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Substrate viscosity plays an important role in bacterial adhesion under fluid flow

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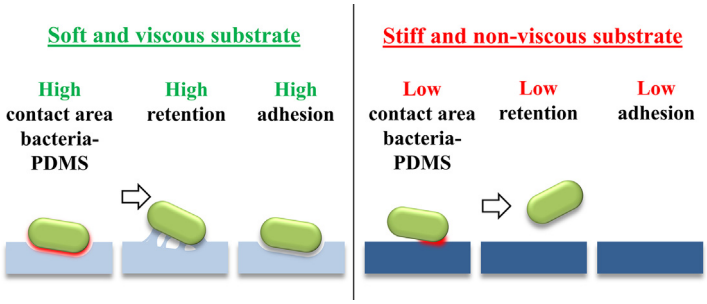
GRAPHICAL ABSTRACT



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ABSTRACT

Many materials used in the medical settings such as catheters and contact lenses as well as most biological tissues are not purely elastic, but rather viscoelastic. While substrate elasticity has been investigated for its influence on bacterial adhesion, the impact of substrate viscosity has not been explored. Here, the importance of considering substrate viscosity is explored by using polydimethylsiloxane (PDMS) as the substrate material, whose mechanical properties can be tuned from predominantly elastic to viscous by varying cross-linking degree. Interfacial rheology and atomic force microscopy analysis prove that PDMS with a low cross-linking degree exhibits both low stiffness and high viscosity. This degree of viscoelasticity confers to PDMS a remarkable stress relaxation, a good capability to deform and an increased adhesive force. Bacterial adhesion assays were conducted under flow conditions to study the impact of substrate viscosity on *Escherichia coli* adhesion. The viscous PDMS not only enhanced *E. coli* adhesion but also conferred greater resistance to desorption against shear stress at air/liquid interface, compared to the PDMS with high crosslinking degree. These findings highlight the importance to consider substrate viscosity while studying bacterial adhesion. The current work provides new insights to an improved understanding of how bacteria interact with complex viscoelastic environments.

Abbreviations: AFM, Atomic Force Microscopy; c-di-GMP, cyclic diguanylate; CFU, colony forming unit; LB, lennox broth; PAAm, polyacrylamide hydrogels; PBS, phosphate-buffered saline; PDMS, polydimethylsiloxane; PPP-NCST, PointProbe® Plus Non-Contact/Soft Tapping Mode - Au coating (Detector side); SEM, Scanning Electron Microscopy.

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1. Introduction

Among nosocomial infections, infections associated to implanted materials are the most frequent and severe due to biofilm formation [1]. As a heterogeneous community embedded into a matrix of polymers, bacteria in biofilms are highly resistant to environmental stress [2], antibiotics [3] and host immune response [4,5]. To avoid biofilm formation, prevention of the first step of biofilm development (i.e. bacterial adhesion) should mitigate this infection problem [6,7]. Different approaches have been explored to modify surfaces to limit bacterial colonization, including usage of antibacterial agents, controlling of surface chemistry and surface topography. It has been demonstrated that bacterial adhesion is influenced by the physicochemical properties of materials, such as chemical composition [8], hydrophobicity [9], charge [10], surface roughness [11], and topography [12].

Recently, a new approach has been investigated to influence bacterial adhesion by modifying mechanical properties of the materials. Using agarose, agar and poly(ethylene glycol) dimethacrylate (PEGDMA) and 2-methacryloyloxyethyl phosphorylcholine polymer (PMPC) hydrogels bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pseudoalteromonas* sp. and *Bacillus* sp.) were demonstrated to adhere preferentially on stiff rather than soft hydrogels [13–16]. In contrast, when polyacrylamide (PAAm) was used, more *S. aureus* adhered on the soft than stiff PAAm hydrogel [17]. Song et al. reported that *E. coli* adhesion and biofilm formation are reduced with an increase of polydimethylsiloxane (PDMS) stiffness [18]. These seemingly different results might be caused by different physicochemical properties of the used hydrogels, different bacterial strains and different experimental conditions, as excellently explained by Wang and co-workers [17].

Furthermore, Song et al. also reported that *E. coli* exhibited higher motility on stiff PDMS than on soft PDMS when PDMS samples with Young's moduli in the range of 0.1–2.6 MPa were compared [19]. It has been suggested that bacterial mechanosensing of stiffness is related to different degrees of deformation of the bacterial cell membrane upon contact with PDMS substrates of various stiffness [20]. Cell membrane deformation was proposed to create more membrane stress to bacteria on stiff than on soft PDMS, which was found to correlate with a decreased level of cyclic diguanylate, an important regulator for biofilm formation [20]. Very recently, our group revealed that the intrinsic physicochemical properties associated with PDMS substrates of different cross-linking content, rather than active bacterial mechanosensing, strongly influenced bacterial adhesion [21].

However, while stiffness is used to describe materials that exhibit elastic (solid-like) properties, most biological tissues (human tissues and cells) [22–24] and medical devices (e.g. silicone-based materials, contact lenses, catheters) [25,26] exhibit both solid- and fluid-like properties and are categorized as viscoelastic. Bacteria are known to interact with, besides synthetic materials, living organic surfaces, such as biofilm matrix or host cells, that are inherently viscoelastic [23,27]. Probing bacterial cellular responses by substrate stiffness alone may oversimplify the mechanisms by which cells interact with their surroundings. It has been reported that the viscosity of a polymer solution can influence the bacterial flagella propulsion and thus bacterial swimming speed [28].

In this work, we seek to gain new mechanistic insight into how materials with inherent viscosity impacts bacterial adhesion under dynamic flow conditions. Such knowledge can improve our understanding of bacterial behavior in complex viscoelastic environments present in the human body. Being a well-characterized polymer network, PDMS is used here as model of viscoelastic materials. Showing good cytocompatibility and biodurability, PDMS has been intensively used for biomedical devices (catheters, drains, contact lenses) [25] and fluidic devices [29], and it is therefore relevant to study for understanding its interaction with bacteria. Here, we synthesize PDMS materials with the mechanical properties from predominantly elastic to viscous by altering the cross-linker content, and systematically quantifying the viscoelasticity, surface adhesive force (stickiness) and stress relaxation properties of PDMS substrates using dynamic rheology and atomic force microscopy (AFM). Furthermore, the influence of material viscoelasticity on *E. coli* desorption from surfaces at air/liquid interface was investigated under flow conditions, which has not been addressed so far in literature. Another novelty of this work lies in the demonstration that material viscosity plays a predominate role in bacterial adhesion and retention, which highlights a novel concept so far not yet revealed in the field of bacterial colonization. For the first time, we find that the inherent viscosity in PDMS substrates is strongly linked to bacterial adhesion. A model is thus proposed to explain the underlying mechanism of impact of substrate viscosity on bacteria adhesion.

