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Laminin motifs inserted into a recombinant spider drag-line silk protein increase the proliferation of human dermal fibroblasts

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Summary

Spider silk is a promising biomaterial. It is highly biocompatible, non-toxic, most likely naturally degraded by the host organism over time and strong and resilient. Importantly, it consists of proteins and new motifs can relatively easily be added. While native spider silk is not possible to produce in available systems, a shorter segment of the protein, 4repCt, coding for 4 repetive alanine blocks and the C-terminal, can be inserted in vector and expressed in E-coli. Three laminin-motifs were added to 4repCt; RGD, IKVAV and YIGSR, in order to test how this affects cell proliferation of dermal fibroblasts. The results, although not confirmed with statistical certainty, strongly suggests that dermal fibroblast proliferation increase when grown on fibers with said motifs inserted compared to unmodified fiber.

Fibers made with 4repCt are not as strong as native spider silk fibers. Therefore, cysteine was inserted into 4repCt, which allows production of potentially stronger fibers that can be subjected to tensile strength tests and disulphide brigde formation tests.

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Introduction

Laminim motif insertion into spider silk

Spider silk have physiological properties that makes it interesting for possible implantation into the human body. It is a foreign protein, yet does not cause inflammation in pigs [1]. Primary studies done also suggests it naturally degrades in the body, likely degraded by the same processes that degrades ECM [2], which depending on the purpose of the implantation may be desireable. It has high tensile strength [3] but is at the same time soft, meaning it should be able to handle the stress the host body will exert on it by the hosts movements and at the same time not damaging surrounding cells through shearing damage. Historically, spider silk have proved difficult to produce en masse due to the cannibalistic nature of spiders which makes spider farms unpractical. Through gene transfer, alternative hosts have been attempted, but the spider silk proteins have proved difficult to express, most likely because they are highly hydrophobic and have highly repetitive sequences rich in glycine and alanine which results in a low yield. Researchers at the department of Anatomy, Physiology and Biochemistry at SLU have developed a method that allows them to produce large quantities of a shorter segment of the MaSp1 gene, a major compenent of dragline silk, from *Euprosthenops Australis* [4]. *Euprosthenops Australis* have one of the strongest dragline silks, and it is also a water spider, which means the dragline silk will retain it's function and structure even during wet conditions, such as inside the human body. This recombinant protein is named 4repCt, which is short for four repetitive alanine / glycine blocks and the C-terminal. See appendix I for the complete 4repCt sequence. 4repCt is fused with a water soluable protein (thioredoxin, TRX). When TRX is cleaved off, 4repCt self assemble into fibers. 4repCt is expressed in *E. Coli*. Most biomaterials on the market today have a structural function and do not provide any biological function beyond this. Adding protein motifs with a biological function is a way of trying to make the materials “smarter”. 4repCt have a high potential as a biomaterial, not only due to its inherent physiological properties, but also because it is protein based. Where other biomaterials must be surface treated and the motif added, the spider silk sequence can be altered to include small protein motifs, eliminating surface treatment steps. Three laminim binding motifs, RGD, YIGSR and IKVAV, short polypeptides which binds to specific sites on laminin responsible for binding the extracellular matrix, have been shown to affect cell proliferation and even differentiation [5]. While these motifs individually each have been shown to have cell proliferation stimulating capabilities, it has been seen that in combination they provide an even stronger effect [6].
This project aim to investigate if introduction of RGD, YIGSR and IKVAV into 4repCt will result in similar stimulation of profileration. To meet this end, these motifs will be introduced at the N-terminus of 4repCt using cloning techniques, the threads will be expressed, plated on wells, and finally, the 4repCt threads will be covered by Primary Dermal Fibroblasts and proliferation progress measured using alamar blue at day 1,2,3,4 and 7. 4repCt can be expressed not only as threads, but also as films and foams.. The cells were grown on a foam of 4repCt with introduced RGD, YIGSR and IKVAV motifs.

Insertion of cysteine in 4repCt

4repCt is weaker than native spider silk. The C-terminal domain of spider silk proteins form dimers. The repetitive blocks form beta sheets, and these then form clusters of beta sheets with beta sheets from both other spider silk proteins and itself. It is likely that 4repCt is weaker than native spider silk because it is a shorter protein. In an attempt to increase the intra- and / or intermolecular binding strength of 4repCt, cysteines have been added to 4repCt previously. These experiments showed a slight increase in tensile strength and and increase in the elastic modulus [7]. Herein, a cysteine was added to the N-terminus of 4repCt in order to investigate whether this positioning will affect fiber formation or strength.

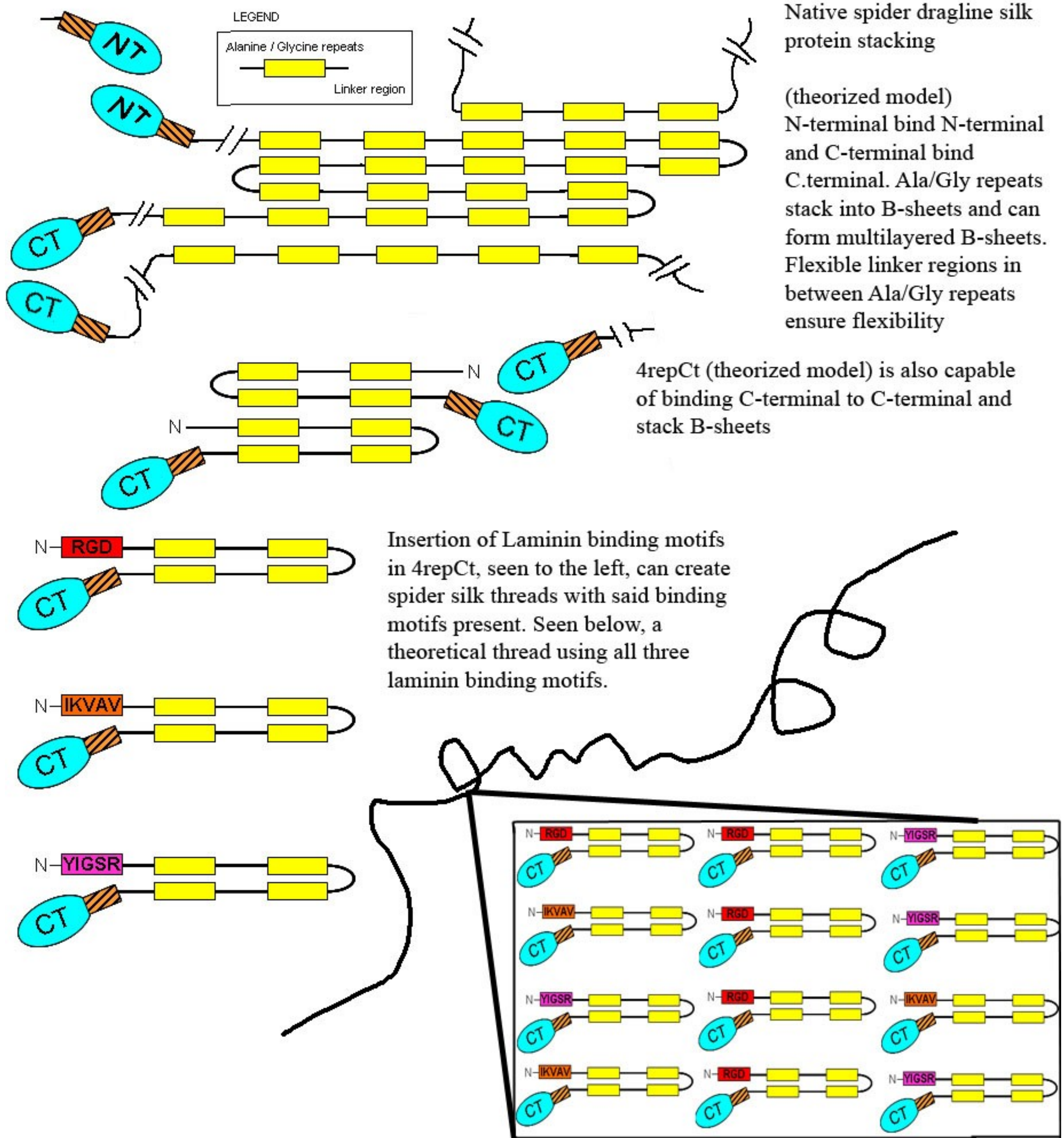

Fig1: A visual representation of how 4repCt stacks based on analysis of structural data obtained through various experiments. This representation is the belief of the author and does not necessarily represent the true stacking properties of 4repCt or dragline spider silk. The thread in the bottom of the picture is constructed through mixing equal proportions of YIGSR4repCt, RGD4repCt and IKVAV4repCt, and results in a thread with random dispersion of laminin binding motifs. This is the fundamental idea of this entire experiment, a strong and flexible biomaterial moldable into complex threedimensional structures that mimics the ECM through laminin binding motifs. These motifs, RGD, YIGSR and IKVAV are native to the ECM.

