In situ determination of refractive index and size of *Bacillus* spores by light transmission

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Light-extinction measurements in the wavelength range of 400 to 1000 nm are performed *in situ* on *Bacillus subtilis* endospores during heat-shock-induced activation. Simultaneous information on particle size and refractive indices during activation is calculated from the transmission spectra by use of the Gaussian ray approximation of anomalous diffraction theory. During activation the refractive index of the core decreases from 1.51 to 1.39, and the size increases from 0.38 to 0.6 μm. © 2005 Optical Society of America

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Light scattering has long been investigated as a tool for identifying bacteria size and shape,[1–4] and quasi-elastic light scattering has been used to study endospore (ES) structure.[5,6] The Gaussian ray approximation (GRA) of anomalous diffraction theory has been used to determine the size and shape of different species of bacteria from light transmission.[3,7] Bacterial cell size typically varies from the submicrometer level to several micrometers; thus their scattering properties in the visible are strongly dependent on wavelength, size, shape, and refractive index. The bacteria genera *Bacillus* and *Clostridium* can differentiate to form an ES, a dormant cell type, in response to hostile environments and can revert to vegetative cells by germination when conditions become more receptive. The structural changes occurring during germination are important to biologists[5] and in bioagent detection. In the past, fluorescence was shown to be an effective technique to detect ESs *in situ*.[8] Combining light scattering, extinction, and fluorescence might offer an ideal tool to determine the type of agent present.

In this Letter light extinction is used *in situ* to monitor *Bacillus subtilis* ES activation and to simultaneously retrieve the refractive index and size of the ES during this process. The optical techniques and methods described allow for the real-time visualization of the ES activation process and the initial stage of bacterial differentiation. The results may have important significance in bioagent identification systems and modeling applications.

ESs are significantly denser than and their refraction index is greater than that of vegetative cells. ESs consist of a high-density, dehydrated core surrounded by a lower-density spore coat composed of cross-linked polypeptides. Germination begins with activation, during which the coat is shed and the core hydrates with a subsequent decrease in density and refractive index and an increase in size.

From the recent statistical interpretation of anomalous diffraction theory[9,10] light extinction by soft particles is mainly determined by the mean and mean-squared size of the particles in the GRA. The scattering cross section can be approximated by

$$C_s = \pi r^2 \left[\frac{4n^2\pi^2}{\lambda^2}(m-1)^2(\mu^2-\sigma^2) - \frac{4n^4\pi^4}{3\lambda^4}(m-1)^4(\mu^4+3\sigma^4+6\mu^2\sigma^2) \right], \quad (1)$$

keeping only the leading two terms in the GRA [Eq. (10) in Ref. 9]. Here λ is the wavelength, n is the refractive index of the background media, m is the relative refractive index of the particle, $\mu=\langle l\rangle$ is the mean light path through the scatterer, and σ is given by $\sigma^2=\langle l^2\rangle-\langle l\rangle^2$. The first term on the right-hand side of Eq. (1) is the scattering cross section in the intermediate case limit.[11,12] The second term is a correction introduced by the GRA when the condition $(2\pi r/\lambda)|m-1|<<1$ is not met. In the absence of absorption, optical extinction K is given by

$$K = C_s NL = \pi r^2 \left(\frac{4n^2\pi^2}{\lambda^2}\alpha r^2 - \frac{16n^4\pi^4}{\lambda^4}\frac{\beta r^4}{12} \right) NL, \quad (2)$$

where N is the concentration and L is the path length. For uniform spheres, α and β are given by

$$\alpha \approx 2(m-1)^2, \quad \beta \approx \frac{460}{81}(m-1)^4. \quad (3)$$

For soft particles with a size smaller than or comparable with λ the first term in Eq. (2) is sufficient to describe K, which is linear in λ^{-2}. For particles with a larger size or refractive index the second term in Eq. (2) must be included to accurately describe K.

In the experiment *B. subtilis* ESs (strain NCTC 3610) were isolated from their cellular debris,[13] heat activated,[14] and placed in a germination medium.[15] The germination medium had a minimal carbon source and limited amino acid content to restrict cell growth and prevent reversal of activation.

Transmission was measured from 400 to 1000 nm, a region in which the bacteria have little absorption, and losses are due to scattering. The concentration was 1.03×10^8 ESs/ml in a 1-cm quartz cuvette. The

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transmission spectra were measured with a halogen light source (HL2000, Ocean Optics, Dunedin, Florida) and compact spectrometer (HR2000, Ocean Optics), both coupled to optical fibers. The spectra were acquired at 1-min intervals for the first 3.5 h after heat shock and at 30-min intervals between 3.5 and 20 h. Each spectra was integrated for 5 s.

