

ORIGINAL ARTICLE

Lyotropic liquid crystal as a real-time detector of microbial immune complexes

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bacteria, detection, immune complex, liquid crystal, spores.

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Abstract**Aims:** To design a simple method for the detection of microbe-immune complexes exploiting the optical and elastic properties of a biocompatible liquid crystalline material.**Methods and Results:** Aqueous solution of disodium cromoglycate (DSCG), a lyotropic chromonic liquid crystal (LCLC), was aligned in a glass cell so as to be optically dark in polarized light. Immune complexes of at least three to four organisms altered the DSCG alignment such that polarized light was subsequently transmitted to reveal the presence of pathogens as optically bright regions around the immune complexes.**Conclusions:** This work describes the first method to detect viable micro-organisms in real time using LCLC.**Significance and Impact of the Study:** This technique provides a powerful tool for the detection of microbes in minutes, exploiting the optical and elastic properties of LC.**Introduction**

The near immediate detection of micro-organisms has been a high priority for clinical and environmental microbiologists for some time. The need to assess suspicious materials, not withstanding, faster microbe detection and identification will have significant impact on health care, food and water purity, and homeland security. Technologies using DNA chips (Martin-Casabona *et al.* 1997; Wang 2000), PCR-amplified nucleic acid (Schmidt 2002; Oberst *et al.* 2003), time of flight mass spectroscopy (Noble and Prather 2000), Raman microscopy (Petrich 2001; Scully *et al.* 2002), quartz crystal microbalance immunosensors (Cooper *et al.* 2001; Eun *et al.* 2002), and pizelectric-surfacewave transducers (Deisingh and Thompson 2001) are making their way from the laboratory to the field and have reduced microbe detection time to hours instead of days. These techniques can be highly selective, but may require pure cultures, pristine samples, large numbers of organisms, substantial computational analysis, and/or a highly trained workforce to achieve reliable results. Alternative technologies are therefore sought

to detect and identify microbes in real time (i.e. total elapsed time of minutes), from a variety of environments, with little or no sample preparation. These technologies must be highly selective, sensitive, and have a low incidence of false reporting (being able to discriminate between similar agents and other 'background noise').

Graham and Sabelnikov (2004) have concluded that sensors based on antibody (AB)-antigen binding (plus a transducer) are much more general and versatile than sensors based on complementary binding of nucleotides, such as PCR-based and DNA chips. Furthermore, they also reported that recently developed technology to discover and design AB with extremely high affinities and specificity for antigens (e.g. combinatorial AB library, or phage display AB library techniques) should significantly improve the selectivity of antigen selection. Specificity and selectivity aside, sensitive, real-time detection and identification of micro-organisms by AB requires a reporting system, which can evaluate discrete microbe-AB binding events, amplifying these reactions, and transducing them into signals. The aim of this study was to exploit the elastic and anisotropic properties of liquid

crystalline materials to amplify the specific capture of microbes by AB to optically report discrete immune aggregates in real time.

Liquid crystals (LC) are thermodynamically stable soft-matter phases, intermediate between solids and isotropic liquids (Collings 2002). LC consist of anisotropic molecules oriented along a common direction (called the director). In the absence of external forces or internal additives, the director configuration is uniformly aligned in space. However, this uniformity is readily disrupted by small external fields (the effect that is used in display devices) or by foreign particles in the bulk of the LC. These director distortions occur easily since the orientational molecular interactions are weak (for example, of van der Waals type). Furthermore, these distortions, when at micrometre scale or larger, cause drastic changes in optical properties of the liquid crystalline medium, because the director usually serves as the optical axis of the LC. Because of this, individual microbes and antibodies, being relatively small by themselves, will remain 'invisible' in the dark LC background, while microbe immune aggregates distorting LC alignment will be amplified by director distortions and identified by optical transmission. Interestingly, and for many LC, the critical particle size, which causes the change in optical characteristics is in the range of 1–10 μm , depending on the elastic properties of the LC and the properties of the LC–particle interface (Shiyanovskii *et al.* 2005).

