University of Twente

Assessment of the immunoprotective character of β-cell laden PEG-TA microgels over time

Thesis to conclude the bachelor assignment of Max Visser

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Contents

Summary

Diabetes Mellitus type 1 is a disease where patients don't produce enough insulin because their own immune system attacks the insulin producing islet cells. Treatment these days consists of regular insulin injections, either manually or by insulin pump. Researchers have been looking for a permanent cure for decades but the disease has proven to be difficult to cure. Islet cell transplantation is a way to cure the disease but they would need to be protected against the immune system. This can be done by encapsulating the cells in a material that can protect them from the immune system. This encapsulation would need to able to let glucose, insulin and other small molecules diffuse freely, while blocking antibodies and immune cells from entering.

A novel way to encapsulate cells is by using a microfluidic device made from PMMA. Using the Polyethylene glycol polymer functionalized with tyramine groups (PEG-TA), single step encapsulation is possible. By forming polymer droplets in the microfluidic device and enzymatically crosslinking them from the outside in small 100-200 µm microgels can be made. These microgels have been found to be protective against the immune system within the research group, although a small number of antibodies had penetrated the microgels after 30 days.

In this thesis I evaluated the immunoprotective character of these cell laden microgels over time. Mouse insulinoma cells (MIN6) were encapsulated in 10% PEG-TA microgels and cultured for 30 days. At various timepoints a portion of these microgels were fixed. These fixed gels were stained for insulin and glucagon surface receptors using antibodies and the gels were exposed to FITC labeled IgG antibodies. The microgels were found to be immunoprotective compared to the control but the development of the immunoprotective character over time was inconclusive.

To assess how the cells proliferate and develop over time in the microgels, viability and actin stainings were performed at the same timepoints. It was found that cell viability remains larger than 60% over a period of 30 days and that the cells aggregate into dense spheroids after a few days which grow in size over time.

Samenvatting

Diabetes Mellitus type 1 is een ziekte waarbij patiënten niet genoeg insuline produceren omdat hun eigen immuunsysteem de insuline producerende β-cellen aanvalt. De behandeling bestaat tegenwoordig uit regelmatige insuline injecties, hetzij met de hand, hetzij met een insulinepomp. Onderzoekers zijn al tientallen jaren op zoek naar een permanent geneesmiddel, maar de ziekte blijkt moeilijk te genezen. β-celltransplantatie is een manier om de ziekte te genezen, maar deze zouden beschermd moeten worden tegen het immuunsysteem. Dit kan door de cellen te encapsuleren in een materiaal dat ze kan beschermen tegen het immuunsysteem. Deze encapsulering zou glucose, insuline en andere kleine moleculen vrij moeten laten diffunderen, terwijl antilichamen en immuuncellen worden geblokkeerd.

Een nieuwe manier om cellen te encapsuleren is door gebruik te maken van een microfluidische chip gemaakt van PMMA. Met behulp van het polyethyleenglycolpolymeer, gefunctionaliseerd met tyraminegroepen (PEG-TA) is inkapseling in één stap mogelijk. Door polymeerdruppeltjes in de chip te vormen en ze enzymatisch van buiten naar binnen te *crosslinken* kunnen microgels van 100-200 µm gemaakt worden. Deze microgels bleken binnen de onderzoeksgroep beschermend te zijn tegen het immuunsysteem, hoewel er na 30 dagen enkele antilichamen in de microgels waren binnengedrongen.

In deze thesis heb ik het immunoprotectieve karakter van deze celbeladen microgels in de loop van de tijd geëvalueerd. Muis insulinoma cellen (MIN6) werden geëncapsuleerd in 10% PEG-TA-microgels en gedurende 30 dagen verzorgd. Op verschillende tijdstippen werd een deel van deze microgels gefixeerd. Deze gefixeerde gels werden gekleurd op insuline- en glucagon-celmembraanreceptors met behulp van antilichamen en de gels werden blootgesteld aan FITC-gelabelde IgG-antilichamen. De microgels bleken immuunbeschermend te zijn in vergelijking met de controle, maar over de ontwikkeling van het immuunbeschermende karakter in de tijd kon geen conclusie getrokken worden.

Om te beoordelen hoe de cellen zich in de loop van de tijd in de microgels prolifereren en ontwikkelen, werden er levensvatbaarheid en actinekleuringen op dezelfde tijdstippen uitgevoerd. Er werd vastgesteld dat de levensvatbaarheid van cellen gedurende een periode van 30 dagen groter blijft dan 60% en dat de cellen na een paar dagen aggregeren tot dichte spheroïden die in de loop van de tijd groter worden.

Acknowledgements

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1 Introduction

1.1 Diabetes

Diabetes Mellitus type 1 is a very prevalent disease in the world, especially in western countries. The prevalence is almost one in 1700 people [1] and is growing worldwide. It is an auto-immune disease, meaning the immune system attacks and kills insulin producing β-cells in the pancreas[2][3]. The β-cells in the pancreas are the only cells in the body that can produce insulin. Insulin, together with its antagonist glucagon, is used by the body to regulate the blood sugar level[4]. The release of insulin causes a decrease in blood sugar level as cells either take up or convert glucose for storage from the blood stream[5]. The release of glucagon causes an increase in blood sugar level as muscle and liver cells release stored glucose back into blood stream. A shortage of insulin can thus cause hyperglycaemia(too high blood sugar level)[6]. Chronic hyperglycaemia can eventually lead to life threatening vascular diseases and all sorts of problems in the kidneys, brain and eyes.

The exact cause is not known yet, but certain genes make a person more susceptible for the disease. Genetics alone, however, cannot explain the disease's prevalence and certain environmental factors might also cause the autoimmune reaction. Factors such as diet and viral infections during youth might play a large role in the development of the disease[3].

The exact immune reaction is also not known, but it is suspected both the innate and the adaptive immune system play a role in the reaction. Many pathways are involved and not one part of the immune system is solely responsible for the disease. It seems especially killer t-cells of the adaptive immune system and autoantibodies for certain components of islet cells are the largest contributors to islet cell death[3].

1.2 Treatment options

Insulin therapy
One way or another the pancreas doesn't produce enough insulin. The pancreas might still produce some insulin, which is useful for the prevention of other associated problems such as retinopathy[2], but not enough to regulate the blood sugar level properly. These days the most common treatment[2][7] of the disease is the injection of exogenous insulin to the body to regulate blood sugar levels. This is usually done manually, but these days devices such as insulin pumps and continuous glucose monitors are slowly becoming the norm[2]. This helps patients regulate their blood sugar level more properly, but not perfectly and it does not provide permanent a cure for the disease.

Pancreas transplantation
The search for that permanent cure has been ongoing for decades and pancreas transplantation provides the way of (temporarily) curing diabetes type 1[8]. This method still has some major flaws; however, the donor pool is very small, it requires major surgery and even then, only 80% of the transplanted patients achieve a 3-year survival rate[8]. You also still face the same problems which caused the disease in the first place since the immune system will continue to attack the transplanted pancreas, so very heavy immunosuppressive therapy is needed. Despite its potential benefits researchers have thus been looking at different methods of curing the disease.

Cell transplantation
The mass of cells in the pancreas that actually produce insulin is very low (only 2%) and scientists have been looking at the transplantation of islet cells or other insulin producing cells as an alternative[8]. In 2000 a breakthrough protocol[2] in islet cell transplantation was found but unfortunately the initially positive results deteriorated after 5 years to the point where only 10% remained independent from external insulin. Currently, with advances made in isolation techniques and immunosuppressive therapy, islet cell transplantation provides the best approach for the cure for diabetes type 1[8] since it requires significantly less invasive surgery than a pancreas transplantation. It is plagued by some of the same drawbacks of pancreas transplantation; only 20% of patients remain insulin dependent after 5 years, islet cell extraction from pancreases is very low and the donor pool is still very small[8]. Patients also still need to take heavy immunosuppressive medicine.

1.3 Cell encapsulation

A way to mitigate some of these drawbacks is to encapsulate the transplanted cells in a protective hydrogel network with a biocompatible character[8]. Encapsulation also allows the transplantation of allogeneic or even xenogeneic cells. In the future human stem cells or pig islet cells might be used for transplantation, thereby solving the limited donor problem.

Such hydrogels would need to be able to allow the diffusion of oxygen, glucose, insulin, nutrients and waste products, while also protecting its contents from the immune system by repelling antibodies and immune cells (see figure 1). Microencapsulation of cells has a few distinct advantages and disadvantages[8]: Its size allows the ready diffusion of particles and due to the number of microgels present a transplantation is not immediately compromised if one of the gels breaks or is removed by the immune system.

