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Enhancement of Podocyte Attachment on Polyacrylamide Hydrogel with Gelatin-based polymers

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Abstract:

The biological activities of cells such as survival and differentiation process are mainly maintained by a specific extracellular matrix (ECM). Hydrogels have been recently progressed in tissue engineering applications. In particular, scaffold made of gelatin methacrylate based hydrogels (GelMA) showed a great potential due to their biocompatibility, biofunctionality and low mechanical strength. The development of a hydrogel having tunable and appropriate

mechanical properties as well as chemical and biological cues was the aim of the work. A synthetic and biologic hybrid hydrogel was developed in order to mimic the biological and mechanical properties of native ECM. The combination of gelatin methacrylate and acrylamide (GelMA-AAm) based hydrogels was studied and showed tunable mechanical properties by changing the polymers concentrations. Different GelMA-AAm samples were prepared and studied by varying the concentrations of GelMA and AAm (AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%}). The swelling behavior, the biodegradability, the physicochemical and the mechanical properties of GelMA-AAm were also characterized. The results showed a variation of swelling capability and a tunable elasticity ranging from 4.03 kPa to 24.98 kPa depending on polymers concentrations. Moreover, the characterization of podocyte cells morphology, cytoskeleton reorganization and differentiation were evaluated as a function of GelMA-AAm mechanical properties. We concluded that AAm_{2.5%} + GelMA_{3%} hydrogel sample having an elasticity of 4.03 kPa can mimic the native kidney glomerular basement membrane (GBM) elasticity and allow podocytes cells attachment without the functionalization of gel surface with adhesion proteins comparing to synthetic hydrogels (PAAm). This work will further enhance the knowledge on podocyte cells behavior in order to understand their biological properties in both health and disease states.

Keywords:

GelMA-Aam scaffold, Stiffness, Podocyte, Glomerular basement membrane, Cells Mechanosensitivity.

I. Introduction:

Currently, the concept of tissue engineering is based on the development and the improvement of a biomimetic extracellular matrix (ECM) which provides structural and mechanical support for cells¹. ECM is defined as non-cellular bioactive component with well-organized dynamic structure network that provides a biological environment and a suitable mechanical support to control and modulate cells activities such as survival, proliferation, and differentiation^{2,3,4}. Therefore, the main goal in tissue engineering field is to design ECM substitutes able to restore, maintain and enhance tissue functions^{5,6,7,8}. Nowadays, a variety of biological (such as chitosan, collagen and gelatin) and synthetic (such as polyethylene glycol and polyacrylamide) hydrogels were proposed as suitable candidates to mimic the native ECM. Hydrogels were widely used as biomaterials because of their capacity to hold a large amount of water essential for an optimal transport of both oxygen and nutrients. The mechanical properties mimicking the cells local environment and the biocompatibility of the hydrogel are tunable to facilitate cells attachment and proliferation^{9,10,11,12,13,14}.

Biological hydrogels derived from native ECM provide biological active sites promoting and regulating various cellular properties such as cells attachment, proliferation and differentiation. However, their limited mechanical properties are considered as a main disadvantage for tissue engineering applications¹⁵. Conversely, synthetic hydrogels have robust mechanical properties¹⁶ but the lack of biological active sites remains a problem which limits the proliferation and the migration of the cells. Synthetic hydrogels act as passive scaffolds for cells and do not promote cells interaction. Polyacrylamide is one of the synthetic hydrogels that has been widely used as scaffold matrices for cell culture with robust tunable mechanical properties. However, the deficiency in biological active domains requires their functionalization with ECM proteins such as collagen (type I and IV) and fibronectin^{17,18}. To

restrict this issue, the use of synthetic polymer in association with the biological polymer is considered as a good solution, since the latter allows cellular attachment due to the presence of biological sites. Therefore, the combination method is advantageous because the synthesis of biofunctionalized hydrogels does not necessitate the grafting of ECM proteins.

Gelatin is a biocompatible, biodegradable and non-immunogenic biological polymer¹⁹. Therefore, they were widely used in biomedical applications including scaffolds engineering. Gelatin is a natural polymer derived from collagen hydrolysis. After collagen denaturation, the bioactive properties are maintained in the gelatin structure, characterized by the presence of cell-binding sequences (Arg-Gly-Asp or RGD sequences) essential for cells attachment and matrix metalloproteinase degradable (MMP) sequences. The gelatin based hydrogels have restrictions toward the *in vivo* applications due to their poor mechanical properties and undergo rapid degradation with enzymes like collagenase^{11,18,20,21}. Thus, gelatin-based hydrogels have to be chemically crosslinked to improve their mechanical properties and to avoid their degradation. For instance, glutaraldehyde²² and diisocyanate²³ were commonly used but their cytotoxicity limits their applications in tissue engineering²⁴. The acrylate modified gelatin, known as gelatin methacrylate (GelMA) is chemically tunable, biocompatible and biodegradable. They provide an appropriate environment for a wide variety of cells²⁵. GelMA-based hydrogels contain cell-binding sequence such as RGD sequence and MMP sequence implicated in cells remodeling. Due to these properties, GelMA-based hydrogels are considered good candidates that mimic the microenvironment of natural tissues^{26,27,28}. However, improvement of their mechanical properties by incorporating various biomaterials is required in order to get suitable hydrogels mimicking the target tissue. Such GelMA hybrid was used as a scaffold material for numerous tissue engineering applications, including kidney, bone, adipose, and cartilage tissues. Consequently, the combination of both biological and

synthetic hydrogels, known as biosynthetic scaffolds, is a promising way to enhance the development of ECM mimicking the heterogeneity of native ECM for tissue engineering application^{29,30,31}. Nowadays, tissue engineering approaches for damaged renal tissue have shown a great advancement in the regeneration of kidney functions and activities. Loss of podocytes phenotype and functions is found in many glomerular injuries. Podocytes are highly differentiated renal epithelial cells of the kidney glomerulus and constitute an essential component for a functional glomerular filtration barrier³². Damaging to the podocyte slit diaphragm represents a hallmark for the development of many glomerular diseases such as proteinuric kidney disease. Recently, kidney tissue engineering using hydrogels as scaffolding materials and cultured podocytes introduced major advancement in the regeneration of kidney functions. Moreover, these scaffolds materials were developed with the purpose to study the effect of their mechanical properties on podocytes cells processes such as cell migration, proliferation and differentiation³³.

This study aimed to synthesize biosynthetic hybrid hydrogel combining polyacrylamide (PAAm) and GelMA in order to mimic the *in vivo* microenvironment by providing mechanical support and structure for podocytes. This model permits to study the biological properties of podocytes in both healthy and diseased states. In fact, the GelMA will provide the biological functions needed for cell adhesion, survival and proliferation, while the PAAm chains reinforce the gelatin network. First, we investigated the effect of polymer compositions on the hybrid hydrogel properties. The swelling capacity, the network microstructure, the biodegradability and the mechanical properties of these hydrogels was studied and a correlation was established between these characteristics. GelMA-AAm hydrogels properties are easily tuned by the polymer composition blend over a wide range. Moreover, we evaluate the effect of

hydrogels mechanical properties on podocytes behaviors such as adhesion, morphology, differentiation and cells elasticity.

