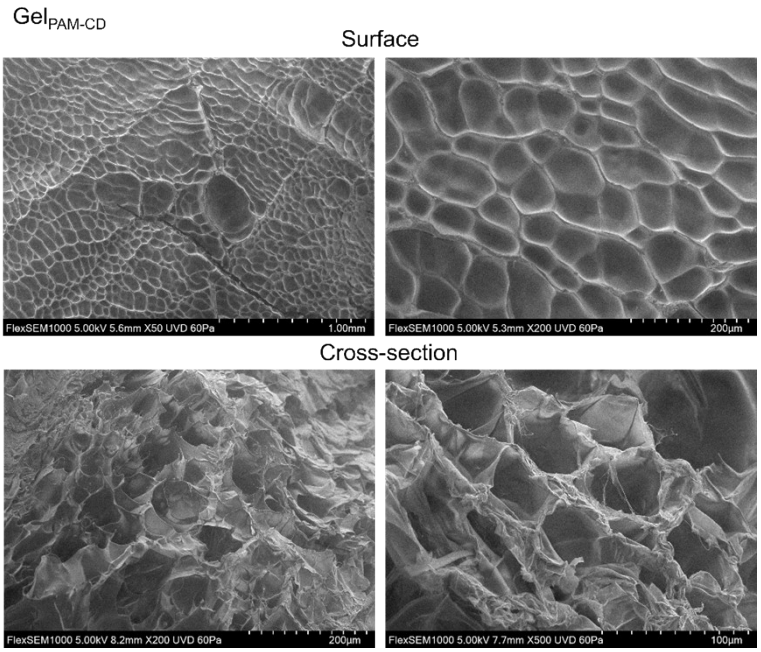

Figure S5. SEM images of surface (scale bar = 1 mm and scale bar = 200 μm) and cross-sectional (scale bar = 200 μm and scale bar = 100 μm) morphologies of freeze-dried Gel$_{PAM-CD}$.

