FINAL REPORT FOR ÅFORSK FOUNDATION PROJECT 17-500
Quality control of next generation biological based medicines

Projekttid: 2017-09-01 – 2019-05-01
Rapporttyp: Slutrapport

Working package 1 NUMERICS *more reliable processing & understanding heterogeneous slow biomolecule kinetics*

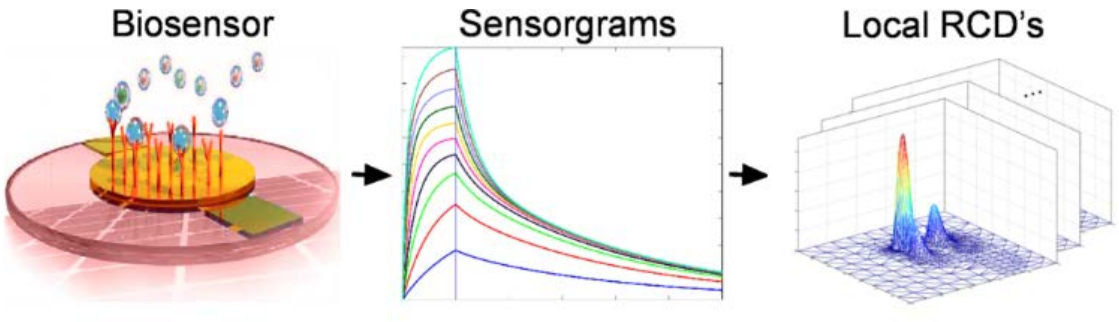

Figure 1

Working package 2 EXPERIMENTS and IMPLEMENTATIONS *improved understanding ultra-high pressure separation processes for enhanced quality control of biomolecules*

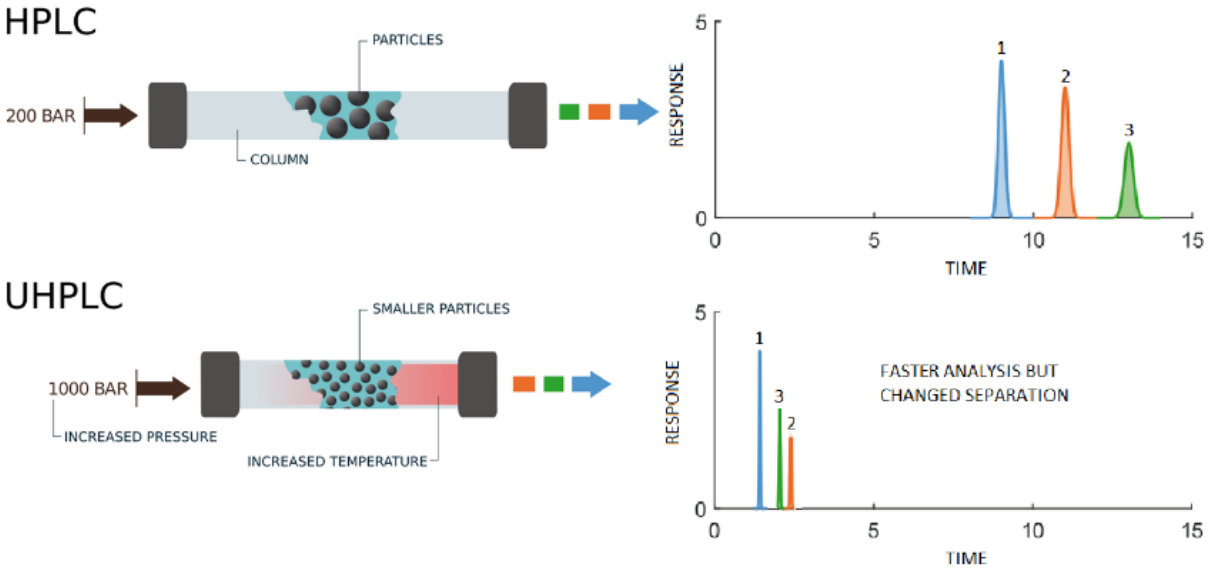

Figure 2

English abstract

We are most grateful for the ÅForsk grant 17/500 from ÅForsk Foundation, the equal amount of co-financing from the units developing next generation medicines at new drug modalities at AstraZeneca Gothenburg and also for the support from the Knowledge foundation (www.KKS.se) that all together made this project possible.

We are happy to report a most successful and specific AForsk 17/500 final report were we achieved the goal to build up the necessary knowledge for to successfully launch the Enhanced-Quality Control (E-QC) process of next generation drug as applied this year 28 February. As can be seen in the publication list at bottom, thanks to this grant six scientific articles have been published in highly ranked scientific journals and five lectures + seven posters have been presented at well renowned international scientific conferences. As can be seen in these publications the grant ÅForsk 17/500 was specifically mentioned and thanked for with also the title of the project, in the acknowledgement sections. Furthermore, as also can be seen in the publication list below, the ÅForsk 17/500 project – together with the corresponding KKS project - was especially mentioned by CEO Teodor Aastrup as an excellent example of successful academic – industrial cooperation at an IVA (Royal Academy of Engineering Sciences) breakfast meeting 8 November 2019.

