Bacterial Spores Respond to Humidity Similarly to Hydrogels

Gennady Y. Gor[a,b,1], **George W. Scherer**[c], **and Howard A. Stone**[b]

This is a draft version, the final version is available at www.pnas.org/cgi/doi/10.1073/pnas.2320763121

Bacterial spores have outstanding properties from the materials science perspective, which allow them to survive extreme environmental conditions. Recent work by Harrellson et al. [Nature 619, 500-505 (2023)] (1) studied the mechanical properties of *Bacillus subtilis* spores and the evolution of these properties with the change of humidity. The experimental measurements were interpreted assuming that the spores behave as water-filled porous solids, subjected to hydration forces. Here we revisit their experimental data using literature data on vapor sorption on spores and ideas from polymer physics. We demonstrate that upon the change of humidity the spores behave like rubber with respect to their swelling, elasticity, and relaxation times. This picture is consistent with the knowledge of the materials comprising the bacterial cell walls – cross-linked peptidoglycan. Our results provide an interpretation of the mechanics of bacterial spores and can help in developing novel synthetic materials mimicking the mechanical properties of the spores.

Bacterial spores | Hydrogels | Adsorption | Absorption | Swelling

Bacterial spores are of interest due to their unique survival capabilities. Understanding their physical properties is valuable for several reasons. On the one hand, there is a demand to be able to kill spores of pathogenic bacteria (e.g., anthrax). On the other hand, understanding the physical properties that provide outstanding protection can help in creating nature-inspired materials with similar properties. Additional interest in bacterial spores, and in particular their response to water sorption, is driven by the observed significant swelling upon an increase in humidity. Since this response to humidity is fast and reversible, the materials that comprise spores may be promising materials for actuators (2).

Harrellson et al. studied dormant *Bacillus subtilis* spores from the perspective of materials science (1). They utilized atomic force microscopy (AFM) and cantilever-bending experiments and measured: (i) swelling of spores as a function of relative humidity (RH), (ii) elastic properties of spores at various RH, and (iii) characteristic relaxation times of spores when RH is perturbed, which appeared strongly dependent on the RH (Fig. 1b). Ref. (1) interpreted these measurements assuming the spores are solids, non-porous at dry conditions, and porous when exposed to humidity. The pore widths x were assumed to change from zero at RH = 0 to 1.5 nm at RH = 100% proportionally to the measured linear expansion of the spore. The deformation was assumed driven by the hydration force expressed as $A\exp(-x/\lambda)$, where A and λ are constants. Harrellson et al. showed that the measured relaxation time cannot be rationalized within the framework of classical poroelasticity (3), since agreement between experiment and theory would have required the viscosity of pore water to exceed the viscosity of bulk water by five orders of magnitude. While nanoscale confinement can alter the properties of water, including viscosity (4), five orders of magnitude increase of viscosity does not appear plausible, and suggests possibilities of additional or alternative mechanisms. This Brief Report proposes such a mechanism based on literature data on sorption of water and nitrogen in *B. subtilis* spores, as well as on knowledge of the spore composition.

Nitrogen adsorption is a standard technique for characterization of nanoporous materials. Nitrogen adsorption isotherms (Fig. 1c) on dry *B. subtilis* spores look similar to isotherms on non-porous solid surfaces (5), consistent with the assumptions made in Ref. (1). The BET (Brunauer–Emmett–Teller) surface area calculated from this isotherm is $5\,\mathrm{m}^2/\mathrm{g}$, which is noticeably lower than for a typical nanoporous material, and comparable to the specific surface area of a non-porous solid the size of a spore. However, adsorption isotherms for water at room temperature on *B. subtilis* spores show qualitatively different behavior (Fig. 1d): the mass of sorbed water exceeds the mass of nitrogen by an order of magnitude and cannot be explained by

Author affiliations: [a]Otto H. York Department of Chemical and Materials Engineering, New Jersey Institute of Technology, Newark, NJ, USA.; [b]Department of Mechanical and Aerospace Engineering, Princeton University, Princeton, NJ, USA; [c]Department of Civil and Environmental Engineering, Princeton University, Princeton, NJ, USA

G.Y.G., G.W.S. and H.A.S. designed the research, performed the calculations, interpreted the experimental data, wrote and proofread the manuscript.