A least-squares fit to a function of the form

$$K = C_2\lambda^{-2} + C_4\lambda^{-4} \tag{4}$$

was applied to each transmission spectrum. Figure 1 shows K plotted as a function of λ^{-2} before heat shock and at representative times after heat shock: $t=1$, 30, 60, 120, and 360 min. The respective least-squares fits are also plotted in Fig. 1. Coefficients C_2 and C_4 are plotted at all time intervals in Fig. 2. As seen in Fig. 1, the spectrum from the spores immediately after heat shock is nearly identical to that of the spores before heat shock, indicating that the response of the spores to heating is not instantaneous. It can also be observed in Figs. 1 and 2 that the largest reduction in scattering occurs in the first 2 h, primarily caused by a dramatic decrease in the refractive index as the ESs shed their coat and hydrate the core. From 2 to 3 h the rate of reduction in refractive index slowed while the radius continued to increase. The scattering cross section remained relatively constant from 3 to 8 h, indicating that the refractive index and spore size were not changing significantly. After 8 h, K decreased slowly as the ESs decayed, releasing their contents into solution.

With a uniform spherical model for the ESs, which neglected the contribution of the coat, the radius and index were calculated from C_2 and C_4 with Eq. (2) and expressions (3). It was observed that the radius (plotted in Fig. 3) remained relatively constant in the first 30 min after heat shock. The ES radius was also calculated with only C_2 and making the assumption that the refractive index was equal to that of vegetative cells, 1.39 (Refs. 3 and 16; also plotted in Fig. 3). The radius, calculated by both methods is in agreement with $t>3$ h, confirming that by 3 h the ESs have lost their coat and the index is close to that of vegetative cells. For $t<3$ h the radius calculated by

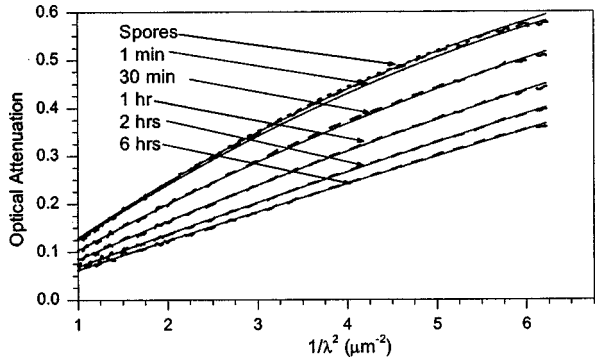

Fig. 1. Light extinction as a function of wavelength plotted for spores before heat shock and at $t=1$ min, 30 min, 1 h, 2 h, and 6 h after heat shock (dashed curves). The least-squares fit to $K=C_2\lambda^{-2}+C_4\lambda^{-4}$ is shown for each spectrum (solid curves).

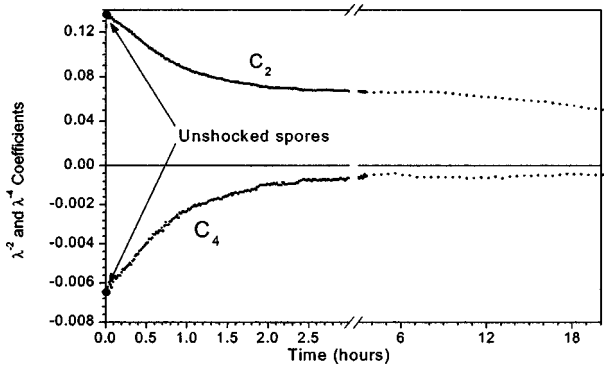

Fig. 2. Least-squares fit coefficients C_2 and C_4 plotted as a function of time.

use of a fixed index of 1.39 is substantially larger than the radius calculated from C_2 and C_4 and incorrectly predicts that the radius is decreasing over time. This lack of agreement demonstrates that the ES refractive index during the early time period is greater than 1.39. The ES radius, calculated from C_2 and C_4, is 0.35 μm for the unshocked spores and 0.34 μm at 1 min after heat shock. The radius gradually increases to a maximum of $\sim$0.6 μm after 6 h. In a nutrient-rich medium the ESs would continue to germinate and grow, eventually reaching the size of vegetative cells. The refraction index, calculated from Eq. (2) and expressions (3) and plotted in Fig. 4, is 1.55 for both the unshocked spores and at 1 min after heat shock. The index decreases steadily in the first 3 h, after which it remains constant at 1.39, showing that by this time the core has hydrated and become closer to vegetative cells in density and composition.

