Design of Biomimetic Cell-Interactive Substrates Using Hyaluronic Acid Hydrogels with Tunable Mechanical Properties

ARTICLE *in* BIOMACROMOLECULES · MAY 2012

Impact Factor: 5.75 · DOI: 10.1021/bm300324m · Source: PubMed

5 AUTHORS, INCLUDING:

Marc R Block
University of Grenoble
87 PUBLICATIONS **3,519** CITATIONS

Article

pubs.acs.org/Biomac

Design of Biomimetic Cell-Interactive Substrates Using Hyaluronic Acid Hydrogels with Tunable Mechanical Properties

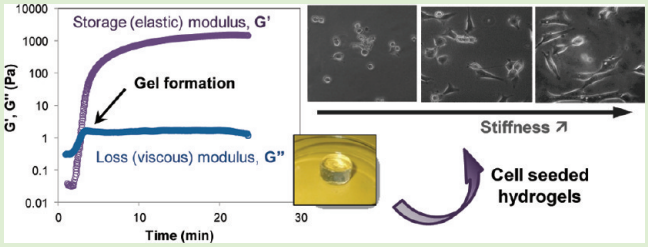
Emilie Hachet,[†] Hélène Van Den Berghe,[†] Eric Bayma,[†] Marc R. Block,[‡] and Rachel Auzély-Velty*[,†]

[†]Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), affiliated with Université Joseph Fourier, 601 rue de la Chimie, 38041 Grenoble, France

[‡]Institut Albert Bonniot, CR INSERM U823, Equipe DySAD (ERL CNRS 5284), Université Joseph Fourier, 38042 Grenoble, France

Supporting Information

ABSTRACT: Hyaluronic acid (HA) is a natural polysaccharide abundant in biological tissues with excellent potential for constructing synthetic extracellular matrix analogues. In this work, we established a simple and dependable approach to prepare hyaluronic acid-based hydrogels with controlled stiffness and cell recognition properties for use as cell-interactive substrates. This approach relied on a new procedure for the synthesis of methacrylate-modified HA macromers (HA−MA) and, on photorheometry allowing real time monitoring of gelation during photopolymerization. We showed in this way the ability to obtain gels that encompass the range of physiologically relevant elastic moduli while still maintaining the recognition properties of HA by specific cell surface receptors. These hydrogels were prepared from HA macromers having a degree of methacrylation <0.5, which allows to minimize compromising effects on the binding affinity of HA to its cell receptors due to high substitution on the one hand, and to achieve nearly 100% conversion of the methacrylate groups on the other. When the HA hydrogels were immobilized on glass substrates, it was observed that the attachment and the spreading of a variety of mammalian cells rely on CD44 and its coreceptor RHAMM. The attachment and spreading were also shown to be modulated by the elastic properties of the HA matrix. All together, these results highlight the biological potential of these HA hydrogel systems and the needs of controlling their chemical and physical properties for applications in cell culture and tissue engineering.



INTRODUCTION

Matrix elasticity or stiffness has been recently shown to have a dramatic effect on many aspects of cell functions including adhesion, migration, proliferation, and differentiation. In many cases, cells appear to have a preferred substrate stiffness which correlates well with that of the tissue environment from which they are derived.[1−5] To mimic native tissue architecture for applications in biotechnology and tissue engineering, various polymer scaffolds have been developed as a substitute for the body's natural extracellular matrix (ECM).[6,7] Among these polymer scaffolds, hydrogels are highly attractive for developing synthetic ECM analogues due to their ability to simulate the microenvironment of most soft tissues. These three-dimensional networks made of cross-linked polymer chains possess high water contents allowing for easy solute diffusion. Furthermore, many hydrogels can be formed under mild, biocompatible conditions and can be straightforwardly modified to exhibit specific cell adhesion ligands, desired elasticity, and degradability.[8] As it is now well established that the cell microenvironment plays a crucial role in cell physiology and fate, the needs for hydrogels with tunable mechanical properties which can be optimally fitted for a wide variety of cell cultures are becoming more apparent, along with identifying the essential biochemical signals to incorporate into these synthetic ECM analogues.[9]

Hyaluronic acid (HA) is a natural glycosaminoglycan that is composed of the repeating disaccharide unit of D-glucuronic acid and *N*-acetyl-D-glucosamine. This polysaccharide is a major component of the ECM in animal tissues, particularly in the brain, the synovial fluid, and the vitreous body of the eye. HA plays an important role in many biological processes including cell proliferation, cell differentiation, morphogenesis, inflammation, and wound healing.[10−13] Although not completely understood, the bioactive properties of HA originate from its capacity of specifically interacting with membrane receptors present on most mammalian cells (such as CD44 or RHAMM),[14−16] as well as with dedicated soluble ECM proteins (named hyaladherins).[17,18] Because of its unique viscoelastic and biological properties, HA finds itself in many biomedical applications such as ophthalmological surgery, treatment of osteoarthritis of the knee, and prevention of postsurgical adhesion.[19,20] In addition, HA has excellent potential for constructing synthetic ECM analogues.[21] However, chemical modification of HA is required to prepare covalently cross-linked hydrogels and to modulate in vivo

Received: February 29, 2012
Revised: May 1, 2012
Published: May 4, 2012

degradation rates and cell attachment abilities of the resulting biomaterials.

An effective strategy for cross-linking HA under mild conditions is the photopolymerization of pendent methacrylates, previously introduced through functionalization of the hydroxyl[11,22−31] or carboxyl[32,33] groups of HA. This methodology provides some advantages such as increased spatial and temporal control over cross-linking and biocompatibility with in situ polymerization.[34,35] Another benefit is that the properties of the formed networks can be tailored by the modification of the HA molar mass, the macromer concentration, and the degree of methacrylation (DM).[23] In this respect, the synthesis of HA−methacrylate conjugates with various DM, from 0.1 to 0.9, was recently reported.[22] In vitro studies of myoblast adhesion indicated that the resulting hydrogels possessing various cross-linking densities do not support alone cell spreading as previously reported for fibroblast and endothelial cells.[25,33,36] When functionalized with the GRGDS peptide, a consensus sequence found in various ECM proteins that trigger the interaction with integrin family cell surface receptors, the hydrogels promoted cell interactions; however, the degree of methacrylation had a minimal effect on myoblast cell attachment, spreading, and proliferation. On the other hand, by using a HA macromer with ∼100% modification of the hydroxyl groups and varying the methacrylate consumption through a dual cross-linking method, Marklein et al. demonstrated the ability to control stem cell spreading and proliferation on HA hydrogels modified with RGD adhesion moieties.[37] These latter findings indicate that the mechanical properties of HA gels can impact some cell types in terms of morphology and functions. As a result, they underscore the importance of fine-tuning gel strength in a broad range of network cross-linking density in order to trigger specific changes in cellular responses. Beside integrin engagement through RGD peptides, as the HA−cell receptor interaction is critically dependent on the molar mass and conformational freedom of HA, care must be taken regarding the conditions for its chemical cross-linking. Indeed, if the aim is to additionally exploit HA biological potential, it must be taken into account that HA interactions with cell surface receptors require minimum sequences of HA repeating units.[38] Thus, HA_6, which is three HA disaccharide units, is the minimum size of HA chain required to occupy the CD44 binding site, while HA_{10} or greater is the optimal length.[39] Indeed, it has already been reported that the degree of substitution of modified HA has also an impact on its biological activity.[40] Therefore, in the design of ECM mimics that are "cell instructive" through specific HA/HA cell surface receptors interactions, it is highly critical to control the conditions for HA modification and gelation so the gel mechanics can be controlled over a broad range without altering the ability of HA to induce cell signaling.

