Endotoxin Testing as a Detection Method for Bacterial Biofilms

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Introduction

Microbial biofilms - structured consortium of bacteria that are embedded in layers of self-produced polymer matrices, largely composed of polysaccharide, protein and DNA – are well described and known problems for pharmaceutical water systems and medical devices. What is less well-researched is the association of biofilms with endotoxin, especially within the pharmaceutical and medical device context. Here the association of biofilms and endotoxin is of significance to the risks presented by biofilms to water systems and for patient risks in relation to medical devices. With water systems the detection of endotoxin may provide an early warning of a biofilm problem. While the screening of Water-for-Injection systems for endotoxin is a GMP requirement, other types of pharmaceutical grade water are not commonly sampled for endotoxin testing. The introduction of this type of testing may prove useful where there is a concern about biofilm formation. The same may also apply to medical devices, especially given the risk posed from endotoxin. Detachment of cells or cell aggregates, production of endotoxin, increased resistance to the host immune system, and provision of a niche for the generation of resistant organisms are all biofilm processes which could lead to infection.[1]

In both cases the impact of endotoxin will be variable given that lipopolysaccharide size and composition are highly dynamic and vary according to the strain and growth conditions which contribute to the way by which bacteria adapt to changing environments.[2] Nevertheless, endotoxin can potentially provide earlier warnings about biofilm developments than are possible with techniques like bioburden testing and assessments can also assist with the design of materials, especially those that do not readily bind endotoxin, and with in-use assessments.

This article discusses the association of biofilms and endotoxin; looks at the challenges this association poses for water systems and medical devices; and considers whether tests for endotoxin can function as part of a detection method to support an endotoxin control strategy.

Biofilms

Microorganisms are often found in dense communities called biofilms, and the biofilm is recognized as the most common state of bacteria given it is an adaptive mechanism against environmental stresses. Protection is provided to the microbial community through an array of secreted molecules termed extracellular polymeric substances that lead to a three-dimensional architecture, made up of polysaccharides, proteins, lipids, and extracellular DNA.[3]

Within a biofilm the behaviors of organisms are often different compared with the non-biofilm state (particularly planktonic cells) as a result of different genes being turned on or turned off. Differences with this stable microenvironment include the mechanisms of communication (like quorum-sensing-regulated mechanisms); development of mutations; and with the competition and co-operation between strains and species, both of which impact upon community function.[4] Another change that can occur with biofilms is with modifications occurring in lipopolysaccharide, the major component of all Gram-negative bacterial outer membranes and the release of which is commonly referred to as 'endotoxin'. Modification to the molecule can occur through the incorporation of a palmitate acyl chain into the lipid A part of lipopolysaccharide, as shown with Pseudomonas aeruginosa strains.[5] The significance of this to reduces host inflammatory response (of importance to the discussion about medical devices below) and to enhance the survival of biofilm communities when subjected to treatments.

Biofilms are not easy to detect, which is a factor of the slow release of cells and a reflection of conventional cultivation methods only being capable of measuring the number of living cells capable of growing on the chosen agar under the selection incubation conditions. Conventional bioburden methods are generally considered to be unreliable methods for assessing biofilms, especially from water systems.[6] Assessment of ATP content may be more reliable, in that research has shown biofilm ATP is proportional to the number of living cells in the biofilm and hence this can provide information on biofilm metabolic activity. However, data obtained for freely suspended cells does not provide a meaningful assessment of immobilized biomass growth (that is, the biofilm structure itself). An alternative means of assessment are endotoxin assays, as discussed below.

Biofilms and Endotoxin

Given that biofilms tend to contain predominantly Gram-negative bacteria it is unsurprising that there is an association between biofilms and endotoxin. Viable, non-dividing Gram-negative bacteria will harbor endotoxin, but because they are not detectable by culture they would have been sometimes overlooked as a source of lipopolysaccharide. Vincent et al. showed that bacterial counts within biofilms on hemodialyzer tubing correlated with endotoxin levels;[7] hence, it can be inferred, that surface associated endotoxin levels correlate with biofilm levels.[8] It also stands that certain molecules, like lipopolysaccharide, can be more particularly produced under biofilm conditions.[9] However, an association with endotoxin levels and biofilm release cannot be quantifiably determined.[10] Nevertheless, a level of endotoxin can be detected using established methods like the LAL test[11] and this has led some researchers to suggest the use of endotoxin detection to indicate the presence of biofilms.

