Biomaterials 30 (2009) 4695–4699

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The influence of hydrogel modulus on the proliferation and differentiation of encapsulated neural stem cells

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ARTICLE INFO

Article history:
Received 27 March 2009
Accepted 19 May 2009
Available online 17 June 2009

Keywords:
Alginate
Hydrogel
Modulus
Stem cells
Proliferation
Differentiation

ABSTRACT

There has been an increasing interest in understanding how the mechanical properties of the microenvironment influence stem cell fate. We describe studies of the proliferation and differentiation of neural stem cells (NSCs) encapsulated within three-dimensional scaffolds – alginate hydrogels – whose elastic moduli were varied over two orders of magnitude. The rate of proliferation of neural stem cells decreased with increase in the modulus of the hydrogels. Moreover, we observed the greatest enhancement in expression of the neuronal marker β-tubulin III within the softest hydrogels, which had an elastic modulus comparable to that of brain tissues. To our knowledge, this work represents the first demonstration of the influence of modulus on NSC differentiation in three-dimensional scaffolds. Three-dimensional scaffolds that control stem cell fate would be broadly useful for applications in regenerative medicine and tissue engineering.

1. Introduction

Considerable recent work has focused on understanding the factors that influence stem cell fate, motivated by both a fundamental interest in stem cell biology as well as by the potential applications of stem cells in regenerative medicine [1–3]. While a majority of such studies have concentrated on the influence of biochemical cues on stem cell behavior, it has become increasingly clear that the mechanical properties of the microenvironment also influence the behavior of a wide variety of cells including stem cells [4–16]. In particular, in a recent seminal study, Engler et al. demonstrated that the elasticity of the matrix influences the differentiation of mesenchymal stem cells (MSCs) into neurons, osteoblasts, and myoblasts [12]. Saha et al. have also studied the influence of the mechanical properties of the substrate on the behavior of neural stem cells (NSCs) [15]. NSCs have the ability to differentiate into the three major neural lineages – neurons, oligodendrocytes, and astrocytes [17,18] – and are thus of interest for a range of neuroregenerative applications [2,17,19–22]. Saha et al. found that NSCs optimally differentiated into neurons when on substrates of intermediate stiffness (500 Pa) and that hard surfaces favor astrocytic differentiation, whereas soft surfaces promote neuronal differentiation [15]. These previous studies were carried out by culturing stem cells *on* hydrogel substrates with controllable stiffness. It would, however, be particularly important to understand the influence of the mechanical properties of the microenvironment on the behavior of stem cells cultured *in* three-dimensional scaffolds, as cell behavior can differ in 2 vs. 3 dimensions [23]. Such an environment would not only be extremely relevant for clinical applications of stem cells, but would also more closely mimic the stem cell microenvironment *in vivo*.

Alginate is a particularly promising material for generating three-dimensional cell-encapsulating scaffolds [24,25]. Alginates are salts of alginic acid, a linear heteropolysaccharide consisting of (1 → 4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) [26,27]. Alginate has been studied in past as a 3-D scaffold for encapsulating NSCs because of its compatibility with central nervous system tissue [24,25,28,29], In the presence of divalent cations – for example, Ca^{2+}, Sr^{2+} or Ba^{2+} – these alginate salts form gels due to the ability of the divalent ions to bridge G residues on different chains [19,26,30–32]. In general, the elastic modulus of an alginate gel depends on the number of cross-links and the length and stiffness of the chains between cross-links, and the modulus of the gel can thus be tuned by varying the molecular weight of the alginate used, the G content in the alginate, and the calcium ion concentration [33].

Here, we report the effect of the modulus of alginate hydrogels on the proliferation and differentiation of encapsulated NSCs. By

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doi:10.1016/j.biomaterials.2009.05.050

modulating the concentration of alginate and calcium ions, we were able to vary the modulus of the hydrogels by more than two orders of magnitude, which impacted both the proliferation and the differentiation of encapsulated NSCs. In particular, we observed the greatest enhancement in expression of the neuronal marker β-tubulin III within hydrogels having an elastic modulus comparable to that of brain tissues. The ability to influence stem cell behavior by tuning the mechanical properties of three-dimensional scaffolds should be broadly useful for applications in regenerative medicine and tissue engineering.