In this study, we aimed to establish a simple and reliable methodology for the synthesis of hyaluronic acid-based hydrogels that encompass the range of physiologically relevant moduli while maintaining binding affinity to HA receptors, as biomimetic ECM platforms for cell culture and other tissue engineering applications. To this end, we first developed new conditions for the methacrylation of HA, based on its reaction with methacrylic anhydride, allowing us to prepare in a short time a series of macromer precursors with DM in the range of 0.1−0.5. Photorheometric measurements were then used to monitor in situ gelation of methacrylated HA derivatives under various photopolymerization conditions, including the concentration of the photoinitiator, light intensity, concentration of macromer, and the degree of methacrylation. This in-depth rheological study allowed us to define the optimal conditions to prepare hydrogels with a broad range of elastic moduli. Lastly, we determined the effect of HA material rigidity on the attachment and the spreading of different cell types. All the cell types tested had the ability to interact to various degrees with these HA hydrogels. Surprisingly, the cells spread on these substrates in a manner that was dependent on the hydrogel storage modulus. Using preosteoblasts, we determined that attachment and spreading were specifically triggered by both CD44 and its coreceptor RHAMM, the two major cell receptors of HA at the plasma membrane.

MATERIALS AND METHODS

Materials. Hyaluronic acid (M_w = 100 000 g/mol) was purchased from Lifecore (Chaska, MN). Methacrylic anhydride (AMA), *N*,*N*-dimethylformamide (DMF), phosphate buffer saline (PBS, pH ∼ 7.4), (3-aminopropyl)trimethoxysilane (APTS, ODTS), and hyaluronidase (HAse type VIII, ∼300 units/mg, ref H3757) were purchased from Sigma-Aldrich-Fluka (L'isle d'Abeau, France). 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) was kindly provided by Ciba Specialty Chemicals (Basel, Switzerland). All chemicals were used without any further purification. The water used in all experiments was purified by a Elga Purelab purification system, with a resistivity of 18.2 MΩ cm.

Synthesis of Methacrylated HA (HA−MA). Hyaluronic acid was modified with methacrylic anhydride either in water as reported elsewhere[28] or in a mixture of water/DMF (1/1, v/v). In the following, we describe the latter procedure which was optimized in this work and which allowed us to obtain HA−MA derivatives with higher DM.

HA (0.20 g, 0.50 mmol) was dissolved in ultrapure water (10 mL) at 4 °C and the resulting mixture was kept at 4 °C under continuous stirring overnight for complete dissolution. DMF (6.7 mL) was then added dropwise in order to have a water/DMF ratio of (3/2, v/v). AMA (0.5, 1, 2, or 3 mol equiv with respect to the moles of repeating unit of HA, i.e., 0.039 g (0.25 mmol), 0.077 g (0.5 mmol), 0.154 g (1 mmol), or 0.231 g (1.5 mmol), respectively) was then added while maintaining the pH between 8 and 9 (by adding 0.5 M NaOH) for 4 h. The reaction was kept at 4 °C under continuous stirring for one night. After this time, NaCl was added to the reaction mixture to have a NaCl concentration of 0.5 M. The polymer was precipitated by the addition of ethanol (with a water/EtOH (v/v) ratio of 2/3). After removal of the supernatant, the precipitate was successively washed with mixtures of water/EtOH (3/7, 1/4, 1/9, v/v) and finally dissolved in ultrapure water for a final purification by diafiltration (ultramembrane Amicon YM10) with ultrapure water. The purified product was recovered by means of freeze-drying and characterized by ^{1}H NMR spectroscopy (Supporting Information, Figure SI-1).

Characterization of Methacrylated HA by ^{1}H NMR. ^{1}H NMR spectra of the HA−MA derivatives dissolved in deuterium oxide (6 mg/mL) were performed at 80 °C using a Bruker DRX400 spectrometer operating at 400 MHz. Deuterium oxide was obtained from SDS (Vitry, France). All spectra were recorded by applying a 45° tip angle for the excitation pulse, and a 10 s recycle delay for 128 scans. The DM of the samples was determined by digital integration of the anomeric protons signals or methyl protons signals of HA and of the methacrylate proton signals at ∼6.1, ∼ 5.7, and ∼1.9 ppm.

Rheometry. An AR2000Ex rheometer (TA Instruments, Inc.) fitted with a UV-curing cell (λ = 365 nm) and an aluminum plate (diameter 20 mm) was used for the in situ measurement of the viscoelastic properties of the HA−methacrylate gels. Following the deposition of 250 μL of HA−MA solution, the gap between the flat quartz plate and the aluminum plate was initially 0.7 mm (measuring at room temperature). It was controlled during the experiments by maintaining the normal force at 0 ± 0.1 N. During the oscillatory time

sweep tests, drops of the G' modulus were observed a few min (~5–10 min) after its sharp increase (as a result of network formation) for the hydrogels with a high stiffness. These were attributed to grip-slip caused by the release of water from the hydrogels. Therefore, the flat quartz plate was sanded which allowed us to overcome this experimental problem (Supporting Information, Figure SI-2). It should be noted that the treatment of the quartz plate had no effect on the values of both moduli (Supporting Information, Figure SI-3).

On each methacrylated HA hydrogel, oscillatory time sweep and frequency sweep experiments were performed. All the dynamic rheological data were checked as a function of strain amplitude to ensure that the measurements were performed in the linear viscoelastic region.

In the oscillatory time sweep experiments, the storage modulus (G') and loss modulus (G'') were measured for a period of 23 or 43 min (depending on the macromer concentration) at a fixed frequency of 1 Hz and a fixed deformation of 3.5%. Typically, after deposition of the solution of methacrylated HA in PBS between the plates and equilibration for 1.5 min, the solution was illuminated (λ = 365 nm) for 20 or 40 min at a fixed light power (50 mW/cm^2 in most experiments) leading to gelation. Light illumination was then stopped for 1.5 min. All measurements were done in triplicates. For comparisons with other tissues and cell culture materials, the hydrogel storage modulus, which dominates for the resulting elastic polymer networks ($G \approx G'$ when $G' > G$), was converted to Young's modulus (E) using rubber elasticity theory, where $G = E/2(1 + \nu)$, assuming a Poisson's ratio (ν) of 0.5.[41,42]

Enzymatic Degradation of Hydrogels. Hydrogel disks (~0.7 mm thickness, 20 mm diameter) prepared by photopolymerization of solutions of HA–MA were immersed into PBS (2 mL) containing 500 U hyaluronidase/mL. The degradation was carried out at 37 °C for 20 h, then 95 °C for 20 min. The resulting solutions were freeze-dried and analyzed by ^{1}H NMR at 25 °C after solubilization in deuterium oxide to determine if unreacted methacrylate groups were present.

Hydrogel Immobilization. For the cell culture experiments, hydrogels were covalently linked to a coverslip during photopolymerization by methacrylation of the coverslip prior to use. The methacrylated coverslip was prepared by treating the slip previously modified with ODTS/APTS with methacrylic anhydride. The glass slip was modified with a mixture of ODTS and APTS as previously described.[43] Briefly, the modified slip with $-NH_2$ groups was immersed in PBS (5 mL) containing AMA (0.1 mL) and stirred at room temperature for half a day. The slip was then rinsed by EtOH/water (1/1, v/v) mixtures, water and dried in air before use. HA–MA solutions (250 μL), deposited on the nonsanded quartz plate of the rheometer, were gelled in contact with the methacrylated glass coverslips. Under such conditions, hydrogels with different stiffness (G'/E modulus varying from 600/1800 to 17000/51000 Pa) were prepared. After preparation, hydrogels fixed on the coverslip and having a thickness of ~0.7 mm were transferred to cell culture media (Dulbecco's modified minimal essential medium, DMEM, Invitrogen) supplemented with 50 U/mL penicillin, and 50 mg/mL streptomycin and left for 12 h for equilibrium before cell culture studies. Media was refreshed after 12 h to remove any remaining monomer or initiator.

