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Application of a real-time biosensor to detect bacteria in platelet concentrates☆

Boris Rotman* and Mindy A. Cote

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912, USA
BCR Diagnostics, Inc., 1062 E. Shore Road, Jamestown, RI 02835, USA

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Abstract

A spore-based biosensor for detecting low levels of bacteria in real-time has been recently developed. The system (termed LEXSAS, label-free exponential signal-amplification system) exploits spore's ability to produce fluorescence when sensing neighboring bacterial cells. We studied the LEXSAS as a possible approach for identifying bacterially contaminated platelet concentrates prior to transfusion because the system offers rapid analysis, high sensitivity, and low cost. If successful, this approach could reduce the risk of morbidity and mortality from transfusion-related bacteremia and sepsis. In this study, we used the LEXSAS for detecting bacteria in platelet concentrates spiked with *Bacillus cereus*, *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Streptococcus pyogenes*. Bacteria were separated from platelets using a 2-min procedure based on bacterial resistance to detergents and osmotic shock. The results indicate that the LEXSAS could be used to design a practical biosensor for identifying bacterially contaminated platelets in real-time.

Keywords: Bacteremia; Bacteria; Biosensor; Nanodetector; Platelet; Sepsis; Spore; Transfusion

Microbial surveillance of blood products intended for transfusion is essential for safeguarding public health. For example, routine screening of blood units for HIV and other viruses has presently reduced the risk of transfusion-related viral infections to less than 1 in a million [1,2]. On the other hand, the risk of morbidity and mortality from transfusion-related bacteremia and sepsis remains high [3,4] presumably because screening for bacterial contamination has not been generally instituted. In this regard, platelet concentrates (PCs) are of special importance because they are stored at room temperature for two to five days before transfusions. Under these conditions, PCs contaminated with even a single bacterial cell during phlebotomy may become heavily contaminated due to bacterial proliferation. Statistically, approximately 1 in 2000 PCs is known to be bacterially contaminated [4].

Although various strategies to identify bacterially contaminated PCs have been reported, no methodology combines rapid completion, high sensitivity, and low cost [5]. For instance, culturing methods can detect single bacterial cells but require one or more days for completion, while tests based on molecular biology are rapid and specific but labor intensive and relatively insensitive [5].

We report a study in which a newly developed real-time biosensor system (LEXSAS) was used to identify PCs spiked with different bacteria.

Principle of the spore-based biosensor. Microbial spores are ideal biosensor elements because their level of functionality far exceeds that of other cells previously used in biosensors [6]. A spore can be metabolically inactive (dormant) for prolonged periods of time while remaining responsive to environmental signals that initiate within seconds a germination cycle during which

☆ *Abbreviations:* Ala–Ala, L-alanyl-L-alanine; CFU, colony forming unit; IAS, image analysis system; LEXSAS, label-free exponential signal-amplification system; PC, platelet concentrate; SD, standard deviation.

* Corresponding author. Fax: 1-401-423-9701.
E-mail address: rotman@bcrbiotech.com (B. Rotman).

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different de novo biological activities emerge [7]. In the biosensor, dormant spores respond to presence of neighboring bacterial cells (the analyte) by producing fluorescent light signals [8].

The LEXSAS consists of microbial spores suspended in buffer containing a "germinogenic" substrate and diacetate fluorescein (DAF). The term "germinogenic" defines an extended group of enzyme substrates that are *not* themselves germinants, but produce a germinant upon enzymatic catalysis. DAF, the other substrate in the system, is a fluorogenic compound which produces fluorescent products when hydrolyzed by esterases.

The germinogenic substrate used in the study was L-alanyl-L-alanine (Ala–Ala), a dipeptide that does not itself induce spore germination, but produces L-alanine (a germinant) when hydrolyzed by aminopeptidases. Ala–Ala was selected under the premise that aminopeptidases could serve as universal bacterial markers. Consequently, the LEXSAS responds to bacterial cells (analytes) capable of hydrolyzing Ala–Ala. The system's response is a chain reaction caused by reiteration of the following sequential events:

1. The bacterial analyte hydrolyzes Ala–Ala to produce L-alanine, a specific spore germinant. A critical point is that spores do *not* recognize Ala–Ala as a germinant.
2. The newly formed L-alanine triggers spores surrounding the bacteria to generate de novo aminopeptidase activity and also de novo acetyl esterase activity.
3. The de novo aminopeptidase activity causes a chain reaction because it generates more L-alanine (from Ala–Ala) which in turn produces more de novo aminopeptidase activity. The de novo acetyl esterase activity is used as a reporter.
4. The chain reaction is monitored at time intervals using the fluorescent products from DAF hydrolysis by de novo acetyl esterase activity.

A feature of the LEXSAS is that assay sensitivity can be increased exponentially by reducing the system's volume [8]. We found that GF/A glass fiber filters (Whatman) provide ideal matrices for the LEXSAS apparently because the fibers form a mesh of discrete microscopic spaces within which spores and bacteria interact. The optimal number of spores in the filters was established empirically (data not shown).

