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ABSTRACT

The detection of microbial species and communities has always been an area of great importance. Rapid methods for microbial detection are especially important in the medicine, pharmacological, and food industry. The lack of rapid and cost-effective detection methods creates a challenge to control possible epidemics. New pathogenic microbes can cause a worldwide outbreak of diseases, pandemics. The majority of currently used detection methods for pathogens have low sensitivity and specificity. Human microbiota has been demanding and mostly unexplored area before modern high-throughput methods were established. These methods have elucidated a lot of connections between different diseases and composition changes of intestinal microbiota. However, sequencing a large number of samples takes a great deal of time, work and it requires centralized facilities. For microbiota, there are no actual comprehensive and rapid sensors. Biosensors have a great potential to respond to the challenges of microbial detection. The small physical size of the equipment, ease of use and operability outside centralized hospitals are appealing characteristics of this emerging class of diagnostic devices.

In this thesis, the applicability of newly developed rapid biosensors were evaluated as a tool for the detection of urinary tract infection and biomarkers. The studied methods are not based on traditional immunoassay detection technology, which usually relies on detecting a single antigen from the sample. Instead, a sensitive long lifetime luminescent europium label was used with different modulating probes to nonspecifically interact with simulated samples, but also with hospital samples. Chemical probes, or phages as biological probes, were used successfully to provide multiparameter luminescence data —a fingerprint from each sample. Phage-based methods were designed to meet the challenges of rapid detection of a single bacterial species. The second phage-based study was based on lysogenic phages and tested with hospital samples the assay time was reduced. The proof-of-principle method showed sensitivity and specificity at the 90% mark when compared to the standard culture method. The method was further developed and applied to detect specific biomarkers in a controlled chemical environment and finally to classify lethal prostate cancer against non-lethal ones from urine samples. The assay demonstrated a statistically significant difference between the two groups ($p<0.0014$). All these results indicate that the new method has potency for a variety of screening targets or research questions.

KEYWORDS: Biosensor, microbiota, microbe, lanthanide, biomarker

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TIIVISTELMÄ

Lääketieteessä, lääkeaineiden puhtaassa valmistuksessa ja elintarviketeollisuudessa mikrobien nopea havaitseminen on ensisijaisen tärkeää. Nopeille ja edullisille havaitsemismenetelmille on tarvetta varsinkin epidemioiden hallitsemisessa. Uudet patogeeniset mikrobit voivat aiheuttaa pandemioita, jotka alkuvaiheessa etenevät hyvin nopeasti maanosasta toiseen. Tämän päivän havaitsemismenetelmillä on yleisesti haasteita herkkyydessä ja spesifisyydessä. Ihmisen mikrobiota on ollut pitkään haastava ja tutkimaton alue. Tämä johtuu yksinkertaisesti siitä, ettei tähän tutkimusalueeseen ollut riittäviä tutkimusmenetemiä. Nämä nykyaikaiset menetelmät ovat selventäneet paljon yhteyksiä eri sairauksien ja suolen mikrobiotan koostumuksen muutosten välillä. Koko mikrobiotan testaukseen ei ole käytännössä todellisia pikatestijärjestelmiä. Biosensoreilla on mahdollista vastata mikrobien havaitsemisen asettamiin haasteisiin. Pieni koko, helppokäyttöisyys ja käytettävyys kenttäolosuhteissa ovat houkuttelevia tekijöitä orastavalle diagnostiikkalaitteiden luokalle.

Tässä väitöskirjassa arvioitiin erilaisten nopeiden biosensorien soveltuvuutta mikrobien ja biomarkkerien havaitsemisessa. Työssä tutkitut menetelmät eivät perustuneet perinteisiin immunomääritystekniikoihin, jotka perustuvat yleensä yksittäisen antigeenin havaitsemiseen näytteestä. Tämän sijasta käytettiin näytteen kemialliselle ympäristölle herkkää europium-leimaa, jota käytettiin ei-spesifisesti vuorovaikutuksessa sekä simuloidun näytteen että potilasnäytteiden kanssa. Bakteriofagit toimivat biologisina koettimina ja tuottivat moneen eri parametriin perustuvaa luminesenssidataa -sormenjäljen jokaisesta yksittäisestä näytteestä. Bakteriofageihin perustuvat menetelmät kehitettiin ensin yksittäisten bakteerilajien havaitsemiseen ensin simuloiduissa olosuhteissa ja tämä jälkeen potilasnäytteistä. Menetelmää sovellettiin pidemmälle tunnistamaan spesifisistä biomarkkeria säädellyssä kemiallisessa ympäristössä. Lopulta menetelmää käytettiin luokittelemaan tappavat eturauhassyöpänäytteet ei-tappavista näytteistä. Luokittelutestin avulla havainnollistettiin tilastollisesti merkittävä ero näiden kahden näytetyypin välillä ($p<0.0014$). Yhteenvetona nämä havainnot osoittavat sen, että lantanidi-leimaan ja bakteriofaageihin perustuvaa menetelmää voidaan käyttää spesifisesti biomarkkerin, tai yksittäisen bakteerilajin havaitsemiseen.

AVAINSANAT: Biosensori, mikrobiota, mikrobi, lantanidi, biomarkkeri

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Abbreviations

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CFU	Colony-Forming Unit
CRP	C-Reactive Protein
DNA	Deoxyribonucleic acid
E-nose	Electronic nose
ELISA	Enzyme-Linked Immunosorbent Assay
FCM	Flow cytometry
GC-MS	Gas Chromatography–Mass Spectrometry
GFP	Green Fluorescent Protein
GG	Grade Group
HITChip	Human Intestinal Tract Chip
HIV	Human Immunodeficiency Virus
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IMS	Immunomagnetic separation
LFIA	Lateral flow immunoassay
MALDI–TOF	Matrix-Assisted Laser Desorption/Ionization - Time-Of Flight
MPN	Most-Probable-Number
mPCR	multiplex PCR
MRSA	Methicillin-resistant *Staphylococcus aureus*
NTA	4,4,4-trifluoro-1-(2-naphthalenyl)-1,3-butanedione
PCa	Prostate Cancer
PCR	Polymerase Chain Reaction
POC	Point Of Care
PSA	Prostate Specific Antigen
qPCR	quantitative PCR
QCM	Quartz Crystal Microbalance
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic acid
(RT-PCR)	Reverse transcription polymerase chain reaction

SPR	Surface Plasmon Resonance
TOPO	Tri-n-octylphosphine oxide
TRFIA	Time-Resolved Fluorescence Immunoassay
TRL	Time-Resolved Luminescence
wt-m13	wild type-m13

List of Original Publications

The thesis is based on the following original publications, referred to in the text by Roman numerals (I–III).

I Kulpakko J., Kopra K. and Hänninen P. (2015). Time-resolved fluorescence-based assay for rapid detection of Escherichia coli. *Anal Biochem*. **470**:1–6.

II Kulpakko J., Rantakokko-Jalava K., Eerola E. and Hänninen P.E. (2019). Rapid time-resolved luminescence-based screening of bacteria in urine with luminescence modulating biosensing phages. *Anal Biochem.* **570**:21–26.

III Kulpakko, J., Juusti, V., Rannikko, A., & Hänninen, P. E. (2022). Detecting disease associated biomarkers by luminescence modulating phages. *Scientific reports*, **12**:2433.

1 Rationale

Microorganisms, or microbes can be divided into six major types: bacteria, archaea, fungi, protozoa, algae, and viruses (Sattley et al., 2015). Microbes are the most numerous, diverse and essential species with regard to carbon and nutrient cycling in earth's biosphere (Pedrós-Alió and Manrubia, 2016). Even though, most of microbes are harmless or even useful to humans, some specific species can cause severe diseases. These epidemic causing pathogenic microbes are constantly monitored and managed by different international organizations (Wilson, 1994). Over the years the significance of microbes for human health has been elucidated. Well functioning microbial communities in human intestines are forcibly related to health. Dysbiosis is a negative perturbation of normal microbiota. It can cause mild temporary problems, but growing research data has linked it to severe health problems (Fraher et al., 2012).

Single microbial pathogen species can cause severe life-threatening diseases and even pandemia. In that case, the speed of detection is very crucial since the early start of treatment increases the chance of a positive outcome. Single microbial species can be detected with relatively simple assays. The very old plating technique is still a “gold standard” method for microbe detection. However, it is clearly too stagnant for rapid biosensor applications. More rapid methods are continuously being developed. For example, relatively simple lateral flow assays can be operated in a variety of conditions.

The composition of microbial species in the human gut, the microbiota, has been connected with many different diseases. From birth, the complexity of human microbiota increases as it is prone to environmental, nutritional and genetic elements influencing the microbial composition. All this is further controlled and modified by e.g., the immune system (Dixit et al., 2021). Only 30 percent of microbes from the human gut are culturable. Microbiota studies remained halted until modern sequencing methods were available (Fraher et al., 2012). Currently, there are robust methods to detect single microbial species in a relatively short time. Hitherto, there are no actual rapid biosensors for microbiota. The reason is the immense diversity of microbiota that generates a challenging task. The majority of microbiota screening techniques are based on detecting 16S ribosomal RNA genes from microbiome.

Being technically very demanding, these sophisticated screening assays lack applicability for wide-scale screening functions. Rapid detection or very accurate determination of microbiota and microbiome is so far not reachable with current technology (Panek et al., 2018).

Biosensors are relatively recent analytical devices, that provide fast, or in some cases even real-time data from the sample. Biosensors are a versatile group of different detection systems that are ever more popular. They fulfill many requirements that rapid detection technologies have to meet. Many of these sensors are portable and applicable for challenging working conditions and sample matrices (Zhao et al., 2014; McNerney, 2015).

Apart from conventional assay systems, the sample can be analyzed with unconventional methods such as liquid fingerprinting technology illustrates (Hänninen et al. 2013). This type of technology is based on different nonspecific interactions of the liquid sample and its components. These interactions are detected as sample causes modulation of the signal via sensitive lanthanide labels. The measurement is based on time-resolved fluorescence, which lowers background signals and the measurement of weak nonspecific interactions in differently arranged chemical environments. Interactions can be modulated even with organisms such as bacteriophages, that may function as natural target binders.

This thesis reports on the development of three different types of rapid biosensors for screening microbes and microbiota. The experimental data ranges from simulated samples to validated hospital samples. The intent of this thesis work was to compare these developed biosensors with established validated methods.

2 Review of the Literature

2.1 Need for detection

There are several important reasons why rapid microbe detection is needed. The definition of rapid test result varies according to user. Rapid test can stand for real-time detection, or a test, which result is achieved after 5 minutes to 24 hours depending on the method used (Shintani et al. 2011). Sensitivity and specificity requirements depend on the test type. Laboratory tests for microbes have obviously higher requirements compared to field tests or point-of-care (POC) tests, which are used for screening before performing confirmatory tests (Lim et al., 2005; McNerney 2015).

The common testing method of cultivating single microbial species on agar plates is clearly time-consuming. Still, many detection methods require this long enrichment step before the actual assay is performed. Microbe cultivation gives a good, but not absolute estimation of microbial viability, which is very important information in the case of medically important pathogenic microbes. This is still a very challenging quest for any modern real-time or near real-time detection methods (Law et al., 2014; Nemati et al., 2016).

The human intestinal microbiota has been linked to many disease conditions both in developed and developing countries. Thus, there is a clear demand for rapid testing in different socioeconomic and environmental conditions. Unfortunately, there are no established rapid biosensors for whole gut microbiota screening. Current microbiome analysis is based on 16S rRNA screening and it can take several days to complete the full genome data analysis (Fraher et al. 2012).

For developing countries, rapid detection systems have special needs. Harsh weather, high temperatures, dust, humidity, shelf life, and lack of cold chain create challenging operating conditions for detection devices. Overall, the device or biosensor should be relatively simple and small. Furthermore, the basic requirements are equipment durability and long-lasting battery system that is independent of mains electricity. Ideal biosensor system would detect multiple targets at the same time (McNerney 2015).

All detection technologies have limits regarding to sensitivity and, first of all, specificity. Effort to minimize the incidence of false positive test results is very

important for an effective biosensor. This is evident especially then when the detection question is related to microbial diseases, that have low prevalence (Altman and Bland, 1994).

For example, in the food industry, the presence of a minute dose of infectious microbes must be detected. If this is neglected, the ramifications can be economically catastrophic. In general, food is a challenging matrix to detect low levels of pathogens. A broadly applicable method should be eligible to detect microbes from the complex matrix. This is one of the major considerations for testing microbes successfully. Furthermore, the system should withhold mixtures of organic and inorganic interfering materials (Lim et al., 2005).

The ideal rapid detection method should be functioning in a miniaturized system with the possibility of automation for the high-throughput system. The simplicity of use leads to a reduction of training and the use of labor. Along with specificity and sensitivity requirements, the test has to provide data that is easy to interpret (Lievens and Thomma 2005; Nemati et al., 2016). Emerging new biothreats force authorities to estimate how quickly a new assay can be constructed. There is a clear need not only for rapid assay but a rapidly assembled detection system for new microbe whether it is a bacteria or virus (Lim et al., 2005).

2.1.1 Single microbial species

Specific cultures for single microbes are not often available. Therefore, old technologies as serological tests are usually performed. This type of immunotesting may take over a week. Obviously, the earlier detection and the medical intervention the better prognosis. Human physiology responds immediately to foreign microbial threats. This is usually a generic response not classifying the target microbe. It takes several days before symptoms occur. To detect the cause of symptoms commonly used antigen or nucleic acid methods are used among culture methods (Figure 1). According to World Health Organization, the spread of resistance to antimicrobial drugs is a global problem. Many common bacterial species causing infections e.g., urinary tract infections have a high occurrence of resistance. Therefore, there is an urgent need for rapid detection of these resistant strains along with emerging new microbial threats (World Health Organization, 2014). Developing countries have further needs for detection. These special requirements call for rapid, inexpensive, robust, and accurate diagnostic tests for diseases like malaria, dengue fever and other tropical diseases (Institute of Medicine (US) Forum on Microbial Threats, 2007).

The pharmaceutical and medical industry has a demand for rapid testing for microbiological contamination. Contaminant microbes must be detected early to avoid major economic losses due to withdrawal and disposing of expensive products (Mugoyela, et al., 2010; Nemati et al., 2016). To detect microbes, a properly

functioning test system has to meet many requirements. The first requirement is the time of assay. It consists of the sample preparation time and the time that performing the assay itself takes. The second demand is assay sensitivity. Pathogenic microbes are usually in low numbers mixed with non-pathogenic microbes which makes detection even more challenging (Mandal et al., 2011).

