D5.5

Integration of multiplex LFA into the SciFiMed biosensor

Project title: Screening of inFlammation to enable personalized Medicine: *SciFiMed*

Grant Agreement No.: 899163

Call identifier: H2020-FETOPEN-2018-2020

Coordinator: Philipps Universität Marburg – UMR

Project start date: 1st of January 2021

Duration: 48 months

Deliverable type:	Report
Task and Work package contributing to the deliverable:	WP5 Task 5
Due date:	Aug. 31st, 2024
Actual submission date:	

Responsible lead organisation:	UREG
Lead Author:	Antje J. Baeumner / UREG
Dissemination level:	Public
Revision Date:	
Document Reference:	

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 899163.

Revision/ Review No.	Date [month/day/year]	Work performed	Contributor
1.0	07/23/2024	Report on efforts to integrate LFA into the SciFiMed biosensor	M. Loessl/UREG
2.0	07/24/2024	report review/ revision	A. Baeumner/UREG
3.0	08/01/2024	Report review/ revision	D. Pauly/UMR
4.0	08/05/2024	revision	M. Loessl/UREG
5.0	08/06/2024	Revision	C.Spitzenberg/UREG
6.0	08/06/2024	Final review	A. Baeumner/UREG

Disclaimer

Abbreviations and acronyms

EDTA	Ethylenediaminetetraacetic acid
ELTE	Eötvös Loránd University
FCN	Ferrocyanide
FH	Complement factor H
FHR	Complement factor H-related proteins
GOx	Glucose oxidase
HBT	Hycult Biotech
HRP	Horseradish peroxidase
LCB	Liposome complement buffer
LFA	Lateral flow assay
LIG	Laser-induced graphene
LOD	Limit of detection
LPS	Lipopolysaccharide
mCL	*m*-carboxy luminol
MES	2-(N-morpholino)ethanesulfonic acid
MICRO	Microcoat Biotechnology GmbH
MTP	Microtiter plate
OG	*n*-Octyl-β-D-glucopyranoside
PB	Prussian Blue
PBS	Phosphate-buffered saline
PMMA	Polymethyl methacrylate
POC	Point-of-care
RT	Room temperature
RuHex	Ruthenium hexamine
SAN	Sanquin
SciFiMed	Screening of inflammation to enable personalized medicine
SRB	Sulforhodamine B
tL	Total lipid
TMB	3,3′,5,5′-Tetramethylbenzidine
UCM	Complutense University of Madrid
UMCG	University Medical Center Groningen
UMR	University Marburg
UREG	University Regensburg

Summary

Proof-of-concept of complement-induced liposome lysis on lateral flow assays (LFAs) was successfully accomplished with the option for pathway- or ligand-specific lysis by liposome modifications.

We furthermore showed proof-of-principle for effective multiplexing in microfluidic chips via the integration of polystreptavidin-functionalized nitrocellulose membranes and detection of biotinylated liposomes.

A multiplexed electrochemical sensing platform with the possibility of adaptation towards the detection of FHR was demonstrated in D5.4. Since high background signals and quenching of the electrochemical signals were observed when applied to human serum, new encapsulants, namely enzymes such as horseradish peroxidase (HRP) or glucose oxidase (GOx) were also investigated. Such markers cannot be used in a simple LFA format, but can be applied in microfluidic chip technologies. For HRP and its electrochemical substrates, human serum hindered detection as well. GOx liposomes showed increased permeability of the lipid bilayer, leading to glucose penetrating into liposomes and generating hydrogen peroxide without lysis of liposomes. Thus, liposome lysis cannot be detected with these kinds of liposomes.

As the SciFiMed-project, in agreeance with the scientific board, pivoted towards the detection of factor H (FH) using a microtiter plate (MTP) assay format, the decision was made to cease all other endeavors within this project. Instead, all resources were redirected towards the advancement of a FH assay.