Material and methods

Construction of insert

Cloning techniques were used to insert the short polypeptides into the 4repCt sequence. The short polypeptides are RGD, IKVAV, YIGSR and C, and will hereby be called Motif(s), short for laminin-binding motifs, C is also included in this definition despite it not being a motif. The 4repCt sequence already exists in a vector fused with TRX. There is also a his tag in front of and after the TRX. The cleavesite (protein), which is either for a protease denoted A or B, is situated right in front of the 4repCt sequence, effectively removing TRX and the HIS-tags. (Either HIS-TRX-HIS-A-x-4repCt or HIS-TRX-HIS-B-x-4repCt, where x denotes the site where the motifs and cysteine is inserted.) These vectors were used as templates. The downstreams primers consists of a region which contain a cleavesite for EcoR1, the motif, and native 4repCt sequence. The motifs had two versions of primers, one with only the motif, and one with the motif plus some of the motifs native sequence, the reasoning behind is that perhaps the motifs will more readily form active fold with longer native sequence. The longer primers are denoted long. The same upstreams primer is used for all downstreams primers. The upstream primer consists of native 4repCt and a HindIII cleavesite. Primer sequences are avaible in appendix II. The primers were then run with a standard PCR-program using Taq-DNA Polymerase. Due to a secondary binding site (see Cysteine and laminin motif insertion into 4repCt on page 8), polymerase was changed briefly to Amplitaq Gold DNA polymerase due to deceptive initial succes but then finally changed to Advantage GC Melt. The PCR-product was purified with either GelExtraction Kit or PCR reaction cleanup Kit and then cleaved with EcoR1 and HindIII. The PCR-produkt should now be the entire 4repCt with the EcoR1 cleavesite and new motif in front and the HindIII cleavesite behind and cleaved so that it is ready to be ligated into vector. A vector (HIS-TRX-HIS-A-4repCt, HIS-TRX-HIS-A, HIS-TRX-B-4repCt or HIS-TRX-HIS-B-4repCt) were opened up with EcoR1 and HindIII at the same time. These vectors are constructed so that the EcoR1 site is directly upstreams of 4repCt and HindIII directly downstreams of 4repCt, and the old 4repCt sequence is effectively cleaved off (the vector lacking 4repCt sequence is merely opened up). After cleavage, the unwanted cleaved off parts are removed by purification of vector and the PCR-product with GelExtraction Kit. The purified opened up vector and the PCR-product are ligated for 1h-ON. If succesfull, the vector should now contain HIS-TRX-HIS-A or B-Motif-4repCt, or HIS-TRX-B-Motif-4repCt as one vector did not have the second HIS-tag. Sometimes, the PCR-products were ligated with A/T-vectors instead because they have a higher succesrate for ligation. After ligation, the vectors were transformed into *E-coli* cells using heatshock, either BL21 or NovaBlue *E-Coli* was used. NovaBlue can not express 4repCt and is used for cloning work only, while BL21 can be used for both cloning work and for protein expression. After transformation they are plated on petridish containing medium and ampicillin and allowed to grow ON. All surviving, or randomly chosen if they are numerous, colonies are then screened using Taq-DNA polymerase. There is no need to use Advantage GC-Melt since this step is merely to confirm 4-repCt presence; if the ligation is unsuccesfull, there should be none. After screening, if 4repCt presence is confirmed, minipreps are prepared from randomly chosen confirmed colonies grown ON in 3-6 ml medium. Before miniprep preparation, 1 ml cell culture is mixed with glyceraldehyde and frozen in -70°C for potential further use. Purified vectors are sequenced with forward upstreams primer FHisA and reverse downstream primers RNT4repCt and reverse in sequence primer RNT7repCt. These primers cover the entire 4repCt sequence. The sequence and location of these primers are not provided herein. After sequencing confirmed motif presence and retained 4repCt sequence integrity, if not already present in a suitable vector and BL21, they were moved using standard cleavage, ligation and transformation protocolls, and 1 ml cellculture is frozen together with glyceraldehyde in -70°C. At this point, the motif4repCt constructs can be stored almost indefinately in the freezer and are ready to be expressed.
All relevant deviations from standard protocolls are avaible in appendix III.

Formation of scaffolds

Cell culture, BL21 with expression vector coding for any of the 4repCt variations, is grown for around 4 h in 37°C 220 rpm or until the optical density is around 1. Half an hour before induction, the temperature is lowered to RT and rpm lowered to 180. Protein production is induced with IPTG. Production is then allowed to progress at RT 180 rpm for 2 h. The proteins are extracted from the cells using lyzosyme and DNAse and cell debris harvested through use of centrifugation. The supernatant is loaded on columns, where the HIS-tagged 4repCt can be separated from the other proteins. The HIS-tags along with TRX is then cleaved off using either A or B, and the 4repCt (including motif) is isolated. 4repCt spontaneously self assemble to form solid polymer. At present, using various methods, three basic scaffolds can be produced, threads, films and foam. Threads of all new constructs were created to verify retained function. Foams were created for all constructs, and in all combinations, and applied to wells (see Proliferation measurement below). Before 4repCt and variations thereoff are allowed to selfassembly, they are sterile filtrated and kept in sterile conditions. The cell culture plates come sterile from the company. In addition to foams, films were formed of 4repCt (without motif) as well, to investigate the importance of structure. Foams were also formed. 96 well plates with 100 µl 4repCt, or 4repCt with motifs and combinations thereof, with an absorbance at 280 nm of around 0,230 were used (approx. 0,5 mg/ml). Foams were made in the wells and excess water removed. Films were made by applying 4repCt-solution directly to the well. The film will spontaneously form on the water surface and sink to the bottom as water evaporates. After application to the wells, all films and foams were dried over night. Before cell seeding, the scaffolds were washed three times with PBS. 6 wells for each construct were prepared in hydrophobic plates, the surface is designed to be hostile to cells to elimate any cell proliferation due to the plate itself. A negative control of 6 wells of the hydrophobic plate was prepared as well. Also used is 6 wells from a hydrophilic 96 well plate for optimal attachment and growth of adherent cells, as a positive control.

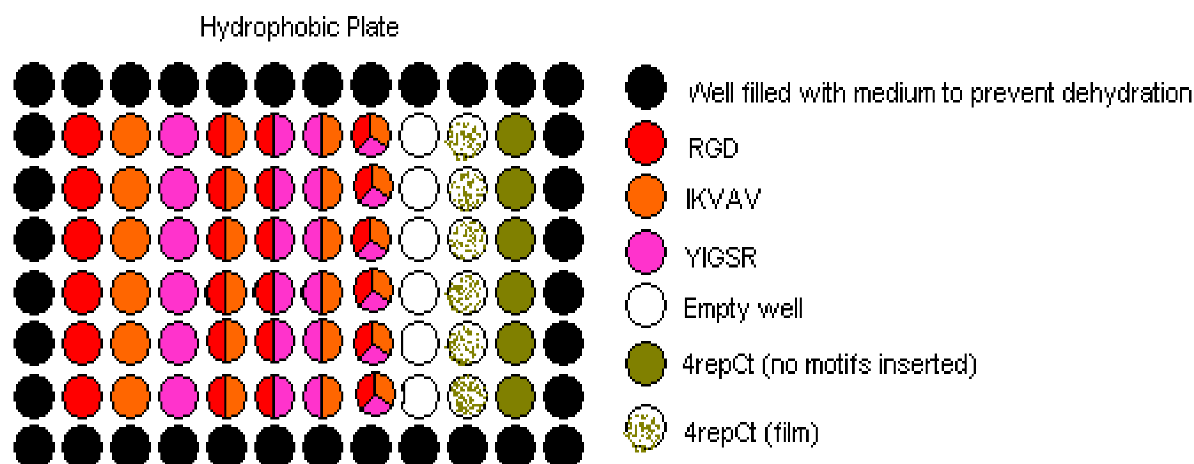

Fig2: There are 10 rows with 6 wells each on the hydrophobic plate. The ten rows are hydrophobic (nothing added), 4repCt (film), 4repCt (foam), RGD4repCt (foam), IKVAV4repCt (foam), YIGSR4repCt (foam), RGD + YIGSR4repCt (foam), IKVAV + YIGSR4repCt (foam), IKVAV + RGD4repCt (foam) and RGD + IKVAV + YIGSR4repCt (foam). Around the rows wells are filled with medium to prevent evaporation of water. On the hydrophilic plate (not pictured), a 3x2 area is surrounded by medium filled wells, and the six wells inside is a negative control using hydrophilic wells.

Proliferation measurement

After the plates had been prepared (see Formation of scaffolds above), wells were preincubated 1 h

in 37°C, five thousand cells were placed in every well. The cells used, Primary Dermal Fibroblast, were harvested from a human donor (HDFn). The cells were grown in complete medium (DMEM nutrient mixture F12 HAM supplemented with %5 foetal calf serum and 1% PEST) for 7 days in 37°C 5% CO_2 incubator, and at day 1, 2, 3, 4 and 7 was exchanged for alamar blue (Invitrogen) diluted 1:10 in complete medium for four hours, before 100 µl of alamar blue dilution (from each well) was transferred to a new plate for fluorometric measurements. The fluorescence (excitation 544 / emission 595) was measured on a FarCyte. Alamar blue was then completely removed from the wells and complete medium was reapplied. Alamar blue is taken up, and is reduced as a secondary effect of mitochondral enzyme activity. ($NAD^+ + 2e^- + 2H^+$ --> $NADH + H^+$). Alamar blue in unreduced form is as the name implies blue, but when reduced, it will become bright red (emission at 590 nm). Note that Alamar blue is non-toxic and it is in fact the proliferation in the very same wells that are measured each day [8].