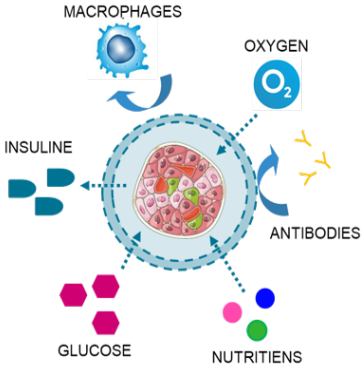


Figure 1: Properties of cell encapsulation

A major disadvantage of microgels is the limited number of biomaterials that is sufficiently protective against the immune system and suitable for encapsulation. Alginate is currently the most used polymer for cell encapsulation purposes[9]. It has a good track record on biocompatibility and biodegradability. The material[10], is derived from natural sources (usually algae) and is crosslinked via ionic reactions. The pores of alginate gels are too large to provide immunoprotection[10] (protecting against the effect of an antigen) so researchers usually coat these gels with other synthetic polymers. Since alginate is derived from natural sources the gels also need to be purified extensively before transplantation[9][10], otherwise specific molecules such as proteins or toxins can elicit a strong immune response.

For these reasons researchers have been looking for another suitable material for encapsulation. One of these is the synthetic polymer polyethylene glycol (PEG)[11], which is already being used as a coating for alginate microgels[10]. It is a biocompatible, low protein fouling and inert material. It doesn't degrade very fast and has mechanical properties closely resembling soft tissue. Due to its high hydrophilicity, it is very hydrated and therefore allows easy diffusion. Finally, it's synthetic nature allows for easier production and more consistent batches compared to natural polymers.

The host immune response remains, even with the right biomaterial, a difficult challenge to overcome and will be discussed in more detail in subsection 1.5.

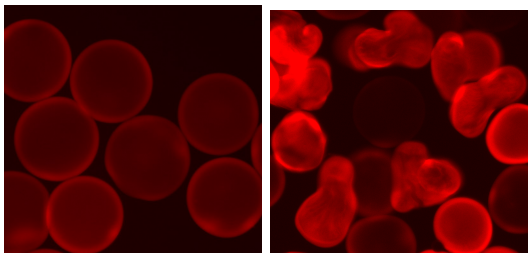
Another current disadvantage of encapsulation is the suboptimal production of the microgels for therapeutical purposes. One needs a large number of cell laden microgels to achieve any effect on the body's metabolism. Batches are also usually polydisperse, meaning it is very hard to consistently get a single size of microgel. Microgel production is also usually associated with harsh solvents and UV/ionic based crosslinking reactions which can damage cells.

A new method used by van Loo et al.[12] uses a microfluidic device to produce microgels. He was able to encapsulate relatively high number of cells in monodisperse microgels based on single step enzymatic crosslinking, thereby eliminating many of the drawbacks mentioned. I am using a similar single step encapsulation method using the 8-arm PEG polymer, functionalized with tyramine groups (PEG-TA).

1.4 Microgel production

To create microgels using the microfluidic method a precursor polymer solution with horseradish peroxidase (HRP) is flowed together with an oil through the microfluidic device. The two fluids are immiscible and the polymer therefore breaks up in the oil and forms small droplets of about 100-200 µm. The workings of microfluidic device will be discussed in detail in the methods section. These droplets are captured by an exchangeable silica nozzle which allows for easy replacement of the nozzle without replacing the entire chip. These hydrophobic nozzles are handcrafted by a design of Tom Kamperman[13]. The droplets are then led through a hydrogen peroxide (H2O2) bath. The HRP enzyme mediates the coupling of the phenol groups of the tyramine groups[12][14]. Since H2O2 diffuses inwards from the bath, the crosslinking happens from the outside in, thus leaving a hollow core. By varying the H2O2 concentration, residence time in the bath and the concentration of the PEG-TA different types of microgels can be made. This can vary between incomplete crosslinking to soft core microgels[12](see figure 2). By varying the flow speed ratio of the polymer and oil, differently sized microgels can be made.

Actual cell encapsulation can be done by mixing the cells with the precursor polymer solution[12]. Due to the outside in crosslinking the cells become encapsulated in a polymer microgel. The H2O2 diffuses inwards from the outside and is consumed in the process of crosslinking so the cells are not exposed to toxic concentrations[12]. Most cells reside in the middle of the microgel, but some can be entrapped in the crosslinked polymer which can lead to earlier cell death or a compromised microgel. It is also important that the microgel shell is not too thick since this would impede the diffusion of nutrients and waste products. Long term cell culture is possible with encapsulated cells[12]. Cells usually aggregate into dense spheroids after 24 hours and have been found to be remain viable after periods of 28 days.



(a) *(b)*

Figure 2: Microgels stained by Ethidium Homodimer-1. (a) shows an example of crosslinked hollow core microgels. (b) shows an example of broken microgels which is the result of incomplete crosslinking

1.5 Immune response

The first thing the immune system encounters is the surface of the microgel. Naturally the microgel material should be biocompatible and not react with any circulating antibodies or immune cells[9]. The material also shouldn't degrade quickly since that attracts and activates macrophages that clean up the debris. If the material is derived from natural sources (such as alginate) it needs to be thoroughly purified to remove any proteins or pathogen associated molecular patterns (PAMP's) which can activate the immune system[9]. The pores of the microgel should also be sufficiently small to prevent immune cells or free-floating antibodies to enter the gels and elicit an immune response. Finally, the encapsulated cells themselves need to be considered[9] since they can activate the immune system in various ways while being encapsulated. Certain products of metabolism can be recognized as a danger associated molecular pattern (DAMP's) which can also activate the immune system. Cell death also releases DAMP's and inflammation stimulating cytokines that attract immune cells.

2 Aim and methodology

2.1 Research objectives

Following the work of my supervisor, dr. N.M. Araújo da Cunha Gomes, I have tested the immunoprotective character of cell laden PEG-TA microgels over time. He found that some antibodies were able to penetrate the microgels after 30 days. This was a snapshot in time, however, so I was tasked to assess this antibody penetration over a period of 30 days. To do this I have encapsulated cells in a 10% PEG-TA microgel for the assessment. Encapsulated cells in a 2.5% PEG-TA microgel provided the control since these microgels are experimentally tested to not be immunoprotective.

We hypothesized that when the cell aggregates inside the microgel become sufficiently large they might push against the microgel, stretching and enlarging it. This stretching might also enlarge pores in the microgel shell enough to allow antibodies to enter the microgels. Using fluorescent IgG antibodies and immunofluorescence I tried to quantify this penetration over time. I have also experimented with the production of the microgels and have performed a live/dead and actin staining on the encapsulated cells at various timepoints.

2.2 MIN6 Cells

To perform these experiments a suitable cell type is needed. The eventual goal is to transplant these microgels with glucose responsive insulin producing cells, such as islet cells, so it is useful to load the microgels with such cells during research. An alternative is to use stem cells differentiated to islet cells. For the purpose of research about the encapsulation material, however, both are a very costly method. Another alternative is the MIN6B1 cell line. This is an established insulinoma beta cell line derived from a transgenic mouse[15]. According to Ishihara et al.[15] This cell line closely resembles the glucose responsiveness of human islet cells and is much more available which make them an attractive choice for proof of principle research. Because of these reasons I will be using the MIN6 cell line. They have a very distinct morphology and have the tendency to grow on top of each other and readily form aggregates. This means that confluency is usually higher than it looks to be under a microscope and the aggregate formation needs to be considered during encapsulation since such cell clumps can clog the fluid lines of the microfluidic device mentioned above.

2.3 Experiment outline

Microgels and encapsulated cells can be characterized in a large variety of ways. I have visualized the microgels by staining the tyramine groups of the microgels with Ethidium Homodimer-1 (EthD-1). The cells were characterized with a live/dead staining, immunofluorescence staining, actin staining and incubation with fluorescent FITC labelled IgG antibodies.

The microgels themselves can be see clearly under a phase-contrast microscope but can also be seen under a fluorescent microscope using EthD-1 which stains the tyramine groups of PEG-TA[12][14]. This can then be seen as a red fluorescent signal. This method showed the outline of the crosslinked shell much more clearly than the image under a phase-contrast microscope could.

The live/dead staining, along with the actin staining, was done to show how the cells proliferate and develop at specific intervals over time. The used cells have the tendency to aggregate over time so this should be visible with these stainings.

