Lab–on–a–disc Screening

Purpose:

To finalize the Synthesizer project we thought about a screening method for our MAGE method to distinguish bacterial colonies producing non-ribosomal peptides (NRPs) of interest. A few NRPs we worked with, such as tyrothricin, tyrocidine and surfactins distinguish toxic properties towards red blood cells [1-3].

The purpose of this experiments was to check toxicity of pure tyrothricin and tyrocidine produced by *Bacillus brevis* that can be synthetized through the MAGE method, towards red blood cells using measurements of the UV-visible absorption spectrum.

Protocol

Absorbance depicts the amount of light absorbed by a measured solution or compounds included in a sample. Additionally, the absorbance can show cell lysis of red blood cells. [4-5]

To perform the experiments, we measured the UV-vis absorption spectra by DS-11 + Spectrophotometer (DeNovix) to detect red blood cell lysis. The measurements were performed in cuvettes and nanodropped.

Materials:

DS-11 + Spectrophotometer (DeNovix)

Cuvette 2mm

Red blood cells (fresh) in Heparin, EDTA, sodium citrate 3,2%, Pottasium Oxalate

Tyrothricin

Tyrocidine

0.15 NaCl

100mM NaCl, 50mM Glucose, 25mM Mannitol buffer

Experiment 1

Purpose:

Investigation of the influence of types of anticoagulants in blood samples and specifying if tyrothricin causes red blood cell lysis.

Procedure:

Absorbance spectra of blood diluted 1:50 in 0.15M NaCl or Tyrothricin solution were recorded between 300-800nm.

1. Add 1000ul of 0.15M NaCl into a 2mm cuvette as a blank to calibrate the spectrophotometer.
2. Add 980ul of 0.15M NaCl or tyrothricin (unknown concentration) and 20ul of blood X (ID) sample into a 2mm cuvette. (According to the table below.)
3. To mix the sample, gently flip the cuvette 10 times and put it into the spectrophotometer.

4. Measure.

	Volumes (uL)				
ID blood	0.15M NaCl	Tyrothricin	Blood	Cuvette Pathlength (cm)	Comments
	500	0	0	0,2	Blank
EDTA	490	0	10	0,2	EDTA
EDTA	490	0	10	0,2	EDTA
EDTA	980	0	20	0,2	EDTA after 30s
EDTA	980	0	20	0,2	EDTA after 120s
EDTA	980	0	20	0,2	EDTA after 2min
sodium citrate 3,2%	980	0	20	0,2	measured after 30sec
sodium citrate 3,2%	980	0	20	0,2	measured 2 after 120sec
sodium citrate 3,2%	980	0	20	0,2	measured 2 after 2min
Heparin	980	0	20	0,2	measured after 30sec
Heparin	980	0	20	0,2	measured 2 after 120sec
Heparin	980	0	20	0,2	measured 2 after 2min
Pottasium Oxalate	980	0	20	0,2	measured after 30sec
Pottasium Oxalate	980	0	20	0,2	measured 2 after 120sec
Pottasium Oxalate	980	0	20	0,2	measured 2 after 2min
Unknown conc.of Tyroth	0	980	20	0,2	measured after 30sec
Unknown conc.of Tyroth	0	980	20	0,2	measured 2 after 120 sec
Unknown conc.of Tyroth	0	980	20	0,2	measured 2 after 30sec
Unknown conc.of Tyroth	0	980	20	0,2	measured 2 after 120 sec
Unknown conc.of Tyroth	0	980	20	0,2	measured 2 after 30sec

Results and Conclusion:

Most of the UV-vis absorption spectra of red blood cells in different anticoagulation substances that were resuspended in 0.15M NaCl did not show great difference between each other. Thus, in the rest of the experiments the blood cells with heparin were used.

All of the curves at the plot were very noisy and unreadable. Therefore, only two curves were selected to observe an indication that the blood cells lyse in the presence of an antibiotic. Figure 1 displays the UV-vis absorption spectra of red blood cells suspended in the absence and presence of antibiotic. The drop of green curve around 600nm indicates less absorbance of red blood cells in the sample, as the cells are being lysed due to toxicity of tyrothricin.