2. Materials and methods

2.1. Materials

Sterile high molecular weight (200–300 kDa MW, 69% guluronate content) PRONOVA SLG 100 sodium alginate was purchased from FMC BioPolymer (PA, USA). Live–dead and cell proliferation assay kits were purchased from Molecular Probes.

2.2. NSC culture

Adult NSCs isolated from the hippocampi of 6-week-old female Fisher 344 rats were cultured as described elsewhere [18]. Briefly, cells were grown on poly-ornithine/laminin-coated dishes in DMEM/F12 medium with N2 supplement (Invitrogen) and 20 ng/mL FGF-2 (Promega). The medium was changed every other day, and upon reaching ~80% confluency, cells were subcultured on poly-ornithine/laminin-coated plates using Accutase (Phoenix Flow Systems).

2.3. NSC encapsulation inside alginate hydrogel

Neural stem cells were grown in alginate hydrogels at a density of 4000 cells per well in a 96-well plate. Briefly, sterile sodium alginate was dissolved in DMEM/F12 medium and mixed with 4000 cells resuspended in DMEM/F12 medium to the final volume of 40 μL, yielding final alginate concentrations of 0.25% or 1% wt/vol. 40 μL of this solution was added to each well of a 96-well plate, followed by the addition of 100 μL of a chilled $CaCl_2$ solution (10 mM, 50 mM, or 100 mM). The plate was kept on ice for 10 min, 50 μL of liquid were then removed from each well, and 150 μL of DMEM/F12 media containing 20 ng/mL FGF-2, 1% penicillin streptomycin, and 5 μg/mL laminin were added. In some wells, NSCs were allowed to grow without any alginate hydrogel (negative control). We performed a 50% media change every other day for 7 days. We cultured NSCs within hydrogels having different values of modulus, prepared by varying the concentrations of alginate and $CaCl_2$. Specifically, the following combinations were tested: 1% alginate/100 mM $CaCl_2$ (1–100), 0.25% alginate/100 mM $CaCl_2$ (0.25–100), 0.25% alginate/50 mM $CaCl_2$ (0.25–50) and 0.25% alginate/10 mM $CaCl_2$ (0.25–10).

2.4. Testing the viability of NSCs

A live–dead assay (Molecular Probes) was used as per the manufacturer's instructions. Briefly, the medium from the wells was removed and cells in the hydrogel were washed once with phosphate buffer saline (PBS) followed by incubation with the dye. Live cells stained with Calcein AM and dead cells marked with Ethidium homodimer-1 (EthD-1) were visualized using a Zeiss LSM 510 Meta Confocal microscope.

2.5. Measuring the proliferation of NSCs

After 7 days of growth inside the hydrogel, with an initial seeding density of 4000 cells per well, medium was removed, and NSCs were washed once with PBS. To release encapsulated cells, the alginate hydrogel was incubated with 100 μL of a solution containing 15 mM sodium citrate and 150 mM NaCl for an hour [17,30]. Using a pipette, the contents of each well were mixed until a homogenous solution was obtained. The 96-well plates were stored overnight at −80 °C. Cell quantities in all hydrogels were assayed in parallel using the Molecular Probes CyQuant™ Assay.