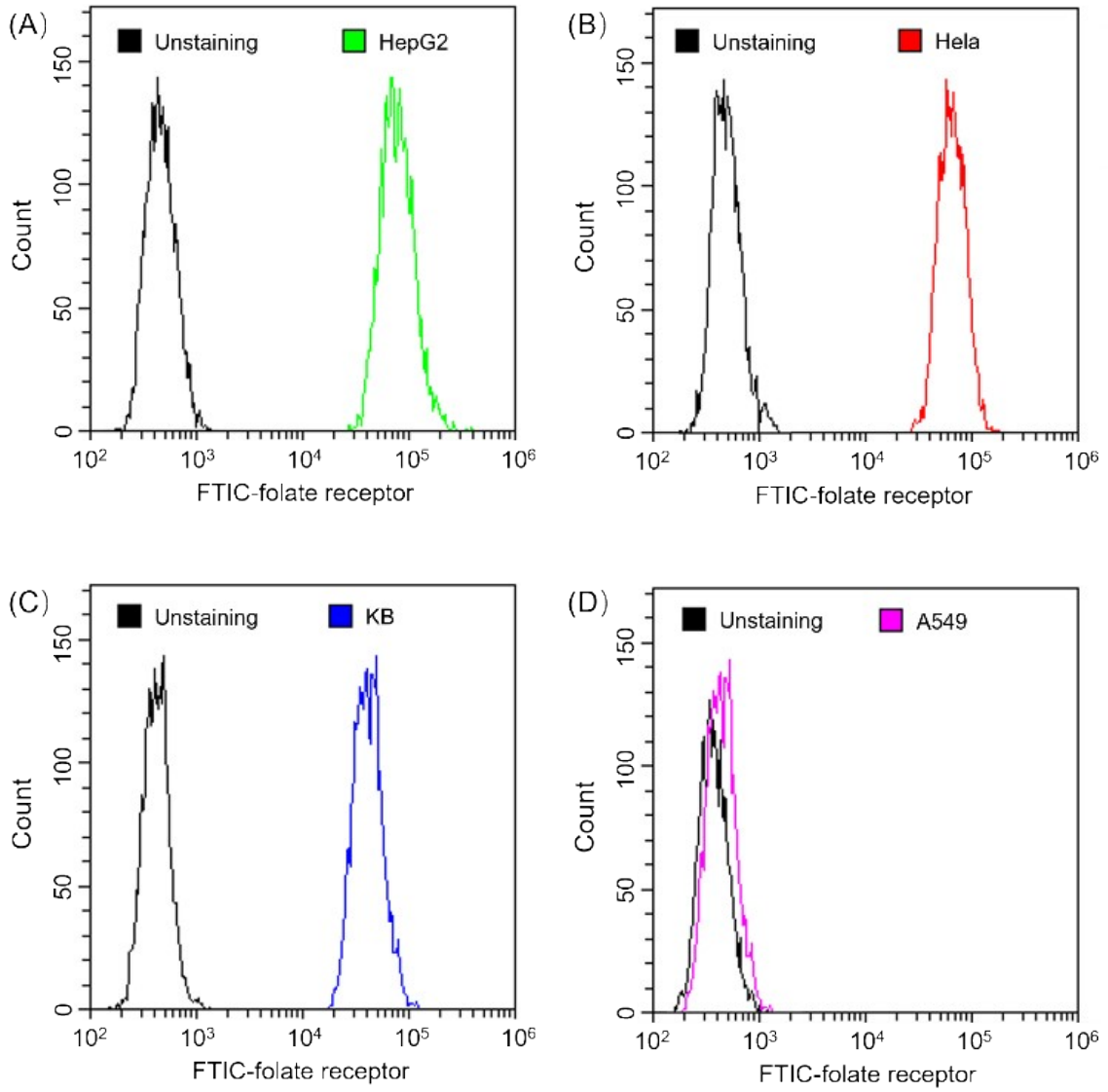

Figure S6. Folate receptor expressions of (A) HepG2, (B) HeLa, (C) KB and (D) A549 cells, confirmed by detection of immunosfluorescence of folate recetpor- alpha (FR-α), measured by flow cytometry. Flow cytometry analysis of FR-α in cells compared to correponding non-staining control. Breifly, cells were harvest and adpated to 1 x 10^7 cells/mL, followed by 4% parafomaldehyde fixation. Cells were block with 2% BSA-PBS solutions at on ice for 30 min, incubated with folate receptor alpha (ThermalFisher Scientific) with a dilution of 1:50 for 1 h on ice. Cells were then incubated for 60 min using 488-conjugated secondary antibody and resuspended in PBS for FACS analysis.