2. Materials and methods

2.1. Preparation of PDMS substrate

PDMS substrates were prepared using a silicone elastomer kit (Dow Corning Corporation, SYLGARD 184, USA) following a protocol described previously [18]. To obtain PDMS substrates of different stiffness, various ratios of silicone base and cross-linking agent were used: 10:1, 20:1 and 40:1. After thorough mixing of both components, air bubbles were eliminated by application of vacuum for 30 min. Sixteen μL of each mix was poured in duplicate into wells of an in house-made transparent polycarbonate slide (8 wells/slide, 6 mm diameter, 0.8 mm depth) and allowed to cure for 24 h at 60 °C. PDMS sample surfaces were flat and even with the top of the six well plates. For biological experiments, the prepared PDMS were sterilized with or without UV irradiation for 20 min (254 nm, 100 $\mu W/cm^2$, Kojair Tech Oy, 18,541 UV-Valo, Finland), followed by 30 min immersion in 70% ethanol (Honeywell, 02860-2.5L, ≥99.8%, Ukraine), subsequently washing with sterile phosphate-buffered saline (PBS) for 1 h and final resuspension in PBS. The same trend of bacterial adhesion was observed for the UV-treated and the untreated samples. PBS was prepared with 0.144 g L^{-1} sodium phosphate dibasic (Sigma-Aldrich, 71640–1 KG, ≥99.0%, United Kingdom), 8 g L^{-1} sodium chloride (Sigma, S3014-5 KG, ≥ 98%,Germany), 0.2 g L^{-1} potassium phosphate monobasic (Sigma-Aldrich, 60220–2.5 KG, 99.5–100.5%, Germany), 0.8 g L^{-1} potassium chloride (Sigma-Aldrich, 60130, ≥99.5%, Germany), adjusted to pH 7.2.

2.2. Characterization of PDMS substrates

The hydrophobicity of the PDMS substrates was characterized by water contact angle measurement (KRÜSS GmbH, DSA25E, Germany). The topography and roughness of the PDMS substrates were analyzed using atomic force microscopy (FlexBio-AFM, Nanosurf, Switzerland). Standard tapping mode with a PointProbe® Plus Non-Contact / Soft Tapping Mode - Au coating (Detector side) probe (Nanosensors, Switzerland) cantilever was used. PDMS samples were immersed in PBS. Scanning area was 25 μm × 25 μm.

The gel fraction was determined by measuring the sample mass before and after extraction in ethanol for 24 h. Specifically, punched PDMS samples (diameter 10 mm) were weighed and the values were recorded as W_d. The samples were then immersed in ethanol (99.8%) for 24 h with 3 changes of solvents. After extraction, the samples were dried under vacuum at 50 °C for overnight.

Finally, the samples were weighed and the values were recorded as W_g. The gel fraction is defined as:

$$\text{Gelfraction} = \frac{Wg}{Wd} \times 100\% \quad (1)$$

The viscoelasticity of PDMS samples (20 mm in diameter, 1 mm in thickness) were analyzed by plate-plate oscillatory rheometry (Anton-Paar, MCR301, Austria). Three important parameters are determined: elastic modulus (G'), loss modulus (G"), and loss factor (tan δ) = G"/G'. Dynamic strain-sweep measurements were performed to determine the linear viscoelastic range at a fixed angular frequency of 10 rad/s and strain of 0.01–500%. For time-sweep, the samples were analyzed at constant angular frequency of 10 rad/s and strain of 0.5%. For dynamic frequency-sweep, the samples were analyzed at constant strain of 0.5% and angular frequency of 1–300 rad/s.

The stickiness of PDMS substrates was evaluated (1) by force curve measurement using AFM (FlexBio-AFM, NanoSurf, Switzerland) and (2) by adhesion failure energy measurement using interfacial rheology (Anton-Paar, MCR301, Austria). (1) Following the cantilever deflection/indentation upon contact with the surface gives information about the deformability (approach step) and the attraction/adhesion force (withdraw step) of the substrate [30]. Experiments were conducted with a PPP-NCST AuD probe (Nanosensors, Switzerland) on PDMS substrates immerged for 2 h in PBS to be able to compare with bacterial adhesion assay. (2) Rheology measurements are detailed in Figure S1. Shortly, each sample is pressed at a constant normal force, then the adhesion failure energy (E_{ad}, J/m^2) is determined during the removal of the upper plate. Stress relaxation properties of PDMS samples were assessed by *in situ* rheology on MCR301. Specifically, the samples were positioned between the plates and conditioned at a strain of 1% for 3 min. The strain was suddenly increased to 20% and held constant. Subsequently the stress was recorded as a function of time. The stress relaxation rate was defined as the half time for stress-relaxation.

The gel mesh size (ε) in cross-linked PDMS networks is calculated based on the results from rheological analysis. The complex modulus (G) is determined by equation:

$$|G| = \sqrt{G'^2 + G''^2} \quad (2)$$

where G is the complex modulus, G′ is the storage modulus, G″ is the loss modulus.

The mesh size (ε) is calculated according to classical models for polymer physics [31] using equation:

$$\varepsilon = \left(\frac{6RT}{\pi N_A G}\right)^{1/3} \quad (3)$$

where R is the gas constant, T is the absolute temperature, N_A is the Avogadro's number, G is the complex modulus.

2.3. Bacterial preparation

E. coli K-12 strain BW25113 was selected in this work due to its wide usage for biofilm study [32–35]. Pre-cultures of *E. coli* BW25113 were grown in Lennox broth (LB, Roth, Germany). For the inoculation of flow systems, an exponential grown bacterial suspension was centrifuged (3000 rpm, 5 min, 4 °C) and the pellet was washed with PBS three times before dilution in PBS to an optical density of 0.01 at 600 nm, corresponding to about 5×10^6 colony forming units (CFU) per mL.

2.4. Bacterial adhesion on PDMS under dynamic condition

The adhesion behavior of *E. coli* to PDMS substrates was studied under laminar flow using a millifluidic device of the design developed by Zhao et al. [36]. The flow circuit is detailed in Figure S2. Characterization of the millifluidic devices was performed to ensure a laminar flow in the flow chamber (Figure S3). To quantify bacterial adhesion to PDMS substrates, *E. coli* suspensions were perfused through the flow chamber at 100 μL min^{-1} (wall shear rate of 2 s^{-1}). After 30 min and 2 h, numbers of adhering bacteria were quantified on three randomly selected areas of 0.0069 mm^2 per sample (duplicates of samples with a specific stiffness per experiment, three independent experiments conducted). Samples were distributed on two rows in an antiparallel direction on the slide (Figure S2).

2.5. Bacterial retention force on PDMS

Experiments were conducted to investigate whether cells adhering on different PDMS stiffness exhibit different mobility and retention forces. First, shear stress was increased from a wall shear rate of 2 to 40 s^{-1}. After the aforementioned 2 h of bacteria perfusion, adhering bacteria were subjected to washing with PBS at a flow rate of 2 mL min^{-1}. Images were captured at a rate of 1 image every 5 min and trajectories of 100 bacteria were tracked with the plugin Manual Tracking for ImageJ [37,38] for a period of 30 min under flow on each substrate. Afterwards, air was injected to empty the flow chamber completely which was then refilled with PBS at a rate of 2 mL min^{-1} in order to increase physical stress toward adhering bacteria. The percentage of strongly adhering bacteria was calculated by quantifying bacterial number before and after air injection.