Biofilms and Pharmaceutical Water Systems

Biofilms present a potential problem to pharmaceutical water systems, and a very real problem to poorly designed or maintained water systems.[12] Biofilms can cause blockages, such as biofilm-induced clogging limiting the efficiency of water flow (as measured by the Darcy scale) and the operation of filtration systems;[13] and biofilms cause increases to levels of microbial and endotoxin contamination.

Once a biofilm develops then an out-of-control situation is likely to emerge.[14] Of concern to pharmaceutical manufacturers is that a biofilm is often only detected sometime after its formation, from point-of-use samples, and even then, several excursions will be required to alert of the probability of a biofilm. In other words, by the time a biofilm is detected it will most probably have been significantly established for some time.

Biofilms in water systems arise from poor design, used as too low a water flow velocity in general, or the presence of areas where water flow reduces, such as 'dead legs'.[14] Biofilms also arise through contamination arising during poorly-executed maintenance, such as following changes to valves or where water systems are cut-into, such as to shorten, lengthen or alter

the direction of pipework.[14] Biofilms are difficult to treat, showing resistance to disinfectant chemicals.[15]

Biofilm and Endotoxin Risk to Surgical Implants

As well as water systems, biofilms also affect medical devices. Biofilm formation is of particular concern to the field of orthopedic surgery, with risks stemming during procedures from microorganisms found on the skin, hair, nose, and oral cavity of both patients and the operating room staff.[16] It stands that if various physical and chemical interactions between the surfaces of an implant and any infecting microorganism are favorable, then biofilm formation will commence, and infection is likely. There is also a chance of infection arising from host-circulating microorganisms. Treatment is very difficult, since bacterial biofilms show decreased tolerance to antibiotics (bacteria within biofilms are 20-1000 times less sensitive to antibiotic than free-living planktonic organisms);[17] and problems are most often addressed by removal of the implant; however, medical evidence indicates that contamination and biofilm risk is higher for a replacement implant in comparison with the risks associated with the initial implant.[18]

Bacterial endotoxin, in association with implants, presents a considerable risk (not least because Gram-negative bacteria have a high association with implant contamination).[19] Aside from the well-described dosage dependent stimulation of various inflammatory cytokines and signal transduction pathways (which can cause mild fever through to septic shock)[20] endotoxin, which binds well to a range of material surfaces, can additionally stimulate osteoclast differentiation[21] leading to bone resorption and ultimately loosening of the implant.[22] This has been shown through an association of abrasion particles and cytokine reactions, suggesting a connection between micro-breakdown of the implant and the presence of endotoxin. Different bacterial species have different potencies, in terms of the nanogram-scale quantities required in order to trigger a pyrogenic response as shown using techniques like capillary electrophoresis.[23] Moreover, endotoxin levels are generally high with greater Gram-negative bacteria species diversity.

In a different area, endotoxin released from biofilms has been shown to increase the bioincompatibility of dialysis liquids, leading to long-term inflammatory complications among dialysis patients. Biofilms have been found on the inner surface of silicone tubing inside dialysis machines. Endotoxin releasing from those biofilms increases the bioincompatibility of dialysis liquids and leads to long-term inflammatory complications among dialysis patients.[24] There are also similar biofilm risks with dentistry and the materials used to construct dental implants, such as acrylic resin which is commonly used as a temporary material, and endotoxin has a high affinity for titanium (which is often present in dental implants).[24] Bacterial endotoxin also has an affinity for the types of adhesives used to fix implants.

The phenomenon has triggered research into biomaterials that help to prevent the binding of both bacteria and lipopolysaccharide, such as oxidized zirconium alloys. However, most research is focused on resisting the binding of bacterial cells rather than endotoxin complexes. The finding also stresses the importance that such medical devices are 'free from endotoxin' (or at least below a clinically safe level), since a contaminated implant could lead to the same effect occurring. Such findings also emphasize the importance of establishing measures for the control of water used for processing medical devices, and of biodecontaminating materials.

Endotoxin Detection as a Signal for Biofilm Presence

Test for bacterial endotoxin, such as the *in vitro* Limulus amoebocyte lysate (LAL) assay, have several useful properties that make for a rapid method: sensitivity, specificity, and potential for adaptation to a quantitative format. These tests can be used to aid to identify the presence of Gram-negative bacteria earlier than conventional methods of bioburden testing and such tests overcome the limitations of bioburden tests in relation to culturability.