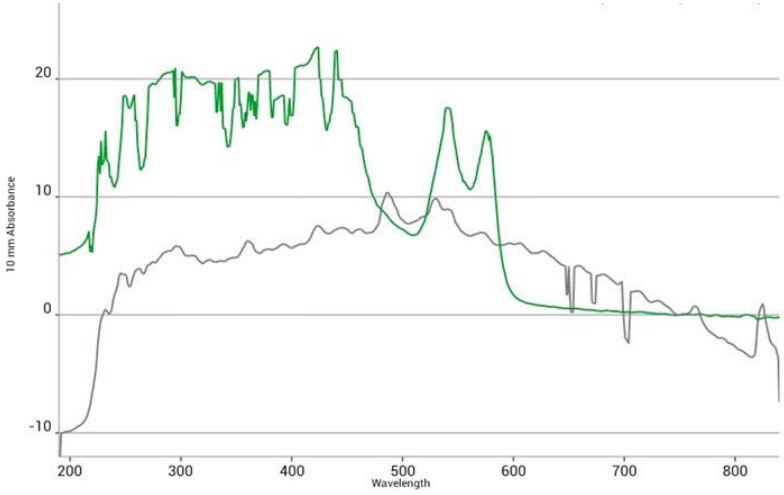

Figure 1. Comparison of blood cells different buffers.
grey line: blood cells anticoagulated in heparin with 0.15M NaCl;
green line: blood cells anticoagulated in heparin with unknown concentration of Tyrothrycin

Additionally, to prove the toxicity of the antibiotic of its different concentrations over time Figure 2 depicts decrease of absorbance around 600nm in all cases what decreases over time.

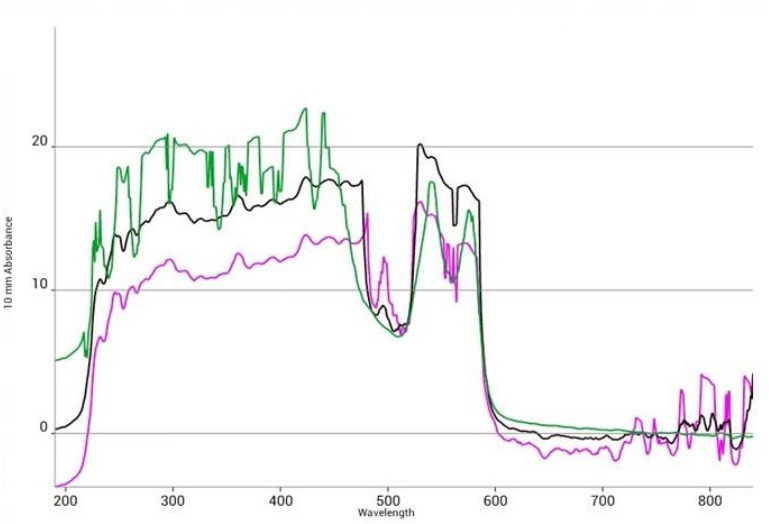

Figure 2. Comparison of blood cells in presence of antibiotic over time.
pink and black line : unknown concentration of tyrothrycin measured after 30sec with blood cells anticoagulated in heparin; green line: unknown concentration of tyrothrycin measured after 120sec with blood cells anticoagulated in heparin.

Experiment 2

Purpose:

Investigation of sensitivity of blood cells to different concentrations of tyrothricin; UV-vis absorption spectra of red blood cells measured by a cuvette method in spectrophotometer and nanodrop.