II. Materials and Methods:

1. Synthesis of GelMA:

According to the method previously described, the preparation of gelatin methacrylamide is based on gelatin - methacrylic anhydride reaction³⁴. Briefly, 5 g of gelatin (Gelatin from Porcine Skin, Sigma Aldrich, 48722) was dissolved in 45ml of phosphate buffer saline (PBS, Sigma Aldrich, P4417) at 60°C. Then, 1ml of methacrylic anhydride (MA, Sigma Aldrich, 276685) was gently added to gelatin solution under vigorous stirring for 3 H at 60°C. Afterwards, the mixture was dialyzed for 7 days against distilled water at 40°C using dialysis membrane with a molecular weight cut-off of 12-14 kDa³⁵. Finally, the resultant GelMA solution was pre-freeze at -20°C for overnight and then freeze-dried for 7 days.

2. GelMA-PAAm hydrogels preparation:

The GelMA-AAm hydrogels were prepared by co-polymerizing Acrylamide (AAm) and GelMA. Acrylamide (AAm, Sigma Aldrich, A8887, 79-06-1, purity >99%) (5% (w/v) and 2.5% (w/v): AAm concentrations) and GelMA solutions (5% (w/v) and 3% (w/v): GelMA concentrations) were prepared. Different GelMA-AAm samples were performed and studied: AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%} (Table 1). The reaction of polymerization was initiated Ammonium persulfate (APS, 25% (w/v), Sigma Aldrich, 248614, 7727-54-0, purity > 98%) and *N, N, N', N'* Tetramethylethylenediamine (TEMED, Sigma Aldrich, T7024, 110-18-9) initiates and catalyzes respectively the polymerization reaction. The mixture was poured in glass molds (6 cm x 2 cm x 0.8 cm) and the polymerization was achieved at room temperature for 3 H. After

complete polymerization, the hydrogels were gently removed and fully swelled in distilled water (DW) then in phosphate buffer saline (PBS).

Table 1: Composition of GelMA-Aam Hydrogels

Samples	Acrylamide (ml)	GelMA (ml)	APS 25% (μ l)	TEMED (μ l)
AAm _{2.5%} + GelMA _{3%}	1	1	2	2
AAm _{5%} + GelMA _{3%}	1	1	2	2
AAm _{5%} + GelMA _{5%}	1	1	2	2

3. Swelling Measurements:

GelMA-AAm hydrogels were fully swelled in distilled water (DW) and in phosphate buffer saline (PBS) until reaching the equilibrium state at room temperature. The swelling ratio degree was determined by measuring the difference between the weight of a fully swollen hydrogel and the weight of dried samples after freeze-dry for overnight (Measurements were repeated 3 times). The swelling ratio was calculated following the below equation:

$$S = \frac{W_s - W_d}{W_d} \quad (1),$$

where W_s is the weight of the fully swollen gel (in deionized water and PBS) and W_d is the weight of dry gel.

4. Physicochemical Characterization:

a. Fourier Transform Infrared Spectroscopy (FT-IR):

The FT-IR spectra of dry GelMA-AAm gels were analyzed by NEXUS instrument, fitted with an attenuated total reflection (ATR) accessory. The spectra were recorded in the wavenumbers ranging from 500 to 4000 cm^{-1} at 4 cm^{-1} resolution.

b. Nuclear magnetic resonance (NMR):

¹H NMR spectra of GelMA sample was recorded using Bruker Avance 300 spectrometer operated at 300 MHz ¹H frequency. GelMA sample was dissolved in DMSO d6 solvent and the resultant solution was used to record the NMR spectra.

c. Scanning Electron Microscopy (SEM):

SEM technique (SEM ZEISS, EVO I HD15) has been used to evaluate the microstructure of GelMA-AAm hydrogels. The samples were freeze-dried in a vacuum system (LABCONCO®, FreeZone 4.5) under a pressure of 0.02 mBar and at -54°C for overnight. For the analysis, the dried GelMA-AAm samples were gold sputter-coated.

5. Hydrogels Degradation:

The enzymatic degradation process was carried out on GelMA-AAm hydrogels. A solution of collagenase type I was prepared in PBS having a concentration of 6.6 units/mg (Collagenase type I, GIBCO™, 17100-017, 9001-12-1). The GelMA-AAm gels were incubated in the collagenase solution at 37°C. The enzymatic solution was removed at several time points, the samples were freeze-dried and then weighed. The percentage of degradation rate of GelMA-AAm samples was determined from the difference of weight between the dry initial and degraded GelMA-AAm gels (Equation 2).

$$DR\% = \frac{W_0 - W_t}{W_0} \times 100 \quad (2),$$

where W_0 is the initial mass of the dry samples and W_t represents the dry mass of the remained polymer.

6. Mechanical Characterization:

a. Atomic Force Microscopy (AFM):

According to our previous work, Asylum MFP-3D AFM (Asylum Research, Santa Barbara, CA, USA) was used for force-spectroscopy measurements. Briefly, triangular silicon nitride cantilevers (MLCT, Veeco) with a nominal spring constant of 30 pN/nm, length of 225 μm , width of 20 nm, resonance frequency of 15 kHz, half-opening angle of $17.5^\circ \pm 2.5^\circ$ and a nominal radius of 20 nm were employed. The cantilever spring constant was determined in liquid using the thermal noise method. Hydrogel samples were attached to a Petri dish by means of double-faced adhesive tape, and covered with 0.5 ml of deionized water. A range of loading forces on the hydrogel surface, in liquid and at room temperature, were tested and a maximum loading force of 5nN corresponding to a maximal indentation depth of 0.3 μm was chosen to perform the measurements. Young's modulus (E) was calculated for each force, as described before.

b. Rheology:

The mechanical properties of GelMA-AAm samples were determined using Anton Paar Physica MCR 301 rheometer. In this study, the viscoelastic properties were measured by applying an oscillatory force on the surface of GelMA-AAm samples using a metal plate (Anton Paar PP25, 25mm in diameter). The rheology measurements were recorded by setting the following parameters: frequency = 1 Hz, strain amplitude = 1 % and force = 0.5 N.

$$G = G' + G'' \quad (3),$$

where G' represents the storage modulus that corresponds to the elastic property and G'' represents the loss modulus corresponding to the viscous behavior.

