Biosensors and Bioelectronics 41 (2013) 538–543

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Visible, colorimetric dissemination between pathogenic strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* using fluorescent dye containing lipid vesicles

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ARTICLE INFO

Article history:
Received 4 June 2012
Received in revised form
15 August 2012
Accepted 13 September 2012
Available online 23 September 2012

Keywords:
Pseudomonas aeruginosa
Staphylococcus aureus
Carboxyfluorescein
Lipid vesicles
Selective sensitivity
Toxins

ABSTRACT

This paper describes a biosensing concept for exotoxins secreted by *Staphylococcus aureus* and *Pseudomonas aeruginosa* based on the toxin mediated breakdown and subsequent fluorescent dye release from phospholipid vesicles (liposomes). The sensitivity of vesicles to toxins was tuned by altering the lipid and fatty acid composition of the membranes such that vesicles could be engineered to respond to toxins/enzymes from *S. aureus* only; *P. aeruginosa* only; and both *S. aureus* and *P. aeruginosa*. Nineteen types of vesicle were made with varying compositions of phosphocholine (PC), phosphoethanolamine (PE), cholesterol and the photo-polymerizable ampiphile 10,12-tricosadiynoic acid (TCDA). The selectivity of the vesicles was measured via a simple fluorescence "switch on" assay. Sensitivity of the vesicles to 40 clinically derived strains of *S. aureus* and *P. aeruginosa* was also demonstrated. This work suggests that this technology could be utilised in a diagnostic tool to discriminate between the species of *S. aureus* and *P. aeruginosa* in wound dressings.

1. Introduction

Bacterial infection in wounds, especially burn-related wounds in children, is a major clinical concern in hospitals (Church et al., 2006). Young children suffering from burns are particularly vulnerable to, and at risk of infection, largely due to an immature immune system and thin dermal layer. Early detection of infection in children with burns is difficult, as the common symptoms: pyrexia, raised C-reactive protein and white blood cell count are all found in children with systemic inflammatory response syndrome (SIRS) following a major burn. Technology exists for diagnosing infection, but this is generally based on culturing of wound swabs, with results coming from central hospital reference laboratories in 24–48 hours (in the UK). However, young children can become acutely sick in as little as 8 hours, due to complications resulting from infection such as toxic shock syndrome (Young and Thornton, 2007; Jones, 2006; Laabei et al., 2012). Nucleic acid based sensing of bacteria such as PCR (Kubista et al., 2006) has a role, but requires highly skilled operatives, expensive equipment and is prone to contamination. Again, due to the organisation of hospital microbiology services, results from such a test often take 24 hours or more. This requires a reliable and robust sensor that can monitor the microbial state of wounds to detect the infection, and if possible to provide the identity of pathogenic species involved in case of wound infection *in situ*.

Two main species of pathogenic bacteria commonly found in burn wound infections are *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Branski et al., 2009). *S. aureus* is the gram-positive pathogen persistently colonized on human skin as a part of the normal flora. Most *S. aureus* strains are known to be pathogenic due to their secreted virulence factors including Pore Forming Toxins (PFTs) such as alpha, gamma and delta toxins (Song et al., 1996; Potrich et al., 2009; Verdon et al., 2009), Panton–Valentine Leukocidin (PVL) exoproteins (Tseng et al., 2009), epidermolytic toxins (Bailey and Redpath, 1992) and Toxic Shock Toxins (TST) (Dinges et al., 2000; Frame et al., 1985). *P. aeruginosa* is the gram-negative opportunistic pathogen responsible for fatal infections in patients with cystic fibrosis and immuno-suppression (Branski et al., 2009). It is also found in human skin flora and associated with virulence factors for the suspected lysis of healthy eukaryotic cells and tissue matrices upon infection. PFTs of *S. aureus* and lipid degrading enzymes such as esterase and phospholipases of *P. aeruginosa* are shown to be able to lyse the cell membrane of healthy mammalian cells *in vivo* and *in vitro* (Songer, 1997; Liu, 1974; Dinges et al., 2000; Arpigny and Jaeger, 1999). The different mode of action of toxins associated with *S. aureus* and *P. aeruginosa* in terms of their interaction with phospholipid membranes has been investigated previously using a Tethered Bilayer Lipid Membrane (TBLM) on gold surface using spectroscopic and optical analysis (Thet and Jenkins, 2010; Thet et al., 2011). The differential response of bacterial toxins with the membrane gave information on the mechanism of membrane degradation by primarily *S. aureus* toxins and primarily *P. aeruginosa* toxins.

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http://dx.doi.org/10.1016/j.bios.2012.09.019

Recently the development of a prototype wound dressing using lipid membranes to detect bacterial toxins and thus detect wound infection by pathogenic bacteria was demonstrated by chemical attachment of the lipid vesicles containing fluorescence dyes onto nonwoven fabrics (Zhou et al., 2010, 2011; Jenkins et al., 2011; Jenkins and Young, 2010). In this paper, we report that by changing the lipid and fatty acid composition of phospholipid vesicles it is possible to 'tune' their response such that they are primarily susceptible to secretion toxins such as phospholipase from *P. aeruginosa,* or delta toxin secreted by *S. aureus* or to both toxin types. Hence, it is possible to obtain some initial information on the toxins produced by the bacteria – information that could be used by clinicians to help inform diagnosis and choice of antibiotic/antimicrobial in the treatment pathway.