Beta cells have insulin surface receptors[16] which can be visualized with immunofluorescence (IF). The used MIN6 cells have also been experimentally tested to have glucagon surface receptors. The technique of IF uses specific antibodies to bind to the desired target to be stained. Fluorescent secondary antibodies then attach to these primary antibodies which then show a signal under a fluorescent microscope. Another way to test antibody diffusion is to expose the gels to non-specific fluorescent labelled IgG antibodies. Since my aim was to prove the immunoprotective character of the microgels, I have performed immunofluorescence staining for insulin and glucagon surface receptors and exposed the gels to FITC labelled IgG antibody to prove the antibody diffusion inhibition to the cells in the core of the microgels.

3 Methods

3.1 MIN6 cell culturing

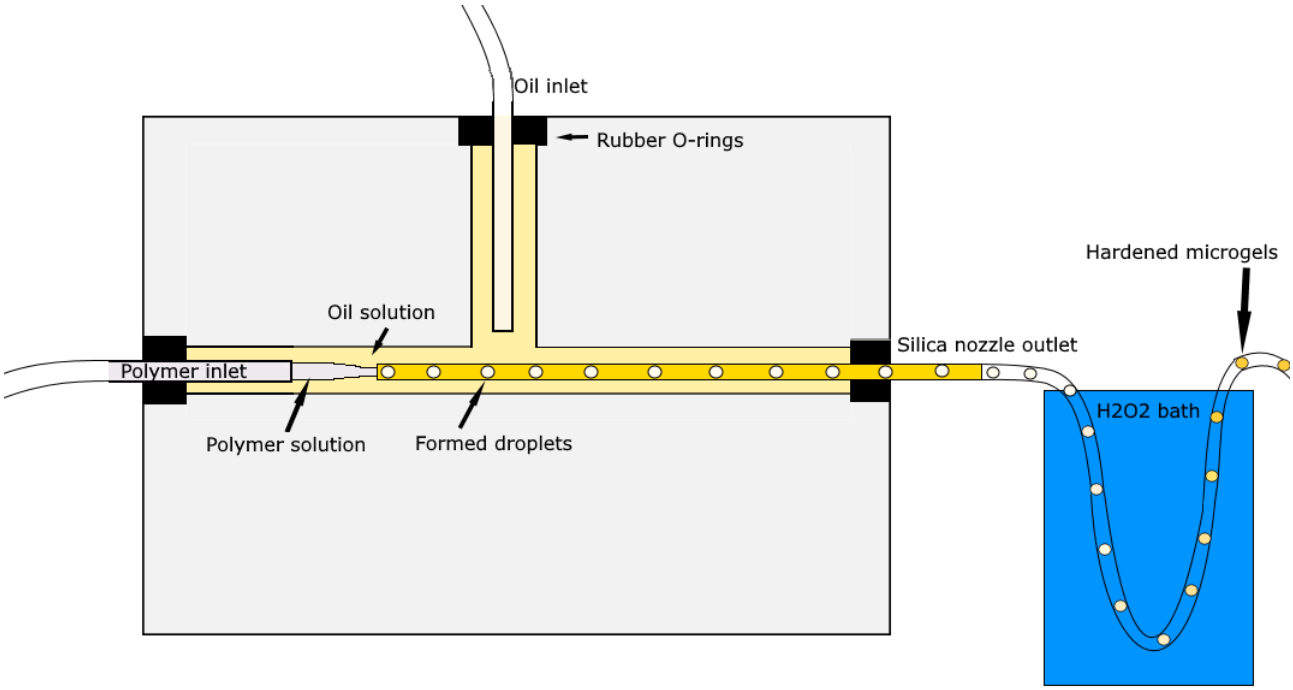
MIN6B1 cells were cultured in standard culture medium containing DMEM (Gibco), 10% FBS and 1% Penicillin-streptomycin (P/S). Betamercaptoethanol(Gibco) was added to the culture to a total molarity of 71mM whenever the medium was changed. Whenever confluency was reached (about 70-80%) the cells were subcultured. The medium was removed and the cells were washed with 1x PBS(Gibco). The PBS was removed and cells were trypsinized with 0,25% trypsin-EDTA(Gibco) and incubated for 3 min to detach the cells(37°C , 5% CO2). The trypsin was inactivated by culture medium with FBS and this was centrifuged at 300g for 5 minutes. The supernatant was removed and the cells were resuspended in fresh culture medium. Cells were counted if necessary and 1.5 million cells were seeded on a new T175 flask. The cells were kept in an incubator (37°C , 5% CO2). The medium was changed every 2-3 days.

3.2 Microgel fabrication

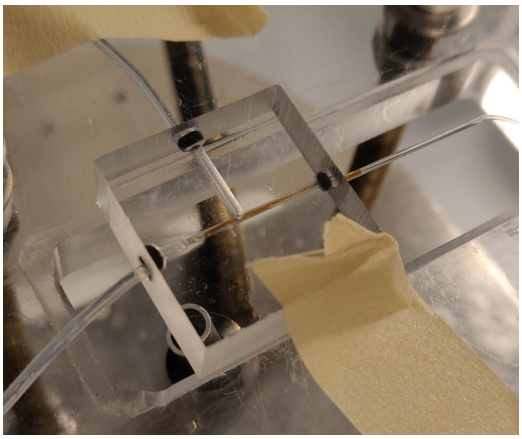
Stock solutions
solid 8-arm PEG-TA 20 KDa polymer batches were made by dr. B. Zoetebier-Liszka. Solid PEG-TA was dissolved in PBS for a final concentration of 10% PEG-TA. HRP(263 U/mg, Sigma Aldrich) was dissolved in this precursor polymer mixture for a final concentration of 80U/ml. The same was done to make a 2.5% PEG-TA solution, also with an HRP final concentration of 80U/ml. A 3% SPAN80 solution was made by dissolving SPAN80(Sigma Aldrich) in hexadecane oil(99%, Acros Organics) which was sterilized by passing through a 0.2 µm filter. SPAN80 is an emulsifier which helps to stabilize the formed polymer droplets in the oil phase.

Nozzle fabrication
The nozzle consists of a silica string with an inner diameter(I.D) of 200µm and outer diameter (O.D) of 350µm (Polymicro Technologies). Pieces of about 4cm were cleaned in a plasma cleaner (FemtoScience CUTE) and made hydrophobic by filling with a water repellent (AQUAPEL Glass Treatment). The nozzles were blown dry with N2. The nozzle was inserted into the end of silicone tubing (I.D. 0.30 mm, O.D. 0.64 mm, Helix Medical) and a glass capillary (I.D. 0.70 mm, O.D. 0.87 mm, VitroCom) was slid over the connection to seal it. A drop of glue was used to secure this glass capillary and the silica nozzle to the silicone tubing.

Microgel fabrication
Droplets were formed by flowing the polymer mixture, either 2.5 or 10 % PEG-TA, together with the SPAN80/hexadecane solution through the T-junction chip (see figure 3). The flow was provided by a syringe pump (neMESYS, Cetoni). Oil flowed from the top from a 5ml Hamilton syringe and polymer from the left from a 0.5 ml Hamilton syringe. The connections to the chip were sealed using rubber O-rings. The chip was held in place under a camera with the chip illuminated from below. The polymer is forced with the oil through the nozzle, and since both fluids are immiscible the polymer breaks up into small droplets. By varying the flow speed ratios droplets of different sizes could be made. The flow rates used were 7.5 µL/min for the polymer and 40 µL/min for the oil. These flow rates were experimentally tested by Nuno Gomes to produce microgels of about 150-200µm. Other flow rate combinations were also tested. The formed droplets were led through silicone tubing of about 30 cm going through a 30% H2O2 bath at room temperature. Outside-in crosslinking of the Tyramine groups, creating hollow microgels, is done by the HRP enzyme, catalysed by the H202 which diffuses through the silicone tubing. The residence time in the H2O2 bath was about 6-10 seconds. Collection of the microgels happened when a stable droplet train was observed by the camera. After collection the SPAN80 was removed from the microgels by repeatedly washing in pure hexadecane until the emulsion broke. PBS was added and the solution, now free of oil, was stored in the fridge.



(a)



(b)

Figure 3: A schematic picture (a) and a photo (b) of the used microfluidic device. Polymer solution comes from the left and oil from the top. These phases are forced through the hydrophobic silica nozzle (yellow colour) together. Since these two solutions don't mix, small microsized droplets are created which are transported though silicone tubing through a H2O2 bath were crosslinking of the polymer happens by HRP, thus forming hollow microgels.