Cell Culture and Biological Reagents. NIH-3T3L was a subclone of ATCC NIH3T3 selected for its high spreading ability and HeLa cells were from ATCC and these were grown in αMEM and DMEM, respectively, supplemented with 10% fetal calf serum and antibiotics in a humidified 5% CO_2 atmosphere. Primary osteoblasts (passage 2) were immortalized by transduction with a retrovirus expressing the large SV40 T antigen,[44] cloned, and tested for their ability to express alkaline phosphatase upon differentiation,[45] as previously described.[46] They were cultivated overnight (i.e., approximately 14 h) under the standard conditions in DMEM supplemented in 10% fetal calf serum. Blocking HA receptors CD44 and RHAMM were from Beckton Dinkinson (Le Pont de Claix, France) and Santa Cruz Technologies (Tebu Bio, Le Perray en Yvelines, France), respectively, and the toxic preservative NaN_3 was removed by spin desalting prior to use.

Cell Labeling and Quantification of Cell Spreading. For quantifying the projected areas of spread cells, the cells were fixed with a solution of 4% (w/v) paraformaldehyde in 0.2 M phosphate buffer (pH = 7.2) for 10 min at room temperature. After 3 washes in PBS, the cells were incubated in DMEM supplemented with 0.5% of Vybrant DIL cell labeling solution for 15 min at 37 °C according to the manufacturer (Life Technologies, St Aubin, France). The cells were washed 3 times in PBS and mounted under a coverslip with 10 μL of mounting solution for epifluorescence observations. Observation were carried out with a Provis 70 microscope (Olympus Europe Hamburg, Germany) equipped with a Plan NeoFluar 20× (N.A. 0.5) objective. Areas were determined with Metamorph software (Molecular Devices, Sunyvale, CA) at a magnification of 200×. Statistical analyses were carried out with R software (The R Project for Statistical Computing, http://www.r-project.org/)

RESULTS AND DISCUSSION

Synthesis of HA–Methacrylate Macromers. Several methods have been developed for synthesizing methacrylate-modified HA, such as using methacrylic anhydride[23,28–30] or glycidyl methacrylate (GMA).[11,24–27,31,47] In both cases, the degree of methacrylation did not exceed 0.3 when methacrylation was performed in aqueous solution due in part to the low solubility and hydrolysis of AMA and GMA in water. Therefore, in order to enhance the DM, alternative methods involving the reaction of HA with GMA in organic solution or in a hydro-organic mixture were developed.[22,27] Although Bencherif et al. showed the ability to increase the DM up to 0.9 when the reaction of HA with GMA was performed in a water/DMF mixture at physiological pH, it required a long reaction time (10 days).[22] Different esters resulting from a reversible transesterification through the primary hydroxyl group and an irreversible ring-opening conjugation through the carboxylic acid group toward the highest substituted carbon of epoxide were assumed to be formed simultaneously. Their respective amounts were reported to be time-dependent as the concentration of ring-opening products increased with time.

In this work, we synthesized methacrylated HA derivatives by the reaction of methacrylic anhydride with the hydroxyl groups of HA, leading to only one type of ester (Figure SI-1).

As previously reported,[23,28,30] when the reaction of AMA with native HA was performed in aqueous solution, we obtained HA derivatives with low DM (≤0.20), although AMA was added in large excess (see Table 1). By comparing the molar ratio of AMA to HA in the reaction medium and the DM of modified HA, it can be noticed that for the two feed ratios, HA was modified with 1–5% of the added AMA. Interestingly, the degree of methacrylation could be enhanced by using DMF

Table 1. Reaction Conditions for the Modification of HA with Methacrylate Groups through the Hydroxyl Functions

			solvent (vol %)		
reaction[a]	sample	AMA[b]	water	DMF	DM
1	**1a**	2	100	0	0.10
2	**1b**	20	100	0	0.20
3	**2a**	0.5	50	50	0.12
4	**2b**	1	50	50	0.20
5	**2c**	2	50	50	0.28
6	**2d**	3	50	50	0.49

[a]All reactions were carried out at 4 °C during 16 h; they were performed in duplicate to ensure reproducibility. [b]Number of molar equivalents with respect to the HA repeating unit.

as a cosolvent. As a result, the percentage of modification of HA for the four feed ratios was between 14% and 24% of the added AMA after 16 h of reaction (Table 1). Actually, such conditions allow the solubilization of methacrylic anhydride in the reaction medium resulting in better control over the DM. This method compared favorably with that using GMA in PBS/DMF[22] which required much longer reaction times (5−10 days) and higher GMA/HA molar ratios (with respect to the AMA/HA ratios) to obtain DM in the range of 0.32−0.60.

Hydrogel Formation and Rheological Properties. The gels were formed by photopolymerization at 365 nm from HA−MA macromers in the presence of the photoinitiator, Irgacure 2959, which was selected for its known biocompatibility with cells.[48] The gelation process was monitored in situ by photorheometry. To optimize the conditions for the synthesis of HA networks, we investigated the effect of various experimental parameters that control photogelation, that is, photoinitiator concentration and power of light used for irradiation. Figure 1 shows the effect of (A) photoinitiator concentration and (B) power of light used for irradiation on the time sweep profiles of the storage modulus (G') and loss modulus (G'') obtained from a solution of HA−MA **2b** with a DM of 0.20.

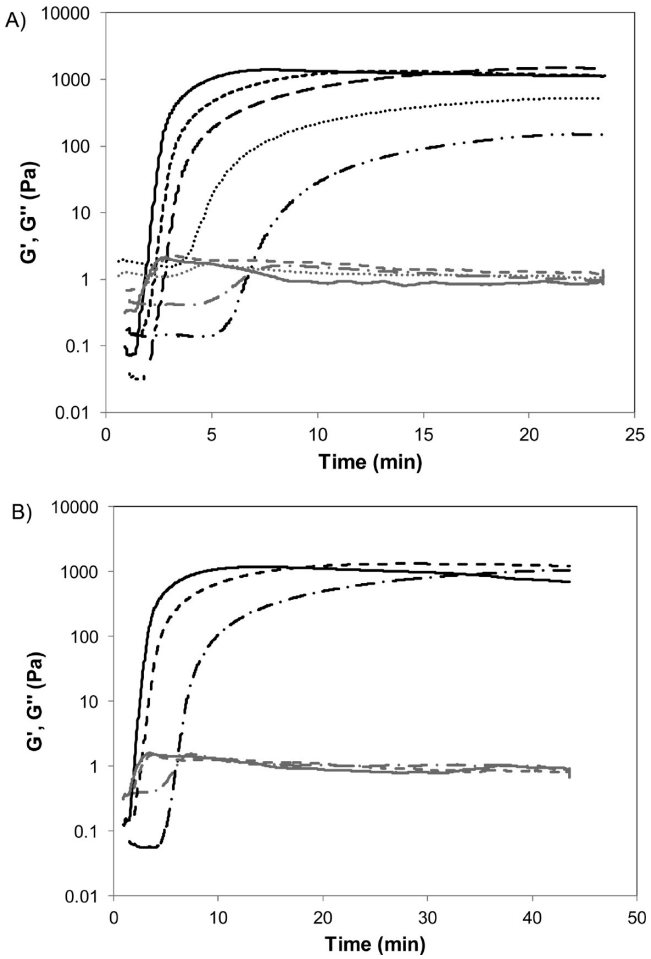


Figure 1. In situ photorheometric measurement of hydrogel formation from methacrylate modified-HA: effect of reaction parameters on photogelation of the macromer **2b** at a concentration of 15 g/L in PBS. The G' and G'' curves are represented by black and gray lines, respectively, and the black and gray arrows indicate the crossover of the G' and G'' moduli. (A) Effect of photoinitiator concentration (%, w/w): 0.2 (—), 0.1 (---), 0.05% (− − −), 0.025 (····), 0.01 (− ·· −); (B) effect of power of light used for irradiation (mW/cm^2): 144 (—), 50 (---), 15 (− · −). As the loss modulus, G'', increased negligibly for all hydrogels, some of the G'' curves are superposed.

