Microrheology of Mucin: Tracking Particles and Helicobacter Pylori Bacteria

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Abstract
The gastric ulcer and cancer causing bacteria, *Helicobacter Pylori* have uniquely adapted to swim across the viscoelastic mucus gel that lines the stomach epithelial surface and colonize in the harsh acidic environment of the stomach. In this paper we first briefly review results of bacteria tracking and oscillatory shear rheology studies to suggest how the bacteria get across the viscoelastic mucus gel by using a chemical mechanism to raise the pH from acidic to neutral which also triggers a gel to sol transition of mucin. We then present new microrheology studies to show that the bacterium influences the Brownian motion of spherical tracer particles in culture broth solution and in solutions of gastric mucin. The elastic and viscous moduli obtained by tracking particles in the mucin solutions are found to decrease in the presence of bacteria. We also examined the Brownian motion of the bacteria themselves and find that motile bacteria display super-diffusive anomalous Brownian motion while the immotile bacteria exhibit regular diffusive Brownian motion.
Keywords: Microrheology, particle tracking, bacteria tracking, mucin, *H. pylori*
Kulcsszavak: Mikroreológia, részecskemozgás, bacterium mozgás, mucin, *H. pylori*

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1. Introduction

Mucus, the material that lines the epithelial surfaces of all organs exposed to the outside, such as the respiratory tract, gastrointestinal tract, oral, ocular, and cervical surfaces etc. provides a fascinating example of the importance of viscoelasticity to lubricate, hydrate and protect the underlying organs from contamination with undesirable materials. In this paper we focus on how the rheological properties of gastric mucus are influenced by the presence of the bacterium *Helicobacter Pylori* (*H. pylori*). Mucus is about 95% water and owes its viscoelastic, lubricant and water-retention properties to a highly negatively charged poly-electrolytic glycoprotein called mucin (about 3% in concentration, the remaining 2% being fats, lipids and other small proteins). For a review of the structure and properties of mucin and mucus see *Bansil* et al. 2013 [1] and *Bansil* and *Turner* 2006 [2]. Understanding the rheology of mucin is especially relevant to its protective and lubricating function, and many rheology studies have been reported on both commercially available gastric mucin [3] and purified porcine gastric mucin, PGM [4, 5] which is analogous to human mucin, MUC5AC. Previous work from our group and others has established that purified gastric mucin and mucus forms a viscoelastic gel under acidic conditions at pH below 4 [4, 5, 6, 7, 8]. Using oscillatory shear rheology *Celli* et al. [5] showed that PGM undergoes a sol-gel transition at pH 4 forming a viscoelastic gel below pH 4 with frequency dependent elastic and viscous moduli, G' and G" respectively. *Celli* et al. [5] also showed that mucin is a shear thinning fluid with a yield stress of such a magnitude that a layer of mucus 1 mm or thicker would yield under its own weight.

The acidic pH induced gelation coupled with the finding that acid secreted by the glands in the stomach is transported via the mechanism of viscous fingering [9] as opposed to diffusion provides a plausible explanation for why the acidic gastric juice does not digest the stomach itself. The jet-like finger of secreted acid punctures through the mucus layer and causes the mucus surrounding the finger to gel forming a viscoelastic tube that confines the acid [10]. Furthermore, mucin is a negatively charged polyelectrolyte and thus swells tremendously in the hydrated state due to electrostatic repulsion. The elasticity of the gel network opposes this stretching, and thus the gel-network exerts an electro-osmotic pressure on the positively charged H+ preventing them from diffusing out. This interplay of a hydrodynamic instability (viscous fingering) with gelation and electrostatics is exquisitely tuned to prevent the stomach from being digested by its own secretion.

