Hydrogel platform capable of molecularly resolved pulling on cells for mechanotransduction

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ARTICLE INFO

Keywords: Hydrogel platform Near-infrared light Macromolecular actuator Cell-ECM interaction Mechanotransduction

ABSTRACT

The active forces exerted from the extracellular matrix (ECM) to mechanoreceptors have crucial impact on many cell functions and disease development. However, our understanding of the underlying mechanisms is held back due to the lack of ECM mimicking platform able to apply molecularly resolved forces to cells. Herein, we present novel hydrogel platform capable of generate pN range forces to specific cellular receptors, at molecular scale. This capability was achieved through near-infrared (NIR) light regulated macromolecular actuators functionalized within the platform. This platform enables us to reveal cell responses to molecularly resolved forces under controlled magnitude (150–400 pN) and frequency (up to 100 Hz) on matrix with varied stiffness. We find the stiffness of the matrix has a large influence on the applied force transduction to cells. This versatile platform holds the potential for establishing correlation between receptor signaling pathways and cellular responses closer to physiological conditions.

1. Introduction

Cells sense their physical environment by translating mechanical cues into biochemical signals through a process termed mechanotransduction [1–3]. The biochemical feedback would trigger many cellular functions as diverse as proliferation, differentiation, migration and apoptosis, and is crucial for organ development and homeostasis [4–7]. Creation of artificial cellular physical environment with well-defined mechanical cues, such as extracellular matrix (ECM)-like hydrogels, represents one of the most efficient approaches to investigate the processes [8–13]. These analogues allow on-demand tailoring of their mechanical properties to deliver passive mechanical cues to cells, and thus have helped us to develop most of the current knowledge about mechanotransduction [14–17]. Besides the passive mechanics, cells also respond to the mechanical forces exerted from ECM [18–20]. Any molecular disruption in these force transmissions and subsequent cell signaling events potentially lead to diverse diseases including atherosclerosis, muscular dystrophy and cancer [21–25]. Therefore, it is highly desirable to develop advanced hydrogel platforms that are able to apply exogenous force to specific cellular receptors at molecular scale for gaining a deeper understanding of mechanotransduction [26–28].

Many methods have been developed to apply forces to cells for mechanotransduction investigation at different scales, including 2-dimensional (2D) stretchable substrates [29,30], atomic force microscopy (AFM) [31] and responsive micropillars etc [32–34]. Stretchable substrates [35] and flow chambers [36,37] can apply shear stress globally to cells, but there is no user control over which cells on the surface are stimulated. The established single cell micro-manipulation techniques, such as AFM [38,39], optical tweezers [40] and magnetic bead [41] cytometry allow for high spatiotemporal precision in the application of forces. In contrast, hydrogel systems can provide physiological environment and have also been designed to apply cyclic stress on cells, for example, using thermoresponsive poly (N-isopropyl acrylamide) (PNI-PAM) to achieve reversible and repetitive small deformations [42] or bending of micropillars [43]. However, due to lack of receptor specificity, the forces generated by such deformation have been limited to the nN and μN range. Moreover, all those methods typically apply forces to cells at cellular or subcellular scales. In contrast, most important aspects of mechanotransduction events occur in pN range at molecular scale [44]. Recently, several approaches have been proposed to generate molecular scale force to cells. These approaches use molecular tools that can undergo controlled motion or contraction, such as light-driven molecular
motor rotation [45] and phase transition of cross-linked thermo-responsive polymer nanoparticles [46,47]. Although these elaborate tools can deliver forces to special receptors on cells, they are designed on stiff glass coverslips, rather than ECM-like substrates, and thus fail to resemble the passive mechanics of the in vivo matrix. To the best of our knowledge, no soft hydrogel platforms are available that allow for molecularly resolved force application in pN range to specific cellular receptors.