The samples were equilibrated for 1.5 min before being exposed to light for a time of 20 or 40 min (see Materials and Methods). Initially, G'' is larger than G', which reflects the viscous behavior of the sample. After an induction period, which depends on the photoinitiator concentration and power of light, the storage modulus increases sharply due to the formation of elastic effective intermolecular cross-links, whereas the loss modulus increased negligibly during this time. Consequently, there is a crossover point where G' becomes higher than G''. The time required for this crossover to occur is sometimes referred to as the gelation time, although this point depends on the applied frequency.[49,50] In the postgelation period, the G' curve levels off indicating the end of the gelation process. In this context, the steady-state value of G' was used as a measurement for hydrogel elasticity. The loss modulus increased negligibly during this time for all hydrogels.

From Figure 1A, it can be seen that the time required for the beginning of gelation, which corresponds to the time where G′ becomes equal to G'' as mentioned above, is dependent on the photoinitiator concentration. In these experiments, the power of light used for irradiation was fixed at 50 mW/cm^2. For concentrations in the range of 0.05−0.2% (w/v, i.e., [photoinitiator]/[MA] ratios ranging from 0.30 to 1.23), the gelation times are around 2 min and 40 s. However, when the initiator concentration was decreased to 0.025% or lower, one can notice a significant increase in the gelation time going from 3 min and 20 s for the concentration of 0.025% (i.e., a [photoinitiator]/[MA] ratio of 0.15) to 6.5 min for a concentration of 0.01% ([photoinitiator]/[MA] ratio = 0.06). Moreover, a significant decrease in the G' modulus can be observed for these two concentrations. For the lowest initiator concentration, the G' modulus thus attains a value which is ∼10 times lower than those measured for the three higher concentrations. This suggests that the initial photoinitiator concentration is too low to activate all methacrylate groups for radical polymerization. The activation of the methacrylate groups is particularly delicate as it strongly depends on the diffusion of the photoiniator in the HA chemical network. On the other hand, the slight decrease in the G' values with the time observed for the higher concentrations may be related to the slight degradation of the HA network due to the high concentration of radicals produced by the photoinitiator. Indeed, the same experiment performed with a film of silicone oil leads to a similar curve indicating almost no solvent evaporation during the experiment performed under these conditions (Supporting Information, Figure SI-4). All together, the data suggest that below a critical [photoinitiator]/[MA] ratio situated between 0.06 and 0.15, the completion of network formation requires much longer times of irradiation.

The decrease in the light power from 144 to 15 mW/cm^2 also delayed the beginning of gelation while it did not have a striking effect on the steady-state value of G' (Figure 1B). As can be seen in Figure 1B, the time required for the G' modulus to reach a plateau was significantly increased when the light power was decreased to 15 mW/cm^2. Indeed, the G' modulus was found to level off after 40 min of irradiation, whereas the steady-state value of G' was reached after ∼20 min of irradiation for light powers >50 mW/cm^2. Therefore, from these results, all subsequent oscillatory time sweep experiments were performed

using a photoinitiator concentration of 0.05% and a light power of 50 mW/cm^2. One can notice a progressive decrease in the G' modulus after reaching its maximum for the sample illuminated with UV light at a power of 144 mW/cm^2. Such conditions are likely to result in sample heating, causing partial evaporation of water. When the same experiment was carried out with a film of silicone oil, the decrease was much less pronounced (Supporting Information, Figure SI-5). This result supports our hypothesis that almost no solvent evaporation occurs when polymerizations are conducted with UV light at a power of 50 mW/cm^2.

Next, we focused our attention on some factors that may affect the mechanical strength, namely, the macromer concentration and the degree of methacrylation.

Figure 2 shows the influence of the irradiation time and the macromer concentration on the storage modulus of hydrogels

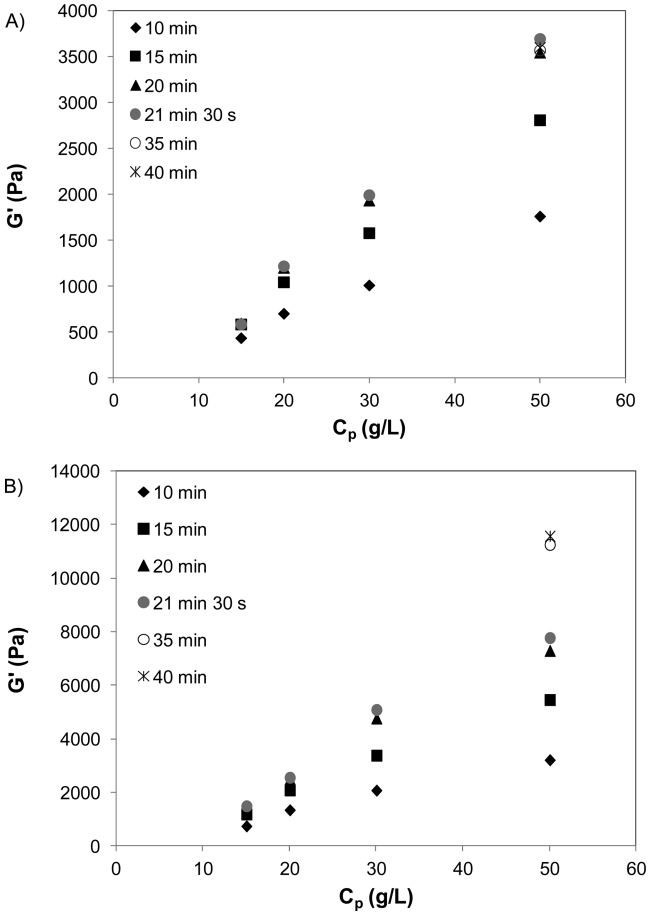


Figure 2. Effect of time of irradiation and macromer concentration on the storage modulus of hydrogels prepared from HA−MA (A) **2a** and (B) **2b**.

prepared from the derivatives **2a** and **2b**. As one can see from this Figure, the G' values become constant ∼20 min after the beginning of irradiation (i.e., 21.5 min after the beginning of the oscillatory shear experiment) for the hydrogels made from the macromer **2a** independent from its concentration. On the other hand, it appears that for the hydrogel prepared from **2b** at a macromer concentration of 50 g/L, the G' values level off only ∼35 min after the beginning of experiment. Moreover, a similar behavior to **2b** was observed with HA−MA **2c**. These results indicate that from DS values ≥0.2 and macromer concentrations ≥50 g/L, the time of irradiation performed using a photoinitiator concentration of 0.05% must be increased from ∼20 min to ∼40 min to reach a plateau for G'. It should be noted that in the case of **2b**, this time could be decreased to ∼30 min by increasing the photoinitiator concentration by 2-folds. However, the steady-state value of G' was found to be 20% lower than that obtained with a photoinitiator concentration of 0.05%. This was attributed to the degradation of HA at high radical concentrations.

To verify if the time for G' to level off corresponds to the total conversion of the methacrylate groups, we analyzed by ^{1}H NMR samples of HA−MA **2b** (C_p = 50 g/L) which were photopolymerized for 20 and 40 min and subsequently degraded by hyaluronidase. After a 20 h incubation at 37 °C in 500 U hyaluronidase/mL, the swollen networks were converted into solutions due to the cleavage of internal *β-N*-acetyl-D-glucosaminidic linkages in the HA chains. After 20 and 40 min of irradiation, traces of methacrylate double bonds could be observed from the NMR spectra (Supporting Information, Figure SI-6). Digital integration of the proton signals of the double bonds and the anomeric proton signals of HA indicated that time of irradiation increased from 20 to 40 min, and an increase in the cross-linking conversion from 80 to 90% occurred. This result suggests that the time required for G' to become nearly constant can be considered as an indication of the time for the photopolymerization to reach near 100% conversion. This method can have some advantages compared to ^{1}H NMR analysis considering its simplicity, rapidity and sensitivity. It should be noted that Fourier Transform Infrared spectroscopy failed to reveal traces of methacrylate double bonds in the sample. The fact that 100% conversion is not strictly obtained can be attributed to severe mobility restrictions after the gel point is reached, which hamper reactions between all methacrylate groups especially when their concentration is very high as discussed below.