Contents

Integration of multiplex LFA into the SciFiMed biosensor

1. Introduction

This deliverable D5.5 ‘Integration of multiplex LFA into the SciFiMed biosensor’ is related to work package 5 ‘Combined quantity and activity biosensor’. To develop point of care (POC)-friendly sensors for more complex systems such as the complement system, new pathways for effective multiplexing, quantitative and functional detection have to be explored.

Lateral flow assays (LFAs) are well established and easy-to-use devices for qualitative or semi-quantitative analyte detection in at-home settings. However, their simplicity limits the flexibility and complexity regarding possible assays[1].

Capillary pump-driven microfluidic chips offer more possibilities such as (stop) valves, mixing and retention elements, thereby offering tools to control the fluidic environment at the microscale. Hence, more complex assays can be performed in a miniaturized setting while still retaining the simplicity of use and single-use products needed for POC settings[2]. After previous efforts to develop a multiplexed electrochemical sensing platform[3], it was found out that redox markers such as ruthenium hexamine(III), potassium hexacyanoferrate(II) and *m*-carboxy-luminol could not be detected sensitively enough in presence of human serum. Thus, a new strategy, using enzyme-encapsulating liposomes, aiming to exploit their ability to generate large amounts of electrochemically active substances even at low concentrations, was investigated, with the goal of using these liposomes in the previously established multiplexed sensing platform.

In the SciFiMed-project, we aimed to develop a fully functioning POC biosensor for the detection of factor H (FH) or factor H related (FHR) proteins. To do so, we aimed to combine knowledge gained by the proof-of-principle of liposome lysis detected by LFAs with capillary pump-driven microfluidic chips, effective liposomal multiplexing and electrochemical detection techniques. Furthermore, the antibody-based LFAs were supposed to be integrated into the microfluidic biosensor. In the end, a personalized biosensor for personalized medicine for complement-related diseases was envisioned.

2. Methods and Materials

2.1 Liposome preparation and characterization

Liposomes were synthesized and characterized as described previously in D5.1.

2.2 Complement-induced liposome lysis on LFAs

Liposome complement buffer (LCB) was prepared using 10 mM HEPES, 150 mM NaCl, 135 nM $CaCl_2$ and 1 mM $MgCl_2$ at pH 7.4. Running buffers consisted of LCB with added 0.4 w% PVP, 0.1 w% Tween-20 and 200 mM $CaCl_2$. For inactive serum samples, 220 mM $CaCl_2$ were used in the washing buffer.

UniSart CN150 membrane was purchased from Sartorius. Membranes Fusion 5, Nytran SPC, FF120HP, FF120HP Plus and FF120HP Plus Thick were obtained from Cytiva. Glass conjugate pad 8980 was bought from Ahlstrom Munksjö. The waste pad 270 was obtained from Kenosha.

Double-sided adhesive tape (5 cm) was applied on a plastic foil (laminated foil without filling, 8 cm) that was used as backing. The membrane (2.5 cm) was glued on the foil leaving 2 cm distance to the bottom edge. Then the sample pad (2.2 cm) and the waste pad (1.6 cm) were glued on the foil, both overlapping with the membrane for 2 mm. Test strips with a width of 0.5 cm were then cut with a paper cutter.

Liposome assays were carried out in either a LCB sample, a 25 vol% inactive serum sample (iaS), a 25 vol% active serum sample (aS) or a 30 mM OG + 25 vol% active serum sample (OG), with each condition tested in triplicates.

Serum was thawed and the necessary volume was inactivated by mixing 2.5:1 with inactivation complement buffer. The samples were incubated for 60 min at 37 °C in an incubator. After incubation, 25 vol% active serum was added to the LCB sample. Liposome solutions were diluted 1:1 in the respective running buffers and added to the wells of a clear 96-well microplate (MTP, 50 µL per well). In all cases, MTP wells contained 50 µM tL of anionic, biotinylated SRB-encapsulating liposomes (44 mol% Cholesterol; 2 mol% DPPE-Biotin). Test strips were placed into the wells (t = ‘0 min’) and the respective washing buffer (100 µL per well) was added after 5 minutes. Pictures of the dry test strips were taken and evaluated the next day.

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 899163.