Lytotropic liquid crystals (LLC) were chosen for this study because they are aqueous-based materials (that provide a more physiologic environment for the microbes) whose chemical and optical properties are well studied. The lyotropic chromonic liquid crystal (LCLC) subset of LLC was subsequently chosen for biosensor capabilities, as it is composed of nonsurfactant, aqueous LLC that are nontoxic to bacteria (Woolverton *et al.* 2005). LCLC represent a wide variety of dyes, drugs, and nucleic acids (Lydon 1998, 2004). LCLC molecules are plank-like rather than rod-like, aromatic rather than aliphatic, and rigid rather than flexible (in contrast to surfactant-based LLC). The so-called nematic *N*-phase of LC order results because the LCLC molecules stack face-to-face, producing elongated aggregates that align parallel to each other in water (Lydon 1998, 2004). Using a protein-coated latex bead system, we previously identified that immune aggregates had to exceed a critical size of 2–3 μm to alter the LCLC director and to produce a measurable optical event (Shiyanovskii *et al.* 2005). It is well known that antigen–microbe binding results in immune complexes of growing size and shape (Eggs 1997). When such complexes grow in the LCLC bulk and become larger than some critical size, director distortions result, and light transmittance through the LCLC system will report the presence of

microbe complexes. In other words, microbe detection is based on the director distortions around immune complexes that grow inside the LC above a critical size. This paper reports the real-time capture and detection of *Bacillus atrophaeus* (BA) spores using the LCLC, disodium cromoglycate (DSCG), to report AB capture of an anthrax surrogate. Although *Bacillus cereus* is most closely related phylogenetically to *Bacillus anthracis*, BA (formerly known as *Bacillus globigii* or *Bacillus subtilis* var. *niger*) has historically been used as a surrogate of *B. anthracis* due to its unique colonial morphology and lack of pathogenicity (Christensen and Holm 1964; Nakamura 1989; Ash *et al.* 1991; Fritze and Pukall 2001; Xu and Cote 2003; Helfinstine *et al.* 2005). BA spores exhibit similar morphological characteristics as *B. anthracis* spores, permitting their use in this paper to demonstrate the utility of the LC biosensor to detect spores.

Materials and methods

Bacillus endospores

Bacillus atrophaeus was obtained from the American Type Culture Collection (ATCC, #9372, Herndon, VA) and passaged on trypticase soy agar (TSA) supplemented with 5% sheep blood (Becton Dickson Co. Sparks, MD). Bacteria were aseptically harvested, grown subsequently on nutrient sporulation medium (Dang *et al.* 2001) at 36°C for 48 h, and then at room temperature. The spores (1.0–1.5 μm in diameter) were harvested with 5-ml cold (4°C) sterile distilled, deionized (18 M Ω) water (DDW), once >90% spore content was observed. This was usually after 1 week. The spore suspension (70 ml) was then centrifuged at 2504 g, 10 min, 25°C (IEC Centra MP4R, Needham Heights, MA); the pellet re-suspended in 70-ml phosphate-buffered saline (PBS, pH 7.2), supplemented with 0.05% Tween 20 (Mallinckrodt Baker, Inc, Paris, KY); mixed for 5 min and centrifuged as described. The spore pellet was then washed four times with 70-ml sterile DDW each time to remove the detergent. Finally, the spores were suspended in 40-ml sterile DDW with serial tenfold dilutions prepared in 1-ml sterile water. Dilutions of 100 μl were incubated in nutrient agar (NA) pour plates ($n = 3$) to determine the concentration of spores in the stock suspension, which was found to be $9.6 \pm 0.1 \log_{10}$ CFU ml⁻¹. A 10-ml spore suspension was adjusted to $9.0 \log_{10}$ CFU ml⁻¹ and stored at 4°C for experimental use.

Antibodies

Polyclonal rabbit anti-BA endospore AB was provided by Tetracore, Inc. (Gaithersburg, MD). Fluorescein isothio-

cyanate (FITC) labeling of anti-BA AB was with the E-Z Label Kit from Pierce Biochemicals (Rockford, IL) as per manufacturer instructions. An immunoblot was performed to confirm the activity and specificity of the unlabelled primary anti-BA AB ($1.0 \mu\text{g ml}^{-1}$) to the spores. The secondary AB ($0.5 \mu\text{g ml}^{-1}$) used in the immunoblot was goat anti-rabbit IgG (heavy- and light chain) (Zymed Laboratories Inc., San Francisco, CA) conjugated with alkaline phosphatase. A BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium salt) substrate kit (Zymed Laboratories Inc.) was used to visualize the conjugate.