Herein, we report a class of novel PEG-based hydrogel platform, functionalized with NIR light-responsive macromolecular actuators. The platform not only allows for on-demand variation of matrix stiffness but also enables molecularly resolved pulling on cells through NIR-induced contraction of the macromolecular actuators. We demonstrate that the system can deliver forces with adjusted amplitude (150–400 pN) and frequency (up to 100 Hz). These molecularly resolved pulling forces are varied by exposure profiles and the actuators’ molecular weights, which can be utilized to finely mediate cell behaviors, including cell spreading and migration. Furthermore, the platform allows us to investigate the applied force transduction on matrices with varied stiffness and correlate cell responses to key signaling pathways.

2. Material and methods

Preparation of CD-PNIPAM-TTC by RAFT polymerization: NIPAM (226.32 mg, 2 mmol), CD-TTC (4.86 mg, 0.005 mmol) and AIBN (1.64 mg, 0.01 mmol) were dissolved in 0.5 mL of DMF in a Schlenk tube. The mixture was degassed with 3 freeze-evacuate-thaw cycles, backfilled with nitrogen and heated at 80 °C. After 48 h, the Schlenk tube was quenched into ice to stop the polymerization. The crude solution was precipitated into excess solution of acetic ether for three times. Then the obtained brown powder was dried overnight under vacuum at room temperature (145.9 mg, 62.7%).

Hydrogel platform preparation: CD-PNIPAM-TTC (33.3 mM, 30 μL) were first reacted with the 20 kDa 8-arm PEG-azide macromers (50.0 mM, 20 μL) through Cu-catalyzed azide-alkyne reactions to provide a prepolymer. 20 μL solution containing 8 μL of 27.5 mM prepolymer, 2.1 μL of 69.4 mM 3,3’-[2,2-bis-[2-propyn-1-yloxy]methyl]-1,3-propanediyl]bis (oxy)]bis [1-propyne] (4-arm alkynyl in DMF, 0.6 μL of 0.2 M CuSO₄ in H₂O, 0.6 μL of 1 M sodium ascorbate aqueous solution and DMF (8.7 μL) were added to azide-functionalized coverslips and sandwiched with a superhydrophobic microscope slide. The reaction was allowed to proceed for 5 h at 70 °C to form a stable hydrogel. To remove the copper catalyst, the hydrogel was immersed in 0.05 M EDTA-2Na for 30-min and repeated for 3 times. PEG hydrogels (E = 49 kPa) functionalized with 33 kDa CD-PNIPAM-TTC (contour length: 64 nm) were used for cell experiments unless otherwise mentioned.

Cell culture: NIH-3T3 Fibroblasts (National Collection of Authenticated Cell Cultures, China) were cultured in DMEM medium (high glucose, BBI) with 10% fetal bovine serum (PBS, Biological Industries) and 1% antibiotics/antimycotics (BBI) at 37 °C under 5% CO₂ concentration. Cells were passaged at 70–90% confluence and plated at a density of 20%. After culturing the cells on hydrogels for 12–36 h, SPYS555-Actin (1 μM) were added for actin staining and the cells were further incubated for 12 h in the above imaging medium [48]. The cells were then directly imaged using Nikon microscope. The pEGFP vinculin was a gift from Kenneth Yamada (RRID: Addgene_50,513). The jetOPTIMUS® transfection reagent kit (Polyplus-transfection SA, Illkirch, France) was used to transfer plasmids into cells as described by the manufacturer’s protocol. All procedures are based on a standard protocol provided by Polyplus-transfection SA and incubated for 24–48 h before imaging. For experiments with inhibitors, cells were pre-incubated with ROCK inhibitor Y27632 (10 μM) for 30 min or piozon channels inhibitor gadolinium (30 μM) for 15 min at 37 °C, respectively. When the cells were exposed to near-infrared light, the incubation temperature was set as 28 °C and the cells side for exposure were selected randomly.