2.6. Bacterial interaction with PDMS analyzed by SEM

The interaction between *E. coli* and PDMS surfaces was observed using scanning electron microscope (SEM, Hitachi, S-4800, USA). Bacteria were allowed to adhere on PDMS samples with different stiffness for 2 h in PBS before being washed and fixed with 4% paraformaldehyde (Sigma, 252549, 37% solution, The Netherlands) and 2.5% glutaraldehyde (Sigma Aldrich, G5882, 25% solution, USA) for 1 h at room temperature. Dehydration was performed by immersion in different concentrations of ethanol (50, 70, 80, 90 and 100%). Samples were stored under vacuum overnight and sputtered to obtain a 10 nm gold layer (Leica, EM ACE600, Switzerland) and finally analyzed by SEM.

2.7. Statistical analyses

Statistical analyses were performed by utilizing unpaired and two-tailed Student's *t*-test for comparison between two groups. Statistical significances were indicated by asterisks in the figures (*p < 0.01). Microsoft Excel was used for all statistical analyses. Standard deviations generated from samples replicates and independent experiments were detailed in individual figures and tables.

3. Results and discussion

3.1. Characterization of PDMS samples

The mechanical properties of PDMS can be varied from a "liquid-like" viscous gel to a "solid-like" elastomer by tuning the cross-linker content. Using oscillatory rheometry analysis, the stiffness values (Young's modulus) of PDMS substrates were

determined and varied from 565 ± 20 kPa, 186 ± 9 kPa to 21 ± 1 kPa, for the base:cross-linker agent ratios of 10:1, 20:1 and 40:1 (wt/wt), respectively (Table 1). Using dynamic mechanical analysis, higher stiffness values have been reported in the range of 2100 ± 100 kPa, 1000 ± 100 kPa and 100 ± 20 kPa for the silicon base:cross-linker ratios of 10:1, 20:1 and 40:1, respectively [18]. It is important to note that these testing methods use different testing principles and therefore may generate variation in absolute moduli values. Nevertheless, in both cases 40:1 PDMS substrates are 20 to 30 times softer than 10:1 PDMS. In the following experiments, three groups of substrates were used: stiff (10:1 base: cross-linker agent ratio), intermediate (20:1) and soft (40:1).

All prepared PDMS materials showed similar hydrophobicity with an average water contact angle of 111 ± 4°. Although the soft PDMS (119 ± 6°) appeared to be significantly more hydrophobic than the stiff PDMS (109 ± 4°) (Table 1), the difference is only 10°. Roughness analysis by AFM revealed that all prepared PDMS surfaces exhibit low average roughness values (below 3.1 nm) and that the surfaces do not exhibit topographical patterns (data not shown). These findings are in agreement with previously published results [18,39,40].

3.2. Properties of viscoelastic PDMS substrates

3.2.1. Characterization of PDMS viscoelasticity

To probe the viscoelastic properties of PDMS substrates, we analyzed the loss factor (tan δ), which is calculated as the quotient of the lost and stored deformation energy: G''/G'. It reveals the ratio of the viscous and the elastic proportion of viscoelastic deformation behavior. The higher value in loss factor, the more viscous (liquid-like) a material is. Fig. 1A shows the loss factor of the three groups of PDMS sample types. Soft PDMS had a loss factor of approximate 0.22, while the loss factor in the other two groups was 0.09 and 0.08, respectively. There is a clear trend that the increase of cross-linking content results in decreased loss factor. Consequently, the viscous proportion of viscoelastic deformation behavior in PDMS decreased.

Furthermore, the complex shear modulus (G) is determined by small-angle rheology measurements at a constant shear strain within the linear viscoelastic range as illustrated in Fig. 1B. A variation in complex modulus can be found. The G values increased about 2 orders of magnitude with the increase of cross-linking content (i.e. from the PDMS ratio 40:1 to the ratio 10:1).

To minimize the influence of uncross-linked free molecules, PDMS samples were extracted in ethanol for 24 h. The gel fraction was determined by comparing the relative mass before and after extraction. The results show that soft PDMS substrates have a gel fraction of ca. 90% (Fig. 1C), indicating that ca. 10% uncross-linked molecules were removed after extraction. By contrast, significantly higher levels in gel fraction were observed in the intermediate and stiff PDMS substrates with 95% and 98%, respectively. The difference in gel fraction suggests a variation in amount of cross-linking chains and dangling chains, which may result in variation in viscoelasticity.

3.2.2. Mesh size of PDMS network

The mesh size in PDMS networks is determined by fitting the complex moduli values into classical polymer physics models (see Materials and Methods). As shown in Table 2, the mesh size in the three PDMS sample types is in the range of 4–12 nm. The

Table 1
Physical characteristics of the prepared PDMS substrates.

PDMS ratio (base:cross-linker, wt/wt)	Stiffness (Young's modulus kPa) [a]	Water Contact angle (°) [b]	Average Roughness Ra (nm) [c]	Mean Square Roughness Rq (nm) [c]
10:1	565 ± 20	109 ± 4	1.3 ± 0.2	1.8 ± 0.2
20:1	186 ± 9	113 ± 4	1.8 ± 0.5	2.5 ± 0.5
40:1	21 ± 1	119 ± 6	3.1 ± 0.8	4.7 ± 1.3

[a] Three individual samples per PDMS type were analyzed for stiffness measurement.
[b] Water contact angles were measured on three different PDMS samples and three droplets per sample which gives a total of nine per PDMS type. Based on Student's *t*-test, significant differences ($p < 0.05$) between all PDMS types have been found for stiffness and water contact angle values.
[c] Roughness values were generated from at least two measurements for each sample (scanning area 25 μm × 25 μm) and two different samples were used for each PDMS type.

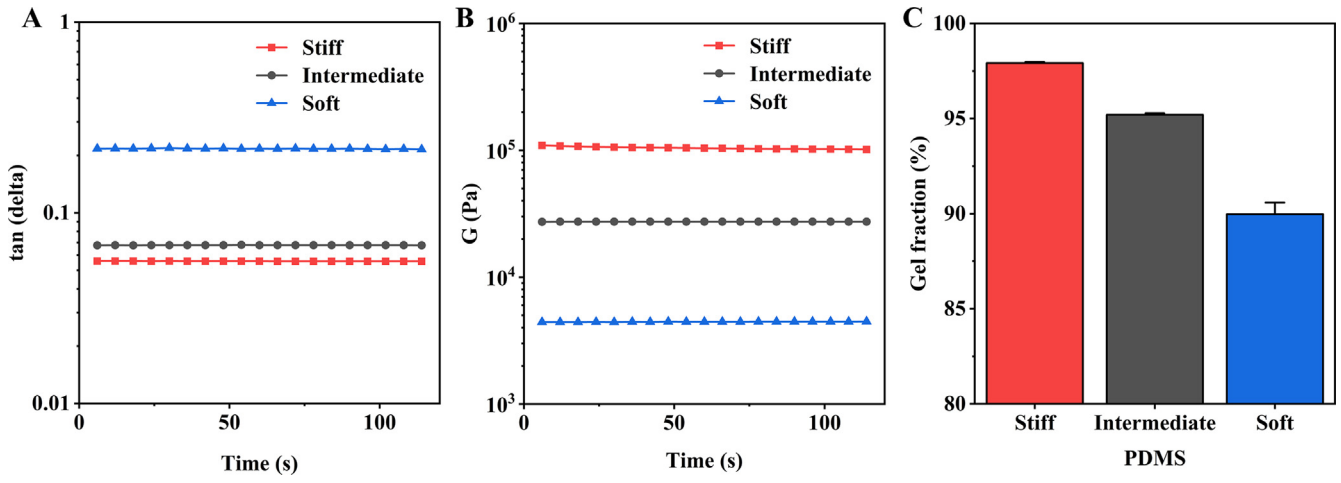

Fig. 1. (A) Influence of cross-linking degree on loss factor (tanδ) of PDMS. (B) Effects of cross-linking degree on complex modulus (G) of PDMS. (C) Influence of cross-linker content on gel fraction. Gel fraction was defined as the mass ratio (M1/M0 × 100%), where M0 and M1 refer to sample mass before and after washing. Error bars refer to the standard deviation over three measurements.