A better model for the spore structure that takes into account the spore coat is two concentric nonabsorbing spheres consisting of a thin outer shell—the spore coat—that slowly dissolves and an inner sphere—the spore core—that changes in index and size. The ratio of spore coat thickness to cell radius is designated ε. The initial spore coat thickness is taken to be 70 nm,[17] and, for an approximate initial radius of 400 nm, ε is initially set to 0.175. Since the calculated spore radius (see Fig. 3) was unchanged during the first 30 min, our model assumes that the spore coat also remains unchanged during this time. This model assumes that from $t=0.5$ to $t=3$ h the coat thickness uniformly decreases from its initial value to zero. Microscopy confirmed that the spore coat completely dissolved within 3 h of heat shock.

In the GRA for concentric spheres the geometric ray inside the particle may bisect both the core and the coat, with relative refractive indices m_i and m_o, respectively. The modified geometric path, $l'=l_i(m_i-1)+l_o(m_o-1)$, is given by

$$\begin{aligned} l' &= 2(m_o-1)(r_o^2-h^2)^{1/2} + 2(m_i-m_o)(r_i^2-h^2)^{1/2}, \quad h < r_i \\ &= 2(m_o-1)(r_o^2-h^2)^{1/2}, \quad h > r_i, \end{aligned} \tag{5}$$

with the distribution function for h given by $p(h)=(2h/r_o^2)$ and normalized to unity. Evaluating $\langle l'\rangle$ and

$\langle l^2 \rangle$ with Eq. (5) and keeping only the first-order terms in ε allows α and β to be approximated by

$$\alpha \approx 2(m_i - 1)^2 - 8(m_i - 1)(m_i - m_o)\varepsilon + \ldots,$$

$$\beta \approx \frac{460}{81}(m_i - 1)^4 - \frac{544}{27}(m_i - m_o)[(m_i - 1)^3 - 2(m_i - m_o)^3]\varepsilon + \ldots, \quad (6)$$

where m_o is taken to be 1.39. The radii and refractive indices were recalculated with this model and are also plotted in Figs. 3 and 4, respectively. The recalculated spore radius is 0.37 μm before heat shock. For the first 30 min after heat shock the radius remains essentially unchanged at 0.38 μm and then slowly increases. The refractive index is 1.515 for the unshocked spores, which is in agreement with the value of 1.52 reported by Tuminello *et al.*[4] After heat shock the index drops linearly to 1.39 over a 2-h period.

Confocal microscopy finds an ellipsoidal shape for the ESs before heat shock with a long axis of 1.25 μm and short axes of 0.75 μm, equivalent in light extinction to a sphere of radius 0.43 μm, which is in agreement with our concentric sphere model.

In conclusion, the GRA has been used to measure refractive-index and size changes in ESs during heat-shock activation from light-extinction measurements. Within 2 h of the start of activation the refractive index decreased from 1.51 to 1.39. The radius began to increase after the first half hour from 0.38 to 0.6 μm at 4 h. This work demonstrates that light extinction can monitor *in situ* both the refractive index and the size of ESs, may potentially be part of an optical bioagent detection scheme, and can be used for modeling applications.

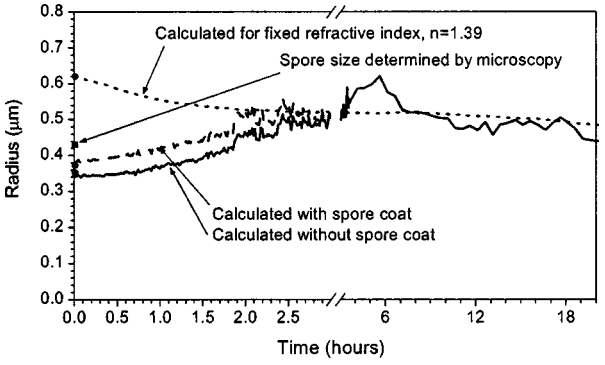

Fig. 3. ES radius plotted as a function of time after heat shock. The radius was calculated by three methods: (1) from C_2 and C_4 assuming a uniform sphere (i.e., without a spore coat), (2) a uniform sphere with $n = 1.39$, and (3) two concentric spheres with the outer sphere (spore coat) dissolving as described in the text.

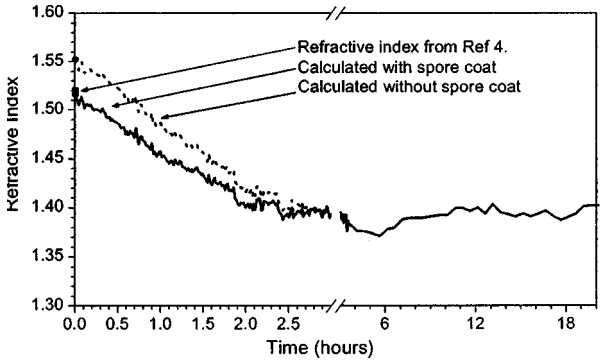

Fig. 4. ES refractive index calculated for uniform spheres (no coat) and two concentric spheres with the outer sphere (spore coat) dissolving as described in the text.

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