When comparing the HA−MA hydrogels prepared from solutions of macromers at the same concentration (15 g/L) but with different DM, an increase in the storage modulus values measured 21 min after the beginning of irradiation was observed as the DM of macromers was increased from 0.12 to 0.28 (Figure 3). This is consistent with the theory which predicts that for cross-linked rubberlike networks, the elasticity is related to the concentration of cross-links by the equation

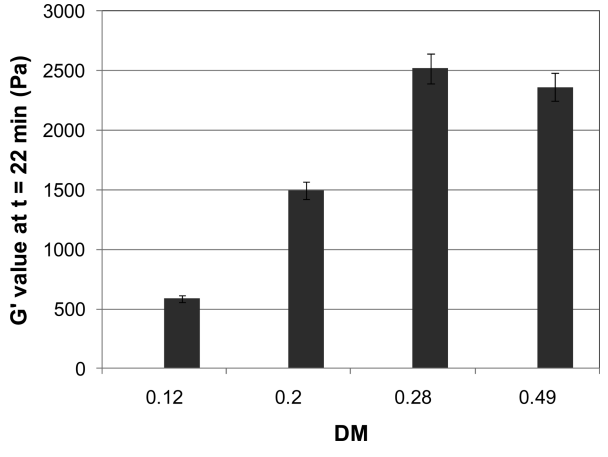


Figure 3. Effect of the degree of methacrylation on the mechanical properties of HA hydrogels prepared from solutions of macromers at a concentration of 15 g/L.

$$G = nRT/V \quad (1)$$

Where n is the number of moles of elastic chains per volume V, and R is the gas constant.[51] It should be noted, however, that in this work, n cannot be directly related to the DM of HA macromers due to the complexity of the network structure consisting of HA chains cross-linked by oligo(methacrylate) chains.

A lower storage modulus value was found when the DM reached 0.49. As such, DM means that on average one over two repeating units is substituted by a methacrylate group; it can be reasonably assumed that the 100% conversion of methacrylate groups is hindered by accessibility problems due to the high DM and severe mobility restrictions after the gel point is reached. This assumption was supported by other experiments performed at higher macromer concentrations which resulted in hydrogels with significantly lower storage modulus values compared to those of hydrogels prepared from the derivatives **2a–c**. ^{1}H NMR analysis of hydrogels after degradation by hyaluronidase also supports this assumption. Indeed, signals corresponding to the protons of methacrylate double bonds could be observed from the NMR spectra of samples prepared from **2c** at concentrations of 15 and 30 g/L (Supporting Information, Figure SI-7). Digital integration of these signals and the anomeric proton signals of HA indicated cross-linking conversions of 82 and 61% for gels made at macromer concentrations of 15 and 30 g/L, respectively.

To determine the conditions needed to prepare gels in the physiologically relevant stiffness, we compared the evolution of G' by varying the concentration of macromers **2a–c**. Matrix elasticity can vary from hundred to a few thousands Pa depending on the natural tissue.[5,52] Figure 4 plots the storage modulus as a function of methacrylate concentration (C_{MA}, calculated from the average molar mass of the repeating unit of HA–MA and the DM) for hydrogels made from solutions of HA–MA **2a–c** as well as a HA–MA derivative (**1b**) prepared according to a literature procedure[28] at concentrations in the range of 15–50 g/L. As can be seen in this figure, a large range of physiologically relevant stiffness can be produced from solutions of HA–MA derivatives with a DM between 0.12 and 0.28. Interestingly, a unique curve is obtained for C_{MA} values ranging from 4.4×10^{-3} to $\sim 20 \times 10^{-3}$ mol/L, thus, indicating that HA macromers **1b** and **2a–c** can independently be used for preparing hydrogels having well-defined storage-moduli in the range of 500–21 000 Pa. According to the relationship $E = 2G'(1 + \nu)$ (see Materials and Methods), this corresponds to Young's moduli in the range of 1500–63 000 Pa. It should be noted that for $C_{MA} < 4.4 \times 10^{-3}$ mol/L, the G' modulus dramatically decreased from 165 Pa (for $C_{MA} = 2.9 \times 10^{-3}$ mol/L) to ~0 Pa (for $C_{MA} = 0.6 \times 10^{-3}$ mol/L, Supporting Information Figure SI-8). The G' value of ~0 Pa was actually obtained for very low concentrations of HA–MA (i.e., 2 and 1.1 g/L for **2a** and **1b**, respectively). All together, these results demonstrate that from both optimized procedures for the synthesis of HA–MA derivatives and their photopolymerization, hydrogels with tunable stiffness could be prepared with good reproducibility by varying either the DM or macromer concentration.

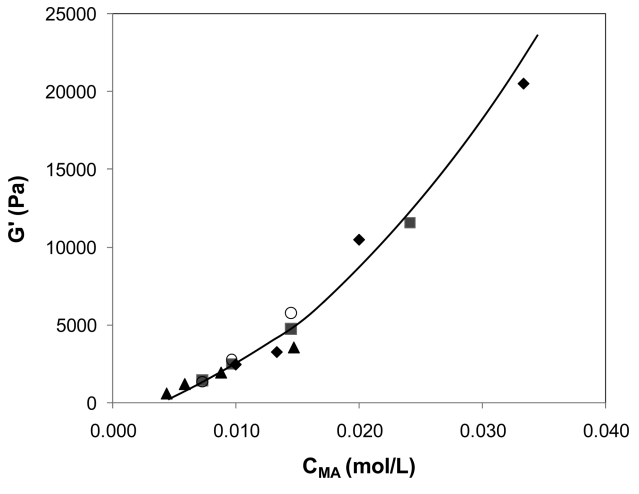


Figure 4. Storage modulus of hydrogels made of methacrylated HA **2a** (▲) **2b** (■), **2c** (◆), and **1b** (○) as a function of MA concentration (C_{MA}).

Cellular Responses in Vitro. Next, we wanted to check the ability of mammalian cells to interact with our methacrylated HA hydrogels. In order to provide stability during the manipulation and the experimentation with the cells, the hydrogels were immobilized to glass substrates during their synthesis by photopolymerization under the optimal conditions discussed above. Therefore, from the unique curve depicted in Figure 4, several macromer solutions were prepared, deposited on the nonsanded quartz plate of the rheometer, and gelled (without applying shear stress) in contact with glass coverslips modified with methacrylate units. Three different cell lines were subsequently seeded on the hydrogels as described under Materials and Methods and cultured overnight in DMEM supplemented with 10% fetal calf serum (the full cell spreading steady state is usually reached after 4 h for most mammalian cells under these experimental conditions). Figure 5 shows that, under these experimental conditions, the different cell types were able to adhere firmly on the hydrogels and were no longer floating in the medium. Surprisingly, all the cell types were able to spread to various degrees onto these substrates in a manner that was favored by the increase in the storage modulus, a property that was similarly observed in the classical integrin mediated adhesion to ECM proteins.[53]

Cell spreading is the read-out of cytoskeleton reorganization and the establishment of internal tensions. It was markedly dependent on the cell type. HeLa cells were poorly spread even at the highest storage modulus, although some small membrane extensions were detected at G' = 17 000 Pa suggesting some initiation of the spreading process. However, the quantification of the distribution of the cell projected areas (n = 60) showed that those epithelial cells did respond to the storage modulus, although to a lesser extent. Conversely, NIH 3T3L cells were fully spread at this storage modulus and exhibited a polarized morphology suggesting a high motility activity. Finally, the preosteoblast exhibited an intermediate situation with some spreading that was increased from G' = 600 to G' = 17 000 Pa. The differences observed among the cell types suggested that the attachment and the spreading observed were not due to some nonspecific binding of adsorbed ECM proteins since all cell types attached and spread similarly on ECM protein coated surfaces (Supporting Information Figure SI-9). Cell type dependence also ruled out a possible cell surface interaction with unreacted methacrylate moieties. This was further confirmed by the fact that no difference was observed by comparing the biological response of HA–MA gels having the same stiffness (G' = 2500 Pa) but prepared from different HA–MA samples, that is, **2a** (40 g/L) and **2d** (15 g/L), for which