Table 2
Viscoelasticity characteristics for the different preparations of PDMS. N = 3.

PDMS ratio (base:cross-linker)	Elastic modulus (G'/kPa)	Loss modulus (G"/kPa)	Complex modulus (G/kPa)	Loss factor (tanδ)	Mesh size (nm)
10:1	102.9 ± 1.4	11.5 ± 0.2	104.0 ± 1.4	0.08 ± 0.01	4.2 ± 0.4
20:1	27.1 ± 0.9	3.7 ± 0.2	27.3 ± 0.9	0.09 ± 0.01	6.6 ± 0.8
40:1	4.1 ± 0.2	1.8 ± 0.1	4.5 ± 0.2	0.22 ± 0.01	12.1 ± 0.9

higher the cross-linking degree, the smaller the mesh size is. It is important to note that PDMS mesh size values are much smaller than the size of bacteria (about 1–2 μm) but are comparable to the scale of structural components of microorganisms such as flagella and pili, facilitating potentially bacterial appendages to be trapped on the surfaces with larger mesh size.

3.2.3. Deformation and stress relaxation of PDMS network

The viscoelastic properties of PDMS samples were further tested by *in situ* dynamic rheology measurements. Upon changing oscillatory shear angular frequency by two orders of magnitude, the amplitude of G' and G'' varied as shown for different samples in Fig. 2A– C. Soft PDMS samples exhibited frequency-dependent moduli, showing characteristics of viscoelastic materials. The amplitude of storage moduli (G') varies by more than 200% across the frequency range. The storage moduli of intermediate samples showed negligible frequency-dependence at low frequency but remarkable frequency-dependence at high frequency, indicating the increased elastic proportion during deformation. By contrast, stiff PDMS samples exhibited nearly frequency-independent storage moduli. The amplitude of storage moduli increased by <10%.

We next studied the stress relaxation properties of PDMS samples as a function of cross-linking degree. Stress relaxation describes the decreased ability of a material to return to its original shape after physical stress. Stress relaxation has been proved to impact fibroblast cells spreading by allowing mechanical remodeling of the viscoelastic hydrogel network [41]. Currently, the impact of substrate stress relaxation on bacterial adhesion is not known, thus deserves an investigation. Fig. 2D shows the stress relaxation profiles of three PDMS samples. Both the stiff and intermediate samples exhibited low extent of relaxation after 25 min with final stress values in the range of 0.90–0.95. However, the soft PDMS samples displayed a stress relaxation extent of more than 50% at the same timescale, which is much higher than for the other two groups. We hypothesize that the varied stress relaxation properties of PDMS substrates may lead to different bacterial adhesion behavior when bacterial cells approach surfaces with inherent viscosity and the capability for dissipating forces.

3.2.4. Stickiness of PDMS network

While dynamic rheology allowed us to quantify the viscoelastic and stress relaxation properties of PDMS, measuring the exact stickiness on PDMS surfaces provides more information about the interfacial forces that might control bacterial adhesion. A material is defined as tacky, or sticky, if an appreciable force is needed to separate a subject from its surface immediately after contact [42]. Interfacial rheology and AFM were selected as two complementary approaches to quantify the forces needed for separating an object

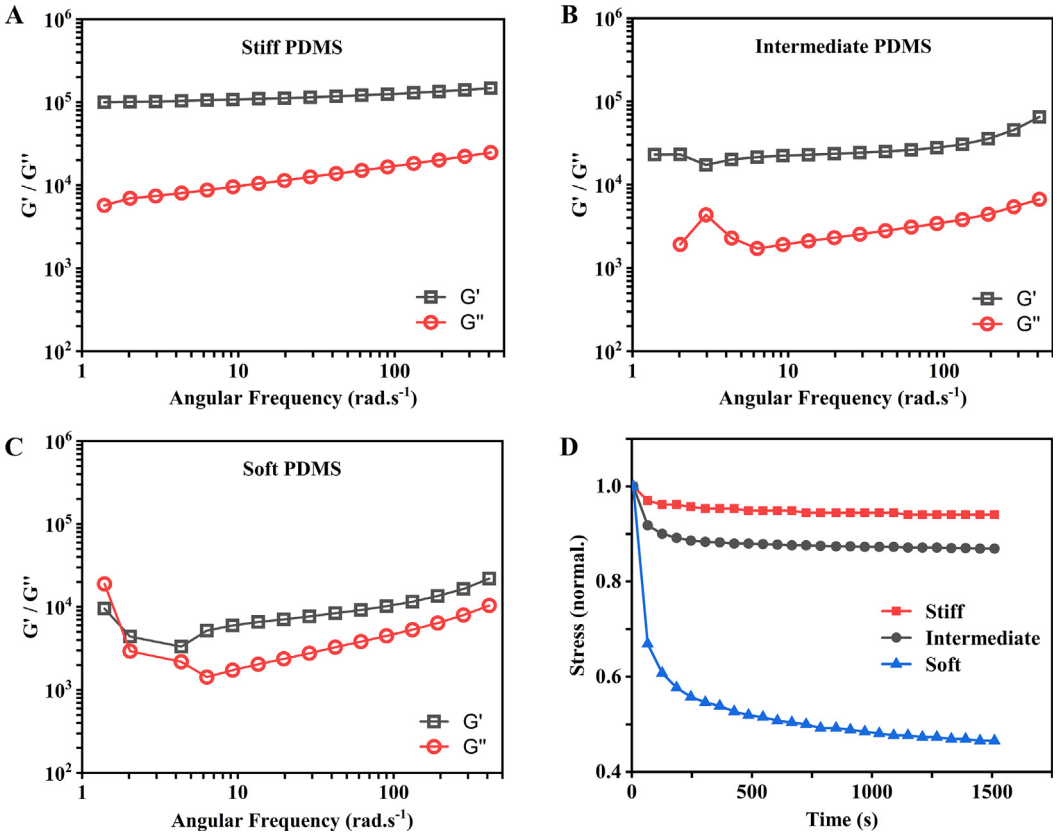

Fig. 2. Dynamic rheological analysis of viscoelastic properties of PDMS: (A) stiff, (B) intermediate and (C) soft. D) Stress relaxation properties of PDMS substrates measured by *in situ* dynamic rheology.

from PDMS surfaces. Fig. 3A shows the quantification of surface stickiness by *in situ* rheology in which the separation force is recorded as a function of distance when moving a metal plate upwards from PDMS surfaces. In a typical measurement, the upper metal plate is pressed onto the sample at constant normal force (F_N) of 10 N. Subsequently the plate is moved upwards to detach from the sample surface and the required F_N is recorded. The representative path-force curves of PDMS samples showed a remarkable difference. The curve for stiff PDMS only exhibits a small peak area while the intermediate and soft PDMS show a substantially increased area underneath the peak. The maximum adhesion force ($F_{N\ max}$) values were 32.1 ± 2.5 N, 17.2 ± 2.1 N, and 2.0 ± 0.3 N for the soft, intermediate and stiff PDMS, respectively. Through integration of the area of each force curves, the adhesion energy (E_{ad}) was determined as 23.0 ± 4.5 J/m^2, 3.1 ± 0.4 J/m^2 and 0.9 ± 0.2 J/m^2 for the soft, intermediate and stiff PDMS, respectively. The adhesion energy required to detach an object from the soft PDMS is nearly 30 times higher than that from the stiff PDMS.