Laser actuation setup and exposure conditions: A custom-made setup, using an optical microscope equipped with a NIR laser, which can simultaneously activate the molecular actuators at 808 nm, as well as monitoring cells, was designed to perform the experiments (Fig. S1). The laser was incident directly above the sample stage and focused on the hydrogel through a collimator. A port on the microscope (Nikon) was especially modified to introduce a diode laser (808 nm, maximum power 2 W) through a fiber optic cable, while a collimator was used to focus the laser beam on the sample (footprint diameter ~16 μm). A short-pass IR filter was added in the optical path to prevent the IR light from reaching the high-speed camera. The laser power reaching the gel was experimentally measured using a power meter and the intensity was calculated from the spot size observed on the hydrogel. The laser footprint was kept the same for all experiments. Different laser settings were applied and a DDS signal generator/counter LE (OPTICS) was used to control the laser. Typical illumination conditions: 15.8 μW μm⁻² power density, 100 Hz, 10% duty cycle with a laser spot size of 201 μm² were used for cell experiments unless otherwise mentioned.

More details about materials and methods such as photothermal actuation and temperature measurement of the hydrogel on surface, quantification of cell protrusion, molecular dynamics simulation details etc., can be found in the Supporting Information.

3. Results and discussion

3.1. Design of the hydrogel platform and synthesis of the macromolecular actuator

Fig. 1 shows our design. The hydrogels are obtained by mixing macromolecular actuators CD-PNIPAM-TTCs with 8-arm PEG-azide and then cross-linked with 4-arm alkynyl crosslinker through copper catalyzed alkynyl-azide cycloaddition (CuAAC). The choice of PEG enables us to tailor readily the biophysical properties of the gel, while eliminating non-specific interactions [49]. The macromolecular actuators are made from single polymer chains CD-PNIPAM-TTCs containing a croconaine dye (CD) chromophore, a photothermal group showing intensive and narrow absorption at around 800 nm [50]. The main components PNIPAM are thermo-responsive [51]. Upon NIR light illumination, the CD group would convert light to heat and induce the PNIPAM chains to shrink, thereby applying a mechanical load. In our design, the CD-PNIPAM-TTC chains are caged with an alkynyl terminal group on one end and a triithiocarbonate (TTC) group on the other end. The alkynyl allows for easy immobilization of the macromolecules into PEG hydrogels through CuAAC reaction [52], while the TTC can be converted into thiol and linked with various functional groups through thiol-maleimide (Mal) reactions, such as arginine-glycine-aspartic acid (RGD) peptide in this study, making the mechanical actuation molecularly selective [53]. Thus, real-time monitoring of cell response to controllable molecular forces applied at specific receptors at cell-ECM interface can be realized using a home-built optical microscope equipped with NIR laser (Fig. S1).

Under this design, we first synthesized CD-conjugated 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid 3-azido-1-propanol ester (CD-TTC) from CD-dialkyne as the chain transfer agent and got a series of macromolecular actuators precursors (CD-PNIPAM-TTCs) with different molecular weights via reversible addition-fragmentation chain-transfer (RAFT) polymerization (Fig. 2a and Scheme S1). Thermo-inert poly (N, N-dimethyacrylamide) CD-PDMA-TTCs and CD-free PNIPAM-TTCs (see Scheme S2 and Scheme S3 for synthetic details) with variant molecular weights were also synthesized as control groups for studying the contribution of simple photothermal heating and NIR light exposure to the systems, respectively. Gel permeation chromatography (GPC) indicated that the obtained CD-PNIPAM-TTCs have number-average molecular weights (Mn) of 15, 33 and 40 kDa (Fig. 2b; Fig. S2; Tables S1–S3), respectively, with narrow distributions (PDI<1.3). Their structures were confirmed by 1H NMR spectroscopy (Fig. S3). The presence of the 1,2,3-triazole ring and CD group was established by the characteristic chemical shift values at 7.47–7.58 ppm [50] and 8.68–8.84 ppm [54], indicating
the successful conjugation of CD group into the macromolecules. CD-PNIPAM-TTCs in N, N-dimethylformamide display strong NIR absorption band centered at 825 nm as characterized by UV–vis–NIR absorption spectrum (Fig. S4). All CD-PNIPAM-TTCs showed lower critical solution temperatures (LCST) at 30–32 °C in aqueous solutions as determined by dynamic light scattering analysis (DLS) and UV absorption spectroscopy (Fig. 2c and d).