The project reported here was based on the results from a previous ÅF-project (ÅForsk 15/497). In the previous project the goal was to introduce the Enhanced Quality Control Process (E-QC) concept for traditional drugs based on small molecules (< 1000 Da), such as Losec® and Nexium® and Viagra®. This previous project was based on the idea that if the company can prove they have achieved a deeper scientific knowledge about the underlying mechanism in the quality control process it should simplify considerably the necessary continual improvement of regulatory approved QC methods without the today necessary tedious filing of papers to regulatory authorities. We were extremely successful with this ÅF-project which resulted among others in a pioneer paper [1] and in the first prize for best thesis 2017 and AstraZeneca has now implemented the concept Enhanced Quality Control Process (Enhanced -QC) for recently approved drug application for the anti-depressant Mianserin®.

However, the next generation of drugs are expected to be based more on up to hundreds or thousands rime larger biomolecules, ranging from short nucleotides and peptides to very large mRNA and proteins (up to one million Da). To achieve a similar level of scientific knowledge for these interactions as for the small molecules is very challenging as biological drugs are extremely more complex than traditional small medicine molecules, with several more possibilities for interactions and contaminations, risk of formation of aggregates between large biomolecules, slow disassociation kinetics, more complex heterogeneity in the thermodynamics/kinetics of the interactions. Ultimately this results in that equilibria are not established in the systems such as the case for the small molecules why now numerical methods must be developed accounting for that steady state is not reached.

Therefore, the aim of the application reporter here (17-500) was to building up the scientific knowledge for being able to expand the previous successful Enhanced-QC process concept to biomolecules, in next grant we hope to get approved and which was applied for this year 2019.

This project that aimed to build up the necessary fundamental knowledge using model molecules, such as peptides and nucleotides. The project was divided into two work packages, WP1 and WP2, respectively, according to as follows:

WP1. Here we intended to develop models and algorithms improving the inverse solver algorithms to account for much more complicated interactions such as slow and heterogeneous kinetics at multiple binding sites between the complex biomolecules and surfaces of chromatographic phases or chips surfaces of biosensors (as illustrated in Figure 1 above) – used for quality control processes. We also intended to implement an improved

“inverse solver” algorithm that can be used to account for slow kinetics when we do not have establish equilibria, i.e. we have not reached steady state. We also intended to implement simulation algorithms based on finite volume methods for the inverse solving and evaluation of putative experimental separation systems *in silico* for finding the optimal experimental quality control processes.

WP2. Here, we intended to systematically investigate separation of peptides under analytical, linear conditions to act as a starting point for development of models and computer algorithms. We also wanted to build up knowledge about separation of a similar class of large biomolecules, oligonucleotides, that is most probable in the pipeline as next drug applications. For oligonucleotides we need also the same knowledge as for the new type of peptides and they have multivalent negative charge biomolecules. Moreover, we found that small classical size medicine molecules selective adsorption is dependent on the pressure which generates temperature and adsorption gradients affecting the separation selectivity. This can take place as illustrated in Figure 2 above when we transfer from the classical high-pressure liquid chromatography (HPLC) method to the most modern ultra-high-pressure liquid chromatography (UHPLC) method, see ref [1] as a result of the previous ÅForsk 15/497 resulting in 1st prize for best doctoral thesis. Today we know these effects will to be much larger for large molecules with multiple charges and must therefore found a technique to visualize the temperature effects whish we did in WP2 (see below).

We can report an excellent result, both quantitatively and qualitatively. From publication list of achievements for ÅForsk 17/500 (see pages 15-17 at bottom) six scientific articles have been published (**A1-A6** below) in highly ranked scientific journals and five lectures + seven posters (**L1-L5** + **P1-P7** below) have been presented at well renowned international scientific conferences. In addition, we have been invited to a highly respected international conference, in the summer of 2019, to give a keynote lecture to present our research in this area (L6 below) Also, worth mentioning is that our research in this area has received attention in other contexts, e.g. it was mentioned as a good example of successful academic – industrial cooperation at an IVA (Royal Academy of Engineering Sciences) breakfast meeting (see **S1-S2** below).

Svensk sammanfattning

i är mycket tacksamma för ÅForsk-bidraget 17/500 från ÅForsk-fonden, samt för den lika stora medfinansiering från enheterna Modalities vid AstraZeneca Göteborg som utvecklar nästa generations läkemedel samt för stöder från kunskapsstiftelsen som alla tillsammans det här projektet såväl möjligt som mycket lyckat.

I ett föregående ÅForsk projektet (15/497) var målet att introducera ett helt nytt flexibelt kvalitetskontroll (E-QC) för traditionella läkemedel som bygger på små molekyler (<1000 Da) som de farmakologiskt aktiva, såsom Losec® och Nexium® och Viagra®. Detta tidigare projekt grundades på tanken att om företaget kan bevisa att de har uppnått en djupare vetenskaplig kunskap om den bakomliggande mekanismen i kvalitetsstyrningsprocessen, bör vi avsevärt kunna förenkla den nödvändiga kontinuerliga förbättringen av godkända certifierade QC-metoder utan den idag tidsödande pappersexercisen och påföljande produktionstopp på ibland flera månader pga. kommunikationen med kontrollmyndigheter i flera olika länder måsta skeppas klart innan produktionen kan återstarta. Vi var mycket framgångsrika med detta ÅForsk-projekt som bland annat resulterade i ett pionjärpapper [1] och i första priset för årets bästa avhandling inom området 2017 till vår doktorand Dennis Åsberg som delvis finansierades ur ÅForsk projektet. AstraZeneca har nu implementerat konceptet Enhanced Quality Control Process (Enhanced -QC) för det nyligen godkänd läkemedelsapplikationen för anti-depressiva Mianserin®