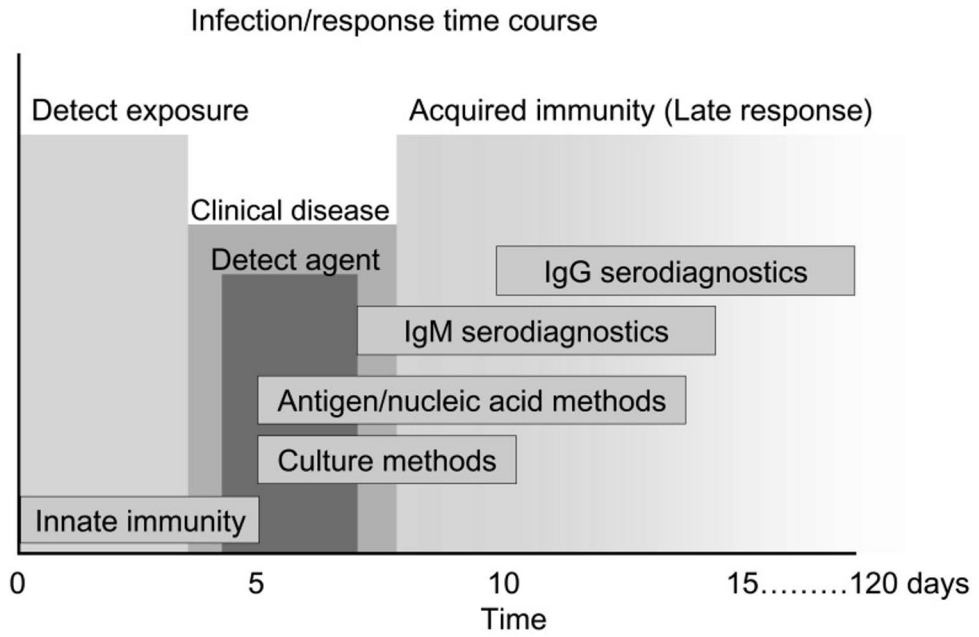


Figure 1. Diagnosis entry time (Wolcott, 2006). Rapid detection should be performed immediately after exposure before symptoms occur.

2.1.2 Human gut microbiota

Perturbations in human intestinal microbiota are suggested to contribute diseases like intestinal cancers, obesity and type 2 diabetes, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). This imbalance of gut microbiota is called dysbiosis (Bull and Plummer, 2014). Early studies of human gut microbiota relied on culture methods. Because of difficulties with culturing microbes, new techniques were developed. The first major technique was based on small subunit ribosomal RNA (16S rRNA) analysis and it was supportive for studying the diversity of gut microbiota and estimating generally bacterial species in the gut and finally searching for links between diseases and imbalances of gut microbiota (Fraher et al., 2012).

New methods have shown links between dysfunctional microbiota and diseases such as IBD, obesity and diabetes. This is just one reason why there is an even higher need for faster, high-throughput methods and the need to identify carefully specific

microorganisms from the gut. There is a strong ambition to find causative mechanisms for disease states and certain microbes residing in the microbiota. New research data may eventually lead to probiotic administration to treat dysbiosis. It is even speculated, that all new pharmaceutical products should be tested with human microbiota before they are accepted (Valdes et al., 2018).

2.2 Modern detection of microbes and microbiota

Detection of microbes and especially clinical detection of pathogenic microbes has been a slowly evolving science. The detection has strong agar plate-based history. Microbes can be also enriched in liquid culture and used for classical biochemical assays. Modern molecular approaches like ELISA and PCR have started to reach the status of established methods (van Belkum et al., 2012). The analysis of vast entities of microbiota is considerably more time-consuming than detecting single microbial species. This has not been possible until recently with modern techniques such as qPCR, microbiome shotgun sequencing, and sequencing of 16S rRNA amplicons (Fraher et al., 2012).

2.2.1 Detection of single microbial species

Conventional methods to detect microbes are time-consuming and high effort is required from trained laboratory personnel. The gold standard of these methods is based on different plating techniques on an agar plate. Modern molecular methods have accelerated the detection of microbes, although some methods still require precultivation. Most established molecular methods are ELISA, immunomagnetic separation and different versions of PCR method (Liotti et al., 2019; Weile J. and Knabbe, 2009). Microbial samples are still often enriched before analysis step and concentrated. One of this kind of method is immunomagnetic separation (IMS), which involves attaching usually specific antibodies to superparamagnetic iron oxide particles. These particles allow selective isolation of microbes similarly as selective enrichment of microbes but faster and without any growth stage. Isolation is easily performed when the sample is placed under external magnetic field. IMS is usually coupled to ELISA and PCR-method (Jordan et al., 2004; Yakub and Stadterman Knauer, 2004).

Different rapid methods to detect single microbial species have their advantages and drawbacks. The needs of the user define what is the required level of specificity, sensitivity, detection time and several other parameters for each use. Usually, needs are met by a tradeoff between different detection parameters (Institute of Medicine (US) Forum on Microbial Threats, 2007).

Table 1. Example of different established techniques employed for foodborne pathogen detection.

Techniques	Assay time before result	Detection limit (cfu L-1 or g)	Target microbe	References
Plating technique	1–3 days	1	*Escherichia coli*	(Frampton and Restaino, 1993)
Quantitative PCR	3 h	10^{3}	*Listeria monocytogenes*	(Rodriguez-Lazaro et al., 2004)
Multiplex PCR	9–29 h	10^{3}	*Listeria monocytogenes*	(Li et al., 2021)
Enzyme-Linked Immunosorbent Assay	3 h	10^{3}	*Salmonella Typhimurium*	(Wang, Y. and Salazar, 2016)
Lateral Flow Immunoassay	10 min	10^{5}	*Escherichia coli* O157	(Jung et al., 2005)
Immunomagnetic Separation Assay	3 h	10	*E. coli* 157:H7	(Zhu et al., 2011)
Flow cytometry	½ days	10^{4}–10^{8}	*Bacillus subtilis, Staphylococcus aureus, Escherichia coli*	(Kerstens et al., 2014)

The detection of single microbial species depends on the type of microbe detected and the matrix of the sample. Furthermore, each matrix requires own type of treatment procedures. For instance, liquids and solid samples are prepared differently (Nemati et al. 2016). The most common detection method is the culture based-method, which requires a long incubation period before the resulting colonies can be interpreted (Frampton and Restaino, 1993). Considerably faster and technologically more advanced methods for microbial detection are: (polymerase chain reaction) PCR, (enzyme-linked immunosorbent assay) ELISA, lateral flow immunoassay, immunomagnetic separation assay and flow cytometry-based methods. Each of these techniques has a certain range of assay times and limits of detection (Table 1). PCR-methods are constantly improving. Especially (Quantitative real-time PCR) qPCR is commonly used to detect microorganisms. The PCR reaction proceeds in real time while amplified DNA is detected. Specific detection is achieved via DNA probe labelled with a fluorescent reporter (Kralik and Ricchi, 2017). This type of method has a broad range of applications. For instance, highly specific genes can be detected. This is important in studies whether bacterial strain has an antimicrobial resistance gene or not. Different versions of PCR method are available: Reverse transcription polymerase chain reaction (RT-PCR) is an extremely sensitive method for detecting RNA from viruses. Multiplex PCR offers an opportunity to amplify a set of chosen

DNA sequences and therefore to detect several pathogenic microbes simultaneously (Liotti et al., 2019; Alanio and Bretagne, 2017).

2.2.1.1 Plating method

The common method to identify microbes is referred to as Standard Plate Count. The rather uncomplicated method consists of sample preparation and dilution steps before the sample is infused with agar. The following incubation temperature and time depend on the type of microbe is detected. For instance, *E.coli* growth in an agar plate to clear colonies takes over 48 hours in 37 °C (Nemati et al., 2016).

Conventional plating can give both qualitative and quantitative information on the microbes present in the sample. Qualitative plating can separate the presence of bacterial species by using selective media. Usually, enrichment steps are required before plating the samples. After pure microbial colonies are obtained, colony samples can be studied with a series of biochemical and serological tests (Mandal et al., 2011). The culture technique reveals only those bacterial cells that survived from sample preparation process and remained viable (Nemati et al., 2016).

The most-probable-number (MPN) technique is used to measure target microbe from natural samples. The sample is diluted into several test tubes each tube having different dilution. In order to gain high confidence in results, several replicates are needed. The presence or absence of the microbe is verified after the incubation period. Modern versions of this enumeration method have been automated (Kuai et al., 2001).

Even though microbial culture methods are convenient, It is estimated that only 0.001–1% of bacteria can be cultured on a Petri dish. Many culture methods require additional chemicals or conditions to favor only desired microbes to grow. For instance, Campylobacteria is typically enriched in selective agar that has 5 different antimicrobial agents. Favoring the growth of the wanted microbe, temperature, oxygen level and growth time need to be adjusted. Since 1960's, microbiologists have also been able to grow cell wall-less Mycoplasma with special conditions and mediums including antibiotics and serum (Lagier et al., 2015).

2.2.1.2 Nucleic acid-based methods

PCR

The PCR method has significantly changed the microbiological detection technologies. The basic principle of PCR was introduced in 1985 (Kralik et al, 2017). This technique multiplies a specific DNA region of the target microbe in an exponential multiplication heat-cool cycle. For this reason, the technique requires

short DNA primers and polymerase enzyme capable of working under high temperatures. The amplification of DNA can be monitored after each cycle in real-time by measuring fluorescence intensity. The amount of microbial DNA sequence data is growing steadily each year. This data allows for designing new qPCR assays for various microbes including the subtypes of particular microbes (Yang et al., 2004).

An especially effective application of qPCR is the detection of viruses as culture methods for viruses are very laborious. Furthermore, using reverse transcriptase enzyme the qPCR can be used to detect RNA viruses. Most important respiratory viral pathogens influenza A and B belong to this viral class (Procop, 2007). High sensitivity of PCR-methods has downsides as well. Contamination of the specimen or laboratory equipment can give false-positive results. Furthermore, many of these methods are dependent on enzymatic activity. Unknown samples may contain inhibitors of the used enzyme. Usually, the sample is pre-enriched in a growth medium in order to diminish the effect of possible PCR inhibitors (Institute of Medicine (US) Forum on Microbial Threats, 2007). Additionally, the PCR method does not differentiate between viable and nonviable cells. This increases the risk of false positive results (Margot et al., 2015).

New PCR technologies are emerging continuously. One of the promising applications is the combination of qPCR with enzyme-linked immunosorbent assay. This immuno-PCR can enhance the limit of detection 100 to 1000-fold (Adler et al., 2008). Overall, different PCR methods have proven to be effective tools for microbial diagnostics. Many of the methods have been validated and standardized for clinical use (Kralik and Ricchi, 2017).

Multiplex PCR

Multiplex PCR (mPCR) allows amplification of multiple targets simultaneously by using multiple primer pairs in a single reaction (Alanio and Bretagne, 2017). mPCR reveals the presence of specific microbial nucleic acids from the sample but cannot give information about the viability or infectivity of the pathogen. mPCR designed to detect several different pathogenic microbial species should also be able to amplify all the microbial species with the same efficiency. That requires careful estimation of reagents for the reaction. mPCR is especially useful for screening of large number of samples in a cost-effective way. Obtaining the result from the beginning of sample treatment takes usually 1 to 6 hours (Sint et al., 2012; Krause et al., 2014; Zhao et al, 2014). As an example, Liotti, et al, (2019) developed a sensitive and rapid multiplex PCR method for the identification and detection of 20 pathogenic microbes and antimicrobial resistance genes directly from blood samples.

2.2.1.3 Antibody-based methods

ELISA (Enzyme Linked Immunosorbent Assay)

ELISA is a widespread method for detecting various targets. For detection of Salmonella and *E.coli,* it can be considered as one of the main methods and it is usually performed in a 96-well microtiter plate. The most effective type of ELISA is sandwich type. It applies two antibodies targeting the analyte. The primary antibody is immobilized onto the surface of the well and the other freely moving secondary antibody is conjugated with an enzyme. The antigen originated from a pathogen binds both the primary and the secondary antibody forms a sandwich structure after unbound reagents are washed away. Finally, the specific colorless substrate for the chosen enzyme is added and color formation is seen. This formation is relative to the concentration of the particular antigen in question. Elisa test usually requires overnight incubation before the actual assay procedure. Even though ELISA is a lengthy test, it is very commonly used to detect microbiological targets. Modern ELISA tests can be automated. Furthermore, improvements of the assay can be achieved by replacing the chromogenic substrate with a fluorogenic substrate. The result is then measured with a fluorometer (Law et al., 2014; Nemati et al., 2016). Advantage of ELISA is quite often achieved with high sensitivity, but pretreatment usually requires many hours of incubation. Microbial samples are often separated by immunomagnetic separation method, in that method, target microbes are captured with paramagnetic beads. Capturing agent is coated on beads and has an affinity towards target (Nemati et al., 2016).

Lateral flow immunoassay (LFA)

Main parts for LFA are sample pad, conjugate pad, membrane and adsorption pad. The membrane is usually made of nitrocellulose. It contains immobilized reagents for the reactions. Addition of liquid sample to the one end of the strip, sample pad, activates immobilized reagents as the flow of liquid proceeds by capillary force (Ngom et al., 2010). The differential partitioning between the mobile sample and stationary membrane phases partly separates interfering substances and therefore reduces assay interference. The sample pad is usually made of cellulose (Posthuma-Trumpie et al., 2009). The test strip has usually two separate lines. One is the test line and the other one is the control line. LFA for bacterial detection is regularly installed as a sandwich format. Analyte-specific antibody is placed on the test line and the conjugate release pad holds antianalyte antibody conjugated with i.e., colored latex particle. The LFA can also be constructed as a competitive format in which the target is conjugated with the label. Target in the sample competes with the known amount of labelled target and

this reaction is used to estimate the presence of the target antigen. The competitive format is mainly used for small antigens or analytes. LFA is obviously a cost-effective and easily operated disposable test. It is very suitable in point-of-care and challenging field conditions to obtain quick results. Major pitfalls for LFA's are the qualitative or semiquantitative nature of the assay. Without a reader, the LFA relies on visual interpretation, which makes it subjective. In the competitive format of LFA, the concentration of the analyte and signal response are inversely proportional. This makes interpretation hard for an untrained user. A lot depends on the constituents of the sample matrix. Viscosity and surface tension have an influence on assay time (Posthuma-Trumpie et al., 2009; Koczula and Gallotta, 2016).

