RESEARCH ARTICLE

A preliminary comparison of two different polyacrylamide hydrogel fabrication methods demonstrate differences in stiffness measurements and adhesion abilities of osteosarcoma cells

Authors
Anita K. Luu[1], Rachel E. Macdonald[1], Richard Parg[2], John R. Dutcher[2], Alicia M. Viloria-Petit[1*]

Affiliations
[1]Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
[2]Department of Physics, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

***Corresponding Author:**
Alicia M. Viloria-Petit
Email: aviloria@uoguelph.ca
Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
(519) 824-4120 ext 54925

Abstract

The purpose of this research was to compare two different polyacrylamide hydrogel fabrication methods described in the literature and assess their use in mechanotransduction studies in osteosarcoma. Both methods employ succinimide chemistry to functionalize the hydrogel surface for cell response studies, one in the form of NHS, while the other in the form of Sulfo-SANPAH. Six hydrogels of two different stiffness were created for each method and were evaluated on their receptiveness to cell seeding and Young's moduli with atomic force microscopy. Both hydrogel fabrication methods lack reproducibility as significant differences were observed in stiffness measurements between six hydrogels at both 0.5 kPa and 50 kPa stiffnesses. Despite the Sulfo-SANPAH method of preparation being more receptive to cell seeding and generating the expected effects on known mechanotransducers, it appeared to have larger variabilities in stiffness measurements for both 0.5 kPa and 50 kPa prepared hydrogels. Researchers may employ the Sulfo-SANPAH method to study the impact of ECM of varying stiffness on osteosarcoma cell mechanotransduction, but should remain cautious when interpreting results as a function of the expected stiffness. Studies should be accompanied by measurements of Young's modulus whenever possible.

Keywords: polyacrylamide hydrogels, mechanobiology, hydrogel fabrication, extracellular matrix, tumor microenvironment

List of Abbreviations

AFM	atomic force microscopy
APTMS	(3-Aminopropyl) trimethoxysilane
ECM	extracellular matrix
kPa	kilo Pascals
NHS	N-hydroxysuccinimide ester
PA	polyacrylamide
PNP-TR	Pyrex-Nitride Probe -TRiangular
Sulfo-SANPAH	sulfosuccinimidyl 6-(4'azido-2'-nitrophenylamino) hexanoate
TAZ	transcriptional coactivator with a PDZ binding motif
TEAD	TEA domain family member
UV	ultraviolet
YAP	yes-associated protein

1. Introduction

The extracellular matrix (ECM) plays a key role in the formation of cell-to-cell contact and communicates external signals to a cell.[1] During pathophysiological conditions, such as cancer, the physical properties of the ECM are altered through various mechanisms.[2] These physical changes can lead to alterations in the activity of certain transcription factors, gene expression, and ultimately cell behaviour through a process known as mechanotransduction.[3] For instance, the mechanotranducers TAZ (transcriptional coactivator with a PDZ-binding motif) and YAP (yes-associated protein) are stabilized and translocate to the nucleus in response to stiff matrices. Once in the nucleus, they act as transcriptional co-activators of TEA domain family member (TEAD) responsive genes to promote cancer progression.[4,5] Both TAZ and YAP have been shown to enhance metastatic ability of tumour cells,[6,7] in agreement with their association with poor patient prognosis in a variety of epithelial cancers and sarcomas; notably osteosarcoma.[8,9] Given that metastasis is the primary cause of death from cancer worldwide (https://www.who.int/news-room/fact-sheets/detail/cancer), and the demonstrated role of mechanotransduction in this process[6], it is imperative to use reliable methods for in vitro mechanotransduction research. This is a required initial step to identify biomarkers and/or therapeutic targets to improve the clinical management of metastatic disease.

One method used to study the impact of matrix rigidity on cell behaviour in *vitro* is through the use of polyacrylamide (PA) hydrogels.[10] PA hydrogels are relatively inexpensive and can be made in-house by sandwiching an acrylamide and bis-acrylamide solution between two glass coverslips. By adjusting the concentrations of acrylamide and bis-acrylamide, the researcher can directly manipulate the stiffness of the underlying matrix. Before cell seeding, PA hydrogels first need to be functionalized with an ECM protein such as collagen, fibronectin, or laminin, to permit cell adhesion. To do this, chemical or photoreactive crosslinkers are employed to crosslink the PA hydrogel surface to the ECM protein of interest.

This study compares the Young's Moduli, as determined by atomic force microscopy (AFM), and cell adhesion abilities of two different PA hydrogel preparation methods to assess their suitability for *in vitro* mechanotransduction studies in osteosarcoma cells. For each method, we constructed gels of two expected stiffnesses, 50 kPa and 0.5 kPa, resembling Young's moduli of collagenous bone and lung, respectively, based on relevance to osteosarcoma progression[9]. Both methods use succinimide to act as a crosslinker between the hydrogel surface and ECM protein.