7. Cell culture:

According to the supplier protocol (Faculty of Medicine, University of Bristol, UK), immortalized human podocytes cell lines were cultured, proliferated at 33°C and then differentiated at 37°C using a RPMI – 1640 Medium (Sigma Aldrich, R8758). 10 % of fetal bovine serum (Sigma Aldrich, F7524), penicillin streptomycin solution (Sigma Aldrich, P4333) and 1 % of insulin-transferrin-selenium liquid media supplement (Sigma Aldrich, I3146) were added to the cell medium. Cells were collected and plated on the GelMA-AAm polymer hydrogels having different compositions (AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%}) for 2 weeks using 24 well cell culture plate. For immunocytochemical characterization, podocyte cells were fixed with 2% of paraformaldehyde (PFA) (Sigma Aldrich, P6148, 30525-89-4) for 15 min at RT. The permeabilization of podocytes cells was done by using 0.5 % Triton X-100 (Sigma Aldrich, X100, 9002-93-1) for 15 min at 37°C. Then, the blocking solution composed of 1 % of bovine serum albumin (BSA) (Sigma Aldrich, A2153, 9048-46-8) and 0.5% of Triton-X in PBS was used to block the non-specific protein binding sites. Then, cells were incubated overnight at 4°C with a primary anti-podocin antibody (Sigma Aldrich, P0372) and with secondary anti-rabbit antibody (Alexa Fluor® 594, Cat: ab150080) for 1h in dark at room temperature. The DAPI (Sigma Aldrich, D9542) and phalloidin staining (Invitrogen, Cat: A12379) were used to visualize the nucleus and the actin cytoskeleton, respectively. Staining images were taken using Nikon TE2000 microscope.

III. Results:

1. Hydrogel synthesis:

Three GelMA-AAm hydrogels having different composition: AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%} were prepared. Their characterization was performed using ¹H NMR and FTIR spectroscopies. Methacrylic anhydride undergoes nucleophilic substitution

reaction with primary amine groups available in gelatin and hydroxyl groups³⁶. ¹H NMR spectrum of gelatin and GelMA was recorded to evidence the grafting of acrylate on gelatin backbone (Fig. 1a). For GelMA sample, the presence of vinyl ($\delta = 5.3$ ppm and 5.7 ppm) and methyl protons ($\delta = 1.8$ ppm) confirms the grafting of methacrylate groups on the gelatin backbone.

FTIR spectra of gelatin, uncrosslinked polyacrylamide gels and polymerized GelMA-AAm hydrogels are shown in the Figure 1b. Gelatin and AAm were used as control samples. The characteristic peaks of gelatin are as follows, amide I, amide II and amide A, which were observed at 1630 cm^{-1} , 1525 cm^{-1} and 3280 cm^{-1} respectively³⁷. The symmetric NH_2 stretching of primary amide was found at 3192 cm^{-1} and $>\text{C}=\text{O}$ stretching vibration of primary amide was detected at 1660 and 1604 cm^{-1} for uncrosslinked PAAm hydrogel (5% AAm-uncrosslinked)³⁸. The spectra of GelMA-AAm showed the presence of all these vibrations confirming the existence of functional groups characteristics of the polymers of study. Thus, the appearance of amide II (1540 cm^{-1}) peak and symmetric NH_2 stretching in GelMA-PAAm hydrogel confirmed the formation of GelMA-AAm matrix.

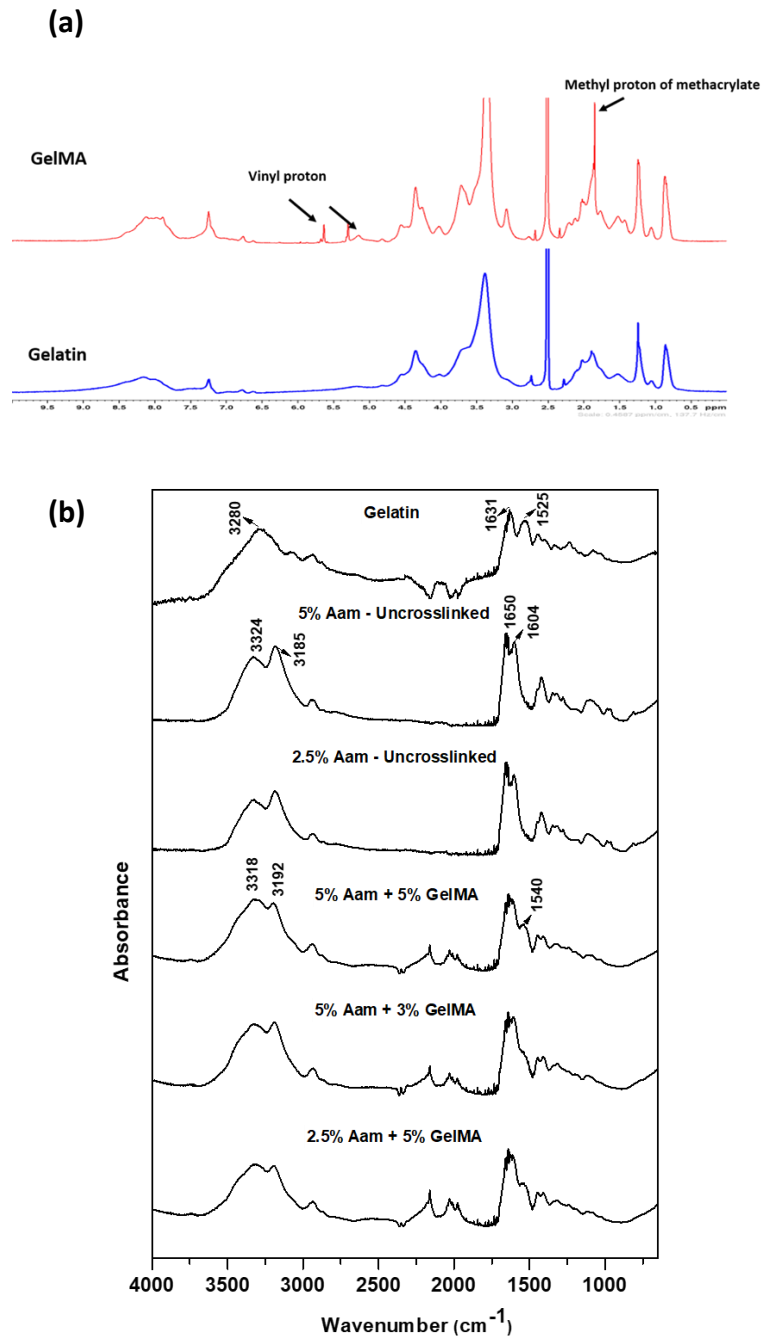


Figure 1: (a) $^1\text{H-NMR}$ of Gelatin and GelMA samples (Black arrow indicates the vinyl proton). (b) FT-IR of pure gelatin and GelMA-AAm interpenetrated polymer network.

2. Swelling and Network Microstructure:

The swelling ratio determines the capability of hydrogels to absorb an important quantity of suitable solvent without dissolving. The swelling equilibrium is reached according to the osmotic and elastic forces of the material. Many factors are involved in the swelling properties

of polymer network such as crosslinking density, molecular weight of polymers and polymer-solvent interactions. The solvent uptake is correlated with the network structure of hydrogels which has an effect on the nutrients and oxygen transport.