The effective use of an endotoxin test infers that biofilms are primarily composed of Gram-negative bacteria, which is especially likely in water systems.[25] The usefulness of this approach, in addition to rapidity, is that the level of endotoxin as measured by the assays likely closely parallels the density of bacteria throughout logarithmic growth,[26] since the shedding of cell-free endotoxin occurs spontaneously in addition to endotoxin release through cell lysis.[27] Therefore, atypical levels of endotoxin coupled with rising levels of endotoxin may signal establishment and development of biofilms; albeit that endotoxins have the ability to form agglutinate or micelles and micellar structures reduce the reactivity relative to LAL.[28] There are further limitations with such test methods, such as LAL method only providing the general activity of endotoxins in the sample, instead of the detailed structure or distribution profile.

Nevertheless the use of endotoxin test methods may help with the assessment of biofilm formation in water systems. Additionally, several researchers have used the LAL test to assess biofilm association with medical implants. For the assessment of medical devices, modifications to endotoxin testing may be required in order to enhance recovery. For example, Rioufol and colleagues found recovered endotoxin was greater when the biofilm was treated with a 1% sodium dodecyl sulfate solution.[29] As an alternative to LAL, researchers have used whole blood assays to examine scraping from medical devices, to look for endotoxin (given that stimulated mononuclear cells release IL-1beta in response to endotoxins). IL-1beta levels can be measured using ELISA methods. An advantage with alternative methods is that they can potential measure for the presence of "endotoxin-like" compounds, which differ from the lipopolysaccharides detected by the LAL assay.[30]