Live / Dead cell stainings

In addition to of the 96 well plates, one foam of each kind were also applied to glass cover slides, with one glass cover slide where nothing was added serving as positive control. Unlike the 96 well cell culture plates, the glass cover slides are hydrophilic. The glass cover slides are sterilized under UV-light for half an hour. Just as with the foams and films in 96 well plates, the foams and films were dried over night and washed three times with PBS. The scaffolds were preincubated 1 h with 3 ml complete medium at 37°C in 5% CO_2. 250 000 cells suspended in complete medium were added to each glass plate. Cells are grown for four days in 37°C 5% CO_2 incubator. Medium was changed once on day 2. On day four, fluorescence microscope pictures were taken. The medium was replaced with a PBS containing Live / Dead solutions (Invitrogen) and the cells were analyzed using a microscope. A digital camera mounted on the microscope was used for taking photos. Photos of live cells were taken under green filter, and dead cells where analyzed under red filter.

Disulphide-bridge formation test

The C4repCt threads were dissolved in pure formic acid with SDS added. All liquids were then evaporated through speedvac and the remaining proteins solved in 60 µl gel loading buffer (containing SDS) and analyzed on a gel. Three volumes of protein loaded in solvent were analysed in the gel, 5, 10 and 15 µl, to make sure the bands are not to strong and not to weak. Also loaded on the gel was C4repCt treated with Betamerkaptoethanol, which will disrupt any S-S-bridges, with the same volumes, 5, 10 and 15 µl. Different concentrations were use to avoid concentrations to small to be visible, or so high that all that can be seen is a smear. If C4repCt cluster in larger units, a band on untreated C4repCt which is larger than the largest band on C4repCt treated with B-merkaptoethanol should be present.

Results

Cysteine and laminin motif insertion into 4repCt

There is a secondary binding site for all insert primers, giving rise to a 400 bp long PCR-product, which made working with 4repCt difficult (see appendix I for the entire 4repCt sequence and appendix II for the location of the secondary binding site). The amplification and/or ligation step had to be repeated many times due to problems caused by this secondary binding site. The main problem with the secondary binding site is that it decreased the yield of the 800 bp band to such a low amount that subsequent ligation attempts failed. Finally, the polymerase was changed to Advantage GC Melt, which completely eliminated the problem with the secondary binding site. Advantage GC Melt is designed to more readily denature sequences rich in GC than other polymerases, which could be the reason Advantage GC-melt worked where Taq DNA Polymerase did not.

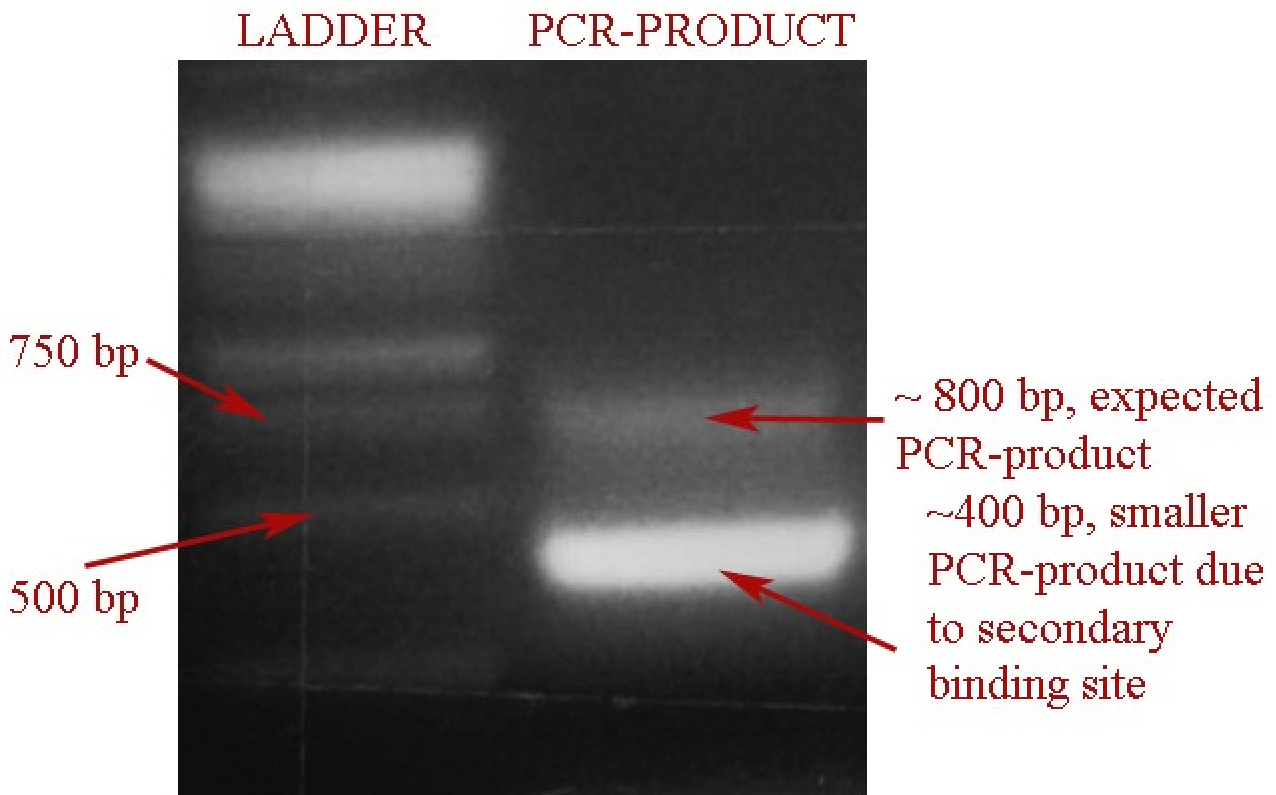

Fig 3: The first four steps from the bottom in the ladder are 250, 500, 750 and 1000. An 800 bp band and a 400 bp band can be seen, a problem encountered repeatedly. Only Taq DNA Polymerase had the problem with a secondary binding site, this problem was solved through the switch to Advantage GC Melt never.

The following construct were made and sequenced:
HIS-TRX-B-C(ysteine)4repCt
HIS-TRX-HIS-A-C4repCt
HIS-TRX-HIS-B-C4repCt
All these constructs came from the same amplification. They therefore all share the same mutation, a deletion of an alanine, alanine 116-123. This alanine repeat all share the same three nucleotides, GCT, for this reason it is not possible to determine where the deletion took place.
HIS-TRX-B-C2repCt
This is Cysteine successfully inserted in a 400 bp band. This construct have been saved for comparison with C4repCt:s disulphide bridge formation. See appendix II for location of secondary binding site.
HIS-TRX-HIS-A-YIGSR4repCt

HIS-TRX-HIS-B-YIGSR4repCt
The YIGSR construct is without mutations.
HIS-TRX-HIS-B-RGD4repCt
The RGD-construct had one mutation in the N-terminal section of the sequence, M193V. It also had two silent mutations, G17G and G78G.
HIS-TRX-HIS-A-IKVAV
HIS-TRX-HIS-B-IKVAV
The IKVAV-construct had two mutations, S157G and A173T.
Spider threads were successfully produced using all constructs above, indicating that these mutations do not hinder the ability to form spider silk. If the structure integrity is maintained, it is also unlikely that it would be these mutations rather than the motif insert that affects cell proliferation. See appendix I for complete 4repCt sequence.

A mutation free RGD construct was constructed as well, but it was not be used due to lack of time to create foam for cell experiments.
Using the RSN7repCt, RGD(long), IKVAV, IKVAV(long), C, RGD(long) and YIGSR(long) constructs cloned into A/T vectors which were sequenced. However, like the RGD construct, they could no be used due to time restrictions.

Disulphide bridge formation test

There was only one attempt made at a disulphide bridge formation test, but it failed and it was not repeated due to time constrictions. Therefore, no real conclusion can be drawn from this experiment.

Cell proliferation

Cells proliferate on all scaffolds. It is important to note that the scaffolds are applied on hydrophobic wells; since all scaffolds have significantly higher values than the hydrophobic wells, any incomplete coverage would decrease, not increase, the proliferation. Any proliferation seen can therefore be directly linked to properties of the scaffold itself. The different combinations, for instance YIGRS+IKVAV, means there is an equal amount of YIGSR and IKVAV that added together have the same total volume and concentration as any other threads and films (see fig1 on page 5 and Formation of scaffolds on page 7). The hydrophilic 96 well plate is designed to allow good adhesion, it is therefore important to note that the (unmodified) 4repCt foams and films have values comparable to the hydrophilic wells. This indicates that 4repCt is a material which has cell adhesive properties. There is a sudden increase of cell proliferation on day 3. This is likely a measurement error, as it is not concurrent with day 1, 2 & 4 (apply an imaginary ruler over the top of the bars on fig4 on day 1, 2 & 4 and day 3 is clearly the odd one out.) When viewing the diagrams, mind that this experiment have not been repeated and any and all results should be considered not statistically verified.