However, one method involves adding this compound in the form of N-hydroxysuccinimide ester (NHS) to the acrylamide and bis-acrylamide solution directly (Cretu et al 2010),[11] while the other employs sulfosuccinimidyl 6-(4'azido-2'-nitrophenylamino) hexanoate or Sulfo-SANPAH which requires activation by ultraviolent (UV) light (Minaisah et al 2016).[12] A thorough explanation of the chemistry behind these methods is described by Kandow and colleagues (2007).[13]

2. Materials & Methods

2.1 Hydrogel Preparation

Twenty-two-millimeter (22 mm) Fisherbrand® round coverslips (Fisher Scientific) were activated with 500 μL of 0.1M NaOH for 3 minutes, aspirated, and incubated with 1 mL of 97% (3-Aminopropyl) trimethoxysilane (APTMS) (Sigma Aldrich) for 3 minutes. Coverslips were then washed 3 times (10 minutes each) with sterile deionized water with rocking, and aspirated dry. Each coverslip was then coated with 0.5% glutaraldehyde (Sigma Aldrich) for 30 minutes to complete the activation step. Coverslips were gently blotted dry with a kim wipe and 140 μL of the gel solution was pipetted onto each coverslip (see Supplemental Table 1 for recipes) and a siliconized coverslip (Cretu et al 2010) or non-treated coverslip (Minaisah et al 2016) was immediately placed on top. After the gel solution was polymerized, the top coverslip was gently removed, and the hydrogel was placed into a 35 mm tissue culture dish with sterile PBS. For the Minaisah et al (2016) method, the PBS was aspirated and 500 μL of a 1 mg/mL Sulfo-SANPAH solution in sterile MiliQ water (stock solution purchased from CovaChem and reconstituted to 50 mg/mL with DMSO) was added to the dish and activated by UV light for 20 minutes. For the Cretu et al (2010) method, coverslips were washed two times with sterile PBS, and then 1 mL of 3 mg/mL neutralized collagen type I (Corning) solution was added to the well and allowed to incubate overnight at 4°C. After the Sulfo-SANPAH reaction, hydrogels were similarly washed with PBS twice and the 3 mg/mL collagen type I solution was added. The following day, hydrogels were equilibrated to room temperature and washed with sterile PBS before cell seeding or AFM measurements.

2.2 AFM Measurements

Collagen type I coated PA hydrogels were measured using a JPK NanoWizard 3 AFM (JPK Instruments; Berlin, Germany) with PNP-TR pyramidal contact mode cantilevers (NanoAndMore; USA) having a nominal spring constant of ~0.08 N/m as determined from thermal calibration. Samples were indented to a peak force of 2 nN and elastic modulus, E, or stiffness was determined using the Hertz model for a four-sided pyramidal indenter. Samples were indented 25 times in a 10 μm by 10 μm grid pattern over two areas separated by more than a millimeter on each sample (see Figure 1 for schematic).

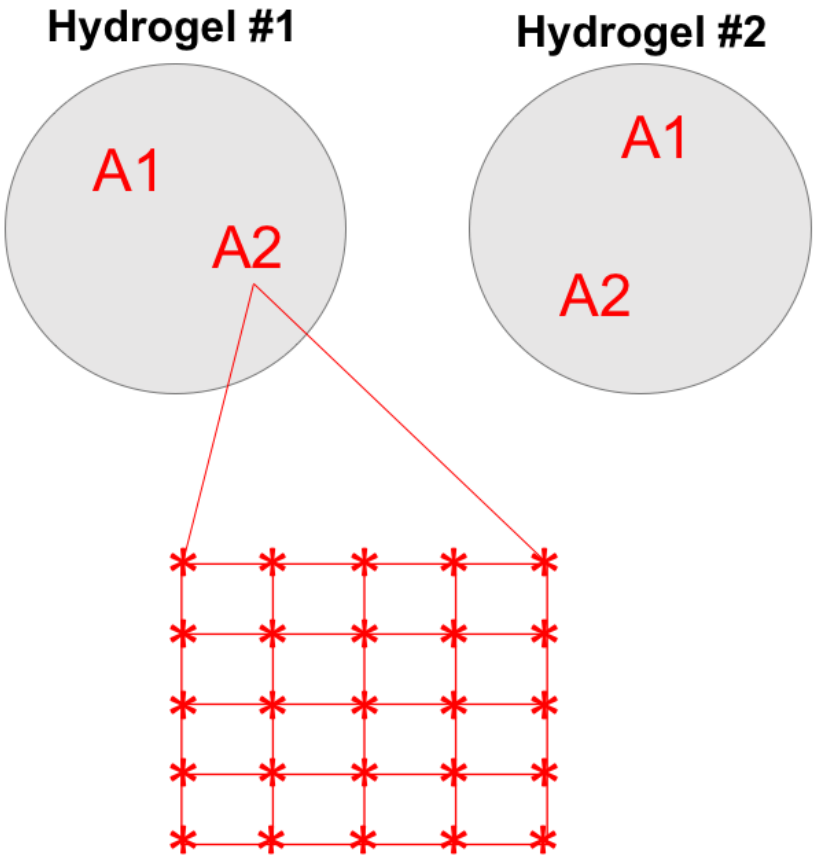


Figure 1: Schematic of approach for AFM readings
Schematic diagram of location of AFM measurements on the two types of hydrogels. For each hydrogel, AFM measurements were performed in two different regions, area 1 (A1) and area 2 (A2), separated by more than a millimeter. In each region, samples were indented 25 times in a 10 µm X 10 µm grid pattern.

2.3 Cell Seeding onto Hydrogels

Canine osteosarcoma cells OVC-cOSA-31 (created in-house by Dr. Geoffrey Wood, Department of Pathobiology, University of Guelph) or commercially available D17, both derived from secondary canine osteosarcoma tumours localized to the lungs, were seeded at 0.225 x 10^6 cells/ 2 mL of cell culture media (DMEM High Glucose supplemented with 10% fetal bovine serum and 100U/mL Penicillin, 100 µg/mL Streptomycin) for 24 hours under standard conditions before visual analysis using bright field microscopy, or confocal microscopy after immunolabeling.