3.2. Preparation of hydrogel platform and NIR-light responsiveness

The obtained macromolecular actuators were introduced into PEG hydrogels by first bonding to 8-arm PEG-azide macromer (20 kDa) through CuAAC reactions, followed by curing in the presence of 4-arm alkyne (Scheme S4). The gelation can be conducted at 70 °C and results in the formation of stable hydrogels within 60 min. By tuning the concentration of the crosslinker or PEG macromer in the mixture, hydrogels with Young’s modulus between 0.6 kPa and 49 kPa were prepared (Fig. 3a and Fig. S5). The copper catalyst was completely removed by immersing the hydrogels in EDTA solution. The obtained hydrogels functionalized with CD-PNIPAM-TTCs or CD-PDMA-TTCs showed strong absorption at 850 nm, indicating the successful conjugation of the polymers (Fig. 3b). We investigated the photothermal effect of the hydrogels functionalized with the macromolecular actuators by monitoring the apparent temperature (A. T.) with a commercial thermal camera. As shown in Fig. 3c, upon 808 nm laser irradiation, A. T. of the irradiated spots increased in the hydrogel substrates containing CD-PNIPAM-TTCs and CD-PDMA-TTCs but not that with PNIPAM-TTCs, suggesting an efficient photothermal effect from the CD group. When pulsed NIR illumination (6.6 μW μm⁻², 1 Hz, and 10% duty cycle) was used, rapid temperature fluctuation was observed even in the millisecond range (Fig. 3d and Fig. S6). The photothermal effect leads to a new equilibrium temperature at 32 °C (~2.5 °C higher than the ground state before light exposure), around which the temperature of hydrogel substrate oscillates (~±2.5 °C), synchronized with the laser pulse. Thus, we concluded that, upon the operating temperature set lower than 32 °C, NIR pulsed illumination could induce thermo-responsive expanded/globule conformation changes in CD-PNIPAM-TTCs. Since PNIPAM chain would experience the microscopic temperature (M. T.) around CD group for conformation changes, we incorporated temperature-feedback core-shell upconversion nanoparticles (csUCNPs, NaYF₄:Yb,Er@NaLuF₄:Yb,Nd) within the hydrogels for detecting the accurate M. T. (Fig. 3e). And the homogeneously-dispersed csUCNPs in hydrogel exerted no obvious influence on the swelling ratio of the PEG hydrogels (Fig. 3f and Table S4). The csUCNPs in hydrogel were irradiated with 808 nm laser to record the upconversion spectrum. The intensity ratio of the two upconversion emission bands generated by Er³⁺ ions in csUCNPs at 525 (2H₁₁/₂ → 4I₁₅/₂) and 545 nm (4S₃/₂ → 4I₁₅/₂), were changed correspondingly with temperature, which could be described by Boltzmann distribution equation because the energy difference of the two emissive excited states were less than 1000 cm⁻¹, meeting a thermal equilibrium [55](Fig. 3g). As a result, the temperature can be interpreted by the measurement of the intensity ratio of the upconversion emissions (I₅₂₅/I₅₄₅), independent of laser power density (Fig. 3h and Fig. S7). It has been demonstrated that the thermometric strategy using Er³⁺ doped csUCNPs exhibited high spatial and temporal accuracy [56]. This feature was utilized to detect the M. T. during the thermo-responsive conformation change of CD-PNIPAM-TTCs. As shown in Fig. 3i, the M. T. determined by csUCNPs (55 °C) was ~19 °C higher than the A. T. (36 °C) captured by IR camera under a laser intensity of 5.7 μW μm⁻². Fourier transform infrared spectroscopy (FTIR) was also applied to prove the shrinkage of PNIPAM chain within hydrogel during heating (Fig. S8). We therefore confirmed that microscopic hotspot caused by CD group could efficiently induce the shrinkage of PNIPAM chain (55 °C > 32 °C) [57] without significantly increasing the A. T., thus minimizing thermal effects on cells.
3.3. Molecularly resolved mechanical actuation to cells and force simulations

