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COMMUNICATION

Control of cancer cell phenotypes *via* supramolecular hydrogels: the role of extracellular matrix stiffnessVirginie Baylot,^a Bruno Alies,^a Palma Rocchi,^b and Philippe Barthélémy^{a,*}Received 00th January 20xx,
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Malignant cell lines are frequently used as a model to understand cancer and to identify potential new treatments. However maintaining the initial cancer cells phenotypes in culture remains an important challenge, which strongly impact on the proper evaluation of novel therapeutic approaches. The mechanical properties of the extracellular matrix (ECM) are known to strongly impact on the cells fate and phenotype. Herein, we report on tunable artificial ECM based on supramolecular gels allowing the control of cancer cell phenotypes.

As one of the important components of the tumor microenvironment the modification and in particular the increase of the ECM stiffness is a remarkable feature of cancers¹. During the tumor formation, cancer cells increase the ECM stiffness, which in return has an impact on their characteristics and fate. Many cancer cells have been cultured in Matrigel, a complex biological system extracted from the secretion of *Engelbreth–Holm–Swarm* mouse sarcoma cells and enriched for extracellular matrix proteins². Despite its massive use in laboratories, Matrigel™ is a complex mixture with more than one thousand components³, which induces batch to batch variabilities. Moreover, this ECM may not contain the proper components, cues and mechanical properties adapted to the cancer cells of interest. Given these limitations, there is an urgent need to develop artificial ECM allowing to improve cancer cell culture 3D methods⁴. Supramolecular gels for cell culture are three-dimensional networks formed by reversible supramolecular interactions, such as hydrogen bonding, van der Waals forces, and π - π stacking, rather than covalent bonds⁵. These materials provide supportive scaffolds that mimic the extracellular matrix (ECM), allowing for better cell attachment, growth, and differentiation compared to traditional two-dimensional systems⁶.

Herein, supramolecular gels based on Glycosyl-Nucleo-Bola-Amphiphile (GNBA) are emerging as an attractive class of artificial ECM in cell culture owing to their key features including biocompatibility, dynamic properties and modularity⁷. These gels can be easily tailored in terms of composition, mechanical properties, and degradation rates, enabling customization for specific cell types or applications^{8,9}. Also their reversible nature of the supramolecular interactions allows for a gel that can change its properties in response to environmental stimuli, which can be beneficial for studying cellular behavior¹⁰. Thanks to their intrinsic properties GNBA can be used for a variety of applications, including drug delivery⁸ and tissue engineering, offering an innovative approach for experimental research.

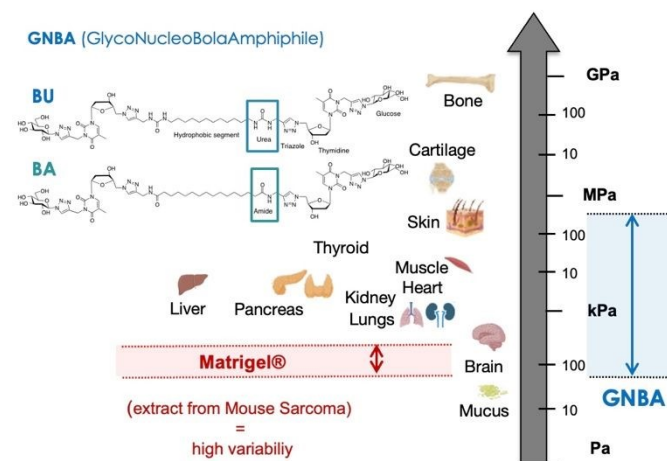


Figure 1. Left) Chemical structures of the two Glycosyl-Nucleo-Bola-amphiphile (GNBA, BU for bisurea in blue and BA for bisamide in green) used in this study. Right) Illustration showing the elastic moduli of different organs and tissues. The stiffest tissues of a body are tooth and bones ($G' > 1$ GPa) lung and brain are among the softest ($G' > 100$ Pa). Matrigel (in red) show stiffness values G' around 400 Pa,¹¹ whereas GNBA supramolecular gels elastic moduli can be modulated from 100 Pa to 100 kPa¹⁰ (in blue). The stiffness of PC3 and LNCaP cellular environments are shown on this scale.