2.4 Immunofluorescence of Hydrogels

Hydrogels were washed with PBS and fixed with 4 % paraformaldehyde (16 % stock solution from Fisher Scientific) for 15 minutes. Hydrogels were washed with PBS, and permeabilized for 15 minutes with 0.1 % Triton-X. Hydrogels were washed again with PBS and blocked for 1 hour at room temperature with 5% normal donkey serum (Sigma Aldrich) diluted in PBS, before incubation with a 1:200 dilution of primary antibody, TAZ (Sigma Aldrich HPA00741) or YAP (Cell Signalling Technologies 14074) overnight at 4ºC in a humidified chamber. The next day, hydrogels were washed with PBS and incubated with a 1:500 dilution of donkey anti-rabbit Alexa Fluor 488 (Invitrogen) to detect TAZ or YAP for 1 hour in the dark, and then a 1:1000 dilution in PBS of Phalloidin-iFluor 594 (Abcam ab176757) for 2 hours at room temperature. Hydrogels were washed with PBS and counterstained with 0.3 µM DAPI (stock solution from Fisher Scientific) for 15 minutes at room temperature. Hydrogels were washed again and

inverted onto a microscope slide using Dako mounting medium. Cells were visualized using confocal microscopy at 60X with an oil immersion objective. Images were compiled and merged with Olympus Fluoview Version 4.0b.

Statistical Analysis

To determine intra hydrogel variability (within hydrogels) and inter hydrogel variability (between hydrogels), a nonparametric t-test or a non-parametric one-way ANOVA was completed with a post-hoc Dunn's multiple comparison test, respectively. Intra hydrogel variability was determined by comparing the means of fifteen measurements obtained for two separate areas within each hydrogel. Inter hydrogel variability was determined by comparing the means of thirty measurements for each hydrogel and comparing it to the means of other hydrogels fabricated using the same method. A p value of < 0.05 was considered statistically significant for both comparisons.

3. Results

3.1 The degree of cell adhesion varies between fabrication methods

There were obvious differences in the quantity of cells adhered to the hydrogel when comparing both methods (Figure 2). When comparing the 0.5 kPa hydrogels, there were little-to-no cells adhered to the surface of the hydrogels prepared using the NHS (Cretu et al 2016) method, while there was uniform coverage on the Sulfo-SANPAH prepared hydrogel for both cell lines. A larger number of cells on the Sulfo-SANPAH prepared hydrogel had a round morphology, which is expected when cells are placed in a soft matrix. When comparing the 50 kPa to the 0.5 kPa hydrogels, the degree of cell adhesion on the NHS prepared hydrogels was improved. However, the cells were not uniformly covering the hydrogel surface but instead adhered in small clusters or islands and some areas of the hydrogel had a lower cell density or were empty. Of the cells that did adhere to the NHS hydrogel, more had an elongated shape cell morphology, as expected on a stiff matrix, but there were also clusters of rounded cells. In the 50 kPa Sulfo-SANPAH prepared hydrogel, cells uniformly covered the surface, and a majority displayed an elongated cell morphology.