Time-resolved fluorescence immunoassay (TRFIA)

TRFIA is an immunoassay method that uses a similar immunoassay analyte sandwich capture principle as ELISA-methods do. Instead of enzymes, the system uses lanthanide chelates of europium, samarium, terbium or dysprosium. Lanthanide labelled antibodies are applicable both in sandwich and competitive formats. After washing steps, TRF enables sensitive measurements without interfering with background fluorescence. Lanthanides emit light microseconds after excitation compared to fluorescence intensity measurements (nanoseconds). Emission from the lanthanide label is separated from the background signal which contains short decay fluorescence and scattering (Hemmilä et al., 1983; Soini and Kojola, 1983). TRFIA has been used for detection of different globally important pathogenic microbes like *E.coli* O157:H7 and hepatitis B virus. Comparison study found that the precision, specificity, and sensitivity of the TRFIA for hepatitis B were much better than achieved by ELISA-method (Yu et al., 2002; Hu et al., 2012).

Flow cytometry

Flow cytometry (FCM) provides quantitative physical and chemical information from a population of cells or particles. It is based on a narrow laser beam focused on a sample stream and light scattering and emitting from a sample that it hits. Scattering depends on the physical characteristics of the particle or cell and emission from the fluorochromes used. Before FCM analysis cells are usually labeled with fluorescent dye conjugated to the antibody. (Nuding and Zabel, 2013).

FCM results are usually obtained in less than 4 hours and the cost per sample remains low. FCM has been shown to be convenient for urine sample analysis, because the matrix allows free passage of light. The method requires living cells and microbes shouldn't form clusters. Also, the equipment is for laboratory use and detecting each microbial surface antigen requires its own antibody (Alvarez-

Barrientos et al., 2000; Kennedy and Wilkinson, 2017). Recently, FCM has begun to be even more popular method for microbial studies and clinical practice. Highly automated FCM can analyze thousands of microbial cells per second. Viable and nonviable microbes can be separated if fluorescent dyes are used (Vesey et al., 1994; Nuding and Zabel, 2013).

2.2.2 Analysis of gut microbiota

The wide diversity and mass of microbes in the gut creates a task of different scale when compared to analysis of single microbial species (Fraher et al., 2012). The main bacterial phyla comprising human gut microbiota are: Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria. From these phyla Firmicutes and Bacteroidetes. (Tap et al.,2009)

Recent advances in microbial metabolic and phenotypic profiling technologies have enhanced the knowledge of the human microbiome and microbiota (Holmes et al. 2011) Until the 1990s, human microbiota studies were limited to microbes that are culturable. Most species of human microbiota cannot be cultured or are very difficult to culture. Modern techniques such as PCR, high-throughput sequencing and MALDI–TOF (matrix-assisted laser desorption/ionization - time-of-flight) mass spectrometry have elucidated the knowledge of unculturable microbial species. The method of choice depends always on the metabolites that are searched for because each method has its own analytical limitations (Namsolleck et al., 2004). For bacterial identification and phylogenetic mapping of unknown bacteria, 16S rRNA-based techniques are advantageous, fast yet laborious tools. The method is based on ribosomal RNA located in bacterial cytoplasm or attached to the endoplasmic reticulum. Most of the 16S rRNA-based techniques require DNA extraction and amplification of 16S ribosomal RNA genes (Namsolleck et al., 2004; Fraher et al., 2012).

The usage of 16S rRNA subunit as a marker works well when bacterial populations are genetically divergent. Close subspecies should be still studied with other methods (Namsolleck et al. 2004). Compared to fecal analysis, intestinal mucosa-associated microbiota analysis requires biopsies and is therefore more laborious for wide-scale screening (Fraher et al. 2012).

qPCR for 16S rRNA gene

qPCR is a version of PCR in which a reaction mixture contains a compound that fluorescences when it binds to double-stranded DNA. (Kralik and Ricchi 2017). The measured fluorescence signal reveals the presence of target microbe DNA as PCR progresses. The method gives a quantitative result of bacteria according to the amount 16S rRNA gene sample has. Part of bacteria have multiple copies of 16S rna

gene and therefore results may be biased (Nadkarni et al., 2002). One of the major benefits of this method is that its high accuracy allows the detection of those bacteria, which would be missed with other gut microbiota analysis methods. The method requires in advance information about the bacterial target DNA. In general, qPCR is rather fast, accurate and allows phylogenetic identification of specific bacterial groups and species. Usually, it is used along with other methods (Carey et al., 2007).

Direct sequencing of 16S rRNA amplicons

The method of 16S rRNA sequencing uses PCR amplification of one or more variable regions. This consists of a part of 16S rRNA gene amplicon, which is immobilized on beads. The PCR reaction occurs in an oil-aqueous solution in a picotiter plate. Fragments are developed on the surface of these beads — one bead per DNA fragment. Pyrosequencing applies pyrophosphate, that is released as nucleotides are incorporated. The reaction is detected via chemiluminescent reaction. The system can detect bacterial species representing lower than 0.01% of the overall bacterial species present. The method is useful for analyzing the microbiota present and estimating athe bundance of microbial species. However, the method doesn't reveal the functional stat the of microbial community at the moment of sample taking (De Filippo et al., 2012; Jo et al., 2016).

Shotgun metagenomics

Shotgun sequencing is a technique, that randomly breaks up DNA sequences in the sample into masses of small fragments. These fragments of the whole microbial genome are then sequenced and the full sequence data is reasoned computer aided by looking for overlapping regions and reassembling them (Jovel et al., 2016). This type of sequencing is a method of choice when large DNA strands are too difficult to be processed with standard sequencing methods. Furthermore, shotgun sequencing is a potent method, when there is a reference sequence of the microbe available (Quince et al., 2017). Massive parallel shotgun sequencing provides data about the entire community of microbes that the sample contained — species-level and strain-level classification can be made. This type of sequencing does not provide only genetic diversity data of gut microbiota but also information about the functions of microbiota. Nevertheless, shotgun sequencing is a very labor-intensive method and is prone to an incomplete covering of the investigated genome. For results, significant computational power is required and there is a high risk of contaminated samples (De Vos, 2009; Jovel et al., 2016).

DNA microarrays

A DNA microarray is an array of probe molecules immobilized on a solid surface. In the system, probe molecules are short single-stranded nucleic acids with known sequence data. The single stranded nucleic acid is able to form double stranded hybrids, when the complementary single stranded sequence is present in the sample. The detection mechanism is based on fluorescently labeled target DNA (probe) capturing complementary sequences from the sample. Unattached sequences are removed by washing and a laser is applied to excite fluorescent dye, which is combined to the probe. Emission is detected with a scanner, which forms the digital image of the microarray chip —thousands of assays can be implemented at the same time.

For phylogenetic analysis, the array contains probes, that are complementary to the small subunit of rRNA sequences. More specifically, the method focuses to the variable regions of the 16S rRNA gene. This allows the phylogenetical analysis of complex microbial communities (Paliy and Agans, 2012). HITChip (Human Intestinal Tract Chip) is an example of a microarray, that has been specifically developed for human gut microbial communities (Rajilic-Stojanovic et al., 2009). This technology is already used for analyzing microbiota profiles related to irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and microbiota changes related to obesity (Paliy and Agans, 2012).

In combination with bioinformatics, DNA microarray technology offers a high degree of parallelism and gives higher diagnostic resolution than the typical PCR method (Bottinelli et al., 2017). Phylogenetic microarrays have performed better in sensitivity than other high-throughput sequencing methods (Claesson et al., 2009; Roh et al., 2010). On the other hand, short microarray probes have tendency to crosshybridize with similar non-target sequences thus creating compromised data (Fraher et al., 2012).

2.3 Biosensors

Biosensors can be described as analytical devices having sensitive biological element and physicochemical transducer converting sample interactions into a signal. The incorporated biological element can be as small as a single protein (e.g. antibody), DNA probe, cell component, or even a whole organism (Zhao et al., 2014). The biological recognition element is connected to a physical transducer (Figure 2) where biological recognition of the analyte is converted for instance to an electrical or optical signal proportional to the target concentration. Other common types of transducing systems are thermometric, piezoelectric, magnetic, or micromechanical systems (Lazcka et al., 2007; Park et al., 2013). Recent development in electrochemical biosensors has focused on different materials e.g. nanoporous metals, graphene, carbon-based structures (Otero et al., 2020). In

general, biosensors offer fast performance, semi-quantitative analytical information, miniaturized size and the capability to operate outside of the laboratory. These qualities hold promise for individualized medicine and point-of-care detection of socioeconomically important diseases (Farré et al., 2012). Most of the current biosensors are based on specific antibodies and their specific binding to the target antigen. The reaction is then converted to an electric signal by a transducer.

Immunosensors can be applied to many different transducer types, because antibodies are well available and are routinely handled and immobilized. Immunosensors are usually designed to use electrochemical, or optical transducing mechanisms. Albeit highly sensitive and specific, antibody-based biosensors are prone to various facts such as interfering chemicals, temperature and pH (Marazuela and Moreno-Bondi, 2002).

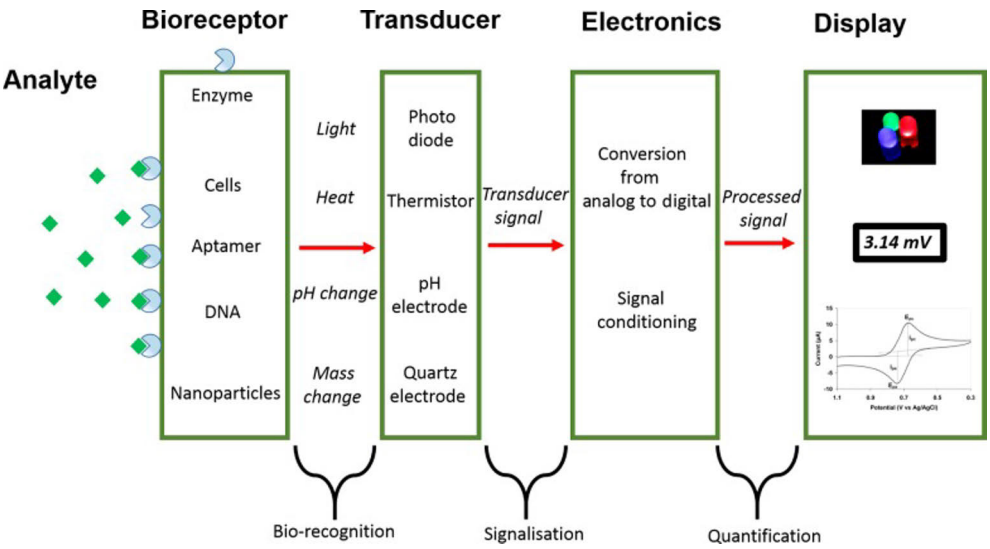


Figure 2. Biosensor classification according to its type of bioreceptor or transducer (Bhalla et al., 2016).

Furthermore, biosensors can be classified in two general types: direct and indirect. Direct biosensors are usually capable of real-time detection provided that the tested solution is continuously changed through e.g., a microfluidic system. Most of these systems are based on antibodies, although phage-based systems have been reported (Singh et al., 2013). Indirect versions require labeling of the bioreceptor and additional detection steps (Ivnitski et al., 1999). In general, biosensors are divided according to their bioreceptor and transducer type (Figure 2). Transducers are

further divided into electrochemical, mass-based and optical transducer types (Figure 3).

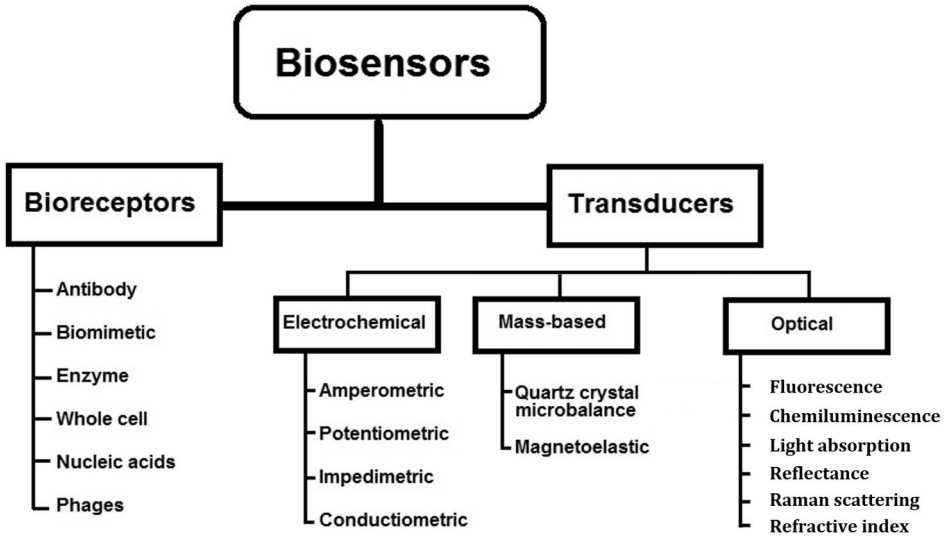


Figure 3. Categorization of various biosensors based on their elements.

Major advantages of biosensors are, that these sensors can be designed to portable format and the data collection is real-time (Zhao et al., 2014). Biosensors are fast growing and competitive detection platforms compared to established detection methods.

Biosensors have potential applications in many fields. Common objective is to detect pathogenic microbes as fast as possible to minimize human suffering and economic losses. The food industry and medical field have shown interest in biosensors, because of almost real-time detection of pathogenic microbes –the measurable signal is usually achieved after seconds of interaction that starts between a biorecognition element and an analyte. This type of direct detection with a special surface is common with optical biosensors like SPR (Surface Plasmon Resonance). Same rapid detection interests apply to the military, where the rapid identification of possible biowarfare agents is of utmost importance (Lim et al., 2005; Zhao et al., 2014). A Specific branch of biosensors is phage-based biosensors. Phages have unique interactions with their host bacteria. This makes them an important category of biorecognition elements (Ertürk and Lood, 2018). Phage-based biosensors have been shown to be effective tool to detect e.g. *E.coli* O157:H7, Methicillin-resistant Staphylococcus aureus (MRSA) and Salmonella. Phages are very versatile elements, and this gives a possibility to different detection formats which can be further coupled to various transducer mechanisms (Olsen et al. 2006; Tawil et al., 2012).

