BIOENG-444 Advanced bioengineering methods laboratory
Practicum: BBIA

Diagnosis of kidney failure through quantification of NGAL by a beads-based immunoassay

Practicum syllabus

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Office hours: Spring semester, on demand

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1 Practicum schedule

Schedule	Experiment	Time	Activity	Room
WEEK 1 2pm-6pm	Experiment Preparation	**14:00-15:30**	Introduction to BBIA and discussion of questions	TP SSV
		15:30-17:00	Preparation of beads	
		17:00-18:00	Antigen Preparation	
WEEK 2 2pm-6pm	Data Acquisition	**14:00-15:30**	Preparation of sample solutions and reagents for antigen binding on beads	TP SSV
		15:30-17:00	Data acquisition	
		17:00-18:00	Data analysis	
WEEK 2 Post practicum	Experiment Follow-up	Sent by email	Collect results	

2 Introduction

Some sections of the following chapter have been taken or readapted from the work of Thiriet, P-E, et al. "Rapid Multianalyte Microfluidic Homogeneous Immunoassay on Electrokinetically Driven Beads." Biosensors 10.12 (2020): 212 and the master thesis of D. Medagoda.

The field of immunoassays is one of the most widely investigated topics in today's diagnostic research. Using beads for immunoassays has proven to be a promising alternative to the already well established lateral flow immunoassays. In this BIOENG-444 laboratory practicum, we will get to know beads-based immunoassays, and we will use them to quantify the acute kidney injury biomarker NGAL.

2.1 Bead-based immunoassays

In Beads-based immunoassays (BBIA), the analyte is captured by the bead decorated with capture antibody (cAb), and detection is performed with the fluorescently labeled detection antibody (dAb). A schematic of this can be seen in Figure 1. A common way to bind the cAb to the surface of the beads is through streptavidin sites on the beads coupling to the biotin sites of the cAb. The beads can be made of different materials, such as latex (e.g., polystyrene), but also metals, which can be useful for manipulating the beads with magnetic fields. The advantage of BBIAs when compared to standard immunoassays, such as the enzyme-linked immunosorbent assay (ELISA), is that they offer a high surface-to-volume ratio increasing the likelihood of molecules encountering a capture agent [1]. Furthermore, the beads are highly versatile and can be functionalized with various capture agents allowing for multiplexing to be performed, compared to each ELISA experiment testing only one single analyte.

For detection, the beads must be localized via image processing or guided to a specific sensing area where the measurements can be taken. Latex beads, made from polymers such as polystyrene, offer a wide range of surface chemistries to suit different capture agents [1]. In the micrometer range, these particles are light and easily suspended in a solution of incubation, diminishing mass-transport issues. Adding a magnetic component to these beads allows for precise handling and separation of these beads for analysis [2]. Manipulating magnetic beads into a specific area for analysis has occurred for decades [3] and provides benefits in terms of boosting the signal and reducing background interference. However, critically, magnetic beads have a strong autofluorescence component [4], requiring complex photobleaching for compensation and interfering with standard assays relying on fluorescence.

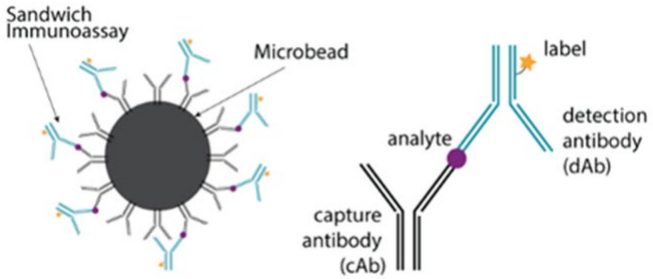

Figure 1: Illustration of a bead-based sandwich immunoassay used for detection of biomarkers. The analyte is captured by the bead decorated with capture antibody (cAb), and detection is performed with the fluorescently labeled detection antibody (dAb).

2.2 NGAL as a biomarker for acute kidney injury

In this practicum, we will focus on trying to quantify the amount of a specific biomarker in a sample. Neutrophil gelatinase-associated lipocalin (NGAL) is a protein biomarker that has gained attention for its role in Acute Kidney Injury (AKI). It's primarily expressed in neutrophils and renal tubular cells and is rapidly released into urine and blood upon kidney injury. AKI is a life-threatening condition characterized by a rapid loss of kidney function [5]. In developed countries, AKI occurs in 20% of hospitalized adult patients and 25% of pediatric patients receiving intensive care [6], and its diagnosis is critical to improving survival. One of the consequences of AKI is the disruption of homeostasis, inducing an accumulation of waste products normally removed by the kidneys, leading to severe damage throughout