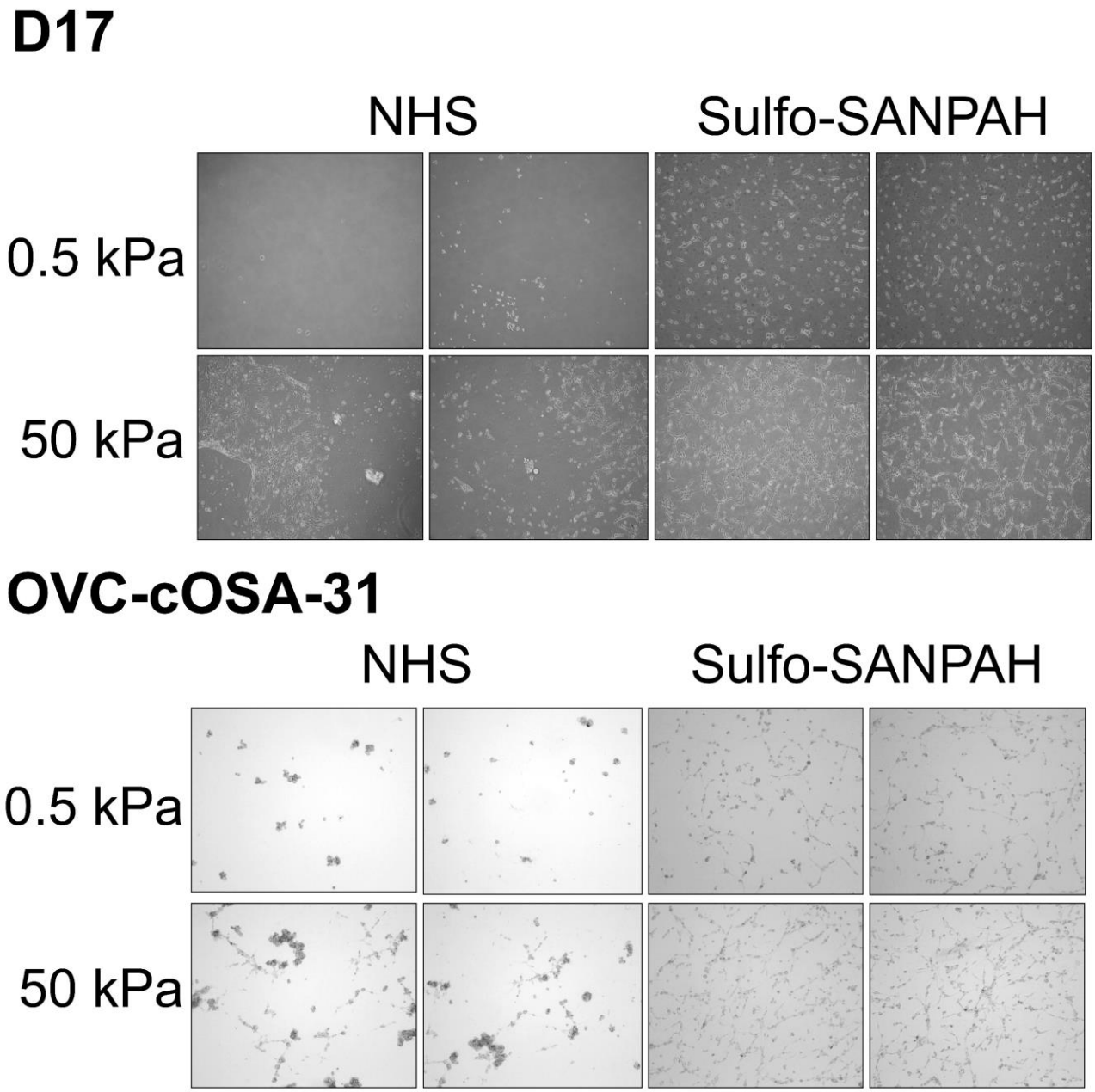
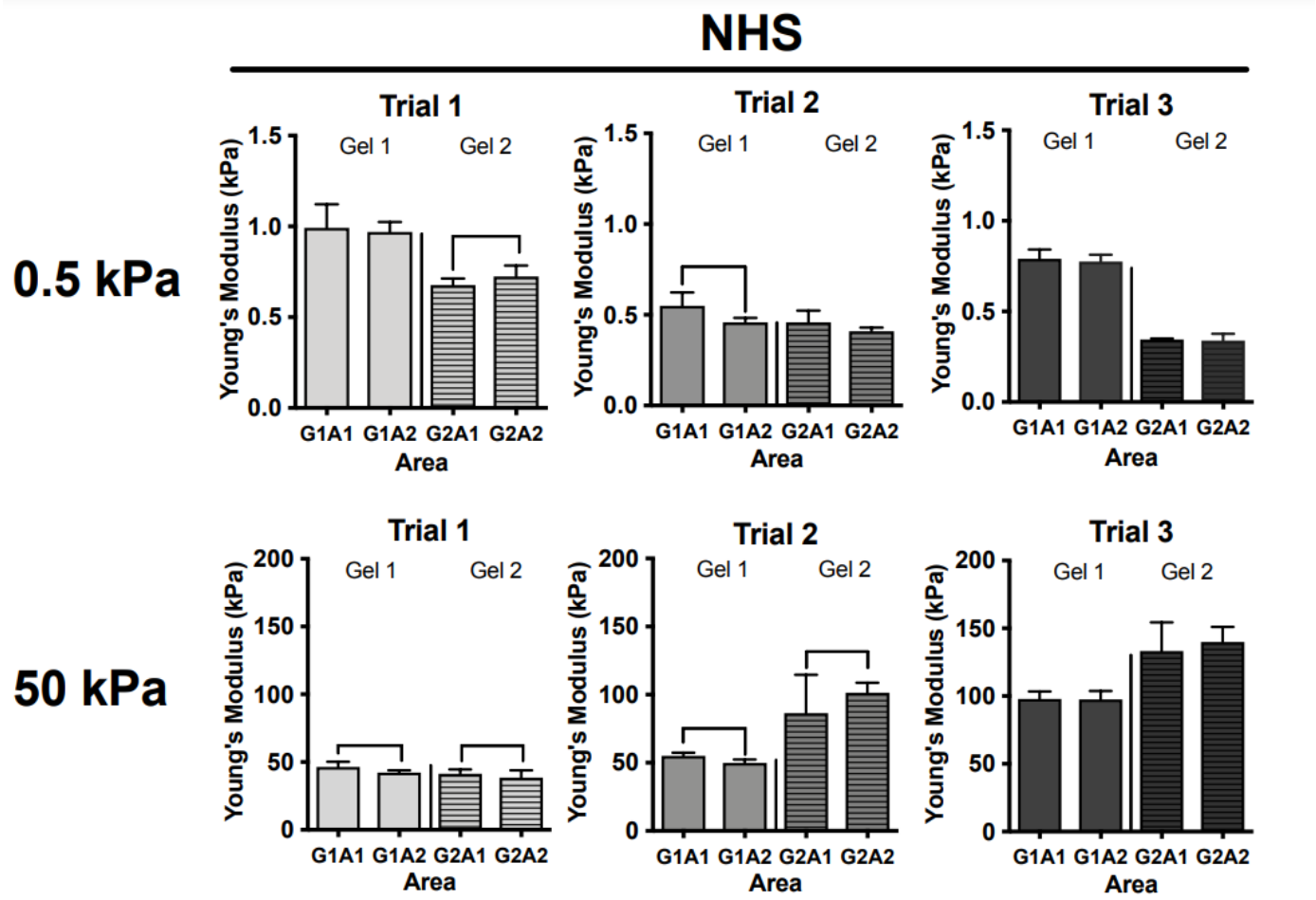


Figure 2: Comparison of hydrogel appearance and D17 and OVC-cOSA-31 cell adhesion abilities on hydrogels prepared using the NHS and Sulfo-SANPAH methods.
Little-to-no cells adhered on the 0.5 kPa hydrogel prepared using the NHS method. Cell adherence was improved on the 50 kPa hydrogel with the NHS method but the cells were not uniformly covering the hydrogel surface. Hydrogels prepared using the Sulfo-SANPAH method had uniform cell adhesion on both 0.5 kPa and 50 kPa stiffnesses. Cells displayed a round morphology on the 0.5 kPa hydrogel, while a more elongated cell shape was observed on the 50 kPa hydrogel. Images were taken a 4X (D17) or 10X (OVC-cOSA-31) objective magnification using an inverted light microscope.