Table 2. Rapid biosensors for microbes. Examples of biosensor performances with a focus on species *Escherichia coli* and *Salmonella*.

Mode of detection	Target microbe	Assay time	Limit of detection	References
Optical biosensor	*Escherichia coli* O157:H7, *Salmonella typhimurium*	45 min	10^4 CFU/mL for both species	(Magliulo et al. 2007)
Surface plasmon resonance biosensor	*Escherichia coli* O157:H7, *Salmonella typhimurium*	<1 h	23 CFU/mL for both species	(Waswa et al. 2006).
Piezoelectric biosensors	*Escherichia coli* O157:H7	18 hours	10 CFU/mL	(Guo et al., 2012)
QCM biosensors	*Escherichia coli* (three strains)	N/A	$8x10^4$ CFU/mL	(Farka et al., 2015)
Electrochemical biosensors	*Salmonella typhimurium*	>1h min	10^2 CFU/mL	(Ro et al., 2005)

2.3.1 Optical biosensors

Optical biosensors are one of the most widely reported biosensor types in pathogen detection. Common classification of optical biosensor types are based on absorption, reflection, refraction, Raman, infrared, chemiluminescence, dispersion, fluorescence, or phosphorescence. In certain models the detection can be real-time and therefore create an appealing opportunity for kinetic estimation of reactions (Velusamy et al., 2010). The most common type of optical biosensor is based on SPR. The SPR is the oscillation phenomenon that exists at the thin metal (gold) interface between the sensor chip and the sample. Usually, chip contains a thin layer of gold coating on the transducer surface. As the target microbe interacts with the surface the refractive index near-surface is altered and can be detected. This sensor type allows label-free detection and the assay chip can be reused (Shankaran et al., 2007)

2.3.2 Quartz Crystal Microbalance (QCM) biosensors

QCM biosensors are devices using the piezoelectric effect, to measure changes in the physical parameter as mass bound on the piezoelectric crystal surface changes the measurable resonant frequency of the crystal (Tombelli et al., 2012). As a crystal is placed under an oscillation circuit the frequency response is measured. The binding of the analyte e.g., microbe to the crystal is observed by a change in the resonant frequency of the system. The decrease in the frequency is proportional to the mass change and this data is used to estimate the bacterial concentration in the sample. Biosensor crystal is usually made of especially suitable anisotropic crystals as quartz, barium titanate, aluminum phosphate or zinc oxide. The surface is

functionalized to immobilize a biorecognition element on it. Common immobilized recognition elements are antibodies for specific antigens. (GarcíaMartinez et al., 2011; Pohanka, 2017). Several microbial species have been detected with QCM biosensors. For example, Salmonella typhimurium, MRSA and HIV (human immunodeficiency virus) and several *E.coli* strains had as short detection time as 10 minutes (Farka et al., 2015; Pohanka, 2017).

2.3.3 Electronic nose biosensors

The concept of electronic nose (e-nose) was reported in 1982 (Persaud, and Dodd, 1982). The main parts of e-nose are a sample delivery system, analyte reacting cross-reactive sensor arrays assembled to a layer, and a computing system, that processes complex multidimensional data from each measurement point. Data processing groups data points to certain classes of interest. In bioelectronic nose systems, the cross-reactive sensor arrays consist of biomimetic parts mimicking olfactory proteins capable of sensing volatile organic compounds (Dung et al., 2018).

Modern e-noses are designed to detect volatile compounds. The compound binding to the sensor surface can take place in various ways like adsorption, absorption, chemisorption and co-ordination chemistry. In older versions, the detection layers had to be regenerated after use. Modern layers are usually continuously operating (James et al., 2005). The sensor part of the e-nose is generally divided into three classes: gravimetric based on mass, electrical based on conductance and impedance, and optical-based on photon/electron interactions. The interactive layer exposed to the sample is often a specific type of conductive polymer or metal oxide. Interaction with the analyte is detected through a change of conductivity, resistivity or voltage change (Fitzgerald and Fenniri, 2018).

E-noses have been successfully used for microbial detection. E-nose applying carbon black composites for array material was able to identify species of Staphylococcus aureus bacteria —both methicillin-resistant S. aureus and methicillin-susceptible *S. aureus*. Complex data generated by an array of 32 composite sensors were processed with three data clustering algorithms providing over 99 % accuracy, though feature extraction still remains a challenge (Dutta et al., 2005). Mycobacterium tuberculosis is another medically important pathogen that has been detected via e-nose. The bacteria were detected both in the original patient source and in vitro using neural network systems for analysis. The array of 14 sensors of conducting polymer detected Mycobacterium tuberculosis from other control bacteria cultured for 5 to 6 hours for the test (Pavlou et al., 2004). A study of anaerobic pathogens with similar conducting polymer-based sensors reported pattern recognition and differentiation of several cultured strains of *Clostridium* spp. and *Bacteroides fragili* (Pavlou et al., 2002).

E-nose detects microbes indirectly via air not directly measuring individual microbial cells. Therefore, they have so far a qualitative nature. For patient related e-nose applications an important notion is to consider interfering factors as consumed food, medication, other diseases and smoking habits. Moreover, the device itself may be prone to humidity, temperature and mechanical errors. The complex data analysis requiring constant calibration and "teaching" is also limiting the extensive use of e-noses (Fitzgerald and Fenniri, 2018).

2.3.4 Electrochemical biosensors

Most electrochemical biosensors deploy specific enzymes as biological recognition elements having specific binding properties. They have catalytic properties that are useful for detecting reduction and oxidation reactions. Electrochemical reaction in the sample can cause current (amperometric), electrical potential, or charge build-up (potentiometric) or change the conductive properties of the measured liquid sample material between electrodes (conductometric). In addition, impedance can be harnessed for electrochemical biosensor applications (impedimetric). Electrochemical reactions occur close to electrode surfaces. The electrode type is chosen based on the intended target that is designed to be measured (Grieshaber et al., 2008; Mishra et al., 2018).

The use of electrochemical biosensor installation usually involves the use of a counter electrode and reference electrode (that is made of silver chloride). The reference electrode is supposed to have a steady potential that is compared to the working electrode interacting with the actual sample solution (Mishra et al., 2018). Amidst all biosensor types, electrochemical biosensors are one of the most sensitive, rapid and easily modified to smaller device sizes. The clear advantage of electrochemical detection is, that the sample can be turbid and the required sample volume can be remarkably smaller than in many optical biosensors. Generally, these sensors are suitable for detecting various microbes (Yang et al., 2008; Cinti et al., 2017).

Recently, an electrochemical biosensor for detecting live or viable bacteria with conductive polymer was reported. This system had an effective detection range of around 6.0×10^{3}–9.2×10^{7} CFU / mL and the biosensor had a real-time signal response (Saucedo et al., 2019). A version of an electrochemical biosensor used aptamers as recognition elements. In addition, this biosensor used gold nanoparticles to detect *Salmonella typhimurium* from pork meat. The detection limit reached 3 CFU / mL (Ma et al., 2014). The earlier study applied single-walled carbon nanotubes among aptamers and the potentiometric measuring technique. Limit of detection was 800 *Salmonella* bacteria per mL of sample (Zelada-Guillén et al., 2012).

2.3.5 Phage-based biosensors

Bacteriophages also called phages are viruses that infect prokaryotes as bacteria and archaea. Phages harness the cell metabolism to produce large amount of copies of itself. Phages are usually highly specific to certain strains of bacteria. Phages can be classified simply by their property of lysing the bacterial cell. Lytic phages lyse the bacterial host and quickly destroy the cell. Lysogenic phages integrate phage genome to bacterial genome. This type of phage may remain dormant until a certain stimulus launches the replication and propagation machinery. In this case, the cell can produce phages long time without noticeably being destructed. Both types of phages mentioned here are widely used in biosensor applications. Additionally, these phages are amenable to easy genetic manipulation that increases their usage for biorecognition applications (Singh, et al., 2013; Schmelcher et al., 2014). Lytic phages release various bacterial cell markers from inside the ruptured cell. These markers can be detected with numerous different methods. Non-lytic phages can be modified via a well-established phage display method. Usually, specific peptide coding genes are introduced to phage DNA and expressed on their surface. The surface is then used for capturing target analytes. Phage-based biosensors withstand harsh conditions and offer a low-cost solution for large-scale screening because phage production and purification is a straightforward processes. On top of this, phages can be coupled to various different transduction platforms (Jones et al., 2020). The most commonly used platform types are: SPR, bioluminescence, fluorescence and the usage of amperometric transducer (Lee et al., 2013; Vinay et al. 2015). Phage-based biosensors for diagnostic purposes are not yet routinely used. The technology is gradually more known and different versions of phage-based biosensors are validated in a larger set of samples (Jones et al., 2020). Some commercial phage-based assays are already available like the detection of mycobacteria in combination with PCR technique (Swift et al., 2020).

Optical phage-based biosensors

Optical biosensors offer high specificity, sensitivity, comparatively small size and low cost per analysis (Damborský et al., 2016). Especially M13 phage is applicable for optical biosensor systems. The ease of genetic manipulation and cylindrical structure of this phage allows its conjugation to optically relevant material (Kim et al., 2016). Phage-based optical biosensors can exploit labels or the system can be completely label-free. SPR-based optical biosensors are potent tools for research concerning biomolecular interactions (Singh et al., 2013). in this application, M13 phages are directly immobilized on the biosensor surface. Near the sensor surface, the refractive index changes as immobilized phage interacts with the bacteria in the sample solution. One of the major benefits of the system is that measurements are

made in real-time. Generally, optical transducer mechanism is suitable for phage mediated detection of microbes (Singh et al., 2013; Schmelcher and Loessner, 2014).

Bioluminescence phage-based biosensors

Phage-based bioluminescence biosensors function by detecting intracellular components like ATP, that lytic phages have released from ruptured cells. The bioluminescent reaction requires a luciferase enzyme. The drawback of the system is the detection of pathogens from food samples because it usually contains ATP. Another way to couple bioluminescence to the phages is to use luciferase reporter phage. As the reporter phage infects bacterial cell luciferase genes activate and produce luciferase enzyme which in turn reacts with intracellular ATP and bioluminescence occurs (Kodikara et al., 1991; Loessner et al., 1997). New version of the bioluminescence-based system used CRISPR-Cas-assisted engineering with reporter phages (Meile et al., 2020).

Fluorescence phage-based biosensors

Fluorescent labeled phages offer straightforward option for detection. For instance, *E. coli* O157:H7 specific phage DNA can be tagged with fluorescent dye. After immunomagnetic separation and enrichment step bacteria can be specifically detected with the use of fluorescent phages and flow cytometer (Vinay et al. 2015).

More sensitive approach to detect bacteria is the use of fluorescent quantum dots and lytic phage. The head of the phage was engineered to contain a biotinylation peptide. Oppositely, quantum dots were designed to have a streptavidin coating. Every lysis generates an amplification of fluorescent signal as streptavidin-coated quantum dots bind to the biotinylated phage heads. Finally, the fluorescence was measured with the flow cytometer. The sensitivity of this system was reported as high as 20 bacterial cells in 1 mL sample (Edgar et al., 2006).

One way to couple fluorescence and phages to a biosensor system is to use fluorescent reporter protein. In this system, a reporter phage carries a gene, that codes (green fluorescent protein) GFP. After the phage infects the target bacteria, the gfp gene is translated to GFP protein. Produced GFP indicates the presence of target bacteria and can then be detected with a fluorescence detector. Vinay et al. reported a successful on-site detection of Escherichia coli TD2158 and Salmonella with GFP reporter phage and the usage of a flow cytometer (Zimmer, 2002; Smartt et al., 2012; Vinay et al. 2015). GFP-coupled phage system has many beneficial characteristics. It has low toxicity and it doesn't require any substrates as luciferase-coupled systems do. As GFP has many different variants, they differ according to their excitation and emission profiles. This allows consideration of multiplex detection for many targets

at the same time (Smartt et al., 2012). Overall, many fluorescence phage-based biosensors require flow cytometer which makes the system impractical as biosensor. Field use of this type of biosensor would require instrumental consideration.

Electrochemical phage-based biosensors

The two most common type of electrochemical phage-based biosensors are amperometric and impedimetric biosensors (Neufeld et al., 2003). Amperometric measurement can be performed after phage infection and bacteria lysis. The lysis releases a myriad of different ions, molecules and enzymes from the bacteria. For instance, one detection strategy is based on the released enzyme that reacts with added substrate and its oxidation is detected with amperometric measurement. Phage interaction with its target bacteria causes changes in the impedance of the sample solution. Usually, this interaction takes place on the surface of the electrode where phages are immobilized. Near the electrode surface conductivity increases and impedance changes due to the lytic release of bacterial cell contents (Singh et al., 2013; Schmelcher and Loessner, 2014).

Magnetoelastic phage-based biosensors

Other successful types of phage biosensors apply magnetoelastic properties. The sensor itself is a free-standing magnetoelastic resonator that functions as the signal transducer. The sensor is assembled by immobilizing phages on the sensor surface. Specific phages binding to target bacteria or spores change sensors mass and therefore cause the change in resonance frequency of the sensor (Chai et al., 2013). Phage-based magnetoelastic biosensors have been successfully deployed with filamentous phages for the detection of Salmonella (Wang et al., 2017). The beneficial feature of the system is that it can be installed to produce real-time data of the sample (Schmelcher and Loessner, 2014).

Table 3. Phage-based biosensors for microbes. Examples of phage-based biosensors. The table shows common pathogenic species like *E.coli* and Salmonella typhimurium.

Transducer	Microbe	Assay time	Limit of detection (cfu)	References
Surface Plasmon Resonance	*E. coli O157:H7*	20 min	10^3 / mL	(Tawil et al., 2012)
Bioluminescent	*Listeria ssp.*	24 h	1 / 25 mL	(Meile et al., 2020)
Fluorescent	*Escherichia coli* TD2158, *Salmonella typhimurium*	1h	10 / mL	(Vinay et al., 2015).
Quartz crystal microbalance	*Salmonella typhimurium*	3 min	10^2 / mL	(Olsen et al., 2006)
Magnetoelastic sensor	*Salmonella typhimurium*	30 min	1.6 x 10^2 / cm^2	(Chai et al., 2012).
Amperometric	*E. coli* K12	6–8 h	1 / 100 mL	(Neufeld et al., 2003).
Impedimetric	*E. coli* K12	8 h	10^3 / mL	(Shabani et al., 2013).