2.3 Preparation of capillary-driven microfluidic chips for multiplexed analyte detection

Microfluidic chips were prepared using a VLS2.30 lasercutter (Universal Laser Systems, Scottsdale, AZ, USA), equipped with a 30 W CO2-laser (10.4 µm). Following parameters were used (see Figure 1): Black: 24.0 % power; yellow: 28.8 % power; cyan: 19.3 % power; magenta: 38.2 % power; all in raster mode with 90% speed. Orange was scribed with 25 % power and 75 % speed in vector mode.

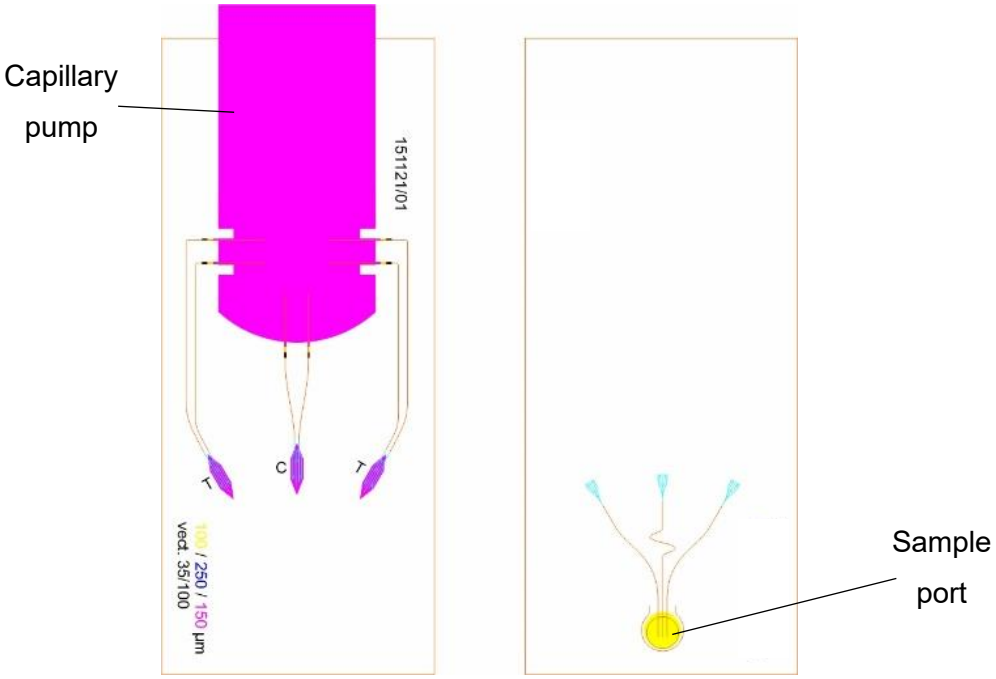

Figure 1: Designs for a multiplexed microfluidic system, laser-engraved into PMMA plates. Left: Top plate; right: bottom plate. Magenta marks the integration of the capillary pump, at violet, (T) polystreptavidin-modified (left and right) or (C) unmodified (middle) nitrocellulose membranes are integrated.

Channels prior to the nitrocellulose membrane were integrated into the bottom plate and channels afterwards into the top plate to ensure proper flow through the membranes. Polystreptavidin was immobilized by dropping 0.2 µL of a protein solution (2 mg/mL poly-streptavidin) on the center of the membrane. Afterwards, the membranes were dried for 1 h at 37 °C. Nitrocellulose membranes were integrated into the microfluidic chip, prior to bonding via the chemical vapor bonding method. To elaborate, PMMA chips were put in a chloroform saturated atmosphere, kept for 3 min 45 s and afterwards pressed onto each other. For the assay, 2 µL of SRB-encapsulating, anionic, biotinylated liposomes (2 % biotin, 44 % cholesterol, c_{tL} = 8.87 mM) were added to the sample port and washed with 480 µL LCB.