3.2 AFM measurements show lack of reproducibility for both fabrication methods

To determine the differences between and within hydrogels, three independent trials were conducted in which two hydrogels were prepared simultaneously. Two areas of each gel were measured using AFM to determine the elastic modulus (see Figure 1 for schematic). Unfortunately, only two independent trials were measurable for the 0.5 kPa hydrogel prepared using the Sulfo-SANPAH method due to issues with the probe adhering to the surface of some of these soft gels. To determine the difference within hydrogels, an average of fifteen measurements were obtained per area for both methods (Figure 3). To determine differences between hydrogels and the reproducibility between methods, the average of all measurements obtained per hydrogel (30 measurements) was used to determine its overall stiffness. Average measurements in kilopascals (kPa) and standard deviations are highlighted in Table 1 and the significant differences between hydrogels are shown in Figure 4.



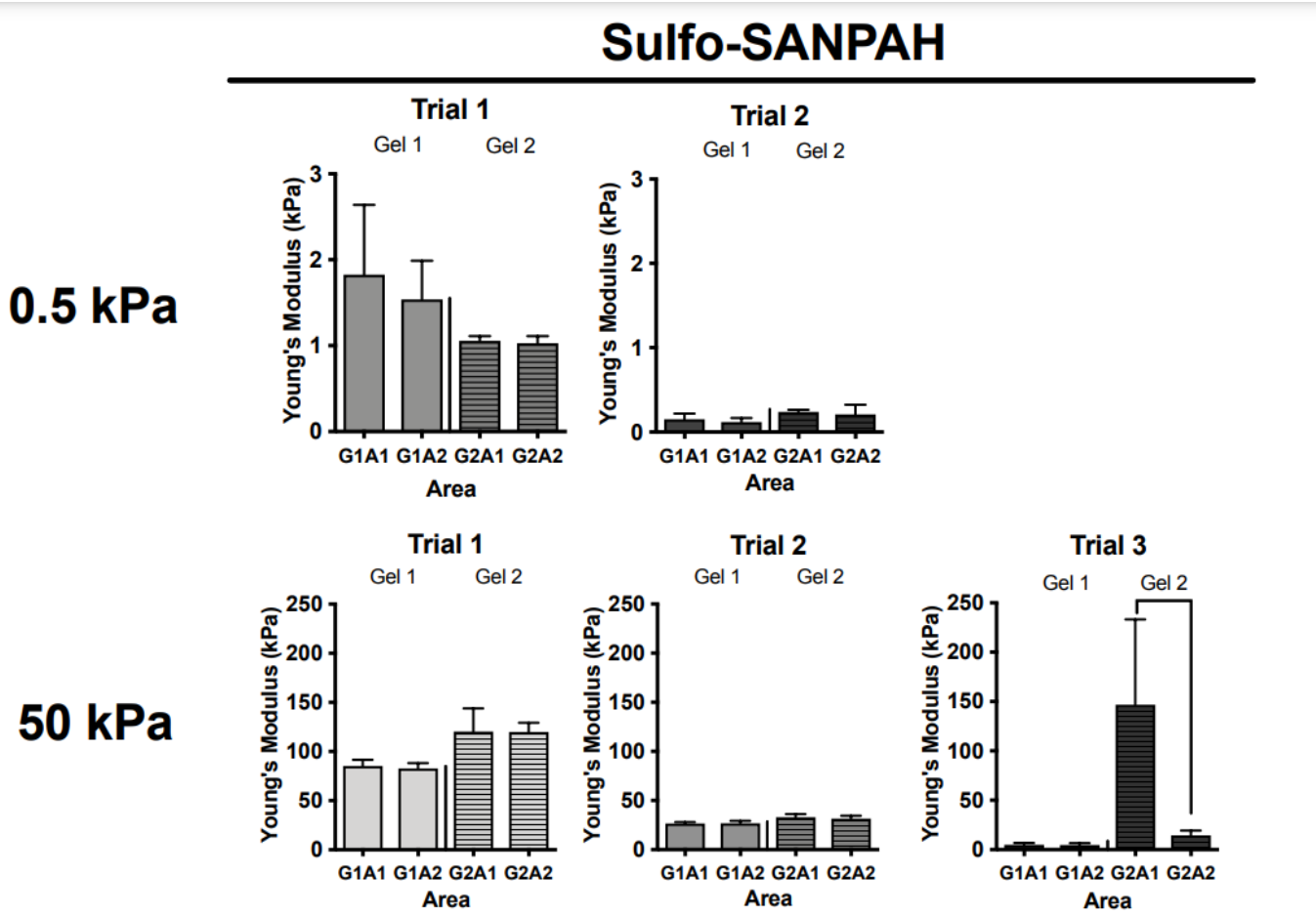


Figure 3: Average AFM measurements for individual hydrogels prepared over three trials using the NHS or Sulfo-SANPAH method

Average Young's Modulus (kPa) for individual hydrogels prepared using the NHS and Sulfo-SANPAH fabrication methods over three independent trials (2 hydrogels/trial). Bars represent the average and the standard deviation for 15 measurements obtained from area 1 (A1) or area 2 (A2) within each hydrogel. Brackets indicate significant differences ($p < 0.05$).