Nästa generations läkemedel förväntas dock till mycket större utsräckning baseras på mycket större biomolekyler, från ett 10-tal större till tusentals större biomolekyler, allt från korta nukleotider och peptider till mycket stora mRNA och proteiner (upp till en miljon Da). Vi syftar nu att bygga upp kunskapen för att uppnå en liknande nivå av vetenskaplig kunskap av kvalitetskontrollmetoderna för dessa stora biologiska molekyler som är extremt komplexare än traditionella små medicinska molekyler, med flera fler möjligheter till interaktioner och kontaminering, långsam disassociationskinetik och med mer komplex heterogenitet i termodynamiken / kinetiken av interaktionerna. I slutändan leder detta till att jämvikt inte uppnås i metoderna system, såsom fallet för de små molekylerna, varför nya algoritmer och expanderade numeriska metoder utvecklas.

Syftet med ansökan (17-500) var därför att bygga upp den vetenskapliga kunskapen för att kunna expandera det tidigare framgångsrika Enhanced-QC processkonceptet till biomolekyler, vars implementering vi planerar i ett tredje ÅForsk som söktes för 28 Februari i år.

ÅForsk 17/500 projekt som syftade till att bygga upp den nödvändiga grundläggande kunskapen för stora biologiska modellmolekyler av peptider och nukleotider, utfördes som två arbetspaket, WP1 respektive WP2 enligt följande:

WP1. Här utvecklade vi modeller och algoritmer för att förbättra lösningen av det s.k. inversa problemet som tar hänsyn till de mer komplicerade interaktioner, såsom långsam och heterogen kinetik pga. komplicerad kinetik på multipla bindningsställen mellan komplexa biomolekyler och ytor. Dessa ytor kan vara sådana som utgörs av kromatografiska faser (se Figur 2 ovan) eller sådana som utgörs av chipsytor hos biosensorer (se Figur 1 ovan) både plattformarna används vis kvalitetskontroll processer. En sådan "invers lösare" -algoritm ska också kunna användas för experimentella situationer då pga. den långsamma kinetik ej uppnår jämvikt, så som illustrerat av sensorgrammen i Figur 1 ovan. Vi planerade också att implementera simuleringsalgoritmer baserade på finita volymer metoden för invers lösning av kromatografiexperiment för att utvärdera de optimala instrumentinställningarna genom att

simulera tusentals syntetiska experiment. Allt detta har gjorts vilken kan läsas i engelska redogörelsen nedan för WP1.

WP2. Här avsåg vi att systematiskt undersöka separering av peptider under analytiska, linjära förhållanden för att fungera som utgångspunkt för utveckling av modeller och datoralgoritmer. Vi ville också bygga upp kunskap om separation av en liknande klass av stora biomolekyler, terapeutiska oligonukleotider, som är mest sannolika som nästa läkemedelsapplikation inom satsningen stora biomolekyler vid AstraZeneca enheten för de framtida satsningarna *Modalities*. För oligonukleotiderna behöver vi också samma kunskap som för den nya typen av peptider och de har multivalenta negativa laddningar att ta hänsyn till i interaktionsmodeller. Dessutom måste vi ta fram en teknisk att synliggöra och kvantitativt bestämma den starka effekten på selektiviteten av kvalitetskontrollmetoden på grand av de temperaturgradienter som alstras av de höga trycken så som illustrerat i Figur 2 ovan när vi övergår från den klassiska högtrycksvätskekromatografi (HPLC) -metoden till den mest moderna ultrahögtrycksmätningskromatografimetoden (UHPLC). Dessa effekter kommer att ha en enormt större effekt för stora molekyler med flera laddningar än för små vi och måste därför hitta ta fram en teknik för att visualisera de temperatureffekter. Allt detta har gjorts vilken kan läsas i engelska redogörelsen nedan för WP2. .

Vi är glada att rapportera en mycket framgångsrik slutrapport från AForsk 17/500 där vi uppnådde målet att bygga upp den nödvändiga kunskapen för att framgångsrikt kunna känna oss mogna att söka för sista applikationsfasen hos ÅForsk för förstärkt kvalitetskontroll (E-QC) för nästa generations läkemedel, vilket vi gjorde i år (28 februari). Som framgår ur den digra publikationslistan längst ner (sid 15-17) så har vi tack vare ÅForsk 17/500 projektbidraget lyckats publicera sex vetenskapliga artiklar [**A1-A6**, sid 15] i högt rankade vetenskapliga tidskrifter och fem föreläsningar + sju väggtidningar [**L1-L5** + **P1-P7** sid 15-17] vid högt rankade internationella vetenskapliga konferenser. I alla dessa publikationer nämndes ÅForsk 17/500 specifikt och tackade för. Därutöver, har ÅForsk 17/500-projektet, tillsammans med motsvarande KKS-projekt, också nämnts av VD Teodor Aastrup som ett ovanligt lyckat exempel på framgångsrikt akademiskt industriellt samarbete vid en IVA (Royal Academy Engineering Sciences) frukostmötet den 8 november 2018 (se **S1-S2**, sid 17].