2.4 Fabrication of LIG electrodes

Laser-induced graphene (LIG) electrodes were fabricated using 125 µm Kapton sheets and a CO_2-Laser (VLS 2.30 system, 30W) with the following parameters: 1% power, 10% speed (13 cm/s), 1000 PPI, image density 6, 2.0”-lens, in focus. The leads of the LIG were coated with clear nail polish to ensure a constant electrode surface.

In case of glucose oxidase detection, LIG working electrodes were coated with 2 µL 5 mM of each $FeCl_3$ and $K_3[Fe(CN)_6]$-solutions and cured for 2 h at 100°C to form Prussian Blue (PB). Afterwards, 2 µL of 0.1% (w/v) chitosan solution were dropped on the electrode on incubated for at least 90 min at room temperature.

2.5 Electrochemical detection of enzyme-encapsulating liposomes

Solutions containing 0-1000 pM HRP, 15 µM TMB and 0.1 mM H_2O_2 with and without 10% human serum in LCB were prepared in triplicates. After enzyme addition, solutions were incubated for 20 min, after which 80 µL 1 M H_2SO_4 were added. Square-wave voltammetric measurements were conducted with the PalmSens4-potentiostat each on fresh LIG electrodes after 15 min incubation on the electrode surface with an external Ag/AgCl electrode. Following parameters were used: Equilibration time: 3 s; potential range: 0.1 – 0.85 V, potential step: 0.005 V; amplitude: 0.025 V; frequency: 5.0 Hz.

For optical glucose oxidase liposome detection, 0.09 µM tL glucose oxidase liposomes in a MES buffer (10 mM MES; 210 mM NaCl; pH=6.5) were incubated with and without 30 mM OG for 20 min, after which 4 µg mL^{-1} HRP, 50 µM Amplex Red and 100 mM or 10 mM glucose were added. Solutions were prepared in triplicates. Fluorescence intensity at 590(10) nm with an excitation wavelength of 545(10) nm was monitored for 1 h at room temperature on a FluoStar Omega MTP reader.

For electrochemical measurements, 0.235 mM tL in phosphate buffer (40.23 mM KH_2PO_4; 34.77 mM K_2HPO_4; 4.54 mM NaCl; 1 mM $MgCl_2$; 135 nM $CaCl_2$; pH=7) were incubated for 5 min with and without 30 mM OG. Solutions were prepared in triplicates. 50 µL of the respective solution were pipetted onto LIG-PB electrodes and amperometric measurements taken with a PalmSens4-potentiostat using an external Ag/AgCl electrode. Following parameters were used: Potential: -0.05 V; run-time: 120 s.

3. Results

3.1 Proof-of-principle for complement-induced liposome lysis in LFAs

Ligand-specific as well as unspecific complement-induced lysis of liposomes has been accomplished in previous studies, see D5.1. Thus, the transfer of this technology towards POC-friendly LFAs was investigated as a proof-of-principle.

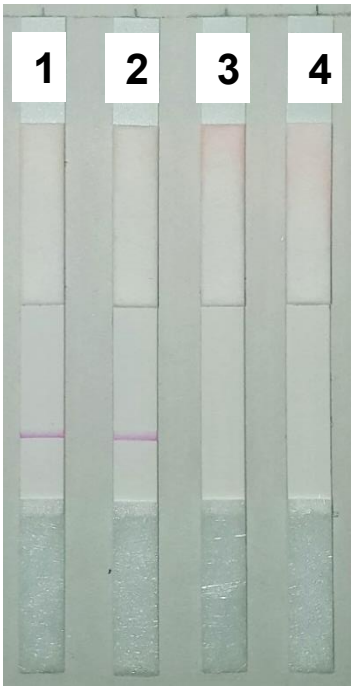

Figure 2: Test strips (1-4 from left to right) of anionic, biotinylated high cholesterol liposomes (50 µM total lipids) run according to the optimized lateral flow assay procedure after 60 min incubation at 37°C in liposome complement buffer w/o serum (strip 1), with 25 vol% inactive serum (strip 2) and with 25 vol% active serum w/o (strip 3) and w/ 30 mM OG (strip 4). 25 vol% active serum were added to the liposome complement buffer sample after incubation prior to addition to the test strip.