Microgel characterization

Characterization of the microgels was done by phase-contrast microscopes. The gels were also stained with EthD-1 (Invitrogen, EX/EM:528/617 [17]) at a dilution of 1:250 and left to incubate for 3 hours at room temperature. The samples were then viewed under the EVOS FL fluorescent microscope (Life technologies).

3.3 Cell encapsulation

The protocol for cell encapsulation was mostly the same as for microgel fabrication but differed slightly. The 0.5ml polymer syringe was cleaned with 70% ethanol and left to dry overnight in sterile conditions. The 10% and 2.5% PEG-TA in PBS polymer precursor solution were also made under sterile conditions.

Cell preparation
The cultured MIN6 cells were trypsinized the same way as mentioned in cell culturing, were resuspended in standard culture medium without FBS after centrifugation and were dropped though a 40 µm grid cell strainer. The MIN6 cells have the tendency to clump together and this could cause complications for the polymer flow in the microfluidic device. The strainer removed the larger aggregates from the cell suspension while the medium without FBS slows down aggregate formation, since FBS accelerates cell aggregation. The strained cells were centrifuged and resuspended in standard culture medium without FBS. Cells were counted with a bürker-türk counting chamber. 10 million cells were then added to the polymer precursor solution. This solution consisted of PBS with 10% PEG-TA, 8,33% optiprep (Sigma Aldrich), 80U/ml HRP and 31.7% standard culture medium without FBS with 10 million cells.

A 2.5% PEG-TA solution was made for the control. This solution consisted of PBS with 2.5% PEG-TA, 8,33% optiprep, 80U/ml HRP and 69% standard culture medium without FBS with 10 million cells. The optiprep is used to match the density of this cell/polymer mixture to the density of the standard polymer solution.

Cell encapsulation
The precursor polymer solution was inserted into the sterile 0.5ml syringe, together with a small, sterilized magnet. This syringe was attached to the microfluidic device and the setup was run at the same flow rate as described for microgel fabrication. The small magnet in the syringe was moved occasionally, to prevent the clumping of cells in the polymer solution. The collected microgels with encapsulated cells were washed with PBS in a laminar flow hood until the emulsion broke and the resulting microgels were seeded on a suspension culture wells plate with standard culture medium and put in an incubator (37°C , 5% CO2). 50% of the medium was refreshed every 2-3 days. The remainder of the polymer precursor solution was seeded on a tissue culture plate to provide a control for polymer toxicity.

Fixation
At the timepoints of 24 hours, 4 days, 7 days, 14 days and 30 days a portion of the microgels were fixated in 4% PFA after being washed twice with PBS. After fixation the gels were put on PBS and stored in the fridge until fixation of all timepoints had taken place after which the different stainings were performed.

3.4 Stainings

Live/dead staining
These stainings were done in parallel with the fixation, so after 24 hours, 4 days, 7 days, 14 days and 30 days after encapsulation a portion of the cultured microgels was removed and stained. Live/dead staining(ThermoFisher) consisting of Calcein AM at a final dilution of 1:2000 and ethidium homodimer-1 at a final dilution of 1:500 was added to the sample and left to incubate for 30 minutes (37°C , 5% CO2). EthD-1 emits at 617nm and Calcein AM emits at 517nm, so the samples were viewed under the RFP and GFP filter cubes of the EVOS FL microscope respectively.

Immunofluorescence staining
The fixed gels were washed with PBS containing 0.25% Triton X-100 and left to incubate for 10 minutes to permeabilize the cell membrane. The gels were washed twice with PBS and blocked with a 1% BSA in 1x PBS solution for at least 20 minutes. The BSA was removed and 1% BSA with the primary antibodies was added and left to incubate overnight at 4°C. The gels were then washed twice with PBS and the secondary antibodies were added in a 1% BSA solution for 1 hour in the dark at room temperature. The gels were washed twice with PBS and PBS with 1:100 DAPI staining (Thermofisher, 62248) was added for 5 minutes in the dark at room temperature to stain the cell nuclei. The DAPI solution was removed and fresh PBS was put on. The samples were then viewed under the EVOS FL microscope and later under the Zeiss KSM 880 laser confocal microscope. DAPI under the DAPI filter, glucagon under the GFP filter and insulin under the CY5 filter.

For the insulin staining the mouse anti-insulin primary antibody (NovusBio, NBP2-34260) at a dilution of 1:200 in 1% BSA was used. The secondary antibody was the donkey anti-mouse AlexaFluor647 antibody (Abcam, ab150107) at a dilution of 1:200 in 1% BSA. The emission of this antibody peaks at a wavelength of 668nm (deep red).
For the glucagon staining the rabbit anti-glucagon antibody (ThermoFisher, PA5-88094) at a dilution of 1:100 in 1% BSA was used. The secondary antibody was the goat anti-rabbit AlexaFluor488 antibody (Abcam, ab150077)at a dilution of 1:250 in 1% BSA. The emission of this antibody peaks at a wavelength of 520nm (green).

Antibody incubation
FITC labelled IgG antibodies (20mg/ml, F9636 Sigma Aldrich) were added to the sample at a dilution of 1:400 in PBS. The sample was then left to incubate for 24 hours at room temperature in the dark. FITC emission peaks at about 519 nm so the sample was viewed under the GFP filter of the EVOS FL microscope and later under the Zeiss KSM 880 laser confocal microscope.

Actin staining
Phalloidin is used for the staining of f-actin filaments. The fixed samples were permeabilized with 0.25% Triton X-100 for 10 minutes. The gels were washed twice with PBS and phalloidin AlexaFluor488 (Invitrogen, A12379), at a 1:40 dilution in PBS was added. The samples were incubated for 30 min in the dark at room temperature. After this time the gels were washed and DAPI at a dilution of 1:100 in PBS was added. The samples were finally washed with PBS and viewed under the Zeiss KSM 880

3.4.1 Fluorescent image characterization

The aggregate formation of the cells prevents regular cell counting for the viability assessment. An alternative method is to calculate and compare the corrected total fluorescence of the microgels (CTF)[18]. Using ImageJ, I could calculate the area integrated intensity of a specific colour channel and region. This is the sum of all the pixel values in that region, or the total fluorescent intensity in that region. The CTF could then be calculated by removing the background noise via:

*CTF= Integrated density - (area of the region * mean fluorescence value of a background reading).*

Although the sum of the intensities of the green (live) and red (dead) fluorescent components don't add up to 100%, for the purpose of a semi-quantification it can be useful to assume this. The viability can then be calculated by:

$$viability = CTF_{live}/(CTF_{live} + CTF_{dead}) \quad (1)$$

The selected area in this case would be the entire microgel and the background noise the autofluorescence of the microgels.

The same CTF technique could also be used for the IF staining. The antibodies randomly stain cells within the microgel so by determining the total fluorescence within a microgel I can compare different microgels of different conditions with each other. This is assuming all microgels are the same size. This value is the CTF mentioned before where the background noise in this case would be the autofluorescence of the microgels. The DAPI staining confirmed that the measured signal was specific and originating from cells.

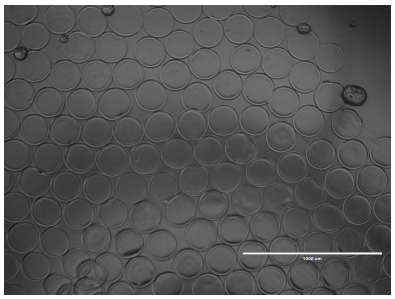
For the fluorescent FITC antibody incubation I looked at the mean intensity of the background signal, and the mean intensity in the microgels themselves. There should be no specific binding to any structure, but a diffused green signal inside the microgels if the antibodies have penetrated. It was therefore useful to compare the mean fluorescent intensity of the inside with the outside of the microgels. By dividing the mean intensity inside the microgels with the intensity of the outside, I got a ratio which could be used to compare different conditions. A higher ratio would mean more penetration of the antibody.

4 Results

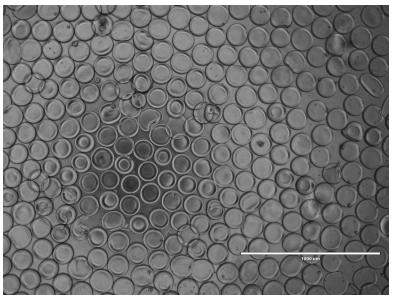
4.1 Microgel fabrication

The main results of experimentation with the microgel creation can be seen in figure 4. The shell thickness and microgel diameter were measured in ImageJ. The results of this analysis can be seen in figure 5. The flow settings eventually used for encapsulation (7.5p/40o 10% PEG-TA) resulted in mostly intact microgels of about 160µm with a shell thickness of about 20µm. Reducing the polymer concentration while keeping the flow speed the same resulted in smaller and thinner shelled microgels. These microgels were also more frequently broken or misshapen. Increasing the polymer to oil ratio resulted in larger microgels with a shell thickness that was slightly thinner than the 10% PEG-TA. These microgels were mostly intact.