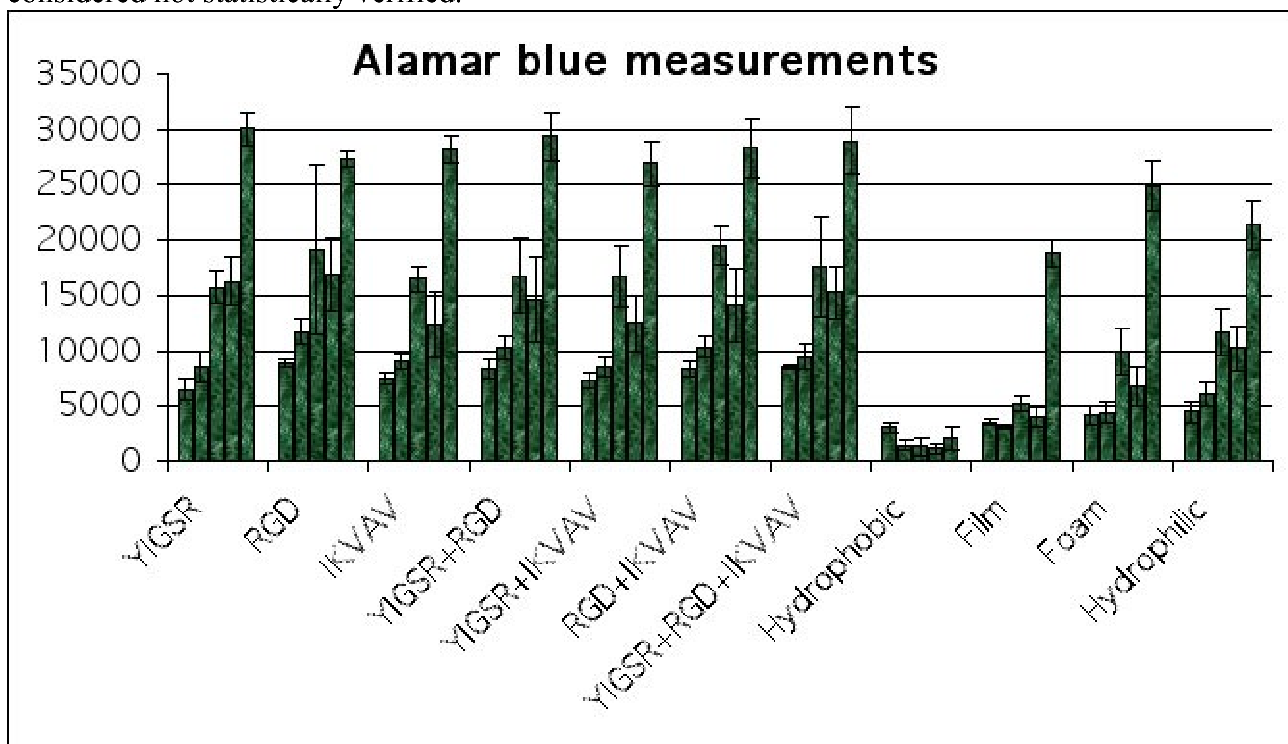

Fig4: A brief overview of the flouremetric measurements of cells growing on each scaffold and the positive and negative controls, the bars represent day 1, 2, 3, 4 and 7. 4repCt is made into a foam and placed on the bottom of these wells, however, 4repCt without motif is also made into a film for comparison. Foam refers to 4repCt without motifs in foam form, while film refers to 4repCt in film form. Note that standard deviations for all functionalized motifs overlap and their internal order can therefore not be determined with any scientific accuracy. What can be determined is the order (in decreasing proliferation): functionalized scaffolds, hydrophilic wells, (unmodified) 4repCt foam, (unmodified) 4repCt films and finally hydrophobic plates.

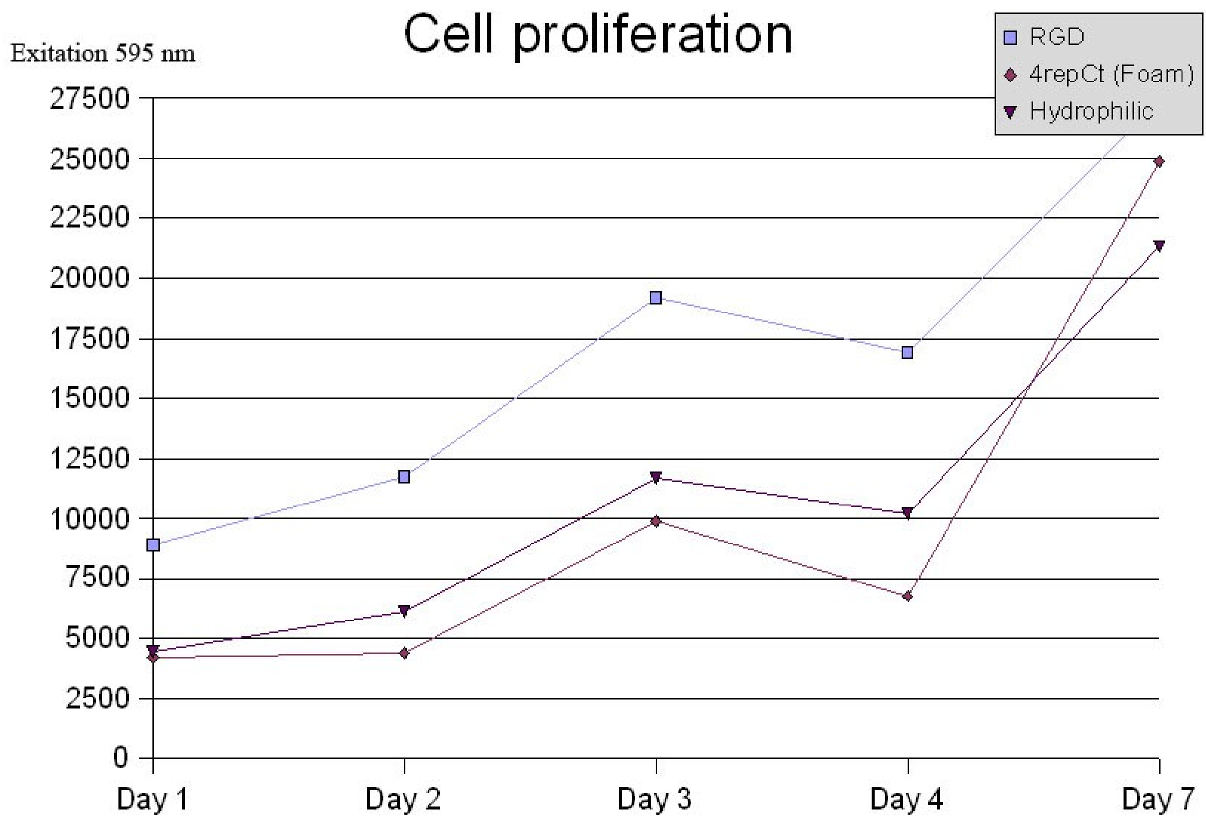

Fig 5: Motifs and motif combinations are represented by RGD in this diagram. Standarddeviations are excluded from this diagram to avoid cluttering, but compare with fig4. Other than day 7, the standard deviations in this case are insignificant.