2. Materials and methods

2.1. Materials and vesicle synthesis

Lipids used in making vesicles were 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) from Avanti Polar Lipids, USA. Cholesterol, 10,12-tricosadiynoic acid (TCDA) and 5(6)-carboxyfluorescein were purchased from Sigma–Aldrich, UK. TCDA is a synthetic polymerizable lipid which has been used to stabilise the vesicles by lateral cross-linking within the lipid bilayer after exposure to UV light. All the lipids, cholesterol and TCDA were used without further purifications. Triton X-100 (Sigma–Aldrich, UK) is a non-ionic surfactant which solubilises, disintegrates and reduces the lipid vesicles into micelles with simultaneous release of encapsulated carboxyfluorescein. A ten-fold dilution of 0.1% solution of triton X-100 was used as a positive control during the plate reading assay experiments. HEPES buffer was prepared according to standard protocol and used as a negative control for the stability of vesicles in the absence of bacteria and Triton X-100.

In the vesicle preparation, lipids, cholesterol and TCDA were individually mixed in chloroform to 100 mmol dm^{-3}. They were then mixed together to a desired composition to a total volume of 100 µl and dried under nitrogen before putting inside the vacuum chamber at 10^{-3} bar for 1 hour. Thoroughly dried lipid mixture was then rehydrated with 5 ml of HEPES buffer with pH 7.3 containing 50 mmol dm^{-3} carboxyfluorescein. After rehydration, the lipid solution was heated in a hot water bath at 75 °C for 10 min before three freeze-thaw cycles. The lipid-dye solution was extruded three times through a polycarbonate membrane of 100 nm diameter pore size using a LF-50 Lipofast extruder (Avestin, USA). Finally, the extruded vesicles were purified using a DNA grade Sephadex NAP-25 column (GE Healthcare, UK). Lipid vesicle compositions containing TCDA were placed in a quartz vial and exposed to UV-light (254 nm), for a total of 12 s in a commercial flood exposure UV source (Hamamatsu, Japan), after storage at 4 °C for at least 2 hours. (Flow chart describing the process of vesicle preparation is presented in Fig. S9 in Supplementary information section).

2.2. Type of vesicles

In order to empirically elucidate the role of the various vesicle components on their sensitivity to different bacteria, five classes of vesicles were studied (A–E, Table 1), each with one component being varied. Type A vesicles were primarily composed of shorter chain DPPC lipids, with varying concentration of cholesterol (0, 10, 20 and 30 mol%); Type B were as Type A but with the addition of a photopolymerizable component TCDA; Type C were composed of longer chain DSPC lipids with varying cholesterol concentration; Type D were also DSPC lipid based, with a fixed mol% of cholesterol and varying TCDA concentration; and finally Type E were composed of DSPC lipids, with a fixed mol% of TCDA and varying cholesterol concentration.

Mean increment in fluorescence of respective vesicles before and after inoculation in Hepes buffer, *P. aeruginosa* PAO1 and *S. aureus* MSSA476 at 37 °C was also tabulated in Table 1 (see Section 2.4 for experimental detail). The rationale of this work is that cholesterol, chain length and presence of TCDA all profoundly

Table 1
Vesicles with mol% of their phospholipid/fatty acid content, and their fluorescent response in Hepes buffer and pathogenic bacteria (Arbitrary classification A–E depending on principal lipid component and variable i.e. cholesterol or TCDA; vesicle 17 belongs to both type D and E, and vesicle 11 belongs to both type C and D).

Vesicles (Types and lipid compositions in mol%)						Mean fluorescent increment after 18 h (a.u.) in medium		
Class	**Vesicle types**	**DPPC**	**DSPC**	**Cholesterol**	**TCDA**	**Hepes (buffer)**	**PAO1**	**MSSA476**
A	Ves 1	88	–	10	–	35,995	1,18,388	76,558
	Ves 2	78	–	20	–	2,035	1,03,842	39,610
	Ves 3	68	–	30	–	1,126	41,546	10,905
B	Ves 4[b]	73	–	0	25	23,593	1,38,137	1,19,276
	Ves 5[b]	63	–	10	25	34,885	1,03,529	84,549
	Ves 6[ab]	**53**	-	**20**	**25**	**13,522**	**99,245**	**83,047**
	Ves 7[b]	43	–	30	25	1,019	35,204	76,162
	Ves 8[b]	33	–	40	25	834	14,087	81,173
	Ves 9[ab]	**23**	–	**50**	**25**	**85**	**11,430**	**83,671**
C	Ves 10	–	88	10	–	863	19,356	5,620
	Ves 11[a]	–	**78**	**20**	–	**758**	**52,905**	**9,818**
	Ves 12	–	68	30	–	563	7,816	3,321
D	Ves 13[b]	–	73	20	5	29	60,060	34,819
	Ves 14[b]	–	68	20	10	531	51,096	41,539
	Ves 15[b]	–	63	20	15	767	42,839	62,152
	Ves 16[b]	–	58	20	20	2,686	55,310	87,173
	Ves 17[b]	–	53	20	25	1,989	60,864	91,885
E	Ves 18[b]	–	63	10	25	3,186	36,058	33,910
	Ves 19[b]	–	43	30	25	2,154	25,689	44,310

All DPPC and DSPC vesicles contain 2 mol% of DPPE and DSPE, respectively. Black bold numbers in table describe the distinctive composition of lipid vesicles which give the best selective fluorescence responses between *S. aureus* and *P. aeruginosa*.

[a] Vesicles used for selective sensitivity tests.
[b] Vesicles required UV cross-linking.