Scientific report – Background and state of the art

The overreaching goal with this ÅForsk application is to introduce scientific-based quality control validation protocols for also the next generation larger biopharmaceuticals, thus increasing the flexibility for the pharmaceutical industry to perform post-approval changes to manage continuous improvements of the analytical procedure in a similar way as shown so successfully in a previous grant. The idea that we by demonstrating a good scientific understanding of the analytical procedure in the application to regulators, i.e. by showing how input parameters affect the method performance characteristics, an increased flexibility for post-approval changes could be gained.

The background is that most analytical method are based on empirical knowhow and filed with regulatory agencies as locked methods [1-4] where continuous improvements are not allowed. This results in a situation where products change over time but with a locked analytical method. An analytic method that was optimized and validated for specific conditions long time ago and that might fail for new products. To handle this problem FDA (Food and Drug Agency) proposed that new analytical methods could be validated and developed within the "Quality by Design" (QbD) framework. QbD is the state of the art in designing modern analytical methods in pharmaceutical environments and is a way of designing analytical methods through scientific understanding rather than empirical knowledge [1, 5]. As a consequence of a more global industry, the differences in how products were regulated in different regions had led to lengthened preparation time and created additional paperwork in order to meet the regulatory requirements. All this is extremely tedious and time-consuming.

This approach was proven very successful for small drug molecules in a previous ÅForsk application generated among others a landmark paper [1] and 1st prize for best PhD thesis 2017 in the area pharmaceutical and biomedicine for our PhD Dennis Åsberg that was financed partly by the ÅF grant, the jury motivation in Swedish was: "*Avhandlingen anvisar vägar till hur farmaceutisk industri kan konvertera existerande metoder till ultrasnabba metoder på ett vetenskapligt hållbart sätt som de läkemedelsregistrerande myndigheter kan acceptera*". This was an excellent example how the Swedish pharmaceutical companies can benefit of enhanced science based understanding of separation methods allowing flexible and easy method approving of method transfers/updates of previous locked methods. AZ is currently implementing this concept for the new drug in Movantik® and estimates that it will save them a significant amount of time and money.

However, the achievements described above were for small size classical medicine drugs. Not for the large biomolecules expected to be next generation biological based medicines.

With this 2nd reported Åforsk 17/500 we aimed - with support also from AstraZeneca and the KKS foundation – to be able to reach the same concept of deeper scientific understanding of the separation methods in the comprising the validation protocols so that we can implement a similar approach in the 3rd ÅForsk applied this year. Thus, this 2nd ÅForsk 17/500 project is an intermediate building up project. Such new larger and multivalent-charged biomolecules shows much more complicated interactions with the separation media use for analysis and purification, as compared to the small classical molecules. Thus, the scope of the project was to focus on building up knowledge concerning the chromatographic separations methods of model molecules similar to the final new drugs for which we plan to implement the flexible quality control concept, in the 3rd ÅForsk application or this year.

The quality control analysis methods we need to gain deeper knowledge about are both based on interaction between the biomolecules and surfaces. In Figure 1 above we see the interactions between the biomolecules and a chips surface immobilized with a ligand that recognize the molecule. In Figure 2 we see chromatographic analysis; here we have a steel/glass column with a certain small diameter (between 1 - 5 mm) and a length of around 100-150 mm which is packed with small porous silica gel beads that have been bonded to a long hydrocarbon chains. The packed material constitutes the stationary phase, spherical

porous or semi porous 2-5 μm particles and the moving phase is a water-based liquid pumped through the packed bed using high pressure pumps. The sample is introduced in the column inlet (to left, in Figure 2) and the chemical substances in the complex sample adsorb more or less strong to the stationary phase particles during their travel along the column and are finally reaching a detector after he column outlet, coupled to a computer that registers when the molecules come out of the column. The separation is successful if one can record several normally distributed peaks well resolved from each other, one for each substance desired to be separated. Then the heights or the areas of the peaks are used to obtain the quantitative information desired.

Most classical analytical chromatographic methods are based on empirical knowhow, and filed with regulatory agencies as locked methods where continuous improvements are not allowed which we want to overcome with this scientific based approach for large biomolecules such as done for small ones in the first ÅForsk project that gave Dennis Åsberg the Phabian Prize for best thesis of the year 2017 from the Swedish Pharmaceutical Society.

More particular report over working package 1, NUMERICS

A. Improving inverse solving algorithms - to process complicated kinetic interactions

The key to understand the complex interactions between the larger biomolecules and the separation surfaces that used in analysis and purifications processes by the pharmaceutical industrial sector, is to characterize in detail the complex thermodynamics and kinetics in these interactions. We have a strong expertise in determination of complicated thermodynamics by using a numerical tool for adsorption energy distribution (AED) calculations in the context of the research reported for ÅForsk 15/497 [1, 6] With AED calculations we can determine the "true" number of different types of adsorption sites and their individual energy of interactions from raw data *a priori* the rival model fits. The Figure 3 left shows raw adsorption isotherm data that are processed by AED calculations revealing we have two energy of interactions (Figure 3 middle) which allowed us to select a binary adsorption model to describe the reality best the raw data (Figure 3, right, lines) instead of leaving that that decision to a statistical report as otherwise normally done and this is why AED calculations has become a so important tool and we have validated and used it for chromatographic [1, 6] and biosensor platforms [7].

