Pathogen enrichment from blood by biosensor linked peptides (PEBBLES) – a case study with challenging to diagnose *Candida albicans* yeast infections

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ABSTRACT

Nucleic acid-based approaches are state of the art when it comes to pathogen detection but there is a strong need for a rapid non-destructive approach for pathogen enrichment. We aimed to setup a novel pathogen enrichment tool by using synthetic human lactoferrin hLF(1-11) peptide in combination with biosensor-technology to detect and enrich *Candida albicans* from blood. Fluorescence microscopy confirmed high sensitivity and specificity of fluorescently labelled hLF(1-11) of *Candida* yeasts within 15 min incubation time in a background of human blood. Click chemistry showed that hLF(1-11) could be used for activation and immobilization on carboxylated and NHS activated surfaces.

Keywords: Pathogen enrichment, biosensor, bloodstream infection, Candida, *yeast.*

1. INTRODUCTION

Annually 5.3 million people succumb as a result of organ dysfunction caused by a dysregulated host response to infection (sepsis) [1,2]. The gold standard to diagnose sepsis is by blood culture that delays targeted therapy with up to 10 days. A third of the blood cultures remain negative, even in highly suspected cases [3,4]. On the contrary, when it becomes positive subsequent tests needs to be performed that further delay treatment by 3 days [5]. Nucleic acid-based approaches are state of the art and rapid when it comes to pathogen detection but although very sensitive, their performance is strongly hampered by the preceding process of nucleic acid purification. As nucleic acid-based approaches are destructive for the pathogen, extensive microbiological testing is ruled out. In the case of a sepsis, on average one microbial cell per millilitre blood is present [6]. During the nucleic acid extraction procedure there is a high likelihood that the few pathogenic cells are washed away, ultimately leading to a false negative result [6,7]. Hence, there is a strong need for a rapid, non-destructive approach for pathogen enrichment from blood.

The here proposed final application of pathogen enrichment by an antimicrobial peptide-coated biosensor to catch and detect microbial cells from blood will overcome the above-mentioned drawbacks of conventional blood culture and nucleic acid-based approaches. This will lead to a drastic reduction in diagnostic-time from many days to a few hours and will have a tremendous positive impact on patient care. To reach that goal we need to experimentally find the optimal peptide-structure for both pathogen-linkage and surface-linkage to the envisioned biosensor.

We aimed to combine a novel approach of pathogen enrichment by using human lactoferrin peptides with existing –but not yet optimal– biosensor-technology [8]. In this project we *i*) optimized lactoferrin peptides by modification of cysteine residues, *ii*) improved binding/absorption by glycosylation, followed by *iii*) investigating the binding capacity of a peptide-coated biosensor-surface using spiked blood samples.

2. STATE OF THE ART

State of the art in sepsis diagnostics are based on classical microbiological culture and nucleic acid detection, and both are entirely depending on a positive blood culture that can take up to 10 days. Nucleic acid detection is rapid

and sensitive for pathogen detection, but it is, unfortunately, strongly hampered by the preceding step of nucleic acid extraction from blood. In the case of a sepsis, on average one microbial cell per millilitre blood is present [6]. During the nucleic acid extraction procedure there is a high likelihood that this sole pathogenic cell will be washed away, ultimately leading to a false-negative result. Due to the use of a multi-copy target gene the sensitivity of nucleic acid pathogen detection is high, often below one microbial cell per reaction, however in those cases resistance detection is excluded as this depends on single-copy target genes. The here proposed final application of pathogen enrichment by an antimicrobial peptide-coated biosensor to catch and detect the microbial cells from blood will overcome the above mentioned drawbacks of conventional blood culture and nucleic acid based approaches. This will lead to a drastic reduction in diagnostic-time from many days to a few hours, a tremendous positive impact on patient care. To reach that goal we experimentally searched for the optimal peptide-structure to establish both a pathogen-linkage and surface-linkage to the envisioned biosensor. Pathogen-specific detection and enrichment of a wide variety pathogens using a single biosensor is the ultimate aim to significantly the turn-around-time of sepsis diagnostics.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