Tissues in the human body comprise diverse extracellular matrix (ECM) components, giving rise to a broad continuum of elastic moduli (Fig. 1). Each tissue exhibits a characteristic

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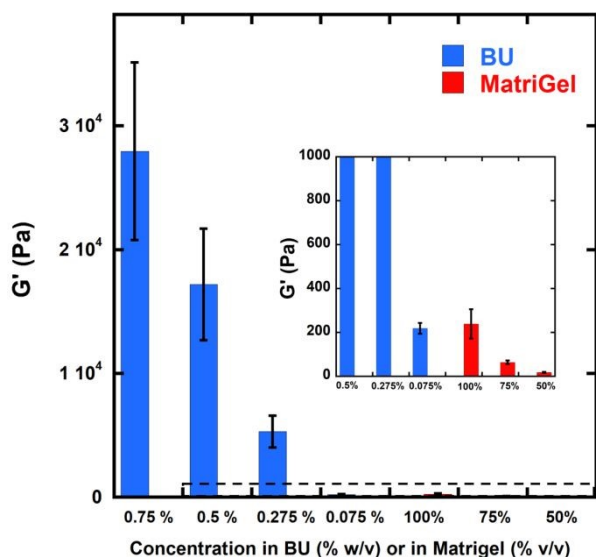


Figure 2: Elastic moduli (G') values recorded at a sweep frequency of 1Hz. G' of GNBA (BU) based supramolecular gels (Blue) versus Matrigel (red) are reported at different concentrations (BU % w/v, Matrigel %v/v).

mechanical profile that supports its physiological role. For example, brain tissue is among the softest (~ 0.1 – 1 kPa), facilitating neuronal plasticity and dynamic cell–matrix interactions, whereas mineralized bone is orders of magnitude stiffer (gigapascal range), enabling structural support and protection of internal organs (Fig. 1).¹² ECM stiffness regulates cellular behavior¹³ and contributes to tissue homeostasis, pathology, and disease progression, including cancer¹⁴.

To further explore and expand the use of the GNBA biomaterials, we here exposed different cancer cell lines, including prostate cancer (PCa) PC-3 and LNCaP lineages into GNBA supramolecular hydrogels. LNCaP cell line was initially established from a lymph node metastasis of a patient with metastatic PCa¹⁵ and PC-3 cell line was initiated from a bone metastasis of a patient with prostatic adenocarcinoma¹⁶. Since these cell lines come from different tissues their actual local environment features different stiffness'. Lymph node tissues (LNCaP) exhibit elastic modulus G' in the range of few kPa (5 to 9 kPa)^{17,18}, whereas prostate cancer metastasis to bone (PC3) show a G' higher than 1 MPa¹⁹. However, to form the secondary tumor in the bone,²⁰ prostate cancer cells have first to interact with collagen rich ECM²¹ such as the one found in osteoid-like tissues (~ 30 to 45 kPa). Hence, as a proof of concept, we hypothesised that modulating the gel stiffness in the 0.2–30 kPa range could maintain the initial phenotype of the cancer cells. Previous studies showed that changes in cell phenotype arising from external environment conditions are irreversible^{22,23}. We thus explored cell behavior modifications due to ECM stiffness in these two PCa cell lines that were derived from tumors progressing in tissues with different stiffness.

First, the mechanical behaviors of GNBA based gels and Matrigel were investigated by oscillatory rheology at different concentrations in RPMI culture media (Fig. 2). In the case of GNBA gels, the observed values for the storage modulus at a sweep frequency of 1Hz dependent on the GNBA

concentrations. At high 0.75% (w/v) the supramolecular gels

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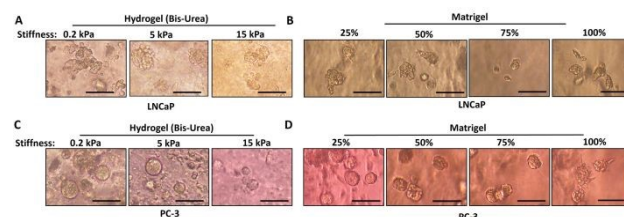


Figure 3: LNCaP and PC-3 cells formed spheroids when cultured in GNBA-based matrix and Matrigel™ at different stiffness. Representative images of the spheroids derived from LNCaP (A-B) and PC-3 (C-D) cell lines, were acquired with a phase-contrast microscope at the 10X magnification, scale bars: 100 μ m.

feature a G' of 28 kPa $\pm$ 7 kPa whereas a G' of 0.2 kPa $\pm$ 30 Pa was observed at a concentration of 0.075% w/v, illustrating the large elastic modularity of the GNBA gels (Fig. 2 and Fig. S14). Of note, very low G' values were measured for the Matrigel samples (from 0.2 kPa $\pm$ 70 Pa at 100% to < 0.02 kPa $\pm$ 5 Pa at 50%), indicating both very low stiffness and weak elastic modularity of the Matrigel materials (Fig. 2, Fig. S15 and Fig. S16).