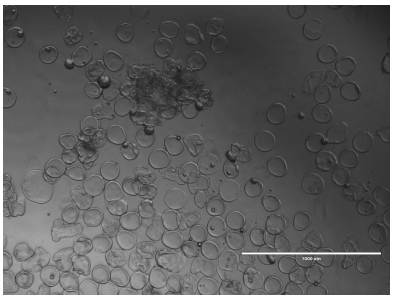
I also found that microgels might swell a little bit over time. Microgels in images made of an ethidium staining 3 weeks after initial production were found to be consistently 10-15µm larger in diameter. The microgels were stored on PBS in the fridge during this time. It could be that the microgels swell a bit over time when kept in PBS. It is unclear if this could also happen to microgels laden with cell in culture medium. It might be of interest to see whether the microgels swell over time due to the influence of the solution medium.



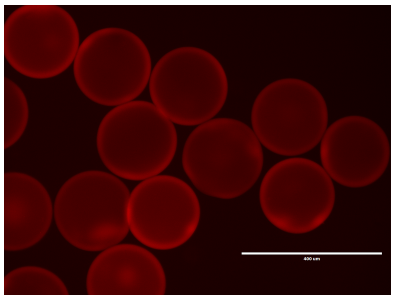
(a) 20p/40o 10% PEG-TA 4x trans. This condition yields microgels of about 220µm in diameter.



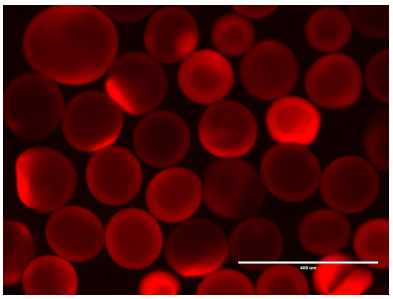
(b) 7.5p/40o 10% PEG-TA 4x trans. This condition yields microgels of about 160µm in diameter.



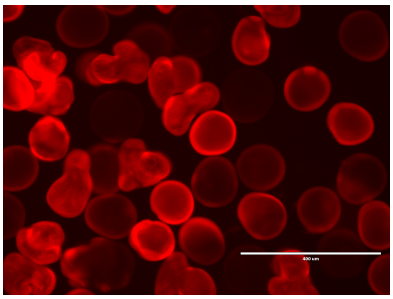
(c) 7.5p/40o 2.5% PEG-TA 4x trans. This concentration yields microgels of about 130µm. Most are misshapen or broken



(d) 20p/40o 10% PEG-TA 10x RFP. The microgel shell, stained more brightly red, is about 18µm thick.



(e) 7.5p/40o 10% PEG-TA 10x RFP. These microgels are smaller and have a slightly thicker shell.



(f) 7.5p/40o 2.5% PEG-TA 10x RFP. The broken microgels can be seen especially well when stained by Ethidium.

Figure 4: microgels of different polymer(p)/oil(o) flow speed ratios in µl/min. Ethidium homodimer-1 stains the crosslinked microgels, staining them red fluorescent under the RFP filter.

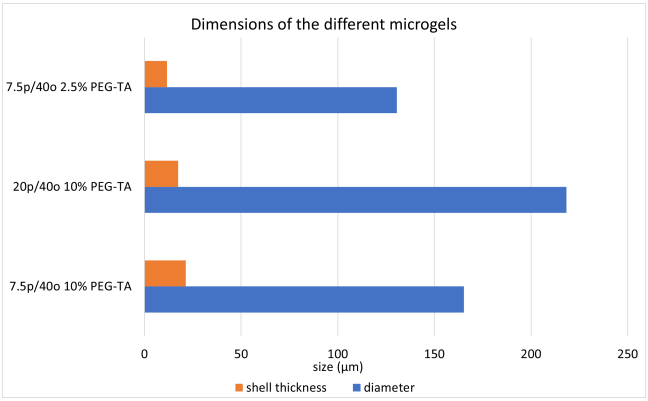


Figure 5: Microgel diameter and shell thickness of the gels resulting from varying polymer flow rates (p/o in µL/min) and varying PEG-TA concentration. For every condition 10 microgels were measured.

4.2 Viability assessment

The results of the live/dead staining can be seen in figure 6. The last two images of figure 6 show the results of the control for polymer toxicity. In general, cell viability in the microgels remains high. One of the outliers is day 1 of the 10% PEG-TA encapsulation. I tried to culture the gels in a conical flask since this would make the changing of medium substantially easier. Due to the high cell mortality on day 1, we moved the encapsulated cells back to a suspension culture plate. This move apparently had a positive effect on the cells since the viability rose afterwards. The 2.5% PEG-TA microgels were cultured on a suspension culture plate from the beginning.

The trans pictures show how the cells form large aggregates over time. After about 4 days most of the cells are part of the aggregate. Cells that are not part of the aggregate usually stain as dead. Furthermore, some cells on the edge of the aggregate stained dead.

The cores of the larger aggregates show weaker staining for both ethidium and Calein AM. This might be attributed to the small penetration depth of both of these molecules or to the extinction of emitted light from the core by higher-lying cell cells in the aggregate. It is unclear whether the cores of the larger aggregates are alive or have become necrotic.

The 2.5% PEG-TA microgels are more frequently empty and contain less cells in general. A reason for this could be that during this encapsulation I didn't have a full flask of cells. According to the cell count there should have been enough cells for encapsulation but there might have been some deviation resulting in more empty microgels. The 10% PEG-TA encapsulation was also done by Nuno Gomes so experience probably plays a role here too. These empty gels posed a problem for the quantification of all stainings, since the sample sizes were generally very small.

The results of the semi-quantification of the viability are shown in figure 7. Figure 7a shows the viability of the cells in the 10% PEG-TA microgels, and figure 7b shows the viability of the cells in the 2.5% PEG-TA microgels. Although this quantification was not very accurate, a trend can be seen in both graphs: in general, the cell viability decreases over time. The initial cell death on day 1 of the 10% PEG-TA microgels mentioned earlier is also represented in figure 7a. There was no data for the viability on day 30 of cells encapsulated in 2.5% PEG-TA microgels, but figure 7b does contain the control for polymer toxicity which shows that the polymer is not toxic for the cells.

The viability results found don't match up entirely with scientific literature on the subject. Bas van Loo et al.[12] for example found encapsulated cells remained 83% viable after a period of 30 days. Suzanne Nijhuis also found a viability of >90% in her thesis. Since I found that viability decreased to <60%, the experiment would have to be repeated.