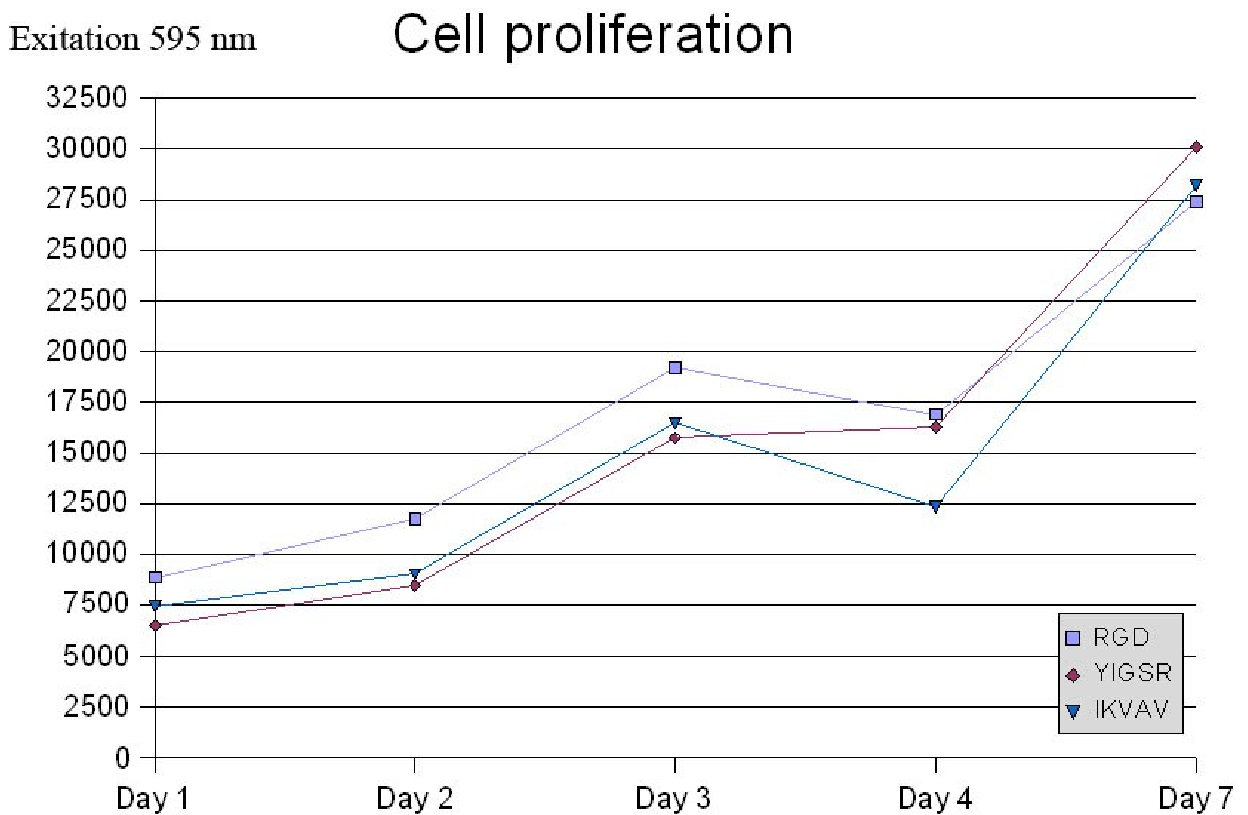

Fig 6: The motifs. Other than day 4 and 7, this order is valid; RGD, IKVAV and YIGSR. However, compare with fig4, these values are not statistically certain.

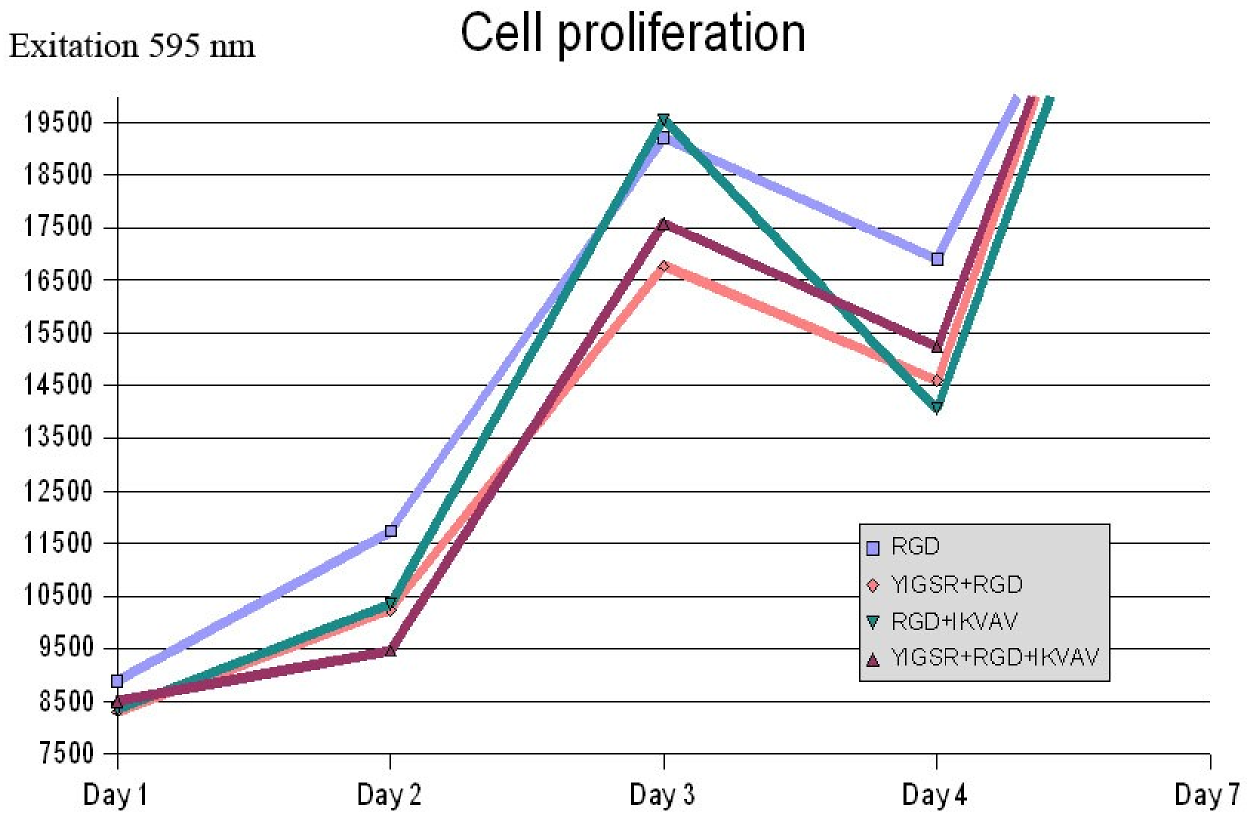

Fig 7: RGD and RGD combinations. There is no appearent difference between the different laminin motif combinations.

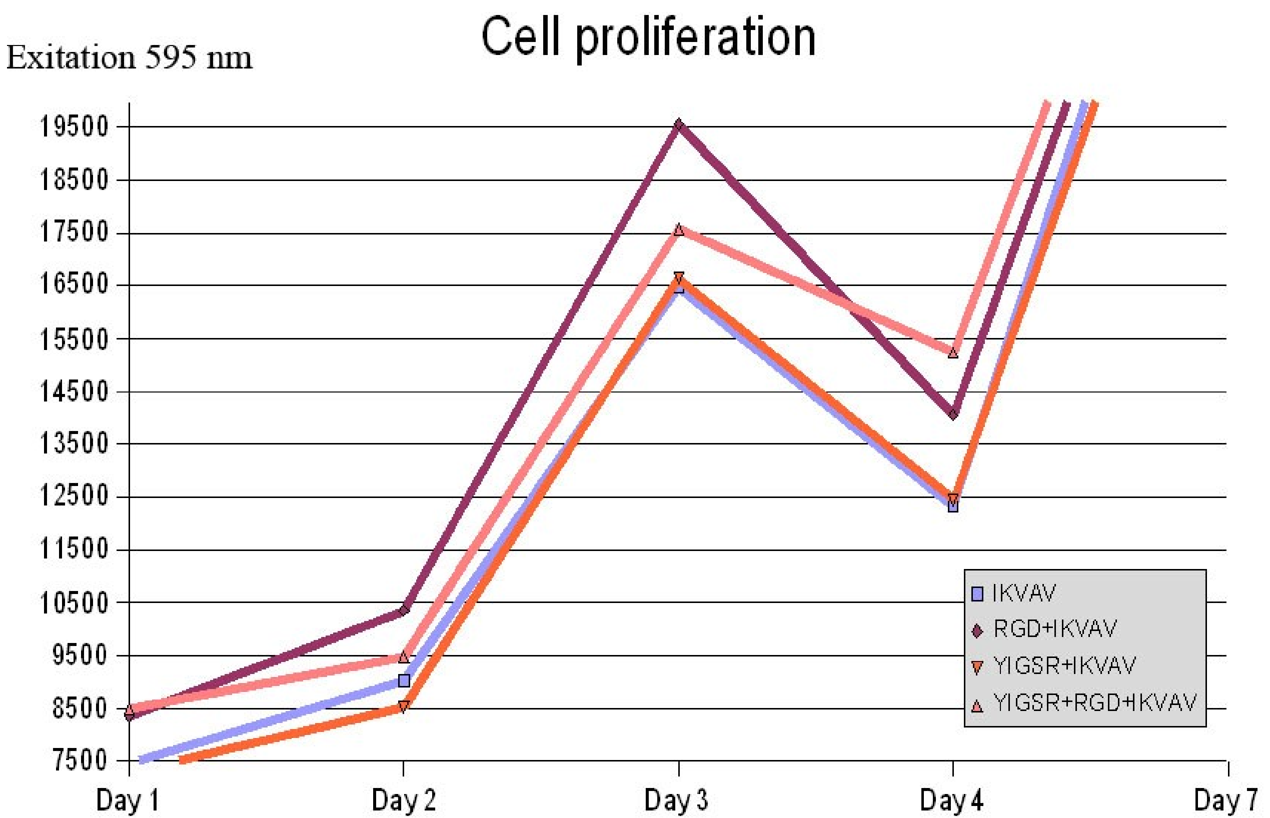

Fig 8: IKVAV and IKVAV combinations. There is no appearent difference between the different laminin motif combinations.