Polystreptavidin-functionalized nitrocellulose-membranes and biotinylated high-cholesterol liposomes for pathway unspecific human complement lysis were used. Thus, if liposomes remain intact, they will bind to the polystreptavidin test line via the biotin-streptavidin interaction, leading to signals visible by naked eye. On the other hand, if liposomes are lysed, either via detergent or complement-induced, the dye will be released, cannot bind to the test line and therefore, no signal should be present. As evident in Figure 2, expected results were accomplished, with the negative controls containing either liposome complement buffer or inactive serum employing clearly visible signals. Both the negative control containing OG as detergent and the complement active human serum sample showed an almost complete decrease in signal. This result proves, that detection of complement activity using liposomes and LFAs is possible. Using modified liposomes containing LPS, mannan or antibodies, or other suitable ligands, pathway-specific complement activity detection as well as

detection of FH and FHR should be possible as well. As multiplexing on LFAs however is in general challenging and leads to loss in sensitivity, capillary-driven microfluidics were investigated with the aim of improving sensitivity while also having higher flexibility in application compared to classical lateral-flow assays.

3.2 Capillary microfluidics as a POC-friendly detection platform for multiplexing

Capillary-driven microfluidics combines the advantages of simplicity of use of LFAs with the advantage of using more sophisticated channels, valves and reaction chambers. Here, we combined the novel approach of using filter paper as high-capacity capillary pumps with rapid prototyping using laser-scribing of PMMA chips. Thereby, a high number of different designs could be evaluated rather quickly compared to other microfluidic techniques such as hot embossing, micro-milling or (photo-)lithography. Similar to the LFA investigated previously, three nitrocellulose membranes, with two of them modified with polystreptavidin test spots, were integrated into microfluidic chips (see Figure 1).

Through all three nitrocellulose membranes, stable flow was achieved. Employed biotinylated liposomes bound to polystreptavidin-modified test spots, as visible by the pink coloration on left and right spots in Figure 3. The middle, control membrane showed almost no coloration after washing away all of the unbound liposomes. This can be seen more pronounced in Figure 3 using inverted colors.

Figure 3: Left: Microfluidic chip with polystreptavidin-modified nitrocellulose membranes (left and right) and unmodified NC membranes (middle) after addition of 2 µL 8.87 mM biotinylated liposomes and washing with 480 µL liposome complement buffer. Right: Zoom-in to the NC membranes (upper picture: colors inverted).

While some unspecific binding is seemingly present, as evident by the coloration of the unmodified nitrocellulose membranes after the washing step, the proof-of-concept was successful and, with further optimization, is expected to outperform LFAs in multiplexed detection.