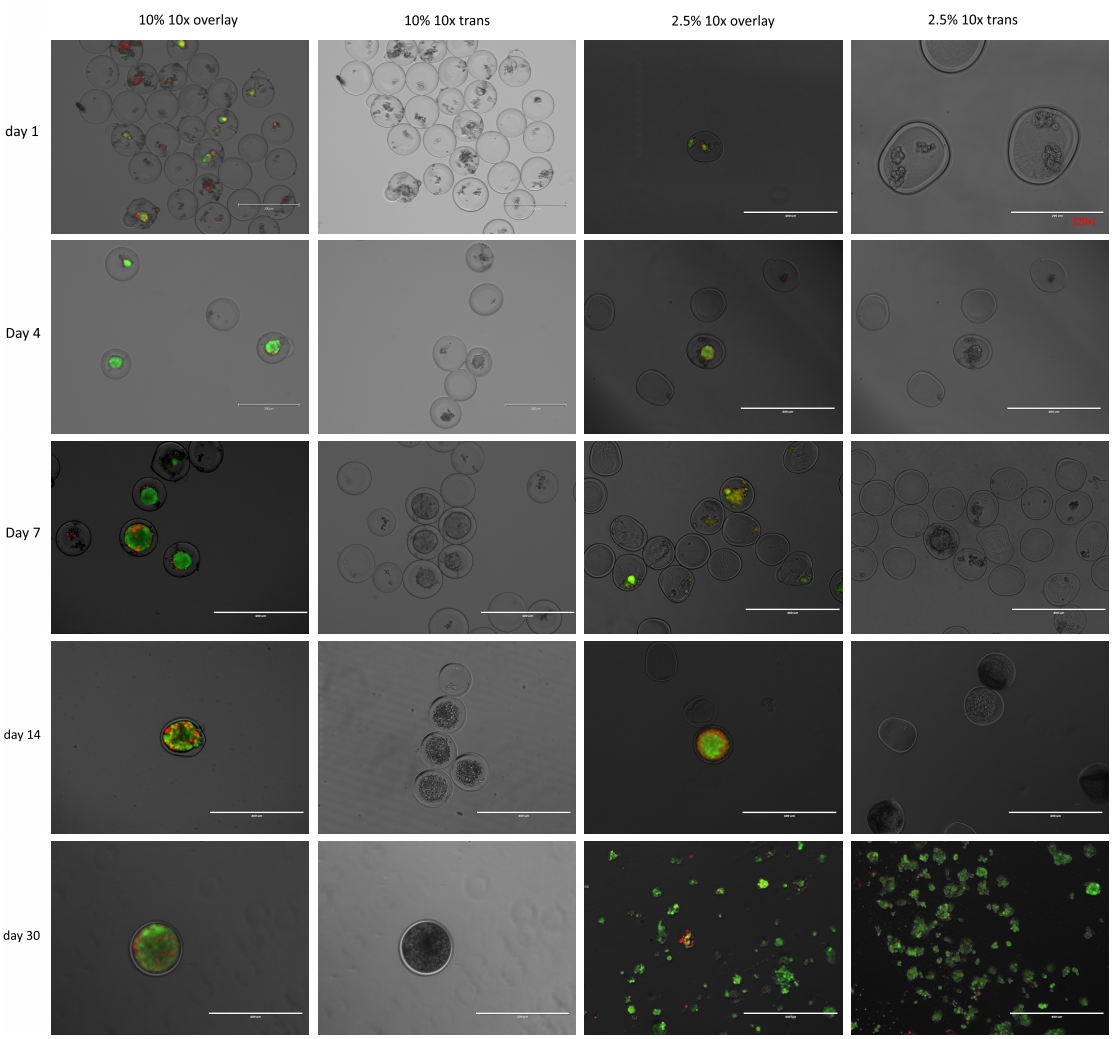
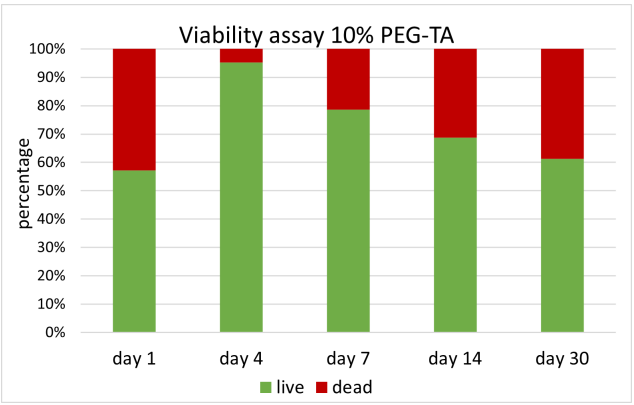
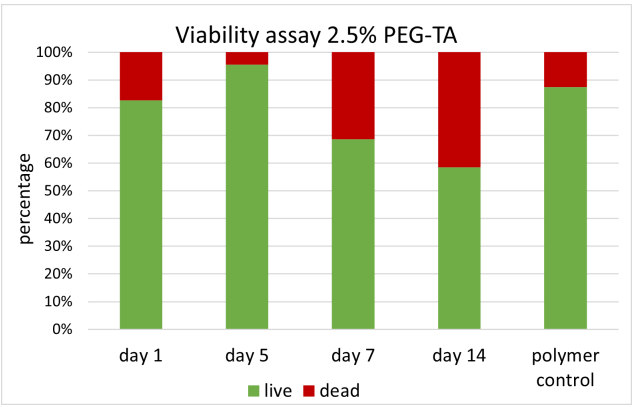


Figure 6: Overview of the live/dead stainings at the different timepoints



(a) Viability assessment of the cells encapsulated in 10% PEG-TA microgels. The viability seems to decrease over time. The cell death on day 1 was initially very high, after which the culture method was changed.



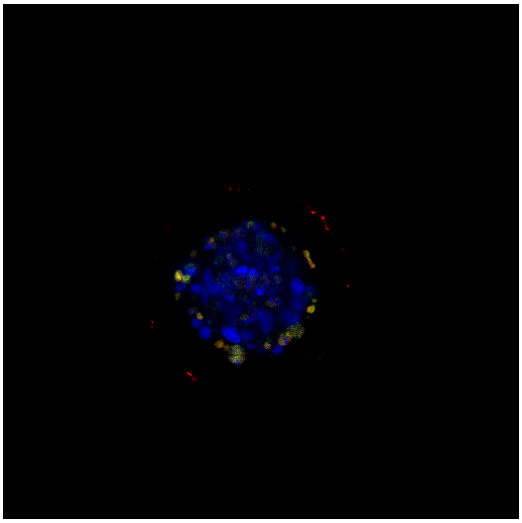
(b) Viability assessment of the cells encapsulated in 2.5% PEG-TA microgels. The viability seems to have the same downwards trend as for the 10%. This graph also includes the control for polymer toxicity.

Figure 7: semi-quantification of the live/dead stainings

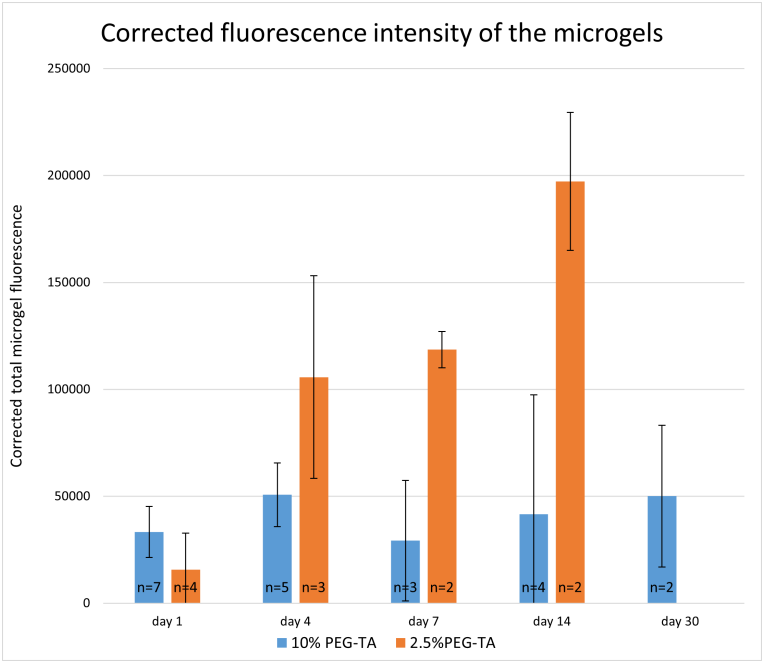
4.3 Immunofluorescence

The results of the IF staining can be seen in figure 8. This analysis is based on the images found in the supplementary data, figure 12. Figure 8a shows a typical image of the IF staining made under the confocal laser microscope. The analysis was done with images made under the EVOS microscope. The green signal (under the GFP filter) represents a glucagon surface receptor. The insulin surface receptor is represented by a red signal (under the CY5 filter). This staining was not visible under the EVOS microscope and only partially under the confocal laser microscope. Since this is a problem under both microscopes there was likely a problem with the primary antibody incubation since the secondary fluorescent antibodies were put on separate for these two experiments. In both cases the insulin staining could not be used for analysis. The blue signal (under the DAPI filter) represents cell nuclei stained by DAPI. Using the DAPI staining as a confirmation of cell presence, I have calculated the corrected total green fluorescence (CTF) of the microgels. The higher the fluorescent intensity the more both antibodies have penetrated the microgel so the less immunoprotective the microgel is.

The results of this analysis can be seen in figure 8b. The 10% PEG-TA microgels are much more immunoprotective than the 2.5% PEG-TA microgels. There also seems to be a slightly positive trend in fluorescent intensity over time, especially in the 2.5% PEG-TA microgels. This could be contributed to a decreasing immunoprotective character over time, or due to the growth and proliferation of the cells inside the microgels. The sample sizes of most of the conditions were also not sufficiently big, especially for the 2.5% PEG-TA microgels, which could result in a misrepresentation. A larger experiment where the intensity is determined relative to the number of cells in the microgel could answer the remaining questions.



(a) An example of an IF image. In this case 10% PEG-TA day 30 under the confocal laser microscope. The green signal represents glucagon surface receptors. The red signal represents insulin surface receptors but is not very visible. It does however vaguely outline the microgel. The blue signals are DAPI stained cell nuclei.