The NHS method resulted in more significant differences between areas within the same hydrogel (Trial 1 and 2 for the 0.5kPa and 50 kPa hydrogels) when compared to the Sulfo-SANPAH method (Trial 3 for the 50 kPa hydrogel), as shown in Figure 3. When considering hydrogels as a whole, the 0.5 kPa hydrogels for both fabrication methods were less variable as compared to the 50 kPa hydrogels when comparing the variations in average values and the magnitude of standard deviations (Table 1). Both methods lacked reproducibility as there were several significant differences between hydrogels, however the Sulfo-SANPAH method of fabrication appeared to have more significant differences between hydrogels compared to the NHS method for the 50kPa hydrogel stiffness (Figure 4).

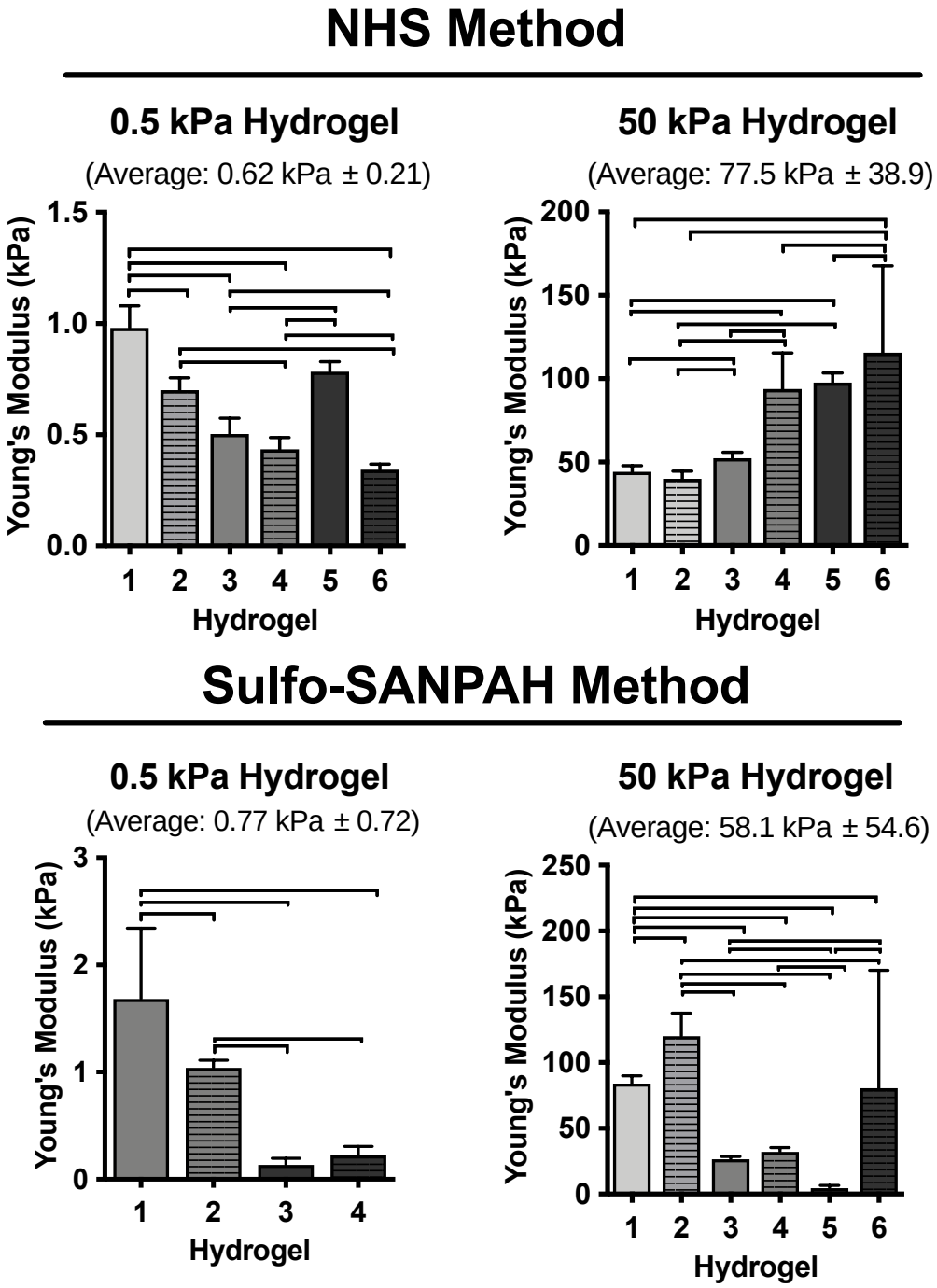


Figure 4: Average AFM measurements for hydrogels prepared using NHS or Sulfo-SANPAH and predicted to be 0.5 kPa or 50 kPa

Average Young's Modulus for six hydrogels prepared using the NHS and Sulfo-SANPAH fabrication methods. Bars represent the average and the standard deviation for 30 measurements obtained from two separate areas of the hydrogel. Brackets indicate significant differences ($p < 0.05$) as determined by a one-way ANOVA and post-hoc Dunn's multiple comparison test. Both fabrication methods and stiffnesses display differences between hydrogels, suggesting a lack of reproducibility for both methods.