2.6. Rheological characterization of alginate hydrogels

Rheological measurements were carried out using a TA instrument AR-G2 stress-controlled rheometer with a parallel-plate geometry. Stress sweeps at a constant frequency of 1 Hz were first performed to obtain the linear viscoelastic region for collecting subsequent data. Frequency sweeps were performed in the linear viscoelastic regime to determine values of the elastic (G') and viscous (G'') modulus. Since the 0.25 wt% gels were very fragile, they were cast directly on the rheometer. In a typical method of sample preparation, 0.25 wt% alginate solutions were poured in a mold placed on top of the bottom plate, quickly followed by the addition of a $CaCl_2$ solution of the desired concentration. After approximately 5 min, the mold was removed, and the sample was cut in the shape of a disc with a diameter of 40 mm. 1 wt% gels were prepared in a similar fashion, though the gels were first cast in a Petri dish prior to loading onto the instrument. A solvent trap was used, and water evaporation was not significant for the temperature and timescales investigated. Gels were gently pressed with tissue paper to remove surface water before lowering the top plate. Samples were allowed to equilibrate for 10–15 min before starting the measurements, which were taken under ambient conditions at 25 °C unless otherwise specified.

2.7. NSC staining and confocal microscopy imaging

After 7 days of culture within alginate hydrogels, the NSCs were washed three times with PBS containing 5% donkey serum and 0.3% Triton X-100 (PBS-DT) and pre-blocked with PBS-DT for an hour at 37 °C. NSCs were then stained overnight at 4 °C with primary antibodies for nestin and β-Tubulin III at a 1:500 dilution in PBS-DT. NSCs were then washed three times with PBS-DT and incubated with secondary antibodies (Alexa fluor labeled), diluted 1:250-fold in PBS-DT in the dark. Cells were then washed three times with PBS-DT. The second to last wash was used to stain nuclei with DAPI. Images were taken on Zeiss LSM 510 Meta Confocal microscope with 40× magnification at constant settings.

2.8. Quantitative gene expression measurements

2.8.1. RNA extraction

Total RNA of the NSCs growing inside the alginate hydrogel was extracted using Trizol Reagent (Invitrogen, USA), according to the single step acid–phenol guanidinium extraction method described by Chomczynski and Sacchi [34]. In brief, after growing NSCs within hydrogels in 96-well plates for seven days, the medium from each well was replaced by Trizol reagent. After 6 h, Trizol reagent and hydrogel from the wells were collected, vortexed, and treated with chloroform. Total RNA was precipitated using chilled ethanol. The acquired RNA samples were treated with RNase-free DNase I, and RNA integrity was tested on a 1.7% agarose gel containing 0.0025% (v/v) ethidium bromide. The RNA concentration was quantified spectrometrically at 260 and 280 nm, and purity was determined at 230 and 260 nm (NanoDrop ND-1000, USA).

2.8.2. Preparation of complementary DNA by reverse transcription

One hundred nanograms of total RNA from each sample was reverse transcribed into cDNA using ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's protocol. At the end of the procedure, cDNA was diluted to a concentration of 10 ng/μL.

2.8.3. Quantitative real-time polymerase chain reaction assay (QRT-PCR)

Gene-specific sense and antisense primer for β-Tubulin III and 18S (house keeping gene) were purchased from IDT (USA), and TaqMan probes were synthesized by Biosearch, USA. The nucleotide sequences and values of the melting temperature (T_m) of the primers and probes are shown in Table 1. QRT-PCR was performed in a LightCycler 489 (Roche, Basel, Switzerland) in a 96-well plate. A 25 μL reaction volume with 12.5 μL Brilliant QRT-PCR Master Mix (Stratagene, USA), 10 ng cDNA as template, and 0.2 mM of the primer pairs and TaqMan probe were used for the reaction.

Target genes were run in triplicate on a single plate, which included different samples as well as no-template controls. PCR conditions were as follows: denaturation (95 °C for 10 min) followed by 70 amplification cycles (95 °C for 15 s, 60 °C for 1 min). The QRT-PCR analysis was repeated using RNA isolated from three independent experiments.

3. Results and discussion

3.1. Alginate hydrogel characterization

We wished to test the influence of the mechanical properties of a three-dimensional scaffold – an alginate hydrogel – on the

Table 1
Primers and probes for quantitative RT-PCR. β-Tubulin III: neuronal marker. 18S (18S ribosomal subunit): internal control. FAM490: FAM490 fluorophore. CAL610: CAL610 fluorophore. BHQ: Black Hole Quencher®. T_m: melt temperature.