Figure 2a reports the swelling ratio and the polymeric network microstructure for different compositions of GelMA-AAm hydrogels. Briefly, the swelling degree increases as the concentrations AAm and/or GelMA decreases. GelMA contains multiple methacrylic moieties necessary to bind to polyacrylamide chains and thus to act as a crosslinker between GelMA and polyacrylamide chains. The increase of GelMA crosslinker concentrations from 3% to 5% decreases the swelling capacity of hydrogels in both water and PBS medium. The density of GelMA-AAm hydrogels was evaluated by Scanning Electron Microscopy (SEM) (Fig.2b). SEM micrographs show that GelMA-AAm based hydrogels exhibit highly connected and compacted network structure for the higher concentrations of AAm and GelMA crosslinker concentrations. This confirms the effect of the crosslinking network density on the swelling capacity³⁹. Thus, the high crosslinking degree of polymer network hinders the swelling of hydrogels. This phenomenon can be described by the effect of GelMA crosslinker on the density of polymer network; the increase of the GelMA concentrations contributes to a highly covalent crosslinked polymer network density³⁰. In addition, the swelling capacity is restricted due to the presence of hydrogen bonds between the gelatin chains. The increase of AAm concentrations from 2.5% to 5% is correlated with a significantly decrease of hydrogels swelling degree and denser pores wall. The increase of AAm concentrations also ensures a highly connected polymer network. To note that GelMA was crosslinked without the use of AAm and it doesn't work due to the low concentrations used during the combination of GelMA and AAm.

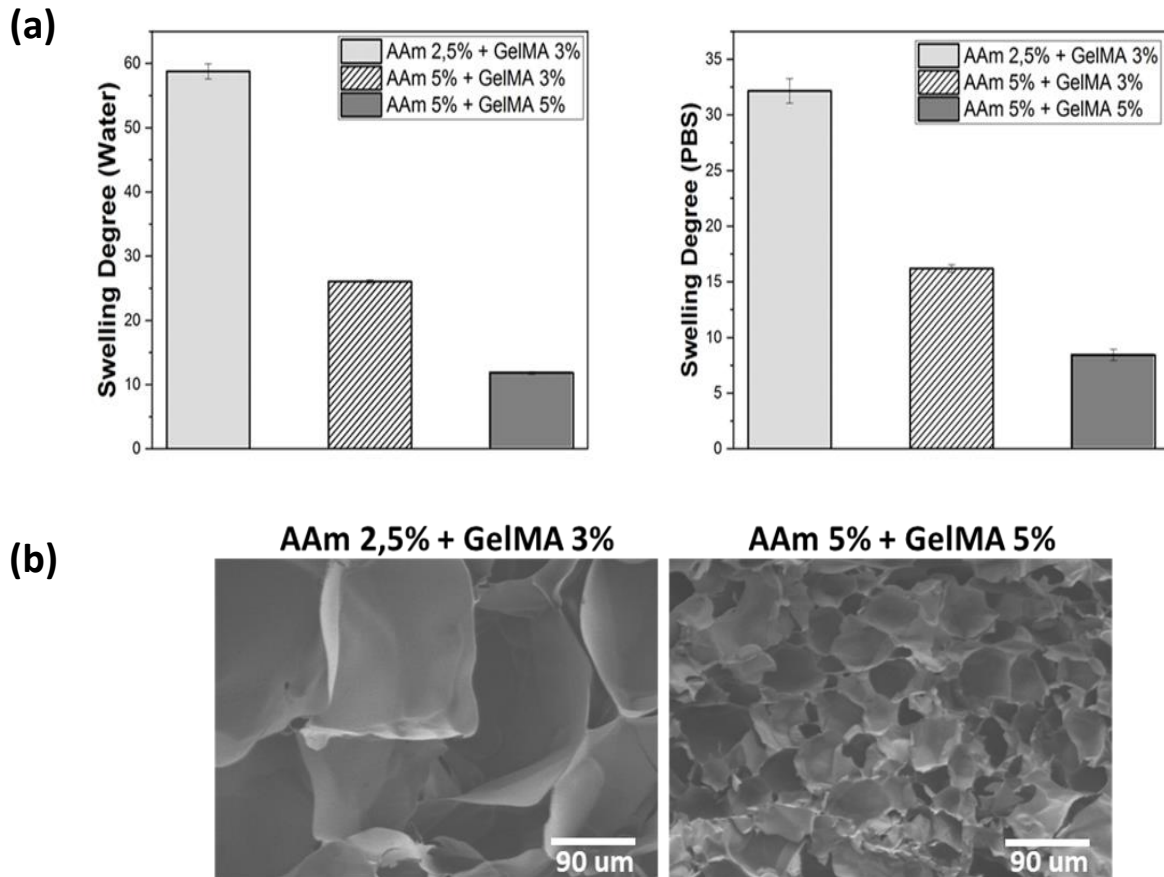


Figure 2: (a) Histogram representing the swelling degree of the gel of study in water and PBS having different polymer (Acrylamide and GelMA) concentrations. (Error Bar = Standard Deviation). (b) Scanning Electron Microscopy for GelMA-AAm based hydrogel having different polymers concentrations.

3. Stability:

The stability of GelMA-AAm hydrogels was evaluated according to its enzymatic degradability at 37°C. The results show a rapid and complete degradation of GelMA in 13 days. Contrarily, the GelMA-AAm hydrogels were partially degraded. Under the same conditions, the AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%} have shown a degradation of 57%, 39% and 29% respectively, after 2 weeks (Fig. 3).

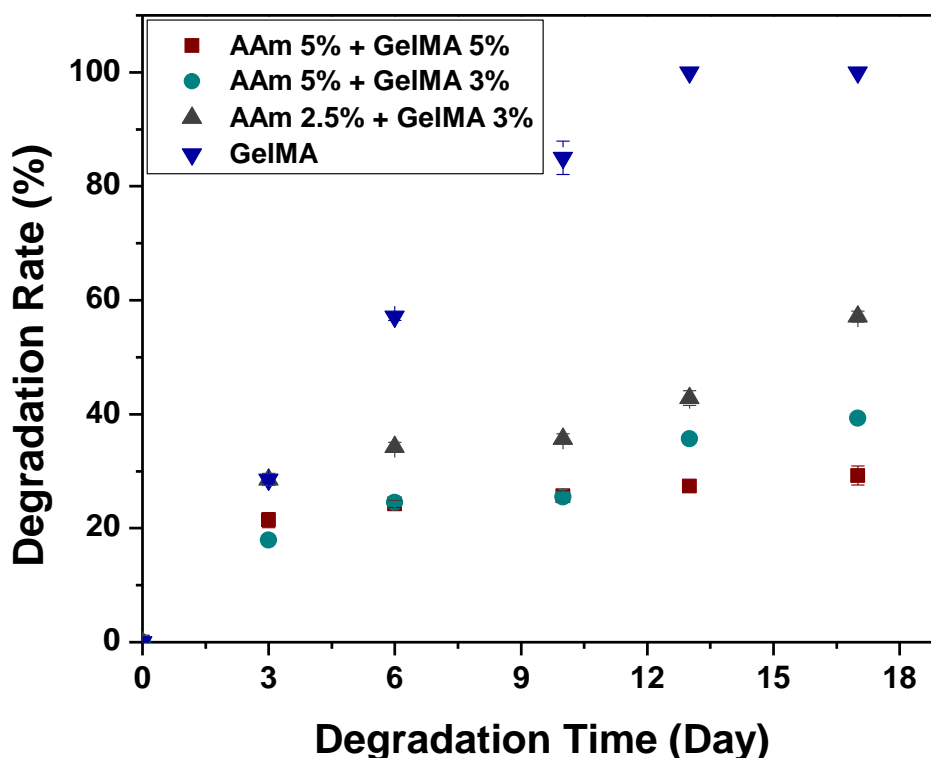


Figure 3: Degradation rate of GelMA hydrogels and GelMA-AAm biohybrid hydrogels over the time.