To probe the force application, the CD-PNIPAM-TTC were first converted to CD-PNIPAM-SH and coupled with RGD-Mal to get CD-PNIPAM-RGD in situ on hydrogel surface to mediate cell adhesion (Scheme S5 and Fig. S9). 3T3 fibroblasts were seeded on the hydrogel for testing the adhesion of RGD ligand to cells. The cells attached and spread on CD-PNIPAM-RGD functionalized hydrogel with spindle-like morphology after being cultured for 72 h, indicating that the platform was not toxic to the cells (Fig. S10). The CD-PNIPAM-SH functionalized hydrogel and the hydrogel incubated with RGD in absence of CD-PNIPAM-TTC were used as the control. After incubation for 24 h, no cell attachment was observed on both control samples (Fig. S10). The density of attached and spread cells increased with the RGD incubation concentration from 0.01 to 0.25 mg/mL on CD-PNIPAM-SH functionalized hydrogels (Fig. S11). These results implied the specific coupling reaction of the RGD ligand to CD-PNIPAM-SH and the solely mediated binding of cell receptors by CD-PNIPAM-RGD on surfaces [46].

We then measured cell recruitment through force application at the RGD/integrin complex. We illuminated circular areas (201 μm²) of the 3T3 cells stained with SPY555-Actin [48] and found that the F-actin localization enhanced over 15 min with light exposure in the illuminated region of the cell (ROI1, Fig. 4a). Quantification of region of interests (ROIs) from multiple cells showed that the cell area in illuminated regions increased by 18.5% in 15 min, whereas in non-illuminated regions (ROI2), a decrease in cell area of 9.0% caused by actin depolymerization was observed during the same period (Fig. 4b). Representative 15 min time-lapse videos of cells on the surface of actuating hydrogels (15.8 μW mm⁻², 100 Hz, 10% duty cycle) are shown in Videos S1–S2. We also visualized the vinculin distribution by plasmid DNA transfection kit (pEGFP-vinculin as reporter gene) and a similar result was observed (Fig. S12). To rule out the interference from adaptive cellular protrusion upon NIR irradiation, comparative experiments were performed with 3T3 cells on control hydrogels conjugated with CD-PDMA-RGD and PNIPAM-RGD (Fig. S13 and Fig. S14). In these cases, no differences were observed in F-actin localization whether or not NIR source illuminated (Fig. 4c–d). These results confirmed that pulling forces could be generated by the photothermal-induced shrinkage of macromolecular actuators to manipulate the cell spreading.
Since cell spreading directly mediates cell protrusion and migration, we further investigated the force-induced cellular migration under various conditions. We first tracked the actin patches’ trajectories on different substrates over the illumination time (Fig. 5a and Fig. S15). Quantification of the maximum displacements showed an actin patches’ migration of about 2.0 μm on hydrogels conjugated with CD-PNIPAM-RGD in 15 min, significantly larger than that of 0.6 and 0.7 μm on control hydrogels conjugated with PNIPAM-RGD and CD-PDMA-RGD, respectively (Fig. 5b). We then varied the laser power and pulses to alter the amplitude and frequency of actuation with these active platforms. In addition, a control where an ROI adjacent but outside the cell membrane was illuminated showing no obvious increase in relative cell area or migration (Fig. S16). With the increase of NIR exposure power density from 0.8 to 49.6 μW μm⁻², the displacement of actin patches increased from 1.2 to 5.3 μm (Fig. 5c). In contrast, the control polymer CD-PDMA-RGD failed to generate a cell response regardless of the NIR power applied. Higher laser powers were expected to induce a higher M. T. temperature, therefore, leading to stronger pulling effect. On the other hand, the temperature of the irradiated portion of the PEG hydrogel functionalized with CD-PNIPAM-TTCs oscillated with the laser frequency detected by IR camera under 808 nm laser exposure (6.6 μW μm⁻², 1 Hz, 10% duty cycle; ground temperature = 28 °C).
hand, three pulse conditions were applied to evaluate the frequency influence on cell migration, including 1, 10, and 100 Hz. They resulted in average actin patches displacements of 0.5, 0.9, and 2μm, respectively, indicating that higher actuation frequency led to more efficient mechanical stimulation. We attributed it to the enhanced mechanical stimulation to the cell receptor by a succession of ultrafast phase transition behavior of the macromolecular actuator at high-frequency (Fig. 5d).