The authors declare no competing interest.

[1]To whom correspondence should be addressed. E-mail: gor@njit.edu

adsorption on the outer surfaces of the spores. Furthermore, Neihof et al. (6) provided clear evidence of porosity appearing in the hydrated state: after the spores were hydrated, water in the spores was replaced by different solvents, and only then dried. Nitrogen adsorption on such solvent-replaced spores showed an order of magnitude higher BET surface area, $74\,m^2/g$ (6). Additional evidence for the appearance of nanopores of $\sim 2\,nm$ in size in the hydrated spores comes from the measurement of diffusion of large molecules through the spore wall material, peptidoglycan (7).

Another type of material showing such a change in behavior is hydrogels. When dry, the solid matrix of a hydrogel can show no porosity, while the addition of water leads to penetration (absorption) of water molecules between the chain-like molecules (free volume) and swelling of the hydrogel. When hydrogels are dried, the porosity vanishes. However, if water in a swollen hydrogel is replaced by a different solvent (e.g., methanol and then carbon dioxide) and then dried, it is possible to maintain the porous structure, and form a nanoporous solid structure: a xerogel or an aerogel. Here we argue that the experimental observations from Ref. (1) can be explained within the framework of hydrogels (8): water molecules penetrate between the flexible, chain-like molecules of the outer layers of the spores. In particular, we contrast adsorption – a process on a surface with absorption – a process taking place in the bulk of the material. While adsorption of vapor in nanoporous solid materials induces deformation, which can be quantified from the solvation pressure in the pores, the resulting strains typically do not exceed 1% (9). Swelling of a polymeric material as a result of solvent absorption can be quantified by assuming that a polymer and a solvent form a solution. The simplest approximation for the swelling can be obtained from an ideal solution model, i.e., the volume of the swollen spore V is equal to the sum of the volume of the dry spore V_0 and the volume of the absorbed water V_w. Using the data on vapor sorption shown in Fig. 1d we can calculate the volume of absorbed water V_w at various RH and compare it to the volume change $\Delta V = V - V_0$ based on the change of the spore height, h, measured in Ref. (1) and geometry shown in Fig. 2a,b (Fig. 2c and Methods).

Results and Discussion

We compare different estimates for the relative volume change $\Delta V/V_0$ of spores as a function of relative humidity (Fig. 2c). The grey dotted line shows $\Delta V/V_0$ calculated from the experimentally measured Δh (1). The red solid line shows V_w/V_0 calculated based on the water sorption isotherm from Ref. (6), and the blue dashed line shows V_w/V_0 calculated based on the sorption isotherm from Ref. (2) (see Methods for details of the calculations). The sorption data from Ref. (2) gives an almost perfect match with the measured swelling, while the calculations based on Ref. (6) slightly over-predict the measured swelling. Note that when calculating the volume of absorbed water we assumed that its mass density equals that of bulk water, which is not necessarily the case, especially at the low loading, owing to pre-existing free volume between the polymer chains.

The rubber-like behavior of hydrated spores is consistent with what is known about the material comprising the spores' outer layers – peptidoglycan (PG) (7, 12). PG, similarly