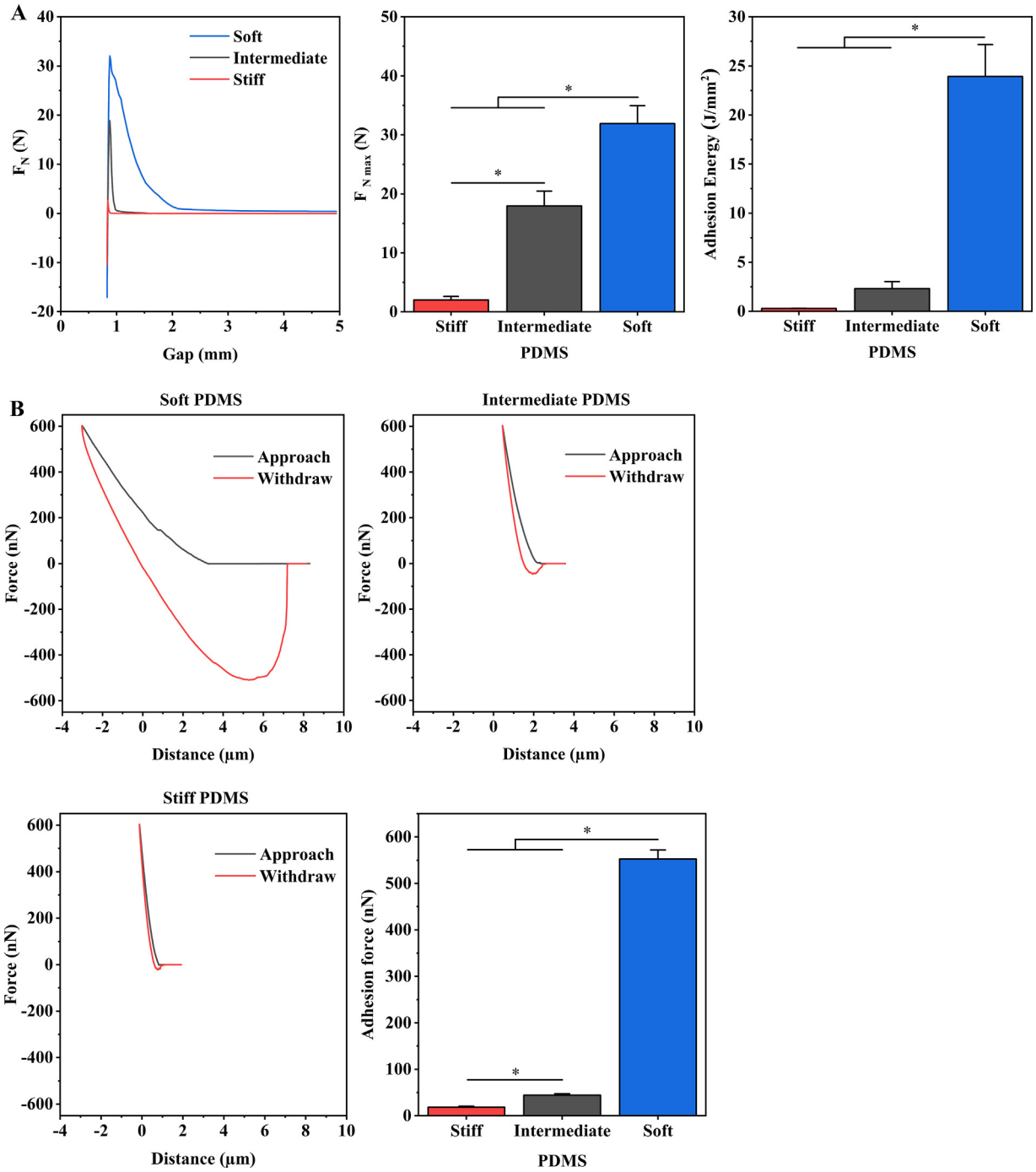

Fig. 3. Quantification of PDMS stickiness (A) by *in situ* interfacial rheology and (B) by AFM. (A) Rheology measurements revealed the interfacial stickiness by measuring the maximum normal force (F_N max) and the adhesion energy, i.e. the area underneath the path-force curve of different PDMS samples. The methods used are explained in the supplemental figures (Figure S1). Error bars represent the standard deviations of three repeated measurements. (B) AFM evaluated the stickiness of PDMS by measuring the adhesion force of the probe. Six hundred curves were measured on stiff (10:1 ratio), intermediate (20:1) and soft (40:1) PDMS substrates, respectively, and representative examples of each PDMS stiffness are shown. * denotes $p < 0.01$ significant difference between all sample types based on Student's *t*-test.

Fig. 3B shows example force curves obtained during force spectroscopy measurements with AFM. It displays the approaching to and withdrawal from PDMS substrates by the probe. Examples of adhesion force curves after contact with soft and stiff PDMS are shown. Following the same trend observed by rheology, stickiness increased with the decrease of cross-linking degree with adhesion forces of 18 ± 2, 44 ± 3 and 552 ± 19 nN for PDMS surfaces prepared with a base:cross-linker ratios of 10:1, 20:1 and 40:1, respectively. Therefore, the force required to separate an object from the soft PDMS is 30 times higher in comparison to the stiff PDMS. In summary, both rheology and AFM test proved that there are remarkable differences in surface stickiness between the PDMS samples. The rheology test measures the interfacial stickiness of PDMS on the mm scale while the AFM indentation measures the local stickiness on the nm scale. These results clearly emphasize that decreasing the cross-linking degree of PDMS network leads to a tremendous increase of stickiness. However, it is important to mention that both the metal plate and AFM tips suffer from remarkable difference from the cell membrane of bacteria. Therefore, quantification of the adhesive force between single bacteria and PDMS surface warrants further investigation.