Gene	Sense and antisense primers/TaqMan probe	T_m (°C)
β-Tubulin III	S: 5′-GCATGGATGAGATGGAGTTCACC-3′	65.2
	A: 5′-CGACTCCTCGTCGTCATCTTCATAC-3′	65.4
	P: 5′-FAM490-TGAACGACCTGGTGTCTGAG-BHQ-3′	59.9
18S	S: 5′-GTAACCCGTTGAACCCCATTC-3′	62.6
	A: 5′-CCATCCAATCGGTAGTAGCGA-3′	62.2
	P: 5′-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3′	67.6

proliferation and differentiation of encapsulated NSCs. To that end, we first prepared and characterized alginate hydrogels having different elastic modulus values, which can be tuned by varying the concentrations of alginate and calcium chloride [35–37]. Previous studies of cell encapsulation in alginate hydrogels utilized 1–2% sodium alginate solutions [17,24,30]; however, we prepared hydrogels using four different combinations of alginate and $CaCl_2$ concentrations to test the behavior of NSCs in softer scaffolds: 1% alginate/100 mM $CaCl_2$ (1–100), 0.25% alginate/100 mM $CaCl_2$ (0.25–100), 0.25% alginate/50 mM $CaCl_2$ (0.25–50) and 0.25% alginate/10 mM $CaCl_2$ (0.25–10).

We characterized the mechanical properties of the hydrogels by carrying out oscillatory shear measurements in a rheometer, and Fig. 1 shows the results of the dynamic frequency sweep tests. The elastic modulus of the hydrogels was weakly dependent on frequency over the entire range studied (Fig. 1a) and significantly larger than the loss modulus (Fig. 1b). Fig. 2, which compares the elastic modulus for hydrogels of different composition, shows that the elastic modulus of the three hydrogels formed using 0.25 wt% alginate decreased with decrease in $CaCl_2$ concentration. The lowest modulus (~180 Pa) was observed for the "0.25–10" hydrogel and lies in the range reported for the modulus of brain tissues (100–1000 Pa) [38]. In contrast, the hydrogel formed using the highest concentrations of alginate and $CaCl_2$ (1–100) had the largest elastic modulus (~20,000 Pa). This dramatic change in the mechanical behavior can be attributed to a higher crosslink density in these gels.

3.2. NSC proliferation inside hydrogels

Having demonstrated the ability to control the mechanical properties of alginate hydrogels, we next proceeded to study the

