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Interaction of modified liposomes with *Bacillus* spores

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The interaction between liposomes modified with a particular peptide sequence and *Bacillus subtilis* spores was experimentally observed as (1) an increase in the average diameter of spore-related particles, and (2) the formation of dense and structured shells around the spores at higher concentrations of liposomes.

Dormant spores of the bacterium *Bacillus* species are resistant to environmental inactivation.[1] The study of structural factors intrinsic to spores that make them resistant to particular decontamination procedures may directly result in the development of safe and efficient inactivation procedures[2] with minimal harm to equipment and personnel. Here we show that spores can be coated with a phospholipid shell formed by liposomes modified with peptide ligands that were discovered[3,4] to bind selectively to *Bacillus* spores.

It is known[5] that a spore's outer membrane is not complete and a deficit of phospholipids always exists in the spore's outermost envelope. This fact leads to the assumption that *Bacillus* spores interact with lipids resulting in a spore coating. However, no literature data on the interactions between spores and phospholipids exist. Our hypothesis is that only phospholipids with entities specific to *Bacillus* spores are capable of interacting with the spore's surface thus initiating the spore coating. Various ligands (peptides, carbohydrates, aptamers, antibodies, lectins, and their conjugates with polymers) have been screened[6] to find one or a group of ligands that are most specific to *Bacillus* spores. Of the various ligand candidates, peptides seem to be appropriate for phospholipid modification, since the amino acid sequences specific to the corresponding receptors on the spore's surface or inside the spore's coats can be obtained by well-established methods.[6,7] A number of different families of short peptides that can bind tightly to specific species of *Bacillus* spores have been discovered[3,4] by screening phage display peptide libraries. In particular, peptides containing the consensus sequence Asn-His-Phe-Leu-Pro (NHFLP) at the N-terminus were found[3] to strictly recognize spores of a few *Bacillus* species which comprise one branch of the *Bacillus* phylogenetic tree, namely, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus globigii*, *Bacillus lentimorbus*, and *Bacillus popilliae*. Another family of peptides was identified[3,4] as interacting selectively with *Bacillus anthracis* spores.

To design modified liposomes that selectively interact with *Bacillus subtilis* 1A700 (originally designated 168) spores, the liposomes, composed of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[3-(2-pyridyldithio)propionate] (N-PDP-PE) in a molar ratio of 10 to 1, were allowed to react with specific peptide chains containing the above mentioned sequence NHFLP (Fig. 1). The number of polypeptides attached to each 170 nm liposome was calculated to be about 11000 (the yield of disulfide bonds formed was 90 ± 2%).

The liposomes served as vehicles for the delivery of modified phospholipids to the vicinity of the spore's surface. Liposome solution was added to a suspension of *Bacillus subtilis* spores to provide different liposome-to-spore ratios. Depending on the relative sizes of the liposomes and spores, thousands of liposomes may interact with the surface of an individual spore. Changes in the size of the resultant assemblage can be directly determined by microscopic or spectroscopic techniques.

Dynamic light scattering (DLS) measurements were carried out with an N4 Plus particle size analyzer (Beckman-Coulter, Fullerton, CA) at a fixed scattering angle of 90°. A He–Ne laser operating at λ = 628 nm and 10 mW power was used as a light source. The temperature was controlled to within ±0.1 °C. DLS measurements showed a bimodal size distribution for a mixture of non-modified liposomes and spores (Fig. 2a). Two peaks at 1070 nm and 170 nm were detected for spores and liposomes, respectively, indicating the absence of interactions between spores and liposomes for any liposome-to-spore ratio. On the other hand, a unimodal size distribution was found for low ratios of modified liposomes and spores (Fig. 2b). The peak shifts to higher values on increasing the concentration of liposomes indicating that modified liposomes bind to the surface of the spores. The average diameter,

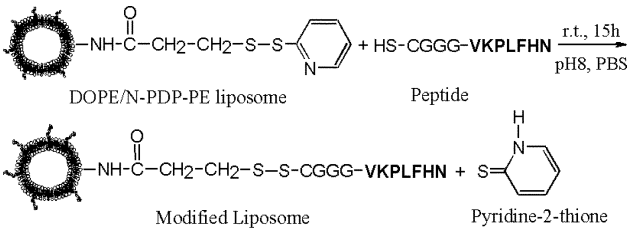

Fig. 1 Covalent coupling of peptide fragments to liposomes.

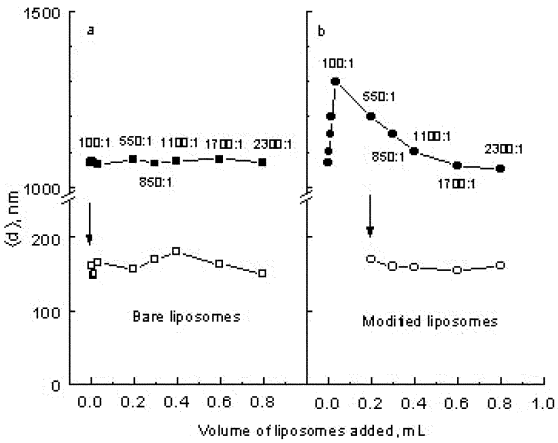

Fig. 2 Interactions of liposomes with *Bacillus subtilis* spores at different liposome-to-spore ratios: (a) DLS data for mixtures of spores and non-functional liposomes; solid squares correspond to the size of the spores, open squares correspond to the free liposomes; (b) DLS data for mixtures of spores and modified liposomes; solid circles correspond to the spore–liposome complexes, open circles correspond to free liposomes. The arrows indicate the amount of added liposomes at which free liposomes appear.