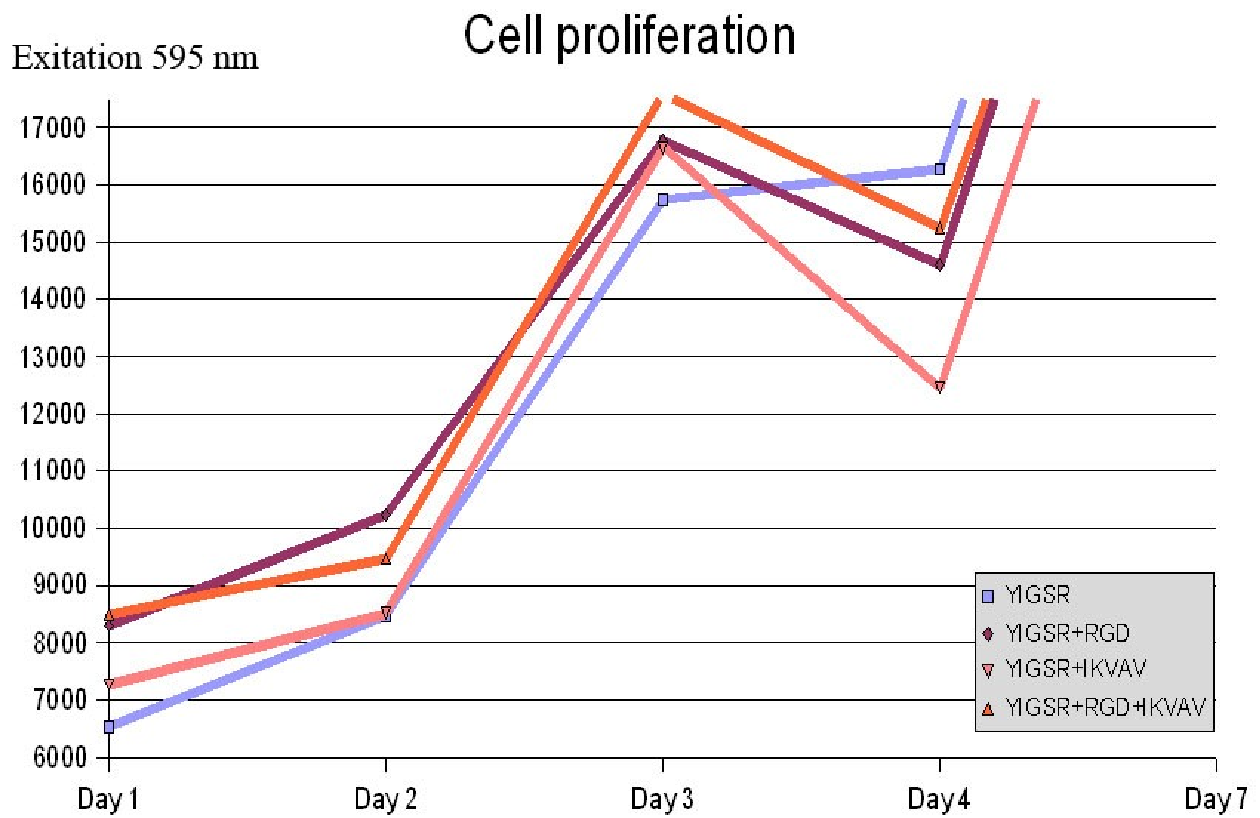

Fig 9: YIGSR and YIGSR combinations. There is no appearent difference between the different laminin motif combinations.

Live / Dead cell staining

When the first pictures were taken, very few cells were dead, a rough estimation would be 1/50. But the cells started to die after two hours of staining, and very few pictures could actually be taken. Because of this limit in photos, any and all results seen could be coincidental. Cells appeared to be rounder (more sickly) and a larger proportion were dead when they were grown on the negative control (glass slide without threads) or on glass slides with threads, but where threads were not visible. Where threads were visible, the cells appeared healthier. Also notably, on areas where threads were visible, it appeared as if there are no cells at all in nearby area lacking threads, and the cells that are present are dead. This suggests living cells move to locate themselves in the presence of the threads. Cells align themselves after the threads, and these cells seem to serve as some kind of anchoring point for neighboring cells; these cells extend and attach one dendrite to a cell bound to a thread, and then the other dendrite is extended towards cell cluster. It is as if the cells in between cell clusters growing on threads form bridges linking the cell clusters (see fig 12).

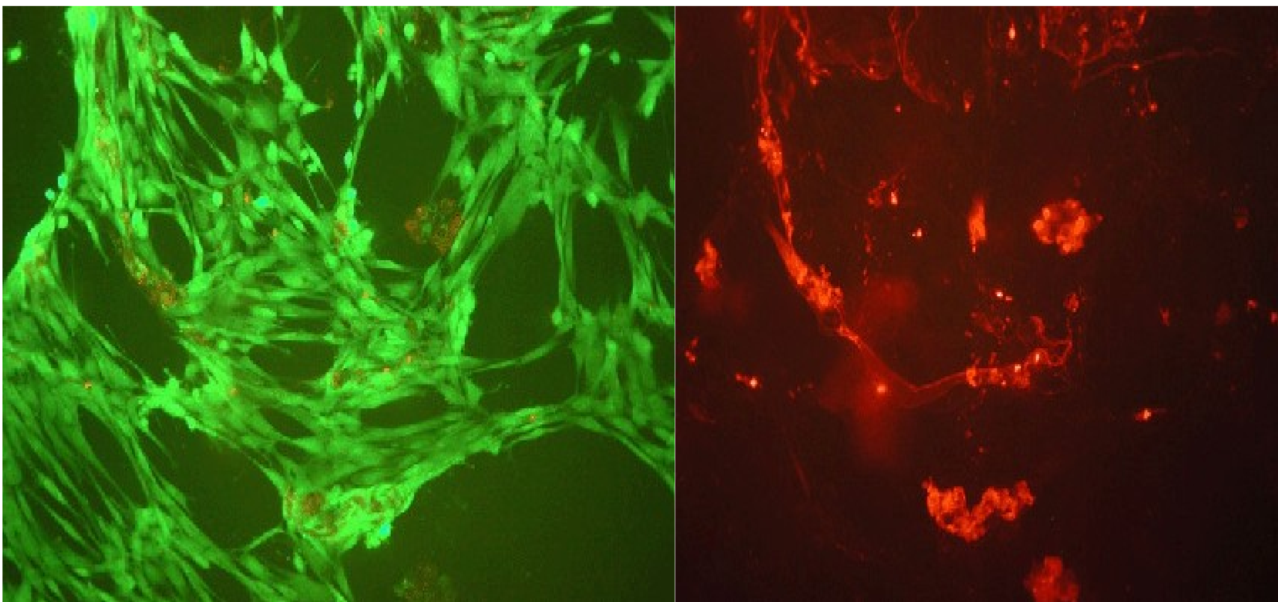

Fig 10: Cells grown on RGD foam. Left: Live. Right: Dead. These pictures are taken using different filters, but without moving the camera, thus depicts the very same area. Dead staining also stains the scaffolds and the longer structural formations seen are indeed spider silk threads.

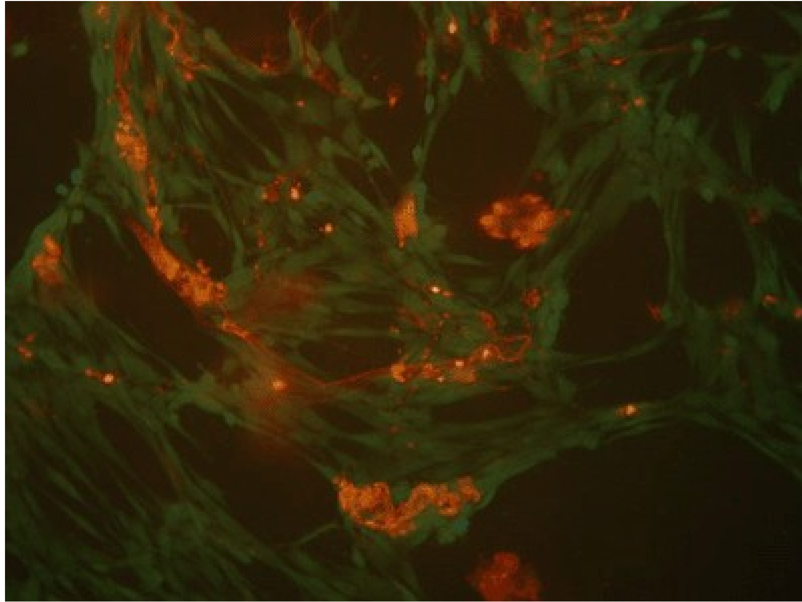

Fig 11: A merge of the pictures in fig 10. Note the strong tendency of the cells to align along the fibers.