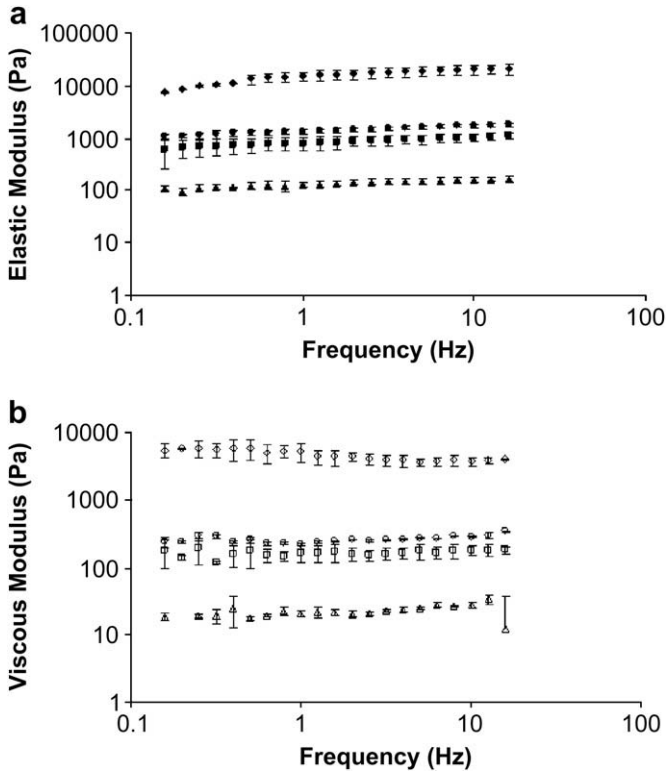

Fig. 1. Characterization of modulus of alginate hydrogels by rheological measurements: (a) elastic modulus of different alginate hydrogels during frequency sweep analysis – 0.25–10 (▲); 0.25–50 (■); 0.25–100 (●); 1–100 (◆); (b) viscous modulus of different alginate hydrogels during frequency sweep analysis – 0.25–10 (△); 0.25-50 (□); 0.25–100 (○); 1–100 (◇).

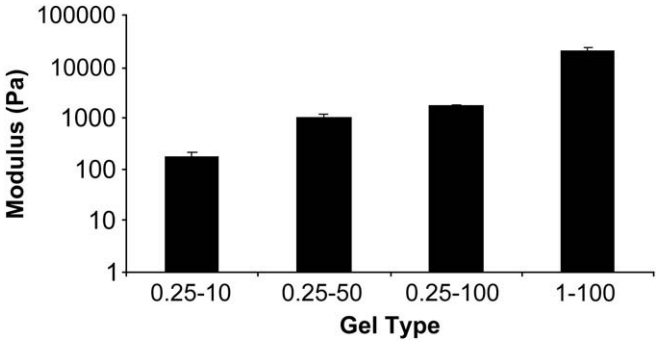

Fig. 2. Influence of composition of alginate hydrogels on the elastic modulus (G') at 8 Hz. Error bars represent the standard deviation.

effect of hydrogel stiffness on the behavior of encapsulated NSCs. We found that the proliferation of NSCs in the hydrogels increased significantly with a decrease in the modulus of the hydrogels (Fig. 3). After 7 days of culture, the NSC number had increased 17-fold in the lowest modulus hydrogel (0.25–10), but only approximately two-fold for the highest modulus hydrogel (1–100). We note that based on previous reports in the literature, we do not anticipate the diffusion of medium components to be a significant limitation for the hydrogels used in this study [39,40], and we found that NSC viability was greater than 80% inside the hydrogels (data not shown). The data in Fig. 3 are consistent with proliferation rate results we had previously obtained for NSCs cultured inside 1% alginate hydrogels [29]. Consistent with prior reports, we also found a greater increase in NSC number (approximately fifty-fold in seven days) in two-dimensional monolayer culture without alginate.

3.3. Effect of hydrogel modulus on neuronal differentiation of NSCs

Next, we tested whether the mechanical properties of the three-dimensional scaffolds influence the differentiation of NSCs. NSCs were encapsulated in alginate hydrogels and cultured in DMEM/F12 media containing FGF-2 (see Methods for details). After 7 days, to test the extent of neuronal differentiation, we first monitored levels of β-tubulin III (a marker for neuronal differentiation) using immunofluorescence analysis (Fig. 4). We also monitored levels of nestin (an immature neural marker); transient co-expression of nestin and NSC lineage markers are seen during the process of differentiation [41]. As seen in the confocal micrographs (Fig. 4), the maximum intensity of β-tubulin III staining was observed in cells grown in the lowest modulus hydrogel (0.25–10), whereas the intensity was considerably lower for cells cultured in the other hydrogels. These results clearly indicate that culturing NSCs in three-dimensional alginate hydrogels having a modulus of ~180 Pa – a value in the range reported for the modulus of brain tissues – promotes neuronal differentiation.