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$\langle d \rangle$, of the modified liposomes–spore assemblies reaches a maximum of ~1300 nm for a ratio of ~100 : 1. At higher ratios, a second peak appears with an average diameter of ~170 nm. This peak has been assigned to excess liposomes which are unable to bind to spores due to saturation of the spore's surface with liposomes.

Atomic force microscopy (AFM) imaging of a mixture of liposomes and spores dried in air on mica (Fig. 3) shows that during water evaporation a thick lipid multi-layered film (1) is formed and the spore (2) is embedded in the surrounding mass. The lipid film conserves some water and prevents complete dehydration of spores. Thus, the spores are close to spherical and their sizes depend on the degree of spore dehydration. Herein, since the spore cortex is a cross-linked peptidoglycan,[8] it is capable of changing volume as the water content changes. Despite all these observations, there is no indication of any interactions between spores and non-modified liposomes (Fig. 3a).

For the modified liposomes (Fig. 3b) there is a shell (3) around each spore (2) indicating that the components of the outermost layers of the spore's envelope do not dissolve in the surrounding phospholipid film (1), but interact with functional phospholipids. If the spore is completely immersed in the lipid layer, only the shell (3′) appears on the surface of the phospholipid film.

At a lower concentration of liposomes (Fig. 4), the thickness of the lipid film was less than the size of the spore, and so all the spores lay on the surface of the mica resulting in their flattening. The spores are not spherical due to dehydration and contraction of the cortex, but the shells around the spores are perfectly round. Imaging at a lower magnification (Fig. 4a) showed that shells surrounded all the spores. AFM imaging of spore shells at different concentrations (Figs. 3b and 4b) revealed a decrease in the diameter of the shell with rising concentrations of modified liposomes. This explains the DLS observations of a decrease in the size of spore–liposome particles at high concentrations of modified liposomes (Fig. 2b).

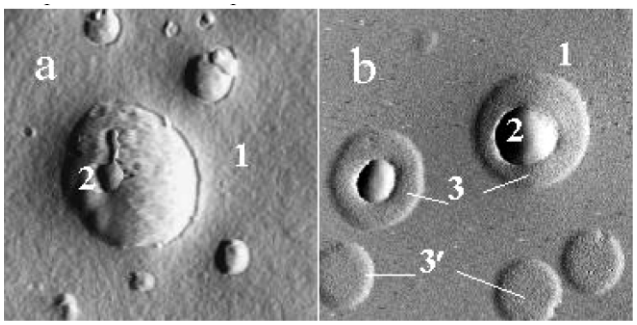

Fig. 3 Tapping mode AFM imaging (amplitude data) of the interactions of the free liposomes (a) and functional liposomes (b) with *Bacillus subtilis* spores at a liposome-to-spore ratio of 1000 : 1: (1) phospholipid film, (2) a spore without exosporium and coats, and (3, 3′) the outermost envelope filled with liposomal phospholipids. The frames are 1×1 μm^2.

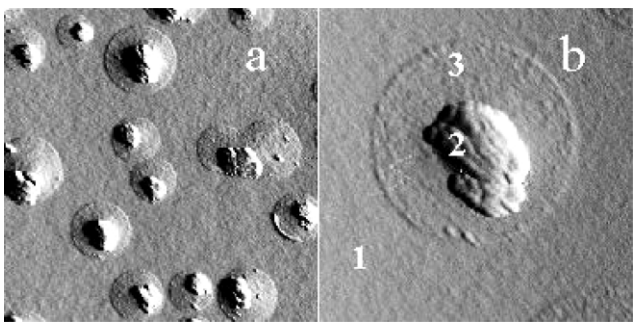

Fig. 4 Tapping mode AFM imaging (amplitude data) of the interactions of functional liposomes with *Bacillus subtilis* spores at a liposome-to-spore ratio of 500 : 1. For notation see Fig. 3. The frames are (a) 3.5×3.5 μm^2, (b) 1×1 μm^2.

The observed formation of a shell around each spore (AFM data) allows us to conclude that a spore attracts functional liposomes into its outer layer probably as a result of the interactions of peptides with a specific protein receptor[4] in the spore's outer envelope. Apparently, this attraction triggers a cooperative penetration of both modified and non-modified phospholipids inside the outer layer of the spore resulting in phospholipid redistribution and formation of a dense and structured shell (3) (Figs. 3b and 4).