3.3. Impact of viscoelastic substrate on bacterial behavior

3.3.1. Bacterial adhesion on PDMS under fluid flow

Previous work investigated bacterial adhesion to PDMS of different stiffness exclusively under static conditions. Assessment of adhesion under static conditions has various limitations such as difficulties in following the adhesion process *in situ*. Therefore, flow conditions were used to analyze bacterial adhesion to different PDMS surfaces, as presented and described in the Supporting Information (Figure S2 and S3). After 30 min of bacterial perfusion at a flow rate of 100 μL min^{-1}, the number of *E. coli* adhering to stiff PDMS was 50% lower compared to that on the soft PDMS (Fig. 4A). Similar observation has been reported previously that the initial deposition rate of *S. aureus* was promoted on soft PAAm (G' of 17 ± 5 Pa) in comparison to stiff PAAm (G' of 654 ± 58 Pa) under flow condition within 3 h [17]. However, after 2 h of perfusion with bacterial suspension, we observed that the adhesion profile on the soft and stiff PDMS surfaces was less distinctive (Fig. 4B). Qualitative analysis by time-lapse microscopy reveals that some bacteria adhering to stiff PDMS are moving over the surface by the flow, which is not observed on soft PDMS (Figure S4). These results suggest that the number of strongly adhered bacteria is overestimated on stiff PDMS. Therefore, bacterial retention force was further studied in order to differentiate the proportion of weakly and strongly adhering bacteria.

3.3.2. E. coli retention force on PDMS under fluid flow

To investigate whether PDMS viscoelasticity has an impact on bacterial retention force, adhered *E. coli* were subjected to different levels of shear stress. First, high shear stress at a flow rate of 2 mL min^{-1} was applied to adherent bacteria (Fig. 5A and B). Relevant movies are available in the supplementary Figure S4. Tracking distances travelled by each bacterium revealed that *E. coli* cells are moved further by the flow on the stiff PDMS than on the soft PDMS. Under the same flow shear stress, bacteria were detached more easily from their initial adhesion spot on stiff PDMS than on soft PDMS. On stiff PDMS 28% of the total adhered bacteria moved more than 2 μm from their initial position after 30 min compared to only 1% of the bacteria on the soft PDMS. The observed movements are aligned with the direction of the flow, which indicated that flow shear stress is likely responsible for the observed bacterial movement. These results suggest that *E. coli* adhere with a weaker adhesion force on stiff than on soft PDMS, and are thus more susceptible to flow shear stress.

Next, adhering bacteria were subject to an air injection followed by a liquid refill at high flow rate (Fig. 5C). The air/liquid interface results in high shear stress and caused bacterial detachment by applying hydrodynamic forces that counteract the bacterial adhesion force to the surface [43]. It has previously been demonstrated that air bubbles in the flow induce detachment of adhering bacteria resistant to constant fluid flow [44]. In our study, a passing air/liquid interface leads to a detachment of 64% of adhering bacteria from the stiff PDMS and 56% from the PDMS of intermediate stiffness. Interestingly, almost no bacteria (<1%) adhering to the soft and viscous PDMS were detached by the action of the air/liquid interface. This difference correlates with the variation in loss factor values (Table 2) of PDMS substrates: 0.08 (10:1), 0.09 (20:1) and 0.22 (40:1). These results clearly demonstrate that a slightly cross-linked PDMS promotes *E. coli* adhesion by considerably increasing bacterial retention force. Furthermore, bacteria exhibit similar retention force on the two highly crosslinked PDMS (20:1

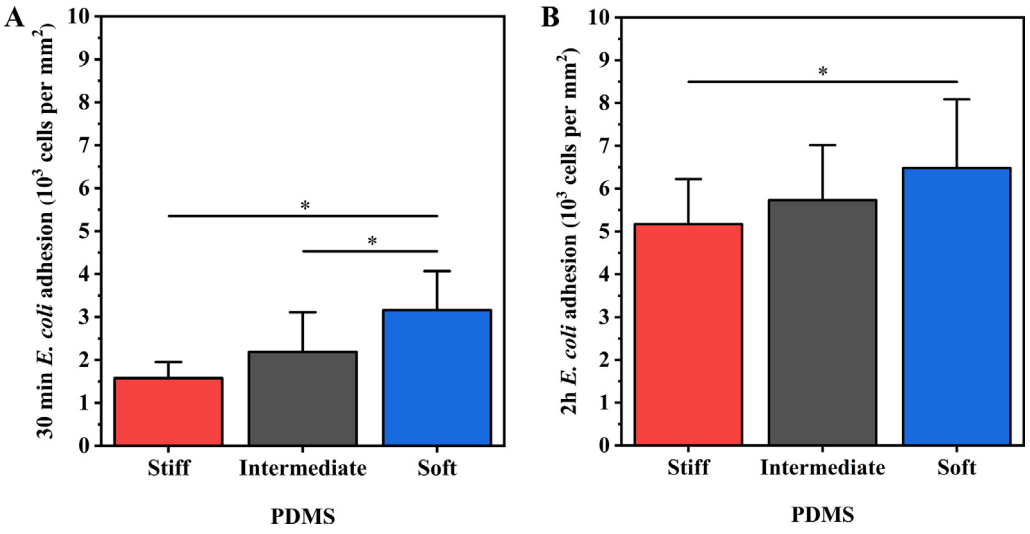

Fig. 4. The number of *E. coli* adhering to PDMS surfaces of different stiffness (A) after 30 min and (B) after 2 h of perfusion with bacterial suspension at 100 μL min^{-1}. Error bars represent the standard deviations of three independent experiments, two samples per experiments and three surface areas taken per sample for bacteria counting. * denotes $p < 0.01$ significant difference between samples based on Student's *t*-test.