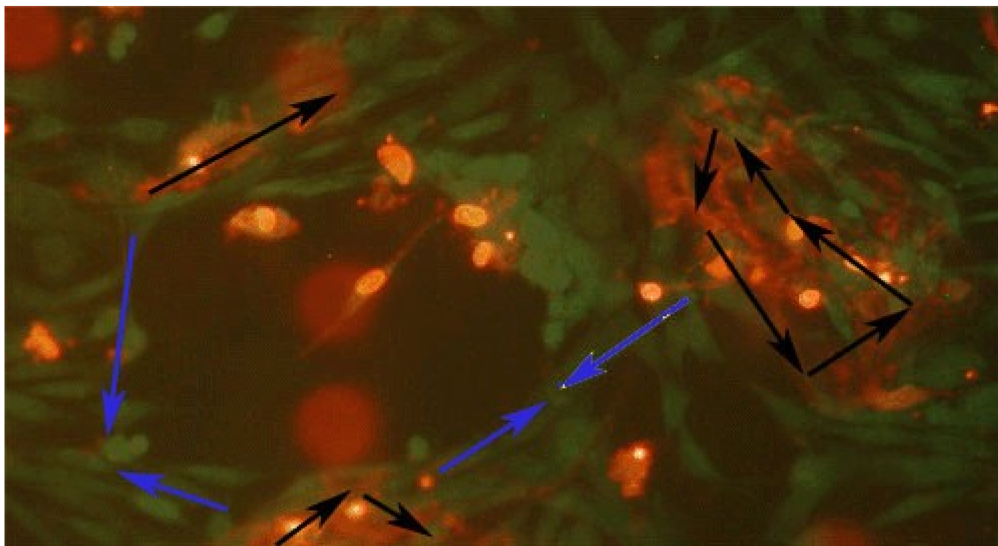

Fig 12: The black arrows indicate the direction of the threads. Note that cells on top of the threads follow the direction of these black arrows. The blue arrows indicate the direction of cells binding cells on top of threads; it appears as if the cells form bridges to the next spider silk thread. Note the absence of cells in the center; cells are always part of the cluster on top of the thread, or a bridge leading to the next cluster, with the exception of the dead cell in the middle.

Discussion

This project aimed at investigating whether addition of laminin motifs onto recombinant spider silk would affect cell proliferation and survival, and to investigate whether addition of a cysteine could make the threads stronger. The results show that the motifs do indeed increase proliferation, but the results from the addition of cysteine are inconclusive.

Motif insertion

The addition of the motif proved very difficult because the PCR-amplification repeatedly yielded an unwanted 400 bp band. Through sequence analysis, it was revealed that the primer had a secondary binding site. The 400 bp bands was by its presence not a significant problem since it could easily be removed through gel extraction kits. The problem was that the 400 bp band decreased the yield of the 800 bp band to the point where it became very difficult to work with it in subsequent steps. Since dragline spider silk proteins are highly repetitive, perhaps the secondary binding site should not come as a surprise. Problems like this probably become more common with more repeats included. While a longer protein chain may be stronger, it is likely even more difficult to work with. Several methods of circumventing this secondary binding site were attempted, mainly the annealing temperature was varied and different concentrations of the ingredients were varied. After much work, it became evident that using Taq DNA Polymerase, the 400 bp band could not be avoided. Two constructs were successful, RGD4repCt and C4repCt, but this was more due to luck and perseverance than anything else. Other polymerases were eventually tried, and Taq DNA Polymerase was abandoned in favor of Advantage GC-melt. Advantage GC-melt eliminated the problem entirely. Advantage GC-melt is designed to more readily open up long GC repeats. The secondary binding site have two mismatches, the primer would likely not prefer this site if the intended binding site were not inaccessible. Despite the supposed proofreading enzyme advantage of Advantage, constructs derived from Advantage GC-melts were much more prone to mutations than Tag DNA polymerase. Another problem was that for whatever reason, the PCR-products resisted cleavage and ligation attempts directly into the wanted vector. For this reason, A/T kits were used as it is easier to cleave out segments from a vector and a higher concentration can be acheived. With both Advantage GC-melt and A/T kits, several new constructs were created in less than a week, something that took 19 hard weeks of trial and error using Taq DNA Polymerase. At least those 19 weeks were not wasted, they served to formulate a way of quickly and efficiently adding new motifs to 4repCt.

The PCR-experiences have led to a protocol which should work for all future attempts at inserting a short sequence to the N-terminus of 4repCt, it is avaible in appendix III. If everything functions as intended, this protocol produce new construct in exactly 1 working week with minimal work load, using the methods with the highest success rate and includes tests to see if each previous step was successful

Thread construction

Despite mutations, all constructs gave rise to proteins that were able to produce threads. The structure of spider silk seem to be quite resistant to change and not so vulnerable. It is the belief of the author that all constructs used, despite mutations, had almost identical structures, and the mutations can largely be ignored. There were some mutationfree sequences constructed, and given more time, especially since a better protocol have now been developed, certainly all constructs can be made mutationfree. It is difficult to say whether this is an exceptionally high mutation rate or if bad luck played a part, but if there is a higher than normal incidence of mutations, this could perhaps be due to the highly repetitive sequence.

Alamar Blue

The cell proliferation experiments proved that all scaffolds are capable of serving as cell growth

scaffolds. It also proved that spider silk scaffolds with laminin motifs are better for growing dermal fibroblasts than hydrophilic plastic designed for cell cultures. The standard deviations are such that it is not possible to conclude which motif or motif combination is the more preferable, and there were certainly no synergestic effects seen. However, the trends show that it is at least feasible that RGD is the laminin-motif that provides the highest proliferation, followed by IKVAV and lastly YIGSR, which is concurrent with articles cited in the introduction [5, 6]. While this is by no means proven, if there were no difference at all in benefit, a more obvious spread of the values could be expected. But future experiments should yield more statistical data before and any conclusion either way should be drawn. It is also the opinion of the author that a method to reduce noise should be used for any such future experiments. One such method could be to create twice the number of statistically needed wells covered in foam, and then remove half of them from the final statistics, representing wells where coverage was incomplete. It is also the author's opinion that hydrophobic wells should be used also in the future; if the wells are hydrophilic and have incomplete coverage, their fluorescence values will deviate less from the fully covered wells, and be much more difficult to spot and remove from the statistics. The 4repCt foams have almost double the Excitation/Emission value compared to the film. From this alone, it can be concluded that structure is also of great importance.
Despite cited articles mentioning synergetic effects when combining two of the three laminin motifs, no such effects could be seen in the statistics. Instead it appears the values are dependant diretcly one the proportions of the different motofs. For example, on day 3, RGD had an average of 12444,5, and IKVAV had an average of 9736,33. RGD + IKVAV had an average of 11056,33, close to 11090,415 ((12444,5+9736,33)/2). If there is any synergistic effect in this case, this experiment failed to see it.
On day 7 the values even out. This is likely one of two reasons, there is no more room for the cells to expand, and regardless of their initial growth rate they will all reach maximun growth at the same time, or the cells have grown to such a number that the amount of alamar blue used will be consumed almost to its entirety during the incubation time, and since you can have no more convertation than around 100%, it is not possible to measure cell proliferation beyond a certain point. If it is case one day 7 should not be taken into consideration if the experiment is repeated. If it is the second case, perhaps the concentration or volume of alamar blue added can be altered.

Live / Dead Staining

The main problem with the Live / Dead cell results is that the cells started to die during staining. It would be better to take one glass cover slide at a time rather than all at the same time. The Live / Dead pictures show that cells cluster on and around threads, and then form bridges to the next cluster. It is the authors opinion that this trend have not been seen in earlier 4repCt Live / Dead staining pictures (unpublished studies), suggesting that this could be a phenomena particular to addition of motifs. Unfortunately, since the cells did start to die and there are so few pictures of 4repCt (without motifs) this can not be claimed with statistical certainty. It is telling that the glass cover slide pictures, and areas in the glass slides covered with scaffolds without visible threads looks rounder (more “sickly”), but again, the statistical validity of this claim is low. All in all, the pictures do show interesting trends, that should be more properly investigated in future Live / Dead cell staining experiments. The photos taken have not been analyzed by an indenpendant observer and blind tests, obviously this needs to happen to assure proper science, however, it is also unnecessary to bring in second opionons while there is still so little material to study.

Future experiments

Other than more statistics as already suggested for both Live / Dead and cell proliferation experiments, neurons could be used and cell adhesion strength could be measured. Any further attempts of inserting a motif or other short peptide sequence to the beginning of 4repCt should use

Advantage GC-melt and A/T-kits. The author considers the protocol that emerged after 19 hard weeks of mostly failed cloning experiments to be a major and important result. In the future it could also be interesting to measure cell adhesion strength. This can be done by applying a water flow and measure at which pressure cells fall of.

In conclusion, it must be said that despite all difficulties, recombinant spider silk is a promising candidate biomaterial for future use in guiding in vivo growth of cells.