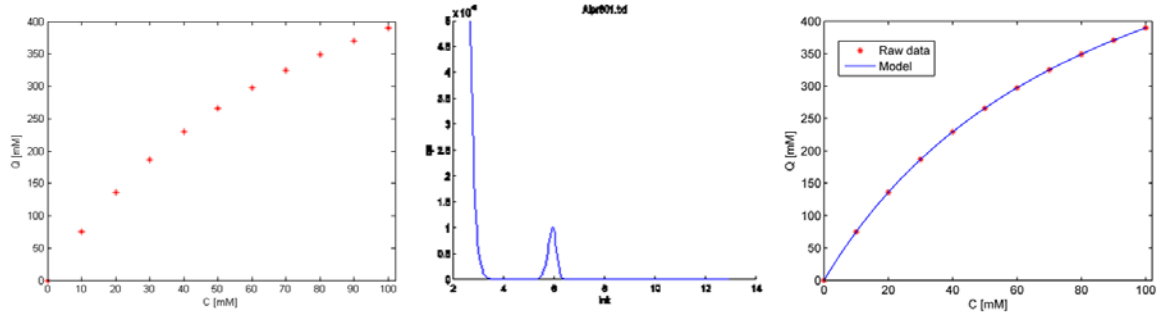

Figure 3

However, AED calculations require that equilibria in the system is established, i.e. steady state in the system is reached, and this is seldom the case for the complex interactions between larger molecules/biomolecules and the separation media aimed for analysis and purification of these, as illustrated in Figure 1 above, middle; here we can see that the sensorgrams does not reach a plateau level before the dissociation phases starts. However, we developed a similar approach to AED that does not require steady state conditions, based on so called rate constant distribution (RCD) calculations developed by the biophysical community [7]. However, there are too many problems associated with solving the inverse problem that was strongly ill-posed and also its implementation was so weak it took several days to process even one single quality control analysis [7].

We therefore focused our mathematical support man possible in WP1 by the grant to improve considerably the inverse problem for the rate constant distributions in WP1. We did so by using a goal-oriented adaptive discretization technique, allowing optimization the formulation for the inverse problem, see publication **A1** in list below. After that we optimized the solver for biosensor process calculations only requires seconds to solve a real-life problem, to be compared with the commercial RCD software that requires 3–30 h to calculate a single rate constant distribution on an ordinary PC [**A1**]. AIDA was validated both the synthetic data and with real experimental peptide model data [**A1**]. In the experiments the human model peptide PTH1R receptor was immobilized on a LNB-carboxyl biosensor chip using amine coupling according to manufacturer's instructions. in Figure 4 below we can see that AIDA could accurately resolve the

two underlying interactions without *a priori* assumptions of either the existence of parallel reaction or the range of the expected kinetic parameters (see also [**A1**] in publication list below]. Thus, AIDA will be a useful tool to determination the true interactions between biological molecules and separation surfaces.

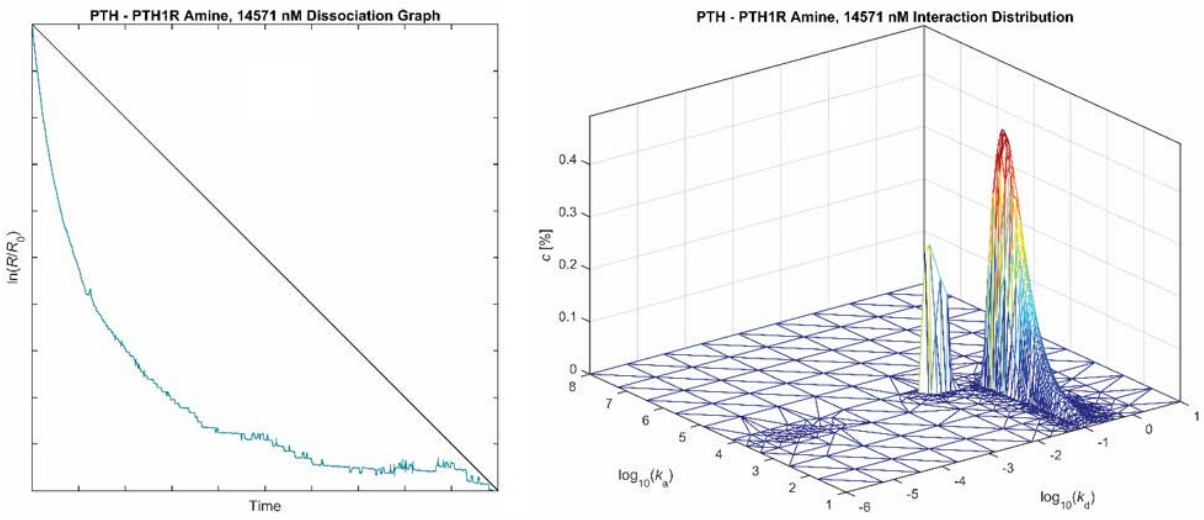

Figure 4

B. Improving inverse solving algorithms - for optimal experimental design

In another study also published recently we improved our chromatographic quality control software based on finite volumes solver using rectangular injection profiles as boundary conditions, for predicting optimal quality control separation operational conditions [see **A2** below]. We used chiral model compounds. In the 3rd ÅForsk application the concept will be used for biomolecules by first expand the chromatographic models with AIDA formulated kernels (see above). At this stage for simplification purposes, we used chiral model drug solutes, to verify if we can accurate predict optimal quality control process experimental conditions.