Single PNIPAM chain collapsing is expected to generate a force in the pN range [57,58], which depends on its molecular weight. It implied that more work would be generated from the shrinkage of CD-PNIPAM-TTC with a longer contour length. Since the actuators’ contour length could be easily tuned by RAFT polymerizations, it was facile to precisely design the force amplitude applied on receptors by varying their contour length. Three different actuators with 29 nm (15 kDa), 64 nm (33 kDa), and 80 nm (40 kDa) contour lengths were employed to demonstrate this assumption. As expected, larger actin patches displacements were observed on the samples with longer contour lengths (Fig. 5e). Together, these results showed that actuators with different molecular weights were capable of applying varied forces to specific cellular receptors.

To further estimate the force generated by macromolecular actuator, we simulated the collapse transition of PNIPAM chain by all-atom molecular dynamics (MD) simulation. PNIPAM with 20, 30 and 40 monomeric units (Fig. 5f–i), comparable to 1–2 Kuhn length [59], were used for simulation (See supporting information for simulation details). As shown in Fig. 5f, at low temperatures (25 °C), the PNIPAM segments with 40 monomeric units adopted an expanded conformation with the radius of gyration ($R_g$) of 2.06 nm and an end-to-end distance of 6.89 nm. When the temperature is increased (40 °C), the entropy rises and intramolecular hydrogen bonding between the amide groups drive the chain’s collapsing into a globular state ($R_g = 1.30$ nm and an end-to-end distance = 1.77 nm). A series of stretching force was then applied to the end of PNIPAM chain, counteracting the tendency to spontaneously form globule. We found the extensions of polymers were rescaled to similar $R_g = 2.09$ nm and end-to-end distance = 6.77 nm (Fig. 5h) at a force of ~40 pN. Thus, we conjectured a ~40 pN force was generated for PNIPAM chain upon heating (Fig. 5i). Following a similar method, we estimated PNIPAM chain with 20 and 30 monomeric units generate ~25 pN and ~30 pN force, respectively (Fig. S17-S19). According to literature [60, 61], the force would aggregate along the polymer chain and the entropic forces arising from stretching of globular chains scale with their contour length (Fig. 5h). The length of actuator we synthesized was about 10 times as we used for simulation, and the pulling forces from macromolecular actuators used for cell experiments are expected to be varied in the range of 150–400 pN. The molecular weights of our actuators could be well controlled via living RAFT polymerization, thus providing a way to control the pulling forces in pN range according to the well-established force measurements and simulations of single PNIPAM chain collapsing [57,58,62].