Liquid crystal

DSCG (Cromolyn, CAS#: 15826-37-6) was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA) and prepared as a solution in DW, as 12–14% w/v, and adjusted to pH 7.2. The solutions were filtered ($0.2 \mu\text{m}$) to remove impurities [the solutions form the nematic *N*-phase between 2 and 22.5°C , as determined by differential scanning calorimetry and textural observations (data not shown), in agreement with prior work] (Lydon 1998).

Optical cassettes

Glass cassettes for evaluating optical events were prepared as follows. Optical quality glass was sonicated in 1% Alconox (v/v in distilled water) for 10 min at 60°C , air dried in a clean room, and spin-coated with 3% v/v polyimide SE-7511 (Nissan Chemical, Japan). The polyimide surfaces were then treated to create an alignment layer for the LCLC by rubbing with 3-mm felt, at 25 mm s^{-1} and 90 kg m^{-2} . The slides were dried at 100°C for 2 min and then baked at 180°C for 1 h. Cassettes were constructed by placing two treated slides together (treated surfaces juxtaposed) with a fixed gap distance set at 20 mm and $2.0 \times 3.5\text{-cm}$ chambers using polymer spacers. LCLC immune aggregate mixtures were allowed to flow (parallel to the rub direction) into preassembled cassettes (LXD, Cleveland, OH).

Microscopy

Fluorescent confocal microscopy was used to track the location and size of fluorescently labelled particles. We used a multi-channel Olympus IX81 Confocal Microscope (Olympus America, Melville, NY) capable of simultaneous imaging of the same region of interest in both fluorescent confocal and polarizing modes. The assembled cassettes were viewed between two crossed polarizers (90° to each other) to image the LC texture and record the transmitted light intensity caused by director distortions around the complexes. The initial director orientation in the

LCLC cassette was aligned either parallel or perpendicular to the polarizer, so that in the absence of distortions, the polarizing-microscope texture was dark (no light transmittance). Cassettes were filled with LCLC-containing microbe or microbe with AB, placed onto the stage of the confocal microscope, and examined for fluorescent, birefringent or fluorescent and birefringent (co-localized) foci. Fluorescence was recorded in one channel while birefringence was recorded in another channel. Fluorescence was used to track the location and size of labelled particles. Polarizing microscopy was used to detect and measure the director distortions around the complexes.

To measure the sensitivity of the AB–spore aggregation, AB samples were mixed with spores of decreasing concentrations. The total number of distortions visualized by polarizing microscopy was counted per spore concentration. Mean numbers of events per square centimetre were reported $\pm\text{SE}$.

Experimental

Serial dilutions (1:10, from $8 \log_{10}$ to $4 \log_{10}$) of BA spores and 1.0 mg ml^{-1} anti-BA AB, respectively, were added to the cromolyn solution, so that the final concentration of cromolyn in water was 12–14 wt% (w/w). The two mixtures were combined in equal proportions to allow for the formation of immune complexes in 12–14% cromolyn. *Bacillus atrophaeus* spores, anti-BA AB, or anti-streptavidin AB served as controls in the LCLC system.

Statistics

Data were evaluated by a one-way analysis of variance (ANOVA). Dunnett's test was used *post hoc* to compare the means of the control group (spores alone) with the other groups to determine significant differences ($P < 0.05$). The null hypothesis was that there is no difference between the experimental and control groups.

Results

We used BA spores (one of several potential anthrax surrogates) as a representative bioterrorism agent. The activity of the anti-BA AB was confirmed in the laboratory by immunoblot prior to use (Fig. 1a). An $1.0\text{-}\mu\text{g ml}^{-1}$ concentration of the anti-BA AB was found to have a detection sensitivity of $6 \log_{10} \text{ ml}^{-1}$. Data obtained using the LCLC detection system (with the same anti-BA AB) suggested a detection sensitivity of $5 \log_{10} \text{ ml}^{-1}$ (Fig. 1b).