In this study, 1500 synthetic random model separation systems were generated, assuming heterogenic two-site Langmuir interactions. We investigated numerically how the maximal productivity was affected by changes in stationary phase adsorption properties. We investigated numerically how changes in the adsorption properties column efficiency, selectivity, total monolayer saturation capacity, relative monolayer saturation capacity and retention factor for component 1 affected the maximum achievable productivity. We could conclude that there is no reason to have columns with more than 500 theoretical plates and larger selectivity than 3 which is a most interesting results for biological processes since here slow kinetics lowers considerably the number of theoretical plates in the separation systems. We could also conclude that substantial gain in maximum achievable productivity can be achieved by increasing the selectivity only of the selectivity is very low at start [**A2**]. Finally, we could conclude that good analytic performance does not necessarily indicate good preparative performance [**A2**] which is a mort important finding for the 3rd and last ÅForsk project in this series since quality control also involve micro preparative purifications (μg - mg) amounts of several fractions for further pharmacological and toxicological tests.

More particular report over working packages 2, EXPERIMENTS and IMPLEMENTATIONS to

Systematically investigation of the impact of temperature gradients in highly pressured modern separations systems aimed at biomolecule separations.

As described in the application of ÅF 17/500 (see page 4/5 under Work Package 2-3" the most important hinder to correlate the biological peptide model molecules analytical retention with their peptides amino acid-composition to use of in data in quality control process optimization, is the tremendous pressure gradients and resulting temperatures gradients (see illustration of this also in Figure 2 above). From the literature we know that the larger and the more charged a molecule the stronger these effects and problems will be why we need to a method for systematically investigate the generation of the temperature profiles under operation in the column, for the quality control process project in the 3rd ÅForsk rgant applied this year.

The method was developed in cooperation with professor Shalliker in Australia who has been guest researcher in our laboratory and thermal images were acquired using a high performance infrared camera from his laboratory and resulted in paper in a highly ranked scientific journal [**A3**]. The experimental set up for this invention is schematic described in Figure 5. The flow path between the devices follow a typical liquid chromatographic system with (i) a solvent container (ii) a high pressure piston pump (iii) a high pressure injection valve and finally (iv) a separation column and a (v) detector coupled to a computer. The column was placed inside a box with an opening on one side to allow viewing of the column with the thermal camera (se bottom of Figure 5 below). The collected thermal images were imported to a computer and processed to define regions of interest for temperature measurements. The bottom subplot to right in Figure 5 is an example if a thermal image that was obtained by using the thermal camera [**A3**]. The temperature changes are seen through the transition of color from dark to light with increasing temperature, specifically from purple to read to yellow to white.

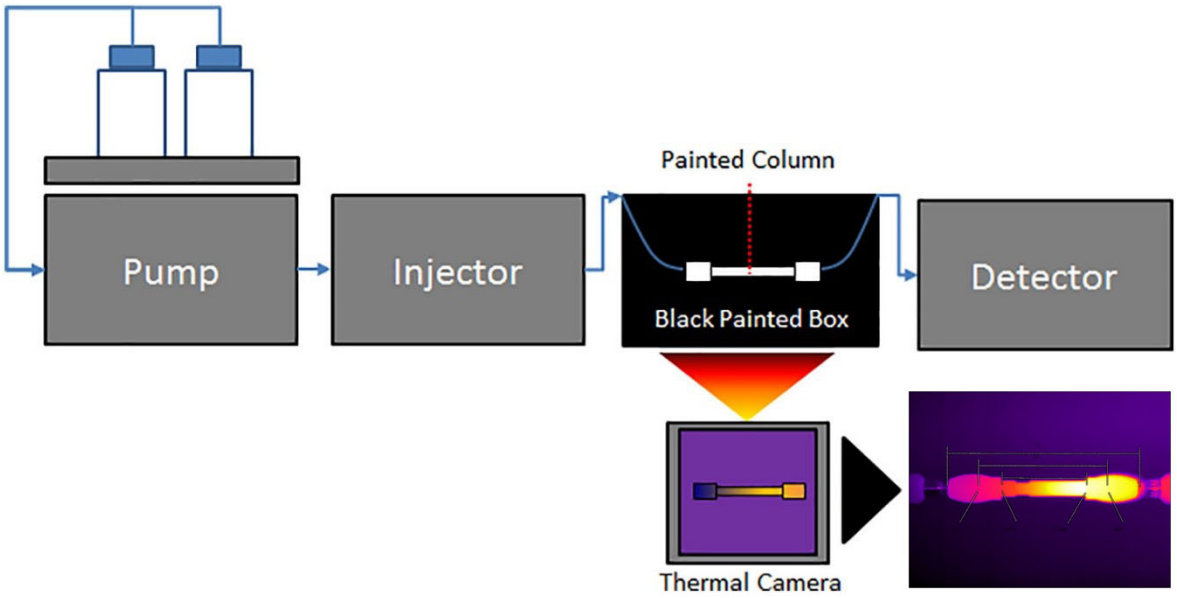

Figure 5

Systematically investigation of separation of peptides for reliable prediction of modern chromatographic methods – under gradient operational conditions – for optimal quality control