3.4. Hydrogel stiffness influence the applied force transduction and signaling pathways correlations

We next investigated the influence of hydrogel passive stiffness on applied force transmission to cells. PEG hydrogels with different Young’s moduli were prepared and functionalized with same amount of macromolecular actuators CD-PNIPAM-RGD (generate ~300 pN force according to simulation) for testing. A threshold stiffness around 4.8 kPa was found for our PEG hydrogels, above which the cell spreading area were not significantly affected by passive stiffness. Numerous reports
suggested that cell-generated forces modulate their spreading on substrates with different stiffness [63–65]. 3T3 cells displayed similar spreading area on substrates with Young’s moduli of 4.8 kPa, 20 kPa, and 48 kPa (Fig. 6a–d), indicating similar cell-generated force transduction on these substrates. In contrast, the transduction of applied force to cells are different from these matrices. Upon 100 Hz pulsed NIR light exposure to selected cell areas, the pronounced force induced actin patch displacement was only observed on the stiff hydrogel with a Young’s modulus of 49 kPa (Fig. 6e). This substrate was much stiffer than the 3T3 itself (Young’s modulus: 20 kPa [66]). Since the shrinkage of the PNIPAM chains stretch both hydrogel matrices and cell receptors, it can be postulated that softer hydrogel scaffolds than 3T3 would be prior.

Fig. 6. (a) Time series of images of SPI555-Actin stained 3T3 cells on PEG hydrogels functionalized with CD-PNIPAM-RGD and CD-PDMA-RGD. Red dashed circles represent the region of NIR illumination. Green, pink, white, and gray circles represent tracked actin patches. The far-right panels show the trajectories of 20 randomly selected actin patches from at least 10 cells for each condition. Scale bars, 10 μm (cells) and 2 μm (trajectories). (b) Scatterplots (mean ± s.e.m.) with each datapoint representing a tracked maximal displacement of the actin patches of cells adhered to PEG hydrogels substrates under typical NIR illumination conditions at 15 min. (c–e) The analyzed maximal displacement of the actin patches from cells on hydrogels functionalized with CD-PNIPAM-RGD was measured to assess the effect of (c) NIR power densities, (d) pulsed frequency, and (e) contour length of the actuator. Incubation temperature = 28 °C. At least 8 cells were analyzed for each experiment. (f–h) Representative molecular dynamics simulation snapshot: (f) periodically replicated syndiotactic PNIPAM chain composed of 40 monomeric units at 25 °C. (g) Collapsed conformation of the PNIPAM without applied force and (h) 40 pN was applied in the z direction for 10 ns to PNIPAM chain at 40 °C. Water was shown only partially in the simulation box (black frame) for clarity. (i) Forces (mean ± s.d.) applied to PNIPAM chain at 40 °C when the end-to-end distance and Rg equilibrium approaching to the value at 25 °C. *, **, *** are determined using Tukey test, and represent statistical significance at p < 0.05, 0.01, and 0.001, respectively.
deformed to balance the shrinkage, leading to less force applied to the receptors (Fig. 6f), although this hypothesis warrants further investigation in follow up studies.

Since our platform allowed force application precisely on integrin at molecular scale without perturbation of other cellular membrane receptors, we demonstrated the potential of using our platform to correlate signaling pathways and cell response. Actomyosin contractility is known to play a major role in integrin-mediated mechanotransduction signaling pathways [67]. We inhibited actomyosin contractility with Y27632 [68], the force-induced actin patches displacement was diminished completely (Fig. 6g–h), confirming actomyosin contractility’s role in the force induced cell migration. Integrin signaling interacts with additional pathways. For example, crosstalk between mechano-sensitive ion channels and integrin signaling has been proposed in sensing of matrix passive stiffness [69]. To elucidate whether ion-channels also involved in this active force transduction, we inhibited ion channel receptors by gadolinium [70], actin patch displacements were largely retained (Fig. 6g–h). Our results suggest that this applied force induced cell response is independent of ion channel-mediated pathway, and mediated solely by components of the integrin signaling pathway.