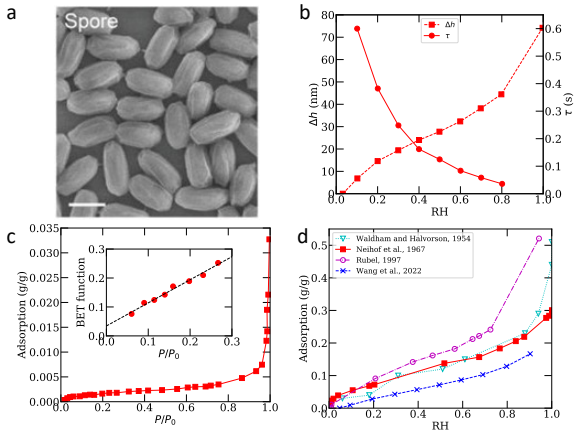

Fig. 1. The effect of relative humidity on the swelling of spores. (a) SEM micrograph of *B. subtilis* spores; scale bar 1 μm, reprinted from H. Wang, et al., Adv. Sci. 9, 2104697 (2022), Wiley-VCH GmbH. (b) Change of the spore height h and relaxation times τ as a function of relative humidity (data from (1)). (c) Nitrogen adsorption isotherm measured on *B. subtilis* spores at 78.15 K from Ref. (5) and a linear fit for the BET surface area calculations (inset). (d) Water adsorption isotherms on spores at room temperature. Data from Ref. (2, 6, 10, 11). Note that there is more than an order of magnitude difference between the mass of nitrogen and water adsorbed.

to rubber, has a cross-linked structure and can swell upon sorption of water, like rubber swelling in organic solvents or hydrogels swelling in water.

Does the physical picture of a swelling hydrogel explain the other experimental data from Ref. (1), i.e., measured relaxation times and their evolution with humidity? The picture proposed here, where the water diffuses into the polymer network, suggests that the characteristic relaxation time (Fig. 1b) should correspond to chemical diffusion rather than the pressure-driven flow. To evaluate the plausibility of the proposed physical picture we can estimate the diffusion coefficient of water in spores as

$$D \simeq \alpha z^2/\tau, \qquad [1]$$

where z is the thickness of the water-permeable layer of the spore, τ is the relaxation time measured in Ref. (1), and $\alpha \simeq 0.24$ is the numerical coefficient corresponding to transient diffusion in a cylindrical annular geometry. This value, obtained from the solution in Crank (13, p. 84-86), differs from the value reported in Ref. (1), which was derived for a planar geometry. The resulting D values as a function of RH are shown in Fig. 2d, along with the diffusion coefficients of water in other types of cross-linked polymers: the commercial hydrogel Hilafilcon A, used for contact lenses, (14), densely cross-linked epoxy resin (15), human stratum corneum (16), and Nylon 6,6 (17). For all polymers, diffusion coefficients change nearly exponentially with RH. D in spores is ≈ 20 times lower than in a hydrogel, however the relative change is the same. Water diffusion coefficients in rubbery polymers are typically of the order of $10^{-8}cm^2/s$, but in glassy, semicrystalline polymers (e.g., Nylon 6,6, and epoxy resin) the diffusion is slower by 1-2 orders of magnitude (17). Slow diffusion in spores is consistent with that result, as the glass–rubber transition in *B. subtilis* is observed at 90–115°C (18). The similar value of D in the outer layer of spores to the D in human stratum corneum

(outer layer of skin) (16) is noteworthy, as both of them perform similar protective functions.

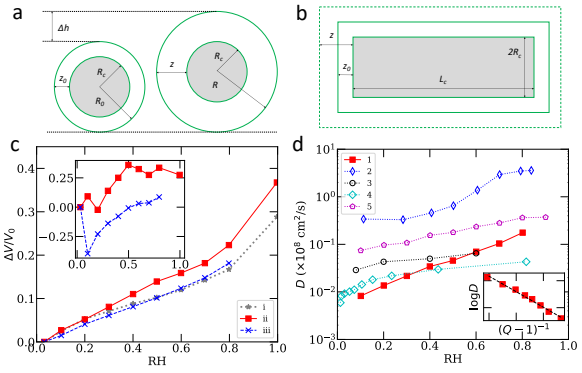

Fig. 2. Comparison of swelling of a spore to hydrogels and other polymeric materials. (a) Schematic of the spore geometry and its change during water absorption: the core remains the same, while the outer layers, cortex and coat, swell; (b) side view. (c) Relative change of the spore volume as a result of water absorption calculated based on the change of the spore height (i), and based on the amount of absorbed water (estimated using sorption data from (6) (ii) and (2) (iii)). The inset shows the relative difference between the data based on the amount absorbed and the measured swelling. (d) Diffusion coefficient of water in various polymer materials as a function of relative humidity: 1 – spores of *B. subtilis* (calculated using Eq. 1, 2) – hydrogel Hilafilcon A (14), 3 – Epoxy resin (15), 4 – human stratum corneum (16), 5 – Nylon 6,6 (17). Inset: dependence of the water diffusion coefficient in spores plotted as function of the volume degree of swelling Q, shown as $\log D$ vs $(Q-1)^{-1}$.