As described in the application of ÅF 17/500 (see page 4/5 under Work Package 2-3" the most important hinder to correlate the biological peptide model molecules analytical retention with their peptides amino acid-composition to use of in data in process optimization of preparative purification of peptide fragments in the mg level for further tests in extended quality control. Preparative liquid chromatography is a there a purification method of rising importance in chemical and pharmaceutical industry especially for quality control of biological molecules such as linear and cyclic peptides, nucleotides, mRNA and therapeutic proteins, in contrast to the small molecules used in current and yesterday's bestsellers. However, the operational conditions in preparative peptide chromatography are much more complex than for analytical chromatography; nonlinear conditions prevail resulting in complex adsorption behavior and overloaded band profiles often having a sharp front and diffuse rear.

We therefore developed a machine-learning (ML) based approach - to determine proper gradient conditions in preparative chromatography utilizing selected tools from analytical linear chromatography [**A4**]. A pre-trained model was used, and it was calibrated with as few as approximately 50 peptides with known retention times for three gradient slopes and two stationary phases, giving us the possibility of predicting retention times for new peptides under these six conditions [**A4**]. A suitable gradient for each column was then calculated using LSS theory to separate peptides of interest. The advantage of this strategy is that no prior experimental screening is necessary once one-time calibration of the stationary phases has been done. Knowledge of the most promising stationary phases and gradients can be obtained in silico, reducing the number of required experiments **[A4**]. The agreement between experimental and calculated elution profiles was excellent and could be used for further optimization of the method.

In Figure 6 below we can see for a C8 column in (aI) the predicted retention times for the gradient elution of the peptides LeuEnk and MetEnk for different gradient conditions and ending fractions of MeCN in the eluent. In (aII) we see corresponding elution profiles for 10-, 20-, and 45-µL injections of a mixture of 10 mg mL^{-1} MetEnk (first eluting) and 10 mg mL^{-1} LeuEnk. The (bI and bII) polts shows the sorresponding results for another column (a CSH column. The vertical dashed lines in (aII) and (bII) are the predicted analytical retention times from (aI). see more details in **A4**.

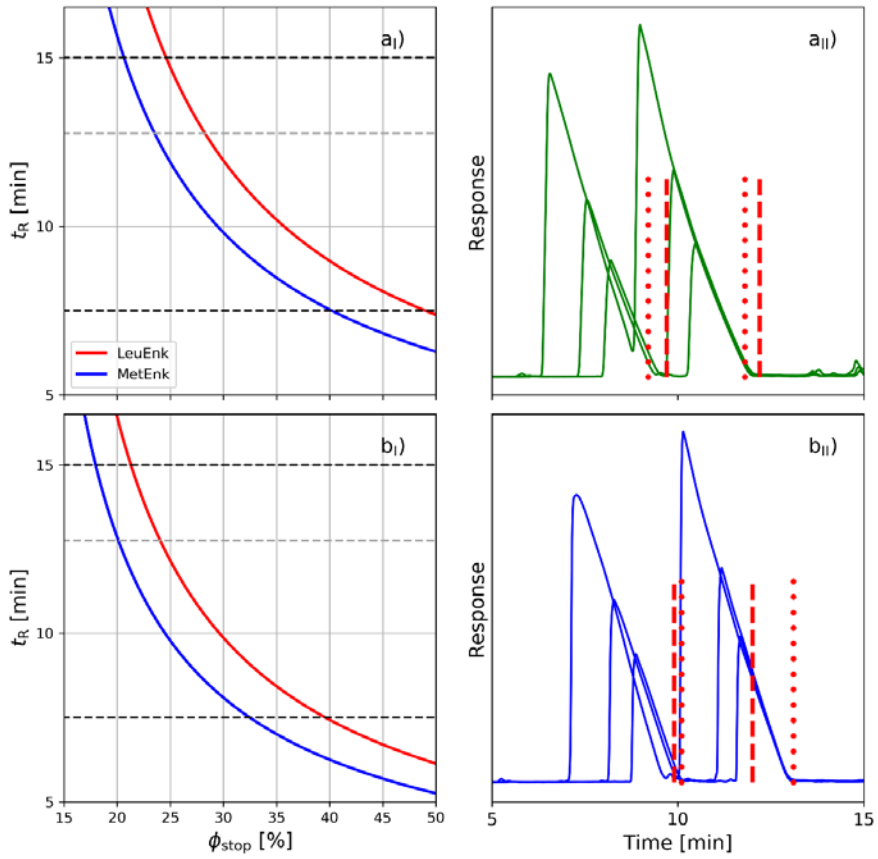

Figure 6

This strategy would be most suitable for staff and personals working with quality control processes and separations in laboratories that conduct many preparative peptide separations under similar separation conditions but have limited time for method development.