Procedure:

Absorbance spectra of blood diluted 1:50 in 0.15M NaCl and tyrothricin solution were recorded between 300-800nm.

a) Cuvette measurement in spectrophotometer

1. Add 1000ul of 0.15M NaCl into a 2mm cuvette as a blank to calibrate the spectrophotometer.
2. Add 980ul of 0.15M NaCl or tyrothricin (A-E) and 20ul of blood sample (heparin) into a 2mm cuvette. (According to the tables below.)
3. To mix the sample, flip the cuvette 10 times and put it into spectrophotometer.
4. Measure.

b) Nanodrop

1. Use 10ul of 0.15M NaCl as a blank to calibrate nanodrop device.
2. Add 9,8ul of 0.15M NaCl or tyrothricin (A-E) and 0,2ul of blood sample (heparin) into a small Eppendorf tube. (Same concentrations as in part **a** of Exercise 2.)
3. To mix the sample, gently move tube around and try to distribute the blood cells within the solution.
4. Take 1uL of the sample and measure.

Name	ID	stock (mg/mL)	final concentration (mg/mL)	final volume (uL)	sample from stock (uL)	buffer (NaCl 0,15M)
Tyrothricin	A	0,8	0,02	1000	25	975
	B	0,8	0,05	1000	62,5	937,5
	C	0,8	0,01	1000	12,5	987,5
	D	0,8	0,2	1000	250	750
	E	0,8	0,4	1000	500	500

	Volumes (uL)				
ID buffer	0.15M NaCl	Tyrothricin	Blood (Heparin)	Pathlength (cm)	Comments
	1000	0	0	0,2	Blank
	980	0	20	0,2	
A	0	980	20	0,2	
A	0	980	20	0,2	after 60-80sec
B	0	980	20	0,2	
B	0	980	20	0,2	after 60-80sec
C	0	980	20	0,2	
C	0	980	20	0,2	after 60-80sec
C	0	980	20	0,2	after 100sec
C	0	980	20	0,2	after 120sec
D	0	980	20	0,2	
D	0	980	20	0,2	after 60-80sec
D	0	980	20	0,2	after 120sec
E	0	980	20	0,2	
E	0	980	20	0,2	after 60-80sec
E	0	980	20	0,2	after 100sec
E	0	980	20	0,2	after 120sec

Results and Conclusion:

a) The final plot did not show the expected results. The absorbance measurements gave many noises on the plot. The most readable curves were achieved after 60-80sec after mixing the blood sample with X buffers. The results are shown in Figure 3. However, all of the curves cover each other, which is unexpected. According to Experiment 1 and other papers (ref X) the blood sample with 0,15M NaCl should be distinguishable from samples with antibiotic. The noisy and unclear data were unreliable – therefore, the nanodrop method was used in part b to compare UV-vis absorption spectra between the same samples and observe changes.

#	Sample Name	Abs (nm1)
2	nacl 0.15M blood	0.448
3	nacl 0.15M blood	0.448
7	tyr 0.02 blood	1.110
13	tyr 0.05 blood	1.105
16	tyr 0.1 blood	1.105
17	tyr 0.1 blood	1.569
21	tyr 0.2 blood	1.105
26	tyr 0.5 blood	1.105