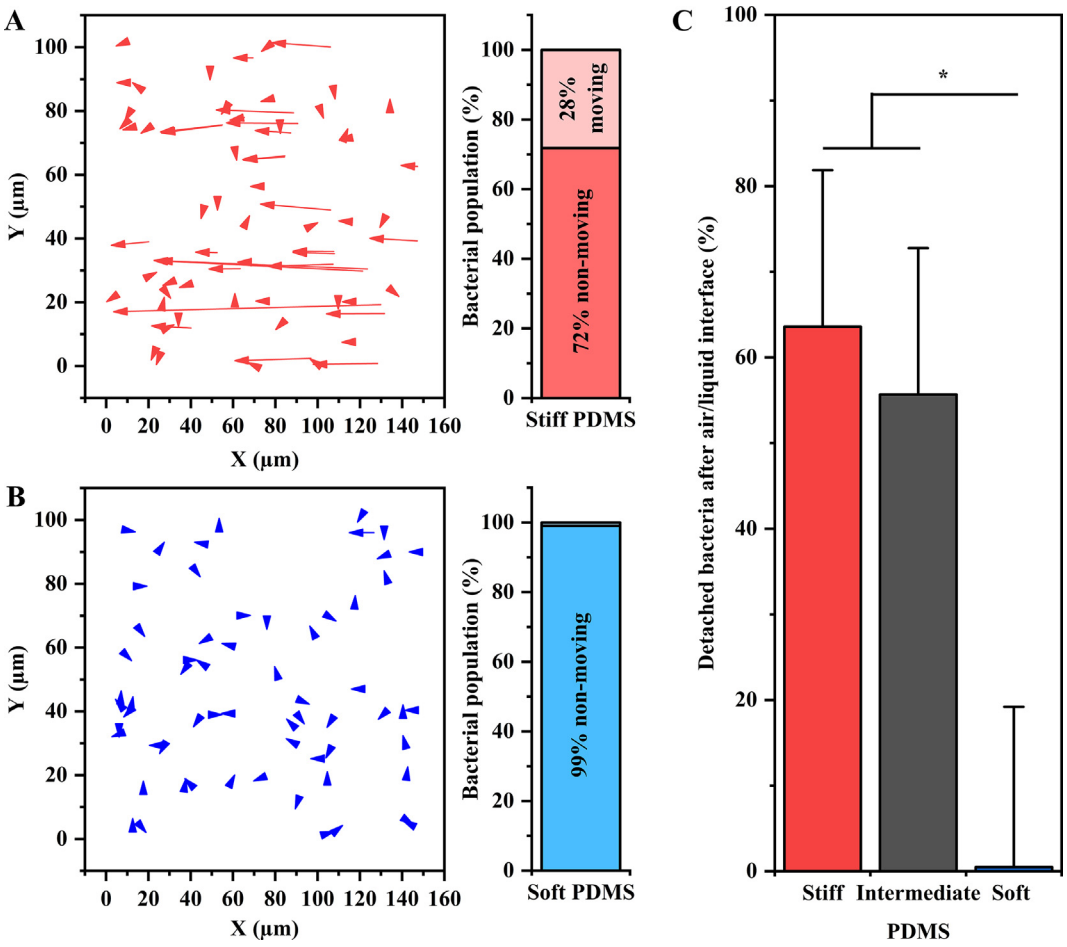

Fig. 5. Bacterial retention force assessed by the ability of adhering bacteria to remain adhering (A&B) after flow shear stress and (C) passing air/liquid interface. (A&B) Bacterial movement on A) stiff (10:1) and B) soft (40:1) PDMS substrates under flow shear stress. Bacterial tracking is processed during 30 min under 2 mL min^{-1} flow (right to left) and the first and last points of each bacterial trajectory are shown (117 bacterial trajectories followed on stiff PDMS, 104 on soft PDMS). Percentage of non-moving i.e. strongly adhering bacteria (moving <2 µm) and moving i.e. weakly adhering bacteria (moving more than 2 µm) are calculated. (C) Bacterial retention studied in a flow chamber by subjecting the adhering bacteria to an air/liquid interface. Air was injected to empty the flow chamber completely, which was refilled with PBS at a rate of 2 mL min^{-1} in order to increase physical stress toward adhering bacteria. Percentage of strongly adhered bacteria was calculated by counting bacteria before and after air injection. Error bars represent the standard deviations of three independent experiments, two samples per experiments and three surface areas taken per sample for imaging counting. * denotes $p < 0.01$ significant difference between samples based on Student's *t*-test.

and 10:1) suggesting a predominant role of viscosity compared to stiffness within this context.

3.4. Underlying interaction between viscoelastic materials and E. coli

By conducting bacterial adhesion assays under flow conditions, we demonstrated that *E. coli* cells adhere preferentially on slightly than highly cross-linked PDMS, and exhibit a stronger retention allowing them to remain adhered even after the high physical stress induced by a passing air/liquid interface. A thorough characterization of PDMS viscoelasticity revealed that decreasing PDMS crosslinking degree leads to not only a decrease in substrate stiffness (565 kPa, 186 kPa and 21 kPa in Young's modulus for a base: crosslinker ratios of 10:1, 20:1 and 40:1, respectively), but also a variation in viscosity from predominantly elastic for 10:1 and 20:1 (loss factor, 0.08 and 0.09) to viscous for 40:1 (loss factor 0.22) (Table 1 and 2, Figs. 1 and 2). Modulating the cross-linker content did not induce a significant difference in surface roughness, which showed low and similar roughness with Ra values in a range of 1.3–3.1 nm, regardless of the cross-linker concentration (Table 1). Also, it is unlikely that the slight difference in hydrophobic will cause the difference in the number of *E. coli* adhering to the different PDMS samples. Previously it has been report that moderate hydrophobicity with water contact angles of about 90° allowed the highest level of *E. coli* adhesion, and further increase of the water contact angle to 120° reduced *E. coli* adhesion of about 0.5% (area fracture of adhered by *E. coli*) [45]. Our results on surface roughness and hydrophobicity are consistent with the conclusions of previous reports that additionally proved that the fundamental chemical composition and charge of the upper layer of PDMS are similar regardless of the cross-linker concentration [40]. However, interfacial rheology and AFM analysis revealed that the adhesion force and separation energy on the soft and viscous 40:1 PDMS substrate increased drastically compared to those on 10:1 and 20:1 PDMS (Fig. 3). Two hypotheses are proposed to illustrate the potential interaction of viscoelastic substrates with bacteria (Fig. 6).

(1) Low cross-linking degree of PDMS substrates leads to high elasticity (Table 1 and 2, Figs. 1 and 2), which could allow material deformation by an interacting object and thus providing a higher contact area [46]. Our dynamic rheology data has proven that soft PDMS was more deformable than stiff PDMS (Fig. 2) and thus supports the hypothesis that bacteria could deform soft PDMS more easily than stiff PDMS. As mentioned in another study [47], such deformation can lead to a superficial niche into the upper layer of the polymer network, increasing contact surface area between bacterium and substrate, and contributing to high bacterial retention force. Using confocal microscopy, Pham et al. showed that a