2.4 Lanthanide luminescence

Specific lanthanide properties are based on transitions between different energy levels of the 4f orbitals. Lanthanides comprise 14 elements. Their atomic number vary between 57 to 71. Lanthanide chemistry is ruled by their +III oxidation state. They have a myriad of scientific and industrial applications. 4f orbitals are shielded in lanthanides and therefore chemical surrounding has a negligible effect on the metal ion. Typical narrow emission peaks are the result of this (Bünzli and Pigue, 2005).

Lanthanide 4f orbitals are shielded by filled 5p66s2 energy levels. Additionally, 4f4f transitions are forbidden by electronic dipole selection rules. This leads to low molar absorbance coefficients. Forbidden orbital transitions cause a long luminescence emission lifetime, which is desired in many applications. Excited-state lifetimes reach up to milliseconds (Pandya et al., 2006). The brightness of lanthanides have remarkable variation. The main reason is the quantum yield, which determines how well the excited state can be depopulated by different non-radiative processes. Europium and terbium are visible emitting and most commonly used lanthanides in many life science applications. Surrounded by water molecules the excited state of lanthanide ion is quenched. Oxygen-hydrogen vibrations have enough energy to disrupt excited state of free lanthanide ion. Altogether, metallic lanthanide ion has low fluorescence due to low energy absorption. However, lanthanide ions are easily chelated by organic ligands that function as a molecular antenna. After excitation, these ligands transfer energy to lanthanide ion (Escribano et al., 2008).

Luminescent lanthanide complexes are commonly used in medical diagnostics to detect small biomolecules or bacteria. Biological material has intrinsic properties of autofluorescence (Bünzli, 2016). This type of fluorescence decays generally in nanoseconds. Lanthanide probes have long luminescence lifetimes and hence their signal can be measured after a time delay (Figure 4). This makes them very useful for diagnostic applications when autofluorescence has already decreased. Metallic lanthanide ion has low fluorescence due to low energy absorption. However, lanthanide ions are easily chelated by organic ligands. After excitation, these ligands transfer energy to lanthanide ion (Bünzli and Pigue, 2005). Lanthanide complexes comprise a vast group of probes that are useful for instance microbial detection from biological sample matrix. Time-resolved luminescent immunoassays are a well-known group of lanthanide-based methods (Hemmilä et al., 1984).

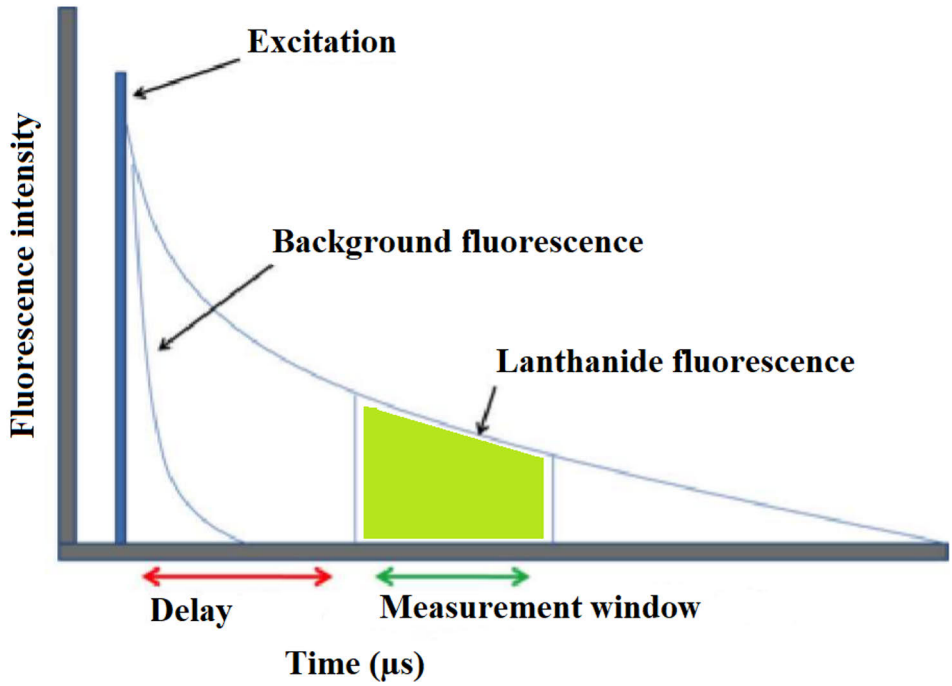


Figure 4. The principle of the time-resolved fluorescence measurement.

3 Aims

Rapid detection of microbes is an outmost need for medical laboratories, food bioanalysis and in general biosecurity. The purpose of the thesis was to study and develop lanthanide-label biosensor applicable to detect single bacterial species, model biomarker and classify lethal prostate cancer from controls.

The specific aims were:

1. To develop lytic phage-based proof-of-principle assay
2. To develop non-lytic phage assay to screen patient samples
3. To assess screening method with prostate cancer samples and model biomarker

4 Materials and Methods

Summary of materials and methods is described in this chapter. More detailed information can be found in the original publications **(I–III)**

4.1 Bacteria and bacteriophages

E. coli strain B ATCC 11303 was used as the host for the lytic phage. Bacillus subtilis (BGA) Spore Suspension DSM 618 (10^7 cfu/ml) was purchased from Merck (cat. no. 1.10649.0001). Wild-type lytic bacteriophage T4 from Carolina Biological Supply (Burlington, NC, USA) was used in this study (**I**). Staphylococcus aureus ATCC 25923 (**II**) The Ph.D.-12 phage display peptide library (E8110S) was supplied by New England Biolabs (NEB).

4.2 Media and buffer solutions

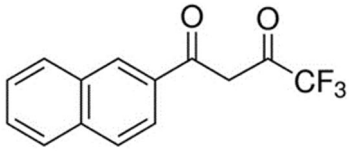
Tryptic Soy Broth (TSB) powder was purchased from Becton Dickinson (Sparks, MD, USA). Phosphate-buffered saline (PBS) from Lonza (Basel, Switzerland) was used for the dilution of bacteriophages. Bacteriophages in SM buffer (10 mM MgSO4, 100 mM NaCl, 0.01 % gelatin, and 50 mM Tris–HCl, pH 7.5) were added to bacterial culture after OD600 reached 0.1 (**I**). Yeast-Tryptone (YT) medium was made of mixing 16 g of tryptone, 10 g of yeast extract and 5 g of NaCl to one liter of MQ water (**II**).

4.3 Instrumentation and measurement parameters

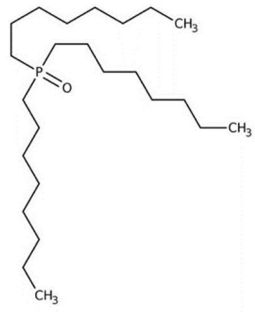
The Time-resolved luminescene signal was measured with a Victor2 multilabel counter (Wallac, PerkinElmer Life and Analytical Sciences, Turku, Finland). The excitation wavelength for europium(III) label was 340 ± 5 nm and the emission filter was 615 ± 5 nm. Luminescence measurements had delay time of 400 μs and measurement time of 400 μs All the measurements (Publications **I–III**) were performed in C12 polystyrene 96-well plates (NUNC immuno plates; Nunc, Roskilde, Denmark).

4.4 Lanthanide label and luminescence modulators

The label utilized in all of Publications **I**, **II** and unpublished was europium (III) chloride hexahydrate with the combination of (NTA) 4,4,4-trifluoro-1-(2-naphthalenyl)-1,3-butanedione and (TOPO) tri-n-octylphosphineoxide (Sigma–Aldrich).



a) NTA



b) TOPO

Figure 5. The structures of the light harvesting ligand a) NTA and b) TOPO, which is used for stabilizing agent.

In the publication **I**, the luminescence modulator for bacterial lysis was citric acid. The modulators used in the publication **II** were: diethyl malonate, 2,3-dichloro-5,6-dicyano-p-benzoquinone and triiosopropylsilane (Fluka, Buchs, Switzerland). Copper (II) chloride (Sigma–Aldrich) functioned both as a luminescence modulator and as a quencher.

4.5 Biopanning procedures

Copper beads (Sigma Aldrich 254177, 2–8 mm). were rinsed with distilled water and autoclaved 120 °C for 60 min before the biopanning experiments. The selected biopanning system, The Ph.D.-12 phage display peptide library (E8110S) was supplied by New England Biolabs (NEB). The library contains 1.5×10^{13} plaque-forming units (pfu) / ml and it has a complexity of 2×10^{9} independent peptide sequences. Each phage used in these experiments contains five copies of the minor coat protein pIII, and each copy of pIII protein has a single peptide displayed at its N-terminus. New England Biolabs, Ph.D.™ Phage Display Libraries: Instruction manual was used to perform the following procedures: Suitable strain *E. coli* ER2738 containing the F+Δ(lacZ) M15plasmid was used to amplify the eluted phage. LB medium was used to culture *E. coli*. According to the instruction manual,

to prevent contaminating phage from the environment, eluted phage were plated on LB agar plates containing 60 µg /ml isopropylβ-D-thiogalactoside (IPTG), and first affinity screening procedure for copper 40 µg / ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). The overall biopanning procedure was performed following a modified protocol from New England Biolabs. A 10µl aliquot of the random peptide library was incubated with copper beads at RT for 30 min with gentle shaking in a microcentrifuge tube containing 1 ml of physiological saline. Unbound phage from metal beads were washed serially and finally eluted as described in publication **II**. In each round, the bound phages were rescued and amplified using *E. coli* ER2738 to make more copies. These were used in the second round of biopanning. After three rounds of biopanning (three rounds of 15 washes) the bound phages were harvested for binding analysis.

Enriched phages from previous screening were used for biopanning experiments against *E.coli* B. The panning procedure was performed according to the following protocol: *E.coli* B cells were grown in tryptic soy broth (TSB; Sigma-Aldrich) medium at 37 °C. The growth medium was centrifuged and the pellet was washed twice with 1 mL of 4 °C PBST (Phosphate Buffered Saline with Tween 20). The random peptide library (1.5×10^{11} plaque-forming units) was mixed with washed cells and incubated in 1 % BSA (Bovine serum albumin) with the washed infectious *E.coli* B cells and left on ice for 1 h. After this phage bound cells were washed twice with 1 mL PBST and three times 1 mL PBS. Washed cells were mixed with 1 mL of phage propagating *E.coli* ER2738 (1.0×10^{9} cells / mL) in 2×YT medium. Before mixing *E.coli* ER2738 cells were grown in intense shaking (250 rpm) at 37 °C. The mixed culture of *E.coli* ER2738 and E.coli B was incubated for 30 min at 37 °C without shaking and following an incubation period of 30 min with gentle shaking (100 rpm). The enrichment of phages was made according to the manufacturers protocol (NEB). After the third affinity selection phages were ready to be used in assays.

4.6 Sample materials

Table 4. Bacterial species and estimated concentration of bacteria detected with the plating method from the urine samples.

Microorganism	CFU Count/mL	No. of Cases
Escherichia coli	≥10^3	29
negative		14
Klebsiella pneumoniae	≥10^3	5
Enterococcus faecalis	≥10^5	4
Citrobacter freundii	≥10^5	3
Proteus mirabilis	≥10^4	2
Citrobacter koseri	≥10^3	2
Pseudomonas aeruginosa	≥10^4	2
enterococcus (non-faecalis species)	≥10^3	2
Hafnia alvei	≥10^5	1
Streptococcus agalactiae	≥10^5	1
Pseudomonas putida	≥10^5	1
Staphylococcus saprophyticus	≥10^5	1
Staphylococcus hominis	≥10^5	1
Raoultella ornithinolytica	≥10^3	1
Staphylococcus saprophyticus	≥10^5	1

Overall seventy patient samples were analyzed at Clinical Laboratory of Turku University Central Hospital (Publication **II**). All samples were stored at 4 °C and collected in tubes, that are specifically designed for single use tubes designed for collection, storage and transport of urine specimens (Vacutainer Plus C&S Boric Acid Sodium Borate/Formate tubes, Becton Dickinson). Markedly turbid urine samples were not excluded from the analysis. Ethical approval for using the patient samples was not required for the reason that the study was considered a basic laboratory screening method development and no additional patient information was collected.

Urine samples from 96 patients were collected at the time of recruitment and stored in -70 °C. Ethical approval for the use of urine samples and clinicopathological data was obtained from the Institutional Ethics Committee of the Hospital District of Helsinki and Uusimaa (HUS/3372/2019 for the PASSIONATE study and HUS/850/2017 for the DEDUCER trial). PASSIONATE study is a retrospective registry-based study utilizing data and samples S obtained from patients participating in the Helsinki Biobank. DEDUCER trial is an ongoing

prospective clinical trial consenting patients with urological malignancies in the HUS Helsinki University Hospital. PCa diagnostics was based on prostate MRI as a triage test followed by targeted ± systematic prostate biopsies.

4.7 Assay principles

In this section, the assay principles of the developed three lanthanide-based assays are defined. The chemical environment sensitive lanthanide label is europium, NTA and TOPO in all three assays (publications **I–II** and **III**). Straightforward assays required no more than 10 minutes of incubation and shaking in room temperature.

4.7.1 Assay with lytic phage

The proof-of-principle-assay in publication I is based on the unstable nature of the lanthanide label and its sensitivity to react a number of molecules leaking from lysed bacterial cells. The specific lysis is gained by T4 bacteriophage, which infects certain types of *Escherichia coli* bacteria.

The method for detecting *E.coli* with lytic phage was performed in 96-well microtiter plates with four replicates. Typically, 10 ul (10^5 phages) of bacteriophages were added in SM buffer and 100 ul of the bacterial sample in physiological saline were added to the plate. After 20 min of incubation at 37 °C with vigorous shaking, 7 µl of 2 mM citrate at pH 7.5 was added to the wells. After 2 min of incubation at room temperature, a 5:3:3 mixture of EuCl3, NTA, and TOPO at Eu3+ was added to the wells in 3 ul of water. The final concentrations of $EuCl_3$, NTA, and TOPO were 6.0, 3.6, and 3.6 µM, respectively. The Eu3+/NTA/TOPO label was prepared in DMSO before dilution to water in order to avoid possible aggregations. After 5 min of incubation, the TRL (Time-resolved luminescence) signals were measured.