the body. If treated quickly, the effects of AKI are reversible, notably through fluid resuscitation and medication [7] but can lead to the patient's death without proper intervention. Currently, AKI is diagnosed by monitoring the patient's urine output volume and measuring serum creatinine level in the blood [7]. Creatinine quantification suffers from diverse limitations, namely, interferences with drugs such as antiretroviral drugs [8], variations in basal creatinine levels between patients, and most importantly, a long delay (36 to 48 h) between the occurrence of AKI and a detectable increase in serum creatinine concentration [9]. This delays the diagnostics, with possibly critical consequences. Numerous potential alternative AKI biomarkers are currently being investigated by research groups worldwide [10]. One of the most promising biomarkers is NGAL. NGAL is an early inflammation marker that can serve as an early indicator of AKI, aiding in timely diagnosis and intervention to prevent further renal damage [11]. Its sensitivity and specificity make it valuable in clinical settings for monitoring kidney function and assessing the severity of AKI.

2.3 Objective

Diagnosis of kidney failure through the quantification of NGAL by a beads-based immunoassay

2.4 Preparation before week 1 of the practical

- Read the protocol for week 1 and calculate all the missing volumes with a question mark (?) indicated in the table in section 3.2.

- Before the first lesson, please answer the questions for week 1 and complete the quiz.

- Please read the following webpage to get a better overview of acute kidney injury: https://www.kidney.org/atoz/content/AcuteKidneyInjury

- Before the first lesson please read:

  Thiriet, P-E, et al. "Rapid Multianalyte Microfluidic Homogeneous Immunoassay on Electrokinetically Driven Beads." Biosensors 10.12 (2020): 212. https://www.mdpi.com/2079-6374/10/12/212

3 Protocol week 1

3.1 Beads preparation protocol

1. Take 25 µL of beads
2. Centrifuge, remove supernatant, resuspend in 25 µL 0.1 M PB (phosphate buffer).
3. Add 26 µL NGAL capture Ab. Incubate 2 hours.
4. Prepare 10% Bovine Serum Albumin (in 0.1M PB) (5 mg in 500 µL PB 0.1M)
   Centrifuge beads and remove supernatant
   Resuspend in 50 µL 10% BSA. Mix well, no vortex. Incubate 30 minutes
5. Centrifugate beads and remove supernatant
   Resuspend in 50 µL 0.05% Tween-20. Mix well, no vortex
   Repeat this step 4 times
6. Resuspend in 25 µL 0.1 M PB (phosphate buffer).

3.2 Antigen preparation

We want to prepare 100 µL of each solution of antigen in order to plot our dose response curve and find out if our patient is experiencing AKI. To do so we have at our disposal a concentrated antigen solution that will dilute according to the following:

$C_{stock} = 0.45\ \mu g/mL$

Solution	C1	C2	C3	C4	C5	C6	Control
Concentration (ng/mL)	200	100	50	10	5	1	0
V_{PBS} **(µL)**	101.7	83	66	128	60	80	100
$V_{dilution}$	? µL C_0	? µL C1	? µL C2	? µL C3	? µL C4	? µL C5	0

4 Protocol week 2

4.1 Sample preparation and data acquisition

1. Add 0.5 µL of dAb to each concentration aliquot. This amount corresponds to a large excess of detection antibody in the aliquot, even at high concentration, and avoid the appearance of Hook effect.
2. Mix the capture antibody decorated beads with the capture antibody-analyte conjugate in an Eppendorf tube
3. Manually shake the tube
4. Put 10 µl of sample in the counting chamber and cover it with a glass slide.
5. Turn on the microscope and open software (Micro-Manager 2.0)
6. Set exposure time to 5000ms
7. Take images in BF and Fluorescence (CY3) of at least 6 beads per concentration
8. Save files
9. After each data acquisition clean the counting chamber and the glass slide

4.2 Data analysis

1. Upload the obtained images from the microscope by dragging them into the Image J software
2. To compare the different spot intensities, use the selection tools (either the ***Elliptical*** or the ***Freehand*** selection)
3. Go to **Analyze/Set measurements** and make sure to tick the box for **Mean gray value**
4. Once you have selected one spot, click ***ctrl t***, and the ***ROI*** (**r**egion **o**f **i**nterest) ***manager*** window will open
5. Every time you select an area, click ***ctrl t*** to add it to the ROI manager. Always select one bead and a background area in the proximity of the bead for normalization
6. By ticking ***Show all***, all the spots indicated with a number in the ROI list will be displayed in the picture with the number they occupy in the list.
7. Click on flatten and save the image so you can backtrack which spot corresponds to which number
8. Once selected all the areas of interest, click on ***Measure*** in the ROI window
9. A new window will open with the results
10. Copy and paste the values to an Excel file
11. Divide the mean gray value of the bead with the background. First, take the values with the known concentrations to plot a calibration curve. Once you have plotted the calibration curve in the Excel file, you can get its equation by right-clicking on one of the data points and selecting **Add trendline…** in the **Format trendline** window, click on the options **Display equation on chart** and **Display R-squared value on chart**.
12. Compare the intensity of the beads you obtained for the unknown concentration and determine the concentration.