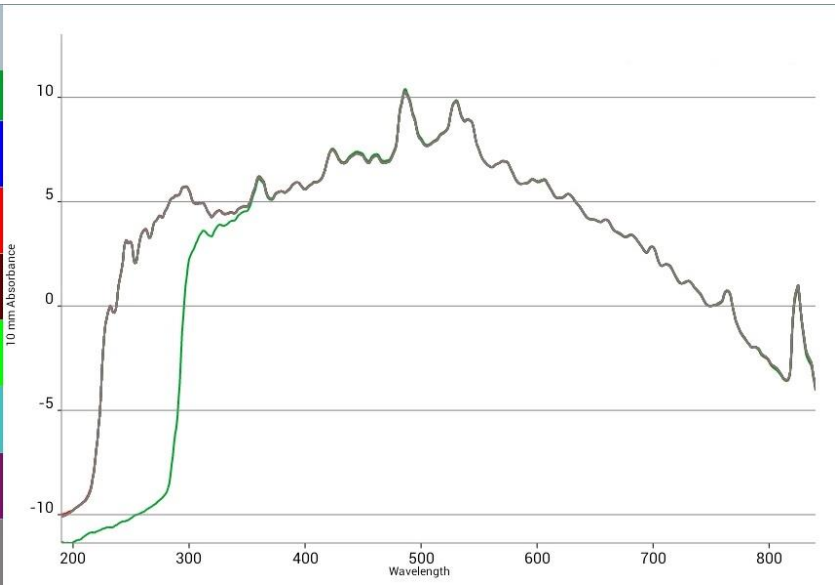

Figure 3. Comparison of blood cells (anticoagulated with heparin) in presence of 0,15M NaCl and tyrothricin after 60-80sec of incubation. Cuvette experiment.

b) The absorbance of spectra in all of the cases (see Figure 4) shows cell lysis, but not full cell lysis. It may have occurred due to a short time of incubation; only 45-60 seconds. Additionally, a blood sample with 0,15M NaCl should not lyse the red blood cells in this case, as it does not have properties that burst them by osmotic pressure. However, the red curve still shows some turbidity around 600-700nm in comparison to other measurements. The cell lysis could happen either due to a narrow orifice of pipette tip that bursts the cells (mechanical forces) or due to hemolysis of the cells, as in the day of this measurement they were 3 days old. [6] We can conclude that the antibiotic lyses the red blood cells but we cannot determine its specificity very much.

#	Sample Name	Abs (nm1)
32	tyr 0.1blood n	2.728
33	tyr 0.2blood n	4.394
34	tyr 0.2blood n	1.770
35	tyr 0.2blood n	4.092
36	tyr 0.5blood n	4.432
37	tyr 0.5blood n	5.538
38	nacl	2.592
39	nacl	8.943

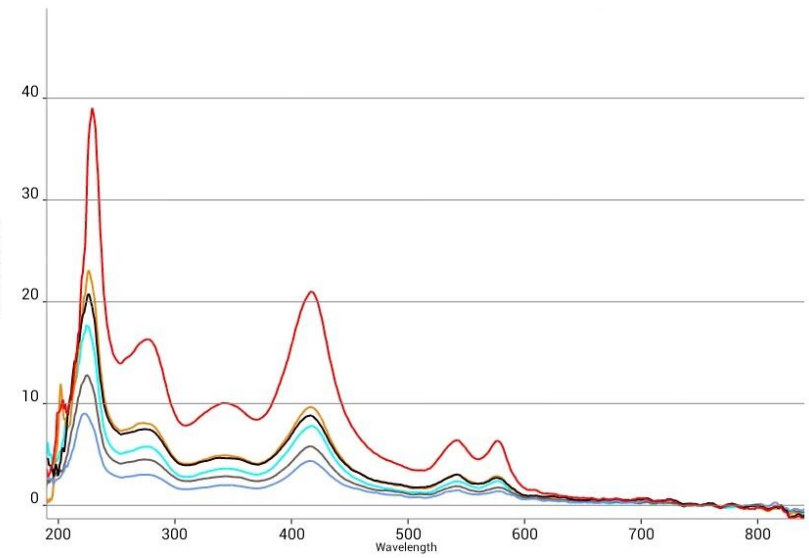

Figure 4. Comparison of blood cells (anticoagulated with heparin) in presence of 0,15M NaCl and different concentrations of tyrothricin after 45-60sec of incubation. Nanodrop measurement.

As the plots in experiment 2b were much better and clearer, it suggests that in experiment 2a the absorbance was wrongly measured due to either a light beam getting through the sample in spectrophotometer or clarity of cuvette. Therefore, the noisy curves are definitely related to the instrument setup or cuvette and we cannot trust the results from experiment 2a. Nanodrop measurements show much better absorbance spectrum curves where we can notice a drop around 600nm of wavelength suggesting red blood cell lysis.

Next time the experiment could be repeated with a use of fresh red blood cells (max 24h old) to exclude a risk of hemolysis of them.