Table 1: AFM measurements in kilopascals (kPa) for hydrogels prepared using the NHS and Sulfo-SANPAH methods

NHS						
Predicted	**Gel 1**	**Gel 2**	**Gel 3**	**Gel 4**	**Gel 5**	**Gel 6**
0.5 kPa	0.98 ± 0.10	0.70 ± 0.05	0.50 ± 0.07	0.43 ± 0.05	0.78 ± 0.04	0.34 ± 0.03
50 kPa	44.3 ± 3.58	40.0 ± 4.55	52.4 ± 3.63	93.9 ± 21.6	97.8 ± 5.72	136 ± 16.9
Sulfo-SANPAH						
Predicted	**Gel 1**	**Gel 2**	**Gel 3**	**Gel 4**	**Gel 5**	**Gel 6**
0.5 kPa	1.68 ± 0.66	1.04 ± 0.07	0.13 ± 0.06	0.22 ± 0.08	N/A	N/A
50 kPa	84.0 ± 5.92	120 ± 17.7	26.6 ± 1.98	32.3 ± 3.03	4.67 ± 1.90	80.6 ± 90.3

3.3 Immunofluorescence demonstrates differences in localization of known mechanotransducers as a function of gel stiffness

As the Sulfo-SANPAH method of hydrogel preparation had the greatest cell adhesion, immunofluorescence was performed to visualize Hippo pathway mediators TAZ and YAP, both of which are well documented mechanotransducers involved in OS progression.[14, 15] Despite the variations in the Young's moduli within hydrogels and differences between the predicted and actual Young's moduli, there were noticeable differences in cell morphology and the localization of TAZ when comparing the 0.5 kPa and 50 kPa hydrogels. In line with the results shown in Figure 1, there were more round, less elongated cells in the 0.5 kPa hydrogel as compared to the 50 kPa hydrogel (Figure 5A). Both TAZ and YAP appeared to be predominately cytoplasmic in the 0.5 kPa hydrogel, and predominantly nuclear in the 50 kPa hydrogel (Figure 5B and 5C).

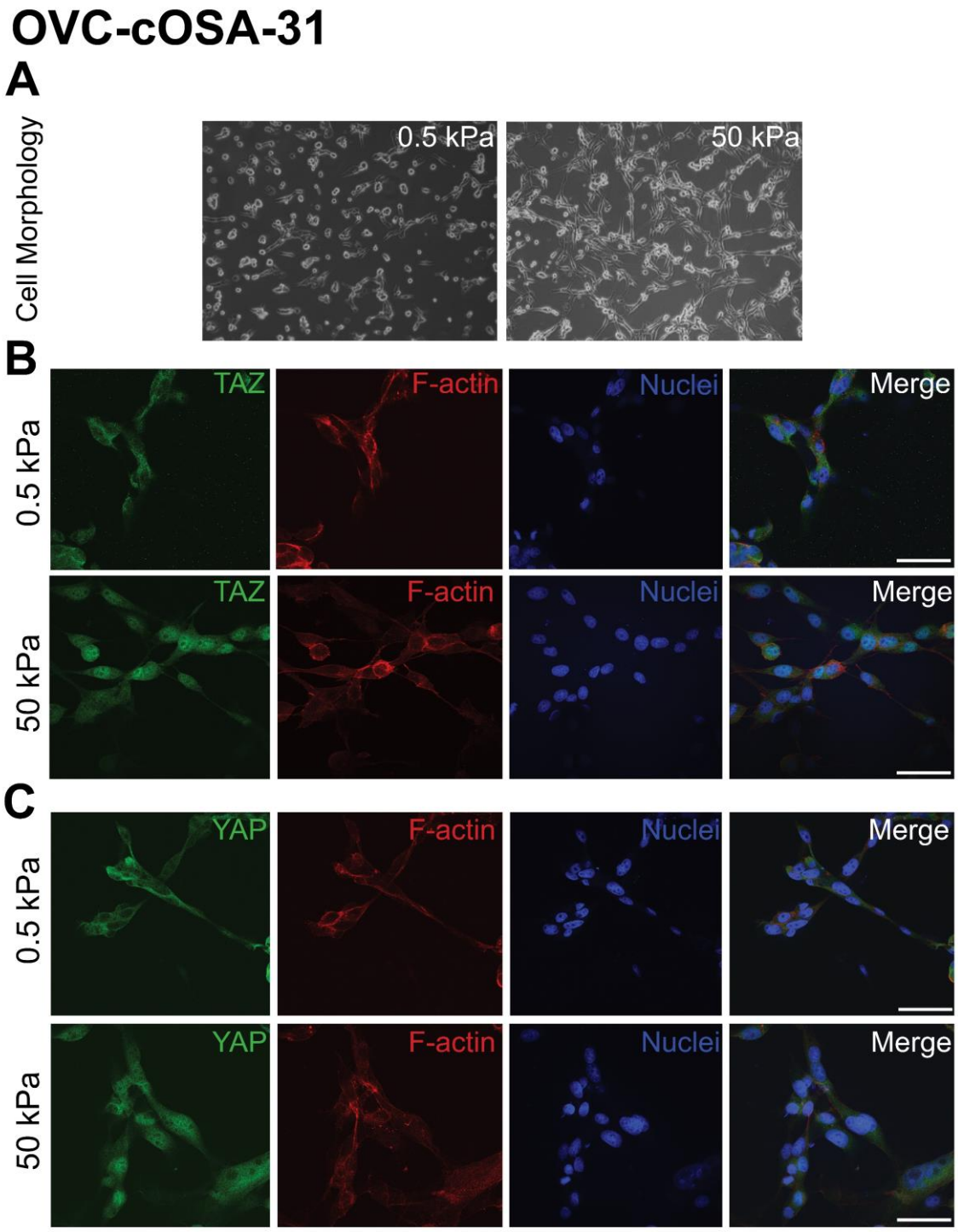


Figure 5: Phase contrast and immunofluorescence images of OVC-cOSA-31 seeded on 0.5 kPa and 50 kPa hydrogels

Hydrogels prepared using the Sulfo-SANPAH method and imaged with a phase contrast microscope demonstrated differences in overall cell morphology. Cells seeded on 0.5 kPa hydrogels had a rounder morphology, while a more elongated morphology was observed on the 50 kPa hydrogels (A). Cells were also immunolabelled for known mechanotransducers TAZ (B) and YAP (C). In the 0.5 kPa hydrogel, both TAZ and YAP appeared to be predominately cytoplasmic. In the 50 kPa hydrogel, TAZ and YAP appeared to be both cytoplasmic and nuclear as demonstrated by the colocalization with the nuclei (refer to Merge image). Images were taken with a confocal microscope (60X objective with oil), scale bar represents 50 μM.