5 Questions

5.1 Week 1

Q1. Name other biomarkers for acute kidney injury

Q2. To make sure that all the streptavidin binding sites of the beads are occupied by the capture antibodies, we want to add the capture Ab in excess. For our experiments, we add 10 µl of cAb to 25 µl of a solution containing 0.125 mg streptavidin-decorated beads. 1 mg of beads can bind 0.14 nmol of biotin. Considering that the molecular weight of the cAb is 150 kDa (1Da=1g/mol) and that c_{stock} of cAb is 0.3 mg/ml, what is the excess ratio of capture antibodies that we add? (Hint: Each streptavidin site will bind to one cAb)

Q3. What happens if we decrease the size of our beads?

5.2 Week 2

Q5. What is the concentration threshold of NGAL at which the patient suffers from AKI?

Q6. Consider the table below (taken from Thiriet *et al.*). Comment on each of the figure of merits (e.g. Limit of detection, Total analysis time, etc.) and how they should behave in an ideal point of care device.

Table 1. Comparison of advantages of our approach with respect to standard methods used for detection of NGAL.

	DEP Surfing	**ELISA**	**Abbott Architect**	**Lateral Flow Assay**
Limit of detection	Low	Low	Low	Average
Total analysis time	Short	Long	Average	Short
Sample processing	Limited	Extensive	Extensive	Limited
Volume needed	Low	Average	Average	High
Multiple analytes	Easy	Difficult	Easy	Average
Translation to PoC	Easy	Difficult	Difficult	Easy

5.3 After practicum

Answer those questions after you did all the experiments.

Q9. Compare your results with the answer of Q5. Does the “patient” of the sample you were measuring suffer from acute Kidney injury?

Q10. What is limiting the reliability of the diagnosis with the method used in this practical.

Q11. Calculate the sensitivity of your calibration curve. Take into account the linear fit acquired from point 11 of the data analysis.

6 Quiz

The quiz will be checked at the beginning of week 1

1. What kind of immunoassay is this?

a. Competitve assay

b. Sandwich assay

c. Direct assay

2. What are the three components of this immunoassay

a. Detection antigen, antibody, capture antigen

b. Detection antigen, antigen, capture antibody

c. Detection antibody, antigen, capture antibody

3. In a sandwich immunoassay, both antibodies should target the same epitope of the antigen. Is this statement

a. true

b. wrong

4. Compared to planar assays, bead-based immunoassays offer

a. higher surface-to-volume ratio

b. lower surface-to-volume ratio

c. the same surface-to-volume ratio

5. What are the most common materials used for beads

a. Latex, polystyrene and ferromagnetic coating

b. Agarose, PDMS and piezoelectric coating

c. Glass, Silicone, and oxide coating

6. Typical symptoms of Acute kidney injury include

a. Too little urine leaving the body, Nausea, Swelling around the eyes

b. Fatigue or tiredness, Seizures or coma in severe cases, loss of smell

c. Nose bleeding, stomach ache, excessive flatulence

7 References

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[5] Makris, K.; Spanou, L. Acute Kidney Injury: Definition, Pathophysiology and Clinical Phenotypes. Clin. Biochem. Rev. 2016, 37, 85–98.

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[7] Rahman, M.; *et al.* Acute kidney injury: A guide to diagnosis and management. Am. Fam. Physician 2012, 86, 631–639. Biosensors 2020, 10, 212 14 of 15

[8] Maggi, P.; *et al.* Novel antiretroviral drugs and renal function monitoring of HIV patients. AIDS Rev. 2014, 16, 144–151.

[9] Wilson, F.P. and Greenberg, J.H. Acute Kidney Injury in Real Time: Prediction, Alerts, and Clinical Decision Support. Nephron 2018, 140, 116–119.

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[11] Meijer, E. *et al.* Association of urinary biomarkers with disease severity in patients with autosomal dominant poly ic kidney disease: A cross-sectional analysis. *Am. J. Kidney Dis.* **56**, 883–895 (2010).