4.7.2 Screening assay with biosensing phage

Clinical urine samples were divided in 100 µL volume per well in three replicates. Immediately after this 10 µl of 10^{13} pfu / mL M13-wt phages or copper/*E. coli* B selected phages and 8 µL of 20 µM copper chloride in MQ was added. The second step was an addition of 4 µl one of three additional chemicals described in publication **II**. The final stage was an addition of 4 µl of label solution to the microtiter wells. After 10 min of incubation, luminescence emission intensities were measured in a 400 µs window after a 400 µs delay time using a Victor 2 multilabel counter.

4.8 Screening assay to detect biomarker and prostate cancer

Model biomarker, CRP was diluted directly to physiological saline. The prostate cancer urine samples were centrifuged at 10 000 rpm for 5 min and the remaining supernatant was then diluted 1 part to 50 parts in physiological saline. For measurement, each dilution or sample was divided as three replicates in 100 µl volume per well to a 96 well plate. Before adding the samples, brilliant green (6 mM) and phage solution (4.0×10^{9} pfu) or chemical modulator, resazurin sodium salt (185 µM), were added to the wells. After adding the sample, the lanthanide-based reporter solution was added to the wells along with the phages and the brilliant green solution described in publication **III**. After 10 min of incubation, luminescence emission intensities were measured in a 400 µs window after a 400 µs delay time using Spark multimode microplate reader (Tecan, Switzerland). After 120 min incubation, absorbance was measured at 623 nm using the same reader.

5 Results and Discussion

5.1 Lytic phages-based assay system

Phages have been used in several bacterial detection assays. Basically, the detection is based on the phages ability to recognize specific bacterial species. Phages can be stained, engineered to produce fluorescent label inside the bacterial cell, or cause rupture of the bacterial membrane. The most used phage is T4, that is capable of binding *E.coli* surface and start ion leakage immediately after phage DNA is injected inside the bacterial cell (Boulanger and Letellier, 1988). Currently, there are several methods of exploiting leaking bacterial ions. Ions can be detected through ion-sensitive chips, or carbon electrode printed microarrays. Applying this type of method for detecting bacteria in urine samples has obvious drawbacks as urine contains high concentrations of different ionic species (Shabani et al. 2008; Nikkhoo et al., 2013). The more engineered version of phage-based detection has green fluorescent protein integrated into its coat protein. This reporter protein multiplies every time phages multiply and therefore fluorescence is enhanced. When ion leakage after phage infection occurs almost immediately, multiplying reporter phages takes several hours (Oda et al., 2004). Another type of engineered phage system took advantage of biotinylated phages and streptavidin (Edgar et al, 2006). Previously mentioned methods that require successful infection and reproduction of phage particles are much slower than methods applying direct phage tail or capsid affinity towards targets (Aliakbar et al, 2020). Recently another type of phage biosensor using coat proteins was developed. The dual-modified phage sensor used different coat proteins having different functions: one peptide region binds the target pathogen and the other binds the fluorescently tagged streptavidin. The detection was performed by flow cytometry and fluorescence microscopy (Wu et al, 2021).

In this part of the thesis work, the lytic phage-based method combining the use of nonspecific lanthanide label and the ability of lytic phages specifically infect a specific range of bacterial species and their subtypes was studied. Practically, the method was designed to distinguish living bacterial cells from lysed ones. The source behind the signal difference between these cell states was discussed.

5.1.1 Preliminary bacterial lysis experiments

Before using phages, the method was studied with sonication, which causes the cell membrane to rupture and release a myriad of different compounds from the cell interior. Europium label with NTA and TOPO was used to detect leaked molecules from ruptured bacterial cells. In addition, citrate was used to enhance the assay by functioning as a quencher.

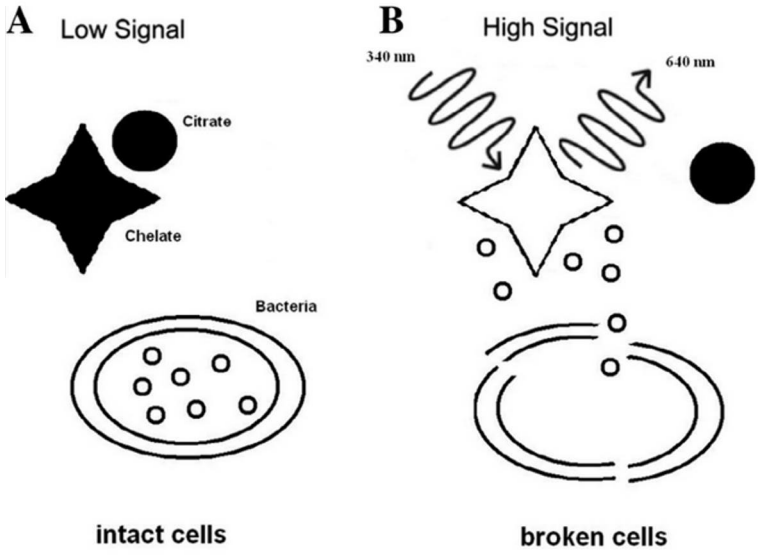


Figure 6. Principle of detecting cell lysis with lanthanide-based label. The Figure was reprinted from **I**.

Low luminescence signal was observed with whole bacterial cells. When the bacterial cell is under rupturing force, cell interior molecules leak outside to the surrounding medium. These molecules create a protective environment for the europium label and prevent citrate ions to capture europium ions. This event was observed as an increased luminescence signal (Figure 6).

Cell rupture can be simulated with sonication without the addition of external chemicals. In publication **I**, the luminescence signal difference between intact and ultrasonically broken bacterial cells was measured more than 20-fold. Different amounts of living and dead bacterial cells were studied with the presence of lytic phages and europium label. Metabolically inactive dead cells didn't react with the phages and a low signal was observed (Fig.7). The test setup was arranged so that for both bacterial species the cell concentration was 1.0×10^5 /ml, and the phage concentration used was 1.0×10^8 /ml. Very high concentration of dead bacteria gave

a slightly higher signal as bacterial mass started to interact with europium label and possibly protected it. Other reasons might be that cell lysis is more difficult to achieve with higher bacteria concentrations, or citrate is mostly chelated at that point. The limit of detection reached 1000 living bacterial cells per milliliter. TRL signal dependence on bacterial cell rupture was further studied with phages. Natural host bacteria, *E.coli* and nonhost bacteria *B.subtilis* were exposed to host-specific phage T4. The TRL signal clearly depended on the specific phage infecting the correct host bacteria (Fig.7)

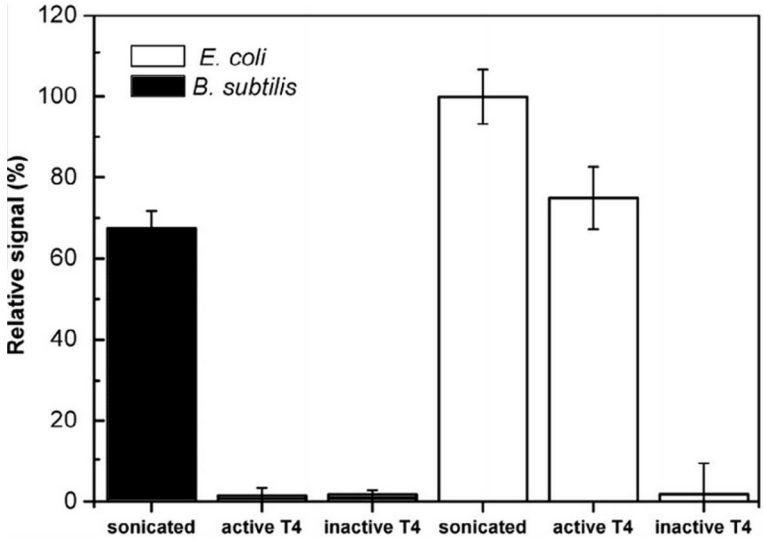


Figure 7. *B.subtilis* and *E.coli* bacteria were studied in three different setups. Bacteria were sonicated, infected with active phages or inactive phages. The Figure was modified from **I**.

Even though bacterial species were used, TRL signals showed a similar tendency when bacterial cell contents were released. Figure 7. shows that both bacterial species gave similar TRL signals when they were sonicated. In addition, the experiment confirmed that only active phages results in a high signal and hence cell rupture.

5.1.2 Detection of *E.coli* from simulated urine samples

The new approach to detecting *E.coli* was studied with simulated urine samples. Many experimental phage-based bacterial detection systems are sensitive to sample ions and consequently not applicable for such challenging sample matrices as urine. The label used in this study is sensitive to various ions, especially metal ions. To clarify possible interferences phages were added directly to urine samples in order

to infect living *E.coli* cells. Inactivated phages were used as a control. The experiment showed that 10,000 cells/mL gave different signal to background than 100,000 cells/mL. The likely reason is that a higher concentration of bacteria brings along interfering metabolites and cellular debris.

Assay parameters as intra-assay repeatability showed that coefficients of variation were in the acceptable range in those concentrations, that are medically relevant. The golden standard laboratory cutoff for determining urinary tract infections is commonly defined as 100,000 cells/mL (Schmiemann et al., 2010). For many applications, the physiological status of a bacterial cell is important to know. Especially fluorescent probes are widely used to detect dead, or living bacteria (Porter et al. 1995).

A broken bacterial cell wall and membrane release a myriad of different molecules. The publication I studies concerned the use of sensitive lanthanide label for bacterial cell lysis or disruption. The interaction consisted of label, citric acid and possibly several different cellular components. Several tests were performed in order to out rule possible cellular components behind the assay TRL signal. Dilutions of DNA and ATP (adenosine triphosphate) concentrations were studied with the presence of the lanthanide label. Among many other molecules, ATP is known to chelate free europium ions (Jagoda and Krämer, 2005).

Neither of these molecules showed to be responsible for the TRL signal with increasing concentration of the studied molecule. Other alternative explanations could be intracellular salts like calcium, sodium, chlorine and potassium. Their concentration is too low to explain the observed phenomena. Cellular content might have a wide variety of different cytosolic proteins, or complex-forming molecular structures that can protect the used lanthanide label.

The usage of whole-phages has its own drawbacks as less specific affinity molecules are available per volume than e.g. antibodies. Another problem is that if the host bacteria are destroyed before the further detection process is finished, the result will not be achieved. (Meile et al, 2020) Nevertheless, this study using whole phages demonstrated a novel method to observe the physiological status of the cell using TRL detection and the possible applicability of using it to detect specific bacterial species. On the other hand, the method has a strict requirement that the target bacteria has living status and active metabolism. One option is to apply a cocktail of lytic phages specific to each bacterial target. This would allow simultaneous detection as a recent study showed (Wu et al, 2021).

5.2 Biosensing phages (II)

Phage display is a over 30 years old method that is a thoroughly established method covering areas from basic research to detailed level molecular investigations. It is

based on screening of phage populations to achieve the best binder to the target. This biopanning process takes various steps before the desired affinity has been achieved. The display method is particularly effective for studying protein-protein interactions (Sidhu et al., 2003; Yu et al., 2009) Overall, the surface of a phage can possess a diverse molecular landscape, that can be harnessed to a variety of biosensing and biosensor applications (Petrenko, 2018). In publications **I** and **II** phages were used as biorecognition elements. In publication **I**, the binding to target cell takes place with natural binding characteristics that lytic phages have. For instance, T4 phage utilizes its own tail fibers in order to bind the target cell, pierce the target cell membrane structures and initiate infection process. (Maghsoodi et al, 2019). In publication II, binding activity was modified and guided by biopanning procedures. The difference between the usage of lytic T4 phage and non-lytic M13 phage is that the latter was used for capture-based assay (Meile et al., 2020). Instead of using an infective process, M13 coat proteins were used and modified.

5.2.1 Biopanning experiments

In order to achieve biosensing properties for phages, biopanning procedures were performed. Each round of biopanning step increased the affinity of phages to bind quencher or target bacteria. Stronger binders are gradually enriched during several repetitive selection rounds and at the same time, weak binders are depleted from the phage population by extensive washing. The biopanning procedures or library screening were performed according to NEB's manual with modifications (New England Biolabs, Ph.D.™ Phage Display Libraries: Instruction manual). The binding property was in the outer minor phage coat protein pIII of the M13 phage. The reason why pIII was used is that the protein is capable of packing large insertions, it is readily available. The aim of the work was not to create a typical high-affinity binder but rather a biosensing surface that interacts with the quencher and target in a suitable chemical environment of the assay.

In publication **I**, it is described how phage tail binding to target receptor started a series of reactions that eventually led to a changed luminescence signal. The next publication **II** introduced the coat proteins, or capsids able to bind target analytes. The second factor was that the phages were able to capture copper ions, which quenched the used europium label. This property enabled to link the event of target analyte binding to the change of luminescence signal. Copper was chosen because it is known to quench the luminescence of lanthanide complexes like Eu3+:TTA:TOPO (Selvin and Hearst, 1994). In order to direct the system toward *E.coli* recognition, rounds of biopanning procedures against this bacteria were required. Even though affinity towards copper naturally decreased in each round of biopanning, the interaction with

E.coli was connected to the level of luminescence. After several phage clones were tested the most suitable for the screening assay was selected.

Further understanding of the copper-binding phage was achieved with kinetic comparison. The assay revealed how different phages decrease the quenching effect of copper chloride solution. It was assumed that copper-specific phages bind copper ions from the solution and therefore had a protective effect compared to wild-type phages. After 10 minutes of incubation, the copper-specific phage continued to protect the lanthanide label from quenching (Figure 8). Whereas controls such as BSA and wt-m13 the relative signal started to decline. BSA is known to bind different metals and the random sequence of wt-m13 was not assumed to bind copper ions (Selvin and Hearst, 1994).