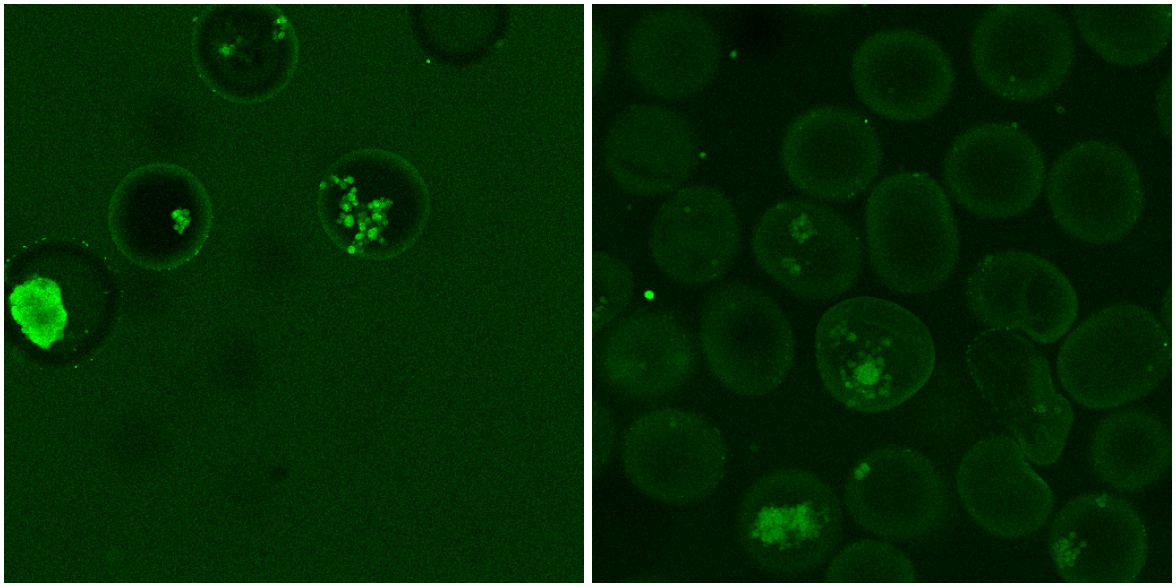
(b) The IF analysis of all timepoints. A higher fluorescent signal indicates more penetration by fluorescent antibodies. In general, the 10% PEG-TA microgels provide more protection for antibodies than the 2.5% PEG-TA microgels. It also looks like the immunoprotective character of the microgels decreases slightly over time.

Figure 8: Immunofluorescence results

4.4 Fluorescent IgG antibodies

The images made with the EVOS microscope were unfortunately not sufficient to perform an adequate analyse. I tried making images under the confocal laser microscope, but all the conditions did not contain enough microgels to perform an analysis. The images made with the confocal laser microscope are shown in figure 9. It looks like the 10% PEG-TA microgels emit less than the background, which would mean they keep the antibodies out. The cells however emit a lot more than the background which is very unexpected. The same happens with cells in the 2.5% PEG-TA microgels. These microgels however emit a lot more than the background, which could indicate the build-up of antibodies inside the microgel. This would mean that the 10% microgels are more immunoprotective than the 2.5% PEG-TA microgels since more antibodies have penetrated the latter.

In both cases the images made did not look like the expected results (see supplementary data, figure 11). The most likely cause for this is the antibody dilution which was probably 10x higher than needed (1:400 instead of 1:40). The strong fluorescent signal emitted by the cells is likely autofluorescence since cells are not usually visible using this technique and the antibodies normally shouldn't specifically stain the cells. It is also known that cellular compounds can cause autofluorescence in the FITC excitation/emission spectrum**AutofluorescenceBio-Rad**. More concentrated antibodies would emit significantly more light, thereby mitigating the effect of cellular autofluorescence. For an accurate analysis the experiment would have to be repeated with a 1:40 antibody dilution.



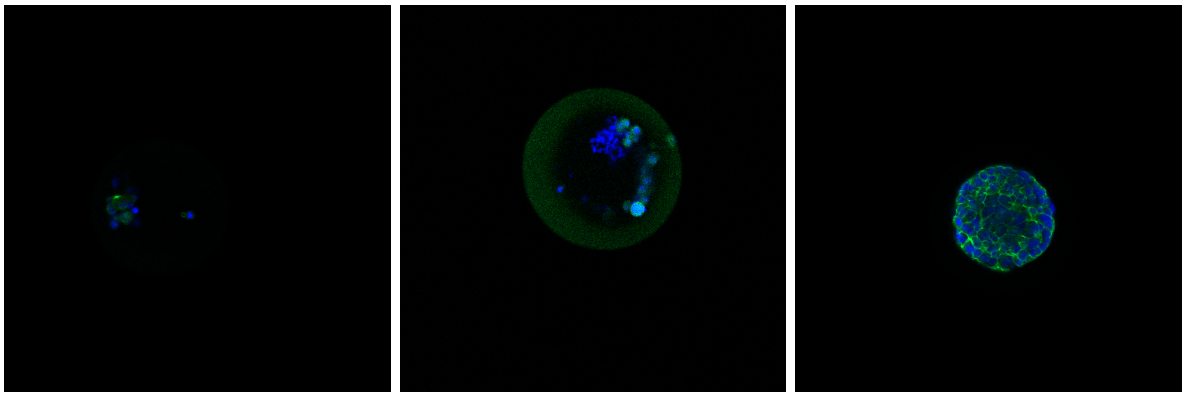
(a) The IgG incubation with 10% PEG-TA microgels on day 14. The insides of the microgels seem to be free of antibodies, although the cells themselves seem to emit a lot and can be seen very well.

(b) The IgG incubation with 2.5% PEG-TA microgels on day 14. The microgel shells emit a lot more than the background, just as the insides of the microgels. The cells also seem to emit a lot, just as in figure 9a.

Figure 9: FITC labelled IgG antibody incubation results

4.5 Actin staining

Figure 10 shows the results of the phalloidin staining. Only the 10% PEG-TA microgels were imaged. The images show that the cells aggregate into dense cell spheroids over time. The actin also appears to reside predominantly around the cell membrane, which is to be expected when cells aggregate together. The day 7 microgel is probably an outlier. As seen in figure 6 cell aggregation already happens by day 4. Although it can't be said definitively most of the cells in that microgel are most likely dead, as suggested by messy clump of blue stained nuclei.



(a) day 1, 20x *(b) day 7, 20x* *(c) day 30, 20x*

Figure 10: The laser confocal images of the actin staining. The green signals are stained actin filaments, predominantly around the cell membrane. The blue signals are DAPI stained cell nuclei. All microgels are 10% PEG-TA. The cells aggregate into dense cell spheroids over time.

5 Discussion

5.1 Aim

The method used to fabricate the used microgels is new and promising but the theoretic properties of these microgels need to be tested for the use in vivo. One of these properties is the ability to be immunoprotective, meaning protective against the effects of antigens. In the context of this research that means antibodies can't penetrate the microgel shell. Research within the DBE group showed that the microgels in general are immunoprotective, but there seemed to be a small amount of antibody penetration after a period of 30 days. This thesis aimed to determine how the immunoprotective character of the cell laden microgels develops over time. This immunoprotective character is very important for experimentation in vivo and eventually for therapeutical purposes. If the microgels do not remain immunoprotective for a period of 30 days, it makes no sense to use them for transplantation. If they are, they could potentially be a way to transplant insulin producing cells into diabetic patients.

5.2 Immunoprotective character

Although the results of the immunofluorescence and FITC antibody incubation were not ideal, it could be seen that the antibody penetration into the 10% PEG-TA microgels was inhibited compared to the 2.5% PEG-TA control, which is known to not be immunoprotective. With the immunofluorescence staining I saw significantly less staining in the 10% PEG-TA microgels. The staining was also a lot less specific compared to the control. With the FITC antibody incubation I saw that the insides of the 10% PEG-TA microgels (ignoring the emission of the cells) emitted significantly less than the background. The 2.5% PEG-TA microgels emitted significantly more compared to the background, so compared to the control the 10% PEG-TA microgels were once again more immunoprotective.

The 10% PEG-TA microgels are known to be immunoprotective *in vivo* and *in vitro* and, in general, my research confirms this. There was some antibody penetration into the 10% PEG-TA microgels but this could be attributed to the stretching of the microgels due to aggregate growth. The effect of this stretching seems to be limited, however, since I could not find a significant increase over time, but the experiments would need to be repeated to conclude this.