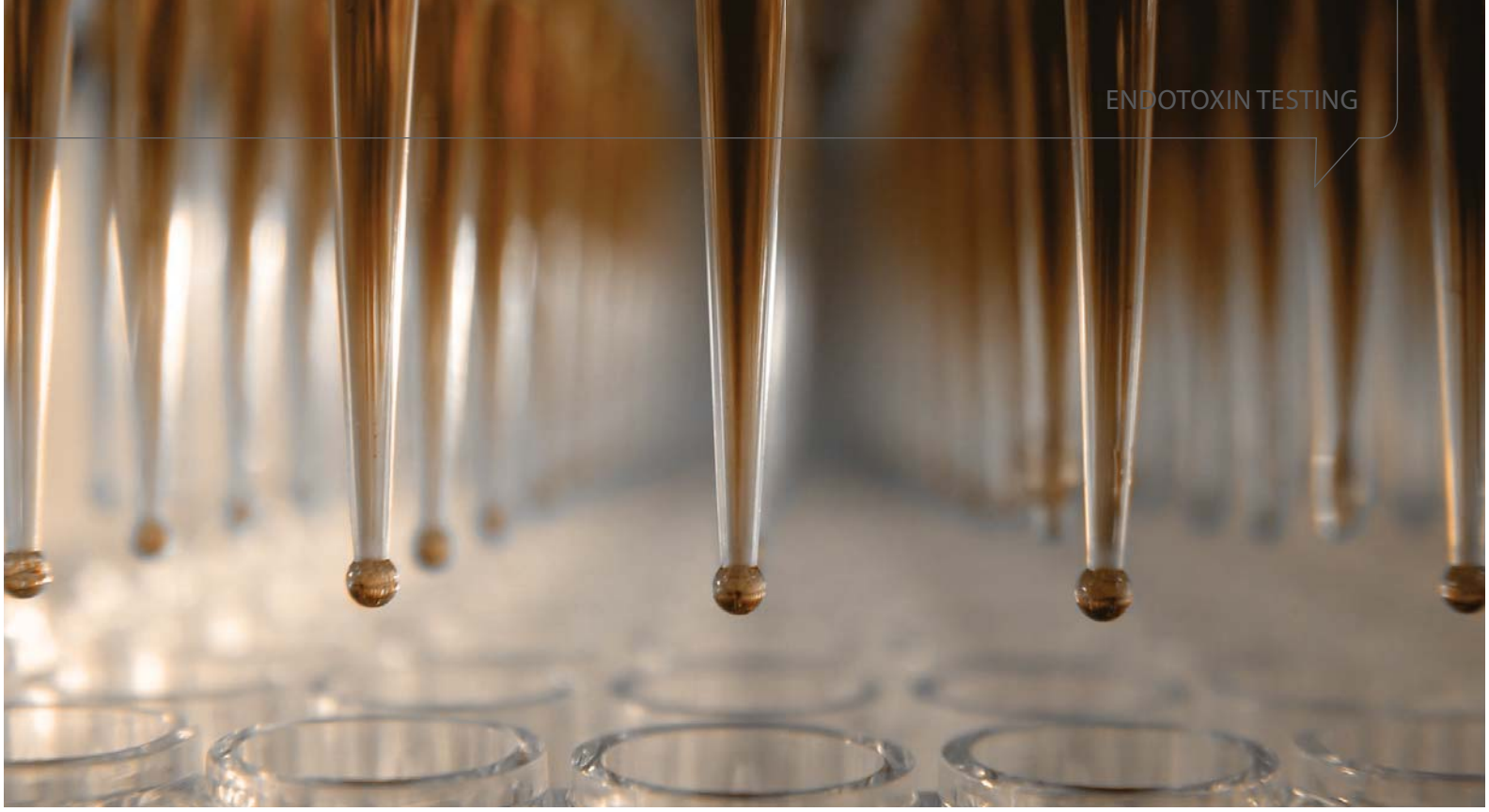
Summary

Endotoxin testing can play a role in the earlier detection of biofilms than is possible using conventional bioburden tests. This is on the assumption, albeit one supported by most literature, that much of the bacterial contamination of water systems, and to an extent medical implants, is by Gram-negative bacteria, which on lysis would release endotoxin.

The use of methods of detection, however, should not be used in lieu of control. As with most microbiological monitoring, assessing the levels of microorganisms and microbial by-products should only be used to confirm that systems have been correctly designed; that procedures have been appropriately followed; and that control is being maintained. With sufficient controls in place, endotoxin testing can provide a useful adjunct to measures to assess biofilms.

References

1. Hapiro, L., and Stallard, R. E. (1977) Etiology of periodontal disease, In Caldwell, R. C. and Stallard, R. E. (Ed.) A textbook of preventive dentistry. W. B. Saunders, Philadelphia, Pa, p. 74-80.
2. Trent, M.S., Stead, C.M., Tran, A.X., Hankins, J.V. (2006) Diversity of endotoxin and its impact on pathogenesis. J. Endotoxin Res. 12: 205–223.
3. Flemming, H. C. and Wingender, J. (2010) The biofilm matrix. Nature Reviews Microbiology 8: 623-633
4. Camilli, A. and Bassler, B. L. (2006) Bacterial small-molecule signaling pathways. Science 311: 1113-1116