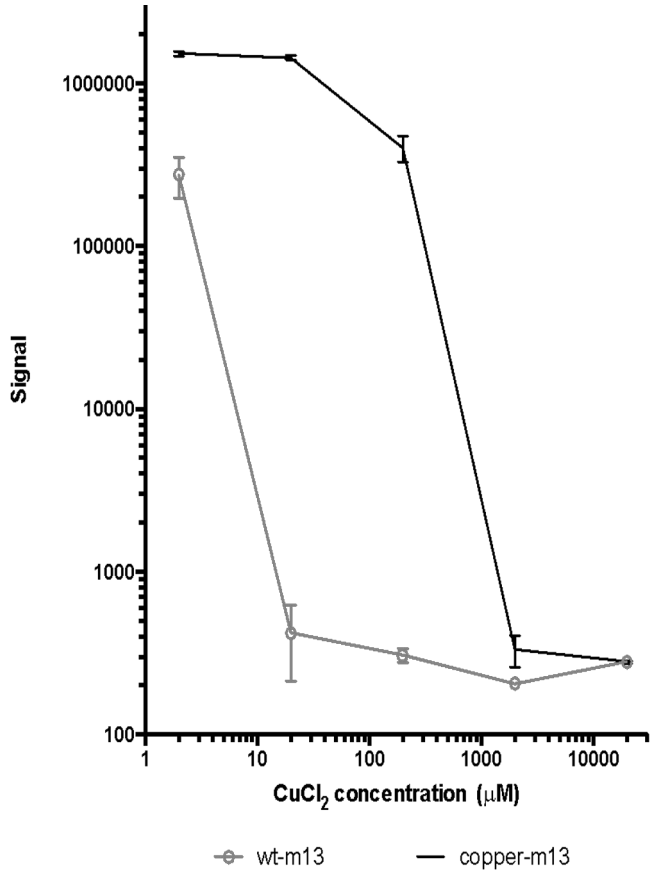


Figure 8. Assessment of copper binding with biosensing phage assay. The assay contained sensitive lanthanide label, phages and varying amounts of copper chloride. Quenching of signal due to copper is seen with different concentrations and phage types. The Figure was reprinted from **II**.

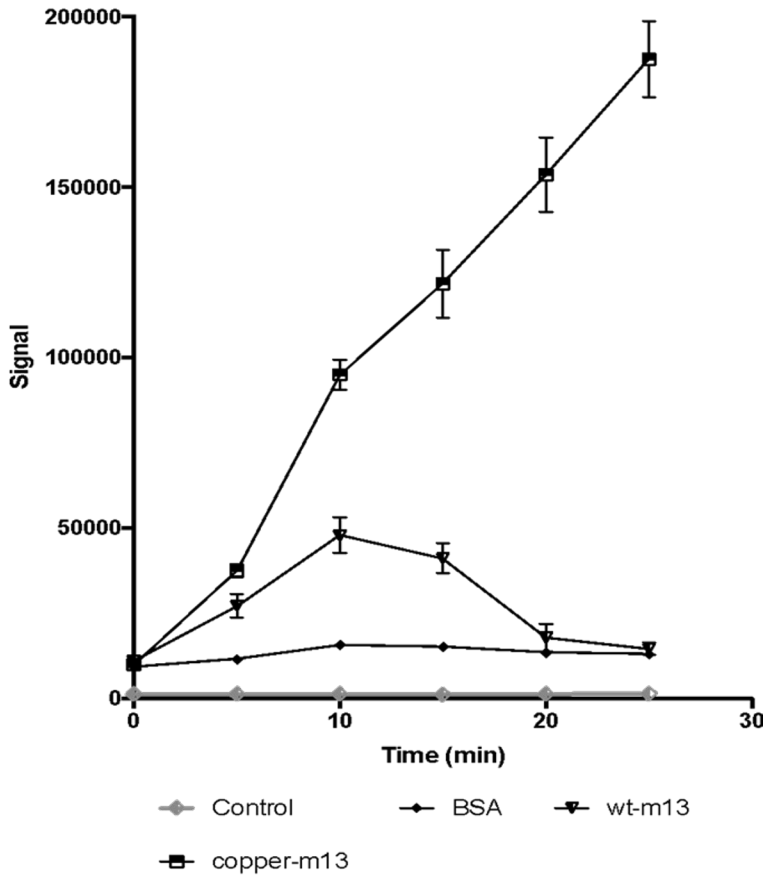


Figure 9. Kinetic comparison of *E.coli* B specific m13 biosensing phage and controls. Controls were wt-m13 and 0.5% BSA solution. The Figure was reprinted from **II**.

After 10 minutes signal-to-noise ratio was observed to increase clearly (Fig.9). 10 minutes incubation time was chosen because the reaction was assumed to have fully started by that time and 10 minutes is an accepted aim for demonstrating a rapid assay with patient samples. The assay time improved when compared with the methods that were based on lytic phages that are dependent on specific lytic cycles (**I**).

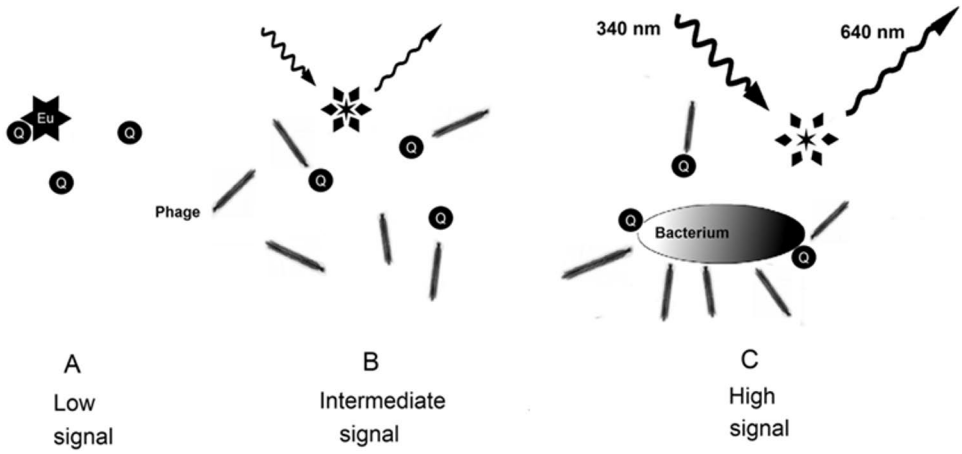


Figure 10. The simplified illustration of biosensing screening system with phages. The Figure was reprinted from **II**.

In Figure 10, Copper is marked as Q that quenches sensitive label (A). Metal quenches easily the europium signal if phages are not present. Higher signal is observed when metal binding phages are distributed throughout the solution and then catching copper ions from the solution (B). If target bacteria are added to the solution, phages are clustered close to the bacterial surface. Simultaneously, copper ions are interacting with phages and possibly bacterial surfaces. A similar formation of clusters or aggregation was seen in a study using copper-specific phages (Korkmaz et al., 2021). Phage display identified and chemically synthesized cysteine-rich peptides were used to capture copper ions. Cysteine amino acids are generally known for their metal-binding properties. In the final biosensor application using these peptides, the aggregation was induced by gold nanoparticles (Korkmaz et al., 2021).

5.2.2 Testing biosensing phages

The possible cross-reactivity of the assay was studied with *E.coli* and *S.aureus*. Both bacterial species were studied in the presence of quenching copper and biosensing phage or wild type phage. The results suggested that phages had more specificity towards *E.coli* than *S.aureus.* The results showed that the difference between wt-M13 and specific phage was significantly higher in the case of *E.coli*, than *S.aureus* (Figure 11).

After biopanning steps, filamentous phage M13 clones were tested with sensitive lanthanide label in the presence of target bacteria, *E.coli* and quenching copper chloride. Overall, from 24 tested phage clones the clone, that had the best biosensing

characteristics, was qualified and enriched. The testing was performed with sensitive label with quenching copper chloride.

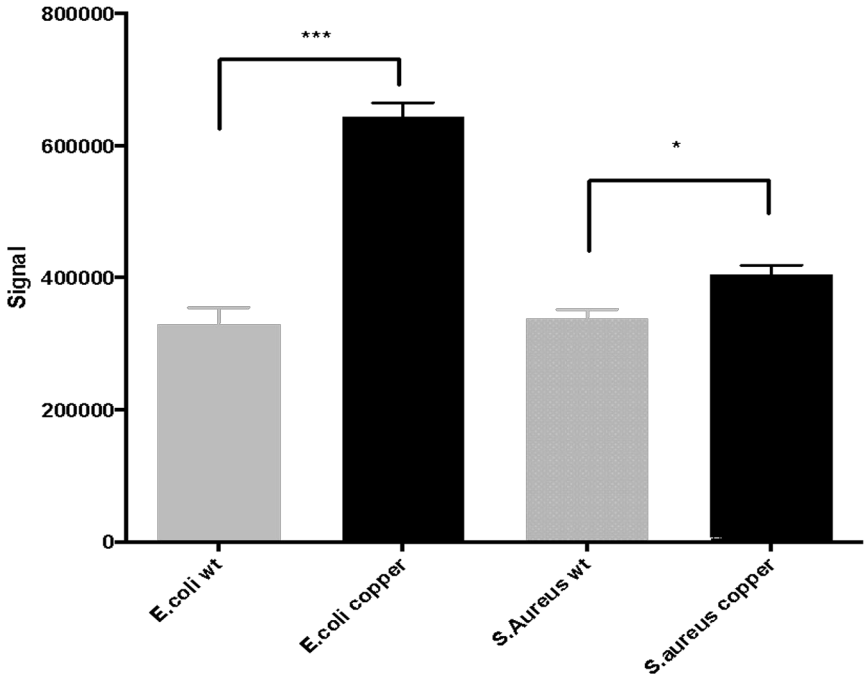


Figure 11. Two different bacterial species compared with the developed screening assay. Copper chloride, europium-label, wt-M13 or biosensing phage was added to the samples. After 10 minutes of incubation with bacterial cell concentration 10 000 cells / mL, time-resolved luminescence signal was measured. The Figure was reprinted from **II**.

The last step for optimizing properties of biosensing phage was to search optimal chemical environment for the screening method (Figure 12). Overall, 80 chemical environments were screened to find possible enhancement of the biosensing capabilities. Two of them: 2,3-Dichloro-5,6-dicyano-p-benzoquinone and triisopropylsilane showed remarkable enhancement. This step was another improvement from the previous method (**I**) as the tuning of the right chemical environment for biorecognition and the proper functioning of lanthanide label was enhanced.

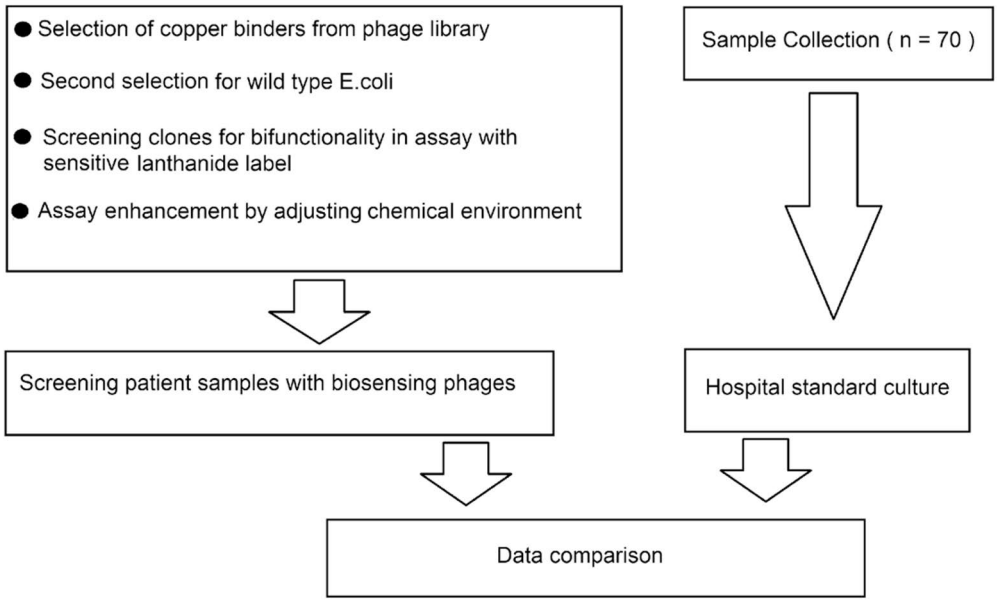


Figure 12. Framework of the development of the phage-based biosensing screening system. The system was then compared with validated culture method. The Figure was reprinted from **II**.

5.2.3 Screening of patient samples with biosensing phages

The ultimate aim of the work was to screen patient samples with the developed biosensing screening method. A panel of 70 urine samples was analyzed and the obtained data was compared with the standard urine culture data considered as golden standard method in hospitals. The proof-of-concept method was tested with patient samples in three different chemical environments. All variants involved specific biosensing phage and wild type reference phage.

The used lanthanide label chemistry combination needed to be sensitive to sample chemical environment in a specific way. That was the reason why 80 different chemicals were used to modify and study the assay chemical environment. Best of three chemical environments were selected for the screening in array form and therefore the data was analyzed with K-Nearest Neighbor (KNN) classification method (Figure 12). The KNN method included artificial random noise data generated from averages from each sample categories.

The assay response was observed to differ between all three tested chemistries, or chemical environments (increase/decrease/magnitude) (Figure 13). It was therefore assumed that each sample-label interaction measured different targets instead of simple repetition of the same measurement. The screening samples with the developed method and analyzing the results with KNN method resulted 90 %

sensitivity and specificity. When considering the results with other rapid methods to detect *E.coli* from urine samples, the outcome is competitive (Zaman et al., 2001). A simple cut-off value comparing between reference and *E.coli* patient samples would have not reached the same outcome.

Considering the overall mechanism behind the assay, it is possible that phages provide protection against the quencher molecules. It is noteworthy that three chemical environments modulated signal differently when measured instantaneously and after 10 minutes of incubation. These time points and the ratio of specific and nonspecific phage was used for signal normalization. Illustrated circled points are from single sample that would have been misclassified by one chemical environment (J30) but was correctly classified by KNN algorithm and the two other chemical environments. Measurement of 3 different at least partly different responses also allowed detection of potential outliers correctly as demonstrated in Fig. 13. Probably each observed TRL-signal is a sum of complex somewhat different networks of nonspecific interactions in the sample material.