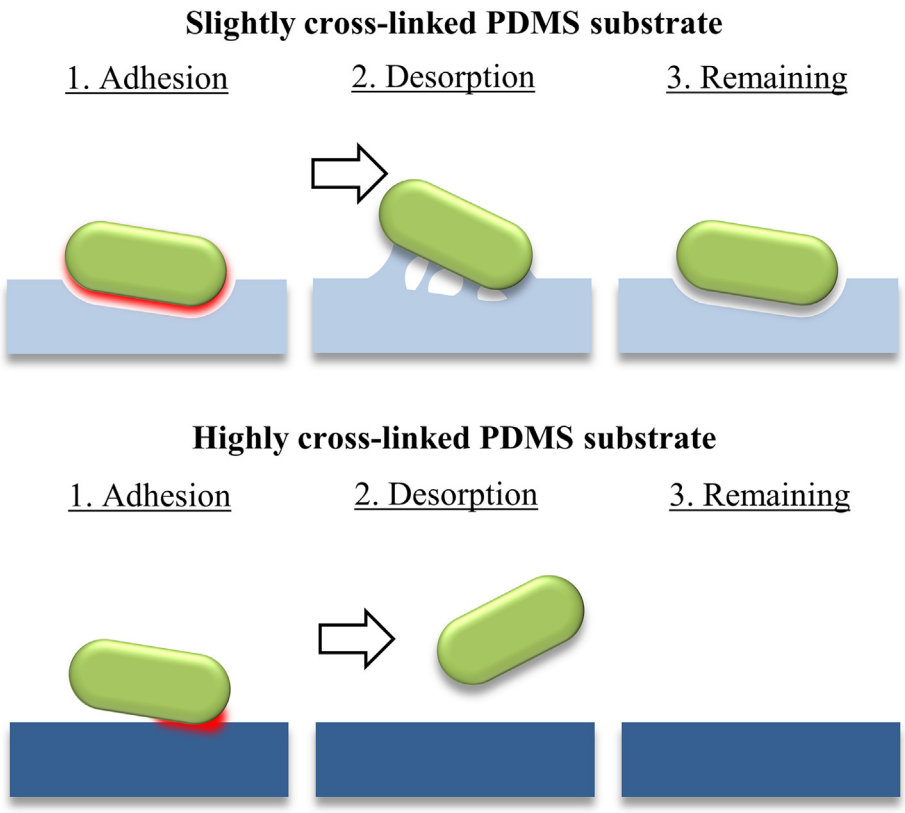

Fig. 6. Hypothetical model of the underlying interaction between *E. coli* and different viscoelasticity properties of PDMS substrates (1) before, (2) during and (3) after shear stress. 1. During the adhesion stage, bacteria adhere weakly on the stiff PDMS (base:cross-linker ratio of 10:1) while bacteria deform the soft and viscous substrate (ratio of 40:1) increasing their contact surface area with the material (representing in red) and their retention force. Indentation of slightly cross-linked PDMS is also expected due to uncross-linked polymer chains. 2. During the desorption stage, a physical shear stress is applied and removes adhering bacteria on the stiff PDMS while the stickiness of the slightly cross-linked PDMS provides an additional force of retention to bacteria through interfacial crack propagation. 3. After shear stress, more bacteria remain adhered on the slightly cross-linked PDMS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spherical probe (radius ≈ 4–6.5 μm) is able to readily deform soft PDMS substrates (prepared with a base:cross-linker ratio of 40:1), but to a less extent toward 20:1 PDMS [48]. It is also reported that surface tension leads to the indentation of the soft matter around the probe upon capillary forces [49,50]. However, bacteria have never been observed deforming PDMS material. As a proof of concept, we used scanning electronic microscopy (SEM) to observe *E. coli* cells on tilted PDMS samples (Figure S5). Tilting the surfaces by 30° allowed us to better observe potential deformation. In comparison to bacteria adhered on stiff and intermediate PDMS (Figure S5 A&B), bacteria appear to sink into the soft PDMS deforming the surrounding polymer (Figure S5 C&D). Deformation of stiff PDMS by bacteria is obviously to a less extent than soft PDMS and thus the contact surface area between a bacterium and a soft substrate is larger. However, it has to be noted that the samples prepared for SEM imaging were treated with a through dehydration process, which might cause artifacts. Hence, using alternative techniques such as cryo-SEM or environmental SEM should be applied in future work.

(2) Low cross-linking degree of PDMS substrates leads to high viscosity and high interfacial stickiness (Fig. 3), further enhancing bacterial retention. The force required to separate a metal object or an AFM probe from the substrate is 20 to 30 times higher upon contact with soft compared to stiff PDMS, similar to what has been reported previously [51]. While both rheology and AFM tests clearly showed that the adhesives forces on the PDMS substrates exhibit remarkable difference, it is important to note that these force values probably differ from the exact forces exerted by bacterial cells when adhering to a surface. Adhesion measurements by AFM can also be subject to bias due to viscoelastic deformation [52]. These limitations should be addressed in future work with advanced force-testing methods in the presence of living bacteria [53]. Such adhesive performance is linked to the viscosity of soft PDMS and high dissipation energy during the debonding process [42]. In other words, PDMS gels follow the Derjaguin-Muller-Toporov (DMT) model used for elastic and adhesive solids [54] and the adhesive properties are explained by the presence of free mobile polymer chains in the slightly cross-linked PDMS network [48,55]. The presence of these dangling chains in PDMS network is investigated and proves to be in a much higher concentration in the soft than the stiff PDMS network (Fig. 1C). In a macroscopic point of view, the debonding of an object from a sticky substrate can lead to substrate deformation such as fracture, cavities and fibril formation which subsequently retain the moving object, and therefore debonding needs more energy than from the non-sticky counterpart [46,56]. Upon retraction of a spherical probe, Pham et al. pictured the stretching of PDMS network at micrometric level [48]. Thus, viscous PDMS can retain adherent bacteria, even after passing an air/liquid interface, by providing an additional and physicochemical-related force of retention.

In this work, investigating the physical behavior of viscoelastic substrates allowed a better understanding of the impact of crosslinking degree in PDMS on *E. coli* adhesion. Other factors such as bacterial mechanosensing might be further involved in the interaction between bacteria and PDMS. For instance, bacterial membrane deformation is known to be one of the triggers used by bacteria to sense a surface [57,58]. Even though some soft hydrogels have been reported to enable less bacterial adhesion

than stiff ones, these hydrogels, unlike the soft PDMS used in this work, are usually hydrophilic. Future investigations are necessary to understand if viscosity of hydrogels may also play a significant role. In this study, we were able to study the trade-off between substrate viscosity and elasticity on bacterial adhesion using PDMS, a substrate whose mechanical properties can be tuned from predominantly viscous to elastic by altering its cross-linking content. However, further investigation is required to decouple the separate contribution of viscosity and stiffness on bacterial adhesion. Considering the omnipresence of viscoelastic substrates in the environment and especially in the human body, material viscosity should be taken into consideration while studying bacterial and biofilm behavior upon substrates.

4. Conclusion

In summary, fabrication of PDMS substrates of different mechanical properties and their detailed characterization allow us to study the impact of substrate viscosity on *E. coli* adhesion under dynamic flow conditions. Observation of bacterial adhesion under flow reveals that *E. coli* adhere approximately 50% more in number on slightly than on highly cross-linked PDMS substrates after 30 min. Furthermore, exposing adhering bacteria to an air/liquid interface shows that almost all bacteria remain attached to the slightly cross-linked PDMS, while more than 60% were detached on the highly cross-linked PDMS. Thus, we demonstrated that soft and viscous PDMS substrates confer to bacteria not only an improved adhesion, similar to what has been reported previously [18], but also additionally a higher resistance to shear stress in comparison to stiff PDMS. Moreover, a thorough characterization of PDMS reveals that decreasing the elastic modulus (stiffness) drastically increased the loss factor (viscous portion). Therefore, we suggest that substrate viscosity should be also considered to understand the mechanisms of bacterial adhesion. Soft PDMS could allow material deformation by the bacterium leading to a larger surface contact area compared to the stiff PDMS. This hypothesis is supported by dynamic rheology showing high deformability of soft compared to stiff PDMS and by SEM pictures of tilted PDMS samples. PDMS viscosity is closely related to un-crosslinked free polymer chains, which could provide an additional physicochemical force for retention. Thus, a thorough characterization of PDMS viscosity and real-time observation of adhering bacteria under flow provided a better understanding of the previously observed bacterial adhesion. [18,21] While our study proves that substrate viscosity can greatly influence bacterial adhesion and retention forces, underlying mechanisms need to be clarified by decoupling the contribution of elasticity and viscosity using more advanced biomaterials that allow independent control of substrate viscosity without changing elasticity. While the impact of hydrogel stiffness on bacterial adhesion is outside the scope of this work, considering the viscosity of hydrogels could also be helpful to understand the observed trend [13–15,17]. Furthermore, bacterial metabolic activity and antibiotic resistance seem to be also affected by the stiffness of PDMS [18] and, thus, further characterization is needed to understand the impact of substrate viscosity. In conclusion, considering viscosity in the context of viscoelastic materials has been proved to be of the upmost importance for bacterial adhesion and should be investigated to improve our understanding of bacterial interaction with their complex mechanical environments.

Author Contributions

The manuscript was designed and prepared through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary material

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