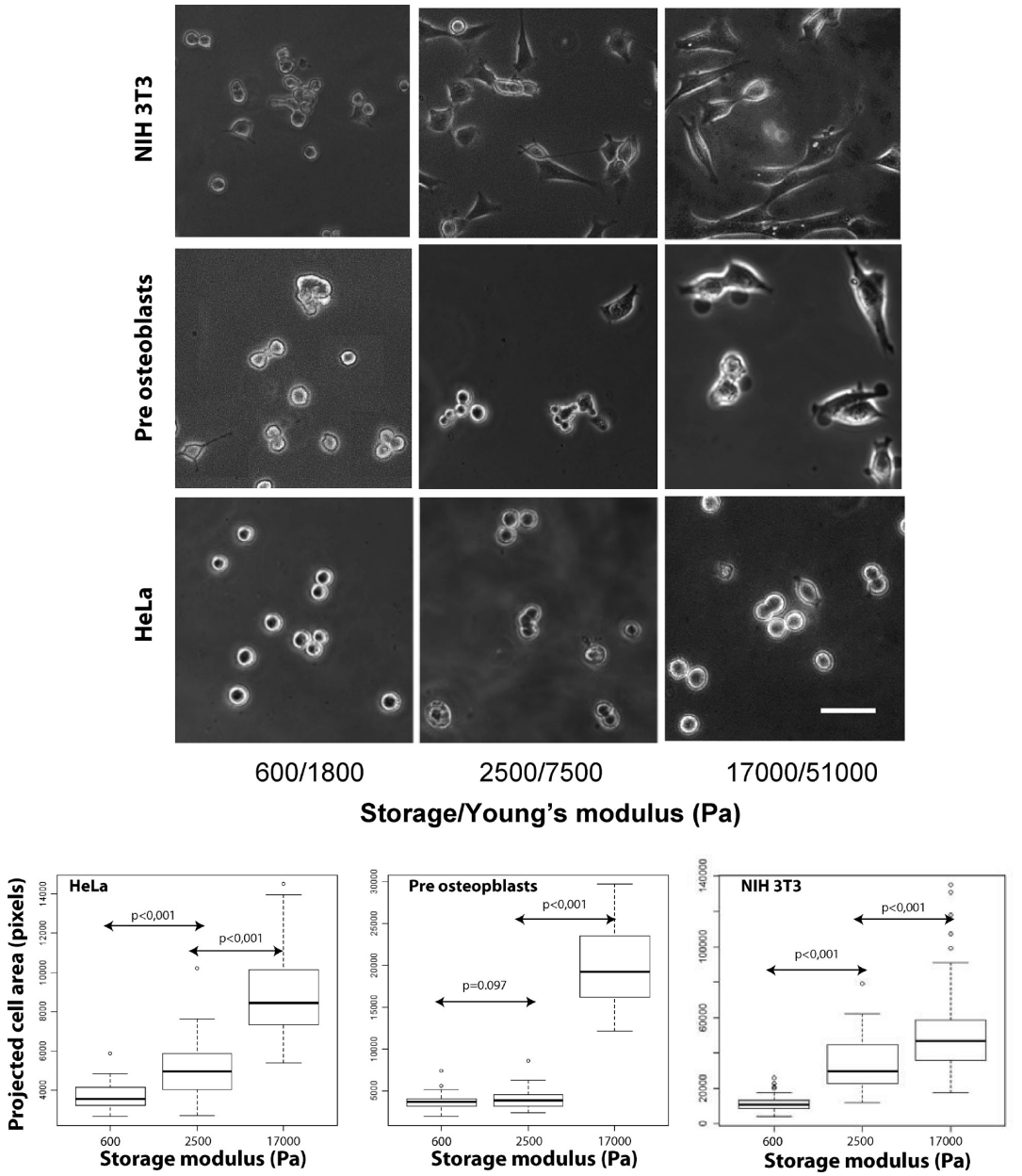


Figure 5. Cell attachment and spreading on methacrylated HA hydrogels as a function of the G' values. Upper panel: phase contrast observations carried out on living cells with a Zeiss 100 M Axiovert microscope equipped with a plan 10× objective (N.A 0.25). Cell Lower panels: cell spreading quantification. After cell fixation with paraformaldehyde, cells were labeled with Vybrant DIL lipophilic tracer. Observations of the fluorescent cells were carried out with an Olympus Provis 70 epifluorescent microscope equipped with a Plan NeoFluar 20× objective (N.A. 0.50). The images threshold value was adjusted to fit the cells limits and the projected cell areas were determined with Metamorph software. Statistical analyses and box plots were performed with the R software (n = 60). The bottom and top of the box are the 25th and 75th percentile (the lower and upper quartiles, respectively), and the band near the middle of the box is the 50th percentile (median). The ends of the whiskers represent the lowest datum still within 1.5 Inter Quantile Range of the lower quartile, and the highest datum still within 1.5 Inter Quantile Range of the upper quartile. Bar is 40 μm.

the methacrylate conversion after photopolymerization was different (∼100% for **2a** and 82% for **2d**) (Supporting Information Figure SI-10). In addition, trypan blue staining showed a good viability (94%) of the cell spread on HA methacrylates gels (not shown). To directly establish that cell behavior on methacrylated HA hydrogels was due to a specific interaction of the cells with HA, we preincubated for 1 h at 4 °C preosteoblasts with blocking antibodies directed against the two major HA receptors at the concentration of 30 μg/mL, a value that is classically used in competition experiments. Subsequently, overnight culture in DMEM containing the antibodies was carried out on HA methacrylate gels. After overnight culture, the cells were fluorescently labeled with a lipophilic tracer Vybrant DIL as described under Materials and Methods, and the fluorescent cell images were taken using a Provis AX70 Olympus upright microscope.

Figure 6 shows that even at high storage modulus, cell attachment and spreading to HA−MA gels could be almost fully inhibited by adding either anti-CD44 or anti-RHAMM. It is worth mentioning that incubation of the cells with the antibodies did not affect the cell spreading on the culture plastic, ruling out a possible toxicity of these antibodies