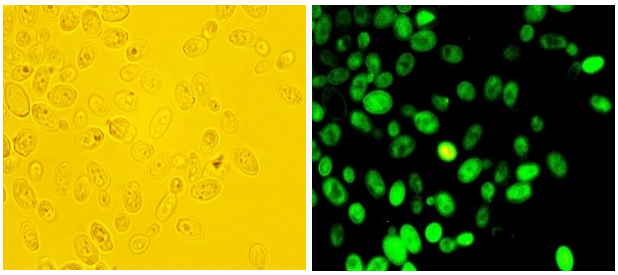
The pluripotent nature of the peptide-coated biosensor will have a high societal impact, not only from a health perspective, but also for food security, quarantine, agriculture, marine, salt and fresh water research as the biosensor device can be used for a plethora of diagnostic and screening purposes. Here we used the tangible example of improved sepsis diagnostics, but it is beyond dispute that there are many more healthcare-related applications for a peptide-coated biosensor. For example, detection of pathogens from cerebrospinal fluid, urine, the 'difficult to culture' pathogens from joint implants or wound swabs. The drastic improvement in early detection, from many days to just a few hours, will significantly reduce direct costs for healthcare in terms of patient care and reduced antibiotics use. The major benefit will be in the earlier detection of pathogenic micro-organisms, which will have the highest impact for the European society as patients can be treated much earlier with the correct antibiotic resulting in less hospitalization days and, in return, lowering the direct and indirect healthcare-related costs.

4. PROJECT RESULTS

Work package 1 – Modification of the lactoferrin peptide. Fmoc synthesis was used to assemble modifications on the N-terminal and C-terminal domains of the hLF(1-11) peptide. Click chemistry cyclocytine (DBCO-labelling) was used to alter the hLF(1-11) sequence by amino-acid substitutions and cyclic and glycosylated modifications. Modified peptides were tested for bioactivity by an *in vitro* killing assay and Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF) detection before immobilization on a surface.

Work package 2 – Binding of peptide to yeasts. Fluorescent microscopy (IF) was used to investigate the binding of the fluorescein isothiocyanate (FITC) labelled hLF(1-11) peptide to the *Candida* yeast, and also bacterial cells were evaluated. This showed that ≥98% of *Candida albicans* cell walls were stained within 15 min incubation time (Fig. 1). Similar results were obtained for other yeast pathogens, namely *Candida auris*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*. FITC-hLF(1-11) also bound to *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* bacteria within 15 min incubation time and in contrast to the yeasts these cells were entirely stained.

Figure 1. FITCH-hLF(1-11) stained *C. albicans* cells



Left: Light microscopy; right: fluorescent microscopy

Work package 3 – Binding of peptide to surface and pathogen. Whole blood samples containing FITC-hLF(1-11) were analysed by IF, confirming no binding between the peptide and human blood cells. The next step, was to detect reference, clinical *Candida* and bacterial species from spiked blood samples after lysis of blood cells. *Candida* and bacterial cells stained with FITCH-hLF(1-11), and Gram-positive and -negative bacteria that also may occur in bloodstream infections were observed to bound too. This observation implies a broader application of hLF(1-11) in pathogen detection.

MALDI-TOF was used to analyze the mass range of peptides and to investigate differences between hLF(1-11) and FITC-hLF(1-11) based peaks. Full protein extraction was performed, and the acquired spectra were analyzed by Flex Analysis prototype software v3.3 and identification was performed by MALDI Biotyper OC v3.1 (all Bruker Daltonik). Peaks occurred in a range between 2700 and 8000 m/z and showed a predominant peak of about 2850 m/z. Additionally, two other main peaks at a range of 3000 m/z with an intensity of about 104 a.u. were detected in FITCH-hLF(1-11) that was not observed in hLF(1-11).