These results confirm that the gelatin is degraded by the collagenase activity, but the PAAm chains might protect the cleavage sites of gelatin from the enzyme activity. Actually, the biosynthetic hydrogels GelMA-AAm with high crosslinking density avoid the penetration of the collagenase which prevent the degradation of the network and become more stable in physiological environment. Thus, the stability of GelMA-AAm is also easily tunable through the gel composition.

4. Mechanical Properties:

The composition of the hydrogels has an impact on their mechanical properties. The measurements of hydrogels elasticity of a fully swollen network in cell medium were conducted using atomic force microscopy (AFM). Force curves were measured on GelMA-AAm hydrogels having different compositions. Quantitative information from these Young's

modulus maps are reported in Figure 4A, the histograms of distribution were best fitted with a Gaussian function. The Young's modulus values of AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%} are 4.03 ± 0.54 kPa, 11.82 ± 0.49 kPa and 24.98 ± 3.68 kPa respectively (Fig. 4B). The hydrogels stiffness increases as the concentrations of the GelMA crosslinker or the AAm are increased.

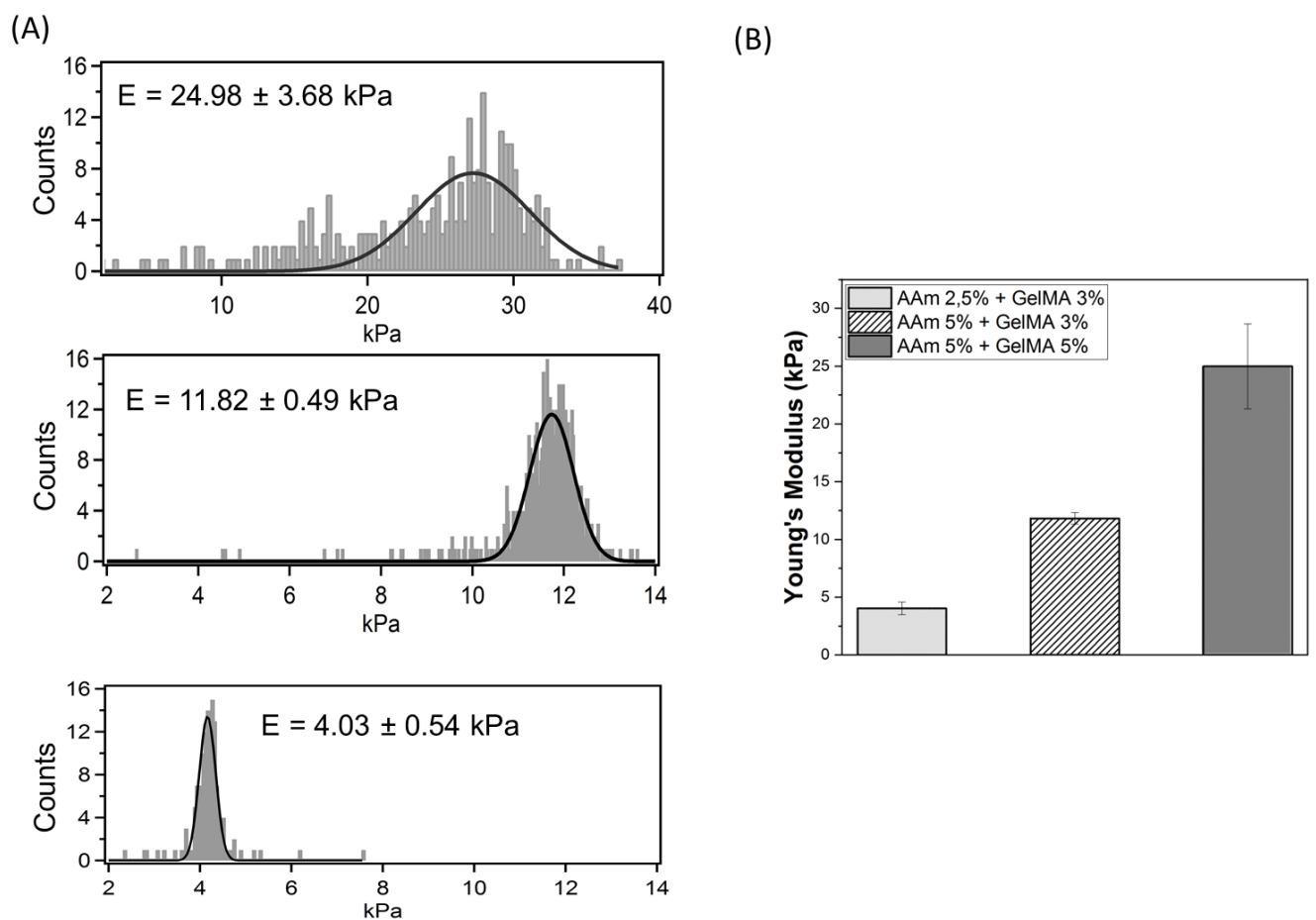


Figure 4: (A) Elasticity distribution of GelMA-PAAm hydrogels swelled in cell medium. Young's moduli were fitted with Gaussian distributions. (B) Variation of Young's modulus "E" with the change of GelMA and AAm polymers concentrations. An increase of Young's modulus values is observed with the increase of GelMA and AAm concentrations.

However, the viscoelastic properties of hydrogels were evaluated by the dynamic shear oscillation measurements. The elastic modulus (G') of GelMA-PAAm, known as storage modulus, represents the capability of hydrogels to have a recoverable energy. The storage modulus (G') are 1.13 ± 0.053 kPa for AAm_{2.5%} + GelMA_{3%}, 1.83 ± 0.048 kPa for The AAm_{5%} +

GelMA_{3%} and 2.14 ± 0.26 kPa for AAm_{5%} + GelMA_{5%} (Fig. 5). Therefore, the increase of the storage modulus (G') is related to the crosslinker and AAm concentrations. As a consequence, the high concentration of AAm and GelMA increases the viscoelastic properties of the hydrogels. These results are coherent with the work represented by Van Den Bulcke *et al.* They have shown that the rheological properties of the gelatin hydrogels depend on the monomer and crosslinker concentrations of the polymer network³⁴.