3.3 Enzyme-encapsulating liposomes for electrochemical complement activity detection

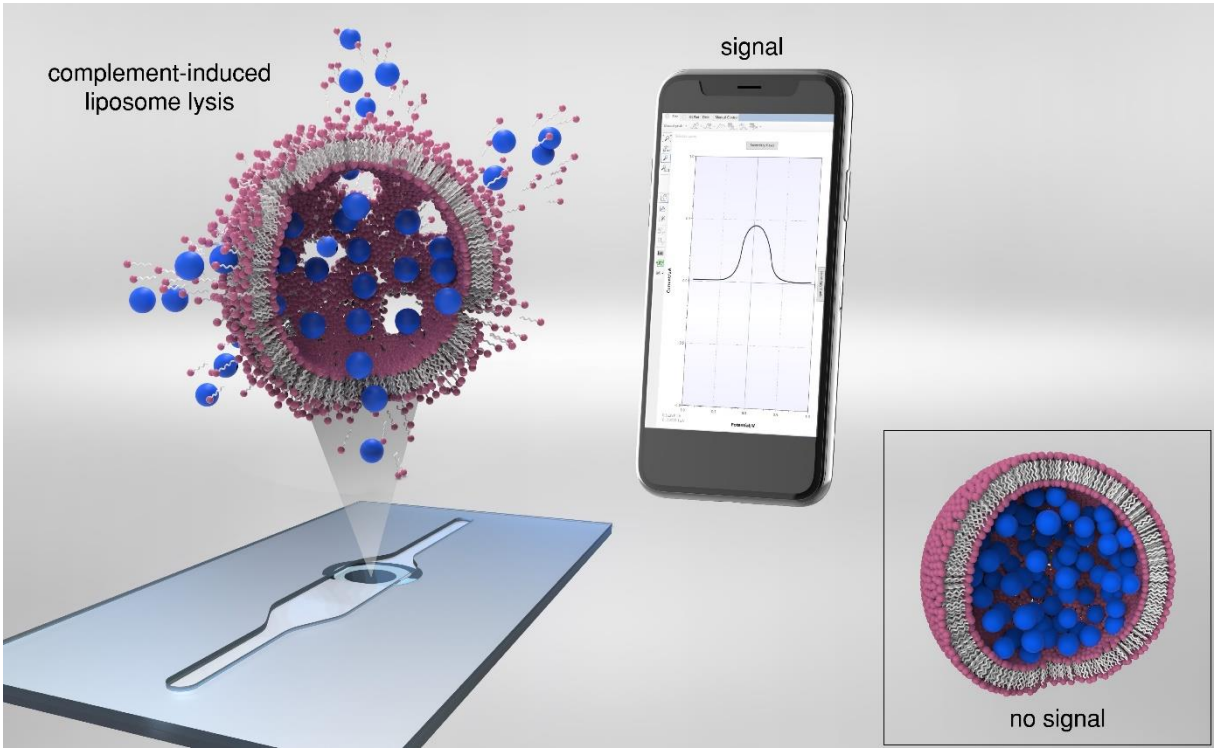

Figure 4: Envisioned assay principle. Enzyme (HRP or GOx)-encapsulating surface-modificated liposomes can undergo complement-induced liposome lysis, upon which the enzyme gets set free, reacts with a suitable substrate, which in turn provides an electrochemical signal. Scheme provided by Vanessa Tomanek

As shown previously in D5.4, different redox markers were investigated for their use in a multiplexed sensing platform. However, when these liposomes were used for detection in human serum samples, it was found that the concentration needed was too high for complement-associated analysis due to a high background signal and a quenching of the electrochemical signal. Hence, other encapsulants that would allow lower limits of detection were needed.

Thus, we investigated the combination of enzyme-encapsulating liposomes with electrochemical detection to develop functional complement assays with low limits of detections (LODs). The use of electrochemical detection in POC microfluidic biosensors offers a cost-effective and miniaturizable solution for rapid and sensitive diagnostics. Enzymes, acting as biocatalysts, can generate substantial amounts of

electrochemically active substances even at low concentrations, thereby amplifying signals. This was investigated especially in regards to measurements in human serum, which often is challenging using traditional electrochemical markers. LIG, a high-performance material fabricated in-house, was utilized due to its cost-effectiveness, excellent properties for integration in microfluidic elements, and suitability for rapid prototyping.

HRP, a stable, low-cost, and versatile enzyme, was encapsulated in liposomes for detection. After screening multiple substrates for HRP, TMB was selected due to its non-toxicity, quick and reproducible reactions, and ability to generate high electrochemical signals. While the detection of OG-lysed HRP-encapsulating liposomes was successful, the addition of human serum completely hindered electrochemical HRP detection (Figure 5). Consequently, a new enzyme was explored.