Next, PC-3 and LNCaP cell lines were cultured in GNBA supramolecular hydrogels with different stiffness values (0.2, 5, 15 and 30 kPa) mimicking lymph nodes, normal prostate, prostate tumor and bone tissues stiffness respectively (Fig. 3). Matrigel™, standard 3D culture ECM, that displays low stiffness values at all the concentrations (100, 75, 50 and 25%) used to embed the PCa cells (Fig. 3). PC-3 and LNCaP cells formed spheroids in both Matrigel™ and GBNA-based hydrogels 7 days after they were embedded in both matrices with no significant differences in spheroids numbers and size (Fig. 3). After 32 days, LNCaP and PC-3 cells were collected and the impact of ECM stiffness on the cellular phenotype was investigated. LNCaP cells that grew in soft matrices (GBNA hydrogel at 0.2 kPa and Matrigel™) maintained their original migratory and proliferative abilities, when compared to the parental cell line (Fig. 4 and Fig. S3 A and B). Whereas LNCaP cultured in stiff substrates display reduced capacities to proliferate (30% of reduction, $P \leq 0.001$, ***) and migrate (30 to 40% of decrease, $P \leq 0.001$, ***) (Fig. 4 A and C and Fig. S11).

In contrast, PC-3 cells show reduced proliferation and migration efficiency when cultured in soft ECM (GBNA hydrogel at 0.2 kPa and Matrigel™) compared to the parental cell line or cells cultured in stiff matrices (hydrogel stiffness values of 15 and 30 kPa) (Fig. 5 and Fig. S3 C and D). Proliferation rates were reduced of 18% ($P \leq 0.001$, ***) and we observed a 33% decrease of migration abilities ($P \leq 0.01$, **) for PC-3 cells that grew in 0.2 kPa GBNA-based gels, compared to their parental counterparts (Fig. 5 A and C and Fig. S2). Interestingly, we showed that PC-3 cells cultured in Matrigel™ lose the original cell line features, illustrated by a 20 to 40% of proliferative capacities reduction and a migration efficiency drop of 25 to 43% ($P \leq 0.01$, **, $P \leq 0.001$, ***, Fig. 5 B and D and Fig. S2). These results demonstrate that Matrigel™, for which the stiffness can't be tuned depending on the cellular or tumor model, is not adapted to grow cells or tumors that were isolated from stiff tissues.