Sorption induces swelling, which increases the volume, providing the path for more molecules, and resulting in the exponential growth of D. In a simple approximation, $\log D$ changes linearly with $(Q-1)^{-1}$, where the volume degree of swelling $Q = \Delta V/(V_0 - V_c)$ (19) (V_c is the volume of the core). Fig. 2d (inset) shows that water diffusion coefficients in spores follow this trend.

To conclude, sorption of water induces swelling and change of the relaxation times of bacterial spores, which is not typical for porous solids. However, such behavior is typical for hydrated polymer networks, i.e., hydrogels. This picture is consistent with the knowledge of the materials comprising the bacterial cell walls – cross-linked peptidoglycan. Our interpretation of sorption-induced swelling and relaxation times of bacterial spores can help better understand their humidity-dependent properties.

Materials and Methods

Estimation of volume of adsorbed water. The volume of adsorbed water and the corresponding relative volume change V_w/V_0 can be related to the adsorption isotherm, measured as the mass of water per unit mass of spore, m_w/m_0:

$$V_w/V_0 = (m_w/m_0)\,\rho_0/\rho_w. \qquad [2]$$

Here ρ_0 is the mass density of dry spores, which we take as $1409\,\mathrm{kg/m^3}$ (5), and ρ_w is the density of liquid water, which we take at 23°C as $998\,\mathrm{kg/m^3}$ (5), since we use the adsorption data from Ref. (6). Note that the data in Fig. 2c are shifted to match zero sorption and zero swelling at the minimum RH.

Estimation of volumetric swelling from measured spore height. Experimental measurements of the spore height and information on the spore geometry and internal structure from Ref. (1) allowed us to estimate the corresponding volumetric swelling. Fig. 2a,b give the approximations for the spore geometry used for calculations of the relative volume change. Following Ref. (1) we assume that a spore has a cylindrical cross-section, the core of the spore is impermeable and the core size (radius R_c) is not changing with RH. The coat and cortex are both water responsive, and their combined thickness z is changing uniformly upon water sorption (z_0 in the dry spore). According to the Methods section in Ref. (1), $z_0 = 140$ nm, and the overall diameter of the dry spore is $2R_0 = 700$ nm, so the radius of the core is $R_c = 210$ nm. The thickness of the swelling annular shell is $z = z_0 + \Delta h/2$, where Δh is the measured change of the spore height.

The SEM micrographs in Ref. (1) indicate that the length of the spore L is approximately twice the spore diameter. Let the length of the core be L_c. If the swelling layer (coat + cortex) has the same thickness over the ends and sides, then the dry length of the spore is $L_0 = L_c + 2z_0$ and the swollen length is $L = L_c + 2z$. The aspect ratio of the dry spore is $(L_c + 2z_0)/(2(R_c + z_0)) \simeq 2$; normalizing by R_c, then $(L_c/R_c + 2z_0/R_c)/(1 + z_0/R_c) \simeq 4$. Recognizing that $z_0/R_c \simeq 2/3$, we find that the aspect ratio of the core is about $L_c/R_c \simeq 16/3$, so $L_c \simeq 1120\,\mathrm{nm}$ and $L_0 \simeq 1400\,\mathrm{nm}$.

The relative change of the volume of the spore can be calculated from the change of the volume of the cylindrical shell (including caps) from the thickness of z_0 to z:

$$\Delta V/V_0 = \left(R^2\,(L_c + 2z) - R_0^2\,(L_c + 2z_0)\right) / \left(R_0^2\,(L_c + 2z_0)\right) \qquad [3]$$

where $R = R_c + z = R_c + z_0 + \Delta h/2$. Eq. 3 is used for calculations in Fig. 2c.