The polyclonal anti-BA AB was next labelled with FITC and used to track BA–anti-BA immune aggregates by fluorescence microscopy. BA–anti-BA immune complexes were concurrently visualized by polarizing microscopy,

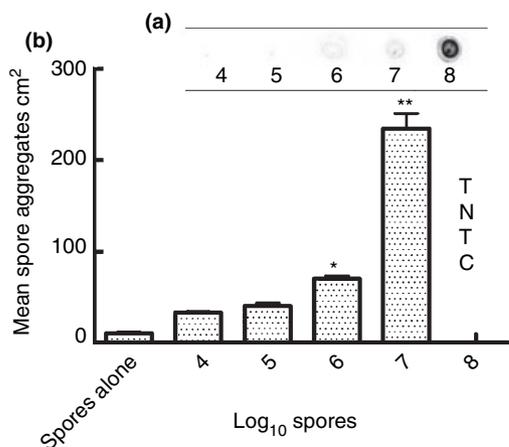


Figure 1 Detection of *Bacillus atrophaeus* (BA) by anti-BA antibody (AB). (a) Immunoblot results using $1.0\text{-}\mu\text{g ml}^{-1}$ anti-BA AB with 1:10 dilutions of BA spores (pinpoint spots above positions 4 and 5 are pencil marks indicating spore deposition site). (b) Mean spore aggregates ($n = 6$) detected by polarized light transmittance on slides at $4\times$ microscopy (statistical differences between group means $**P < 0.01$ and $*P < 0.05$). The 8 \log_{10} column is represented as the spore aggregates were 'too numerous to count' (TNTC).

evaluating the alignment (texture) of the LCLC. BA–anti-BA immune complexes co-localized as fluorescent (Fig. 2a) and birefringent (Fig. 2b) events ($60\times$ magnification) suggesting that BA–anti-BA aggregates were the cause of the LCLC misalignment. Figure 2c demonstrates that immune complexes formed from $7 \log_{10} \text{ ml}^{-1}$ BA spores and 1.0 mg ml^{-1} anti-BA AB could be visualized by low magnification ($4\times$) polarizing microscopy. Importantly, anti-streptavidin AB in DSCG did not aggregate BA spores and did not result in fluorescence or polarized light transmission (data not shown). Furthermore, spores

alone also did not result in fluorescence or polarized light transmission (data not shown).

Conclusions

The LCLC biosensing technology is based on AB capture of microbes, coupled with the sensitive detection and amplification properties of LC. In this real-time bacterial endospore detection technique, the detecting and amplifying medium represents a mixture of the water-based LLC and a highly specific anti-spore AB. When the targeted spores were added to the LCLC–AB sample, spore–AB aggregates (agglutinating immune complexes) formed. Once the growing immune complexes exceed a critical size determined by the elastic properties of the LCLC and the properties of the LCLC–complex interface, they trigger director distortions (Shiyanovskii *et al.* 2005). These distortions are easy to detect optically, by viewing between two crossed polarizers (precisely the way we view the LC-based flat panels in modern TV and laptop monitors). The samples containing spores alone, no spores, and the samples containing an irrelevant AB, did not transmit polarized light (as the director remained uniform in the absence of immune complexes). In contrast, immune complexes, whose dimensions exceed the critical size, are immediately seen as bright director distortions as polarized light passed through the disturbed LCLC around each immune complex. The LCLC-based detection of spores was confirmed using fluorescently labelled AB to co-localize birefringent aggregates with fluorescent ones (Fig. 2a, b). Furthermore, LCLC distortions produce optical events, which are typically larger in size than the actual immune aggregates because the immune aggregate-directed LC alignment extends outwards from the immune aggregate due to the elastic nature of the LC. In