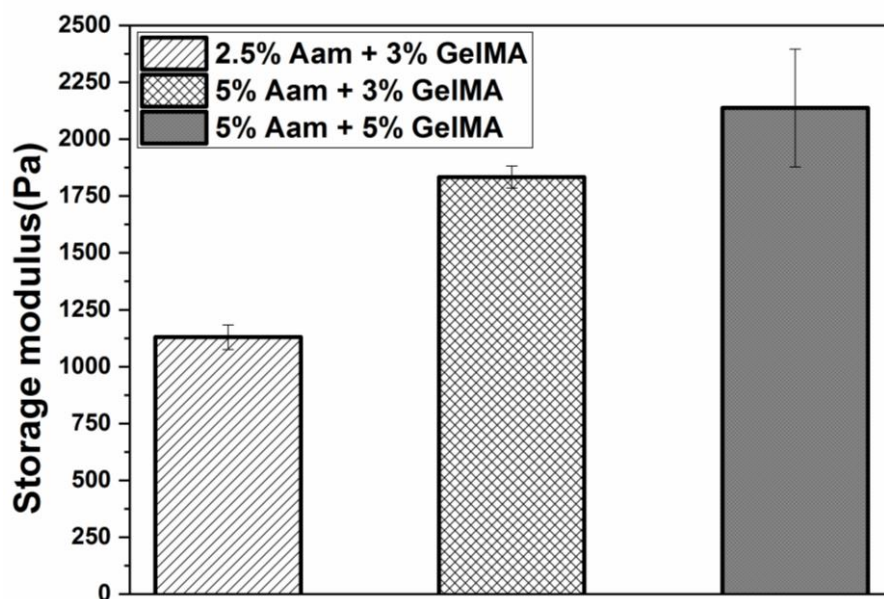


Figure 5: The storage modulus of GelMA-PAAm gels fully swelled in water was assessed as a function of GelMA and AAm concentrations. (Error Bar = Standard Deviation)

To sum up, our results have shown a correlation between the swelling ratio, the network microstructure, the biodegradability and the mechanical properties of GelMA-AAm hydrogels. The increase of AAm and GelMA concentrations contributes to the increase of the mechanical properties and leads to decrease the swelling activity. In addition, GelMA-AAm mechanical properties have shown an adjustable elasticity with a broad range that permits to mimic the elasticity of glomerular basement membrane. Indeed, it has been previously shown that the optimal stiffness for podocyte growth is between 2.1 and 9.9 kPa¹⁷.

5. Podocytes Cells Culture:

The ECM mechanical properties ensure the cells migration, proliferation and differentiation. The alteration of the elastic environment contribute to the development of tissue diseases^{40,41,42}. Podocytes are highly specialized epithelial cells of the glomerulus that are part of the filtration barrier which in healthy setting prevents the passage of plasma proteins into the urine⁴³. The stiffness of the kidney glomerular basement membrane measured by magnetic bead displacement is about 2.4 kPa. The change of GBM stiffness is correlated with the progression of renal diseases⁴⁴. GelMA-AAm hydrogels were developed as a synthetic extracellular matrix support for podocyte cells. Here, we investigated the cells attachment and the influence of GelMA-AAm mechanical properties on podocyte behaviors represented by morphology, cytoskeletal organization, podocin expression and cellular elasticity.

The cell adhesive peptide sequences such as RGD, SIKVAV etc., are prerequisite to favor the improved cell attachment through integrin interaction whereas, cell spreading is controlled by the hydrogel stiffness. Adam *et al.* demonstrated that collagen coated soft acrylamide hydrogel displays poor cell spreading in comparison to collagen coated stiff hydrogel⁴⁵. Inert biocompatible hydrogel such as Polyethylene Glycol (PEG), and some of the polysaccharides such as Alginate and Gellan display no cell adhesive molecules and results to poor or no cell adhesion. Whereas, protein-based biopolymers such as gelatin, collagen, fibrin, etc., inherently contains the cell adhesive amino-acid sequences in their polymer back bone along with matrix metalloprotease degradable amino-acids sequences that facilitates cell attachment on the hydrogel as well as remodeling of the hydrogel.

Moreover, many research studies have worked on biocompatible substrates based on polymers such as polypyrrole modified surfaces and have shown their capability to be used for biomedical purposes due to their significant impact on the adhesion and proliferation of

stem cells^{46,47}. Lan *et al.* have developed a GelMA/PAM biohybrid hydrogel and have demonstrated that GelMA/PAM hydrogel has an appropriate biocompatibility, cell adhesion and proliferation⁴⁸.

In our work, the influence of GelMA-PAAm hydrogels on podocyte cells viability was assessed using Live assay (Calcein) after a 24 hours seeding period. Figure 6A showed podocyte cells adhesion on GelMA-AAm substrates. Podocytes were unable to adhere to non-functionalized polyacrylamide based hydrogels (data not shown). Thus, the adhesion of podocytes to the GelMA-AAm substrates is related to the presence of gelatin cell-binding sequences important for cells attachment. Also, cell viability test confirmed that GelMA-AAm substrate is not toxic to podocytes.

Extracellular matrix mechanical properties have an influence on the cells morphology and cytoskeletal reorganization. The actin cytoskeleton structure of podocyte cells on GelMA-AAm was investigated using Phalloidin staining (Fig. 6B). The results showed an effect of GelMA and AAam concentrations on cells morphology and actin filaments organization. On the softest substrate (AAm_{2.5%} + GelMA_{3%}), podocyte morphology is not fully spread. Conversely on the stiffest substrate (AAm_{5%} + GelMA_{5%}), podocytes exhibited a larger spread area with extended cytoplasm. The actin cytoskeleton organization responds to the mechanical stress of the extracellular matrix through transmembrane receptors named integrin. Actually, cells feel the variation of ECM stiffness and react in a different process. For instance, cells sense the ECM high stiffness which contributes to the loss of cells ability to respond and to contract against the matrix. Thus, cells produce additional forces and the number of focal adhesion and so the number of actin fibers connected to non-muscle myosin will be increased leading to an increase in cell stiffness in order to go with matrix stiffness. Contrariwise, the cells on a matrix with low stiffness generate a small force and show a few number of focal adhesion complex

and thus actin fibers. In conclusion, cell responds and reacts to the variation of substrate stiffness which contributes to regulate the organization of actin cytoskeleton⁴¹. The visualization of actin filaments shows a dense actin cytoskeleton on the stiffest substrate (AAM_{5%} + GelMA_{5%}) compared to the softest substrate (AAM_{2.5%} + GelMA_{3%}) (Fig. 6B). Podocytes express several marker proteins involved in the establishment of the glomerular filtration barrier such as podocin and nephrin⁴⁹. Figure 6B showed that podocytes cultured on the softest substrates (AAM_{2.5%} + GelMA_{3%}) and (AAM_{5%} + GelMA_{3%}) robustly expressed podocin, while podocin expression in podocytes cultured on the stiffest substrate (AAM_{5%} + GelMA_{5%}) is markedly decreased.