These new peaks might appear due to the labelled probe bound to hLF(1-11).

Work package 4 – Development of prototype lactoferrin peptide-coated biosensor. For unique bioconjugation technology (SPR biosensing) an azido (N3)-derivated polycarboxylate sensor coating was used for immobilizing the cyclocytine (DBCO)-labelled hLF(1-11). Antimicrobial activity testing showed that click chemistry modified hLF(1-11) did not show attenuated activity. The specificity of the sensor was evaluated using two different borosilicate glass chips. *Candida* yeasts did not bound to uncoated sensors. The purity of labelled hLF(1-11) peptide was confirmed by HPLC. NHS preactivated sensor chips were activated with EDC/(S)NHS activation mix and incubated with DBCO-labelled hLF(1-11) peptide for 30 min. and repeated to obtain an increased yield of immobilization. Remaining (S)NHS esters were quenched for 15 min. with buffer and thereafter washed with water. Interaction analysis was induced by adding *Candida* yeasts, concentration up to 10^6 cells/mL for 1hour at 30°C. After washing the sensor was dried and calcofluor staining was used for microscopic inspection of the sensors. Results showed that *Candida* yeasts were covalently bound onto the sensor surface. Results indicate that the proposed approach can be effective in the detection of microorganisms and may be useful in the early prevention and treatment of sepsis.

5. FUTURE PROJECT VISION

5.1. Technology Scaling

During the ATTRACT Phase 1 project we worked on the experimental proof of concept (TRL3) to optimize the peptide hLF(1-11) by modifying cysteine residues for targeted attachment of *Candida* yeasts and site-specific orientation to link peptides to a biosensor-surface. We established collaborations within the Netherlands with the Technical University of Twente and the University of Wageningen, and with Xantec Bioanalytics in Germany. With this consortium we are currently fine-tuning the binding efficiency between hLF(1-11) and the biosensor.

As hLF(1-11) is not specific for a single pathogen we want to make use of pathogen-specific antibodies to identify the five most common yeast pathogens from human blood bound to the biosensor. For this, we need to prepare antibody libraries for *C. auris*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *Pichia kuzdriavsevii* (= *C. krusei*). For the most prominent pathogen *C. albicans* we recently generated a library in a joint project between the Westerdijk Institute and QVQ, an SME based at Utrecht that produces and develops llama-based antibodies. QVQ showed interest to be partner in a follow-up project of the current ATTRACT Phase 1.

The technology will be validated (TRL4) in the laboratory using voluntarily obtained human blood spiked with *Candida* cells. As the hospital setting is the foreseen environment we need to validate the technology there (TRL5), this can be primarily carried out at a single academic center, likewise that of our consortium partner UMC Utrecht, followed by a larger group of hospitals. In hospital diagnostic laboratories, samples needs to be tested retrospectively in order to benchmark the biosensor approach versus the gold standard of culture. Thereafter, a blinded and randomized multi-center study has to demonstrate the usefulness of the technology in the relevant environment (TRL6). This will be performed with guidance of professional societies, like the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the European Confederation of Medical Mycology Excellence Centers (ECMM). Next to ESCMID and ECMM pharmaceutical and diagnostic companies might be supportive via educational research grants and by joining the current ATTRACT Phase 1 consortium. To have the final system prototype demonstrated in an operational environment (TRL7) we need to join forces for an ATTRACT Phase 2 project with the current consortium partners mentioned above and ultimately with a commercial diagnostic manufacturer that operates globally. Note that we have excellent collaboration with the clinical diagnostic-focussed company Bruker Daltonik GmbH in Bremen, Germany, that may be interested in this purpose.