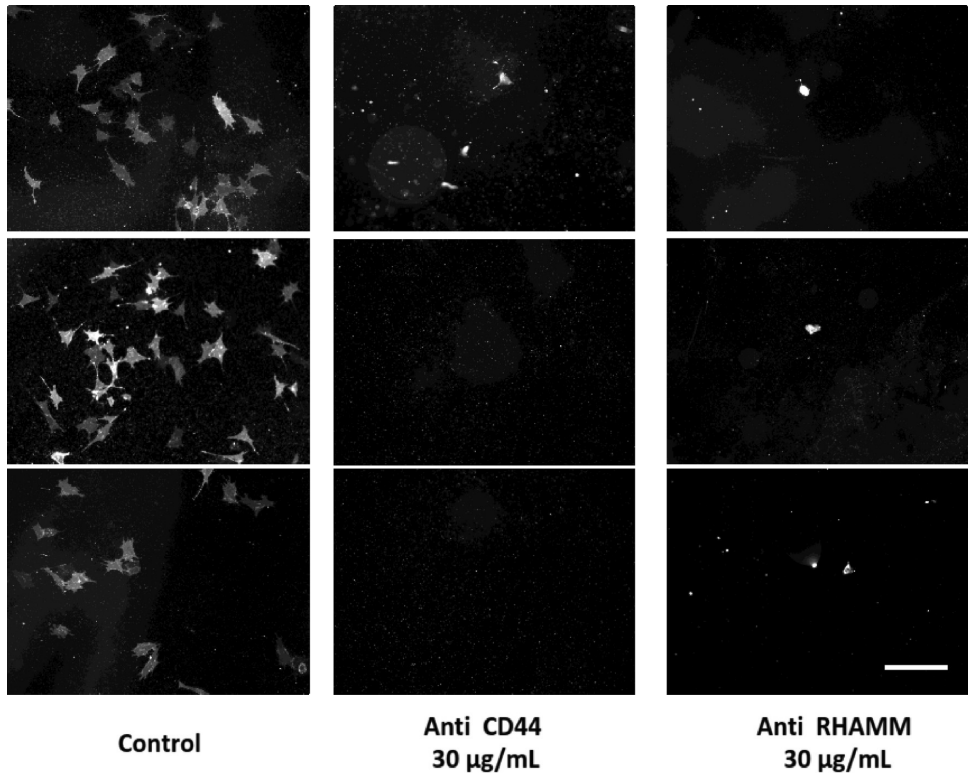


Figure 6. Effect of blocking antibodies rose against CD44 and RHAMM on primary osteoblasts spreading on HA methacrylate gels. The cells were preincubated for 1 h at 4 °C with the antibodies at the concentration of 30 μg/mL and seeded on the hydrogels with the antibodies. Culture was performed overnight under classical conditions, i.e., at 37 °C in a humidified 5% CO_2 atmosphere. After cell fixation with paraformaldehyde, cells were labeled with Vybrant DIL lipophilic tracer. Observations of the fluorescent cells on 3 independent observation fields for each experimental condition were carried out with an Olympus Provis 70 epifluorescent microscope equipped with a Plan NeoFluar 20× objective (N.A. 0.50). Bar is 50 μm.

(Supporting Information Figure SI-11). These results indicated that both receptors were functional on these cells and that there is a direct evidence that cell spreading was mostly mediated by the interaction of the gel HA moiety with those specific receptors and not due to interferences with either methacrylate moieties or adsorbed proteins.

Other reports have mentioned the lack of cell spreading of human dermal fibroblasts and NIH3T3 cells.[33,36] Our data show that cell spreading on HA gels is cell type and storage modulus dependent. In addition, for most of the cells, this spreading however is quite smaller than integrin mediated spreading (for instance, when hydrogels are functionalized with RGD peptides). Such limited spreading has been neglected in former works. Earlier studies with NIH3T3 cells were carried out in the absence of serum.[36] These experimental conditions switched off the regulatory GTPase RhoaA and consequently myosin II activity, resulting in the loss of cellular contractility that is required for cell spreading. Cell interaction with a nonspecific matrix such as poly(L-lysine) results in an isotropic spreading of the cells and indeed, the initial stage of cell spreading seems to be independent from the integrin activation.[54] However, in the case of HA hydrogels, the final stages of cell spreading were strongly anisotropic with highly polarized cells indicating that HA receptors are likely to engage in active processes within the cells that support migration.

■ CONCLUSIONS

In this study, we developed appropriate conditions for the synthesis of HA hydrogels allowing to fine-tune their (bio)physical properties. We first adjusted the procedure for HA-methacrylation, allowing us to obtain HA−MA derivatives with good reproducibility and satisfactory grafting efficiency. Then, using photorheometry for the real-time monitoring of HA−MA photopolymerization, we demonstrated the ability to finely control the conversion of the methacrylate groups and, thus, the mechanical properties of the HA hydrogels. Notably, it was shown that HA macromers with DM in the range of 0.1−0.30 can independently be used for the synthesis of HA matrices with storage moduli included between 500 and 21 000 Pa. This result, which can be related to the full conversion of the methacrylate groups, was then exploited to synthesize biomimetic hydrogel substrates for 2-dimensional cell culture experiments using various mammalian cell types. Very interestingly, our results showed that the cells not only interacted with the HA hydrogels, but also were able to spread on these surfaces in a manner that was favored by the increase in the gel rigidity. On preosteoblasts, we showed that this interaction was specific and was mediated mostly by the HA receptors CD44 and RHAMM. Rigidity dependence of integrin mediated cell adhesion through ECM protein has been extensively described, although the mechanism of integrin mechanotransduction has been only partly unraveled. However, our data indicated for the first time that cell types are sensitive to the substrate rigidity when cells are only using HA receptors. This suggests that these transmembrane proteins also play a role as mechanotransducers and can induce the rearrangement of actin cytoskeleton. In summary, we have developed new conditions for HA cross-linking, affording HA-based platforms

that may prove potent tools for further exploration of mechanotransduction and its effect on cell differentiation and migration for understanding their role in disease and tissue regeneration.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR spectrum of of HA−MA derivatives **2a−d**; in situ photorheometric measurement of hydrogel formation from methacrylate modified-HA **2c** performed on a nonsanded and sanded quartz plate; in situ photorheometric measurement of hydrogel formation from methacrylate modified-HA **2b** performed on a nonsanded and sanded quartz plate; in situ photorheometric measurement of hydrogel formation from methacrylate modified-HA **2b** performed in the absence and in the presence of a film of silicone oil at a fixed light power of 50 mW/cm^2; in situ photorheometric measurement of hydrogel formation from methacrylate modified-HA **2b** performed in the absence and in the presence of a film of silicone oil at a fixed light power of 144 mW/cm^2; ^{1}H NMR spectra of initial HA and HA−MA hydrogels after digestion by hyaluronidase; ^{1}H NMR spectra of hydrogels prepared from HA−MA **2d** at 15 and 30 g/L in PBS after digestion by hyaluronidase; storage modulus of hydrogels made of methacrylated HA **2a** and **1b** as a function of MA concentration ($C_{MA} < 4.4 \times 10^{-3}$ mol/L); attachment and spreading of different cell lines on ECM protein coated surfaces; preosteoblast spreading on HA−MA gels with a storage modulus of 2500 Pa prepared with HA−MA **2a** and HA−MA **2d** (15 g/L); incubation of preosteoblast cells with blocking antibodies raised against CD44 or RHAMM in plastic Petri dishes. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +33 4 76 03 76 71. E-mail: rachel.auzely@cermav.cnrs.fr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank David Israel (TA Instruments) for helpful discussions about the photorheometry measurements and his technical assistance. This work was financially supported by the "Agence Nationale pour la Recherche" (grant ANR P3N 2009 to R.A.-V.). H.V.D.B. gratefully acknowledges the CNRS for a postdoctoral fellowship. R.A.-V. is indebted to the Institut Universitaire de France for financial support.