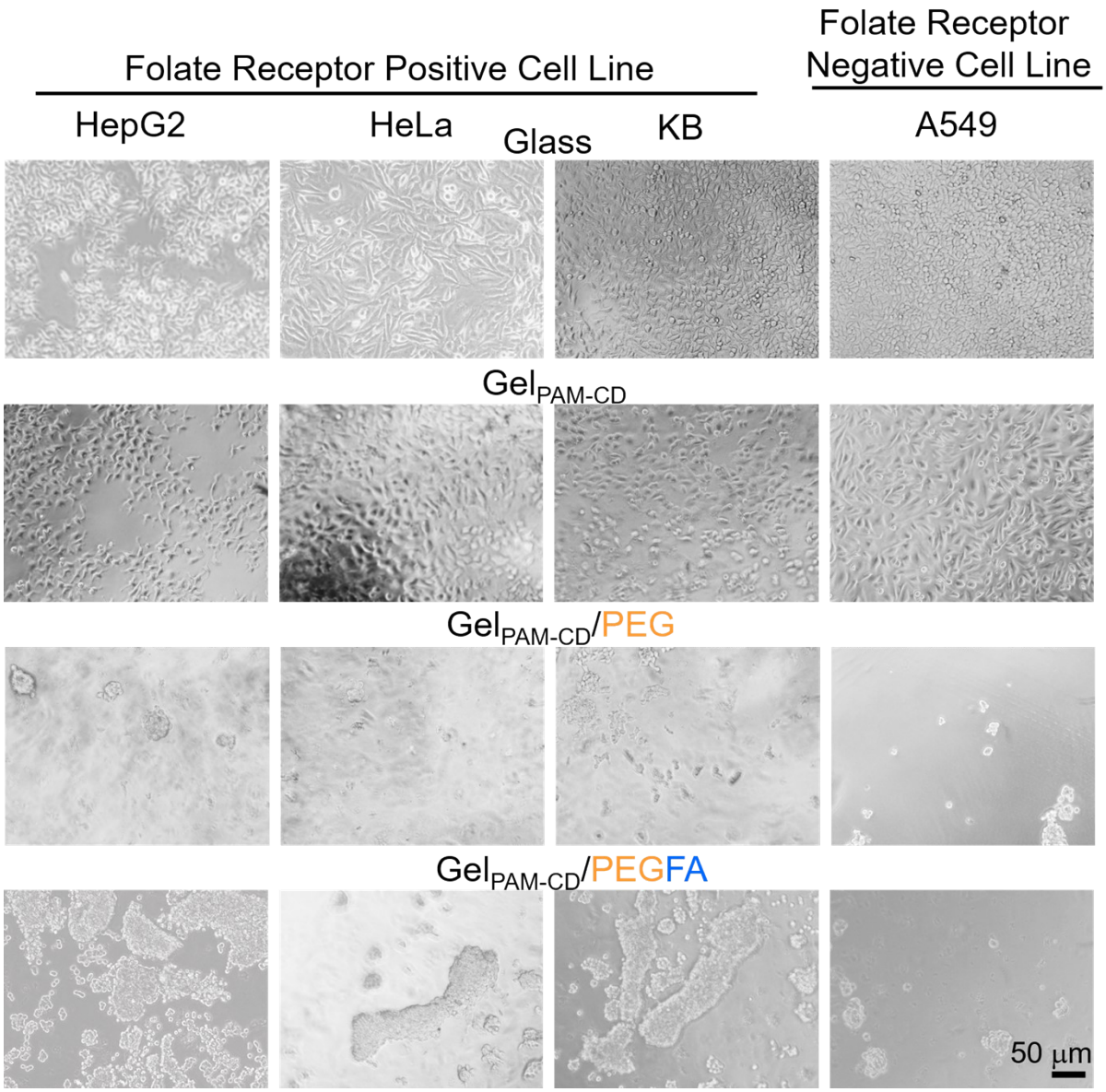

Figure S7. Cell capture of FR+ cell lines (HepG2, HeLa, and KB) and FR- cell line (A549) by different surfaces. Representative bright field microscopic images showing cell adhesions on glass, Gel_{PAM-CD}, Gel_{PAM-CD}/PEG, and Gel_{PAM-CD}/PEGFA (scale bar = 50 μm).

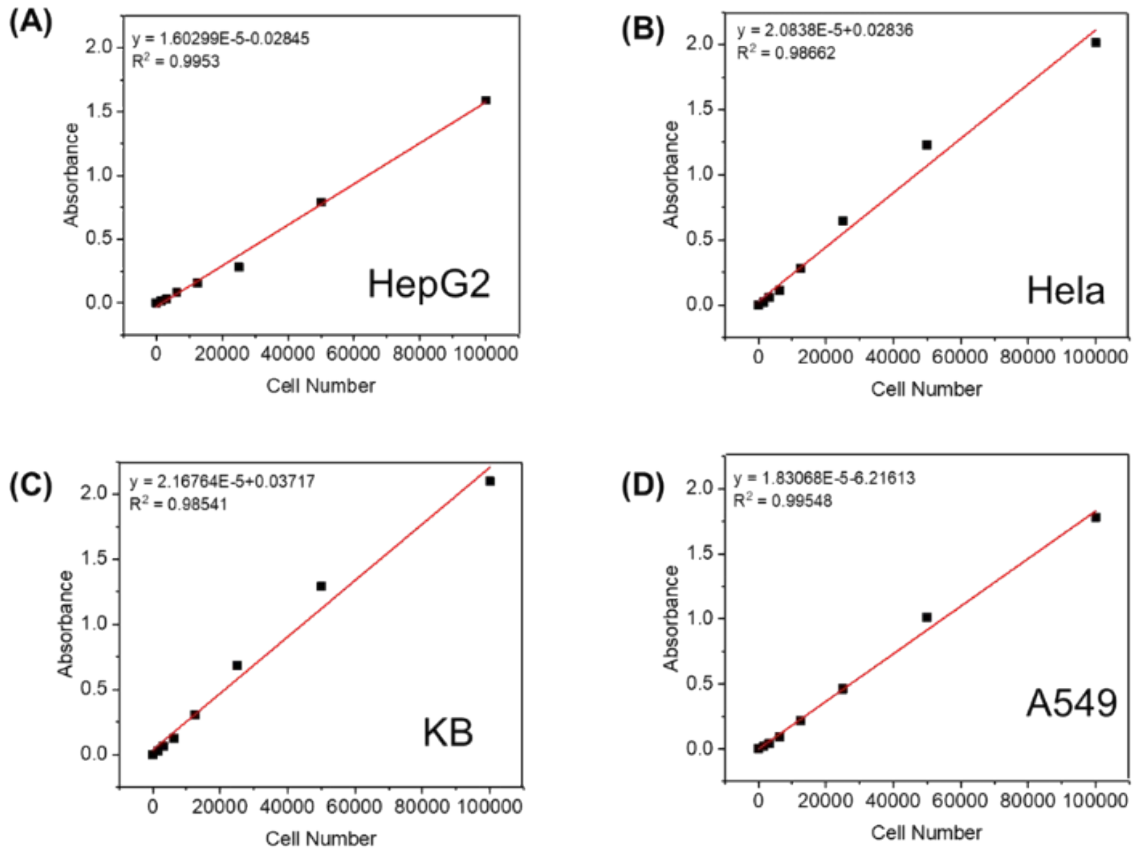

Figure S8. Cell number standard calibration curve. Standard curve of cell number of (A) Hep G2, (B) HeLa, (C) KB and (D) A549 vs absorbance at 450 nm measured by CCK-8. Absorbance means the amount of formazan dye generated by the activity of dehydrogenases in living cells.

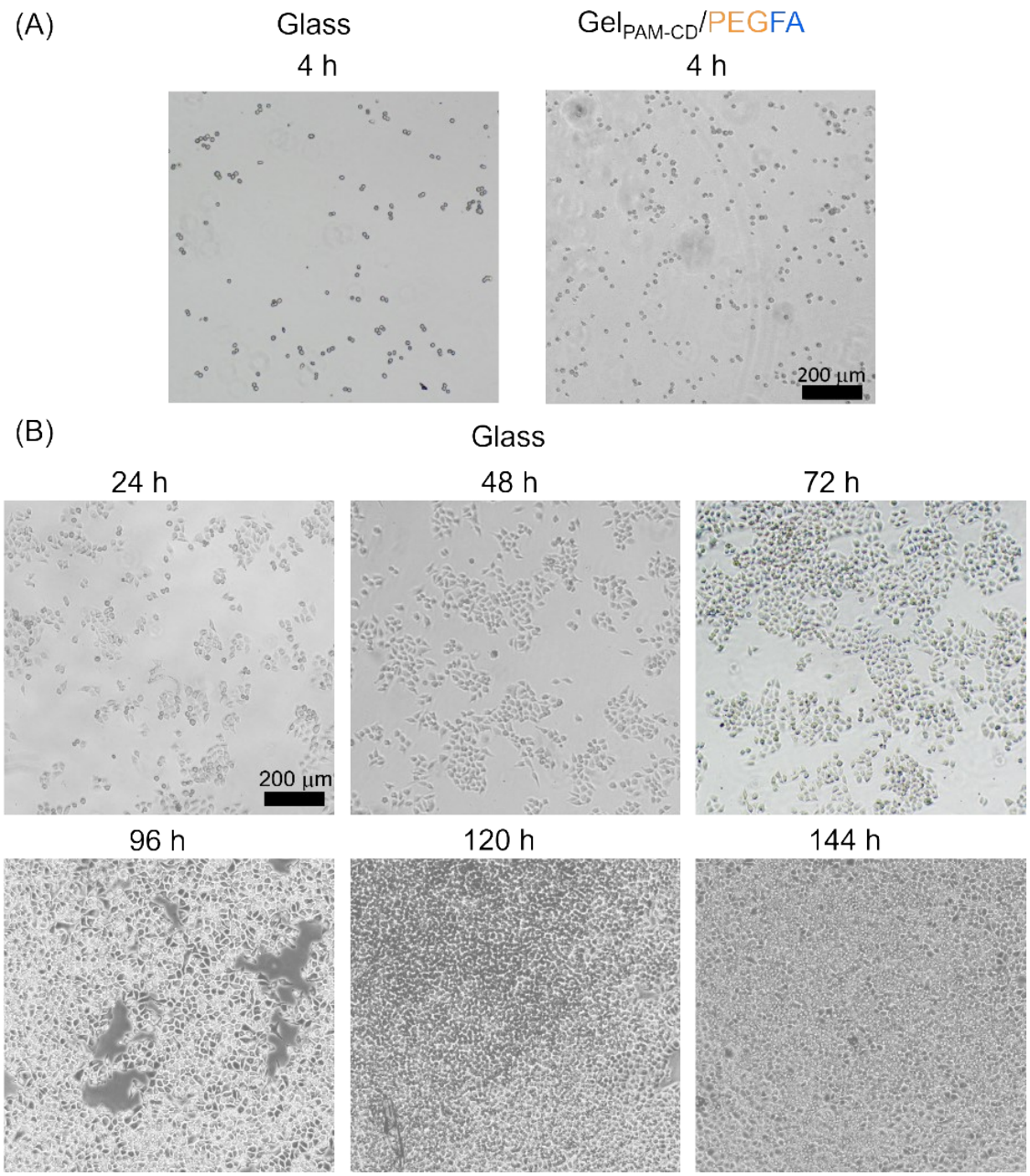

Figure S9. (A) Representative bright field microscopic images of HeLa cells on glass and Gel_{PAM-CD}/PEGFA after 4 h incubation (with starting cell concentration of 2500/well). (B) Representative bright field microscopic images of HeLa cells grown on glass at various incubation time (scale bar = 50 μm).

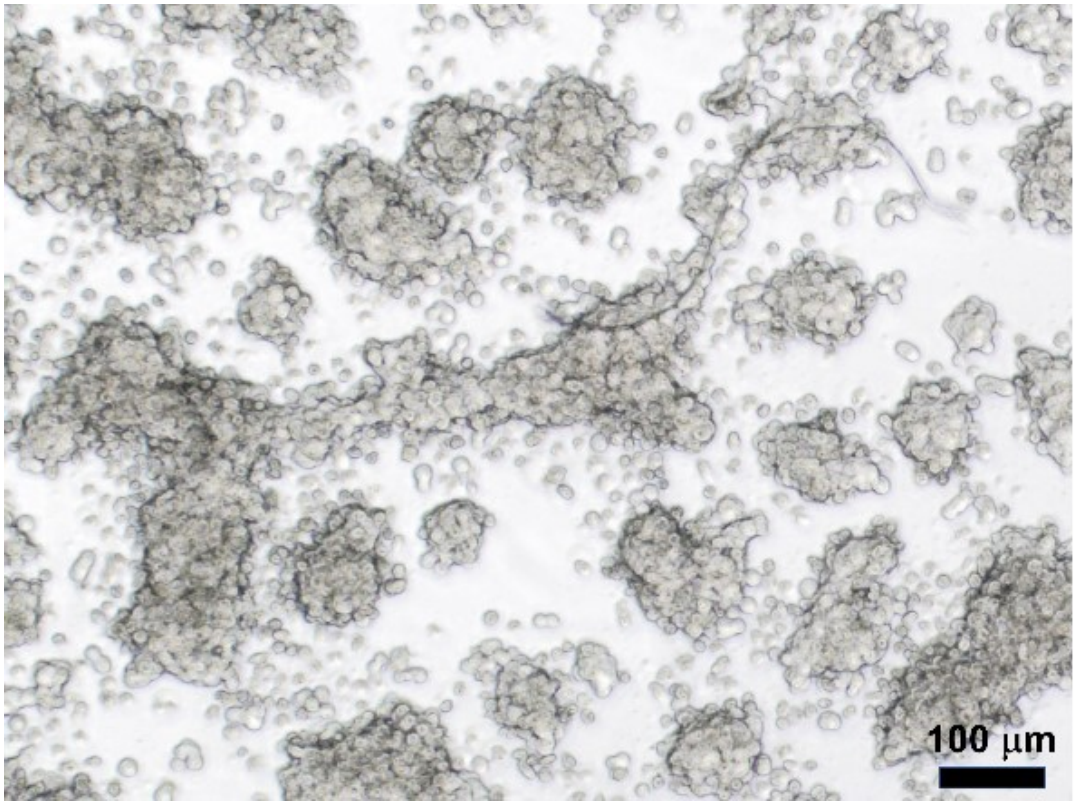

Figure S10. (A) Representative bright field microscopic image of HeLa MCSs released from $Gel_{PAM\text{-}CD}$/PEGFA with HPβCD treatment. HeLa MCSs released from $Gel_{PAM\text{-}CD}$/PEGFA was centrifuged at 200 g for 5 min, resuspended in medium and seeded onto 24 well plate. Image was taken 4 h after seeding, when all the cells settled to the bottom of plate.

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