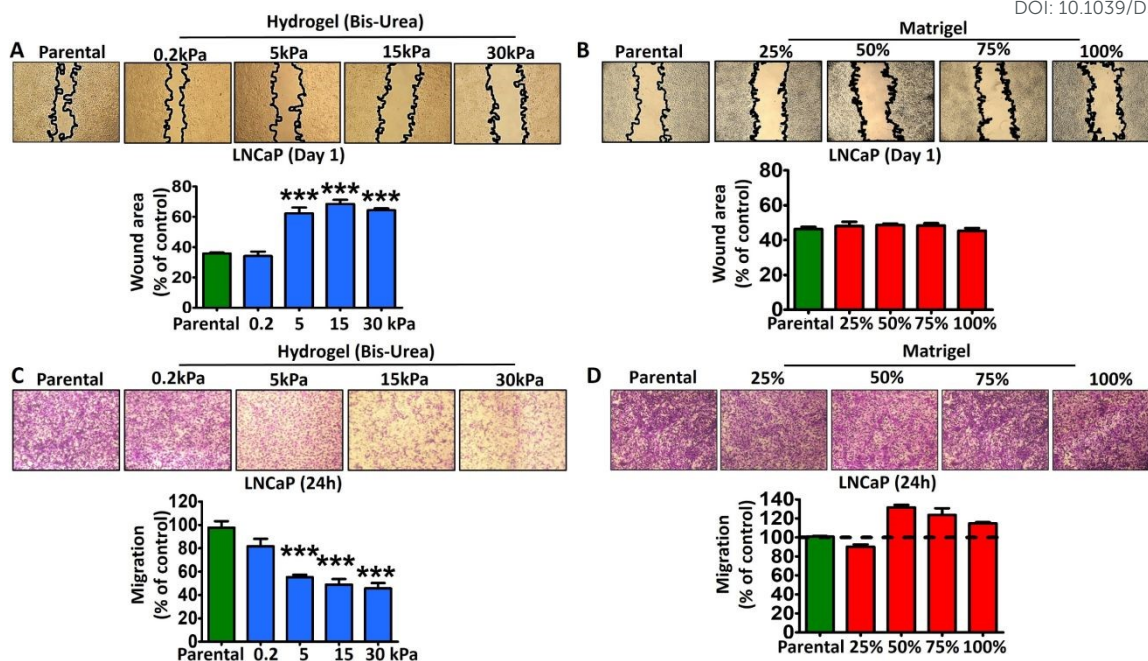


Figure 4: LNCaP migration and proliferation capacities vary according to matrices stiffness. **A and B-** Proliferation rates of LNCaP cultured in GBNA supramolecular hydrogels at different concentrations 0.2, 5, 15 and 30 kPa (**A**) and in Matrigel™ at 25, 50, 75 and 100% (**B**) evaluated by wound healing assay. Images of the wounds (representative pictures are shown in the first line) were acquired 24 hours after the scratch was created ($n=12$ per conditions) and analysed with the software Image J ($n=3$ wells per conditions). **C and D-** LNCaP cells cultured in specified substrates - GBNA based hydrogels (**C**) and Matrigel™ (**D**) - migration abilities were investigated using Transwell assays ($n=3$ wells per condition). Representative images of migrated cells stained with crystal violet are shown in the first line. For quantification (graph bars shown in second line), crystal violet was solubilized in ethanol and the absorbance at 555 nm was measured. For all the *in vitro* experiment, parental LNCaP cell line was used as the reference. Statistically significant differences between the conditions of culture were determined by analysis of variance (one-way ANOVA). p -values of Tukey's post-hoc analyses are presented. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

In conclusion, we have shown that supramolecular gels applied to cancer 3D cell cultures allows to adapt the elastic properties of the artificial ECM to the native tumor environment. As demonstrated for PCa cell lines (PC-3 and LNCaP), the proliferation and migration properties are impacted by the artificial ECM stiffness. LNCaP cells maintained their original phenotype in both weak GNBA gels and Matrigel™ mimicking their native environment. However, PC-3 cells require ECM featuring higher stiffness and lose their native phenotype in the weak Matrigel™, demonstrating that controlling the ECM stiffness in cell cultures is required for maintaining the native phenotypes. 3D cell cultures are often developed to mimic in an accurate fashion the original tumor, for both drug screening and tumor characteristic studies. Such models need to maintain the native clinical features over time to be relevant. In this study, we clearly demonstrate the importance of adapting the ECM stiffness to the tumor or cell models to keep their native phenotypes. Overall, the GNBA supramolecular gels feature key characteristics required for 3D cell-cultures, including i) modularity of the elastic moduli over a large range of values compared to Matrigel™ (Fig. 1), ii) ease of use (see movie) thanks to their intrinsic supramolecular nature, iii) non-toxicity and iv) their applicability to different cells⁶. The possibility of modulating the viscoelastic properties of supramolecular gels are promising tools in cell culture,

providing a more realistic environment for cells compared to traditional methods.

Methods

Cell culture

Human prostatic cell lines LNCaP (castration-sensitive prostate cancer cells) and PC-3 (castration-resistant prostate cancer cells) were cultured in RPMI-1640 (Roswell Park Memorial Institute) and DMEM (Dulbecco's Modified Eagle's Medium) respectively, supplemented with 10% of fetal bovine serum (FBS) at 37°C in 5% CO₂.