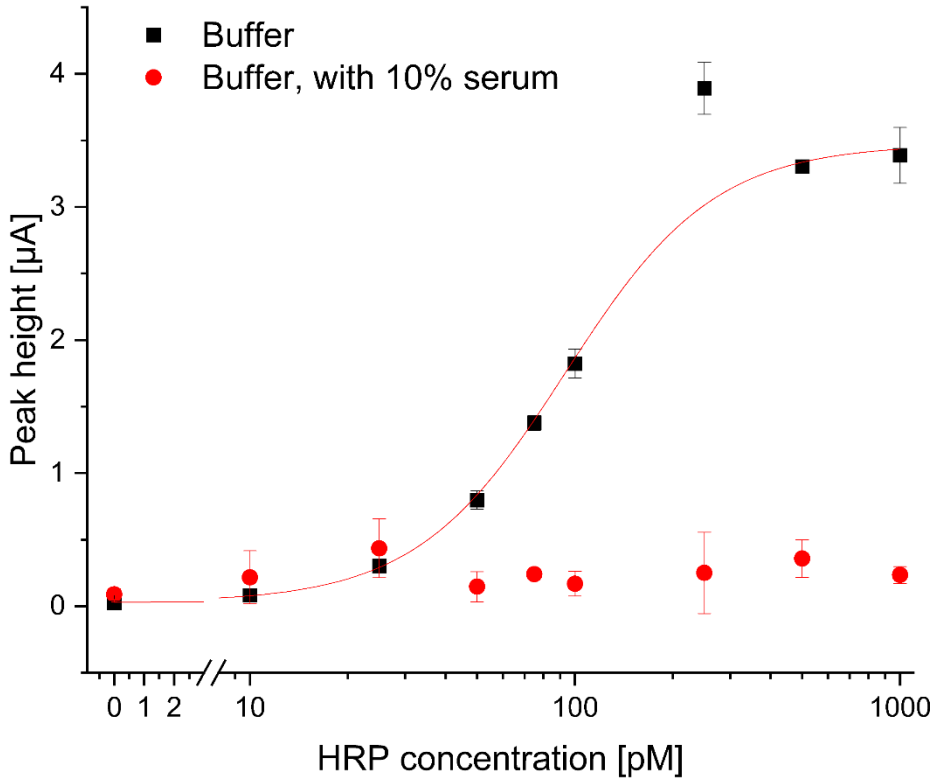

Figure 5: Different concentrations of HRP with 15 µM TMB in LCB with and without 10% human serum (pooled); Peak heights at 0.30 V determined via SWV (square-wave voltammetry). Measurements performed in triplicates.

Glucose oxidase (GOx), similar to HRP in terms of stability and cost, was investigated. The reaction of glucose with this enzyme produces hydrogen peroxide, which generates the electrochemical signal. To facilitate hydrogen peroxide detection, LIG electrodes were modified with PB. Detection of glucose oxidase in various buffer

environments, including 10% human serum, was successfully accomplished, and stable liposomes were synthesized. However, detecting GOx-encapsulating liposomes proved challenging, likely due to the interaction between the lipid shell of the liposome and the enzyme. This interaction, as also reported in literature[4], led to increased substance exchange into the liposome, i.e., penetration of glucose into the liposome and its subsequent reaction with the enzyme prior to lysis. This phenomenon was also evident in the high electrochemical or fluorescent signals stemming from unlysed liposomes (Figure 6).