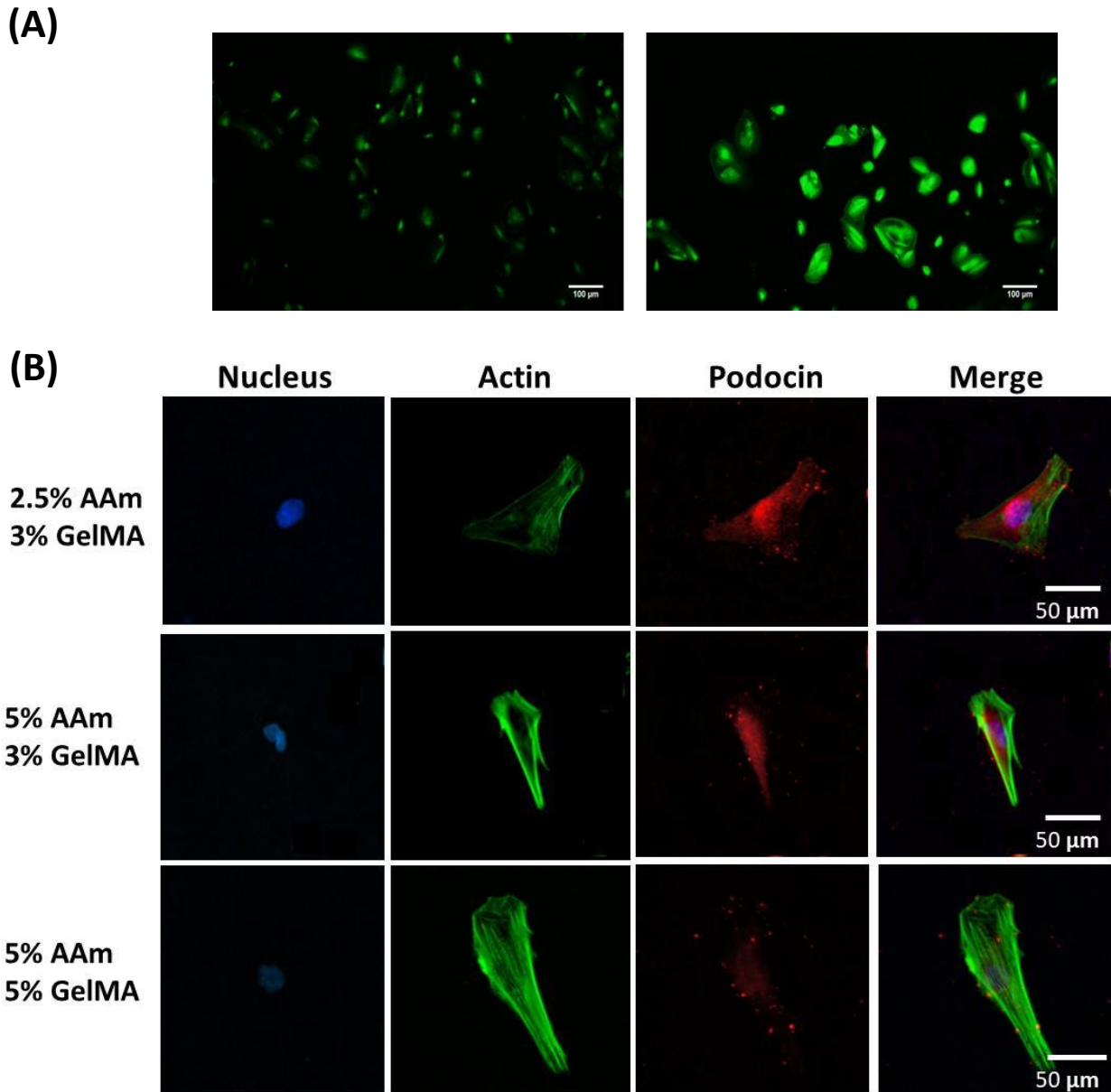


Figure 6: (A) Podocyte cells stained with calcein for the determination of cells viability. (B) Immunofluorescence images of podocytes seeded on GelMA-PAAm substrates having various mechanical properties. The cells were detected by staining the actin cytoskeleton, the nucleus and Podocin protein using Phalloidin (green), DAPI (blue) and anti-Podocin antibody (red) respectively.

Figure 7 showed an increase of podocin expression associated with a decrease of actin expression. Currently, this correlation is still unclear. Importantly, Fan *et al.* reported that podocin might not interact directly with α -actinin. Moreover, Saleem *et al.* showed a functional interrelationship between podocin and actin cytoskeleton. Typically, a perturbation

of podocin distribution is correlated with the loss of cell membrane expression since the actin cytoskeleton is depolymerized⁵⁰. The knockdown of α -actinin induces the increasing of podocin level expression⁵¹.

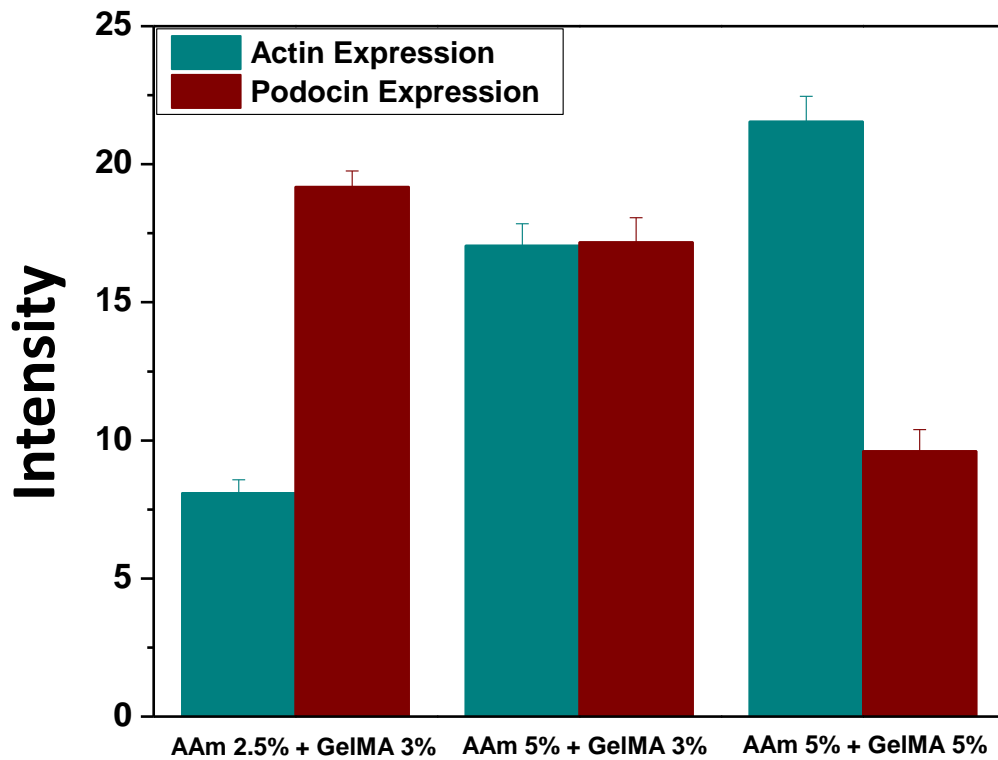


Figure 7: Relationship among podocin and actin levels expressions

Previous investigations reported that podocytes cells cultured on substrates with elasticity of 3 - 5 kPa present similarities of actin fibers and focal adhesions to *in-vivo* ones³³. Consequently, according to their swelling and mechanical properties, AAm_{2.5%} + GelMA_{3%} and AAm_{5%} + GelMA_{3%} are considered good candidates as matrix substrates for podocytes adhesion, proliferation and differentiation.