■ REFERENCES

(1) Discher, D. E.; Janmey, P.; Wang, Y.-l. *Science* **2005**, *310*, 1139−1143.
(2) Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. *Cell* **2006**, *126*, 677−689.
(3) Ghosh, K.; Ingber, D. E. *Adv. Drug Delivery Rev.* **2007**, *59*, 1306−1318.
(4) Paszek, M. J.; Zahir, N.; Johnson, K. R.; Lakins, J. N.; Rozenberg, G. I.; Gefen, A.; Reinhart-King, C. A.; Margulies, S. S.; Dembo, M.; Boettiger, D.; Hammer, D. A.; Weaver, V. M. *Cancer Cell* **2005**, *8*, 241−254.
(5) Yeung, T.; Georges, P. C.; Flanagan, L. A.; Marg, B.; Ortiz, M.; Funaki, M.; Zahir, N.; Ming, W.; Weaver, V.; Janmey, P. A. *Cell Motil. Cytoskeleton* **2005**, *60*, 24−34.
(6) Patterson, J.; Martino, M. M.; Hubbell, J. A. *Mater. Today* **2010**, *13*, 14−22.
(7) Tibbitt, M. W.; Anseth, K. S. *Biotechnol. Bioeng.* **2009**, *103*, 655−662.
(8) Lutolf, M. P.; Hubbell, J. A. *Nat. Biotechnol.* **2005**, *23*, 47−55.
(9) Kloxin, A. M.; Kloxin, C. J.; Bowman, C. N.; Anseth, K. S. *Adv. Mater.* **2010**, *22*, 3484−3494.
(10) Chen, W. Y. J. *Hyaluronan* **2002**, *2*, 147−156.
(11) Jia, X.; Burdick, J. A.; Kobler, J.; Clifton, R. J.; Rosowski, J. J.; Zeitels, S. M.; Langer, R. *Macromolecules* **2004**, *37*, 3239−3248.
(12) Takahashi, Y.; Li, L.; Kamiryo, M.; Asteriou, T.; Moustakas, A.; Yamashita, H.; Heldin, P. *J. Biol. Chem.* **2005**, *280*, 24195−24204.
(13) Toole, B. P. *Semin. Cell Dev. Biol.* **2001**, *12*, 79−87.
(14) Entwistle, J.; Hall, C. L.; Turley, E. A. *J. Cell Biochem.* **1996**, *61*, 569−77.
(15) Peach, R. J.; Hollenbaugh, D.; Stamenkovic, I.; Aruffo, A. *J. Cell Biol.* **1993**, *122*, 257−64.
(16) Yang, B.; Zhang, L.; Turley, E. A. *J. Biol. Chem.* **1993**, *268*, 8617−23.
(17) Day, A. J.; de la Motte, C. A. *Trends Immunol.* **2005**, *26*, 637−643.
(18) Day, A. J.; Sheehan, J. K. *Curr. Opin. Struct. Biol.* **2001**, *11*, 617−622.
(19) Kogan, G.; Soltes, L.; Stern, R.; Gemeiner, P. *Biotechnol. Lett.* **2007**, *29*, 17−25.
(20) Prestwich, G. D.; Kuo, J.-W. *Curr. Pharm. Biotechnol.* **2008**, *9*, 242−245.
(21) Burdick, J. A.; Prestwich, G. D. *Adv. Mater.* **2011**, *23*, H41−H56.
(22) Bencherif, S. A.; Srinivasan, A.; Horkay, F.; Hollinger, J. O.; Matyjaszewski, K.; Washburn, N. R. *Biomaterials* **2008**, *29*, 1739−1749.
(23) Burdick, J. A.; Chung, C.; Jia, X.; Randolph, M. A.; Langer, R. *Biomacromolecules* **2005**, *6*, 386−391.
(24) Jin, Y.; Yamanaka, J.; Sato, S.; Miyata, I.; Yomota, C.; Yonese, M. *J. Controlled Release* **2001**, *73*, 173−181.
(25) Leach, J. B.; Bivens, K. A.; Collins, C. N.; Schmidt, C. E. *J. Biomed. Mater. Res., Part A* **2004**, *70*, 74−82.
(26) Leach, J. B.; Bivens, K. A.; Patrick, C. W., Jr.; Schmidt, C. E. *Biotechnol. Bioeng.* **2003**, *82*, 578−589.
(27) Oudshoorn, M. H. M.; Rissmann, R.; Bouwstra, J. A.; Hennink, W. E. *Polymer* **2007**, *48*, 1915−1920.
(28) Pitarresi, G.; Pierro, P.; Palumbo, F. S.; Tripodo, G.; Giammona, G. *Biomacromolecules* **2006**, *7*, 1302−1310.
(29) Shah, D. N.; Recktenwall-Work, S. M.; Anseth, K. S. *Biomaterials* **2008**, *29*, 2060−2072.
(30) Smeds, K. A.; Pfister-Serres, A.; Miki, D.; Dastgheib, K.; Inoue, M.; Hatchell, D. L.; Grinstaff, M. W. *J. Biomed. Mater. Res.* **2001**, *54*, 115−21.
(31) Trudel, J.; Massia, S. P. *Biomaterials* **2002**, *23*, 3299−3307.
(32) Hahn, S. K.; Park, J. K.; Tomimatsu, T.; Shimoboji, T. *Int. J. Biol. Macromol.* **2007**, *40*, 374−380.
(33) Park, Y. D.; Tirelli, N.; Hubbell, J. A. *Biomaterials* **2003**, *24*, 893−900.
(34) Anseth, K. S.; Burdick, J. A. *MRS Bull.* **2002**, *27*, 130−136.
(35) Wang, D.-A.; Elisseeff, J. H. *Encycl. Biomater. Biomed. Eng.* **2004**, *2*, 1212−1225.
(36) Shu, X. Z.; Ghosh, K.; Liu, Y.; Palumbo, F. S.; Luo, Y.; Clark, R. A.; Prestwich, G. D. *J. Biomed. Mater. Res., Part A* **2004**, *68*, 365−375.
(37) Marklein, R. A.; Burdick, J. A. *Soft Matter* **2010**, *6*, 136−143.
(38) Morra, M. *Biomacromolecules* **2005**, *6*, 1205−1223.
(39) Lesley, J.; Hascall, V. C.; Tammi, M.; Hyman, R. *J. Biol. Chem.* **2000**, *275*, 26967−26975.
(40) Oh, E. J.; Park, K.; Kim, K. S.; Kim, J.; Yang, J.-A.; Kong, J.-H.; Lee, M. Y.; Hoffman, A. S.; Hahn, S. K. *J. Controlled Release* **2010**, *141*, 2−12.
(41) Kloxin, A. M.; Benton, J. A.; Anseth, K. S. *Biomaterials* **2009**, *31*, 1−8.
(42) Vanderhooft, J. L.; Alcoutlabi, M.; Magda, J. J.; Prestwich, G. D. *Macromol. Biosci.* **2009**, *9*, 20−28.

(43) Destaing, O.; Planus, E.; Bouvard, D.; Oddou, C.; Badowski, C.; Bossy, V.; Raducanu, A.; Fourcade, B.; Albiges-Rizo, C.; Block, M. R. *Mol. Biol. Cell* **2010**, *21*, 4108−4119.
(44) Fassler, R.; Pfaff, M.; Murphy, J.; Noegel, A. A.; Johansson, S.; Timpl, R.; Albrecht, R. *J. Cell Biol.* **1995**, *128*, 979−88.
(45) Mansukhani, A.; Bellosta, P.; Sahni, M.; Basilico, C. *J. Cell Biol.* **2000**, *149*, 1297−308.
(46) Bouvard, D.; Aszodi, A.; Kostka, G.; Block, M. R.; Albiges-Rizo, C.; Fassler, R. *Development* **2007**, *134*, 2615−25.
(47) Bencherif, S. A.; Srinivasan, A.; Jiang, A.; Hollinger, J. O.; Matyjaszewski, K.; Washburn, N. R. *Polym. Prepr.* **2008**, *49*, 576−577.
(48) Bryant, S. J.; Nuttelman, C. R.; Anseth, K. S. *J. Biomater. Sci., Polym. Ed.* **2000**, *11*, 439−457.
(49) Kavanagh, G. M.; Ross-Murphy, S. B. *Prog. Polym. Sci.* **1998**, *23*, 533−562.
(50) Bonino, C. A.; Samorezov, J. E.; Jeon, O.; Alsberg, E.; Khan, S. A. *Soft Matter* **2011**, *7*, 11510−11517.
(51) Flory, P. J. *Principles of Polymer Chemistry*; Cornell University Press: Ithaca, NY, 1953; p 672.
(52) Lee, M. H.; Boettiger, D.; Composto, R. J. *Biomacromolecules* **2008**, *9*, 2315−2321.
(53) Berrier, A. L.; Yamada, K. M. *J. Cell. Physiol.* **2007**, *213*, 565−573.
(54) Zhang, X.; Jiang, G.; Cai, Y.; Monkley, S. J.; Critchley, D. R.; Sheetz, M. P. *Nat. Cell Biol.* **2008**, *10*, 1062−1068.