Materials and methods

Materials. Sensor disks (6.35 mm diameter) were cut from GF/A glass microfiber sheets (Whatman). Diacetyl fluorescein (DAF) was synthesized and purified as previously described [9]. All other chemicals were of the highest purity available from Sigma. Reagent solutions were prepared using water distilled from a glass apparatus (Corning).

Bacteria. Six bacterial species known to contaminate PCs [10] were used in this study: *Bacillus cereus* (ATCC No. 27522), *Enterobacter cloacae* (ATCC No. 13047), *Escherichia coli* (K-12 3.300), *Pseudomonas aeruginosa* (ATCC No. 10145), *Staphylococcus aureus* (ATCC No. 12600), and *Streptococcus pyogenes* (ATCC No. 49399). With exception of *E. coli*, the bacteria were obtained from the American type culture collection (ATCC). Bacteria were spread on nutrient agar to obtain separate individual colonies. For testing, 2–3 colonies were suspended in 100 μl of sterile water, washed twice with sterile water, and resuspended in phosphate-buffered saline. The suspension was diluted with phosphate-buffered saline to obtain varying bacterial concentrations. A 20-μl volume of a diluted bacterial suspension was used to spike 5 ml of PC.

Platelet concentrates. Leukocyte-reduced PCs from single donors were obtained from the Rhode Island Blood Center. PCs were used between two and five days after phlebotomy and were kept in their original collection bag under agitation at room temperature (21–22 °C). Bacterially spiked PCs were placed in tissue culture flasks and agitated overnight at room temperature. The number of colony forming units per milliliter in the spiked PCs was determined before testing in the biosensor.

Test sample preparation. Bacteria were rapidly isolated from spiked PCs using a procedure based on intrinsic bacterial resistance to detergents and osmotic shock. Namely, a 0.5-ml platelet sample (in a 1.7-ml microcentrifuge tube) was mixed with 20 μl of 5% Triton X-100, incubated at room temperature for 30 s, and then centrifuged at 13,600*g* (Fisher micro centrifuge) for 15 s to pellet together platelets and bacteria. The supernatant was removed and the pellet was resuspended with 1.5 ml sterile water to osmotically lyse the platelets. The suspension was incubated at room temperature for 30 s and then centrifuged at 13,600*g* for 30 s. The supernatant was removed and the pellet was resuspended with 50 μl sterile water. At this stage, microscopic examinations indicated that the pellets consisted of intact bacterial cells and small particles (under 0.5 μm diameter) most likely derived from platelet debris. A 2-μl sample of the pellet suspension was used for testing in the biosensor. It should be noted that sample preparation is a crucial step for subsequent testing in the LEXSAS because it eliminates potential interfering substances present in either the platelets or the plasma. The entire procedure for sample preparation required about 2 min.

Microbial spores. Spores of *B. cereus* were prepared as previously described [11]. Spore preparations were heated at 80 °C for 10 min in order to reduce fluorescence baseline of the LEXSAS. Heated spore suspensions stored at about 4 °C were used for periods ranging from 3 to 8 months.

Biosensor operation. For testing, each glass fiber disk received a 12-μl volume from a 40-μl reaction mixture containing 2 μl of the test sample, *B. cereus* spores (4.5×10^7 CFU), 100 mM Tris–NaCl buffer (pH 7.5), 0.9 mM Ala–Ala, 0.47 mM adenosine (or inosine), and 27.5 μM DAF. Disks were incubated in a moist chamber at 37 °C for variable time intervals ranging from 10 to 20 min. After incubation, fluorescence images of the disks were captured and quantified using an image analysis system (IAS) previously described [12]. Disk fluorescence is expressed as "sum of fluorescent pixels" measured inside a square region of 3600 pixels in the image center. PCs were tested using duplicate samples.

Bacterial enumeration. Colony-forming units of bacteria were measured in duplicates by plating serial dilutions of spiked PCs on nutrient agar. After overnight incubation, plates showing between 200 and 500 colonies were used for counting and calculating colony forming units per milliliter.

Kinetics measurements. Glass fiber disks received a 12-μl volume each of a *B. cereus* spore suspension (6×10^9 CFU/ml) and then were dried at room temperature overnight. For testing, a disk was placed under the IAS on a thermostated chamber at 38 °C and images were captured every 20 s. The reaction was initiated by adding 12 μl of a mixture (equilibrated at 37 °C) containing 100 mM Tris–NaCl buffer (pH 7.5), 0.9 mM L-Ala, 0.47 mM inosine, and 27.5 μM DAF. A set of disks receiving mixtures with only buffer and DAF were used as controls.