To the best of our knowledge, this is the first attempt enabling ECM-like hydrogel platform with varied stiffness to apply molecular resolved forces to specific cellular receptors at user-defined doses. This capability was based on the unique design of the macromolecular actuators which features several advantages. First of all, the macromolecular actuators with active terminal groups allowed easily conjugation into hydrogel platforms and flexible functionalization with any ligands of choice without additional synthetic effect. Secondly, compared with reported UV-responsive motor-based molecular tool [45], NIR light was used in our system, which was expected to cause minimal cell damage and applicable to long time stimulation. Thirdly, molecularly resolved forces are decoupled from the physical and biochemical background of cells. Reversible volume change of crosslinked PNIPAM network typically use gold nanorods (AuNRs) as photothermal transducers [46,47], which leads to the global collapsing of the polymer networks. The entire contraction of the network not only generate forces to the receptors from diverse direction with complexity over the force amplitude control, but also change the passive stiffness and ligand density concurrently. In contrast, our NIR-photothermal CD group enabled efficient thermal transfer to the linked PNIPAM chain at molecular level. Therefore, our platform enables user-defined forces exerted on specific cellular receptors, while hydrogel passive stiffness and actuator densities can be independently varied. These capabilities are not matched with any other method.

4. Conclusions

We have developed a class of novel ECM-like hydrogel platforms functionalized with NIR-regulated macromolecular actuators for generating forces to specific cellular receptors at molecular scale. By tuning the NIR optical pulse profile, the magnitude and frequency of the molecular force can be precisely adjusted, thus, regulating the spreading and migration of 3T3 fibroblast. Actuators’ molecular weights can be well controlled via living RAFT polymerization, which indicates the possibility of developing a robust toolbox of actuators capable of generating varied forces within applicable ranges to address cellular responses. We have demonstrated the capability of our platform for addressing challenging issues in cellular mechanotransduction, such as how the hydrogel stiffness influences the active force transmission to cells and the correlation between signaling pathways and cell response. This noninvasive,

Fig. 6. (a–c) Representative cell outlines on PEG hydrogels with varied Young’s modulus (4.8 kPa, 27 kPa, 49 kPa), respectively. Scale bars = 20 μm (d) Quantification of cell area on PEG hydrogels with varied Young’s modulus (mean ± s.e.m.). (e) The analyzed maximal displacement of the actin patches from cells on PEG hydrogels after NIR illumination. (f) Schematic showing the shrinkage of the actuator stretching on integrin and matrix, respectively. (g) Maximal displacement of the actin patches from cells on PEG hydrogels (E = 49 kPa) after NIR irradiation. Cells were pre-treated with ROCK inhibitor Y27632 (10 μM) or ion channel inhibitor gadolinium (30 μM). (h) Schematic showing the integrin and ion-channel mediated pathways for mechanical sensing. Incubation temperature = 28 °C. Data were analyzed from at least N = 8 cells. *, **, *** are determined using Tukey test, and represent statistical significance at p < 0.05, 0.01, and 0.001, respectively.
cytocompatible, and tunable approach of using a NIR light illumination holds great potential utility to manipulate and study the cellular responses and mechanotransduction pathways.

**Author contributions**

The project was supervised by Yijun Zheng. Yile Zhang and Xiaoxin Shen designed and built the microscope for simultaneous laser actuation and imaging in Shen’s lab. Yukai Wu and Xingjun Zhu designed the temperature feedback upconversion nanoparticle and established the optical thermometry system for microscopic temperature detection in Zhu’s lab. Hong Wang and Jianxi Cui synthesized the RAFT agent. Yukai Wu and Nan Cheng conducted the photothermal actuation and temperature measurement. Nan Cheng performed the polymer synthesis, hydrogel preparation and cell experiments. Bohan Li quantified the cell results. Bohan Li and Peng Zhao performed molecular dynamics simulations. Shaojie Chen conducted glass functionalization. All the authors contributed to the discussion, data analysis and wrote the manuscript.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

**Acknowledgements**

This work was supported by the Shanghai Pujiang Program (Grant No. 20PJ1411200, 20PJ1410700 and 20PJ141160), the National Natural Science Foundation of China (Grant No. 52073175 and 82001945), and ShanghaiTech University. The authors thank the support from the ShanghaiTech University. The authors thank the support from the National Natural Science Foundation of China (Grant No. 20PJ1411200, 20PJ1410700 and 20PJ141160), the National Natural Science Foundation of China (Grant No. 52073175 and 82001945), and ShanghaiTech University.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100476.

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