5. Ciornei, C.D., Novikov, A., Beloin, C., Fitting, C., Caroff, M., Ghigo, J.M., Cavaillon, J.M., Adib-Conquy, M. (2010) Biofilm-forming Pseudomonas aeruginosa bacteria undergo lipopolysaccharide structural modifications and induce enhanced inflammatory cytokine response in human monocytes. Innate Immun. 16:288–301.
6. Fulford, M.R. Walker, J. T., Martin, M. V. and Marsh., P.D. (2004) Total viable counts, ATP, and endotoxin levels as potential markers of microbial contamination of dental unit water systems. British Dental Journal 196:157–159
7. Wirtanen, G., Storgårds, E., Mattila-Sandholm, T. (2003): Biofilms. Encyclopedia of Food Sciences and Nutrition (Second Edition), pp484–489
8. Vincent, F. C., A. R. Tibi, and J. C. Darbord (1989) A bacterial biofilm in a hemodialysis system. Assessment of disinfection and crossing of endotoxin. ASAIO Trans. 35:310-313
9. Raetz, C.R. and Whitfield, C. (2002) Lipopolysaccharide endotoxins. Annu. Rev. Biochem. 71:635–700.
10. Donlan, R. M. and Costerton, J. W. (2002) Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms, Clin. Microbiol. Rev. 15 (2): 167-193
11. Glorieux, G. Neirynck, N. Veys, N., Vanholder, R., Holland, S. P., Mathias, R. G., Morck, D. W., Chiu, J. and Slade, S. G. (2000) Diffuse lamellar keratitis related to endotoxins released from sterilizer reservoir biofilms. Ophthalmology 107:1227-1234
12. Sandle, T. (2017) The Problem of Biofilms and Pharmaceutical Water Systems, American Pharmaceutical Review, 20 (7): http://www.americanpharmaceuticalreview.com/Featured-Articles/345440-The-Problem-of-Biofilms-and-Pharmaceutical-Water-Systems/
13. Nicolella C, van Loosdrecht MC, Heijnen JJ (2000) Wastewater treatment with particulate biofilm reactors. J Biotechnol 80(1):1–33
14. Sandle, T. (2017) Design and control of pharmaceutical water systems to minimize microbial contamination, Pharmaceutical Engineering, 37 (4): 44-48
15. Sandle, T. (2013). Bacterial Adhesion: An Introduction, Journal of Validation Technology, 19 (2): 1- 10
16. Gallo, J., Kolar, M., Novotny, R., Rihakova, P., Ticha, V. (2003) Pathogenesis of prosthesis related infection. Biomedical Papers. 147 (1): p. 27-35
17. Gristina, A.G., Hobgood, C.D., Webb, L.X., and Myrvik, Q.N. (1987) Adhesive colonization of biomaterials and antibiotic resistance. Biomaterials; 8:423–6
18. Ehrlich, G. D., Stoodley, P., Kathju, S., Zhao, Y., McLeod, B. R., Balaban, N., Hu, F. Z., Sotereanos, N. G., Costerton, J. W., Stewart, P. S., Post, J. C., Lin, Q. (2005) Engineering approaches for the detection and control of orthopedic biofilm infections. Clinical Orthopedic Related Research, 437: 59-66
19. Mizgerd, J.P., Spieker, M.R., Doerschuk, C.M. (2001) Early response cytokines and innate immunity: essential roles for TNF receptor 1 and type I IL-1 receptor during Escherichia coli pneumonia in mice. J Immunol., 166:4042-8
20. Ragab, A. A., Van De Motter, R., Lavish, S. A., Goldberg, V. M., Ninomiya, J. T., Carlin, C. R., Greenfield, E. M. (1999) Measurement and removal of adherent endotoxin from titanium particles and implant surfaces. Journal of Orthopaedic Research, 17(6): p. 803-806
21. Jiang, Y., Mehta, C. K., Hsu, T. Y., Alsulaimani, F. H. (2002) Bacteria induce osteoclastogenesis via an osteoblast-independent pathway. Infection and Immunity, 70 (6): 3143-3148
22. Greenfield, E. M., Bi, Y., Ragab, A. A., Goldberg, V. M., Nalepka, J. L., Seabold, J. M. (2004) Does endotoxin contribute to aseptic loosening of orthopedic implants? Wiley InterScience, p. 179-183
23. Granfors, K., Jalkanen, S., Lindberg, A. A., Maki-Ikola, O., Von Essen, R., Lahesmaa-Rantala, R., Isomaki, H., Saario, R., Arnold, W. J., and A. Toivanen, A. (1990) Salmonella lipopolysaccharide in synovial cells from patients with reactive arthritis. Lancet 335:685–688
24. Marion-Ferey, K., Leid, J.G., Bouvier, G., Pasmore, M., Husson, G., Vilagines, R. Barão, V.A., Mathew, M.T., Yuan, J.C., Knoernschild, K.L., Assunção, W.G., Wimmer, M.A. et al. (2013) Influence of corrosion on lipopolysaccharide affinity for two different titanium materials. J Prosthet Dent. 110:462-70
25. Mahapatra, A., Padhi, N., Mahapatra, D., Bhatt, M., Sahoo, D., Swetlina, J., Dash, D. and Chayani, N. (2015) Study of Biofilm in Bacteria from Water Pipelines. J Clin Diagn Res.; 9(3): DC09–DC11.
26. Hoekstra, D., Van Der Laan, J. W., De Leij, L., and Witholt, B. (1976) Release of outer membrane fragments from normally growing Escherichia coli. Biochim. Biophys. Acta 455:889–899
27. Devoe, I. W. and J. E. Gilchrist, J. E. (1973) Release of endotoxin in the form of cell wall blebs during in vitro growth of Neisseria meningitidis. J. Exp. Med. 138:1156–1167
28. Erridge, C., Bennett-Guerrero, E. and Poxton, I. R. (2002) Structure and function of lipopolysaccharides. Microbes and Infection 4: 837–851
29. Rioufol, C., Devys, C., Meunier, G., Perraud, M. and Goullet, D. (1999) Quantitative determination of endotoxins released by bacterial biofilms, J Hosp Infect. 43(3):203-9
30. Marion-Ferey, K., Leid, J.G., Bouvier, G., Pasmore, M., Husson, G., Vilagines, R. (2005) Endotoxin level measurement in hemodialysis biofilm using "the whole blood assay", Artif Organs.;29(6):475-81