The gelation of mucus prevents large macromolecules and toxic particles such as bacteria from penetrating the mucus barrier of the stomach. However, the bacterium *H. pylori* has evolved to survive in the harsh acidic environment of the stomach and somehow manages to swim across the viscoelastic mucus gel and colonize on the epithelial surface [11]. Infection of the stomach by *H. pylori* is directly associated with gastritis and gastric ulcers, and can lead to gastric cancer [12, 13]. How the bacterium swims across the gel-like mucus is not well understood. The original hypothesis that the helical shape of the bacterium enables it to corkscrew its way through the mucus gel [11] was questioned in another study from our group [14]. *Celli* et al. [14] showed that *H. pylori* bacteria were immobile in mucin gels buffered at pH 2 or 4, but swam freely in mucin solutions at pH above 4. From bulk rheology measurements they showed that the gels at low pH infected with *H. pylori* transformed to

solutions with a higher pH (about 6), provided urea was present in the bacterial broth. From these observations we suggested that *H. pylori* uses the elevation of pH by urease receptor mediated hydrolysis of urea to produce ammonia and neutralize acid *both* for survival in the harsh acidic environment of the stomach as well as trigger a pH dependent gel to sol transition of the mucin gel allowing the bacteria to move across the mucus layer.

Due to the heterogeneous structure of mucin hydrogels and the fact that *H. pylori* may affect the gel in their immediate vicinity either biochemically or by a local gel-sol transition it is important to examine the length scale dependence of the elastic and viscous moduli. These local microrheological properties can be probed by tracking the Brownian motion of micron sized polystyrene latex particles in the medium of interest. From the particles trajectories the mean square displacement, $<\Delta r^2>$, can be calculated from which the complex elastic modulus G(ω) can be obtained using a Fourier-Laplace transform [15, 16]. Using this method, *Celli* [17] found that in a mucin solution at pH 6 the elastic or storage and viscous or loss moduli, G'(ω) and G"(ω) respectively, have similar magnitudes to those obtained in bulk rheology, as expected for particles moving in a viscous solution. However in the mucin gel at pH 2 the moduli obtained by microrheology were significantly lower than those obtained by bulk rheology; see *Fig. 4* of ref. [1]. The decrease of local moduli in the gel implies that the particles in the gel moved in an inhomogeneous micro-environment consisting of water filled pores in the gel, i.e. in a less viscous environment than the bulk gel. Heterogeneities have also been observed in particle tracking experiments in mucin gels by *Lieleg* et al. [18].

The differences reported between bulk and micro-rheology of mucin gels, and the strong influence of bacteria on bulk rheology raises intriguing questions regarding the Brownian motion of particles in the presence of *H. pylori* bacteria in the medium. The first question is whether the rheological parameters determined by tracking the Brownian motion of particles is influenced by the presence of motile bacteria. Moreover, the bacteria can themselves be used as probe particles. In this case, is the Brownian motion different for the motile bacteria which exhibit active swimming as compared to dead or otherwise immobile bacteria which only exhibit passive Brownian motion? In this paper we address both these questions by tracking particles in the presence of *H. pylori* in porcine gastric mucin solutions, and by tracking the Brownian motion of active and passive bacteria in PGM.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

We used the wild-type *H. pylori* strain: LSH100, a derivative of the sequenced human clinical isolate G27 [19, 20]. Bacteria were streaked and grown on horse blood plates after which they were moved to liquid media containing 90% (v/v) Brucella broth (BD Biosciences) and 10% fetal bovine serum (GIBCO) (BB10) in the absence of antimicrobials as previously described [21]. Cells were maintained at 37 °C under microaerobic conditions in a tri-gas incubator equilibrated to 10% CO_2 and 10% O_2. Plates were incubated 24-72 hours and liquid cultures were incubated for 12-16 hours under constant agitation at 200 rpm.

2.2 Preparation of purified PGM

PGM was isolated from mucosal scrapings of pig stomach epithelium and purified by Sepharose CL-2B column chromatography followed by density gradient ultracentrifugation as described in [14]. Lyophilized PGM powder was allowed to reach room temperature before opening tubes to avoid condensation. The powder was weighed and appropriate amount of PGM was dissolved in sterile H_2O to prepare a 15 mg/mL or 30 mg/mL solution. PGM solution was allowed to hydrate and equilibrate for 48 hours at 4°C before use.

2.3 Preparation of bacteria and latex particle solutions

Bacteria were grown in liquid culture broth to an $O.D._{(600)}$ of 0.5 and 10 μL of culture was added to 80 μL of PGM solution. For samples including only bacteria 10 μL of pH 6 buffer (0.1 M phosphate-succinate) was added to produce a 10% bacteria mixture by volume with a final PGM concentration of 15 mg mL^{-1} or 30 mg mL^{-1}. For samples including bacteria and fluorescent beads, 10 uL of fluorescent polystyrene latex beads (1.001 +/- 0.01 μm diameter) (Polysciences Inc.) diluted in pH 6 buffer (0.1 M phosphate-succinate) were added to produce a 10% bacteria mixture by volume with a final bead concentration of 0.05% beads by volume and final PGM concentration of 15 mg/mL or 30 mg/mL. Bacteria were incubated for 45 min in their respective PGM or PGM+bead solutions at 37 °C under microaerobic conditions prior to imaging. After the incubation period, each cell suspension was mixed by gentle pipetting and 10 μL was applied to standard glass microscope slides with secure imaging spacers (9 mm in diameter × 0.12 mm depth, Secure-Seal, Sigma-Aldrich), and secured with a coverslip.

2.4 Phase contrast and fluorescent microscopy

Samples were immediately imaged using an Olympus IX70 inverted microscope (40X Plan N, 0.65 NA Phase lens). Fluorescent beads were excited using an Olympus BH2 Mercury arc source while samples of only bacteria were imaged using phase contrast with light from a halogen bulb. Focus was set to the center and middle Z-positions of the sample in order to minimize edge effects. Videos of beads were captured using a QImagingRolera CMOS camera (10 millisecond exposure at 30 frames per sec, 0.09 μm/pixel). Videos of samples containing only bacteria without particles were taken using an Andor Zylas CMOS (1 msec exposure at 200 frames per sec). Videos were digitally recorded onto a lab workstation using micromanager software (MDS Analytical Technologies).

2.5 Particle-tracking microrheology

Videos were analyzed in MATLAB v7.12.0 using a particle-tracking routine that finds the center of intensity of each bead or bacterium using a polynomial Gaussian fit [22]. Videos and trajectories were inspected to remove any superfluous tracked objects. If drift was present trajectories were de-drifted using a custom MATLAB routine. Using the trajectories data the mean square displacement $<\Delta r^2>$(MSD), storage (G'), and loss moduli (G") were calculated using previously described microrheology routines [15, 16, 23].

3. Results and Discussion

3.1 Particle tracking in the presence and absence of bacteria

We first discuss the tracking of polystyrene latex particles in bacterial culture broth (BB10). Particle tracking data for latex beads in broth and in PGM *without added bacteria* has been reported in ref. [21]. In this work we report the results of two sets of experiments in BB10 with 1 μm particles in the presence of *H. pylori* at optical densities of 0.05, 0.25, and 0.45. *Fig. 1.A* shows that at the lowest concentration of bacteria, OD = 0.05, we observe Brownian diffusion with the MSD being linear in time for particles with and without bacteria present.

However the diffusion constant is slightly larger for particles diffusing in the presence of bacteria, indicating that the broth viscosity is slightly reduced in the presence of bacteria (motile and non-motile). *Fig. 1.B* shows that as bacterial concentration is increased to OD of 0.25 and 0.45 there is a slight decrease in MSD for long lag times. In the literature it has been observed that particles show super-diffusive behavior at small lag times (t < 0.1 sec) in the presence of bacteria [24]. This is followed by diffusive behavior for long lag times (t > 1 sec). Our experiments agree with the long lag time observations but cannot provide any information on the small lag time behavior because of the large frame rate used (30 frames per second).

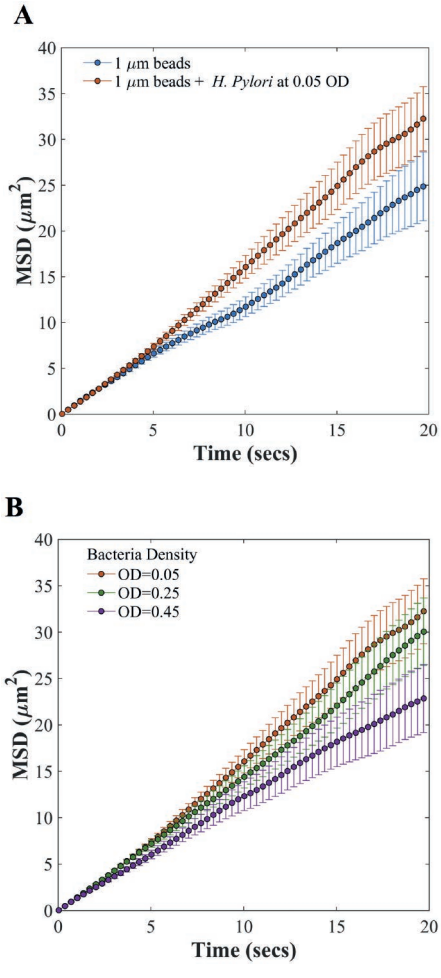

Fig. 1. Average mean square displacement of 1 μm beads in bb10 solution without H. Pylori and with H. Pylori at various concentrations
1. ábra 1 μm méretű részecskék átlagos négyzetes elmozdulása bb10 oldatban, H. Pylori nélkül és különböző H. Pylori koncentrációk mellett

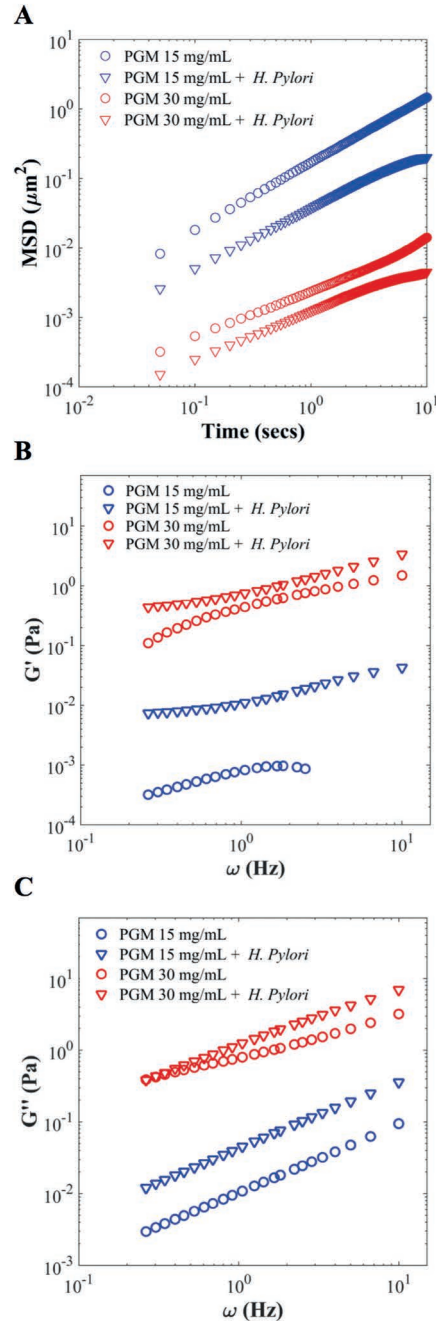

Fig. 2. Average mean squared displacement, G', and G" for 1 μm beads in PGM 15 mg/mL and 30 mg/mL with and without H. Pylori present
2. ábra 1 μm méretű részecskék átlagos négyzetes elmozdulása, G', és G" értéke PGM 15 mg/mL és 30 mg/mL oldatban H. Pylori nélkül és H. Pylori jelenlétében

To examine the influence of bacteria on micro-rheological properties of mucin particle tracking experiments with latex particles were done with and without the bacteria LSH100 added at an OD of 0.05 in PGM 15 mg/mL and PGM 30 mg/mL. *Fig. 2.A* shows log-log plots of the ensemble averaged MSD of the latex particles versus time in PGM at 15 mg/mL and 30 mg/mL both with and without *H. pylori* present. The data without bacteria are averaged from the tracks presented in ref. [21]. As expected the MSD is decreased in the more viscous PGM at 30 mg/ml. At both concentrations we observe that the MSD is decreased due to the presence of bacteria, and moreover the MSD plot shows sub-diffusive behavior at large times (***MSD***

~ t^{α}, α < 1). The MSD was analyzed to get the complex modulus G (ω). *Fig. 2.B* shows the frequency dependence of the storage modulus G' (ω) in PGM 15 mg/mL and 30 mg/mL solutions while *Fig. 2.C* shows the loss modulus, G" (ω). Comparing PGM at the two concentrations without bacteria, we note that at both concentrations G'< G" indicating that the mucin at pH 6 is solution at both concentrations, in agreement with the results of *Celli* et al. [5] and *Georgiades* et al. [25]. As expected both moduli are larger in the more concentrated solution. From the ratio tan (δ) = G'/G" we note that PGM at 30 mg/mL is more viscoelastic than at 15 mg/mL; in fact the storage modulus for PGM 15 mg/mL drops to zero beyond ω ≈ 3 Hz, implying that beyond this frequency PGM at 15 mg/mL is a purely viscous solution. In the presence of bacteria at both concentrations the moduli G' and G" increase, and the ratio also increases, i.e. the particles encounter greater resistance in the presence of bacteria consistent with sub-diffusive behavior. PGM at 15 mg/mL now displays a non-zero G' beyond ω ≈ 3 Hz, i.e. the bacteria are enhancing theviscoelastic response to higher frequencies.

3.2 Brownian motion of active versus passive bacteria

The final results we present explore the use of bacteria as tracer particles to probe microrheology. For these experiments we tracked *H. pylori* in a solution of culture broth (BB10) at a bacterial density of 0.05 OD. These experiments were done using a fast camera at 200 fps and magnification of 40×. Within the bacteria population some bacteria were swimming (referred to as motile) while other are non-motile. *Fig. 3.A* shows the MSD of each bacterium tracked and *Fig. 3.B* shows a histogram obtained by fitting the each MSD to a power law in time with exponent α, ***MSD*** ~ t^{α}. Motile bacteria in the population exhibit super-diffusive motion with α > 1while non-motile bacteria exhibited simple Brownian motion α = 1. To segment motile and non-motile populations we defined any bacteria with α > 1.2 to be actively swimming while bacteria with α < 1.2 were taken to exhibit only passive Brownian motion. Most of the population (83%) was found to be non-motile, i.e. exhibiting regular Brownian diffusion and can be treated as passive tracer particles.

Fig. 4 shows the mean MSD of all the non-motile, diffusive bacteria compared with that of 1 μm fluorescent latex particles in the initial time domain (from *Fig. 1*). While both particles and non-motile bacteria exhibit Brownian motion the diffusion constant of bacteria was found to be approximately half that of a 1 μm spherical particle. This suggest an effective hydrodynamic radius of ~ 2 μm on average, likely due to their different size and shape (bacterium is an ellipsoid with cell length ≈ 3 μm, cell width ≈ 0.5 μm).

From the values of G" at 1 Hz we can obtain the effective viscosity of the medium. We find from the data for 1 μm latex particles in BB10 (G' = 0, G" = 0.012 Pa) which gives an estimated viscosity of 1.2 cP at 1 Hz, close to that of water. From 1 μm particles in PGM 15 mg/mL (G' = 0.025 Pa, G"=0.063 Pa) we get the viscosity of PGM at 15 mg/mL as 6.3 cP, in agreement with previous estimates, and using particles in PGM at 30 mg/mL (G' = 0.44 Pa, G"=0.80 Pa) we get the viscosity of PGM at 30 mg/mL as 80 cP. Comparing the data for 1 μm beads in the presence and absence of motile bacteria we conclude that the effective viscosity of PGM 15 mg/mL decreases from 6.3 cP to 4.5 cP in the presence of bacteria.

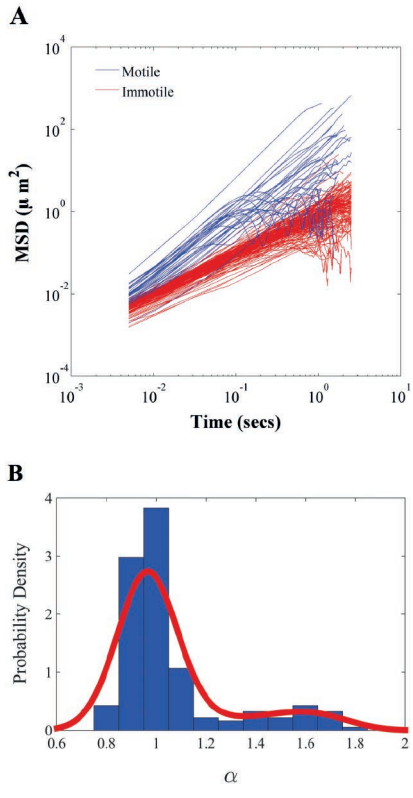

Fig. 3 Mean squared displacement and histogram of power law exponent of bacteria tracked in bb10 solution
3. ábra Átlagos négyzetes elmozdulás és hatványtörvény kitevőjének hisztogramja egy baktérium mozgásából bb10 oldatban

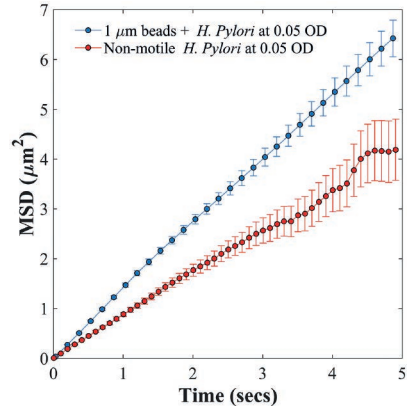

Fig. 4. Comparison of the mean squared displacement when tracking 1 μm beads vs. tracking non-motile H. Pylori cells
4. ábra 1 μm méretű részecskék és nem mozgékony H. Pylori baktériumok átlagos négyzetes elmozdulásának összehasonlítása

4. Conclusions

In summary, our studies show that the presence of the *H. pylori* bacterium alters the microrheology probed by latex tracer particles. In bacterial culture broth solution the latex particles diffuse somewhat faster in the presence of bacteria at the lowest concentration of bacteria tested (OD 0.05) while at higher concentrations the diffusion constant decreases. In PGM solutions that also contain bacteria the latex particles exhibit sub-diffusive behavior with a marked decrease in the MSD and resulting elastic and viscous moduli of mucin. The effect is more pronounced in PGM at 15 mg/mL as compared to 30 mg/mL.

Surprisingly PGM at 15 mg/mL exhibits viscoelastic response up to higher frequencies in the presence of bacteria. We also were able to examine the Brownian motion of bacteria by themselves in culture broth solution. The trajectories could be classified into two groups, those corresponding to motile active swimmers exhibiting super-diffusive motion, while those bacteria which were immotile exhibited regular diffusive Brownian motion. The non-motile population of bacteria were found to have an effective hydrodynamic radius of ~ 2 μm. In future work we seek to use this same method in PGM solutions to test whether PGM alters the Brownian motion of *H. pylori.* Comparing the MSD of immotile bacteria in PGM to that of immotile bacteria in broth solution should allow for immotile bacteria to be used to examine the viscoelastic environment a bacteria experiences.

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