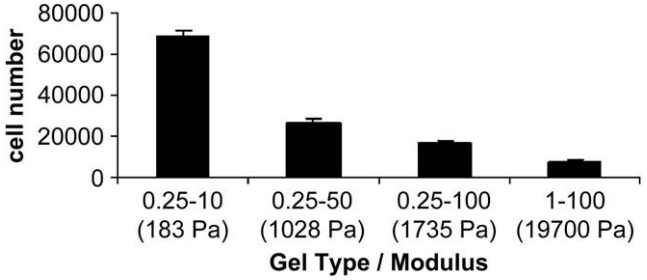

Fig. 3. Proliferation of NSCs after 7 days in alginate hydrogels having different values of the modulus. Error bars represent the standard deviation; $p < 0.01$ based on Student's t-test.

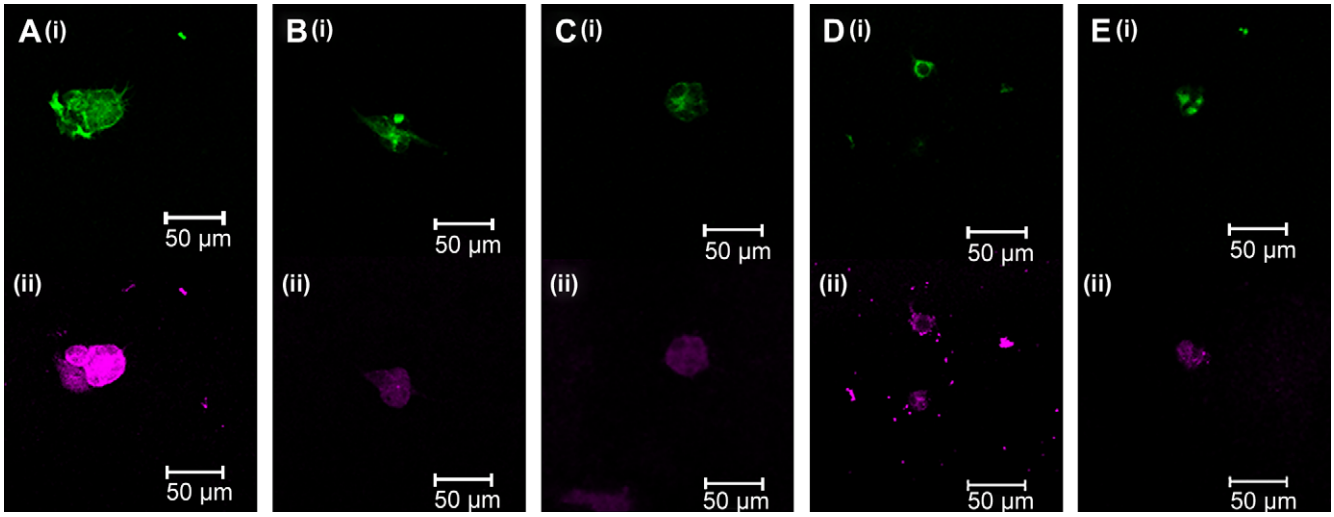

Fig. 4. Confocal images showing nestin (i) and β-tubulin III (ii) staining of NSCs grown inside alginate hydrogels having different values of the modulus ((A) 0.25–10 (183 Pa); (B) 0.25–50 (1028 Pa); (C) 0.25–100 (1735 Pa); (D) 1–100 (19,700 Pa); (E) Control) in medium containing 20 ng/mL FGF-2.

The imaging results were complemented by an analysis of β-tubulin III expression using quantitative real-time polymerase chain reaction (QRT-PCR). Expression of 18S was monitored as an internal control, and Fig. 5 shows the ratio of β-tubulin III and 18S expression levels (normalized relative to the ratio in cells cultured in the highest modulus hydrogel (1–100)). Consistent with the immunofluorescence analysis, the relative expression of β-tubulin III is ~20-fold greater in cells cultured in alginate hydrogels having the lowest value of modulus and decreases with increasing hydrogel modulus.