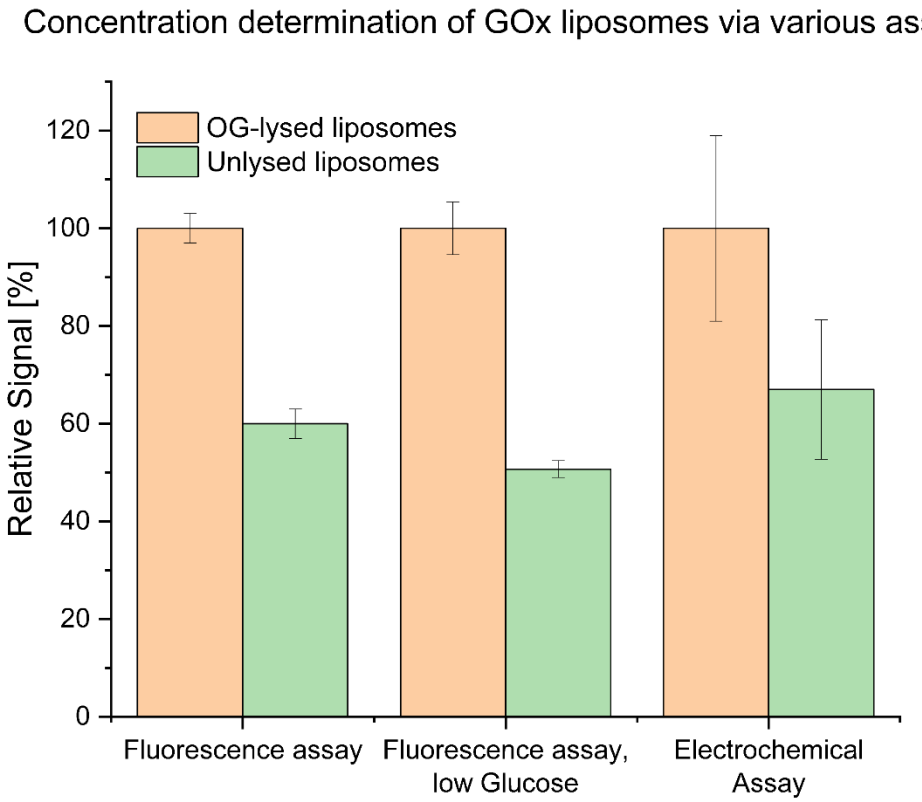

Figure 6: Ratio of OG-lysed GOx -encapsulating liposomes to unlysed liposomes, determined via fluorescent Amplex Red assays or electrochemical detection on LIG -PB electrodes. For fluorescent assays, 94 nM tL GOx-encapsulating liposomes were incubated with 30 mM OG for 20 min, after which 4 µg mL^{-1} HRP and 50 µM Amplex Red were added. After addition of 0.1 or 0.01 M glucose, fluorescence was measured for 60 min, with an excitation wavelength λ_{exc} of 545 nm and an emission wavelength λ_{em} of 590 nm. For electrochemical measurements, 0.235 mM tL liposomes were incubated with 30 mM OG. 0.1 M glucose was added, solutions incubated for 5 min and chronoamperometric measurements at -0.05 V on PB-modified LIG electrodes taken against an external Ag/AgCl reference electrode. Measurements performed in triplicates.

Due to that, liposome lysis with glucose oxidase-encapsulating liposomes cannot be detected. Thus, enzyme-encapsulating liposomes for electrochemical detection did not work as envisioned. With further efforts concentrating on protection of the electrode from serum protein adsorption or investigation of different enzymes, positive outcomes of this part of the project are expected. However, due to a shift in the focus of the

SciFiMed-project, it was ultimately decided to discontinue further efforts on electrochemical liposome detection.

4. Conclusion

Various strategies to develop a fully functioning POC device for FH and FHR detection were investigated. As a proof-of-principle, detection of complement-induced liposome lysis on LFAs was accomplished. Furthermore, a concept for multiplexed detection on microfluidic chips was developed using spatial separation of the solution into different channels and integration of test pads, which in future application can be modified at will. This way, qualitative or semi-quantitative assays for complement activity or, if suitable ligands are available to couple to liposomes, FH and FHR assays could be accomplished. Furthermore, the microfluidic chip technology allows the use of a variety of markers including electrochemical, optical, small molecules and enzyme-based strategies.

Unfortunately, the use of enzyme-encapsulating liposomes with electrochemical detection with the aim of developing a functional, microfluidic assay for detecting human complement activity was not feasible. The human serum proteins hindered electrochemical signal generation which are traditionally overcome through membrane coating, however for this sensor set-up such separation is not feasible. Furthermore, interactions between the enzymes and lipids lead to increased diffusion or substance exchange through the liposomal membrane. In the future, optical approachs should be studied as those substrates will have a lower likelihood of membrane diffusion.

Finally, the focus of the SciFiMed-project shifted based on the reviewers' recommendations. It changed from the multiplexed FHR-sensors to a robust FH-assay due to the inaccessibility of suitable ligands. As a result, multiplexed sensing was no longer required and all resources were applied to the development of FH assays. Nevertheless, based on results in the LFA and microfluidic experiments, a miniaturized, multiplexed assay for the simultaneous detection could be accomplished, provided suitable ligands for FH and FHR were available.

5. References

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