4. Discussion

The purpose of this study was to compare the NHS and Sulfo-SANPAH hydrogel fabrication methods in terms of cell adhesion abilities and elastic moduli within and between hydrogels coated with a collagen type I matrix. These results show that the Sulfo-SANPAH hydrogels are more receptive to osteosarcoma cell seeding (Figure 2), while reproducibility is a limitation for both hydrogel fabrication methods as there were significant differences between hydrogels at both stiffnesses (Figure 4). It appears that the NHS method may be more reproducible as evidenced by the smaller standard deviations between hydrogels, and the large ranges in stiffnesses measured. For the Sulfo-SANPAH method, the stiffness of the predicted 0.5kPa hydrogel ranged from 0.13 kPa – 1.68 kPa versus 0.34 – 0.98 kPa for the NHS method, while the 50 kPa hydrogel for Sulfo-SANPAH ranged from 4.67 – 120 kPa versus 40 kPa – 136 kPa for the NHS method (Table 1). However, despite this larger variation in stiffness measurements among gel replicates prepared with the Sulfo-SANPAH method, immunofluorescence imaging of TAZ and YAP showed that gels prepared by this method are suitable for mechanotransduction studies, since the stiffer gels (50 kPa) promoted TAZ and YAP nuclear localization, as expected.

Although it is not obvious why these differences exist between these methods, some insight could be gathered when considering the experimental procedures. For the Cretu et al (2016) method, NHS ester was dissolved in toluene and combined with bis-acrylamide and acrylamide mixture to permit cross-linking with the ECM. The NHS ester-toluene solution is insoluble in water and as such, creates several bubbles when combined with the bis-acrylamide and acrylamide mixture. Thus, when the mixture solidifies, several bubbles are created within the hydrogel as seen in Figure 2. This non-homogenous mixture could then hinder the ability of collagen type I to uniformly coat the surface of the hydrogel. Some areas of the gel that do contain the NHS-toluene might adequately bind to collagen type I, while other regions may not. This may explain why the cells adhered in small islands or clumps. The Sulfo-SANPAH mode of preparation is more advantageous in this regard, as the Sulfo-SANPAH mixture diluted in water is added to the hydrogel-containing well and is then distributed throughout the hydrogel surface to ensure uniform coating. The variabilities observed between hydrogels with the Sulfo-SANPAH fabrication method could be attributed to the light sensitive nature of the product. Although the activation step was carried out quite quickly, the activation of Sulfo-SANPAH may have been inadequate during some trials, leading to poor functionalization of the hydrogel surface.

We would like to recognize that this is a small characterization and there are limitations to our study. First, the data reported in this manuscript were based off six prepared hydrogels per method. Although the reagents used were the same for their preparation, additional replicates could be completed to determine the reproducibility of different fabrication methods. Next, only canine osteosarcoma cell lines were utilized for seeding onto the hydrogels. Although not suspected to make a difference, other cell lines, including human osteosarcoma and canine and human cells derived from epithelial tumours, could have been employed to determine their adherence ability. Lastly, only collagen type I was used as the ECM protein for coating the hydrogels. Alternative ECM proteins, such as laminin or fibronectin, could have been tested for their affinity to bind to the hydrogel. Additional analyses could have been performed to determine the thickness and uniformity of the hydrogel and matrix coating by using fluorescent beads and cross-sectional confocal imaging as previously described.[16]

Employing these techniques would provide insight on the differences in hydrogel surfaces.

In summary, this comparison of two different hydrogel fabrication methods demonstrated that variability does exist between methods. Although the Sulfo-SANPAH method was appropriate to assess the impact of ECM of two different stiffnesses on osteosarcoma cell mechanotransduction, this method also resulted in considerable differences in stiffness measurements among gel replicates. The variations observed with either method may be inevitable due to natural human error that can occur from improper mixing of the solution and/or poor distribution on the glass coverslip. Research groups employing polyacrylamide hydrogels should exercise caution when utilizing these methods for experimentation. Multiple AFM measurements from different regions and hydrogels should be evaluated and reported. Variations in underlying hydrogel stiffness may also lead to variability in the data obtained. If performing immunofluorescence, it is imperative that different regions of the hydrogels are imaged and analyzed. Additional replicates may also be employed to validate these results. Researchers may also consider purchasing ready-to-use hydrogels to eliminate any potential human error, however the quality control report for these gels might not be available to the researchers, and the range of stiffnesses are limited and not always compatible with the biological system of interest.

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Declarations

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

AKL drafted the manuscript and prepared all figures and tables. AKL and REM both prepared the hydrogels. REM performed the immunofluorescence and performed confocal imaging to generate images in Figure 2. RP performed all AFM measurements. JRD provided expertise and equipment necessary for AFM measurements. AVP guided experimental design; all authors edited and revised the manuscript. All authors read and approved the final manuscript.

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