Experiment 3

Purpose:

Investigation of sensitivity of blood cells to unknown concentrations of tyrocidine purified from *Bacillus brevis* cultures. UV-vis absorption spectra of red blood cells measured by Nanodrop device.

Procedure:

Absorbance spectra of blood diluted 1:50 in 100mM NaCl, 50mM Glucose, 25mM D-Mannitol buffer and six different purified bacterial products diluted in 50ul of the buffer were recorded between 300-800nm.

Nanodrop

1. Use 10ul of 100mM NaCl, 50mM Glucose, 25mM D-Mannitol as a blank to calibrate Nanodrop device.
2. Add 9,8ul of dissolved Tyrocidine samples and 0,2ul of blood sample (heparin) into a small Eppendorf tube.
3. To mix the sample, gently move tube around and try to distribute the blood cells within the solution avoiding their destruction.
4. Take 1uL of the sample and measure.

Results and Conclusion:

Figure 5 shows lysed blood cells in all of the samples, incl. control sample. Very possibly the red blood cells are lysed as they were three days old and it makes the positive results less viable.[6] Next time, the experiment should be run with freshly taken blood samples from a donor. In this experiment we also changed the resuspension buffer to increase the solubility of rehydrated bacterial NRPs. It might also influence the blood cells stability and requires investigation in terms of finding better buffers for resuspension of samples and keeping blood cell stability.

#	Sample Name	Abs (nm1)
39	nacl	8.943
40	S buffer blood	3.812
41	S sample 1 blood	8.480
42	S sample 2 blood	10.148
43	S sample 3 blood	11.386
44	S sample 4blood	6.210
45	S sample 5blood	14.570
46	S sample 6blood	8.240

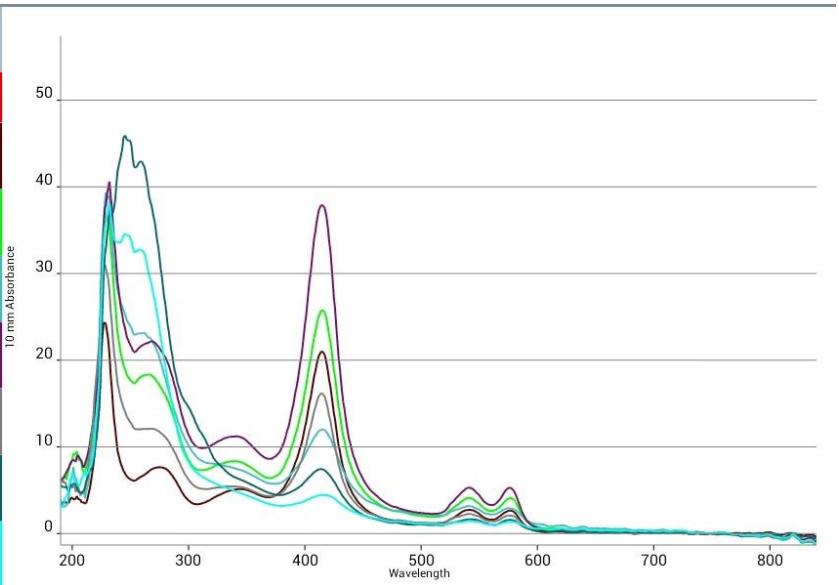

Figure 5. UV-vis absorption spectra of blood anticoagulated with heparin in presence of unknown concentrations of Tyrocidine after 45-60sec of incubation. Nanodrop measurement.

This part of the experiments should be repeated with a fresh blood cells not only by nanodrop but also in spectrophotometer, and considered in broader aspect of toxicity of other NRPs, kinetics of cell lysis to achieve more detailed data, optimization of cultivation time of colony samples and optimization of a disc set up. However, with these results and current knowledge, the lab-on-a-disc screening of NRP products of bacterial transformants for the Synthesizer project is very likely and has potential to be a low-cost, quick and simple way of detection and screening of interesting transformants.

References:

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