6. Cells Elasticity:

AFM is a useful technique consisting to map different locations of cells and to provide quantitative measurements known by the Young's modulus parameter of the local stiffness of

the cell surface. Research studies reported the effect of the cellular biological activities such as cells adhesion, proliferation, actin filaments organization and differentiation on cells mechanical properties as cells stiffness. The indentation responses of cells are mostly affected by the mechanical properties of the cell membrane and the subcellular components such as the cytoskeleton and the nucleus⁵². The mechanical properties of podocyte cells seeded on GelMA-AAm hydrogels were analyzed by AFM. Podocytes cultured on AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%} showed an elasticity of 0.46 ± 0.28 kPa, 4.36 ± 1.14 kPa and 11.65 ± 4.35 kPa respectively. Therefore, podocytes elasticity increases as we increase the mechanical properties of GelMA-AAm substrates. Simultaneously, actin expression levels were found to correlate with cell stiffness (Fig. 8). Typically, podocytes showed a dense actin cytoskeleton on the stiffest GelMA-AAm substrate. Thus, these results showed that the mechanical properties of podocyte cells are influenced by the substrate elasticity.

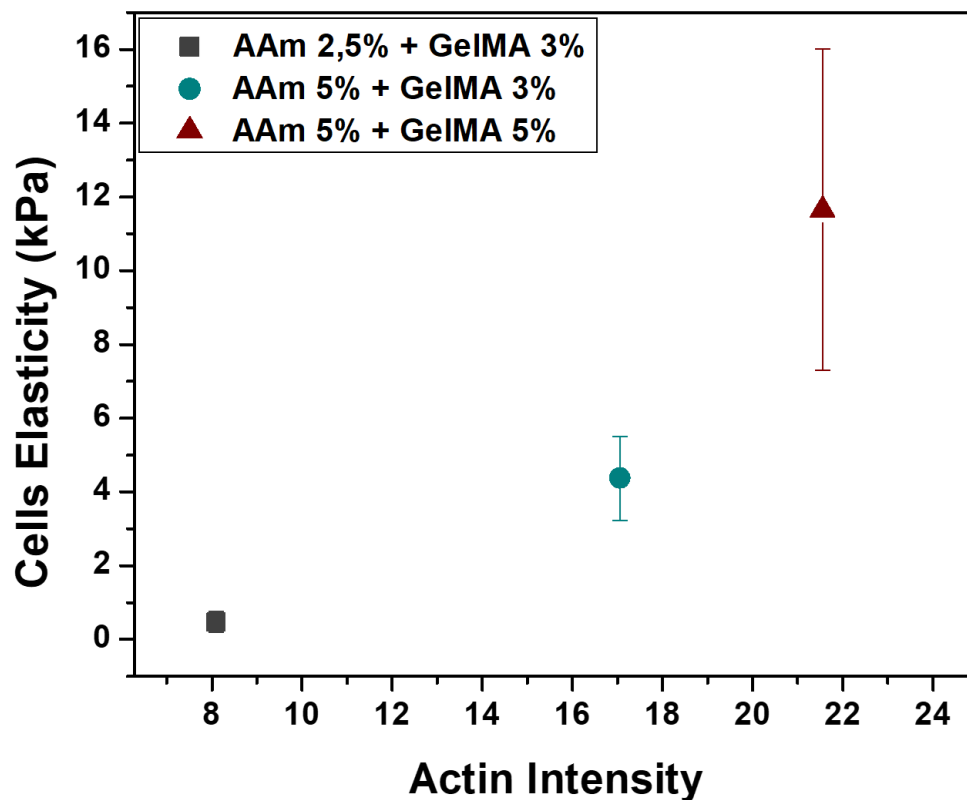


Figure 8: Evaluation of Young's modulus (E) as a function of actin intensity. Bars represent the average of the Gaussian peaks. Error bars are standard deviation of the mean.

IV. Conclusion:

The development of the polymer network based on the combination of biological and synthetic polymers aims to benefit from both the mechanical integrity and the cytocompatibility properties. Here, we studied the properties of GelMA-AAm hydrogels having various concentrations. The characterization of swelling and mechanical properties of these hybrid hydrogels was determined and tuned by adjusting the polymers concentrations. The study of podocyte behaviors was conducted on GelMA-AAm hydrogels having different mechanical properties. The swelling study showed that an increase in the polymers concentrations contributed to a decrease in the hydrogel water uptake and to an increase in hydrogels mechanical properties e.g. elasticity (AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%} are 4.03 ± 0.54 kPa, 11.82 ± 0.49 kPa and 24.98 ± 3.68 kPa respectively). Moreover, the crosslinking density of the GelMA-AAm hydrogel depends on polymers concentrations. This, in turn, also has an effect on the molecular weight amongst the crosslink points. The GelMA-AAm hydrogels are biocompatible for in vitro podocytes cultures and did not alter their proliferation as well as their viability. However, the substrates stiffness influenced podocytes phenotype, morphology and cytoskeleton reorganization. Moreover, evaluation of podocyte cells stiffness showed an increase in cell elasticity by increasing the polymers concentrations (AAm_{2.5%} + GelMA_{3%}, AAm_{5%} + GelMA_{3%} and AAm_{5%} + GelMA_{5%} have an elasticity of 0.46 ± 0.28 kPa, 4.36 ± 1.14 kPa and 11.65 ± 4.35 kPa respectively). We found that AAm_{2.5%} + GelMA_{3%} and AAm_{5%} + GelMA_{3%} having stiffness close to the native glomerular extracellular matrix are optimal for podocytes growth. In conclusion, this work provided a new

tool to combine natural and synthetic polymers to produce an in vitro like GBM with a robust mechanical properties and mimicking the native tissue structure.

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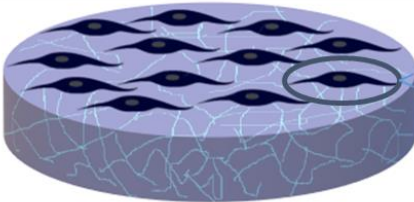
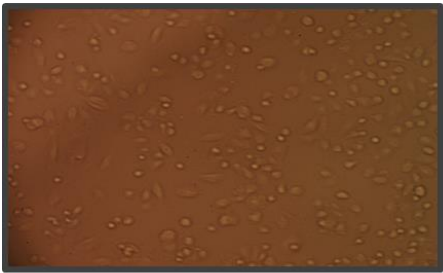
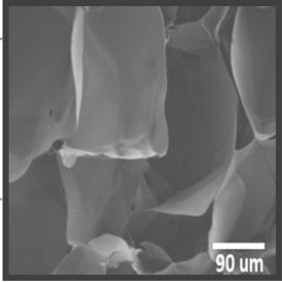
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Graphical Abstract:



GelMA-AAm Hydrogel



**Cultured Podocyte on
GelMA-AAm Hydrogel**