Results

Table 1 shows results of an experiment in which PCs spiked with *E. cloacae* were measured in the biosensor. The data show that significant fluorescence is produced only in disks containing *both* spores and *E. cloacae* (first row). These disks had 47 times more fluorescence than disks with samples from sterile PCs (i.e., $S/N = 47$). From data correlating disk fluorescence with colony forming units per milliliter in spiked PCs, a value of 100 fluorescent pixels/disk corresponds to about 25 CFU/disk.

To evaluate the minimal time required for testing in the LEXSAS, we conducted kinetic experiments as described above in Materials and methods. The results (Fig. 1) show that: (1) a burst of fluorescence occurs 2 min after adding germinants to the system; and (2) the fluorescence increases 167-fold during the subsequent 2 min. Not clearly shown in Fig. 1 is that at the 2-min reading, germinating spores have already 5-fold more fluorescence than control spores.

The ability of the system to identify PCs spiked with different bacterial strains was tested using six microorganisms known to contaminate platelets intended for transfusion [10]. As shown in Table 2, all samples from bacterially spiked PCs have fluorescence values 30–100-fold higher than samples from sterile PCs. Samples from PCs with *S. aureus* gave lower fluorescence signals than the other samples, but still had considerably higher fluorescence than samples from sterile PCs.

Table 1
Bacterial detection using PCs spiked with *E. cloacae*

Tested sample	Sum fluorescent pixels/disk[a]	S/N[b]
PCs + *E. cloacae*	56,896 ± 648	47
Sterile PCs	1204 ± 28	—
Control: PCs + *E. cloacae*—no spores	2136 ± 168	—
Control: sterile PCs—no spores	16 ± 0.0	—
Sterile water	24 ± 8	—

[a] Average sum of fluorescent pixels per disk ± SD of the mean. See Materials and methods for experimental details.

[b] Signal-to-noise ratio: average fluorescence of disks with PCs + *E. cloacae* divided by that of disks with sterile PCs.

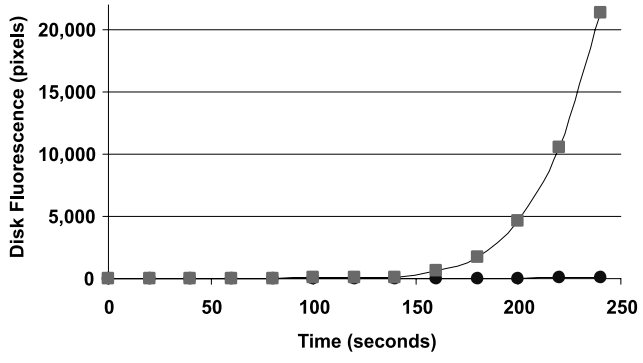

Fig. 1. Kinetics of the biosensor. *Squares*, Fluorescence of spores contacting buffer with DAF and germinants at time zero. *Circles*, Fluorescence of spores contacting buffer with only DAF at time zero. See Materials and methods for experimental details.

Table 2
Bacterial detection using PCs spiked with different bacterial analytes[a]

Bacterial analyte	Sum fluorescent pixels/disk[b]	S/N[c]
B. cereus	24,630 ± 1967	68
E. cloacae	33,737 ± 10,933	94
E. coli	30,810 ± 3527	86
P. aeruginosa	38,098 ± 17,283	106
S. aureus	11,188 ± 136	31
S. pyogenes	28,908 ± 2848	80
Sterile PCs	360 ± 351	

[a] Values represent an average of results from four or more experiments.

[b] Average sum of fluorescent pixels per disk ± SD of the mean. See Materials and methods for experimental details.

[c] Signal-to-noise ratio: average fluorescence of samples from spiked PCs divided by that of samples from sterile PCs.

Discussion

The results presented above show that the LEXSAS is capable of identifying PCs spiked with each of six different bacterial strains of clinical significance as platelet contaminants. The system appears to be robust because PCs spiked with bacteria give at least 30-fold more fluorescent signal than sterile PCs. Moreover, the system's response exhibits remarkable kinetics: seconds after the germination signal, a burst of fluorescent light appears and increases exponentially 167-fold within 2 min (Fig. 1).

It should be noted that the sample preparation developed for this study allowed us to separate bacteria from PCs using a relatively simple method requiring about 2 min. Probably the methodology can be extended to many other bacteria because it is based on intrinsic bacterial resistance to detergents and to osmotic shock.

In addition, the results support our premise that aminopeptidase activity can be used as a general bacterial marker in the LEXSAS. This agrees with results of a 1967 study in which profiles of aminopeptidase activity were used for bacterial identification [13]. In this regard, although Ala–Ala is a suitable germinogenic substrate for the tested bacteria (which included both Gram positives and negatives), it will be necessary to extend the study to about 15 other bacterial strains known to contaminate PCs.

In conclusion, these preliminary results using a spore-based system indicate the feasibility of designing biosensors with considerable better performance than the

glass fiber prototype used for our study. A real-time biosensor sensitive to single bacterial cells would be ideal for surveillance of blood products prior to transfusion.

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