Increasing the number of chemical environments might improve the sensitivity and specificity of the assay. Same might take place if several different phage clones would be used with data classification programs. Wu et al., (2020) used cocktail of phages to detect different pathogens. This approach might work with the assay developed in this thesis using lanthanide label and phages in array. Obvious benefit of using phages for clinical screening is the ease of production and the resistant nature of phage particles. Phages are very tolerant to different organic solvents, ultrahigh temperatures and pH ranges that antibodies fail to operate (Bárdy et al., 2016). Phage evolution during millions of years has modified them to operate in various environments including human body and different biological liquids it contains. Another advantage is that the developed assay does not use any labelled reagents and phages aren't attached to any surface. The challenge for applying phages in clinical screening is the slow adoption of phage research to clinical settings and regulatory issues. There are still work to do with coordination between academics and commercial interests (Jones et al., 2020).

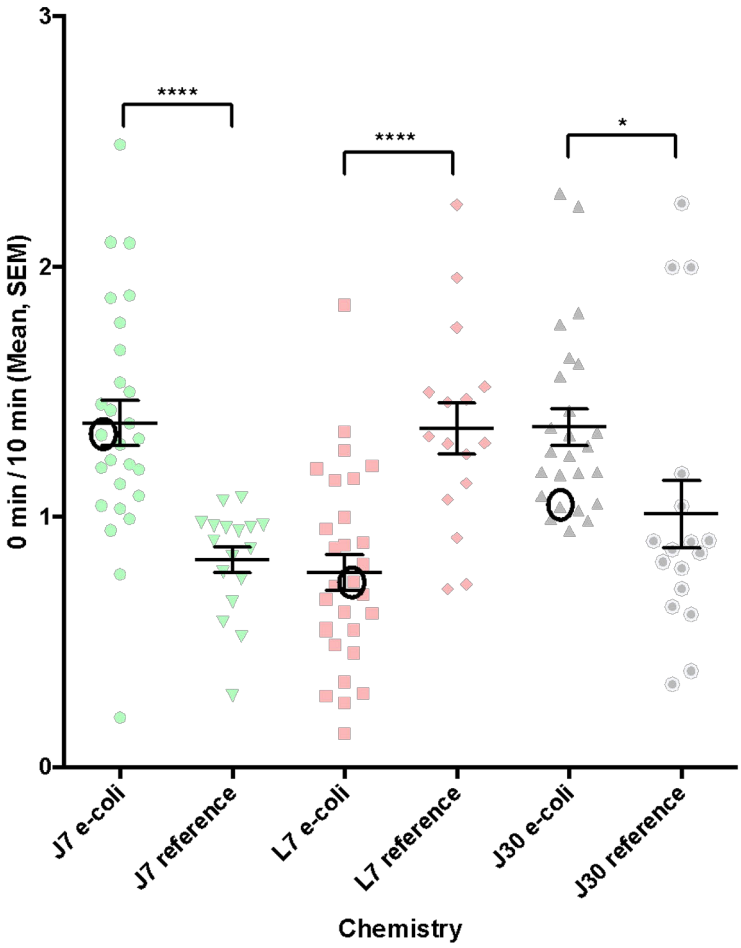


Figure 13. Patient sample screening with the developed method. Chemical environments are noted as J7, L7 and J30. The Figure was modified from **II**.

5.3 Detection of model biomarker and classifying lethal prostate cancer

CRP is one of most commonly detected biomarker form blood and it indicates a variety of different inflammation conditions (Boncler et al, 2019). Therefore, it was chosen as a model biomarker for the development of screening assay. The second purpose of this study was to detect and classify lethal variants of prostate cancer from urine samples. The results were compared with PSA assay that is generally used to diagnose prostate cancer. In this study (**III**) the same lanthanide label was used to screen patient samples as in the publications **I** and **II**. Phages have been employed for cancer detection in several studies. Han, et al. (2016) developed phage-based sensor for whole cancer cell detection. The biosensor was based on electrochemical

impedance spectroscopy changes upon binding of the cancer cell to the golden electrode surface. Earlier Jia, et al. (2007) detected both cancer biomarker and whole cancer cells with label-free potentiometric sensor using silicon nitride surface. More advanced phage-based biosensor detected bladder cancer biomarker proteins from urine samples (Bhasin et al., 2020).

In this thesis work, a modified phage display process was developed for the detection of lethal prostate cancer. The developed phage-based biosensor was a more advanced version than in publication **II** as the reaction was seen not only with time-resolved luminescence, but visible color reaction with the naked eye. Phage-based biosensors are known to produce visible color changes. For instance, when a phage encounters its host bacteria added gold nanoparticles aggregate and the test solution changes its color (Peng et al., 2020).

The development of the method combining phages and lanthanide-label had special incremental steps (Figure 14). In the publication **I**, the system was based on lytic phages binding naturally to the receptor in the surface of bacterial cell. The sensitive lanthanide-label detected leaked interior of the broken cells with the aid of competing chelate for europium (citrate). In the publication **II**, lysogenic phages were used and targeting properties were not natural but made with artificial selection (biopanning). The phages capsids attached to the surface of the target without infection process. This opened a possibility to develop assay to wide range of target analytes.

The quencher used in publication **II** was a simple metal ion, copper that simply replaces europium from the centre of the lanthanide-label. In publication **III**, quenthe cher was changed. Copper used in publication **II**, may bind to chemical groups or binding peptides and proteins that occur in the biological samples therefore are able to hamper the proper functioning of the system. After testing several alternative candidates, brilliant green was chosen. This quencher molecule was selected for quenching lanthanide-label and still being stable in the different chemical environments that different biological samples may have. It was observed that in the presence of phages that were able to bind brilliant green and target analyte, the color of the test solution changed. The phenomenon was observable with absorbance reader. This reaction was probably due to changes in the network of phages and the changed properties of brilliant green in its microenvironment.

Publication	Label	Phage	Target	Quencher	Possible role of quencher	Optical detection type
I	Lanthanide	Phage tail-based lytic infection (natural)	Bacteria	Citrate	Competing chelate for europium	Time-resolved fluorescence
II		Lysogenic, capsid affinity (artificial selection)		Copper chloride	Competing ion for label	
III			Protein, biomolecules	Brilliant green	Sensing chemical microenvironment induced by phages	Time-resolved fluorescence, absorbance

Figure 14. Overview of the method development from the publication **I** to **III**. The key differences of each phage-based biosensor used in different publications are shown.

5.3.1 Detection of CRP

The biosensing system was produced in a way that it recognizes both the brilliant green and CRP creating a competitive microenvironment for the assay. As shown in Figure 15, the CRP biomarker in the sample resulted in a decline of relative lanthanide luminescence. The strongest decline with our set-up is seen around 20 mg/L of CRP which is within the clinically relevant area. An average level of CRP in serum in a healthy person is around 0.8 mg/L but increases as much as to 1000-fold in different types of infections and inflammations (Sproston and Ashworth, 2018). However, it is important to note that the prof-of-principle test was performed in pure physiological saline lacking any interfering components inherent to clinical samples used for biomarker measurements.

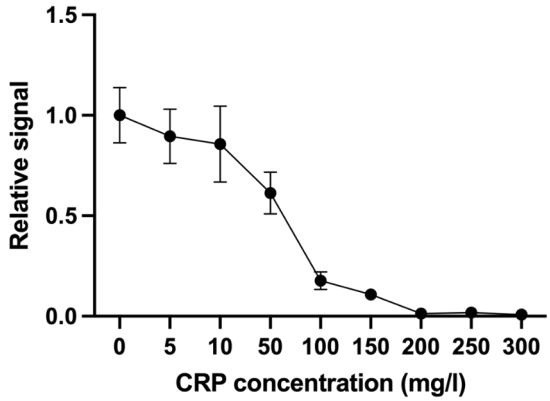


Figure 15. The CRP measurements with phages and lanthanide luminescence. The relative luminescence is decreasing as a function of CRP concentration. Error bars are the coefficients of variance % of the signals from three replicate measurements. The Figure was reprinted from **III**

5.3.2 Classifying lethal prostate cancer

The separation of potentially lethal prostate cancers, Grade Groups 4–5 from clinically insignificant Grade Groups 0–3 has long been a major issue as it may lead to significant overdiagnosis and overtreatment of the disease. The PSA assay for prostate cancers gives both false negatives and false positives because the PSA concentration can rise from various reasons not related to the disease itself. Therefore, there is a need for assay detecting new prostate cancer related biomarkers.

In this thesis work, lethal and non-lethal prostate cancer samples were studied with the developed phage-based biosensor and compared to PSA values (Figure 16). Grade Group for each patient sample was determined by histological analysis of prostate biopsies after MRI and targeted ± systematic biopsies. All the patients were also tested with PSA-antibody based blood test which is currently the gold standard method (Right side). The phage-based method resulted in a clear and statistically significant difference between the groups.

In this doctoral work, the separation between lethal prostate cancer and non-lethal samples was achieved with one phage clone. Instead, a phage-based sensor using twenty designed phages was reported recently (Lee et al., 2021). The method combined neural network analysis, electronic nose and DNA sequences from mammalian olfactory receptor cells. The engineered phages were able to detect lung cancer breath samples from control samples without biopanning processes. Considering this, several phages in array form as in publication **II** may improve the performance of the assay developed in this work.

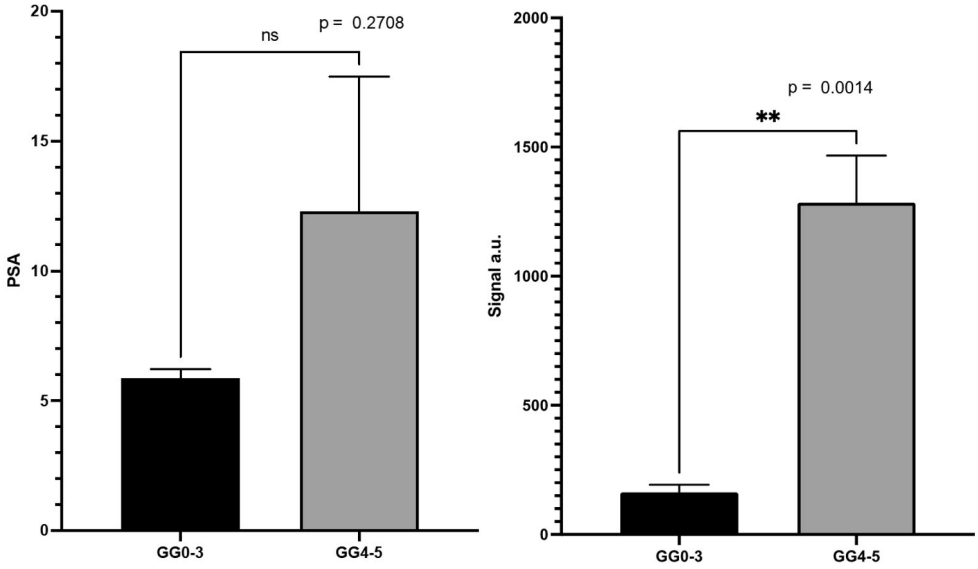


Figure 16. The phage-based assay signal for the potentially lethal and non-lethal PCa (Left side). The Y-axis is the linear combination of the measurements (Mean, N=96, median of 3 replicates, error bars SEM). The left black bar shows PCa GG 0–3 (N=90) samples and the right grey bar shows GG 4–5 (N=6). Mean PSA for the GG 0–3 group (left black bar) and the GG 4–5 group (right gray bar). The Y-axis is the linear combination of the measurements (Mean, N=96, median of 3 replicates, error bars SEM). The Figure was reprinted from **III**.

6 Summary and Conclusions

Biosensors are relatively recently described group of sensors that use various types of bioreceptors as a detection elements. Basically the detection involves biosensing between biorecognition element and analyte. Shortly, biophysical or biochemical property of the sample is converted into an electrical signal. Biosensors are proven to be effective detection methods and devices for globally important pathogenic microbes.

In this thesis, three different assay setups were developed. They were based on luminescent lanthanide label that has clear benefit of sensitivity by elimination of autofluorescence from biological sample material. First two studies concentrated to phages as biological recognition elements for detecting bacteria. Third study studied the applicability of the lanthanide label-based phage biosensor for known biomarker in controlled chemical environment and finally prostate cancer urine samples.

In publication **I**, it was found that bacterial cell rupture can be detected via sensitive lanthanide label. Same could be seen in urine matrix, that contains various metabolites possibly interfering lanthanide label. Furthermore, lytic phage breaking the bacterial wall causes similar results and signal changes. Phages were chosen for the study because they are very resistant to various conditions and offer a natural binding scaffold for target analytes. Model bacteria, *E.coli* was detected rapidly using lytic phages. The results were competitive when compared with other biosensors and established detection methods for detecting *E.coli*. In general, the observation that broken cells induce a change of luminescent signal can be exploited for many bioassay applications. It is likely that many types of cell membrane disruptions can be observed with the method. A clear benefit is that the system has no labelled biomolecules or any washing steps.

In publication **II**, it was demonstrated, that *E.coli* can be detected not only by disrupting the membrane but also with bacterial surface interacting phages and their peptides. This is much more straightforward and faster method than the previously described method for screening *E.coli* from the urine. The performance of the method was tested with a panel of patient samples that had different bacterial pathogens. High sensitivity and specificity of the urine screening resulted within only 10 minutes reaching performance values used in hospital laboratories. In this

study, bifunctional phage offered a new way of detecting targets. Phage binding to quencher and target must be balanced in order to have functioning assay. The desired performance of the biorecognition element, phage can be further enhanced by adjusting chemical environment of the screening assay. This opens possibilities to develop similar systems to detect other important pathogens, or to create a semi-specific phage-based array for detecting multiple microbial species.

Publication manuscript **III** concerning lanthanide label-based screening method for lethal prostate cancer involved challenging sample matrix and no known biomarker to screen for. Main concern was quenching factors but also a myriad of possible unknown interactions in the urine. The novel screening method has high potential because of its simplicity and rapid performance. The results obtained with the developed method were different from the biomarker test for prostate cancer PSA, which is used as a gold standard in prostate cancer diagnostics. This indicates that the developed method interacts with molecules unrelated to PSA. It should be noted that even though assay specificity and sensitivity were high, the number of patients in the study was limited. These results suggest that the study should be conducted in a larger-scale clinical material.

Collectively, these results suggest that the developed luminescent lanthanide label-based methods are suitable for biological matrices and phages are a potent tool for different biosensor platforms.

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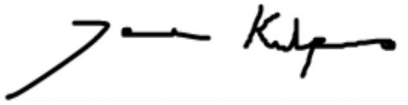

Turku, June 2022
Janne Kulpakko

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