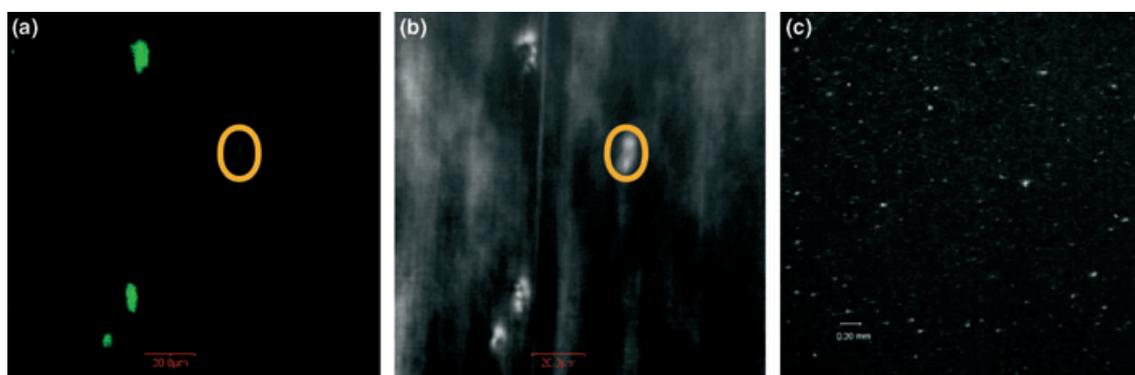


Figure 2 Co-localization of fluorescent (a) and birefringent (b) reactions caused by *Bacillus atrophaeus* (BA)–anti-BA antibody (AB) immune complexes ($60\times$, scale bars $20 \mu\text{m}$). (c) A low-magnification ($4\times$) view of the BA–anti-BA AB immune complexes viewed between crossed polarizers (scale bar 0.20 mm). Circled spots (a and b) identify aggregates undetected by fluorescence, but clearly identified by viewing between crossed polarizers.

other words, the director distortions occur over length scales substantially larger than the size of the inclusion that caused them (Shiyanovskii *et al.* 2005), e.g. the LC distortion is amplified and may permit detection of relatively small immune aggregates long before traditional immunoassay systems can report detection. Thus, most spores and AB, being individually too small to perturb the director field, will remain 'invisible' in the uniform LC background, whereas spore-immune complexes will be amplified by distortions and reported by optical transmission. The LCLC biosensor functions in real time since the formation of immune aggregates occurs within seconds of initiation and the timescale of director distortion, at length scales between 1 and 10 μm , is less than 0.1 s (Shiyanovskii *et al.* 2005).

This assay method, however, can potentially cause false-positive results, as relatively large contaminants (e.g. $>5 \mu\text{m}$), may be able to produce director distortions. We propose two methods to prevent false-positive results due to contaminating particles. First, one could remove particles larger than 5 μm by filtration, prior to sample entry into the optical cassette. Second, assay progress could be evaluated by real-time imaging of the developing immune complexes within the optical cassettes. Because immune complex formation in this assay results from the aggregation of spores by AB, individual spores evolve into aggregates over time. Foreign particles, or preformed aggregates, would typically enter the optical cassette fixed in size, not increasing their size with time. Initial images could be subtracted from final images so as to identify only particles that had evolved from spore aggregation. Both of these methods involve minimal sample preparation.

We have demonstrated that immune complexes formed from BA (an anthrax surrogate) and anti-BA AB can be detected within minutes when formed in LCLC of DSCG, and viewed with crossed polarizers. Decreasing spore concentration resulted in decreased fluorescent and birefringent signals. Individual reactants were not detected by fluorescence or birefringence. Birefringent foci resulting from immune aggregate distortion of the LCLC are larger than the actual immune aggregates that caused them. This distortion beyond the immediate area in which the immune aggregate is formed results in an amplified signal compared with its fluorescent counterpart, thus increasing the optical sensitivity of this new reporting system. The chosen AB guarantees the specificity of the system and use of the LC produces real-time results. The system permits detection of immune aggregates, approaching a detection sensitivity limit of 10^5 spores ml^{-1} . We anticipate that use of high-affinity monoclonal antibodies would increase assay sensitivity. Their use is the subject of ongoing investigations. Other current investigations

centre on assay development using complex and nonpristine sampling conditions. The use of low-magnification polarizing microscopy to image birefringent immune complexes suggests that a simple device, which uses AB capture of spores, and measures birefringence, could be engineered as a portable LC biosensor.

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