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Appendix I: The 4repCt sequence

Please note that for comparison reasons, peptide1 is considered to be the first peptide of the original 4repCt sequence, since the inserted motifs differ in length, and the sequence before 4repCt is varied depending on the protein binding site, either A or B.

```
         A            EcoR1         Insert        4repCt
// L  V  P   R  G  S   G  N  S  [Insert]  A  G  A   A  S  G   6
//ctggtgcca cgcggttct gggaattca [Insert] gcaggagcg gctagcggt

 Q  G  G   Y  G  G   L  G  Q   G  G  Y   G  Q  G   A  G  S   24
caaggagga tatggtgga ctaggtcaa ggaggatat ggacaaggt gcaggaagt

 S  A  A   A  A  A   A  A  A   A  A  A   A  G  G   Q  G  G   42
tctgcagcc gctgccgcc gccgcagca gccgccgca gcaggtgga caaggtgga

 Q  G  Q   G  G  Y   G  Q  G   S  G  G   S  A  A   A  A  A   60
caaggtcaa ggaggatat ggacaaggt tcaggaggt tctgcagcc gccgccgcc

 A  A  A   A  A  A   A  A  A   A  G  R   G  Q  G   G  Y  C   78
gccgcagca gcagcagca gctgcagca gctggacga ggtcaagga ggatatggt

 Q  G  S   G  G  N   A  A  A   A  A  A   A  A  A   A  A  A   96
caaggttct ggaggtaat gctgctgcc gcagccgct gccgccgcc gccgccgct

 A  A  A   G  Q  G   G  Q  G   G  Y  G   R  Q  S   Q  G  A   114
gcagcagcc ggacaggga ggtcaaggt ggatatggt agacaaagc caaggtgct

 G  S  A   A  A  A   A  A  A   A  A  A   A  A  A   A  G  S   132
ggttccgct gctgctgct gctgctgct gctgccgct gctgctgct gcaggatct

 G  Q  G   G  Y  G   G  Q  G   Q  G  G   Y  G  Q   S  S  A   150
ggacaaggt ggatacggt ggacaaggt caaggaggt tatggtcag agtagtgct

 S  A  S   A  A  A   S  A  A   S  T  V   A  N  S   V  S  R   168
tctgcttca gctgctgcg tcagctgct agtactgta gctaattcg gtgagtcgc

 L  S  S   P  S  A   V  S  R   V  S  S   A  V  S   S  L  V   186
ctctcatcg ccttccgca gtatctcga gtttcttca gcagtttct agcttggtt

 S  N  G   Q  V  N   M  A  A   L  P  N   I  I  S   N  I  S   204
tcaaatggt caagtgaat atggcagcg ttacctaat atcatttcc aacatttct

 S  S  V   S  A  S   A  P  G   A  S  G   C  E  V   I  V  Q   222
tcttctgtc agtgcatct gctcctggt gcttctgga tgtgaggtc atagtgcaa

 A  L  L   E  V  I   T  A  L   V  Q  I   V  S  S   S  S  V   240
gctctactc gaagtcatc actgctctt gttcaaatc gttagttct tctagtgtt

 G  Y  I   N  P  S   A  V  N   Q  I  T   N  V  V   A  N  A   258
ggatatatt aatccatct gctgtgaac caaattact aatgttgtt gctaatgcc

 M  A  Q   V  M  G   S T O P  HindIII                       269
atggctcaa gtaatgggc taatgataa gcttct
```

Appendix II: Primer sequences and secondary binding site

Motifs with native sequence

C GAA TTC ATG CGG TCA AGG TGG ATA TGG TGG A
RGD GAA TTC ACG CGG CGA TGG TCA AGG TGG ATA TGG TGG A
RGD (long)GAA TTC AAC AGG ACG AGG AGA CTC ACC AGC AGG TCA AGG TGG ATA TGG TGG A
IKVAV GAA TTC ATG CGG TCA AGG TGG ATA TGG TGG A
IKVAV(long) GAA TTC ACA GGC CGC CAG CAT TAA AGT GGC CGT GAG CGG TCA AGG TGG ATA TGG TGG A
YIGSR GAA TTC ATA TAT TGG CAG CCG CGG TCA AGG TGG ATA TGG TGG A
YIGSR(long) GAA TTC AGA TCC GGG CTA TAT TGG CAG CCG CTG CGA TGG TCA AGG TGG ATA TGG TGG A
Antisense AAG CTT ATC ATT AGC CCA TTA CTT GAG CCA T

More primers were ordered later to acheive higher yield or correct designflaws:
RGD CCG GAA TTC ACG CGG CGA TGG TCA AGG TGG ATA TGG TGG ACT A
C CCG GAA TTC ATG CGG TCA AGG TGG ATA TGG TGG ACT A
Above two primers have slightly longer native sequence
RGD CCG GAA TTC ACG CGG CGA TGC AGG AGC GGC TAG CGG TCA AGG A
IKVAV CCG GAA TTC AAT TAA AGT GGC CGT GGC AGG AGC GGC TAG CGG TCA AGG A
Above two primers used a vector with Nt4repCt, although this is not relevant other than as the N-terminal will not be amplified.

Antisense CCC AAG CTT ATC ATT AGC CCA TTA CTT GAG CCA TGG C

Location of the 400 bp band secondary binding site
Note that there are only two mismatches

```
124                                                         143
 A  A  A   A  A  A   A  G  S  G  Q  G   G  Y  G   G  Q  G   Q  G
gctgccgct gctgctgct gcaggatct ggacaaggt ggatacggt ggacaaggt caagg
                              IIxIIIIII IIIIIxIII III
          gaa ttc a (insert) ggtcaaggt ggatatggt gga
```

Appendix III; Protocols

PCR-protocol using Taq DNA Polymerase
The primers were run in PCR together with a template containing 4repCt.
This program was used both for insertion of motifs and screening.
PCR-program used with Taq DNA Polymerase:

Temperature	Time	
94°C	10 min	
90°C	30 seconds	
66°C	30 seconds	25 repeats of step two to 4
72°C	3 min	
72°C	5 min	
4°C	Infinite	

Alteration of this PCR-program were used occasionally.
Step one of 94°C was included only for screening samples taken directly from monoclonal colonies.

Ligation protocol
2 µl vector
5 µl PCR-product *
2,5 µl ligation buffer
15 µl water *
0,5 µl ligase
*PCR-product was varied depending on estimation from band strength in gel or an OD reading of concentration. In these cases, the amount of water was varied to compensate.

Protocol for sequencing
10 µl PCR-products with big dye kit and PCR-program below was used to sequence the vectors with inserted 4repCt with motif. Combining three different primers, named FHisA, RNT4repCt and WiMa32as, the entire sequence of 4repCt can be covered. Sequence of these primers as well as location are not considered relevant and are not provided in this article. The PCR-sequence-products are then dissolved in HiDi and run in a sequencer.
PCR-recipe
3 µl water
2 µl vector
2 µl Big Dye polymerase (includes dNTP)
2 µl Big Dye buffer
1 µl primer

PCR program
15 sec 90°C
15 sec 50°C Repeat 25 step one to three
4 min 60°C
7 min 72°C
Forever 4°C

Protocoll for solving PCR-sequencing products in HiDi
Add 10 µl water
Move to E-tube
Add 50 µl EtOH 95% and 2 µl NaAc
Vortex shortly and leave for at least 15 minutes
Centrifuge at max speed 20 min

Discard supernatant
Add 250 µl EtOH 70%
Vortex shortly
Centrifuge for 5 min at max speed
Remove supernatant and place E-tube in 90°C heat block for 1 min to ensure all ethanol is removed
Dissolve in 16 µl HiDi and transfer to septa-tube with rubber lock
The sequencing product should be kept at 4°C until it is run in the sequencer

Concludary protocol with high success rate for inserting motifs at the start of 4repCt
1. PCR with Advantage GC-melt
2. SDS-gel to confirm presence of 800 bp band
3. Extraction from gel, and immediate ligation with A/T cloning kits
4. Steps 2 and 3 should be done as quickly as possible, as the overhang degrades over time.
5. Ligation should be allowed 2 h, or over night, in room temperature.
6. Transformation of E. Coli and subsequent plating. Both BL21 or Novablue may be used.
7. Cells should be incubated in 37°C over night.
8. Screening with in-sequence primers to confirm presence of insert.
9. Promising clones are incubated in 37°C during shaking at 220 rpm in 4 ml medium over night.
10. 1 ml medium is transferred to an Eppendorf tube (label the tubes)
11. 20 minutes CF at max speed for the tubes with the remaining 3 ml
12. During the 20 minutes wait, add 0,2 ml glycerol to the eppendorf tubes and rush them to -80C freezer
13. Extract the vectors using a miniprep kit
14. Use a reverse in-sequence primer that will cover the N-terminus of 4repCt and run in sequencer to confirm successful motif insertion
15. Cleave A/T-vector, all of it, and the vector 4repCt should be inserted into with HindIII and EcoR1 for 4 H.
16. Run the cleavage products on gel, extract 4repCt from A/T vectors and the cleaved vector you wish to insert 4repCt into.
17. Ligate them two hours or overnight.
18. Transform into BL21.
18. b: If there are no colonies present. Repeat step 9 but make 100 ml instead, using the 1 ml E-tube with glycerol in the -80 freezer, 11 (100 ml instead of 3), 13 (but use a midiprep kit instead), 15, 16, 17 and 18.
19. Repeat step 8, 9, 10, 12 and 13.
20. Sequence using primers covering the entire length of the insert and evaluate the sequence.
21. If the sequence is adequate, expression can now be started.

Under ideal conditions, one or several new construct can be ready to be expressed in just one week;
Monday; Step 1-7
Tuesday; Step 8-9,
Wednesday; Step 10-18,
Thursday; Evaluate sequence from step 14, eventually throw away the cell culture plates from step 18. Do step 19, but repeat only 8 & 9.
Friday; continue with step 19 and then do step 20.
Monday; Step 21.