Spheroids culture

LNCaP and PC-3 cells were embedded in gels (BU) based on the protocol of El Hamoui *et al.*¹⁰ Briefly, the adequate volume of culture media (RPMI+FBS for LNCaP and DMEM+FBS for PC-3) was added to the specific amounts of the gelator molecule powder to obtain the desired ECM stiffness (from 0.2 to 30 kPa). The mixture was then heated at 80°C for 15 minutes and left at room temperature for 4-5 additional minutes to reduce the temperature before adding the cells. LNCaP and PC-3 cells were counted with an automated cell counter (Cell Countess 3, Invitrogen) and encapsulated in the gel (BU) material at a final concentration of 50 000 cells/ml. The prostate cancer cells were embedded in Matrigel™ at the same final concentration.



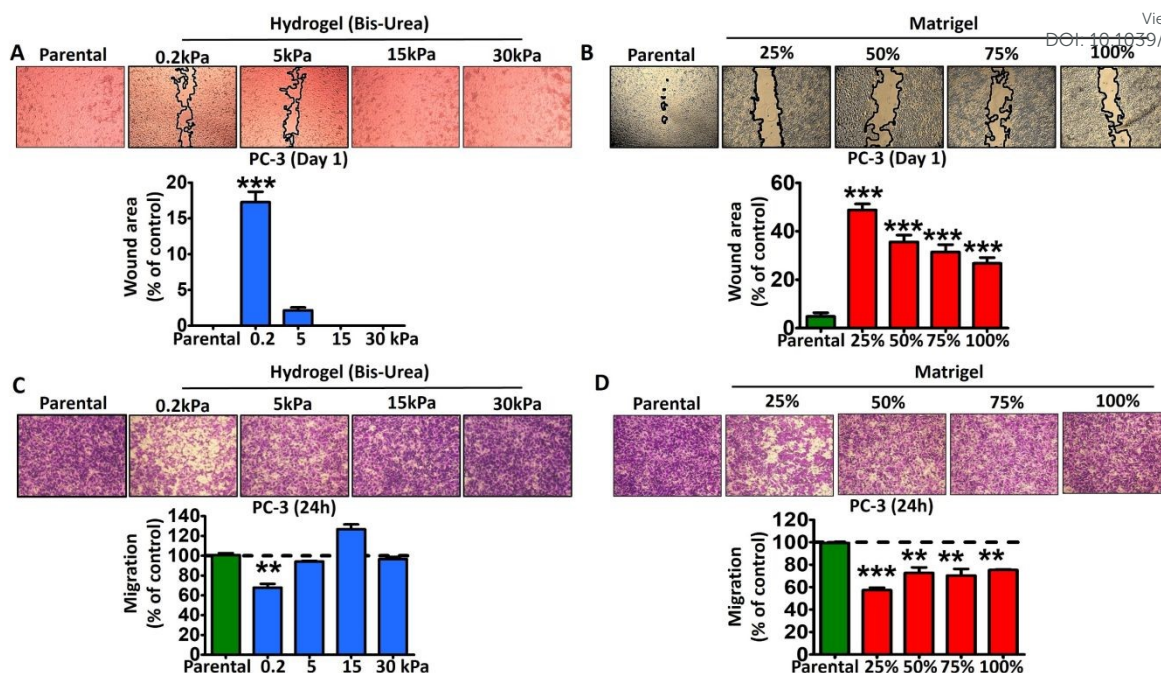


Figure 5: PC-3 display reduced migration and proliferation efficiencies when cultured in soft matrices. **A and B** - Proliferation capacities of PC-3 cultured in the specified substrates - GBNA supramolecular hydrogels at different stiffness values of 0.2, 5, 15 and 30 kPa (A) and in Matrigel™ at 25, 50, 75 and 100% (B) - were evaluated by *in vitro* scratch assay. Images of the wounds (first line, $n=12$ per conditions) were acquired at 0 and 24 hours after the scratch was created and analysed with the software Image J ($n=3$ wells per conditions). **C and D** - PC-3 cells cultured in specified matrices - GBNA based hydrogels (C) and Matrigel™ (D) - migration abilities were investigated using Transwell assays ($n=3$ wells per condition). Representative images of migrated cells stained with crystal violet are showed on the first line. For quantification (graph bars), crystal violet was eluted from cells in ethanol and the absorbance at 555 nm was measured. For all the *in vitro* experiment, parental PC-3 cell line was used as the reference. Statistically significant differences between the conditions of culture were determined by analysis of variance (one-way ANOVA). *p*-values of Tukey's post-hoc analyses are presented. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

Matrigel™ was mixed or not (100 %) with the adequate culture media at different Matrigel™: Medium ratio: 3:1 (75%), 1:1 (50%) and 1:3 (25%). The mixture cells/material was transferred in 96 well plates (100 μ l/well) and left at 37°C for 10 minutes to allow the gelation of the matrices. Finally, 200 μ l of culture media were added on each well. The first spheroids appeared after 7 days and left in culture for 35 days. The culture media was refreshed twice a week.