In a further similar investigation we focused on the quest how to achieve higher degree of robustness under quality control of biological molecules that always require operation under gradient conditions, meaning that he composition of the mobile phase is continuously changed under the chromatographic run [**A5**]. We investigated and compared the robustness of gradient chromatography we used a supercritical fluid chromatographic (SFC) system to separate the peptide gramicidin, using either isocratic or gradient elution. In SFC we have even larger sensitivity to operational changes of the chromatographic run since the mobile phase is something between a liquid and gas and there more compressible which was a good situation for stressing the system. Comparing the elution modes, we found that gradient elution was more than three times more robust than isocratic elution **[A5**]. We also observed a relationship between the sensitivity to changes and the gradient steepness and used this to draw general conclusions beyond the studied experimental system [A5]. Using measurements of the actual operational conditions (not the set system conditions), the isocratic deviation was quantitatively explained using the retention model. The finding indicates the benefits of using gradient elution in SFC as well as the importance of measuring the actual operational conditions to be able to explain observed differences between laboratories when conducting method transfer.

I was recently invited to give a so called key note lecture about this, summer 2019, as the industrial quarters found this so promising, the lecture is scheduled this summer in USA [L6; *this achievement is shaded in the publication list since it is not presented yet*].

Fundamental investigation of separation of oligonucleotides as model solutes for therapeutic oligonucleotides

Therapeutic oligonucleotides represent a recent breakthrough in the pharmaceutical industry [8]. Now at least five therapeutic oligonucleotides have entered commercial phase and hundreds are in clinical trials [9-10]. Synthetic therapeutic oligonucleotides are an important class of therapeutic DNA- or RNA-based biopolymers intended for altering the function of RNA, and one of the therapeutic classes is the so-called antisense oligonucleotides [8-10]. Since we in the 3rd ÅForsk application this year 2019 aim at launching the Enhanced-QC concept for next generation biomolecules more particularly for the next therapeutic oligonucleotide in pipeline we need to complement the fundamental knowledge built up with peptides also on the more specifically differences as compare therapeutic oligonucleotides.

Thus, a fundamental investigation of factors influencing the ion pair HPLC separation of diastereomers of pentameric oligonucleotides as model substances was done and recently published [**A6**]. The properties of the type and concentration of ion-pair reagent in the eluent and the properties of the oligonucleotide model solutes were in focus [**A6**]. Four different tertiary alkylamines in their ammonium acetate form was investigated. Trimethylamine gave the lowest retention and highest diastereomer selectivity, while tributylamine gave no apparent selectivity. However, as also demonstrated in this study, we could show even tributylamine has partial selectivity.

A design of experiments was carried out using tributylamine to evaluate whether any conditions promote diastereomer selectivity. No obvious conditions were found, but retention was shown to increase with the number of phosphorothioate substitutions. Interestingly, the position of the sulfur substitution was found to change the retention. Substitution at the first or fourth phosphate linkage gave higher retention than did substitution at position two or three. Finally, an experimental design treating gradient slope and ion-pair concentration was performed using triethylamine. Analysis showed that diastereomer selectivity increases with both decreasing gradient slope and decreasing ion-pair concentration.

The results have been so promising so we were recently invited to give a lecture about this sponsored by AstraZeneca in the most important conference series developed for these new drugs TIDES and arranged by and for the industrial quarters [**L7]:** *this achievement is shaded in the publication list since it is not presented yet*].

References in the report – other than in the Publication list

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Publication list to final report for ÅForsk foundation project 17-500:

Diarienummer : 17/500

Projekttitel : **Quality control of next generation biological based medicines**

Total projektperiod : 2017-08-01 - 2019-05-01

www.FSSG.se/ÅForsk

A. Referee judged Articles published 2018/2019 in the project and thus thanked in the articles acknowledgement section with "*The work was supported by ÅForsk Foundation by the project "QC of next generation biological based medicines grant number 17/500*".

A1) *An adaptive regularization algorithm for recovering the rate constant distribution from biosensor data*. **By** Y. Zhang, P. Forssén, T. Fornstedt, M. Gulliksson & X. Dai. **In** Inverse Problems in Science and Engineering 26 (2018) 1464-1489. https://doi.org/10.1080/17415977.2017.1411912

A2) Impact of column and stationary phase properties on the productivity in chiral preparative LC. **By** Patrik Forssén, Torgny Fornstedt. **In** Journal of separation science Vol 41 (2018) 1346-1354. https://doi.org/10.1002/jssc.201701435

A3) *Protocol for the visualization of axial temperature gradients in ultra-high performance liquid chromatography using infrared cameras*. **By** C.M. Vera, J. Samuelsson, T. Fornstedt, G.R. Dennis, R.A. Shalliker. **In** Microchemical Journal 141 (2018) 141–147. https://doi.org/10.1016/j.microc.2018.05.004

A4) *Determining gradient conditions for peptide purification in RPLC with machine-learning-based retention time predictions*. **By** Jörgen Samuelsson, Finnur Freyr Eiriksson, Dennis Åsberg, