



Mechanics and Properties of Biopolymer Networks and Cells

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Mechanics and Properties of Biopolymer Networks and Cells

Abstract

Many naturally derived biomaterials have porous structures. These porous structures are closely related to the mechanical performance of the biomaterial, such as the stability and stiffness. In the meantime, these porous structures are also of great biological importance. They serve as essential physical scaffolds to lodge the cells, and they provide various mechanical cues that can affect cell behaviors such as attachment, spreading, and differentiation. In this thesis, we explore the aforementioned two aspects of microstructure by using several porous materials as examples.

We first explore the correlation between the structure network and the mechanical property of fibrin with modulation by Zn^{2+} . We find that as the concentration of Zn^{2+} increases, the diameter of a fibrin fiber becomes thicker, whereas the mesh size of the fibrin network becomes larger. Moreover, bulk rheological measurements show that network shear modulus decreases with the increase of Zn^{2+} concentration. We then adapt the semi-flexible polymer model to bridge the network topology and mechanical property, and we further predict that each fiber is a loosely coupled bundle of protofibrils.

Next, we investigate the influence of hydrogel microstructure on the cell behavior. By culture cell on hydrogels with different microstructure and same stiffness, we investigate the cell behaviors such as attachment, spreading, and differentiation. We find that cell attachment and proliferation are not obviously affected by varying hydrogel pore size, however, cell

spreads bigger and migrates faster on hydrogel with smaller pores. We show that focal adhesion formation and nuclear translocation of Yes-associated protein (YAP) is promoted, indicating the cellular mechanotransduction is modulated by hydrogel microstructure.

Then we investigate the effect of surface topography on the cell behavior. By culturing bone marrow-derived stem cell (BMSC) on nanoporous membranes with controlled nanopore size and roughness. We find that increasing the roughness of nanoporous surface has no noticeable effect on cell attachment, and only slightly decreases cell spreading areas and inhibits osteogenic differentiation. However, BMSCs cultured on membranes with larger nanopores have significantly fewer attached cells and larger spreading areas. Moreover, these cells cultured on larger nanopores undergo enhanced osteogenic differentiation.

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Citations to Previously Published Work

Small portion of Chapter 2 have appeared in the following paper:

In Situ Measurement of Depletion Caused by SDBS Micelles on the Surface of Silica Particles Using Optical Tweezers. Langmuir, 35(42), 13536-13542.

Shuai Liu, Yue Hu, **Jing Xia**, Shenwen Fang, and Ming Duan

Small portion of Chapter 3 have appeared in the following paper:

Zinc promotes clot stability by accelerating clot formation and modifying fibrin structure. Thrombosis and haemostasis, 115(03), 533-542.

Sara Henderson, **Jing Xia**, Huayin Wu, Alan R. Stafford, Beverly A. Leslie, James C. Fredenburgh, David A. Weitz, and Jeffrey I. Weitz

Larger portion of Chapter 3 have appeared in the following paper:

Mechanics of Zn modified fibrin network. --- to be submitted

Jing Xia, Huayin Wu, Yuan Yuan, Liheng Cai, Frederick C. MacKintosh, David A. Weitz

Large portion of Chapter 4 have appeared in the following paper:

Hydrogel microstructure affects collagen assembly and regulates cell behavior. --- to be submitted

Jing Xia, Zongyuan Liu, Zhengyuan Han, Yuan Yuan, Xiqiao Feng, David A. Weitz

Small portion of Chapter 4 have appeared in the following paper:

Tissue and cellular rigidity and mechanosensitive signaling activation in Alexander disease. Nature communications, 9(1), 1-14.

Liqun Wang, **Jing Xia**, Jonathan Li, Tracy L. Hagemann, Jeffrey R. Jones, Ernest Fraenkel, David A. Weitz, Su-Chun Zhang, Albee Messing, and Mel B. Feany

Larger portion of Chapter 5 have appeared in the following paper:

Decoupling the effects of nanopore size and surface roughness on the attachment, spreading and differentiation of bone marrow-derived stem cells. Biomaterials, 120014.

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Dedication

Dedicated to my parents.

Chapter 1 Introduction

1.1 Structure of this thesis

This thesis contains three subtopics, which together are aiming to understand the mechanical aspect of the biomaterial and their effects on cell behavior.

1. A study of mechanics of Zn^{2+} modified fibrin network. By imaging the network morphology using confocal microscope, we quantified the structure change of fibrin network in the presence of Zn^{2+} . By further combining with rheological measurement of fibrin network, we quantify the mechanical property change in the small deformation and large deformation regimes, separately. In addition, by combining mechanical measurement with 3D imaging with a theoretical model, we unveil the nanostructure mechanics changes which cannot be experimentally measured.
2. Effects of hydrogel microstructure on the collagen coating and cell behavior. By adjust the thickness of the hydrogel, we develop a method to fabricate a series of hydrogels with different microstructure but the same stiffness. By characterizing the collagen coating with confocal microscopy, we compare the collagen coating difference as a result of hydrogel microstructure change. After seed hBMSCs onto the hydrogels, we compare the cell behaviors on the hydrogel, including cell attachment, proliferation, spreading, and migration. As last, we correlate collagen coating change with the relative cell behavior changes, which both results from the microstructure change of the hydrogel.
3. Decouple the effect of nanopore size and roughness on the cell behavior. By selecting a series of porous membranes with well defined pore sizes and roughness, and by changing one parameter at a time, we develop a system that enable us to decouple the effect of

nanopore size and roughness on the cell behavior, which includes cell attachment, cell proliferation, cell spreading, and cell migration.

1.2 Introduction and background

Most naturally existed biomaterials are porous in structure, such as skin tissue, intestine tissue and bone tissue. These structures are especially important and relevant in biology tissues. On one side, they are rich in biochemical cues, such as growth factors, and bioactive molecules which play significant roles in regulating cell behavior. On the other side, they provide mechanical support for the human body and scaffold to lodge the cells, misbehavior of which could lead to tissue malfunction. These cues together can stimulate cells and initiate a series of cellular response. Therefore, it is very necessary to understand the mechanical property of the porous material, and how they interact with cells and further regulate cell behavior.

In the human body, the porous biomaterial can be roughly classified into two kinds by their mechanical properties. One kind is relatively soft, such as skin tissues($\sim$ kPa), which are hydrogels constituting of protein fibers with interstitial fluid in between the fibers. The other kind is relatively stiff ($\sim$ Gpa), such as bone tissues, which are mineralized collagen fibers together with mineral crystals. In the past decades, most research has mostly focused on the effect of biochemical cues of biomaterial on the cell behavior. Until recent years, more and more attention has been drawn to how the mechanical aspect of the biomaterial can affect the cell behavior. Mechanical cues of the biomaterial have been found to regulate cell behaviors and even determine stem cell fate. Therefore, it has been regarded as potential treatment method for some disease.

Despite that numerous efforts made to understand the effect of mechanical stimulus on the cells, majority of them are focusing on the stiffness of the biomaterial, which is only one mechanical perspective of the biomaterial. There are many other fundamental mechanical properties, such as pore size, microstructure and surface roughness, remains to be explored as they may play an equal important role in cell fate determination.

In this thesis, we highlight the mechanical aspect of the porous biomaterial, and we investigate the following two perspectives: 1. mechanics of the porous material; 2. Cellular effect of porous structure. More specifically, we first investigated the correlation between the microstructure of the porous structure to its mechanical property by investigating the naturally exist fibrin hydrogel. Then we explore the effect of hydrogel microstructure to the cell behavior, including cell attachment, cell spreading, and cell differentiation. Finally, we explore the effect of topography cues of porous structure on the cell behavior, and we explore the nanopore size and surface roughness specifically.

Chapter 2 Materials and Methods

2.1 Cell culture and cell behaviors assays

2.1.1 Cell culture

Human bone marrow-derived mesenchymal stem cells (hBMSCs) (ATCC, VA, USA) are used in this study. MSC growth medium is prepared by mixing mesenchymal stem cell basal medium (ATCC, VA, USA) with mesenchymal stem cell growth kit (ATCC, VA, USA). Cells are cultured in the MSC growth medium and maintained in the 37 °C, 5% CO₂ infused incubator. All experiments are carried out with early passage hBMSCs (passage 2–passage 6).

2.1.2 Cell focal adhesion immunofluorescence assay

For observation of the cell focal adhesions, cells are seeded onto cell culture membrane assemblies at a density around 5000 cells/cm², and cultured in the 37 °C, 5% CO₂ infused incubator for 16 hours. Cells are fixed with 4% formaldehyde and 0.1% Triton X100 diluted in PBS, followed by PBS wash for 3 times to remove excessive reagents. After cell fixation, cells are triple stained for actin, vinculin, and nucleus. Fixed cells are blocked with 10% bovine serum albumin (Thermo Fisher Scientific Inc, MA, USA) in PBS for 1 hour, followed by a two-step immunostaining process for vinculin. Briefly, cells are first incubated with mouse monoclonal anti-vinculin antibody (Sigma-Aldrich, MO, USA) diluted 200X in PBS with a supplement of 10% normal goat serum for 1 hour at room temperature. Samples are then washed 5 times for 5 minutes each with PBS and incubated with goat anti-mouse alexa fluor plus 488 secondary antibodies diluted 200X in PBS with a supplement of 10% Normal goat serum for 1 hour in the dark. To stain actin and nucleus, Phalloidin-iFluor 555 (Abcam, MA, USA) and Draq 5 nucleus probe (Thermo Fisher Scientific Inc, MA, USA) are diluted at 1:1000 and 1:5000 each to incubate cells for 1 hour. Stained cells are then washed 3 times for 5 minutes each with PBS and imaged with the confocal microscope (see Optical microscopic imaging).

2.1.3 Collagen Immunofluorescence staining

PA hydrogels with rat/bovine collagen coating are blocked with 1% bovine serum albumin (Sigma, MO, USA) in PBS for 1 hour, followed by a two-step immunostaining process for collagen. Briefly, samples are first incubated with mouse monoclonal anti-collagen I antibody (ab90395, Abcam, MA, USA) diluted 200X in PBS with a supplement of 1% bovine serum albumin for 1 hour at room temperature. Samples are then washed 5 times for 5 minutes each with PBS and incubated with goat anti-mouse alexa fluor plus 488 secondary antibodies (Thermo Fisher Scientific Inc, MA, USA) diluted 200X in PBS with a supplement of 1% bovine serum albumin for 1 hour in the dark. PA hydrogels are washed 3 times for 10 minutes each with PBS before imaging. PA hydrogels without collagen coating was Immunofluorescent stained with the same procedure as stated above to be used as blank control. The stained samples are then fluorescently imaged with confocal microscope (see Optical microscopic imaging and image analysis).

2.1.4 Cell attachment and proliferation assay

Cells were seeded onto hydrogel at a density of 4000-6000 cells/cm² and cultured in MSC growth medium supplemented with 0.5uM SiR-DNA(Cytoskeleton Inc.) to stain cell nucleus. When cells are fully attached on the hydrogels (around 4 hours), the 12-well plate are then transferred to a TCS-SP5 confocal microscope (Leica Microsystems Inc., IL, USA) equipped with cell incubator box supplemented with 5% CO₂ at 37 C (OKO lab, NA, Italy). The cell nucleus is fluorescently imaged with confocal microscope (see Optical microscopic imaging and image analysis). Total of 5 random selected locations were imaged in each sample at day 0, 1, 2, 3, 4.

2.1.5 Cell morphology assay

Cells are stained with the celltracker green CMFDA dye(Thermo Fisher Scientific Inc, MA, USA) according to manufacturer's protocol. Briefly, cells are centrifuged at 400 g-force after trypsinization, and re-suspended in the staining medium (2ug/ml celltracker green CMFDA in MSC growth medium). After 40 minutes, stained cells are centrifuged again at 400

g-force to remove the staining medium and re-suspended in the MSC growth medium. Stained cells are then seeded onto the PA hydrogels on 12-wells plate at a density around 5000 cells/cm². To qta for vinculin. Briefly, cells are first incubated with mouse monoclonal anti-vinculin antibody (Sigma-Aldrich, MO, USA) diluted 200X in PBS with a supplement of 10% normal goat serum for 1 hour at room temperature. Samples are then washed 5 times for 5 minutes each with PBS and incubated with goat anti-mouse alexa fluor plus 488 secondary antibodies(Thermo Fisher Scientific Inc, MA, USA) diluted 200X in PBS with a supplement of 10% Normal goat serum for 1 hour in the dark. To stain actin and nucleus, Phalloidin-iFluor 555 (Abcam, MA, USA) and Draq 5 nucleus probe (Thermo Fisher Scientific Inc, MA, USA) are diluted at 1:1000 and 1:5000 each to incubate cells for 1 hour. Stained cells are then washed 3 times for 5 minutes each with PBS and imaged with the confocal microscope (see Optical microscopic imaging and image analysis).

2.1.6 Cell yap measurement

Cells are seeded onto the PA hydrogels on 12-wells plate at a density around 5000 cells/cm², and are subsequently cultured in the 37Co, 5% CO₂ infused incubator for 12 hours. Cells are then fixed with 4% formaldehyde and 0.1% Triton X100 diluted in PBS, followed by PBS wash for 3 times to remove excessive reagents. After cell fixation, cells are triple stained for actin, yap, and nucleus. Fixed cells are blocked with 10% normal goat serum (Thermo Fisher Scientific Inc, MA, USA) in PBS for 1 hour, followed by a two-step immunostaining process for yap. Briefly, cells are first incubated with rabbit polyclonal anti-yap antibody (Cell Signaling Technology, Inc., MA, USA) diluted 200X in PBS with a supplement of 10% normal goat serum for 1 hour at room temperature. Samples are then washed 5 times for 5 minutes each with PBS and incubated with goat anti-rabbit alexa fluor plus 594 secondary antibodies (Thermo Fisher Scientific Inc, MA, USA) diluted 200X in PBS with a supplement of 10% normal goat serum for 1 hour in the dark. To stain actin and nucleus, Phalloidin-iFluor 405 (Abcam, MA, USA) and Draq 5 nucleus probe (Thermo Fisher Scientific Inc, MA, USA) are diluted at 1:1000 and 1:5000 each to incubate cells for 1 hour. Stained cells are then

washed 3 times for 5 minutes each with PBS and imaged with the confocal microscope (see Optical microscopic imaging and image analysis).

2.1.7 Cell osteogenic differentiation assay

For cell differentiation study, cells are seeded on cell culture membrane assemblies at a density around 8000 cells/cm² and cultured in MSC growth medium overnight. Cell culture membrane assemblies are then transferred to a new petri dish to keep only the cells attached on membranes. Cells are cultured in MSC growth media for 5-7 days until reaching confluency. Then the culture medium is switched to the osteogenic differentiation medium for osteogenic differentiation. Osteogenic differentiation medium is prepared by mixing human osteogenic supplement (R&D Systems, Inc., MN, USA) with stemXVivo osteogenic/adipogenic base media (R&D Systems, Inc., MN, USA). Cells are cultured with fresh osteogenic differentiation medium changed every three days.

For staining of alkaline phosphatase protein and collagen type I, cells are fixed after 16-18 days of culture in osteogenic differentiation medium. Briefly, cells are fixed with 4% formaldehyde and 0.1% Triton X100 diluted in PBS, followed by PBS wash for 3 times to remove excessive reagents. After fixation, cells are triple stained for alkaline phosphatase protein, collagen type I, and nucleus. ImmPACT Vector Red Alkaline Phosphatase Substrate (Vector Laboratories, CA, USA) is used to stain alkaline phosphatase protein. Briefly, reagent 1 and 2 from the kit is mixed with Vector Red Diluent and incubate with fixed cells for 1 hour in the dark, followed by PBS wash for three times. To stain collagen type I, fixed cells are blocked with 10% normal goat serum (Thermo Fisher Scientific Inc, MA, USA) in PBS for 1 hour, followed by a two-step immunostaining process. Briefly, fixed cells are incubated with COL1A mouse monoclonal antibody (Santa Cruz Biotechnology, TX, USA) diluted 200 times in 10% normal goat serum for 1 hour at room temperature. Samples are then washed 5 times for 5 minutes each with PBS and incubated with goat anti mouse alexa 647 secondary antibodies (Thermo Fisher Scientific Inc, MA, USA) diluted 200X in 10% normal goat serum for 1 hour in the dark. The cell nucleus is stained with DAPI. Stained cells are then washed 3

times for 5 minutes each with PBS and imaged with Axiozoom.V16 microscope (see Optical microscopic imaging).

For immunostaining of osteocalcin and osteopontin, cells are fixed after 24-26 days of culture in osteogenic differentiation medium. Briefly, cells are fixed with 4% formaldehyde and 0.1% Triton X100 diluted in PBS, followed by PBS wash for 3 times to remove excessive reagents. After fixation, cells are stained for osteocalcin and osteopontin. Fixed cells are blocked with 10% normal goat serum (Thermo Fisher Scientific Inc, MA, USA) in PBS for 1 hour, followed by a two-step immunostaining process. Briefly, fixed cells are incubated with osteocalcin mouse monoclonal antibody (Santa Cruz Biotechnology, TX, USA) and osteopontin chicken polyclonal antibody (Thermo Fisher Scientific Inc, MA, USA) diluted 200 times in 10% normal goat serum for 1 hour at room temperature. Samples are then washed 5 times for 5 minutes each with PBS and incubated with goat anti mouse alexa 647 secondary antibodies (Thermo Fisher Scientific Inc, MA, USA) and goat anti chicken alexa 488 secondary antibodies (Thermo Fisher Scientific Inc, MA, USA) diluted 200X in 10% normal goat serum for 1 hour in the dark. Stained cells are then washed 3 times for 5 minutes each with PBS and imaged with Axiozoom.V16 microscope (see Optical microscopic imaging).

2.2 Membranes characterization and fabrication

2.2.1 Nanoporous membranes characterization

Surface topography of membranes is characterized by Cypher Atomic Force Microscope (Oxford Instruments Asylum Research, Inc., CA, USA). A cantilever with a cone tip (Innovative Solutions Bulgaria Ltd., Sofia, Bulgaria) is used to scan a 30 μm * 30 μm area in the air-tapping mode. The arithmetic average roughness of the solid regions excluding the nanopores of the membranes are measured by Igor pro software (WaveMetrics, OR, USA). To visualize the nanopore distribution on membranes, scanning electron microscopy images are taken with Ultra55 scanning electron microscope (Carl Zeiss Microscopy, LLC, NY, USA). Nanopore size and density are quantified by ImageJ (<https://imagej.nih.gov/ij/>). For characterization of the membrane surface chemistry, X-ray photoelectron spectrometer (XPS)

analysis is performed with a K-Alpha XPS System (Thermal Fisher Scientific, Waltham, MA) to scan the membranes from 0eV to 1350 eV with 1 eV step size.

2.2.2 Cell culture membranes fabrication

To fabricate the cell culture membrane assembly, hydrophilic polycarbonate membranes (Sterlitech Corporation, WA, USA) of different nanopore sizes (10 nm, 80 nm, 200 nm) are sandwiched between stainless steel metal washers (Mcmaster, NJ, USA) and laser cut ring made with 0.004'' polyester shim stock (Mcmaster, NJ, USA). This sandwich assembly is glued between each layer using epoxy NOA81 (Norland Product Inc, NJ, USA) and cured under a 365nm wavelength handheld UV lamp (AnalytikJena, Germany) for 1 hour. Each side of the cell culture membrane assembly is sterilized with a germicidal lamp in the biosafety cabinet for 40 minutes. Cell culture membrane assemblies are subsequently transferred to a 35mm glass-bottom Petri dish (Cellvis, CA, USA) and immersed in PBS for 2 hours before cell seeding.

2.3 Microscopic imaging

2.3.1 Optical microscopic imaging

For cell attachment, proliferation, morphology, migration, and focal adhesion assays, stained cells are observed using 25X/0.95-NA water immersion objective on a TCS-SP5 confocal laser scanning microscope (Leica Microsystems Inc., IL, USA). For cell attachment and proliferation studies, the cell nucleus number is counted at day 0, 1, 2, 3, 4 by using ImageJ. To calculate cell attachment percentage, the attached cell number was then divided by the total seeding number. For cell morphological studies, fluorescent images of the nucleus or cell cytoplasm in focus are taken. The images are then analyzed with Image J to quantify the projected cell or nucleus area. For cell migration studies, cell nucleus are fluorescently imaged and recorded at time series of 5 minutes interval. To prevent the drift of stage in the upright direction, Z stacks of images with 5um z-interval at each position was taken. The movement of each individual cell was analyzed by tracking the nucleus in the fluorescent channel with

particle tracker plugin of ImageJ(<https://imagej.nih.gov/ij/>). The extracted trajectories were then analyzed with customized code in MATLAB (Mathworks, MA, USA) to calculate cell motility parameters. For cell focal adhesion studies, fluorescent images of the nucleus, actin and focal adhesion in focus are taken for each cell. Focal adhesions are quantified according to the previous method¹. For yap quantification assay, fluorescent images of the nucleus, actin and yap in focus are taken for each cell using 63X/1.2-NA oil immersion objective on a LSM880 confocal laser scanning microscope (Nikon Instruments Inc. NY, USA). Nucleus and cytoplasmic regime are manually selected for each cell to quantify the fluorescent intensity of yap with ImageJ software.

For collagen cluster quantification assay, gel surface was carefully focused in the shortest time possible, no obvious photo-bleaching was observed during this process. Same imaging parameters were used to image the collagen on all the gels. The collagen fluorescent images were then quantified with ImageJ to extract the collagen cluster intensity and density.

2.3.2 Scanning electron microscopic imaging

For observation of cell morphology under the scanning electron microscope, fixed cell samples are dehydrated in ethanol graded series (50%, 60%, 70%, 80%, 90%, 100%) for 30 minutes each and eventually immersed in 100% ethanol for 2 hours. After dehydration, samples are transferred to a critical point dryer (Tousimis 931GL, MD, USA) and dried under the critical point of CO₂. Samples are then coated with 5 nm Pt/PD and observed with Ultra 55 scanning electron microscope (Carl Zeiss Microscopy, LLC, NY, USA).

For observation of polyacrylamide hydrogel structure under the scanning electron microscope, gel samples are dehydrated in ethanol graded series (50%, 60%, 70%, 80%, 90%, 100%) for 30 minutes each and eventually immersed in 100% ethanol for 5 hours. After dehydration, samples are transferred to a critical point dryer (Tousimis 931GL, MD, USA) and dried under the critical point of CO₂. Samples are then coated with 5 nm Pt/PD and observed with Ultra 55 scanning electron microscope (Carl Zeiss Microscopy, LLC, NY, USA).

2.3.3 Confocal microscopic imaging

Fibrinogen was fluorescently labeled using TAMRA-SE (c1171, Thermo Fisher Scientific Inc, MA, USA) according to previous protocol [4]. Fluorescently labeled fibrinogen was then mixed with unlabeled fibrinogen at the ratio of 1:6 and polymerized between two #1.5 22mm-diameter coverslips with a 1-mm-thick spacer. Samples were then imaged with a confocal microscope (TCS-SP5, Leica Microsystems Inc., IL, USA) equipped with a 63X/1.2NA water immersion objective. Laser power was set to minimum that no obvious photobleaching was observed during the imaging process. Optical cross-sections are recorded at 0.1 μ m z-axis interval to obtain 3D imaging stacks.

2.3.4 Image processing

To quantify the fibrin network morphology, Amira 6.0 software (Thermo Fisher Scientific Inc, MA, USA) was used to analyze the image stacks. Briefly, image stacks were first deconvolved using point spread function, and then skeletonized with auto skeleton module. The skeletonized image stacks were then used to calculate fiber radius, network connectivity and line density.

To measure the mesh size of fibrin network, each image stack was binarized using otsu's threshold method. Mesh size was measured based on the binarized image stacks as described previously[5]. Briefly, pixels with intensity above the threshold are considered to be part of a fibrin fiber, here we call it 'fiber pixel'. The distance between the 'fiber pixels' defines the fiber spacing. The distribution of fiber spacing was fitted into an exponential function $P(l)=P_0 \exp\left[-l/\xi\right]$ with two independent fitting parameters: scale parameter, P_0 , and mesh size, ξ . Due to the homogeneity in x and y direction, only the fiber spacing along x-axis was used to calculate the mesh size.

2.4 Fibrin network formation

To reconstitute fibrin network, human α -thrombin (Enzyme Research Laboratories, IN, USA) and fibrinogen (FIB 1, Enzyme Research Laboratories, IN, USA) were used in this study. Fibrinogen was subjected to precipitation with 19% ammonium sulfate (Sigma-Aldrich,

MO, USA) to ensure the highest integrity of the α -chains[1]. Polymerization was performed in a Zn^{2+} buffering system (TcBS) containing 10 mM Tricine (Sigma-Aldrich, MO, USA), 150 mM NaCl (Sigma-Aldrich, MO, USA), pH 7.4, 2mM $CaCl_2$ (Sigma-Aldrich, MO, USA), and 0.01 % Tween 20 (Sigma-Aldrich, MO, USA) [2]. Fibrinogen was incubated in TcBS buffer with different concentration of $ZnCl_2$ (Sigma-Aldrich, MO, USA) for 5 minutes, and polymerization was initiated by adding 1NIHU/ml α -thrombin.

2.5 Rheology

Rheological property of fibrin network was measured with a stress-controlled rheometer (Hybrid DHR-3, TA Instrument, DE, USA). Pre-polymer solution was prepared as mentioned previously. The pre-polymer solution was then polymerized at 25°C between a steel cone geometry (40mm diameter, 4°) and a peltier plate. Mineral oil (Sigma-Aldrich, MO, USA) was used to surround the sample to prevent drying. The storage and loss modulus were measured by applying an oscillatory strain with amplitude of 0.5% and frequency of 0.1 Hz during the gel polymerization process. Once the gel has fully polymerized, a frequency sweep (0.01-1 Hz) with strain amplitude of 0.5% was performed to probe the frequency dependence of the storage and loss modulus. To probe the non-linear response of fibrin network at high stress, we used a differential measurement[3]. Briefly, a small amplitude stress oscillation of amplitude $\delta\sigma = 0.1\sigma$ and frequency 0.1 Hz was superimposed on a steady shear stress, σ , that is gradually increased in a stepwise manner. For each applied $\delta\sigma$, the sample will yield a strain response of $\delta\gamma$. The complex differential modulus is given by $K^*(\sigma_0) = \delta\sigma/\delta\gamma$, which can be used to calculate the differential storage modulus, K' , and differential loss modulus, K'' .

2.6 Measurements

2.6.1 Turbidity measurement

To calculate number of protofibrils in each fiber, we measured the mass-length ratio of fibrin fibers in their hydrated state by turbidimetry. These measurements were performed on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, CA, United States). Pre-polymer

solution was prepared as mentioned previously. The pre-polymer solution was then quickly transferred into a disposable cuvette and sealed with cap at room temperature (25 °C). Once the sample has fully polymerized, optical density, D , was measured as a function of wavelength, λ , between 650nm and 800nm. And turbidity, τ , was calculated based on the relation [6]: $\tau = D \ln(10)$. Fibrin mass-length ratio, μ , can be further calculated by the following equation [7]:

$$\tau \lambda^5 = 2\pi^3 C n_s \mu \left(\frac{dn}{dc} \right)^2 \frac{44}{15} \left(\lambda^2 - \frac{184}{154} \pi^2 a^2 n_s^2 \right)$$

Where τ is turbidity, λ is the incident wavelength [cm], μ is the mass length ratio [Da/cm], a is the fiber radius [cm], C is the fibrinogen mass concentration [g/ml], and $n_s = 1.33$, $\frac{dn}{dc} = 0.17594 \text{ cm}^3 \text{ g}^{-1}$. Given that individual protofibril has a mass-length ratio $\mu_0 = 1.44 \times 10^{11} \text{ Da/cm}$ [9], the number of protofibrils in a fiber, N , is calculated as $N = \mu / \mu_0$.

2.6.2 Contact angle measurements

The static water contact angle of the porous membrane is measured by the sessile drop method. Briefly, a 5 μL DI water drop is gently deposited onto the membranes. Images at the interface are captured with a web camera and further analyzed with Image J to calculate the contact angle.

2.6.3 Measurement of ALP activity in cell culture medium

Cells are cultured on cell culture membrane assemblies in MSC growth media for 5-7 days until reaching confluency. The cells are then cultured in osteogenic differentiation medium with medium changing every three days. After 16-18 days, the ALP activity of the cell culture medium is measured using Alkaline Phosphatase Assay Kit (Colorimetric) (Abcam, MA, USA). Briefly, the cell culture medium is mixed with pNPP solution in room temperature, and stop solution is added after 60 minutes. The absorbance of the mixture at 405 nm is subsequently measured with Synergy H1m plate reader (BioTek Instruments, Inc, VT, USA). For quantification of the ALP enzyme activity in the sample, the ALP enzyme standard

is prepared and measured according to assay instructions and further used to calculate the ALP activity.

2.6.4 Membrane hydrogel composite assay

For fabrication of the membrane hydrogel composite, the hydrogel is cast onto the aforementioned cell culture membrane assembly. Acrylamide monomers (Sigma-Aldrich, MO, USA) and N,N'-Methylenebisacrylamide (Sigma-Aldrich, MO, USA) are mixed in deionized water to reach final concentration of 6% / 0.35%, 9% / 0.126%, 12% / 0.065% in a total volume of 1124 μ L. After degassing for 5 minutes, polymer solutions are mixed with 25 μ L 1M Hepes solution (Sigma-Aldrich, MO, USA) and 15 μ L of 10% (wt/vol) ammonium persulfate solution (APS) (Sigma-Aldrich, MO, USA). Then 0.5 μ L N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) (Sigma-Aldrich, MO, USA) is added and mixed for 1 s. To cast the hydrogel onto cell culture membrane assemblies, 500 μ L of the mixed polymer solution is then quickly transfer to the bottom well of cell culture membrane assembly and covered with 17 mm round coverslip (Thomas Scientific, NJ, USA). After polymerization, the round coverslip is removed, and the membrane hydrogel composite is rinsed with PBS. Cell seeding and morphology quantification is then performed the same way as before (see Cell attachment and morphology assay).

2.6.5 Rheometer measurement of bulk gel stiffness

Pre-polymer of polyacrylamide gel was quickly transported to the 1000 μ m gap between 20mm plate geometry and peltier plate of Hybrid DHR-3 rheometer (TA instruments, DE, USA), the rheometer was kept at constant temperature of 25°C. A time sweep of 0.5% shear strain oscillation with 0.1Hz frequency was performed to probe the polymerization process of PA gel and measured the linear rheology property of PA hydrogel, the equilibrium storage modulus was taken as the linear shear modulus of the gel.

2.6.6 Nanoindenter measurement of bulk gel stiffness

All nanoindentation (Agilent G200, Agilent Technologies, Santa Clara, USA) tests were conducted with a cylindrical diamond probe 100 μ m in diameter. PA hydrogels were

fabricate into disk shape of 30mm diameter, 1.2mm using 55mm bottom glass petri dish as the mold.

Two separate hydrogels for each acrylamide concentration were measured, and 6 indents were performed in a location array of 2x3, at least 200 μm spacing between neighboring location. The tests were performed at the frequency of 10 Hz. Samples were immersed in DI water and measured at samples swell for different time period of 0, 1, 2, 5, 12 hours.

Moduli were obtained from continuous stiffness measurement (CSM) indentation at an amplitude of 500 nm, with a preload of 3 μm , and an assumed Poisson ratio of 0.5.

2.7 PA hydrogel

2.7.1 PA hydrogel fabrication

Bottom glass petridish coating:

12-well bottom glass cell culture plates(Cellvis, CA, USA) were cleaned for 60 seconds using plasma cleaner at 100mW (Diener electronic GmbH + Co. KG, Germany). Then the petri dishes were covered with 1% APTES (Sigma-Aldrich, MO, USA), 1% 1N NaOH(Sigma-Aldrich, MO, USA) in pure ethanol(Sigma-Aldrich, MO, USA) for 10 minutes. The petri dishes were then washed with ethanol for 5mins each, and rinsed with DI water for 5mins each. The petri dishes were left to dry in air for 2 hours.

To fabricate PA hydrogel of different thickness on bottom glass plates, polymer solutions containing acrylamide monomers (Sigma-Aldrich, MO, USA) and N,N'-Methylenebisacrylamide (Sigma-Aldrich, MO, USA) are mixed in deionized water to reach final concentration of 6%/0.35%, 9%/0.126%, 12%/0.065% in total volume of 1124 μL . Monodispersed microsphere of diameter 2.5 μm (No.PS4001B-0519, Magesphere), 15 μm (FSDG009, Bangslab, Indiana, USA), 30 μm (84135-5ML-F, Sigma-Aldrich, MO, USA), 200 μm (16435-5ML, Sigma-Aldrich, MO, USA) were added at volume percent from 1/140 to 1/80. After degassing for 5min, polymer solutions are mixed with 25 μL 1M Hepes solution(Sigma-Aldrich, MO, USA). 15 μL of 10% (wt/vol) ammonium persulfate (APS)

(Sigma-Aldrich, MO, USA) is added and mixed for 1 s. Then 0.5 μL N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich, MO, USA) is added quickly and mixed for 1 s. The prepolymer solution was then transferred to the bottom glass petri dishes and then covered with 18mm coverslips. Three magnets(R821, K&J magnets, PA, ISA), two on the top of the coverslips, one beneath the bottom of the petridish, were used to squeeze the coverslip to make sure the coverslip touch the 2.5 μm microsphere spacers. To fabricate other thickness(15 μm , 30 μm , 200 μm) PA hydrogels, a balance weight of 30g was added onto the coverslip.

For all AFM indentation experiments, we fabricate PA hydrogels with different thickness on the APTES coated 55mm bottom glass petridish (Cellvis, CA, USA) and measured the stiffness using Nanowizard atomic force microscope (JPK, Berlin, Germany). 3.5- μm spherical tips, 11-kHz resonant frequencies, and $\sim 6\text{--}15\text{-pN/nm}$ nominal spring constants were used (sQubeCP-qp-CONT-SiO-B-5, NanoAndMore USA Corporation, CA, USA). PA hydrogel samples were indented at an array of 3*3, 17 μm apart at an approach velocity of 5 $\mu\text{m/s}$ until a 2-nN trigger force was registered, and then retracted at 5 $\mu\text{m/s}$. The linear portion of the indentation force curves were analyzed with JPK data processing software to determine Young's modulus using Hertz model.

2.7.2 PA Hydrogel Functionalization with collagen

PA hydrogels for cell culture were further coated with collagen as follows: PA hydrogels were immersed in Hepes (pH 8.5) buffer (Sigma-Aldrich, MO, USA) and then sterilized under germicidal light in cell culture hood for 20 mins. Hepes buffer was then removed, and PA hydrogel was covered with 0.5ml of 0.125 mg/mL N-sulphosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulpho-SANPAH; Thermo Scientific, MA, USA) in pH 8.5 Hepes and were incubated for 30min in dark. The PA hydrogels are then activated under 365-nm UV light (Analytik Jena, Germany) at a distance of 7cm for 30mins. Sulpho-SANPAH was then removed, and PA hydrogels were washed three times with PBS for 5mins each, covered with 1 mL of 200 $\mu\text{g/mL}$ rat collage type I (C3867-1VL, Sigma-Aldrich, MO, USA) or bovine collagen type I (5005, Advanced

BioMatrix, CA, USA) and incubated overnight at 37 °C. This protocol was used for both cell spreading and migration assays.

2.7.3 AFM measurement of PA gel stiffness

To prepare bare hydrogel samples for SEM, PA gels were polymerized in 1.5ml Eppendorf tube. The gels were then flash frozen by immediately immersing them into the liquid nitrogen (-196°C). The frozen gels were then transferred to a freezer dryer (VirTis, SP SCIENTIFIC, PA, USA) to dry in vacuum for 1 day until fully dried. The dried gels were then coated with 5nm gold and imaged under a scanning electron microscope (Ultra 55, Carl Zeiss Microscopy, LLC, NY, USA).

To prepare hydrogel samples with collagen coating for SEM imaging, samples were first washed with DI water for 5 times with 10 mins interval to remove the unbounded collagen. Samples were then fixed using 2.5% glutaraldehyde (Sigma, MO, USA) diluted in DI water to stabilize the adhesion between collagen and PA gels surface. The samples were then washed with DI water for 4 times with 10 mins interval and gradually dehydrate using a series of ethanol concentrations (10% 30% 50% 70% 90% 100%) for 1 hour each. The samples were then immersed in 100% ethanol and incubated in room temperature for 6 hours. Dehydrated PA gel samples were then dried under critical point of CO₂ in a critical point dryer (931GL, Tousimis, Maryland, USA). Samples were then coated with 5nm gold and imaged under a scanning electron microscope (Ultra 55, Carl Zeiss Microscopy, LLC, NY, USA).

For all AFM indentation experiments, we fabricate PA hydrogels with different thickness on the APTES coated 55mm bottom glass petridish (Cellvis, CA, USA) and measured the stiffness using Nanowizard atomic force microscope (JPK, Berlin, Germany). 3.5- μ m spherical tips, 11-kHz resonant frequencies, and ~6–15-pN/nm nominal spring constants were used (sQubeCP-qp-CONT-SiO-B-5, NanoAndMore USA Corporation, CA, USA). PA hydrogel samples were indented at an array of 3*3, 17 μ m apart at an approach velocity of 5 μ m/s until a 2-nN trigger force was registered, and then retracted at 5 μ m/s. The linear portion of the indentation force curves were analyzed with JPK data processing software to determine Young's modulus using Hertz model.

2.8 Statistical analysis

Statistical analysis is performed on Origin software. Statistical tests used one-way ANOVA for multiple comparisons, or Student's t-tests for comparison between two groups. P-values larger than 0.05 are assumed to be non-significant in all analyses; P-values smaller than 0.05 are marked with *; P-values lower than 0.01 are marked with **; P-values smaller than 0.001 are marked with ***.

Chapter 3 **Mechanics of Zn^{2+} Modified Fibrin Network**

3.1 Abstract

Fibrin are the main constituent during the hemostasis due to its unique mechanical properties resulting from its hierarchy structure, malfunction of fibrin can lead to diseases such as thrombosis. Here, by combining mechanical measurement with 3D imaging, we show Zn^{2+} can tune the fibrin network structure and its mechanical properties. In the presence of Zn^{2+} , fibrin protofibrils form huge fiber bundles which are intrinsic loosely coupled. By further comparing with theoretical model, we show that under low stress, the mechanical properties of Zn modified fibrin network are contributed by the thermal fluctuation of the network; at high stress, the mechanical properties are contributed by single protofibril mechanics.

3.2 Introduction

Mechanical property of fibrin gel is essential for the stability of blood clot during hemostasis, it must be mechanically strong enough to withstand pressure of the arterial blood; otherwise it may mechanically break and cause a stroke or heart attack¹. This pressure, however, is not a constant; rather it varies in a wide range depending on locations in the body². Thus, the mechanical response of the fibrin gel to the extent of deformation, or strain, is critical to successful hemostasis.

Similar to other biopolymers such as vimentin, neurofilaments and collagen, the fibrin gel exhibits a strain dependent mechanical response³. Under small strains, the fibrin gel shows linear elasticity and the applied stress is linearly proportional to the strain. By contrast, under large strains, the fibrin gel exhibits strain stiffening and the applied stress increases non-linearly with the strain. Moreover, the stiffness of the fibrin gel increases with the concentration of divalent ions such as Ca^{2+} , which effectively act as a crosslinker to make neighboring filaments form a network⁴. Remarkably, in the presence of Zn^{2+} , a kind of

di-valent ions released by platelet during homeostasis, the stiffness of fibrin gel decreases. Such a decrease is often associated with sparser network mesh and large diameter of fibrin fibers⁵. Despite its physiological relevance, it remains to be understood why Zn^{2+} affects the stiffness of fibrin gel in a way opposite to other divalent ions.

Here, we explore the origin of fibrin gel mechanics in the presence of Zn^{2+} . Using 3D fluorescent confocal microscopy, we find that as the concentration of Zn^{2+} increases, the diameter of a fibrin fiber becomes thicker, whereas the mesh size of the fibrin network becomes larger. Moreover, bulk rheological measurements show that network shear modulus decreases with the increase of Zn^{2+} concentration. Surprisingly, in the presence of Zn^{2+} , both the linear and non-linear mechanics of fibrin gels change in a non-trivial way, but can be captured by a scaling theory of semiflexible polymers accounting for microscopic structural changes. Our results suggest that the mechanics of fibrin gel is strongly correlated with its microscopic structure.

3.3 Results

3.3.1 fibrin network morphology quantification

Fibrin gels are networks made up of bundled fibrin protofibrils that are crosslinked by Factor XIII (FXIII). Protofibrils are formed when thrombin, an enzyme that exists in blood, cleaves fibrinogen, the fibrin monomer, to allow them to assemble into 2-stranded protofibrils⁶. We polymerize fibrin gels in the presence of different Zn^{2+} concentrations while keeping the concentration of fibrinogen the same. We also vary the fibrinogen concentration, while keeping the Zn^{2+} concentration the same. The morphology of fibrin gels has previously been studied using 2D scanning electron microscopy (SEM)⁷. The observed network morphology, however, may be altered because of sample drying; moreover, such 2D measurements cannot provide the 3D structure of the network. To explore the 3D structure of the network in an unperturbed way, we fluorescently label fibrinogen monomer with TRITC, and form fibrin gels using a mixture of labeled and unlabeled fibrinogen in a 1:5 ratio. We then visualize the formed fibrin gels using fluorescent confocal microscopy (see materials and methods). The 3D confocal images are further analyzed to extract the fiber radii and network

topology using a commercial image processing software (Amira). The extracted network structure is used to calculate the fiber line density, ρ , which is defined as the sum of the contour lengths of all fibers per volume, and the mesh size, ξ , which is defined as the average distance between neighboring fibers, a simple illustration of mesh size is shown in FIGURE 3.1D.

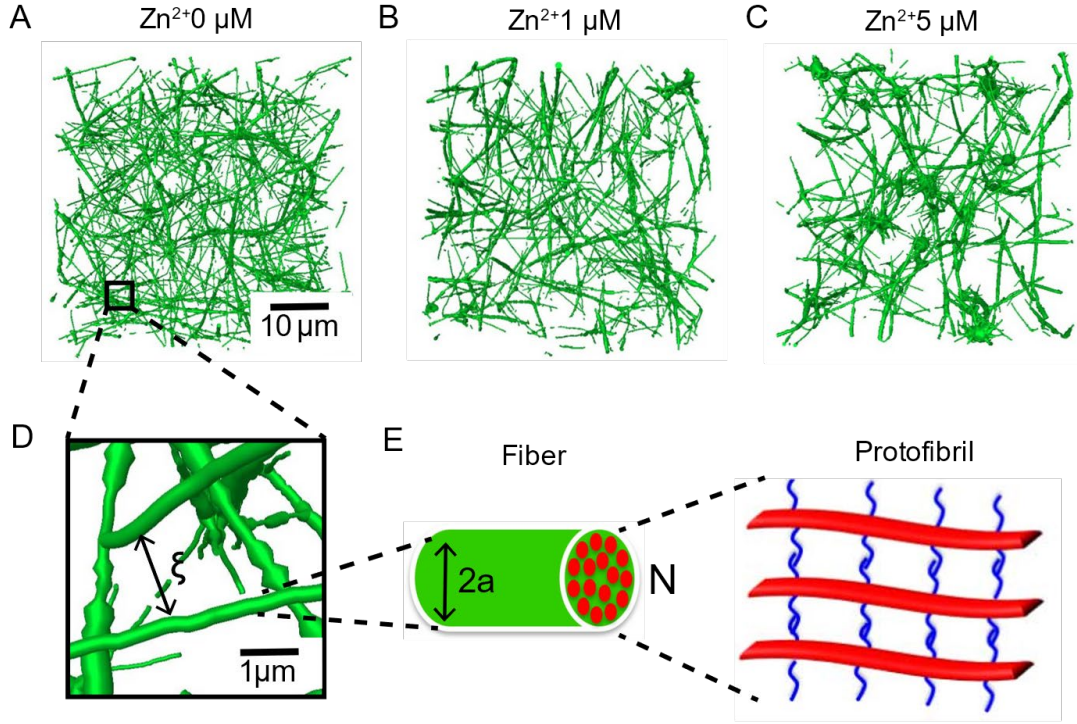


FIGURE 3.1 Morphology of fibrin network.

(A-C) Representative 3D confocal images of 3 μM fibrin network polymerized under different Zn^{2+} concentrations. The Zn^{2+} concentrations is indicated above each image. (D) Local structure of fibrin network from 3D confocal image. Mesh size, ξ , is defined as the average distance between fibers. (E) Each fibrin fiber, with radius of a , consists of N protofibrils inside. (F) Inside each fibrin fiber, protofibrils are aligned with the main axis of fiber.

As a simple consistency check, we verify that the mesh size, ξ , divided by fiber radius, a , decreases with fibrinogen concentration according to a $-1/2$ power law, as shown in FIGURE 3.2C. The rationale is as follows: the fibrinogen concentration, c_{Fg} , is proportional to fiber volume fraction, $\Phi \sim a^2 / \xi^2$, assuming that fiber length is of the same order as mesh size. As a result, the fibrinogen concentration scales with the ratio of mesh size to fiber radius as a $-1/2$ power law, $c_{Fg}^{-1/2} \sim \xi/a$.

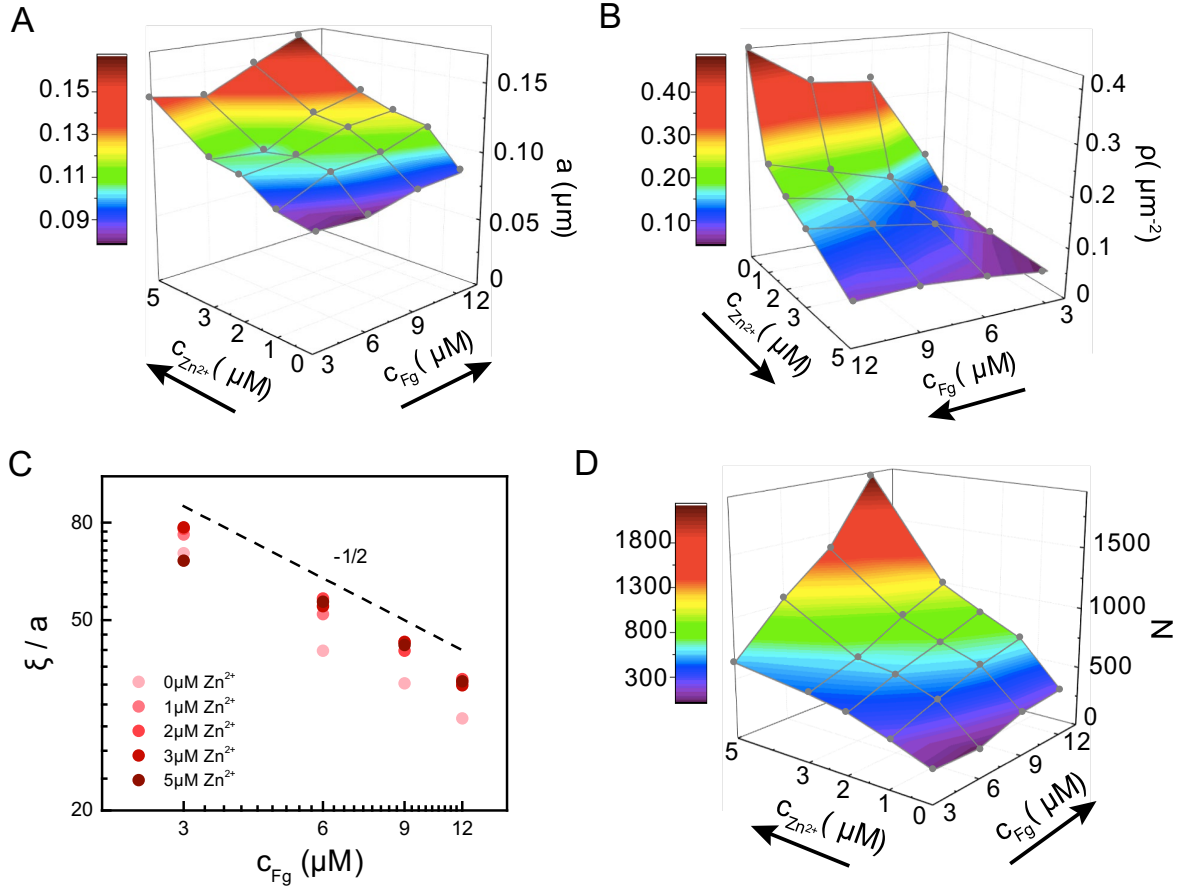


FIGURE 3.2 Quantification of fibrin network structure.

Reconstructed 3D confocal images of fibrin network are quantified as follows: (A) Radius of fibrin fiber, a , is plotted as a function of fibrin concentration, c_{Fg} , and Zn^{2+} concentration, $c_{\text{Zn}^{2+}}$. (B) Fiber line density, ρ , is plotted as a function of fibrin concentration, c_{Fg} , and Zn^{2+} concentration, $c_{\text{Zn}^{2+}}$. (C) Consistency check of fibrin network. Mesh size, ξ , divided by fiber radius, a , decreases with fibrinogen concentration according to a $-1/2$ power law, $\xi/a \sim c_{\text{Fg}}^{-1/2}$. (D) Average number of protofibrils in each fiber, N , is plotted as a function of fibrin concentration, c_{Fg} , and Zn^{2+} concentration, $c_{\text{Zn}^{2+}}$.

To examine the effect of Zn^{2+} on fibrin gel network topology, we keep the fibrinogen concentration the same while varying the Zn^{2+} concentration. As the Zn^{2+} concentration increases, fibrinogen forms thicker fibers while the network becomes more heterogeneous, as shown by the reconstructed 3D images of fibrin gels in FIGURE 3.1A-C. Meanwhile, the fiber radius, a , increases almost 2-fold when Zn^{2+} concentration changes from 0 μM to 5 μM , as shown in FIGURE 3.2A. As the fiber radius becomes larger, the fiber length must become shorter to conserve the volume of the fibrinogen; this is confirmed by 3-4-fold decreases in fiber line density, ρ , shown in FIGURE 3.2B. As a result of volume conservation, the mesh

size must become larger as the line density decreases, as shown in FIGURE 3.3. To better compare fibrin gels under different polymerization conditions, we also vary the fibrinogen concentration while keeping the Zn^{2+} concentration constant. At a fixed Zn^{2+} concentration, increasing the fibrinogen concentration 4-fold only leads to a slight increase in fiber radius of ~ 1.2 fold, as shown in FIGURE 3.2A, but greatly increases line density, ~ 2 -3 fold, as shown in FIGURE 3.2B. These results suggest that Zn^{2+} can promote bundling and increase the fiber diameter, while amount of fibrinogen only affects the fiber line density.

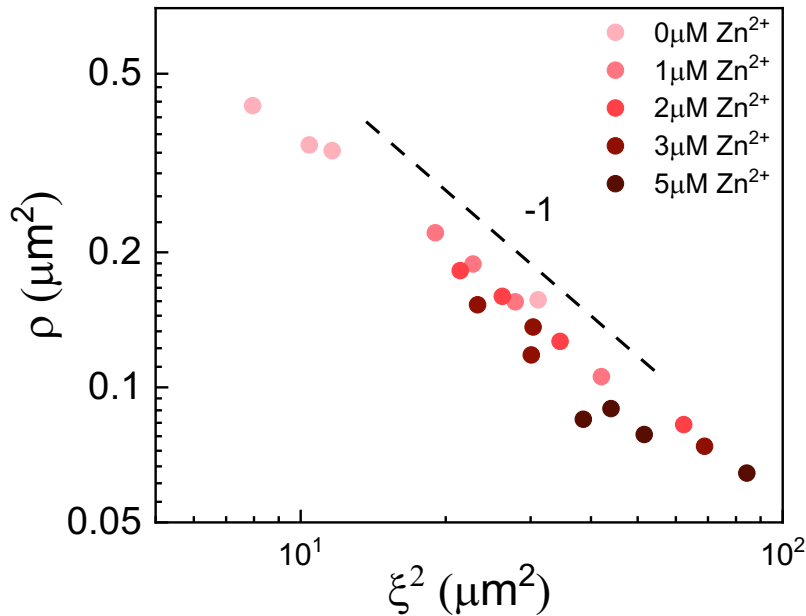


FIGURE 3. 3 Consistency check of the fibrin network. In consequence of fiber volume conservation, fiber line density, ρ , scales with mesh size, ξ , as $\rho \sim \xi^{-2}$.

To further understand the network structure under different polymerization conditions, we analyze the 3D confocal images to calculate network connectivity, Z , which is a measure of network constraint defined as the average number of fibers connected at any branch point. Assuming that we have a fully crosslinked network, higher connectivity indicates a more constrained network, which will deform in an affine fashion, while a less constrained network with lower connectivity will deform non-affinely and exhibit local structural reorganization^{8,9}. We calculate the network connectivity by averaging over all branch points. Surprisingly, the network connectivity of fibrin gels remains the same regardless of changing Zn^{2+} or fibrinogen

concentrations, as shown in FIGURE 3.4. This result indicates that network topology is similar in all fibrin gels independent of polymerization condition, suggesting that the topological structure of the network forms in a robust way.

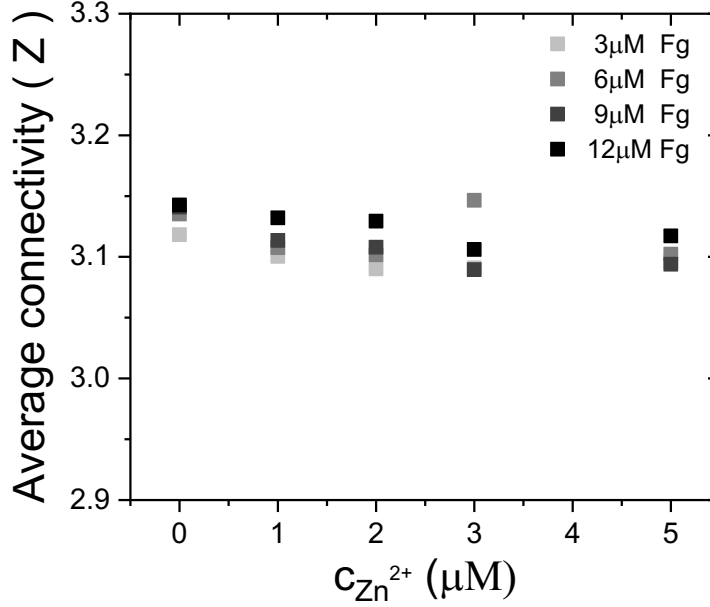


FIGURE 3. 4 Average connectivity of fibrin network, Z , is slightly larger than 3 for all polymerization conditions.

Fibrin fibers are not randomly packed, instead, they consist of protofibrils that are bundled in a parallel manner, as shown in FIGURE 3.1E. To further investigate this bundling inside the fibers, we calculate the number of protofibrils in each fibrin fiber, N , using line density, ρ , protofibril mass length ratio, μ , fibrinogen concentration, c_{Fg} , with the relation $N = \frac{c_{Fg}}{\mu\rho}$. We find that increasing either fibrinogen or Zn^{2+} concentration will lead to an increased number of protofibrils packed (as much as 2000) in each fiber, as shown in FIGURE 3.2D. This trend is consistent with values of N derived empirically from measurements of the network turbidity¹⁰, with magnitudes within a factor of 1.5. A comparison of the results from both methods is shown in FIGURE 3.5.

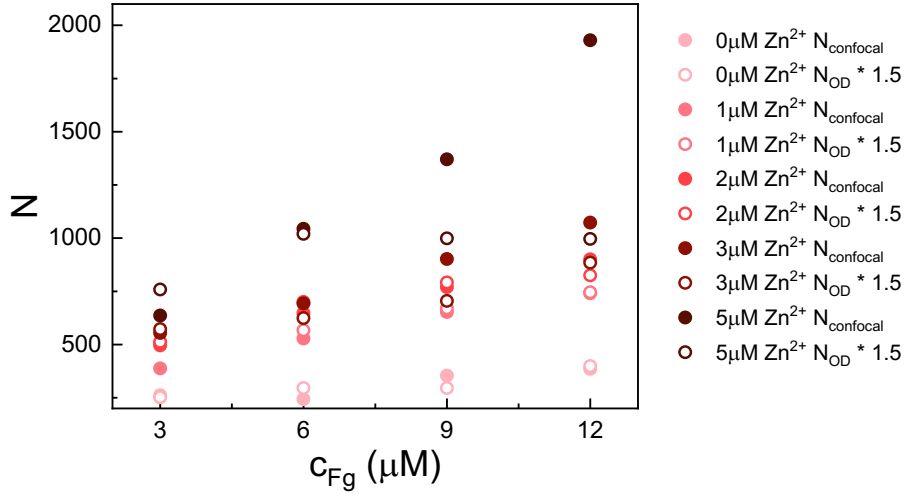


FIGURE 3. 5 Number of protofibrils, $N_{confocal}$, extracted from fibrin confocal images, are similar to number of protofibrils, N_{OD} , derived empirically from the network turbidity, with magnitudes within a factor of 1.5.

3.3.2 Linear rheology of fibrin clots

Based on the dramatically changed network morphology, and especially the large bundles observed in the network, we expect a corresponding change in network mechanics. In our studies, we show that thousands of protofibrils can bundle into one single fiber, yet it remains unclear how such a large degree of bundling will change the mechanical properties of the fibrin networks. To quantify how Zn^{2+} changes the elasticity of fibrin clots and explore the elastic origin of such networks, we measure the rheological properties of the fibrin clots. We probe the rheological properties during the clot formation process by initiating polymerization of a fibrinogen solution, immediately loading it into a cone-and-plate geometry and applying an oscillation of 0.5% shear strain at 0.1 Hz. As the fibrinogen polymerizes and forms a crosslinked network, both the storage modulus, G' , and the loss modulus, G'' , increase with time, and eventually reach a plateau once the network has fully polymerized, as shown in FIGURE 3.6A. As the oscillation amplitude (0.5% strain) and frequency (0.1 Hz) are small, the storage modulus measured at the plateau is also a measure of the linear shear modulus, G_0 . To further investigate the frequency dependence of the storage and loss moduli, a frequency sweep from 0.1 Hz to 10 Hz is performed using the same strain of 0.5%. We find that the

storage and loss moduli are essentially frequency-independent below 1Hz, as shown in FIGURE 3.7. These results suggest that the Zn^{2+} modified fibrin networks are fully crosslinked networks.

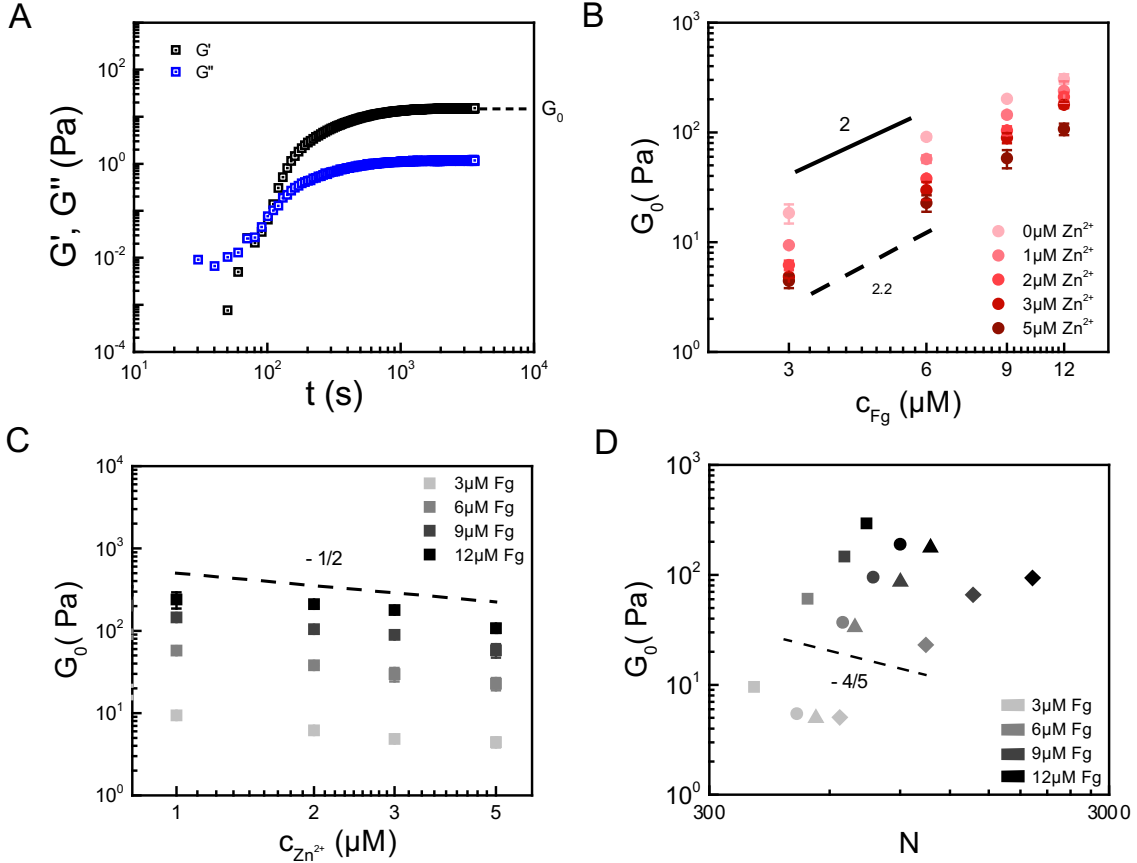


FIGURE 3. 6 Rheological property of fibrin network under low stress.

(A) Storage modulus, G' , and loss modulus, G'' , of fibrin clot during polymerization process are plotted as a function of time. Fibrinogen at $3\mu\text{M}$ with no Zn^{2+} is shown here as an example. (B) For each given Zn^{2+} concentration, equilibrium shear modulus of fibrin network, G_0 , scales with fibrin concentration slightly larger than quadratic. (C) For each given fibrin concentration, equilibrium shear modulus of fibrin network scales with Zn^{2+} concentration as $G_0 \sim c_{\text{Zn}^{2+}}^{-1/2}$. (D) For each given fibrin concentration, equilibrium shear modulus scales with number of protofibrils as $G_0 \sim N^{-4/5}$ when Zn^{2+} concentration varies from 1 μM to 5 μM (Zn^{2+} 1 μM , square; Zn^{2+} 2 μM , circle; Zn^{2+} 3 μM , triangle; Zn^{2+} 5 μM , diamond;).

To explore the elastic origin of Zn^{2+} modified fibrin networks, we next investigate the scaling dependence of the linear shear modulus on fibrinogen and Zn^{2+} concentrations. The linear shear modulus increases approximately quadratically with fibrinogen concentration

from 3 μM to 12 μM for all the Zn^{2+} concentrations that we used. More specifically, the linear shear modulus, G_0 , increases with the fibrinogen concentration, c_{Fg} , as $G_0 \sim c_{Fg}^z$ with an exponent $z \cong 2.2$, as seen in FIGURE 3.6B. This behavior is consistent with the scaling generally observed for semiflexible polymer networks originating from thermal affine deformation, as predicted by the analytical semiflexible polymer model¹¹⁻¹³. This scaling is also consistent with recent network rheology reported for collagen networks, which are generally believed to be athermal, mechanical networks¹⁴⁻¹⁶. Thus, from this behavior alone it is not possible to distinguish between thermal and athermal network elasticity. However, increasing the Zn^{2+} concentration leads to a clear decrease in the linear shear modulus, $G_0 \sim c_{\text{Zn}}^{-0.5}$, as shown in FIGURE 3.6C. This decrease is unexpected because the linear modulus of fibrin gels increases in the presence of other divalent cations such as Ca^{2+} or Mg^{2+} ,^{4,17}.

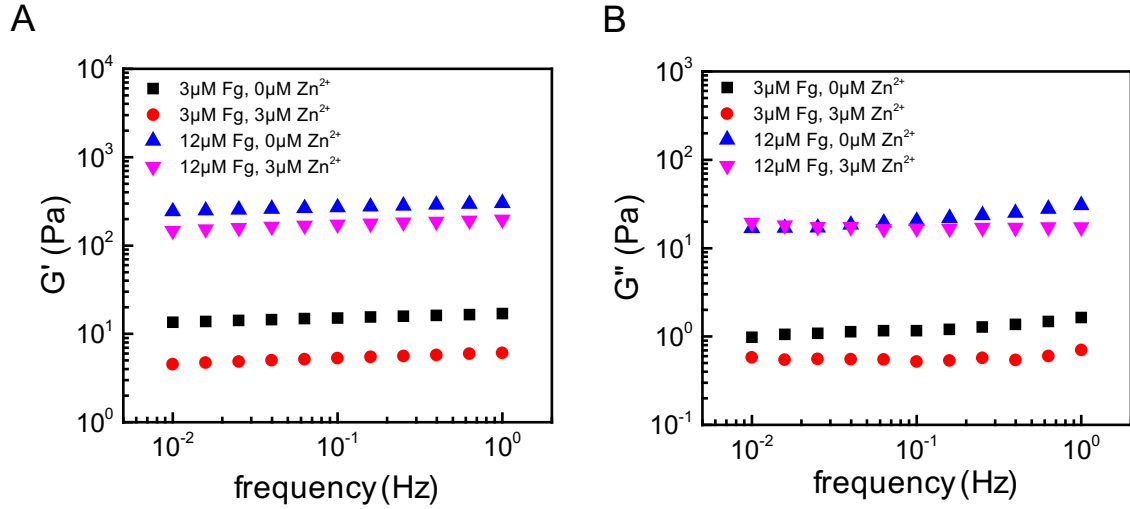


FIGURE 3. 7 Frequency dependence of the storage modulus, G' , and loss modulus, G'' .

To investigate this anomalous behavior, we consider the implications of the strong bundling effect of Zn^{2+} , as seen in Fig. 1. One important effect is essentially structural or geometric: for a given protein concentration, bundling decreases the fiber line density, ρ_f , and increases the mesh size, ξ , as shown in Fig. 1. Thus, for a fiber bundle consisting of N protofibrils, ρ_f is related to the protofibril line density, ρ_{pf} , by the relation

$$\rho_f = \rho_{pf}/N \quad (1)$$

The other effect of bundling is mechanical, in which bundling increases the bending rigidity κ of fibers. The strength of the increase in bending rigidity, however, depends on the nature of the bundling. For so-called *tight bundles*, in which the filaments in the bundle are laterally coupled to prevent relative sliding, the bending rigidity κ is expected to increase quadratically with the number N of filaments in the bundle. For loose bundles, with relative sliding, the bending rigidity is simply additive, with $\kappa \sim N$. Thus, we assume that

$$\kappa_f = N^x * \kappa_{pf} \quad (2)$$

where κ_{pf} is the bending rigidity of fibrin protofibril and $x=1$ or 2 for loose or tight bundling, respectively.

In the thermal affine model, the network shear modulus in the linear elastic regime, G_0 , is calculated as¹¹

$$G_0 \sim \rho_f^{11/5} l_p^f{}^{7/5} \quad (3)$$

Combining equations (1), (2) and (3) allows us to predict the effect of bundling on the linear shear modulus³,

$$G_0 \sim \rho_{pf}^{11/5} N^{\frac{7x-11}{5}} l_p^f{}^{7/5} \quad (4)$$

Where l_p^f is the persistence length of fibrin protofibril. Thus, for a given total protein concentration, the linear shear modulus is predicted to increase with N for tight bundles and decrease with N for loose bundles. Our experiments are consistent with the latter case, where $G_0 \sim N^{-4/5}$, as shown in Fig 3D. A similar conclusion can also be drawn from the model for athermal fiber networks. For purely mechanical, athermal fiber networks, very general considerations would predict an approximate quadratic scaling of the shear modulus with concentration, in which

$$G_0 \sim E \varphi^2,$$

where φ is the volume fraction of polymer and E is the Young's modulus of the fibers¹⁸⁻²¹. This model can also be applied to tightly bundled networks, for which the shear modulus is predicted to be independent of bundling. Since the elastic response is governed by bending, however, the shear modulus for loose bundles is predicted to decrease with N as $G_0 \sim 1/N$. Thus, our observations of a decreasing network stiffness with increased bundling by Zn^{2+} are

clearly inconsistent with tightly bundled networks in either thermal or athermal limits. Moreover, the decrease that we observe in FIGURE 3.6D is generally consistent with the mechanics of loose bundled networks.

3.3.2 Nonlinear rheology of fibrin network

Fibrinogen networks are hierarchical structures and they can exhibit distinct elastic responses in different strain regimes. To explore the nonlinear response of Zn^{2+} modified fibrinogen networks, we perform a prestress test to probe the elastic behavior under large stresses. Briefly, small amplitude stress oscillations of amplitude $\delta\sigma = 0.1\sigma$ and frequency 0.1 Hz are superimposed on a steady shear stress, σ , that is gradually increased in a stepwise manner. The storage modulus at each shear stress is taken as differential modulus, $K'(\sigma) = \delta\sigma/\delta\gamma$. When the fibrin network is subjected to continuous shear stress, the differential modulus is nearly constant at small stress, reflecting linear behavior. But, the differential modulus increases substantially at large stress, as shown in FIGURE 3.8A. Such stiffening is qualitatively similar to the behavior of a wide range of biopolymer networks^{12,22}. These data also agree qualitatively with the theoretical prediction from the thermal affine model, shown as the inset of FIGURE 3.8A. Here we also define a characteristic stress σ_c where the differential modulus increases to 50% larger than its linear value, representing the onset of nonlinear rheological response of the network, as shown by the red dashed lines in FIGURE 3.8A.

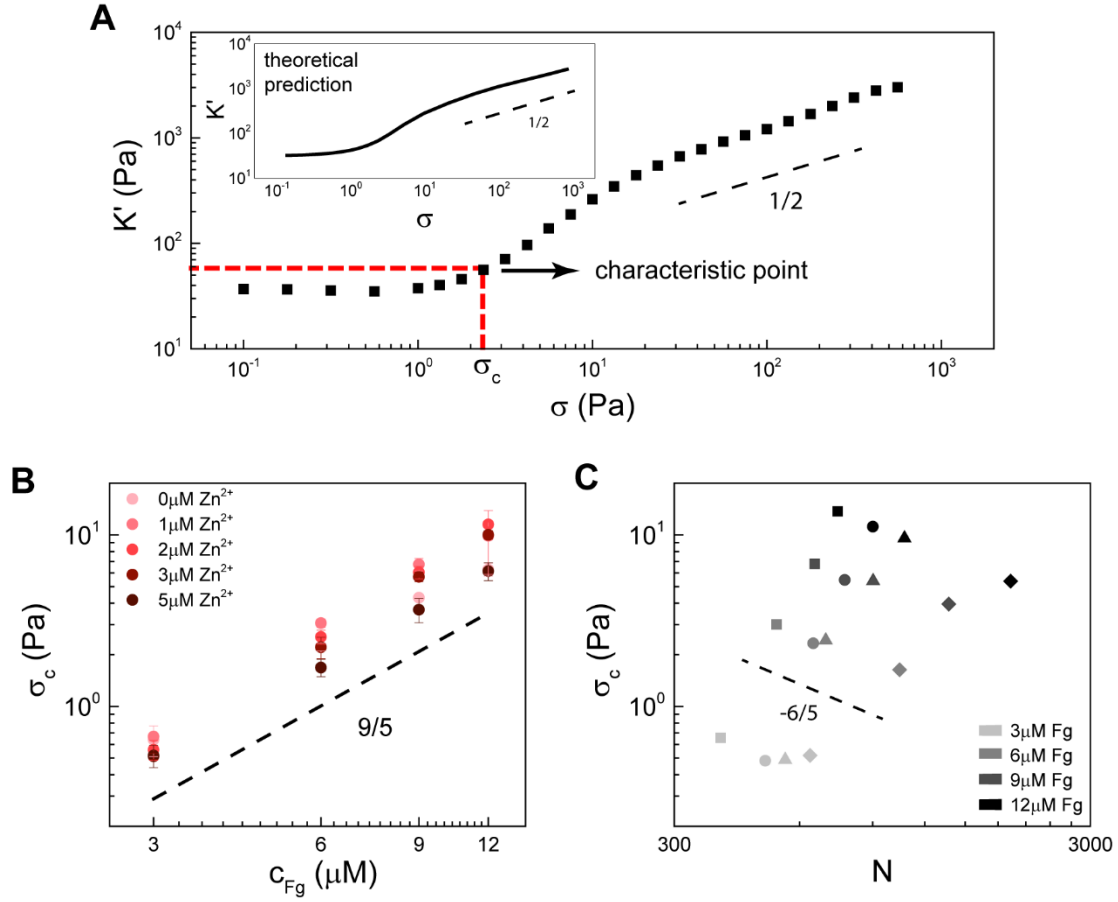


FIGURE 3. 8 Stress-stiffening transitions of fibrin network.

(A) Differential modulus, K' , is plotted as a function of prestress, σ . Characteristic stress at onset of stress stiffening is shown as σ_c . Fibrinogen at $3\mu\text{M}$ with $1\mu\text{M}$ Zn^{2+} is shown as an example here. (B) Critical stress increases with fibrin concentration as $\sigma_c \sim c_{Fg}^{9/5}$ for various Zn^{2+} concentrations. (C) For each given fibrin concentration, critical stress scales with number of protofibrils as $\sigma_c \sim N^{-6/5}$ when Zn^{2+} concentration varies from $1\mu\text{M}$ to $5\mu\text{M}$ (Zn^{2+} $1\mu\text{M}$, square; Zn^{2+} $2\mu\text{M}$, circle; Zn^{2+} $3\mu\text{M}$, triangle; Zn^{2+} $5\mu\text{M}$, diamond;)

This threshold stress is predicted to vary for affine networks as

$$\sigma_c \sim \rho_f^{9/5} (l_p^f)^{3/5} \sim \rho_{pf}^{9/5} (l_p^{pf})^{3/5} N^{\frac{3x-9}{5}},$$

which is consistent with our measured dependence on fibrinogen concentration, as shown in FIGURE 3.8B. Meanwhile, the threshold strain decreases with the fibrinogen concentration, with an exponent of $-2/5$, for various Zn^{2+} concentrations, as shown in FIGURE 3.9. Moreover, as we show in FIGURE 3.8C, with the addition of Zn^{2+} , our measured threshold

stress for c_{Fg} above 3 μM is also consistent with the predicted $\sim N^{-6/5}$ dependence for loose bundling. The weaker N dependence we observe at the lower fibrinogen concentration may be an indication of non-affine behavior, for which no dependence on N is predicted^{3,23}

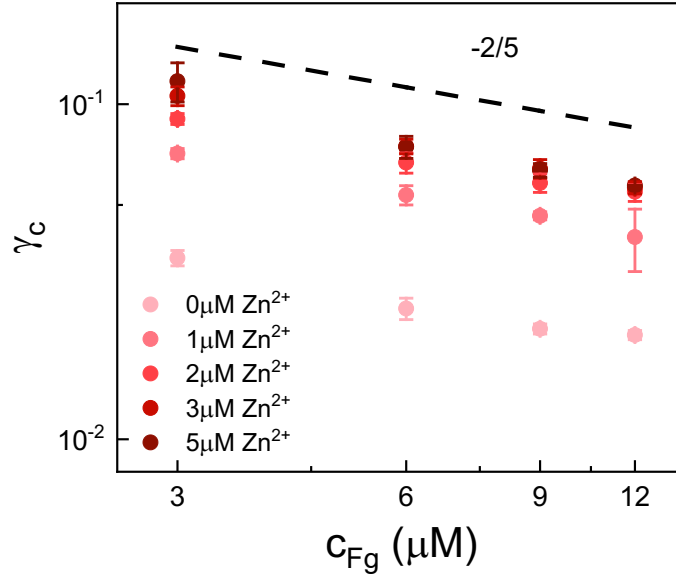


FIGURE 3.9 Critical strain of fibrin network, γ_c , scale with fibrin concentration, c_{Fg} , as $\gamma_c \sim c_{Fg}^{-2/5}$.

Since the thermal affine model seems to capture most of the elastic properties of fibrin gels, at higher fibrinogen concentrations, we normalize the applied stress by the characteristic stress and the differential modulus by the linear modulus to compare response of the fibrin gels under different strain regimes. When there is no Zn^{2+} , fibrin networks show 4 different stiffening regimes as the normalized stress is increased: (I) first plateau regime, (II) first stiffening regime, (III) second plateau regime, (IV) second stiffening regime, as seen in FIGURE 3.10A. In regime I, where the normalized stress is small, the network response is linear and the elasticity is due to thermal fluctuations in a floppy network. In regime II, the fibrin network undergoes a transition to stretching the constituent network fibers, which results in overall stiffening of the network. Once all the thermal slack has been pulled out, the network deforms affinely and enters regime III, where the network elastic response reflects the single fiber elasticity. Regimes III and IV of the fibrin network are due to the stretching of

single fibers, which have a linear stretch modulus under small strains and a much larger stretch modulus under large strains. A possible 5th regime where the protofibril backbone is stretched may be reached if high enough stresses are applied; however, the highest stress we achieve for each protofibril is below 10 pN, which is far less than the average force required (130 pN) to unfold a single protofibril^{13,24}. When fibrinogen concentration is low (3 μ M), the second plateau regime becomes less defined, as shown in FIGURE 3.10A. This transition is possibly due to non-affine deformation resulting from the sparse network; at low enough fibrin concentrations, the network will not enter an affine stretching regime but instead will enter a non-affine mechanical bending regime²⁵.

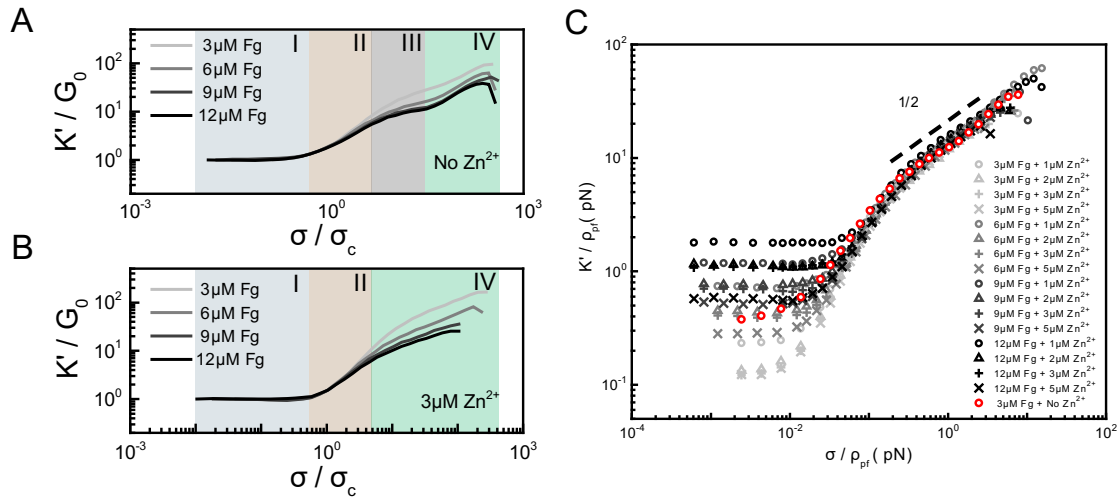


FIGURE 3. 10 Rheological property of fibrin network at high stress.

Stress stiffening curve of fibrin network obtained by normalizing differential modulus and applied stress by equilibrium shear modulus and critical stress separately. (A) Fibrin network with Zn^{2+} shows 3 regimes (I II IV) during stress stiffening, clearly deviates from (B) fibrin network with no Zn^{2+} , which shows 4 regimes (I II III IV). (C) Stress stiffening curves measured at different polymerization conditions, rescaled by protofibril line density, ρ_{pf} . The data collapse onto a master curve at large stress (above 0.1 pN), suggesting the response of the network is a linear sum-up of the elastic response of each protofibrils.

Similar to the elastic response of pure fibrin gels at low fibrinogen concentrations, fibrin formed with Zn^{2+} shows 3 regimes corresponding to regimes (I), (II), and (IV) as described above and indicated in FIGURE 3.10B. In the plateau regime (I), the elastic response of the fibrin gels originates from pulling thermal fluctuations out of the network. Upon increasing the stress, two sequential stiffening regimes [(II) first stiffening regime, (IV) second stiffening

regime] with different slopes are revealed. In the first stiffening regime, the normalized differential modulus increases with the normalized stress as $K'/G_0 \sim \sigma/\sigma_{\text{crit}}$, possibly indicating the transition to mechanically stretching individual fibers, which is dominated by the non-affine mechanical bending of the fibers. In the second stiffening regime, individual fibers are stretched and stiffened in the affinely deforming network, displaying a $1/2$ power law dependence, $K'/G_0 \sim (\sigma/\sigma_{\text{crit}})^{1/2}$, that is similar to the response of collagen network at high stress in a stretch dominated regime¹⁸.

Inspired by the finding that, at high stress regimes, the differential modulus scaling of the Zn^{2+} modified fibrin gels does not depend on the specific Zn^{2+} or fibrinogen concentration, we rescale both the differential modulus and the applied stress by the line density of protofibrils, ρ_{pf} . Since the protofibrils are loosely coupled and deform independently of each other, the bulk stress is uniformly distributed among the protofibrils, and the response of the network is a linear sum-up of the elastic response of each protofibrils. We find that the rescaled data indeed fall onto a master curve at high normalized stresses, as shown in FIGURE 3.10C. Thus, a normalization by the protofibril line density will reveal the intrinsic mechanics of single protofibrils. Interestingly, low concentration fibrin gels (3 μM) also fall onto the master curve even without Zn^{2+} , as seen in FIGURE 3.10C. This suggests that the protofibrils may naturally assemble in a loosely coupled manner when the fibrinogen concentration is low enough. In contrast, high concentration fibrin gels (6 μM , 9 μM and 12 μM) without Zn^{2+} do not collapse when their stiffening curves are rescaled, suggesting that the protofibrils are not loosely coupled and therefore their contribution to high stress mechanics may not be linearly additive, as shown in FIGURE 3.11.

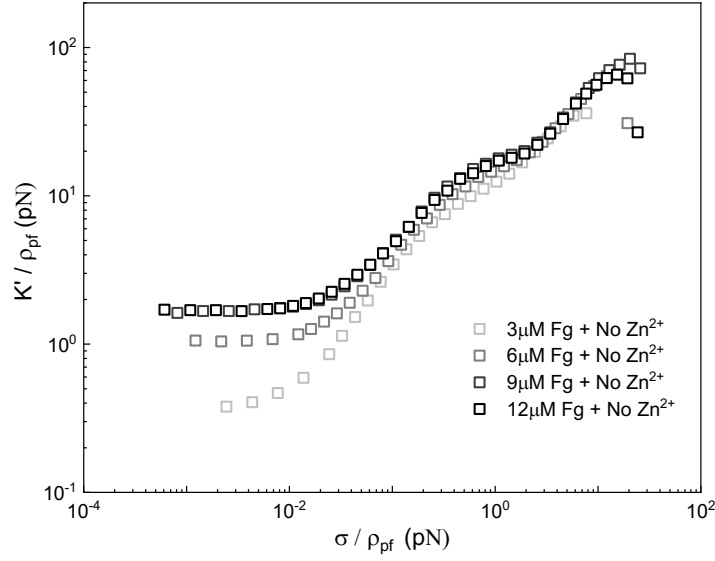


FIGURE 3.11 Stress stiffening curves of fibrin network at different fibrin concentration with no Zn^{2+} , rescaled by protofibril line density, ρ_{pf} .

Further increasing the stress will result in fibrin gel rupture, as shown by the dramatic drop in K' in FIGURE 3.10A. We plot the rupture stress of fibrin under different polymerization conditions, as shown in FIGURE 3.12. For the same fibrinogen concentration, the rupture stress decreases with the Zn^{2+} concentration, indicating a more fragile network. Surprisingly, Zn^{2+} does not affect the concentration dependence of the rupture stress, $\sigma_{rupture} \sim c_{Fg}^{1.4}$, which might indicate that fibrin networks formed with or without Zn^{2+} share the same breaking mechanism.

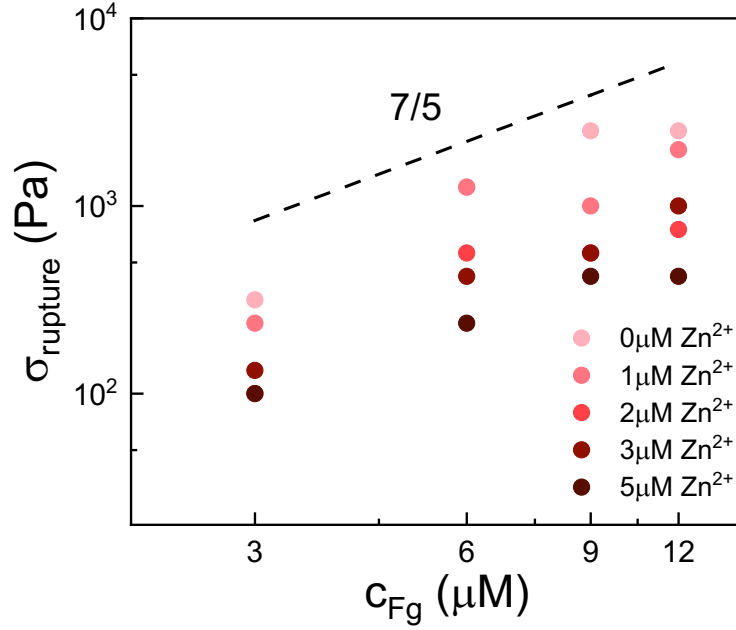


FIGURE 3.12 Rupture stress of fibrin network, $\sigma_{rupture}$, scale with fibrin concentration, c_{Fg} , as $\sigma_{rupture} \sim c_{Fg}^{7/5}$.

3.4 Conclusions and discussion

Here we assemble highly bundled fibrin networks by adding Zn^{2+} ions, and we explore the elastic origins of these networks at both low and high stress regimes. We show that, at low stresses, linear elasticity can be well characterized by the thermal affine model, which says that the mechanical properties originate from pulling the thermal slack out of the network. By combining rheological measurements and network topology, we infer that fibrin networks formed in presence of Zn^{2+} are loosely coupled in nature, where the protofibrils inside fiber bundles fluctuate independently of each other. At high stresses, the elasticity originates from pulling on the individual protofibrils, as confirmed by the data collapsing with the theoretical prediction of protofibril mechanics. Previous research has shown that Zn^{2+} binds to fibrinogen with high affinity, and that Zn^{2+} may increase fibrin fiber thickness at least in part by binding to the alpha-C domain and promoting bundle formation⁷. In this work, we have shown that fibrin bundles are loosely coupled from a mechanical perspective. It would be interesting to

further measure the fibrin bundle mechanics and relate the mechanics with the protein structure in the future.

Our findings have important implications for understanding the origin of fibrin network mechanics and, in particular, the relationship between the fiber structure and its effect on network mechanics. These findings inform our understanding of the structure and physical mechanisms leading to defective or excessive clot stiffness, especially in thrombosis or vascular diseases related to Zn^{2+} ²⁶. Furthermore, this understanding can be used in tissue engineering applications to design hierarchical fibrin scaffolds with multi-scale control over the mechanical properties.

Chapter 4 Hydrogel Microstructure Modulate Collagen Assembly and Regulate Cell Behavior

4.1 Abstract

Mechanical cues from extracellular environment has emerged as non-trivial players in cellular mechanotransduction, in addition to the traditional biochemical cues which have significant effects on cell behavior. Among others, a long-forgotten yet very important cue is the microstructure of the pericellular environment, which has non-negligible effect on cellular response. Here we use a polyacrylamide hydrogel platform, which has excellent control over many mechanical cues, to investigate the effects of hydrogel microstructure on cell behavior. We find that more surface collagen clusters are assembling on hydrogel with smaller pores. Although cell attachment and proliferation are not obviously affected by varying hydrogel pore size, cell spreads bigger and migrates faster on hydrogel with smaller pores. With a further look at subcellular scale, we find that focal adhesion formation is also encouraged as the hydrogel pore size decreases. Moreover, when cells are grown on hydrogel with smaller pores, the nuclear translocation of Yes-associated protein (YAP) is promoted, indicating the cellular mechanotransduction is modulated by hydrogel microstructure. To conclude, our findings highlight the important role of hydrogel microstructure in governing cell behavior and mediating cellular mechanotransduction.

4.2 Introduction

Cells interact and response to the local extracellular microenvironment, which not only serves as essential physical scaffolds to lodge the cells, but also provides various stimulus to regulate cell behavior²⁷. Over the past decades, it has long been dictated that biochemical cues, such as growth factors and bioactive molecules, play significant roles in regulating cell behavior. Recently, in addition to these biochemical cues, mechanical cue has also been recognized as another non-trivial factor that can affect cell behavior²⁸⁻³⁰. Hydrogel systems,

which are controllable over many mechanical cues, are developed as an ideal platform for cellular mechanosensing studies. This system has been used to demonstrate the influence of various mechanical cues on cell behavior, with the most well-studied one being the stiffness of hydrogel²⁹⁻³². Besides stiffness, microstructure, another important characteristic of hydrogel, has not been thoroughly investigated.

So far, there has been evidence suggesting that microstructure of hydrogel involves in the regulation of cell behavior³³⁻³⁵. By culturing cells on collagen hydrogel with different microarchitectures, researchers found that cell spreading, proliferation, and migration are significantly altered³³. A previous study also demonstrated that stem cells differentiate into different cell types on polyacrylamide (PA) hydrogel with different pore sizes³⁵. However, these above-mentioned hydrogel systems are still coupling multiple mechanical cues together, making the effects of hydrogel microstructure inconclusive. Understanding the effects of hydrogel microstructure not only fills in a gap of knowledge in cellular mechanosensing, but also sheds lights into adopting hydrogel microstructure as an approach to control cell behavior.

In this study, we culture human bone marrow-derived stem cells (hBMSCs) on PA hydrogels with different microstructure but the same stiffness, and investigate the corresponding cell responses, such as attachment, proliferation, spreading and migration. Our results suggest that the different microstructure of hydrogel significantly influences the amount and distribution of collagen type-I attached on the surface: hydrogels with smaller pores have more collagen clusters assembled on it. Both the cell attachment percentage and proliferation rate remain the same regardless of changing hydrogel microstructure. However, cells form more focal adhesion assembly, spread bigger and migrate faster as hydrogel pore size decreases. Furthermore, we find more YAP protein translocate from cytoplasm to nucleus in cells grown on hydrogel with smaller pores, indicating a variation of cellular mechanotransduction. In summary, our results demonstrate that hydrogel microstructure has dramatic effects on cell behavior.

4.3 Results

4.3.1 Preparation of hydrogels with identical stiffness but different microstructure

We develop a hydrogel-glass platform with controllable hydrogel microstructure and stiffness. To change hydrogel microstructure, we tune the concentrations of acrylamide monomer and crosslinker N,N'-Methylenebisacrylamide, which are 6%/0.35%, 9%/0.126%, and 12%/0.065%, respectively. Scanning electron microscopy (SEM) images of freeze-dried hydrogels shows significant differences among their microstructures, as presented in FIGURE 4.1a. In this study, the averaged pore size of PA hydrogel is measured to characterize the hydrogel microstructure. Higher monomer concentration results in smaller pores in hydrogel, as shown in FIGURE 4.1b. It is known that SEM may slightly overestimate the pore size of hydrogel due to the drying process in the sample preparation, however, the pore size trend in the dried state still qualitatively match that in the hydrate state ³⁶.

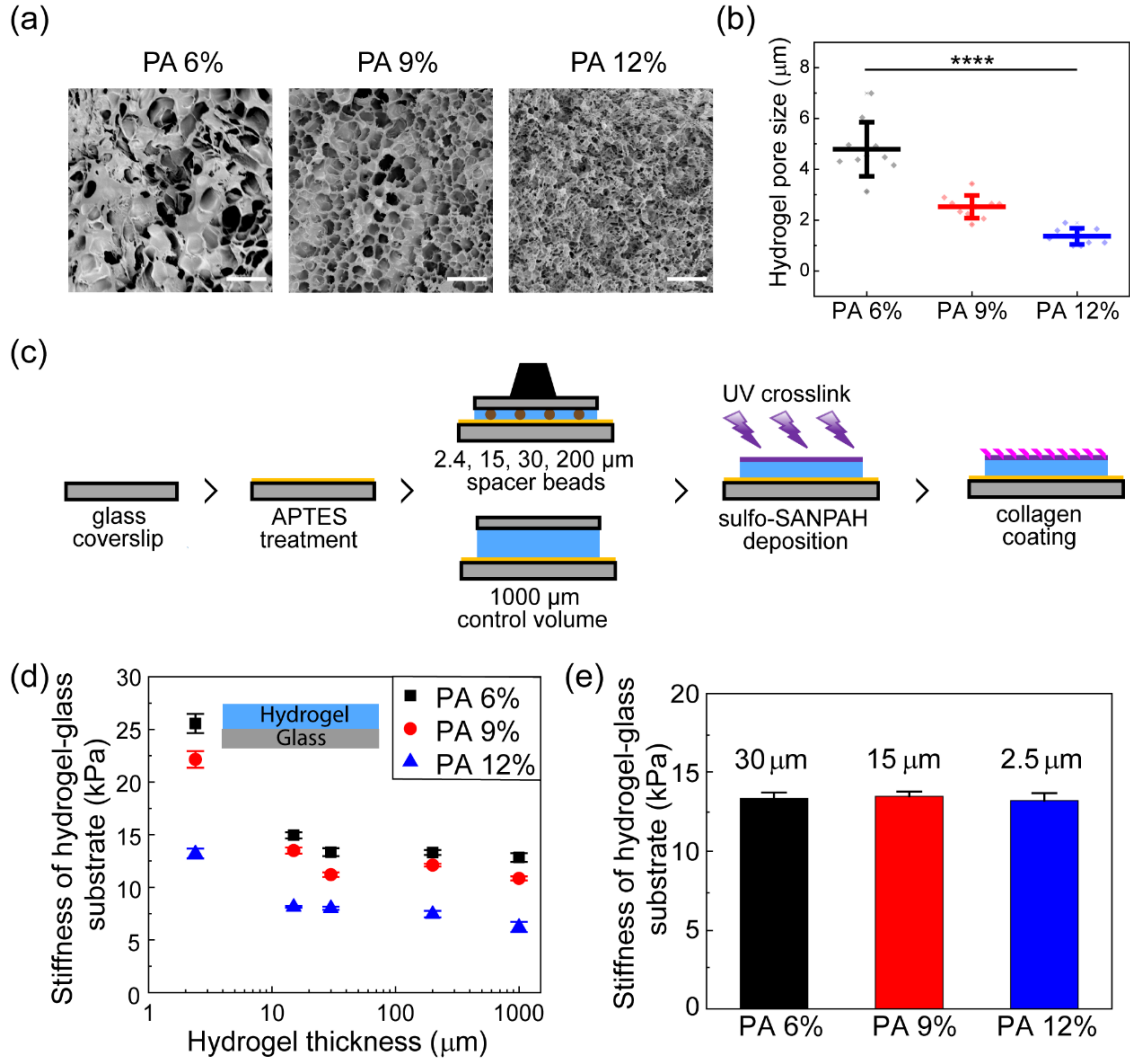


FIGURE 4. 1 Fabrication of hydrogel-glass substrates with different hydrogel microstructure and the same.

(a) Scanning electron microscopy (SEM) images of PA hydrogel with different monomer concentration (scale bar, 5 μm). (b) Pore size of hydrogels measured from SEM images (Mean $\pm$ Std, N=10). (c) Schematic of hydrogel-glass substrate fabrication. (d) Stiffness of hydrogel-glass substrates with different monomer concentration and hydrogel thickness (Mean $\pm$ Std, N=9). (e) Stiffness of three model hydrogel-glass substrates used in the following experiments with indicated monomer concentration and hydrogel thickness (Mean $\pm$ Std, N=9).

Although the microstructure of hydrogel can be easily tuned, the stiffness of hydrogel is also changing during this adjustment³⁷. As acrylamide concentration increases from 6% to 12%, the stiffness of hydrogel decreases from ~ 15 kPa to ~ 7.5 kPa, as shown in FIGURE 4.2.

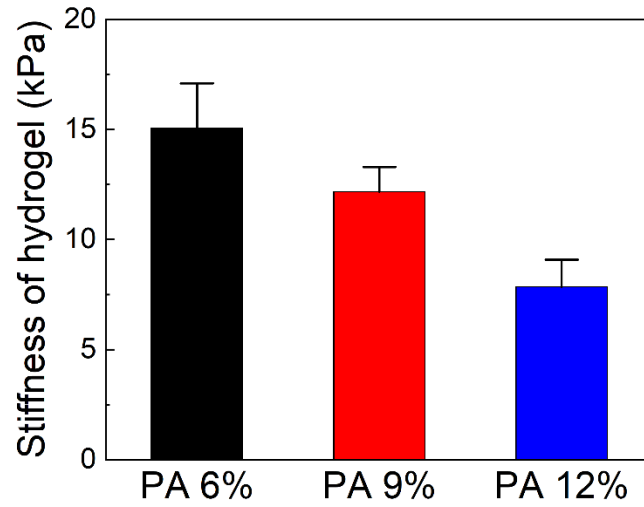


FIGURE 4. 2 Stiffness of three different hydrogels.

However, the stiffness of the hydrogel-glass substrate is not only determined by the stiffness of hydrogel, but also by the thickness of hydrogel. Previous studies have evidenced that by tuning the thickness of hydrogel layer, the same stiffness of hydrogel-glass substrate can be achieved³⁸⁻⁴⁰. In this work, a similar strategy is adopted: by tuning the thickness of hydrogel layer, hydrogel-glass substrates with different hydrogel microstructure and the same stiffness are developed. We use two different methods to vary the thickness of PA hydrogel layers, either by adding spacer beads with certain diameter or controlling solution volume, as shown in Figure 4.1c. To assess their mechanical properties, atomic force microscopy (AFM) was used to quantify the stiffness of hydrogel-glass substrates. Our results show that, for the hydrogel-glass substrates made with the same hydrogel, the stiffness of the substrates decreases with increasing thickness of hydrogel layer and reaches a plateau above a certain thickness, as shown in Figure 4.1d. Our results suggest that the effects of underlying glass on substrate stiffness vanishes when the hydrogel layer is thick enough, in agreement with previous experimental observations^{38,40}. Eventually, we select three substrates with different hydrogel microstructure and the same stiffness as a model system, which are PA 6% of 30 μm thickness, PA 6% of 15 μm thickness and PA 12% of 2.5 μm thickness, and use them to investigate the effect of hydrogel microstructure on cell behavior, as shown in Figure 4.1e.

For simplicity, here we refer these three samples as PA 6%, PA 9% and PA 12%. In this manner, the stiffness-dependent effects of cell behavior are eliminated, thus the hydrogel microstructure being the only changing parameter.

4.3.2 Characterization of collagen self-assembly on hydrogel with different microstructures

Cells do not readily attach to PA hydrogel due to the lack of anchoring sites. Therefore, ECM ligands, such as collagen, need to be coated on hydrogels to provide essential anchoring sites for cells to attach, which is the pre-requisite for the survival of adherent cells^{41,42}. In this study, we first coat hydrogels with sulfo-SANPAH, a protein crosslinker, and then covalently link collagen to sulfo-SANPAH. To avoid introducing other changing parameters, the concentration of sulfo-SANPAH and collagen monomer are controlled the same. To characterize the collagen self-assembly on hydrogels of different microstructure, we image collagen coating with confocal microscopy. Spot-like collagen clusters are randomly distributed on hydrogels with different microstructure, which have size of hundreds of nanometers and spacing of several microns, as shown in FIGURE 4.3a.

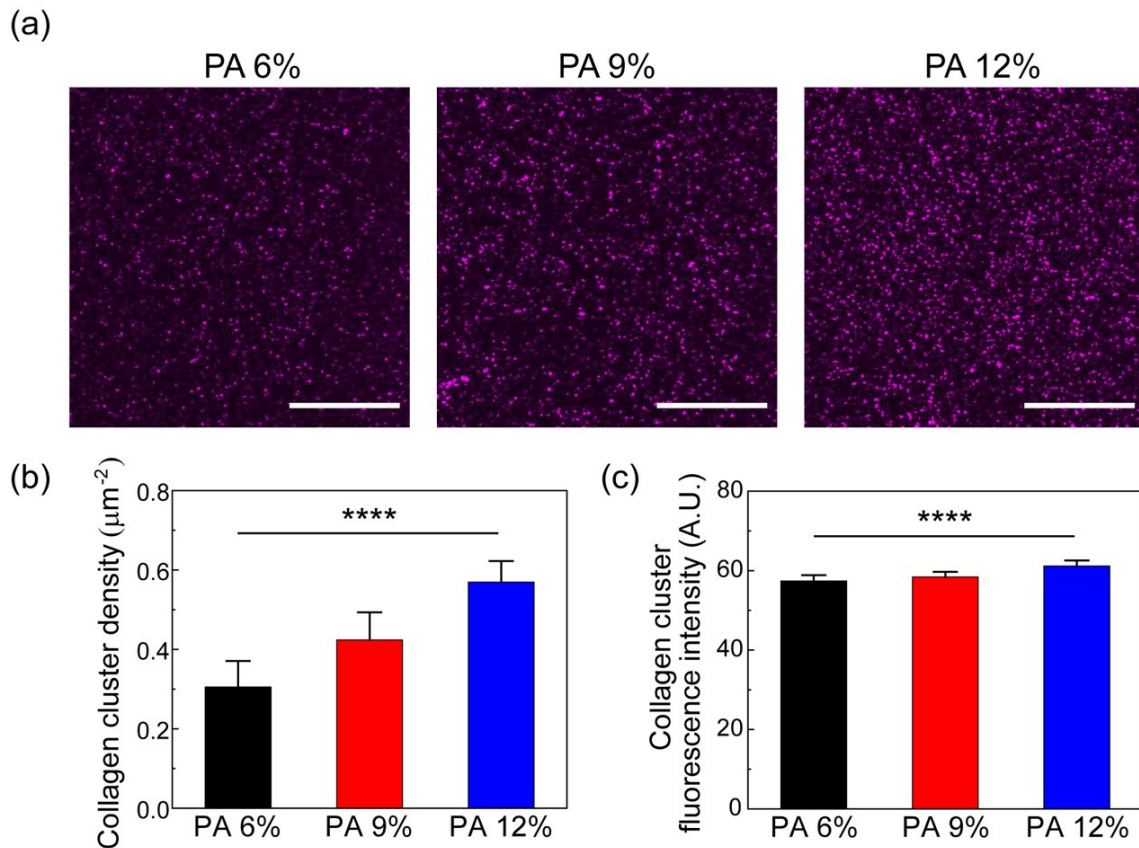


FIGURE 4. 3 Collagen self-assembly induced by varying hydrogel microstructure.

(a) Confocal fluorescent microscopy images of collagen clusters on different hydrogels (scale bar, 20 μm). (b) Density of collagen cluster on different hydrogels (Mean $\pm$ Std, $N>17$). (c) Fluorescence intensity of collagen cluster on different hydrogels (Mean $\pm$ Std, $N>17$).

By contrast, collagen monomers usually assemble into rod-like fibers in physiological condition^{43,44}. In this study, the deposition of sulfo-SANPAH on PA hydrogels establishes stable connections between PA and collagen, but at the same time, it disrupts collagen monomer forming fibers due to the non-specific protein conjugation and the blockade of triple helix formation⁴⁵⁻⁴⁷. Despite forming spot-like collagen clusters on all the samples, the distribution of collagen clusters and the amount of adsorbed collagen are different. The hydrogel with smaller pores induces a significantly increased number (~ 2 folds) of collagen clusters per micron square, suggesting a decreased collagen clusters spacing, as shown in FIGURE 4.3b. Whereas the mean intensity of individual collagen cluster slightly increases ($\sim 5\%$), indicating the collagen amount in each cluster is not significantly affected by hydrogel microstructure, as shown in FIGURE 4.3c. The measured fluorescent intensity of collagen is

approximately two orders of magnitude higher than background fluorescent noise, as shown in FIGURE 4.4.

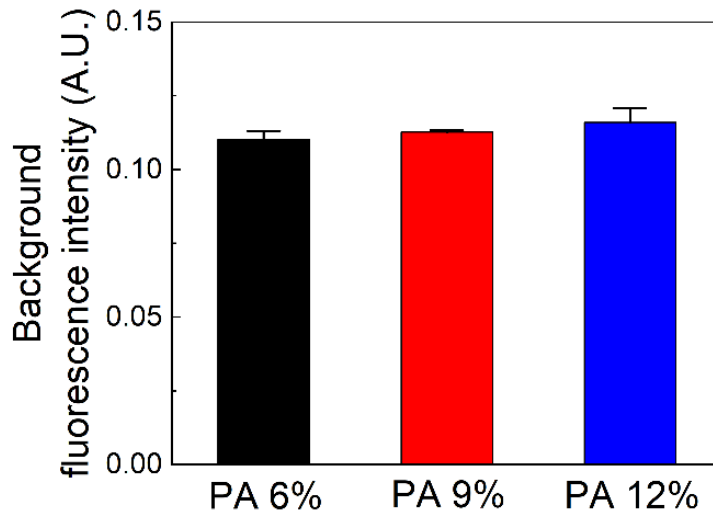


FIGURE 4. 4 Background fluorescence noise without collagen.

Our results show that smaller pores of hydrogel encourage more collagen cluster forming, which can be explained by the increased collagen anchoring sites available on the hydrogels with smaller pores. The overall intensity of collagen staining, which is contributed by both the number density and the individual fluorescent intensity of collagen clusters, shows that there is an increase in the average collagen amount absorbed on hydrogel with smaller pores, as shown in FIGURE 4.5.

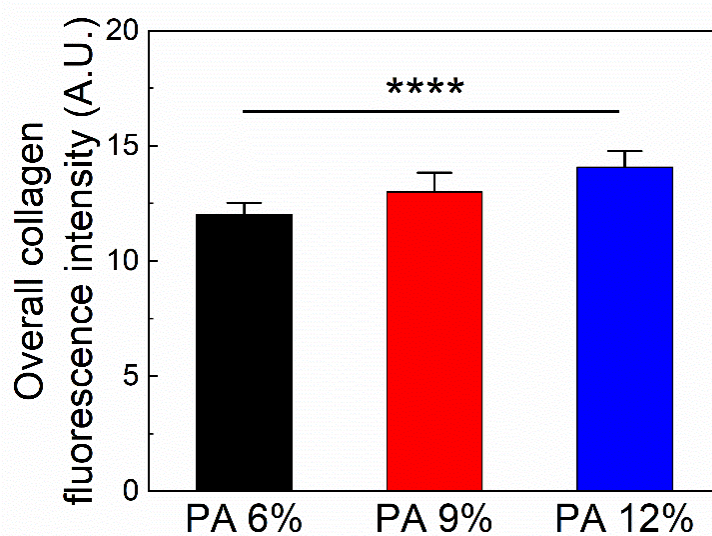


FIGURE 4. 5 Overall fluorescence intensity of collagen.

In summary, our results show that although the collagen amount of each cluster is similar on hydrogels of different microstructure, hydrogel with smaller pores shows an increased number density and a decreased spacing of collagen clusters.

4.3.3 Effects of hydrogel microstructure on cell attachment and proliferation

To investigate the interactions between hydrogel microstructure and cells, we hereby study several typical cell behaviors, including cell attachment, proliferation, spreading and migration. To assess how the changes in hydrogel microstructures affects cell attachment and proliferation, cells are seeded onto three hydrogels at low density (~ 4000 cells per centimeter square), with the purpose to isolate cells and avoid cell-cell contacts. Representative confocal images show that the cells are well separated on different hydrogels at day 0 and day 4, as shown in FIGURE 4.6a and b. The number of attached cells on day 0 are then quantified and normalized by the cell seeding density to calculate the cell attachment percentage. At least 80% percent of seeded cells can attach to the three hydrogels. Additionally, no significant difference is found among different samples, suggesting that cell attachment is not affected by the hydrogel microstructure, as shown in FIGURE 4.6c. The cell proliferation rate from day 0 to day 4 also shows similar trends among different samples, as shown in FIGURE 4.6d, suggesting that hydrogel microstructure has minimal effect on the cell proliferation rate.

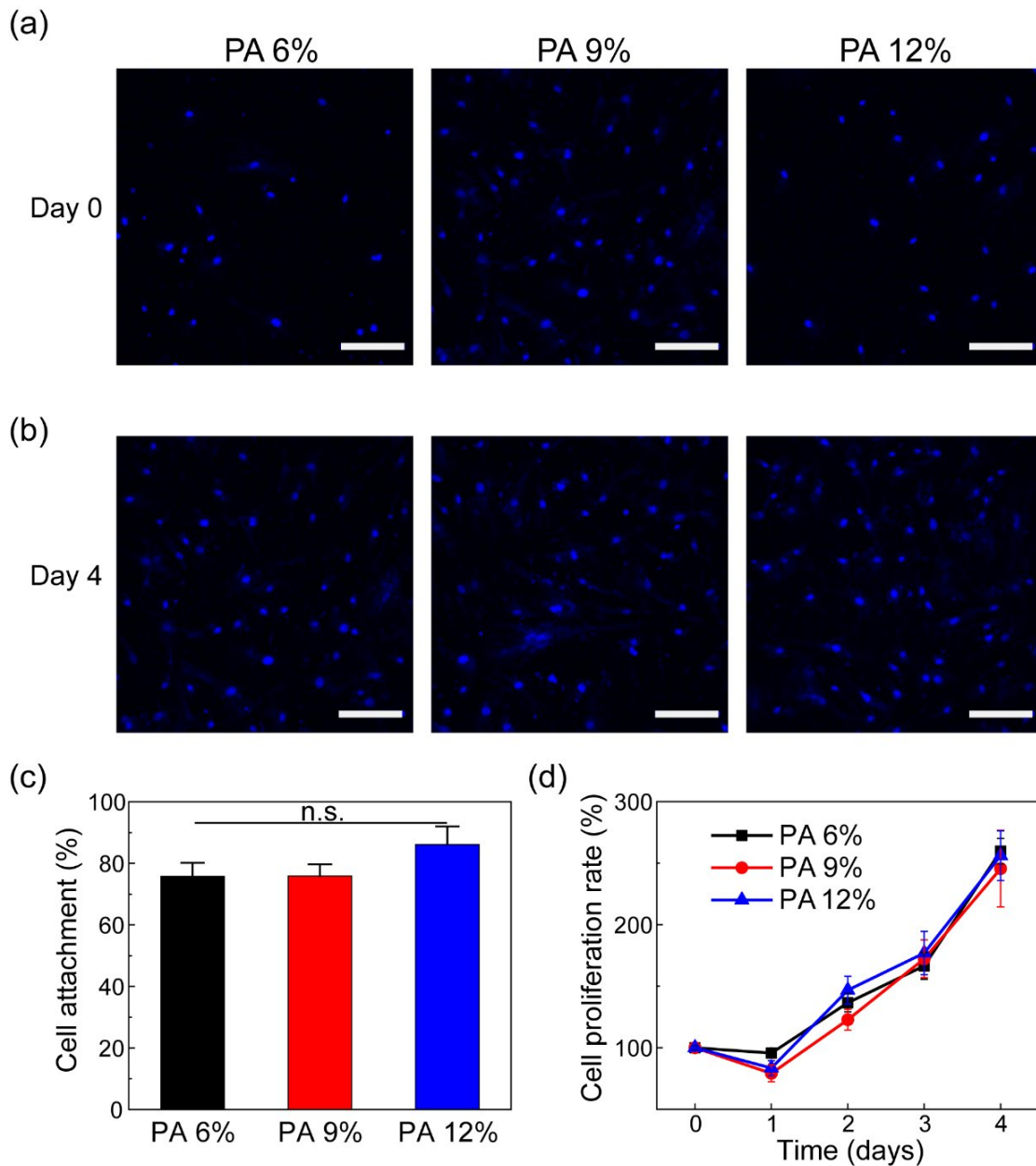


FIGURE 4. 6 Cell attachment and proliferation of hBMSCs on hydrogels with different microstructure.

(a) Confocal fluorescent microscopy images of cell nucleus on different hydrogels at day 0 (scale bar, 200 μm). (b) Confocal fluorescent microscopy images of cell nucleus on different hydrogels at day 4 (scale bar, 200 μm). (c) Cell attachment on different hydrogels (Mean $\pm$ Ste, N=26). (d) Cell proliferation rate on different hydrogels (Mean $\pm$ Ste, N=26).

A previous study showed that cell attachment percentage and proliferation rate increase with more collagen assembled on hydrogels⁴⁸. By contrast, our results show that cell attachment percentage and proliferation rate are similar among different hydrogels, though the absorbed collagen amount are different. This seemingly inconsistency may result from the fact

that there is only slightly difference (~15%) in collagen amount, as shown in FIGURE 4.5, while significant difference (~600%) is observed in other literature ⁴⁸. Taken together, such comparison may suggest that cell attachment and proliferation are not sensitive to the minor difference of collagen amount coated on hydrogels.

4.3.4 Effects of hydrogel microstructure on cell spreading

Next, we investigate the spreading of cells after attachment. After 24h of cells seeded on hydrogels with different microstructures, confocal images of random selected cells with dual staining of cytoplasm and nucleus are taken, as shown in FIGURE 4.7a. Projected cell area and nucleus area are then measured. According to our results, there is a significant increase of cell spreading area on hydrogel with smaller pores, as shown in FIGURE 4.7b. The projected nucleus area follows the same trend as spreading area, as shown in FIGURE 4.7c. Our results demonstrate that by varying hydrogel microstructures, cell spreading behavior can be significantly affected: cell spreading shows preference of hydrogel with smaller pores. In our study, there are more collagen clusters assembled on hydrogel with smaller pores, resulting in increased ligand density presented to cells, which might lead to the increased cell spreading area. Previous studies also reveal a positive correlation between ECM ligand density and cell area ^{49,50}. The similarity between our observations and previous studies suggest that ECM ligand density may act as the key mediator in regulating cell spreading by hydrogel microstructure.

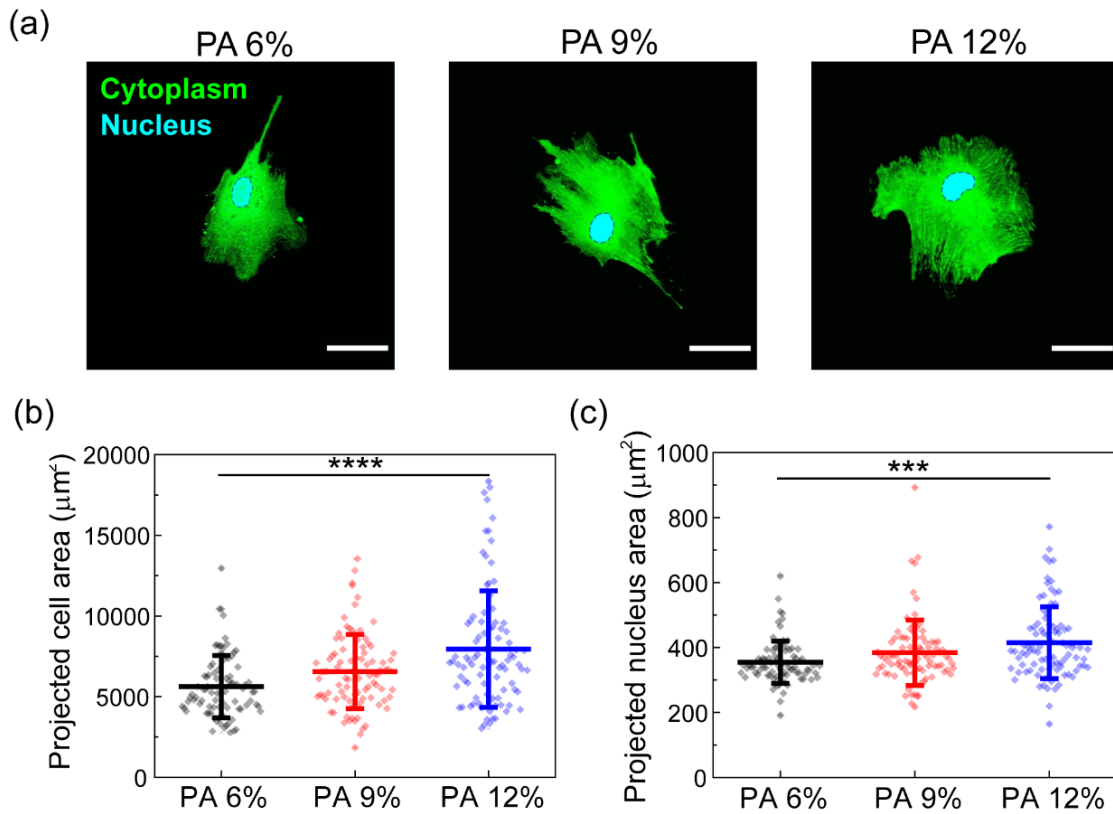


FIGURE 4. 7 Cell spreading behavior of hBMSCs on hydrogels with different microstructure. (a) Morphology of representative cells on different hydrogels (scale bar, 50 μm). (b) Projected cell spreading area on different hydrogels (Mean $\pm$ Std, $N>88$). (c) Projected cell nucleus area on different hydrogels (Mean $\pm$ Std, $N>88$).

4.3.5 Effects of hydrogel microstructure on cell migration

Besides spreading, cell migration is another dynamic process resulting from complex interactions between cell and ECM^{51,52}. To investigate the migration of cells on hydrogels of different microstructures, time-lapse images of cell nucleus are taken to track cell motion within a 48-hour period. Cells are seeded sparsely enough to avoid cell-cell interactions. Overlays of cell migration trajectories shows that cells migrate in a random pattern and without directional preferences on all three different hydrogels, as shown in FIGURE 4.8a. This random migration behavior is different from other studies which use collagen fibrous networks as contact interface for cells, showing that collagen fiber can guide the cells to migrate along the direction of fiber orientation⁵³⁻⁵⁵. We attribute this difference to the random distributed and non-fiberized collagen clusters, which lead to the surface symmetry in spatial dimensions. The average cell speed is calculated based on individual trajectory, suggesting

that cells migrate faster on hydrogel with smaller pores, as shown in FIGURE 4.8b. According to the histograms of cell speed, most of the cells migrate in the range of $0.5 - 1.5 \mu\text{m}/\text{min}$, but the cells on hydrogel with smaller pores have a smaller proportion of cell speed ranging from $0.5 - 1.0 \mu\text{m}/\text{min}$, as shown in FIGURE 4.8d. In addition, directional persistence, a parameter that measures the straightness of migration trajectory, is quantified as the ratio of end-to-end distance to the contour length of a trajectory. In contrast to average cell speed, the directional persistence exhibits no difference among hydrogels with different microstructures, as shown in FIGURE 4.8c, e, suggesting that the migration stability of cells is not affected by the hydrogel microstructure.

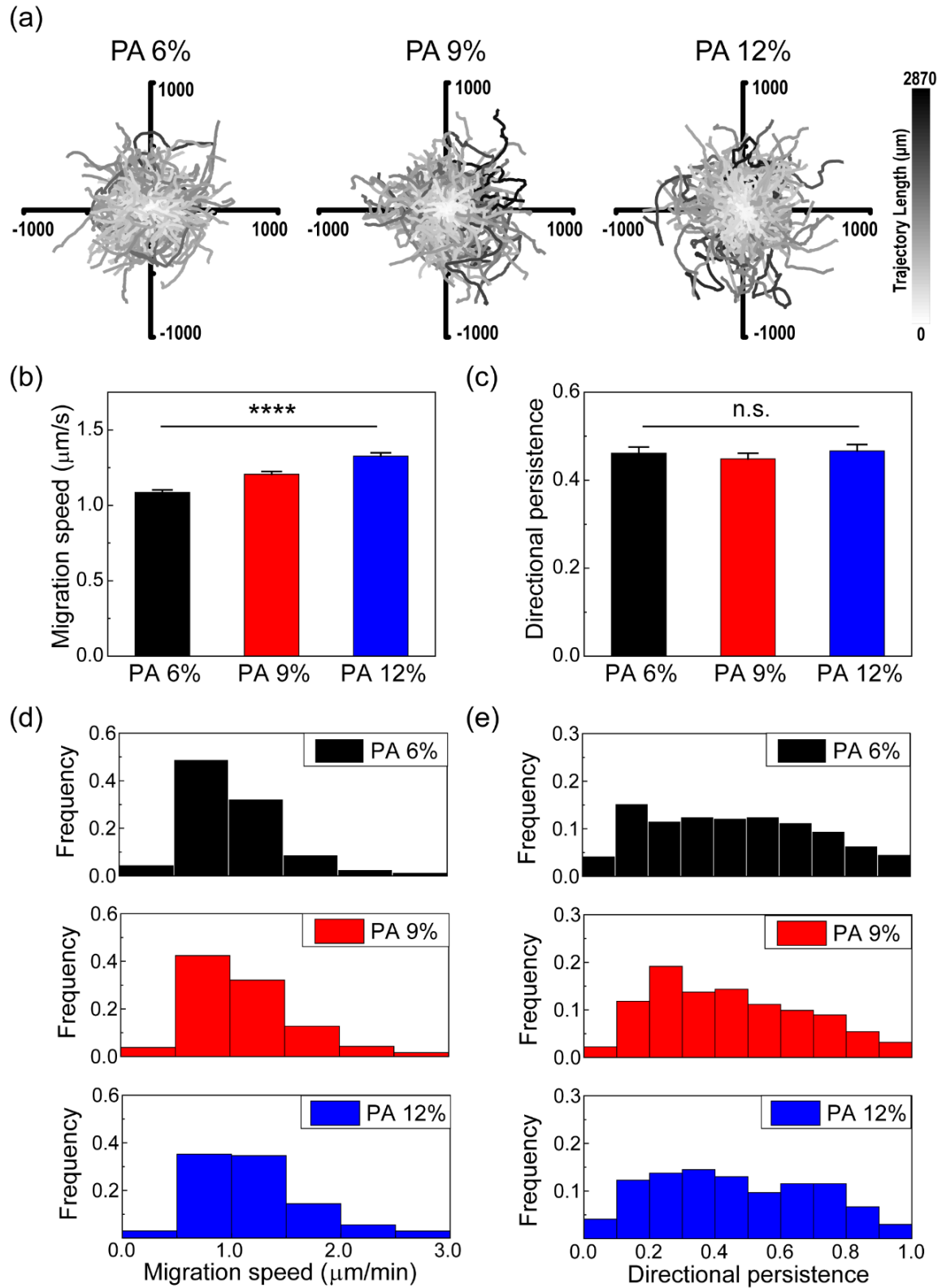
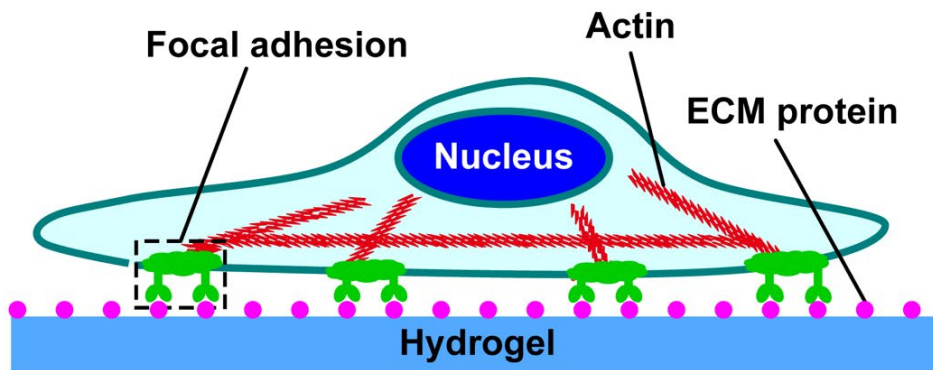


FIGURE 4. 8 Cell migration behavior of hBMSCs on hydrogels with different microstructure. Migration trajectories of cells on different hydrogels. (b) Cell migration speed on different hydrogels (Mean $\pm$ Ste, $N > 1000$). (c) Directional persistence of cells on different hydrogels (Mean $\pm$ Ste, $N > 1000$). (d) Distribution of cell migration speed on different hydrogels ($N > 1000$). (e) Distribution of cell directional persistence on different hydrogels ($N > 1000$).

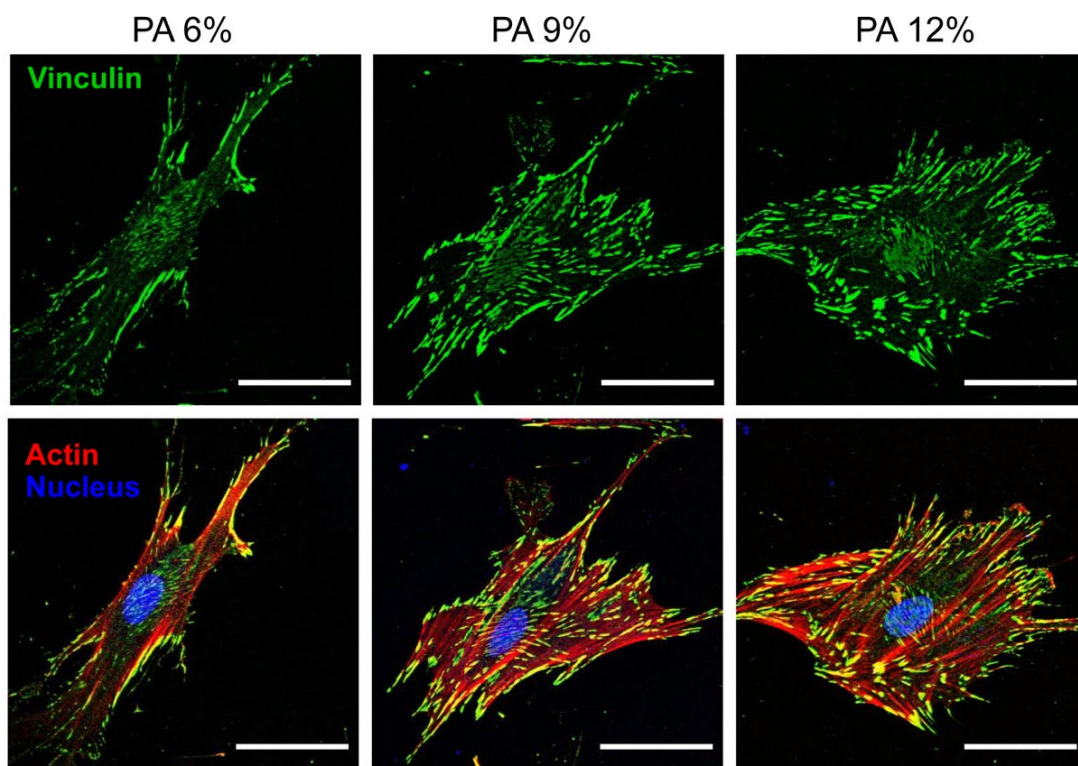
4.3.6 Effects of hydrogel microstructure on focal adhesion formation

Focal adhesion, a key mechanosensor at the cell-ECM interface, is believed to regulate cell spreading and migration^{29,56}. Given that seeing a dramatic change in spreading area and cell migration speed, we hypothesize a corresponding change in focal adhesion. Focal adhesion serves as a bridge between ECM and cells, connecting ECM at one end and connecting cytoskeleton at the other, as shown in FIGURE 4.9a. In our study, confocal microscopy is used to characterize the morphology of actin, a major component of stress fiber, and vinculin, a structural protein of focal adhesion. On hydrogel with smaller pores, more stress fibers are formed in cells, as shown in FIGURE 4.9b. Meanwhile, on all the hydrogels, focal adhesions of cells display an elongated shape at one end of stress fibers with a typical length of 3–5 μm , indicating the maturation of focal adhesions, as shown in FIGURE 4.9b. Moreover, larger focal adhesions are formed on hydrogel with smaller pores, as shown in FIGURE 4.9c. Focal adhesion number per cell also increases as hydrogel pore size decreases, as shown in FIGURE 4.9d.

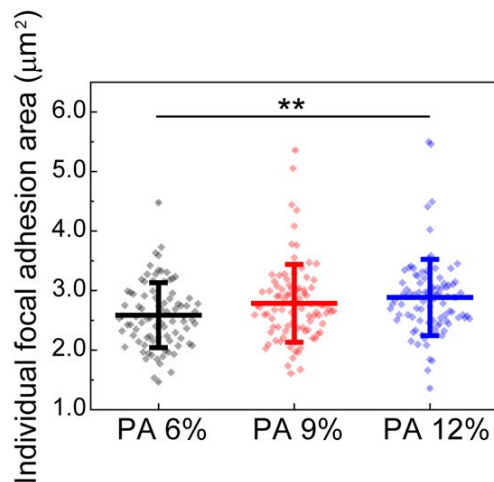
(a)



(b)



(c)



(d)

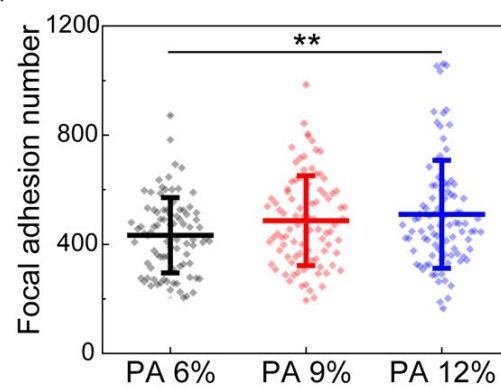


FIGURE 4. 9 Focal adhesion formation of cells on hydrogels with different microstructure.

(a) Schematic view of an adherent cell on hydrogel-glass substrate. Cells adhere to ECM ligands by focal adhesion, which is further connected by actin stress fiber inside cells. (b) Confocal fluorescent microscopy images of cell focal adhesions on different hydrogels (scale bar, 50 μm). (c) Individual focal adhesion area of cells on different hydrogels (Mean $\pm$ Std, N>88). (d) Total focal adhesion area of cells on different hydrogels (Mean $\pm$ Std, N>88).

In the meantime, total focal adhesion area within a single cell follows the same trend of individual focal adhesion area, as shown in FIGURE 4.10. Our results suggest that the ability of cells to form focal adhesions is encouraged on hydrogel with smaller pores. Previous researchers develop ECM patterns with hundreds nanometer size and several micrometer spacing, and show that focal adhesion number increases as the density of ECM nanodots increases⁵⁷. In this study, we find that increasing of focal adhesion number is correlated with the increase in the number density of collagen clusters. Therefore, we believe that the phenomenon observed here shares the same mechanisms that correlate focal adhesion formation with ECM ligand density^{58,59}.

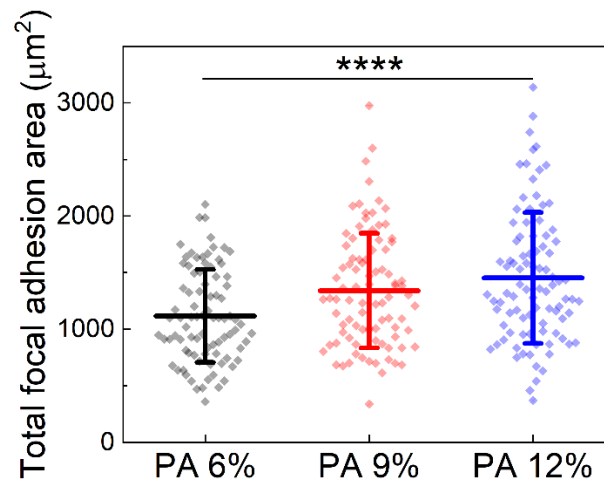


FIGURE 4. 10 Total focal adhesion area of individual cell.

4.3.7 Effects of hydrogel microstructure on YAP nuclear translocation

To test whether the observed differences in focal adhesion distribution is related to cellular mechanotransduction, we quantify Yes-associated protein (YAP), which is a key transcriptional regulator in mechanotransduction⁶⁰⁻⁶². Via translocating from cytoplasm to nucleus, YAP transcriptional activity is enhanced and further influence cellular response such

as cell spreading and focal adhesion formation^{63,64}. Translocation of YAP is regulated by the tension of F-actin cytoskeleton, which is usually revealed by the formation of more stress fibers^{65,66}, as shown in FIGURE 4.11a. YAP and actin in cells are stained and imaged on different hydrogels with confocal microscope. We find that as hydrogel pore size decreases, YAP is more localized in the nuclear region of cell, as shown in FIGURE 4.11c. The nuclear/cytosolic YAP ratio shows that nuclear YAP increases significantly on the hydrogel with smaller pores, as shown in FIGURE 4.11b. Moreover, more stress fibers are also formed in cells grown on the hydrogel with smaller pores, as shown in FIGURE 4.11d. Our results demonstrate that hydrogel microstructure alters YAP translocation, indicating hydrogel microstructure act as a physical regulator which directly modulates cellular mechanotransduction.

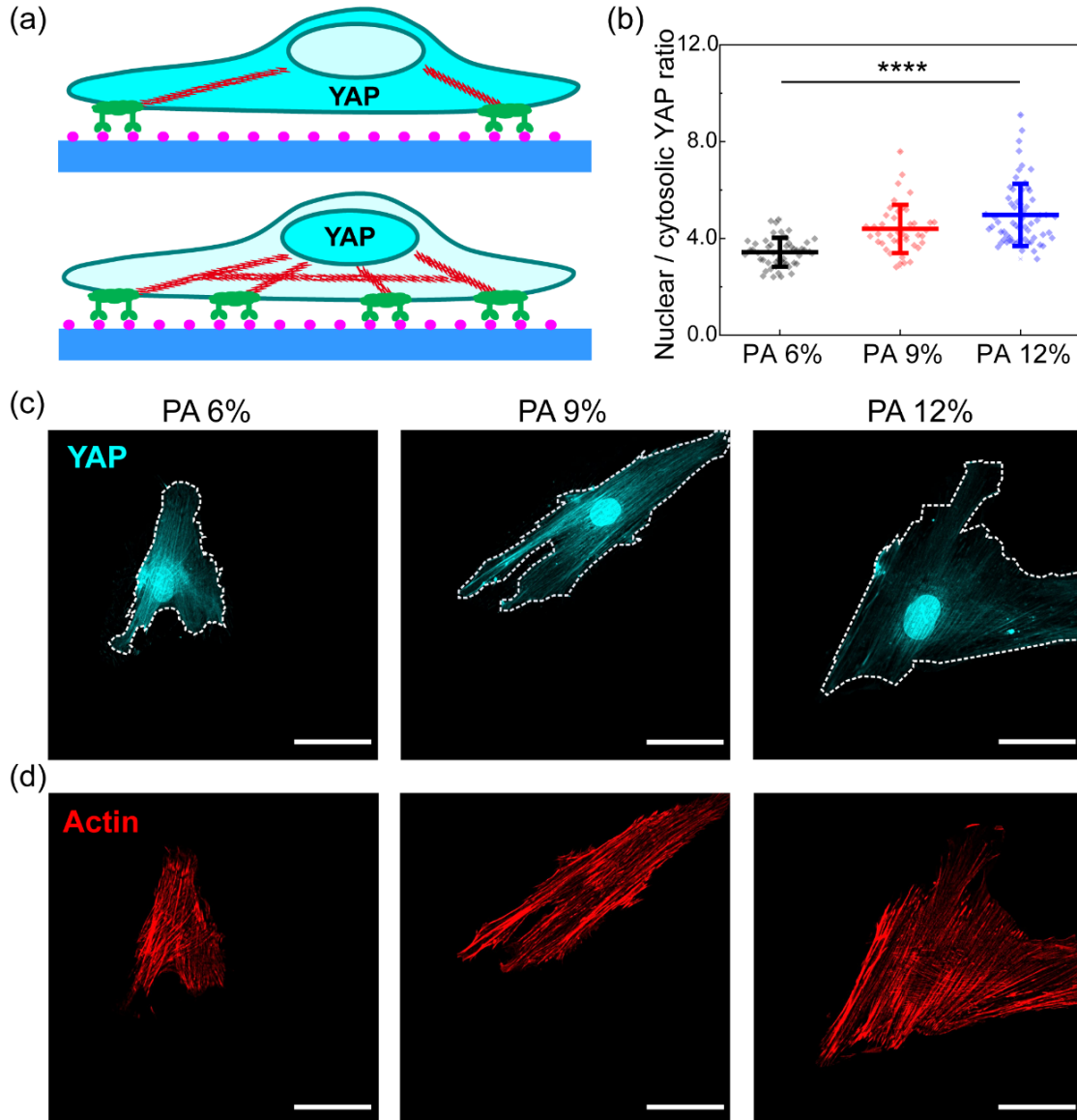


FIGURE 4. 11 YAP translocation of cells on hydrogels with different microstructure.

(a) Schematic view of YAP translocation regulated by the stress fiber. With more stress fiber formed, YAP nuclear translocation is promoted. (b) The ratio of nuclear YAP versus cytosolic YAP on different hydrogels (Mean $\pm$ Std, $N > 42$). (c) Confocal fluorescent microscopy images of YAP localization in cells on different hydrogels. Dashed white line represents the cell outline (scale bar, 50 μm). (d) Confocal fluorescent microscopy images of actin in cells on different hydrogels. (scale bar, 50 μm).

4.4 Conclusions and discussion

In this study, we fabricate hydrogel-glass substrates to investigate the effects of hydrogel microstructures on hBMSCs behavior, including cell attachment, proliferation, spreading and migration. We find that the number density of collagen cluster is larger on hydrogel with smaller pores, while the collagen amount of each cluster is not significantly affected. In

addition, we show that varying hydrogel microstructure can directly affect cell behaviors. Cell spreading area and migration speed increase on hydrogel with smaller pores, while cell attachment and proliferation is not significantly affected. At subcellular scale, both focal adhesion area and number increase as the hydrogel pore size decreases. Moreover, we find significantly increased YAP nuclear localization on hydrogel with smaller pores, suggesting that the mechanotransduction of cell is modulated by varying hydrogel microstructure. In summary, our results reveal that cells response to hydrogel microstructure, which is often overlooked in cell-substrate interactions. Moreover, the correlations between collagen cluster number density and cell behaviors such as cell spreading and focal adhesion formation suggest that, collagen distribution may act as the medium to transmit the mechanical cues such as hydrogel microstructure into cells. This study also demonstrates that hydrogel microstructure can be adopted as a physical regulator to alter cell behavior.

Chapter 5 Decoupling the Effects of Nanopore Size and Surface Roughness on the Attachment, Spreading, and Differentiation of BMSCs

5.1 Abstract

The nanopore size and roughness of nanoporous surface are two critical variables in determining stem cell fate, but little is known about the contribution from each cue individually. To address this gap, we use two-dimensional nanoporous membranes with controlled nanopore size and roughness to culture bone marrow-derived mesenchymal stem cells (BMSCs), and study their behaviors such as attachment, spreading and differentiation. We find that increasing the roughness of nanoporous surface has no noticeable effect on cell attachment, and only slightly decreases cell spreading areas and inhibits osteogenic differentiation. However, BMSCs cultured on membranes with larger nanopores have significantly fewer attached cells and larger spreading areas. Moreover, these cells cultured on larger nanopores undergo enhanced osteogenic differentiation by expressing more alkaline phosphatase, osteocalcin, osteopontin, and secreting more collagen type I. These results suggest that although both nanopore size and roughness can affect BMSCs, nanopore size plays a more significant role than roughness in controlling BMSC behavior.

5.2 Introduction

Bone marrow-derived mesenchymal stem cells (BMSC) are skeletal progenitor cells that originate from bone marrow, which has the ability to differentiate into many cell types such as adipocyte cell, osteoblast cell and chondrocyte cell^{67,68}. Among all three differentiation lineages, osteogenic cells, which are responsible for the bone remodeling and regeneration^{69,70}, have long been regarded as a potential cellular substrate to cure bone diseases such as osteoporosis, or repair bone tissues by growing new bone around artificial implant⁷¹⁻⁷⁵. By designing the two-dimensional surface topography of bone implant on the nanometer scale,

growth of BMSCs around implant can be promoted and thus enable enhanced bone healing⁷⁶⁻⁷⁸. In particular, since nanoporous surface topography of bone plays a key role in guiding bone tissue formation, mimicking this nanoporous surface topography to design artificial implant is thought to direct the fate of BMSCs similar to native bone structure, which can promote better clinical performance⁷⁹⁻⁸².

Nanoporous surface topography can be characterized by many parameters, with nanopore size and surface roughness being two of the most fundamental ones. Both parameters can dramatically affect the behavior of BMSC cells such as attachment, spreading and differentiation, which are all critical to cell survival and function⁸³⁻⁸⁵. By mimicking the nanopore of bones, nanotube of pore size between 15-100 nm has shown significant influence on the differentiation lineage of BMSCs^{81,82,86}. In addition, by culturing cells on non-porous surface with similar roughness to cortical bone (10-100 nm)⁸⁷, it was shown that roughness has no significant influence on cell differentiation, but the rough surface can improve BMSC cell adhesion compared with smooth surface⁸⁸. However, a significant limitation of current studies is that these two factors are often coupled due to the material fabrication processes, which makes it unclear to which extent does each factor contribute to BMSC behavior. Therefore, to better design bone implant surface using nanoporous topography that can precisely regulate BMSC cell behavior, it is necessary to decouple the contribution of nanopore size and roughness.

In this paper, the goal is to distinguish the contribution of nanopore size and roughness to the BMSCs behavior. To achieve this, we culture BMSCs on biocompatible nanoporous polycarbonate membranes with controlled nanopore size and surface roughness, yet with similar stiffness to cortical bones⁸⁹, and study their behavior such as attachment, spreading and differentiation. Our results show that increasing the roughness of nanoporous surface has no obvious effect on cell attachment, and only slightly decrease cell spreading area and inhibit osteogenic differentiation. In addition, BMSCs cultured on membranes with larger nanopores have significantly fewer attached cells and larger spreading area. Moreover, cells undergo enhanced osteogenic differentiation by expressing more alkaline phosphatase, osteocalcin, osteopontin, and secreting more collagen type I. Our results suggest that, compared with

surface roughness, nanopore size plays a more significant role in governing BMSC cell behavior: larger nanopore can significantly enhance cell spreading and osteogenic differentiation.

5.3 Results

5.3.1 Nanoporous membrane characterization, and cell culture device fabrication

Nanoporous polycarbonate membranes are used as a model system to study effect of nanopore size and surface roughness on the BMSC behavior. We select commercially available nanoporous membranes which have randomly distributed nanopores that are mono-disperse in size due to the track etching method by which they are formed⁹⁰. They also have different surface profile on each side, with one side rough and the other side smooth. During fabrication, these membranes are also treated with polyvinylpyrrolidone to make them hydrophilic, which enhances cell attachment⁹¹. We select membranes with different nanopore sizes (10 nm, 80 nm, 200 nm) to mimic the nanopore sizes found within cortical bone⁸⁶. Membranes are examined by scanning electron microscopy (SEM), as shown in FIGURE 5.1a.

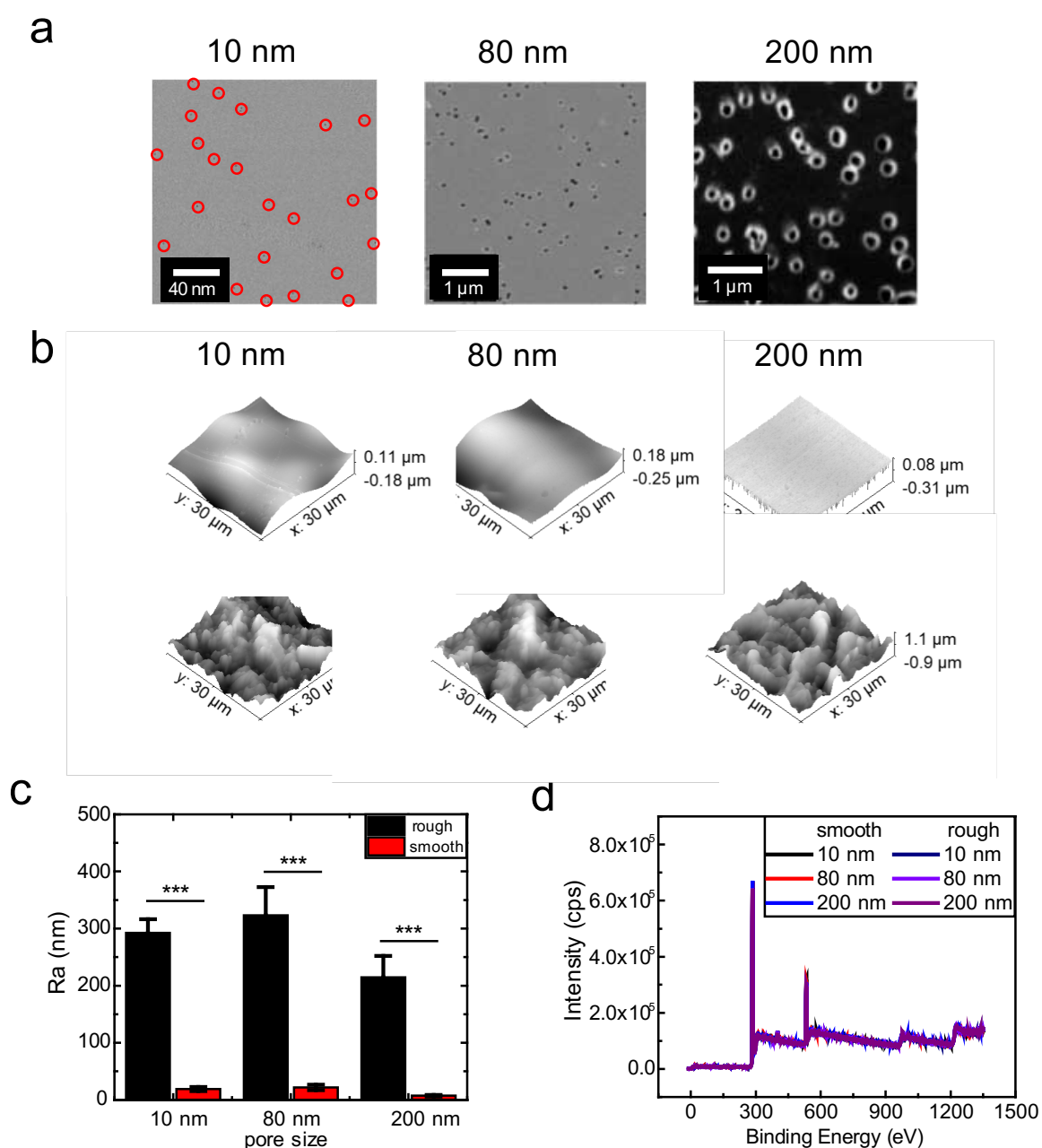


FIGURE 5.1 Surface characterization of nanoporous membranes.

(a) Representative scanning electron microscopy (SEM) images of smooth side of nanoporous polycarbonate membranes. Nanopore locations are marked out with red circles for 10 nm pore size membranes. (b) Representative 3D atomic force microscopy images of smooth (top row) and rough (bottom row) side of different pore size (10 nm, 80 nm, 200 nm) polycarbonate membranes. (c) Mean roughness (Ra) of the polycarbonate membrane surface based on AFM measurement (Mean $\pm$ Std, $n=3$). (d) X-ray photoelectron spectroscopy (XPS) surface chemistry characterization of nanoporous polycarbonate membranes.

Additionally, SEM images of larger field of view are provided in FIGURE 5.2. SEM images are further processed with Image J software to calculate the nanopore density of each membrane. Results confirm that membranes are of similar nanopore density, which are around 4×10^8 pores/cm², ensuring that the same number of nanopores is encountered by each cell. Furthermore, the open area (%) of the membrane is below 11%, suggesting majority of cell body is supported by the solid surface while it still interacts with the tiny nanopores on the surface, as shown in table 1.

Table 1			
Nanopore size (nm)	10	80	200
Nanopore Density (pores/cm ²)	4.38×10^8	4.02×10^8	3.48×10^8
Open Area (%)	0.0344%	2.02%	10.91%

Since the membranes have different roughness on each side, we characterize the topography of the two sides using AFM, as shown in FIGURE 5.1b. The arithmetic average roughness (Ra) is calculated for the solid regions of nanoporous membranes. Results show that the roughness of one of the surfaces is an order of magnitude higher than the other surface, as shown in FIGURE 5.1c; in this paper, we refer to them as rough and smooth, respectively. To confirm that the surface chemistry of the membranes is not affected by the porosity and roughness, X-ray photoelectron spectroscopy (XPS) analysis is performed on the various membranes. The XPS results show no difference among the chemical composition of membranes, as shown in FIGURE 5.1d. Moreover, there are two peaks corresponding to carbon and oxygen with an area ratio of 3:1, as expected for polycarbonate. Furthermore, there is a small peak corresponding to nitrogen, which confirms the presence of a polyvinylpyrrolidone coating.

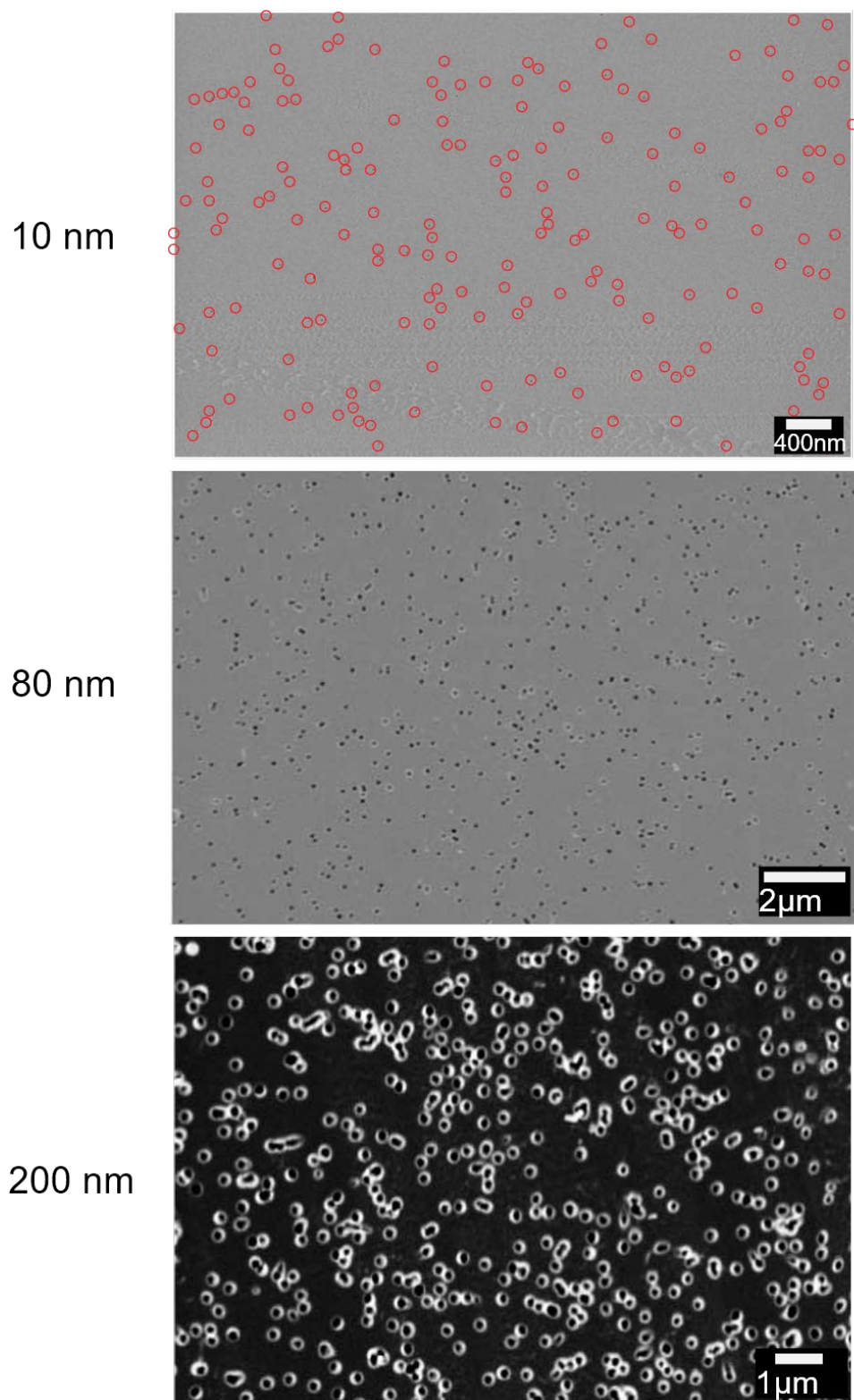


FIGURE 5.2 Additional SEM images of membranes.

Representative scanning electron microscopy (SEM) images of smooth side of nanoporous polycarbonate membranes. Nanopore locations are marked out with red circles for 10 nm pore size membranes.

To culture cells, membranes are sandwiched between two spacer layers to ensure that the membrane is flat and that media can reach the cells from all sides. The sandwich structure is then UV sterilized, immersed in medium, and seeded with cells; a simplified workflow is shown in FIGURE 5.3a. After 16 hours, BMSC cells are examined under SEM after being fixed and dried using critical point drying. Images using an SEM suggest that cells are fully spread out and able to span over thousands of nanopores on the membranes, which shows that the membranes are biocompatible. Examples of BMSCs cultured on rough and smooth membranes with 80 nm pores are shown in FIGURE 5.3b and FIGURE 5.3c.

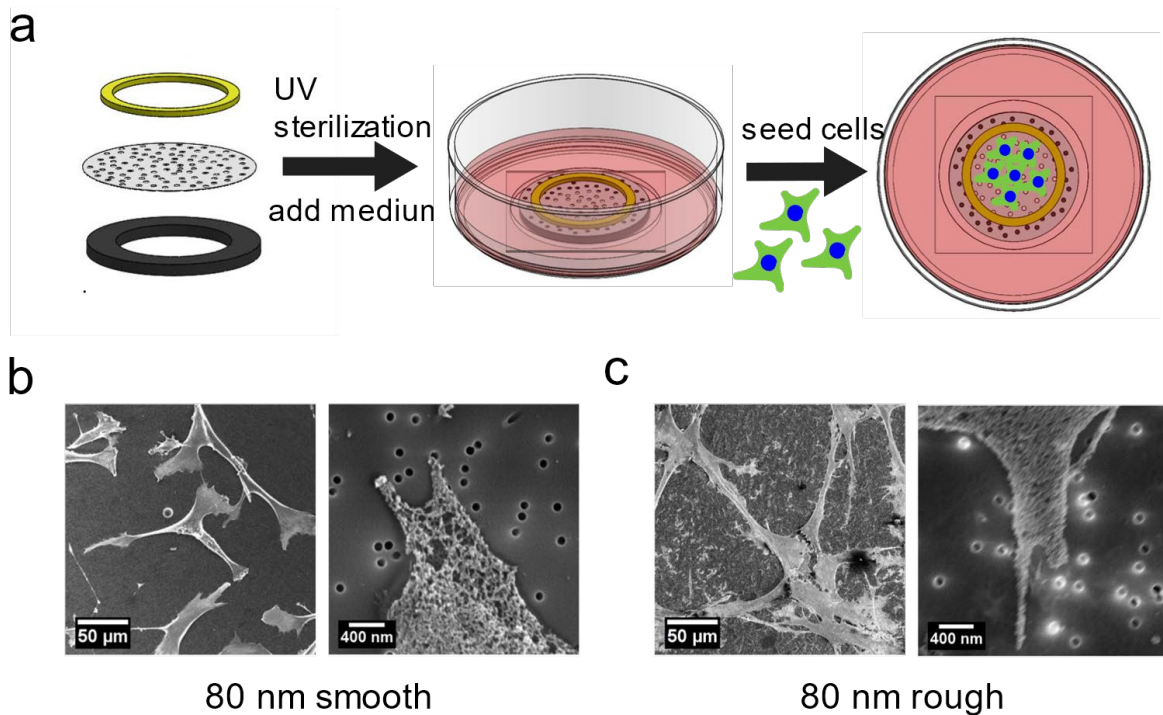


FIGURE 5.3 cell culture device.

(a) Fabrication workflow of cell culture device. (b) Representative scanning electron microscopy (SEM) image of cells cultured on 80 nm smooth membrane (left) and local zoom (right). (c) Representative scanning electron microscopy (SEM) image of cell cultured on 80 nm rough membrane (left) and local zoom (right)

5.3.2 BMSC initial adhesion number on membranes

To investigate the effects of nanopore size and surface roughness on initial cell adhesion of BMSCs, we count the number of cells 16 hours after seeding. For fluorescence imaging, we fix the cells and stain their nuclei using DAPI, as shown in FIGURE 5.4a.

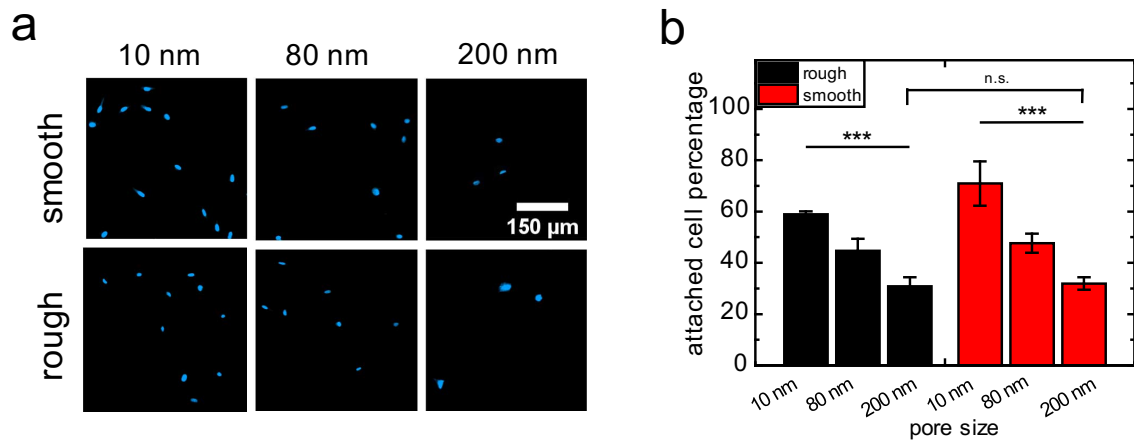


FIGURE 5. 4 Initial cell attachment percentage.

(a) Nuclei stain of BMSC on smooth and rough side of membranes with different nanopore size, 16 hours after seeding. (b) Initial cell attachment percentage after 16 hours (Mean $\pm$ Std, $n=3$).

Additionally, fluorescent images of larger field of view are provided in FIGURE 5.5.

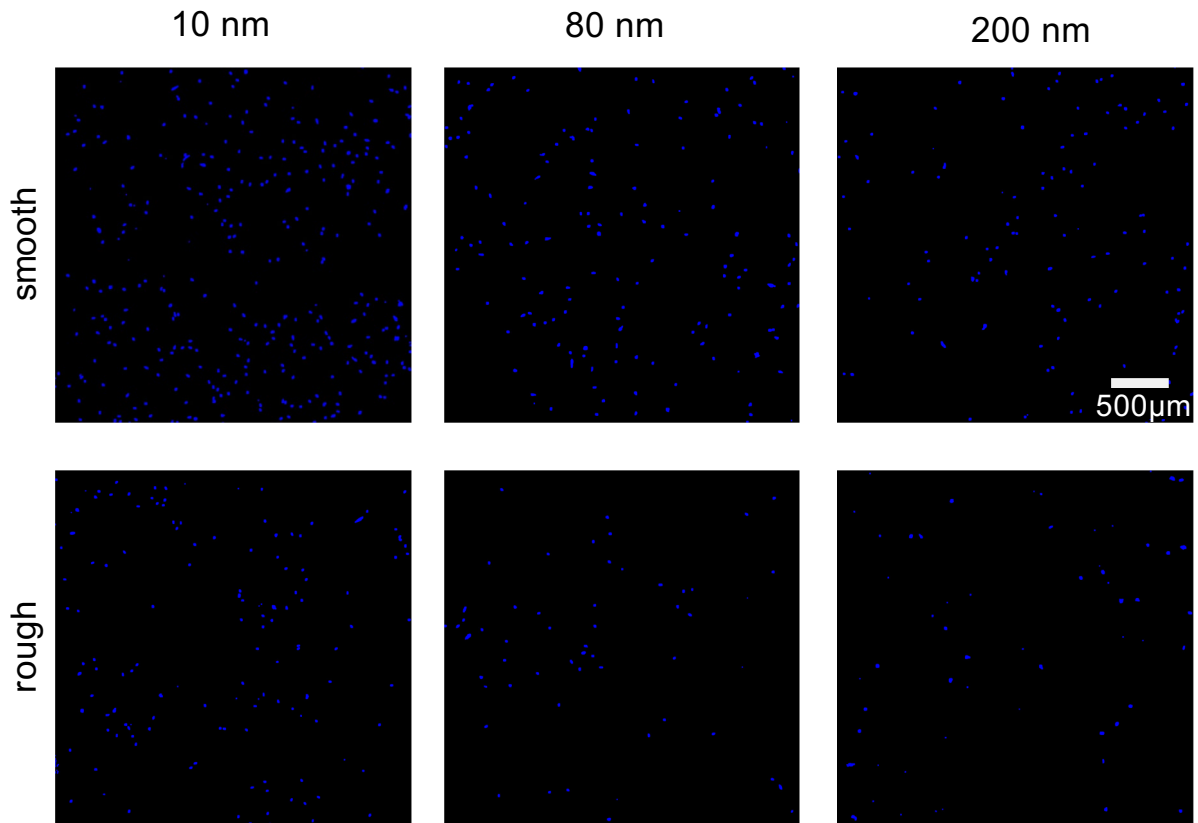


FIGURE 5. 5 Additional images of attached BMSC cells with nucleus stained on membranes with different nanopore size and roughness.

Cell nuclei are then counted to determine the cell number. Surprisingly, our results show that porous membranes with larger nanopores have fewer cells attached, as shown in FIGURE 5.4b. Since more hydrophilic surfaces can promote protein adsorption and thus enhance cell adhesion^{91,92}, we then quantify the hydrophilicity of the membranes by measuring static contact angle using sessile drop method. Indeed, membranes with larger nanopores have bigger contact angles, therefore, are less hydrophilic, as shown in FIGURE 5.6.

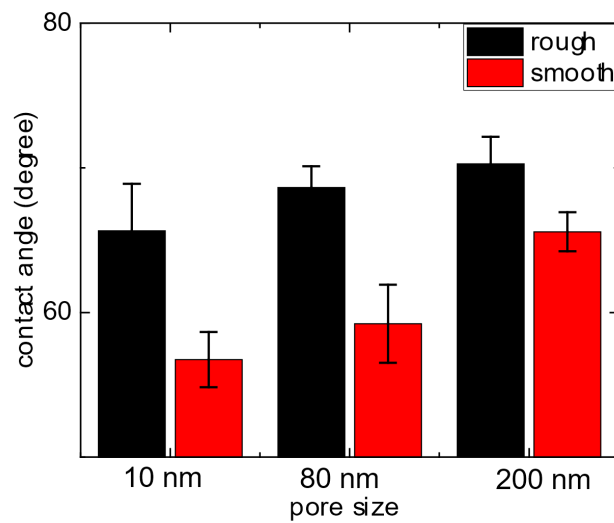


FIGURE 5. 6 Water contact angle of nanoporous membranes, measured by sessile drop method (Mean $\pm$ Std, n=3).

Meanwhile, as nanopore size increases from 10 nm to 200 nm, the solid surface area available for cell attachment decreases from 99.97% to 89.09% (table 1), which may also contribute to decreased cell attachment. On membranes with larger nanopores, the decreased hydrophilicity and available surface area may inhibit cell adhesion, and thereby decrease cell adhesion number. While smooth surfaces reportedly encourage better attachment [12], we do not find that surface roughness significantly affects the initial cell adhesion number, as shown in FIGURE 5.4b. This is different from other researches done on non-porous surface showing that better cell adhesion is achieved as roughness decrease from 105.6 nm to 1.8 nm⁹³. We attribute our results to a larger contribution by nanopore size on cell adhesion, which overshadows the contribution by surface roughness, as surface discontinuity can greatly affect cell adhesion behavior⁹⁴, which is mainly determined by nanopore size in our scenario.

5.3.3 BMSC spreading behavior and focal adhesion size quantification

In addition, we also study the morphology of BMSC cells cultured on the membranes. We fluorescently stain the cell cytoplasm with cell tracker green and fix the cell with formaldehyde after 16 hours of culture. Cells are then imaged by using confocal microscopy to obtain a z-stack images. The confocal images are further analyzed with MATLAB to calculate cell spreading area, volume and height. Top and side views of the 3D cell shape on smooth membranes are shown in FIGURE 5.7a. Results show that the spreading area of BMSCs on the 200 nm pores is almost 2-fold larger than on 10 nm pores. By contrast, the spreading area increases only around 20% when the surface is smoother, as shown in FIGURE 5.7b. Similarly, the volume of BMSCs on the 200 nm pores is almost 2-fold larger than on 10 nm pores, and a smooth surface results in a 25% increase in cell volume, as shown in FIGURE 5.7c.

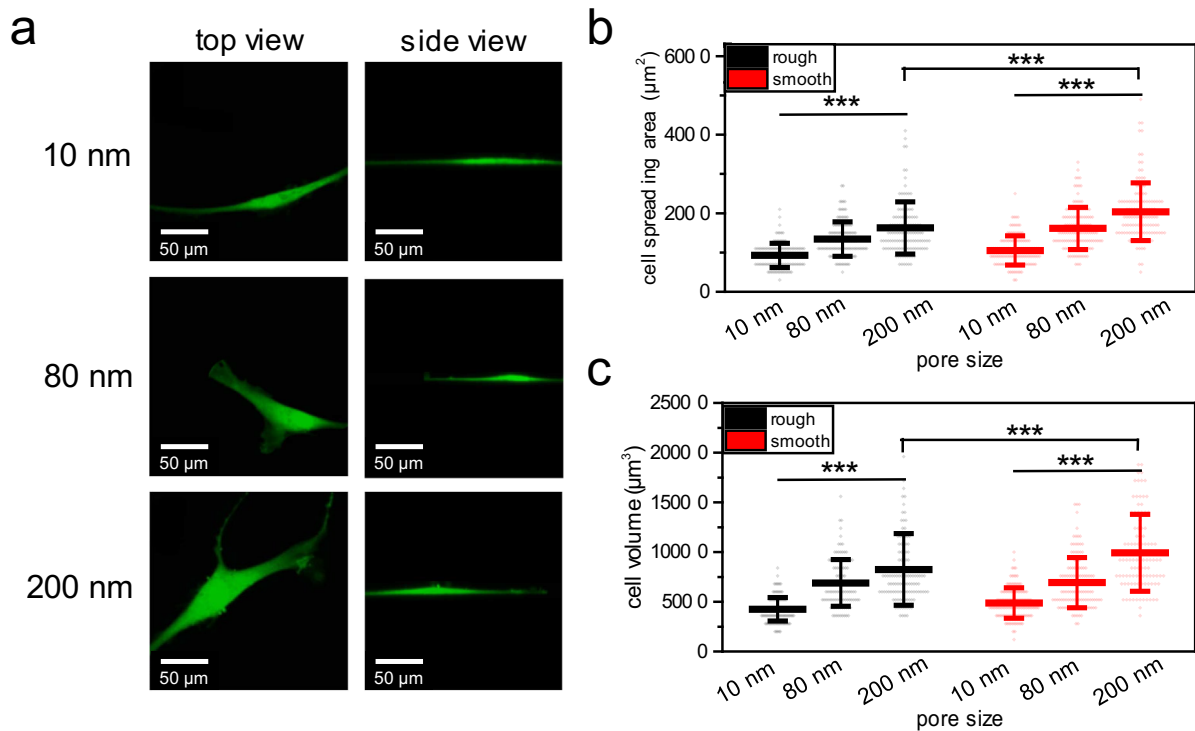


FIGURE 5. 7 Cell spreading behavior on nanoporous membranes.

(a) Top and side view of confocal images of BMSC on smooth nanoporous membranes. (b) Cell spreading area on nanoporous membranes with different nanopore size and roughness (Mean $\pm$ Std, $n > 100$). (c) Cell volume on porous membranes with different nanopore size and roughness (Mean $\pm$ Std, $n > 100$).

The similar trends observed in cell spreading area and volume imply that the cell height is not affected by the different membrane conditions, as shown in FIGURE 5.8.

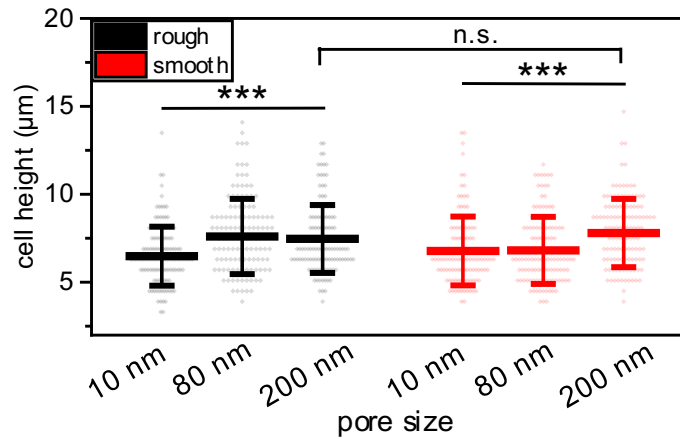


FIGURE 5. 8 Cell height on nanoporous membranes with different nanopore size and roughness (Mean $\pm$ Std, n>100).

Given that we see a dramatic change in spreading area, we hypothesize a corresponding change in focal adhesion structures, which are thought to involve in mechanotransduction process that can affect cell spreading area⁹⁵⁻⁹⁷. Focal adhesions (FA) are structures that mechanically link actin stress fibers, the force transducing unit during mechanotransduction, with the extracellular substrate through proteins such as vinculin⁹⁸⁻¹⁰⁰. A simplified schematic of cell adhesion structure is shown in FIGURE 5.9a. To compare morphology of focal adhesions, we culture BMSCs on nanoporous membranes for 16 hours and fluorescently stain actin and vinculin^{101,102}, followed by imaging them using confocal microscopy. Our results show that cells exhibit different cellular structures on membranes with different nanopore sizes when the roughness is held constant. When the nanopores are larger, cells are in spread-out, polygon shapes with thicker actin stress fibers, and focal adhesions are oval-shaped whose long axis is aligned with the actin stress fibers. The elongated FA shape indicates their maturation and suggests that strong adhesion is formed between cell and substrate^{103,104}. On membranes with smaller nanopores, cells are elongated with fewer stress fibers and focal adhesions are round with no well-defined orientation, which suggests that focal adhesions are immature and that adhesion between the cells and substrate is poor, as

shown in FIGURE 5.9b. Interestingly, after quantifying the focal adhesion size, we find that the average focal adhesion size increases with nanopore size, as shown in FIGURE 5.9c. Similarly, we find that surface roughness has a significant influence on focal adhesion morphology. On smooth surfaces, cells form large, oval focal adhesions, with orientations aligned with actin stress fibers, while on rough surfaces, focal adhesions are mostly small and round, as shown in FIGURE 5.9b-c. Together, these results suggest both nanopore size and surface roughness play a significant role in regulating focal adhesion morphology and size, as larger nanopore and smoother surface promote larger focal adhesion formation, and at the same time, we see an increase of spreading area. This is consistent with previous reports showing a correlation between focal adhesion, morphology and cell spreading area¹⁰⁵.

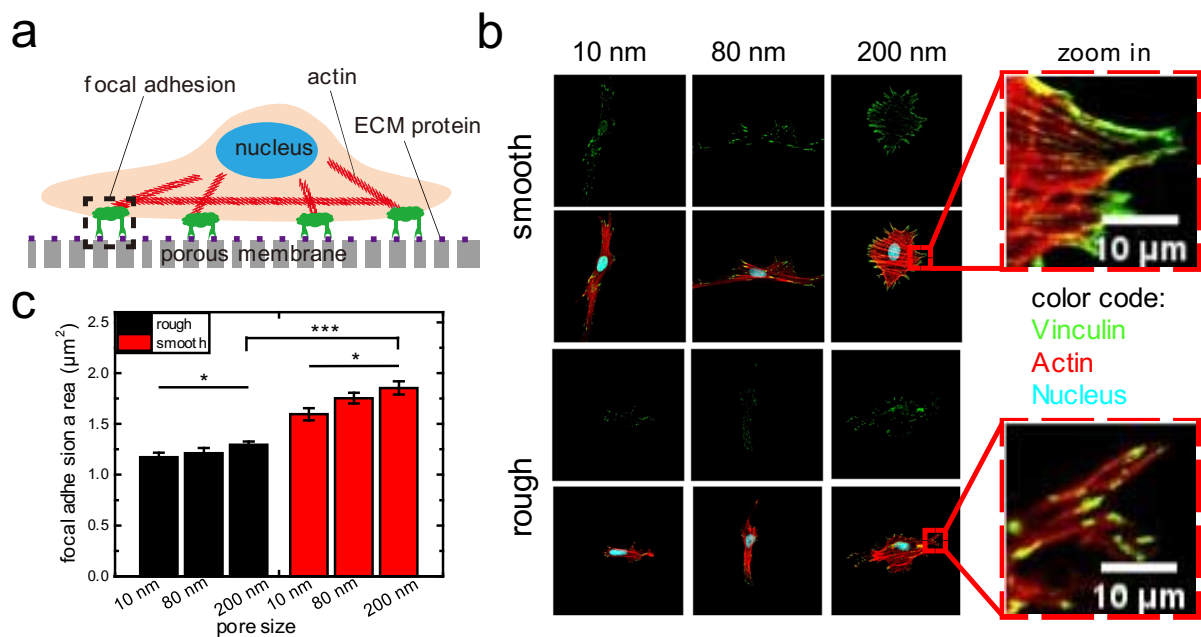


FIGURE 5. 9 cell morphology and focal adhesion quantification.

(a) Schematic cartoon of an adherent cell on nanoporous membranes. Cells adhere to ligands of extracellular matrix (ECM) on nanoporous membrane by focal adhesion, which is further connected by actin stress fiber inside cells. (b) Representative confocal microscope images of cells on different nanopore size and roughness membranes, vinculin (green), actin (red) and nucleus (cyan) are stained. (c) Individual focal adhesion area of cells on different nanopore size and roughness membrane (Mean $\pm$ Ste, n>9, N>700).

In the nanopore sizes used, the total nanopore area ranges from less than 1% to over 10%, which may lead to differences in the diffusion of cell culture medium beneath the cells.

To investigate whether the observed differences in cell spreading area are due to the diffusing profile difference as a result of different nanopore area, we fix the nanopore size of membranes at 200 nm and attach a polyacrylamide hydrogel layer underneath, which enables us to change the diffusion profile beneath the membrane without changing its surface topography, as illustrated in a simplified cartoon in FIGURE 5.10a. The pore size of the gel is tuned by varying the acrylamide and N, N'-Methylenebisacrylamide concentration. As examined by scanning electron microscopy, the apparent pore radius of the dried gel sample change from 3 μm to 1.2 μm for acrylamide concentrations of 6% to 12%, as shown in FIGURE 5.10b. However, it's known that SEM will overestimate the pore size of hydrogel due to the drying process in the sample preparation. Standard procedures such as SDS-PAGE which use acrylamide concentration from 5% to 15% are able to size separate protein from ~ 212 kDa to ~ 13 kDa, which has the same size range as the protein in fetal bovine serum premixed in cell culture medium¹⁰⁶. The hydrogel layer is therefore assumed to hinder the protein exchange between cell and culture medium beneath it. When BMSCs are cultured on the hydrogel-modified system, we find the presence of the hydrogel has no effect on cell spreading area or volume, as shown in FIGURE 5.10c and FIGURE 5.10d. These results suggest cell spreading area and volume are not influenced by the diffusion profile change beneath the membranes, suggesting membrane nanopore size might affect cell spreading area in the way by providing mechanical stimulus other than changing the diffusing profile.

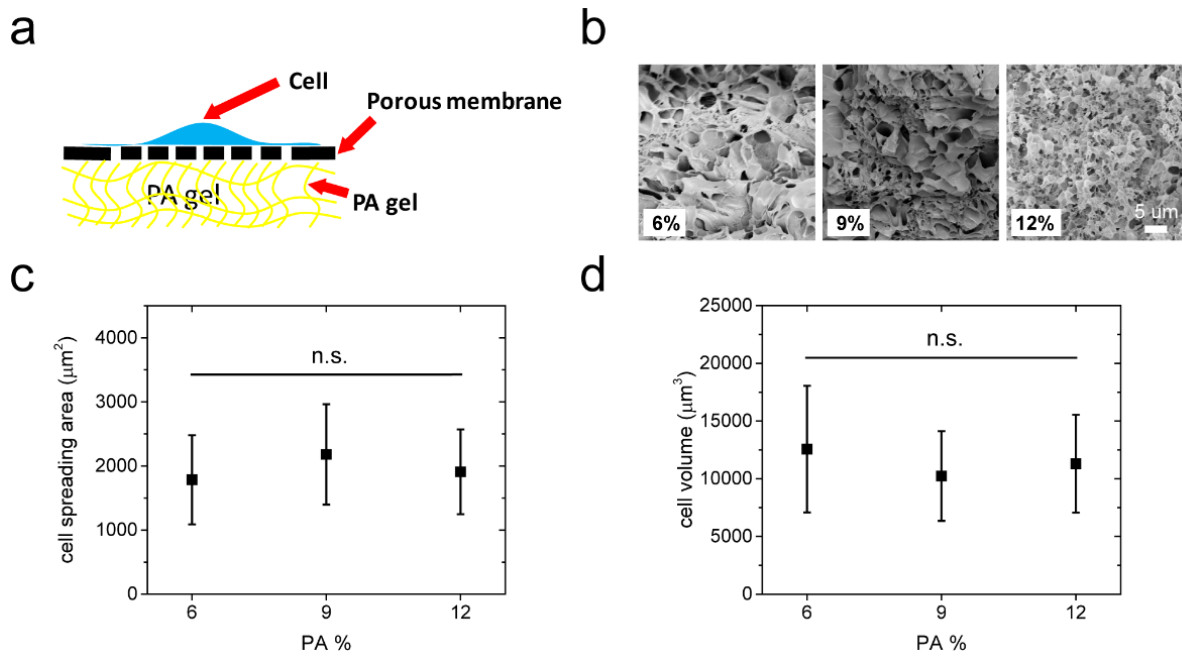


FIGURE 5.10 cell spreading behavior on membrane hydrogel composite.

(a) Schematic of membrane hydrogel composite. (b) Scanning electron microscope images of different hydrogel (6% PA, 9% PA, 12% PA). (c) Cell spreading area on membrane hydrogel composite (Mean $\pm$ Std, $n > 24$). (d) Cell volume on membrane hydrogel composite (Mean $\pm$ Std, $n > 24$).

5.3.4 Osteogenic differentiation of BMSC cells on nanoporous membranes

One of the primary functions of BMSC cells is their ability to differentiate into various lineages, of which osteogenic differentiation is most desirable for bone implant. Given that in short time scale, nanopore size and roughness can dramatically affect the cell attachment and spreading, we expect the long-term behavior of BMSC cells will also be altered. Since the membranes are very stiff ($\sim$ Gpa), our system is particularly suitable to study osteogenic differentiation which is known to occur on stiff substrate^{107,108}. In the process of osteogenic differentiation, ALP, an enzyme localized to the outside of the plasma membrane of cells, will be highly expressed in the early stage¹⁰⁹. Then extracellular matrix proteins such as collagen type I, osteocalcin, and osteopontin will be secreted to initiate the mineralization process¹¹⁰⁻¹¹³. To induce differentiation, BMSC cells are seeded onto nanoporous membranes to grow in MSC growth media till confluency after 5-7 days, and subsequently cultured in osteogenic differentiation media. Then, to quantify the degree of osteogenic differentiation, we perform fluorescent staining on the four osteogenic marker proteins: Alkaline phosphatase protein (ALP), collagen type I, osteocalcin (OCN), and osteopontin (OPN). ALP and collagen

type I are stained after 16-18 days of culture in osteogenic differentiation media. Osteocalcin and osteopontin are stained after 24-26 days of culture in osteogenic differentiation media. ALP is fluorescently stained using ImmPACT® Vector® Red Alkaline Phosphatase (AP) Substrate and imaged under a fluorescence microscope, as shown in FIGURE 5.11a. Other osteogenic differentiation markers, collagen type I, osteocalcin, and osteopontin are immunostained with fluorescent antibody and imaged with fluorescence microscope, results are shown in FIGURE 5.11c, e, g.

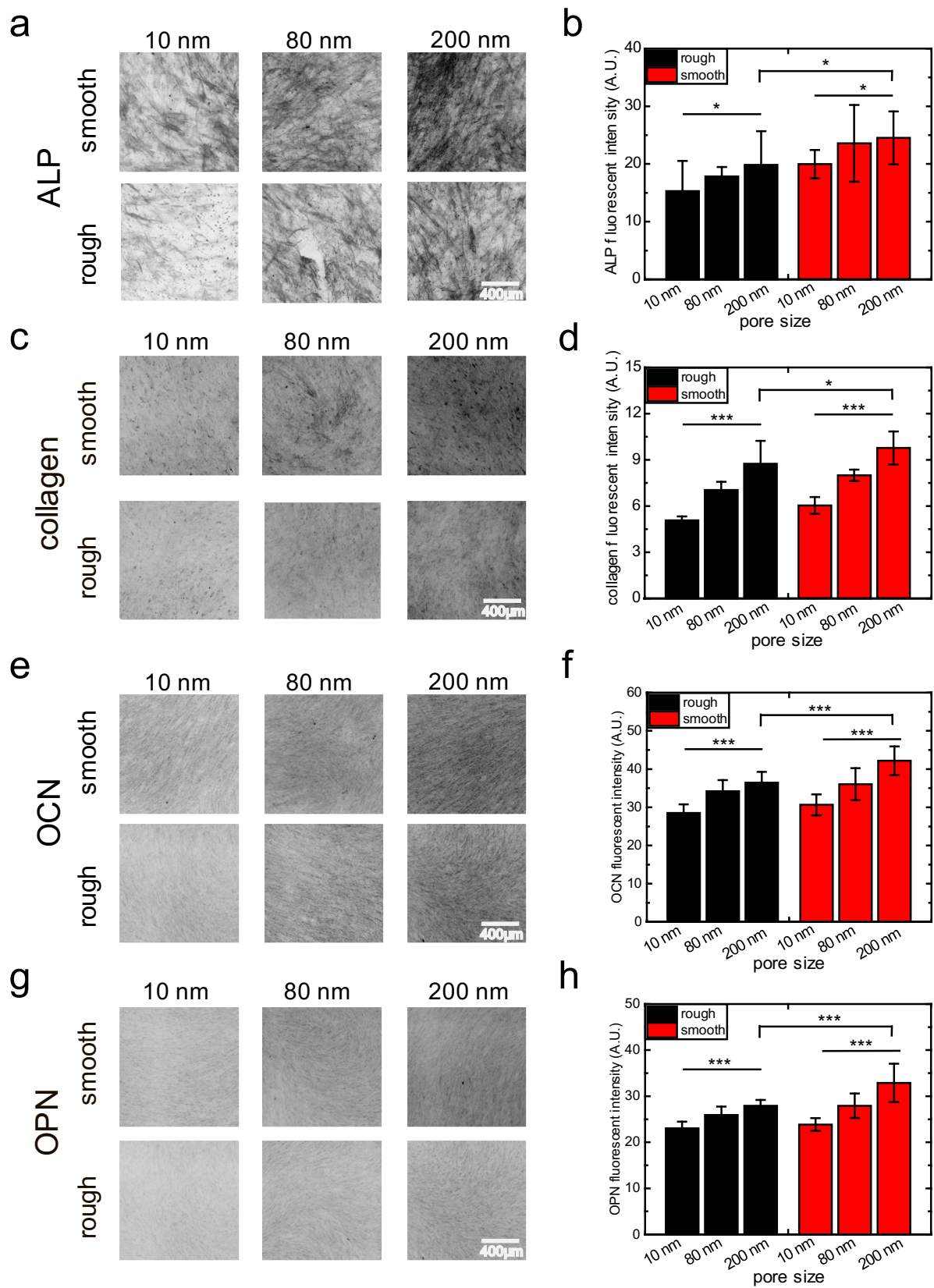


FIGURE 5.11 BMSC differentiation on nanoporous membranes.

(a) ALP fluorescent staining of BMSC monolayer after 16-18 days of culture in osteogenic differentiation media. (b) Fluorescence intensity quantification of ALP in BMSC monolayer (Mean $\pm$ Std, n=3, N=15). (c) Immunofluorescence staining of secreted collagen by BMSC monolayer after

16-18 days of culture in osteogenic differentiation media. (d) Fluorescence intensity quantification of immuno-stained collagen secreted by BMSC monolayer (Mean $\pm$ Std, n=3, N=15). (e) Immunofluorescence staining of secreted osteocalcin by BMSC monolayer after 24-26 days of culture in osteogenic differentiation media. (f) Fluorescence intensity quantification of immuno-stained osteocalcin secreted by BMSC monolayer (Mean $\pm$ Std, n=3, N=15). (g) Immunofluorescence staining of secreted osteopontin by BMSC monolayer after 24-26 days culture in osteogenic differentiation media. (h) Fluorescence intensity quantification of immuno-stained osteopontin secreted by BMSC monolayer (Mean $\pm$ Std, n=3, N=15).

To compare ALP content between different samples, the average fluorescence intensity of each sample is calculated. Results show the fluorescence intensity of ALP is higher on membranes with larger nanopores, suggesting increase of nanopore size may stimulate the ALP expression, as shown in FIGURE 5.11b. The bare membranes are used as negative control groups, which have fluorescence intensity 10-fold smaller than the cell samples, suggesting that the fluorescent signals in cell samples are mostly contributed by cell monolayer, but not membranes, as shown in FIGURE 5.12a.

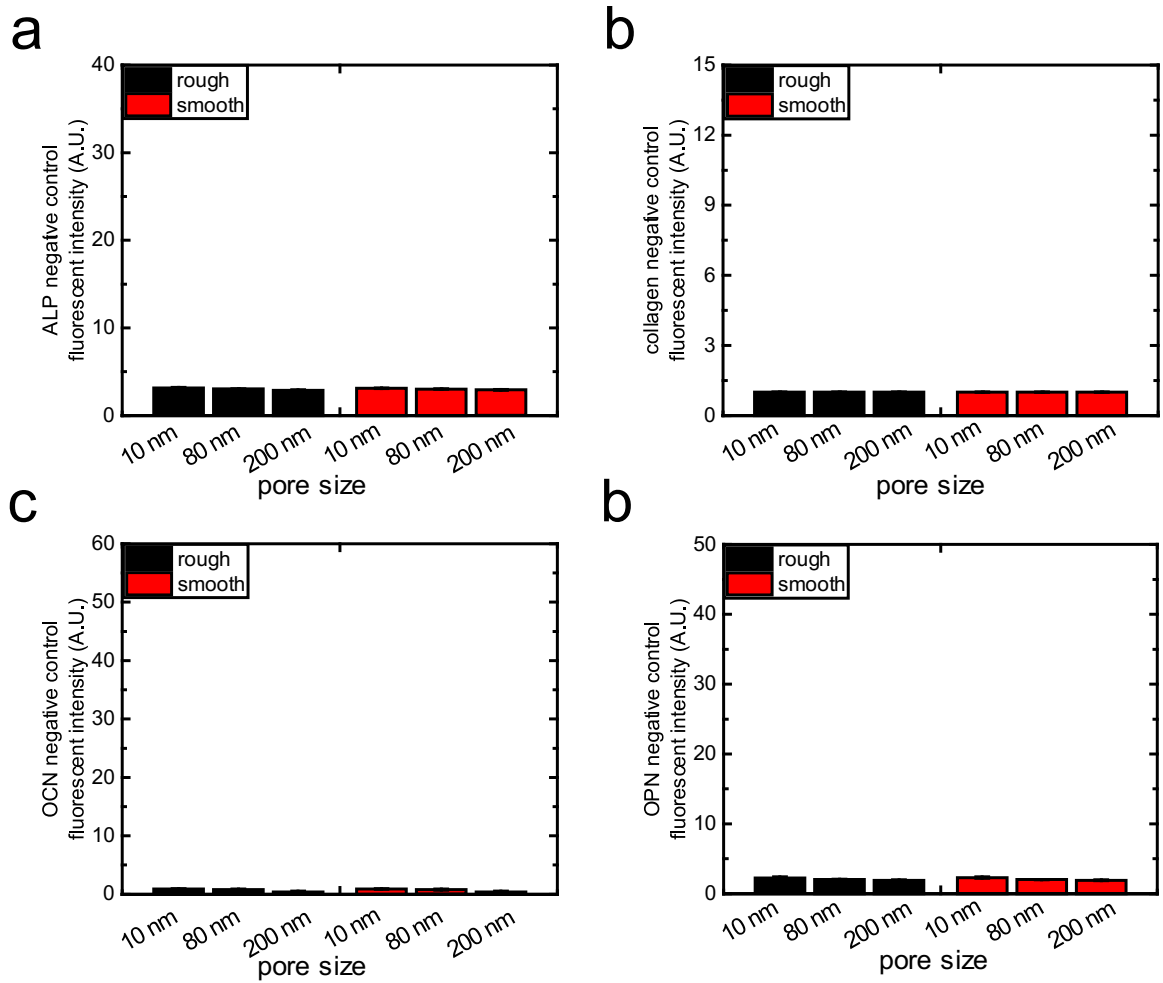


FIGURE 5.12 Negative control of osteogenic differentiation marker proteins.

(a) Negative control of ALP staining (Mean $\pm$ Std, n=5). (b) Negative control of collagen type I staining (Mean $\pm$ Std, n=5). (c) Negative control of OCN staining (Mean $\pm$ Std, n=5). (d) Negative control of OPN staining (Mean $\pm$ Std, n=5).

By further measuring the ALP concentration in the culture media, we find that BMSC cells cultured on larger nanopore size also have higher ALP concentration, as shown in FIGURE 5.13, which is consistent with the cell surface ALP measurement.

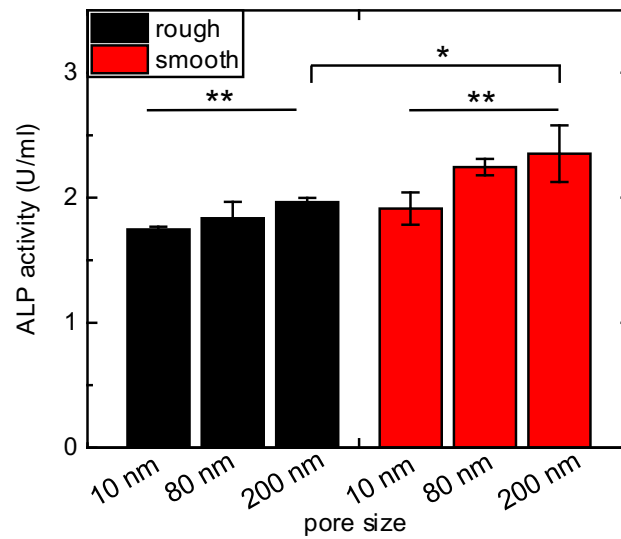


FIGURE 5. 13 Alkaline phosphatase (ALP) activity in cell culture media after culturing BMSC on nanoporous membrane for 14 days (Mean $\pm$ Std, n=3).

Meanwhile, collagen type I secretion increases almost 2 times over the tested nanopore size range, as shown in FIGURE 5.11d. Additionally, both osteocalcin and osteopontin increase almost 1.4 times over the tested nanopore size range, as shown in FIGURE 5.11f, h. Moreover, the bare membranes are used as negative control groups, which have fluorescence intensity 10-fold smaller than the cell samples, suggesting the fluorescent signals in cell samples are mostly contributed by cell monolayer, but not membranes, as shown in figure S6b, c, d.

Since ALP, collagen type I, osteocalcin and osteopontin all increase with the nanopore size of the membranes, we conclude that increases of nanopore size can promote osteogenic differentiation of BMSC cells. Surface roughness, however, does not have a major influence on osteogenic differentiation, as suggested by minor change in fluorescence intensity of stained protein markers, though the change is still statistically significant, as shown in FIGURE 5.11b, d, f, h. This suggests that nanopore size has a significant effect on the osteogenic differentiation of BMSC cells, whereas roughness only have a minor influence. Since nanopore size has significant effect on the short-time behavior of cell such as adhesion and spreading, which might eventually lead to the long-time behavior such as differentiation.

These results suggest that initial cell adhesion number, cell spreading and osteogenic differentiation are all closely related.

5.4 Conclusions and discussion

In this study, we use nanoporous polycarbonate membranes to decouple the effects of nanopore size and roughness on BMSC cell adhesion, spreading and differentiation. We demonstrate that increasing nanopore size will inhibit the initial adhesion number of BMSC cells, but promote larger spreading area and cell volume. Furthermore, we find more osteogenic differentiation on larger nanopore size membranes, as indicated by higher expression levels of ALP, collagen type I, osteocalcin and osteopontin. In addition, changing the surface roughness does not have significant effect on initial adhesion of BMSC cells or their osteogenic differentiation, but instead, surface roughness plays a non-negligible role in regulating cell spreading area and volume. Overall, our results suggest that, both nanopore size and surface roughness can regulate the cell spreading and differentiation behavior, but to different extents; compared with surface roughness, nanopore size plays a more significant role in governing BMSC cell behavior. Not only does nanopore size affects the short-time behavior such as adhesion and spreading, but also affects the long-time behavior such as differentiation, which suggests that nanopore size affect long-time behavior by affecting short-time behavior. This is supported by other studies using different substrate system such as petridish and PDMS, which shows that increased spreading area or decreased initial cell plating density can promote BMSC osteogenic differentiation¹¹⁴⁻¹¹⁶. Surprisingly, our results find similar behavior even though our membrane material is markedly different from other substrate system, suggesting that the regulation of BMSC differentiation by mechanical signals happens by a mechanism that is intrinsic to the cells. These results suggest that initial cell adhesion number, cell spreading and osteogenic differentiation are all closely related. However, the biological mechanism to connect these behaviors is not clear and deserves more study.

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