5.2. Project Synergies and Outreach

The project is based on the development of a biosensor capable of detecting pathogens, i.e. fungal, bacterial, viral, or parasitic. The biological-recognition component can be coated with different materials, including a mixture of hLF(1-11) peptides and pathogen-specific llama-antibodies (QVQ). The biosensor subsurface can be coated with materials that are available as carriers; accordingly a gold layer or silicone coating is preferred (Xantec and TU-Twente). The properties of the material must be in such a format that electrical differences can be measured without loss of power/resistance (Technical University Twente). For the newly developed signal processor that converts the digital signal converted into a binary code, collaboration with ICT/software developers is essential for optimal read-out of the data. Reach out will be realized via all partners, mostly by contributions to

conferences, focused meetings, and public science events. For the latter two we foresee to organize a professional and general public meeting during one larger event close to the end of ATTRACT Phase 2. For the public science event, we will request additional funding from the Royal Netherlands Academy of Arts and Sciences (KNAW). Next to the abstracts at the above-mentioned meetings, a scientific open-access publication is part of this project.

5.3. Technology application and demonstration cases

The objective of the project is to develop a system that can enrich and identify pathogens. It allows the detection of pathogens from blood, cerebrospinal fluid, urine, the 'difficult to culture' pathogens from joint implants, or wound swabs. Indisputable such a biosensor can be of great importance in all aspects of daily life, ranging from monitoring drink water quality, pathogen-detection in the food-chain, monitoring air-quality in indoor-and outdoor environments (for example detection of *Legionella* or SARS-CoV-2 in air).

5.4. Technology commercialization

Click chemistry has gained increasing popularity among nanotechnology companies as this method has fast reaction kinetics combined with excellent selectivity. This make this unique bioconjugation technology available for a plethora of applications, like surface plasmon resonance (SPR) biosensing. We demonstrated that labelled peptides could be used in a fast and selective manner in combination with MALDI-TOF detection. Opening opportunities to strengthen and expand current connections with the private companies QVQ that manufactures llama-antibodies for fast detection of pathogens and Bruker Daltonics for biomolecular characterization using MALDI-TOF and FTIR Both companies have expressed their interest in our approach of pathogen enrichment.

5.5. Envisioned risks

The peptide hLF(1-11) binds to a wide variety of human pathogenic fungi and bacteria. In the envisioned ATTRACT Phase 2 project we focus on targeted detection and enrichments of common sepsis causing fungal pathogens by using hLF(1-11) and llama antibodies. The consortium has extensive experience in generating antibodies to *Candida albicans*, globally the most frequent cause of candidemia together with*C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei* [9]. We identified three core risks for this, namely *i*) aspecificity of the generated antibodies towards other *Candida* species; *ii*) attenuated binding efficiency between pathogen-specific antibodies and the biosensor surface; and *iii*) binding between pathogen and their specific antibodies is irreversible. We can mitigate the impact of aspecific antibody binding towards the development of a broader detection of pathogenic *Candida* yeasts and not individual pathogens. When one of the other two core risks occurs we can convert the foreseen biosensor into a lateral flow assay (LFA), a product that is highly portable, non-laborious and cheap, but has the disadvantage that it is an end-point test that relies on circulating cellular components. At the same time, we would like to develop a targeted enrichment tool in order to obtain pathogenic cells from human blood, so that they can be further investigated by non-destructive (culture, antibodies) and destructive (molecular diagnostic) methods.

5.6. Liaison with Student Teams and Socio-Economic Study

New prototyping will be achieved by chain development between current consortium partners mentioned in section 5.1. The presence of existing parties and those who want to join in an ATTRACT Phase 2, being QVQ (Netherlands), Xantec (Germany), ProteoGenix (France), Bruker Daltonik (Germany) to combine interdisciplinary knowledge bases creativity and resources in robust, long-term intra- and intersectoral collaborations. Sectors with a large health component such as water (*Legionella*) and livestock (mastitis and resistant bacteria/fungi) are opening new options to explore. With NIKHEF, a partner in the ATTRACT Phase 1 project, we see options to explore chain development to form new idea's, using their student teams for technology transfer potential to establish valorisation and implement the product and services. In addition, students from the universities of Amsterdam and Utrecht may get involved.

6. ACKNOWLEDGEMENT

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