5.3 Therapeutic potential

Experimentation within the DBE group showed that smaller molecules, such as BSA, can enter the microgel with ease. As is shown in this thesis, larger molecules such as antibodies are severely inhibited in doing so. This would mean that cells inside the microgels are protected from the immune system. At the same time smaller molecules such as oxygen, insulin or glucose can diffuse through the microgel with ease. Due to the small nature of the microgels (160µm) the cells inside benefit from quick diffusion of nutrients. The viability assessment confirms that cells inside the microgel can survive for a period of at least 30 days. The actin staining, along with the live/dead staining, confirms that the cells aggregate into dense spheroids over time. These kinds of 3D structure generally enhance cell functions[19]. Some cells were found to be dead inside the cell aggregates, but they did not seem to inhibit cellular growth and the aggregate was found to be mostly alive. The cores of the aggregates stain alive in smaller aggregates, but it can't be confirmed if the insides of the larger aggregates are necrotic or not.

The small nature of these microgels also allow for easy transplantation into the body. No surgery is required since the microgels can easily be injected into the body. The total volume of these microgels is also a lot smaller than an entire pancreas so they can be injected into any reasonable location. High vascularization around β-cells in the pancreas is associated with high cell proliferation and strong secretory function[20]. β-cells mostly metabolize glucose almost exclusively via aerobic glycolysis, so they are very dependent on oxygen. Good vascularization around the graft is therefore very important for the survivability of the transplanted microgels. Oxygen diffusion in the body rarely exceeds 200µm and is usually less than 100µm. This would mean that the microgels can be supplied with oxygen in their entirety[21]. However, there seems to be a large downside to good vascularization. Recent research has found that highly perfused microgels with islet cells were more prone to cell death and fibrosis due to the increased accessibility to cytokines and immune cells[22]. Therefore, the increased perfusion might lead to earlier cell death and ultimately transplantation failure.

5.4 Future outlook

This thesis builds upon the research done by the DBE group about the encapsulation method used. It proves that the produced PEG-TA microgels inhibit the diffusion of antibodies and that cells can survive for long periods of time *in vitro*. The next step would be to assess the response against these microgels *in vivo*. It is recommended though to repeat this experiment to determine how the immunoprotective character develops over time. It could also be useful to determine whether the core of the cell aggregates remains viable or becomes necrotic.

These microgels might also benefit other areas of research. Cells were seen to quickly condense into aggregates within a few days. The microgels might therefore be an attractive method to produce cell spheroids. They could also be used in tissue engineering as a scaffold. When packed together these circular microgels leave empty spaces between them, which could be used for vascularization.

6 Conclusion

In conclusion, encapsulating cells in a 10% PEG-TA microgel might be a viable option to protect cells from the immune system. Monodisperse microgels of about 160µm in diameter were successfully made using single step enzymatic crosslinking in a microfluidic device.

The aim was to assess the immunoprotective character of these microgels over a period of 30 days. The 10% PEG-TA microgels appeared to be immunoprotective for this period compared to the control. However, there appeared to be small penetration of antibodies. This might be attributed to stretching of the microgels, but this could not be confirmed. The experiments would need to be repeated to get a definitive conclusion.

The microgels remained intact and the encapsulated cells remained viable for a period of at least 30 days. The cells also aggregated into dense spheroids after a few days of culture. However, it is unknown if the core of the aggregates remained viable or become necrotic.

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Supplementary data

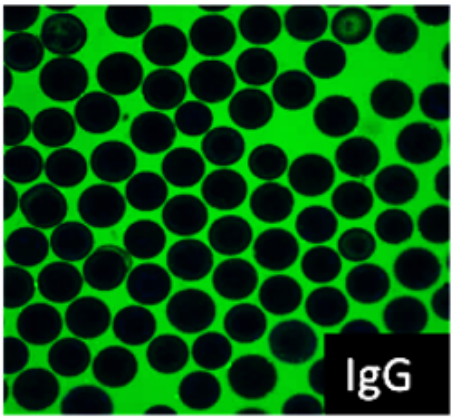
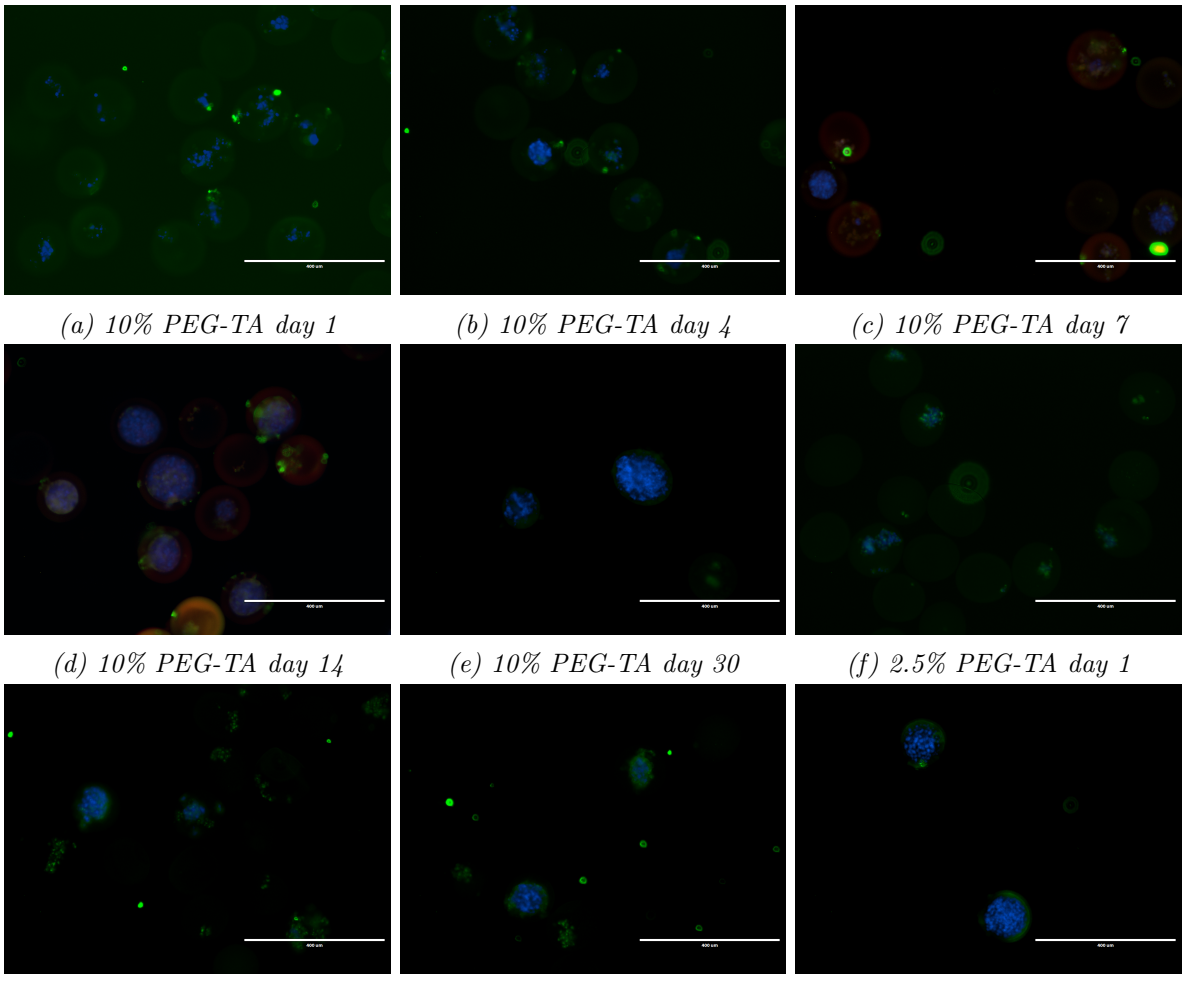


Figure 11: An example of the FITC labeled IgG incubation performed by Nuno.



(a) 10% PEG-TA day 1 *(b) 10% PEG-TA day 4* *(c) 10% PEG-TA day 7*

(d) 10% PEG-TA day 14 *(e) 10% PEG-TA day 30* *(f) 2.5% PEG-TA day 1*

(g) 2.5% PEG-TA day 4 *(h) 2.5% PEG-TA day 7* *(i) 2.5% PEG-TA day 14*

Figure 12: The used immunofluorescence images for the analysis. Green colour represents glucagon receptors, red colour (when visible) represents insulin markers and the blue colour represents cell nuclei.