The geometric dimensions of the pores allow us also to estimate the specific surface area of dry spores as $a = A_0/(\rho_0 V) = 2\,(R_0 + L_0)\,/\,(R_0 L_0 \rho_0) \simeq 5\,\mathrm{m^2/g}$. This value is the same as the BET surface area calculated from nitrogen adsorption data. This agreement may be fortuitous, given the additional surface area of the wrinkles on the spores' surface clearly seen in the SEM micrographs, and the distribution in size of the spores.

ACKNOWLEDGMENTS. The authors thank Prof. Ozgur Sahin for discussions. G.Y.G. and H.A.S. thank NSF CBET-1944495 and DMS-2245850 grants for the support respectively.

1. SG Harrellson, et al., Hydration solids. Nature **619**, 500–505 (2023).
2. H Wang, et al., High energy and power density peptidoglycan muscles through super-viscous nanoconfined water. Adv. Sci. **9**, 2104697 (2022).
3. MA Biot, General theory of three-dimensional consolidation. J. Appl. Phys. **12**, 155–164 (1941).
4. U Raviv, P Laurat, J Klein, Fluidity of water confined to subnanometre films. Nature **413**, 51–54 (2001).
5. E Berlin, H Curran, M Pallansch, Physical surface features and chemical density of dry bacterial spores. J. Bacteriol. **86**, 1030–1036 (1963).
6. R Neihof, J Thompson, V Deitz, Sorption of water vapour and nitrogen gas by bacterial spores. Nature **216**, 1304–1306 (1967).
7. P Demchick, AL Koch, The permeability of the wall fabric of Escherichia coli and Bacillus subtilis. J. Bacteriol. **178**, 768–773 (1996).
8. JC Brinker, GW Scherer, Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing. (Academic Press), (1990).
9. GY Gor, P Huber, N Bernstein, Adsorption-Induced Deformation of Nanoporous Materials – a Review. Appl. Phys. Rev. **4**, 011303 (2017).
10. DG Waldham, H Halvorson, Studies on the relationship between equilibrium vapor pressure and moisture content of bacterial endospores. Appl. Microbiol. **2**, 333–338 (1954).
11. GO Rubel, Measurement of water vapor sorption by single biological aerosols. Aerosol Sci. Technol. **27**, 481–490 (1997).
12. DL Popham, J Helin, CE Costello, P Setlow, Muramic lactam in peptidoglycan of Bacillus subtilis spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc. Natl. Acad. Sci. **93**, 15405–15410 (1996).
13. J Crank, The Mathematics of Diffusion. (Clarendon, Oxford), 2 edition, (1975).
14. C Weinmüller, C Langel, F Fornasiero, CJ Radke, JM Prausnitz, Sorption kinetics and equilibrium uptake for water vapor in soft-contact-lens hydrogels. J. Biomed. Mater. Res. Part A **77**, 230–241 (2006).
15. S Cotugno, G Mensitieri, P Musto, L Sanguigno, Molecular interactions in and transport properties of densely cross-linked networks: a time-resolved FT-IR spectroscopy investigation of the epoxy/H_2O system. Macromolecules **38**, 801–811 (2005).
16. A El-Shimi, H Princen, Diffusion characteristics of water vapor in some keratins. Colloid Polym. Sci. **256**, 209–217 (1978).
17. FM Preda, et al., Investigation of water diffusion mechanisms in relation to polymer relaxations in polyamides. Macromolecules **48**, 5730–5741 (2015).
18. ML Stecchini, et al., Glassy state in Bacillus subtilis spores analyzed by differential scanning calorimetry. Int. J. Food Microbiol. **106**, 286–290 (2006).
19. NA Peppas, CT Reinhart, Solute diffusion in swollen membranes. Part I. A new theory. J. Membr. Sci. **15**, 275–287 (1983).