We note our observed increase in neuronal differentiation of NSCs within soft hydrogels is consistent with other reports on the promotion of neuronal differentiation of stem cells cultured *on* soft substrates. For instance, Discher and colleagues [12] found a ~6-fold increase in the ratio of β-tubulin III and actin expression levels when MSCs were grown on hydrogels with a modulus of 100 Pa relative to the ratio in naive MSCs. Saha et al. have recently shown enhanced neuronal differentiation of NSCs cultured *on* interpenetrating polymer networks having a modulus of 500 Pa; the ratio of β-tubulin III and 18S expression levels was >3-fold greater on these hydrogels than for cells on stiffer substrates [15]. To our knowledge, however, we have shown for the first time the effect of modulus on NSC differentiation within a 3-D microenvironment. Moreover, we observed a ~20-fold increase in relative β-tubulin III expression for NSCs cultured within the lowest modulus hydrogels as compared to control cells, significantly greater than that reported in previous studies. Thus we see a significantly greater influence of modulus on neuronal marker expression on going from a 2-D to 3-D microenvironment. Understanding the mechanisms by which the mechanical properties of the hydrogel influence stem cell fate remains a fundamental challenge in stem cell biology. Discher and colleagues have shown that cytoskeletal motors may be involved in the matrix-elasticity sensing that drives lineage specification in mesenchymal stem cells grown on hydrogels [12]. Probing a possible role for cytoskeletal motors in influencing the function of neural stem cells in three-dimensional environments will be an area of interest for future work.

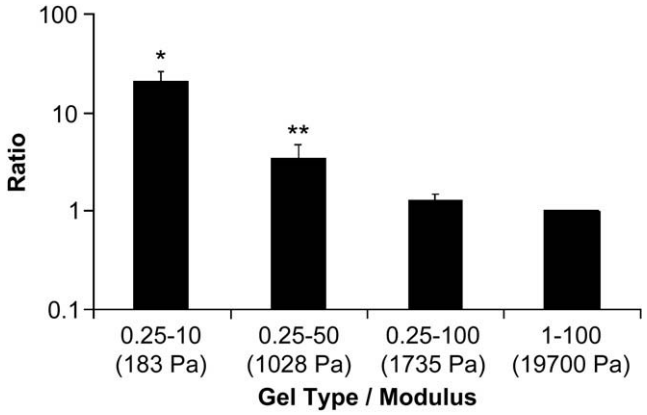

Fig. 5. QRT-PCR results for β-tubulin III after 7 days of growth in alginate hydrogels having different values of the elastic modulus. The data reported represent the ratio of the expression of β-tubulin III and 18S ribosomal RNA normalized relative to the ratio in cells cultured in highest modulus hydrogel (1–100). Error bars represent the standard deviation; $^{*}p < 0.0005$, $^{**}p < 0.05$ compared with highest modulus hydrogel (1–100) based on Student's *t*-test.

4. Conclusions

There has been an increasing emphasis in recent times on elucidating the influence of the mechanical properties of the microenvironment on stem cell fate. This work provides insights into the influence of the mechanical properties of three-dimensional alginate hydrogel scaffolds on the proliferation and differentiation of NSCs, where varying the concentrations of alginate and calcium chloride provided facile control over the elastic modulus of the hydrogels. We demonstrated that the properties of the three-dimensional scaffolds significantly impacted both the proliferation and the neuronal differentiation of encapsulated NSCs. In addition, we observed the greatest enhancement in expression of the neuronal marker β-tubulin III within hydrogels having an elastic modulus comparable to that of brain tissues. We note that the optimal value of the elastic modulus may depend on the stem cell type and the lineage to which differentiation is being directed. Such three-dimensional platforms will undoubtedly be useful for fundamental studies of cell mechanobiology. Moreover, the ability to control stem cell fate – possibly without the use of chemical inducers – would also be broadly useful for applications in regenerative medicine and tissue engineering.

Acknowledgements

We acknowledge support from the NIH (EB007295), a New York State Spinal Cord Injury Research Board CART grant, and from the NSF-sponsored ICE IGERT grant (DGE-0654128).

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