The basic structure of spores of all *Bacillus* species[5,8] explains the formation of the observed shell. The spore's outer membrane is embedded in spore coats and does not contain enough phospholipid to enclose the spore completely. Therefore, when modified liposomes appear in close proximity to the spore's surface, the outer membrane "tries" to complete its structure by "borrowing" phospholipids from the liposomes. This hypothesis is consistent with the physiological role of the coats protecting the spore from mechanical damage and harmful chemicals, mainly by forming a permeability barrier against large molecules.[5]

The number of 170 nm liposomes needed to cover the surface of a 1070 nm spore with a lipid bilayer was estimated to be ~30–50. According to the DLS data, the number of liposomes accommodated by the spore is ~100. This number is sufficient to cover the spore with at least one or two bilayers. Thus, it is the fusion of liposomes and the formation of a shell around each spore that explains the decrease in the average spore/capsule diameter detected by DLS at high concentrations of liposomes.

In conclusion, it is worth noting that the discovery of a phospholipid shell around a spore will spur on further studies in this area because of the great potential for developing a new concept for spore decontamination and a new method for spore identification, and also for furthering the basic knowledge of structural features of the spore's outermost envelope. Whether coating of a spore with a phospholipid membrane can prevent or at least considerably reduce the ability of spores to germinate is a question that is currently under investigation in this laboratory.

Notes and references

1 W. Nicholson, N. Munakata, G. Horneck, H. Melosh and P. Setlow, *Mol. Biol. Microbiol. Rev.*, 2000, **64**, 548.

2 (*a*) R.-M. Cabrera-Martinez, B. Setlow and P. Setlow, *J. Appl. Microbiol.*, 2002, **92**, 675; (*b*) P. Genest, B. Setlow, E. Melly and P. Setlow, *Microbiology*, 2002, **148**, 307; (*c*) E. Melly, A. Cowan and P. Setlow, *J. Appl. Microbiol.*, 2002, **93**, 316; (*d*) B. Setlow, C. A. Loshon, P. C. Genest, A. E. Cowan, C. Setlow and P. Setlow, *J. Appl. Microbiol.*, 2002, **92**, 362.

3 (*a*) C. Turnbough, Jr, *J. Microbiol. Methods*, 2003, **53**, 263; (*b*) J. Knurr, O. Benedek, J. Heslop, R. B. Vinson, J. A. Boydston, J. McAndrew, J. F. Kearney and C. L. Turnbough, Jr, *Appl. Environ. Microbiol.*, 2003, **69**, 6841.

4 (*a*) D. Williams, O. Benedek and C. Turnbough, Jr, *Appl. Environ. Microbiol.*, 2003, **69**, 6288; (*b*) C. Steichen, P. Chen, J. Kearney and C. Turnbough, Jr, *J. Bacteriol.*, 2003, **185**, 1903.

5 (*a*) L. Matz, T. Beaman and P. Gerhardt, *J. Bacteriol.*, 1970, **101**, 196; (*b*) A. Warth, *Adv. Microb. Physiol.*, 1978, **17**, 1; (*c*) W. Murrell, in *The Bacterial Spore*, eds. G. Gould and A. Hurst, Academic Press, London, 1969, pp. 215–274; (*d*) H. Jenkinson, W. Sawyer and J. Mandelstam, *Gen. Microbiol.*, 1981, **123**, 1; (*e*) D. Tipper and P. Linnett, *J. Bacteriol.*, 1976, **126**, 213; (*f*) R. Goldman and D. Tipper, *J. Bacteriol.*, 1978, **135**, 1091; (*g*) A. Aronson and N. Pandey, in *Spores VII*, eds. G. Chambliss and J. Vary, American Society for Microbiology, 1978, pp. 54–61; (*h*) M. Koncewicz, D. Ellar and J. Postgate, *Biochem. Soc. Trans.*, 1977, **5**, 118; (*i*) B. Wilkinson, J. Deans and D. Ellar, *Biochem. J.*, 1975, **152**, 561; (*j*) P. Gerhardt, R. Scherrer and S. Black, in *Spores V*, eds. H. Halvorson, R. Hanson and L. Campbell, American Society for Microbiology, 1972, pp. 68–76.

6 S. Iqbal, M. W. Mayo, J. G. Bruno, B. V. Bronk, C. A. Batt and J. P. Chambers, *Biosens. Bioelectron.*, 2000, **15**, 549.

7 B. Merrifield, *Biopolymers*, 1995, **37**, 3.

8 (*a*) H. Rogers, In *Spore Research*, eds. A. Barker, J. Wolf, D. Ellar, G. Dring and G. Gould, Academic Press, London, 1976; (*b*) A. Warth and J. Strominger, *Biochemistry*, 1972, **11**, 1389; (*c*) D. Tipper and J. Gauthier, in *Spores V*, eds. H. Halvorson, R. Hanson and L. Campbell, American Society for Microbiology, 1972, pp. 3–12; (*d*) D. Popham, J. Helin, C. Costello and P. Setlow, *J. Bacteriol.*, 1996, **178**, 6451.