Dissociation of spheroids

After 35 days in 3D culture, LNCaP and PC-3 spheroids were isolated and disrupted into single cell suspensions. The spheroids embedded in BU hydrogels and Matrigel™ were dissociated by pipetting the mixture cells/gel up and down in 1 ml of cold PBS. The cells were spun down by centrifugation and washed twice in cold PBS. The cells were counted and plated at the same concentration in 6 well plates for 2 days before performing the different *in-vitro* based assays.

Microscopy

LNCaP and PC-3 cells cultured in 3D were imaged at Day 14 using phase-contrast microscope. All images were obtained with a 10X objective.

Migration assays

LNCaP and PC-3 cells were cultured in 3D at the specified conditions. Parental cell lines that never were cultured in 3D

were used as controls. Migration assays were performed using Thermo Scientific™ Nunc™ polycarbonate cell culture inserts (membrane pore size of 8 μ m) in 24 well plates. 75 000 cells/well were plated onto the transwell upper chamber in 200 μ l of serum free culture media and 500 μ l of complete media were added onto the bottom chamber. After 24 hours, the cells were washed twice in PBS, and fixed in PBS + 4% of paraformaldehyde (PFA) for 15 minutes at RT. After 2 washing steps in PBS, the non-migrated cells on the upper surface of the transwells were gently removed with cotton swabs. The migrated cells on the bottom surface of the transwells were stained with 0.2% (w/v) crystal violet for 5 minutes. Migrated cells were observed with a phase-contrast microscope. For the quantification, crystal violet coloration was solubilized in ethanol for 5 minutes, transferred in clean 96 well plates and the optical density was read at 555 nm using a plate reader.

Wound healing assays

LNCaP and PC-3 cells were cultured in 3D at the specified conditions. Parental cell lines that never were cultured in 3D were used as controls. PC-3 and LNCaP cells were seeded at the concentration of $360 \cdot 10^5$ and $500 \cdot 10^5$ cells per well in a 24 well plates respectively. The cell monolayer was scraped in a straight line to create a "scratch" with a p200 pipet tip. Debris and smooth the edge of the scratch were removed by washing the cells twice with PBS. The images of the wounds were acquired on a phase-contrast microscope.



Statistical analysis

Results were analyzed and represented as mean $\pm$ SEM. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc). The one-way ANOVA followed by Tukey-Kramer *post-hoc* test was used to compare the means between the different conditions of culture. Results with $P < 0.05$ were considered statistically significant and are indicated by *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Rheology experiments

Rheological measurements were carried out on a Malvern Kinexus Pro+ rheometer with steel cone-plate geometry (diameter: 20 mm). The lower plate was equipped with a Peltier temperature control system. A solvent trap was used to ensure homogeneous temperature and to prevent water evaporation. BU in cell medium (RPMI + FBS) were heated at 85 °C, and was deposited on the motionless disk of the rheometer, and then the cone-plate was set and the gel could rest and cure between the disks. All rheology experiments were performed at 37 ± 0.05 °C. G' moduli indicated in Figure 2 were taken at a frequency of 1 Hz ($\omega = 6.283 \text{ rad s}^{-1}$) at a strain of 0.5% and error bars represent standard deviation of moduli during frequency sweep (data shown in Fig S4-5). Strain amplitude sweep experiments are displayed in Fig S6.

Author contributions

P.B., V.B. and B.A. conceived and led the project. Experiments were performed by B.A. and V.B., with assistance from P.R. for cell experiments. P.B. wrote the manuscript with input from all other authors.

Conflicts of interest

There are no conflicts to declare

Data availability

A data availability statement (DAS) is required to be submitted alongside all articles. Please read our [full guidance on data availability statements](#) for more details and examples of suitable statements you can use.

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COMMUNICATION

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Data availability

The data supporting this article have been included as part of the Supplementary Information.

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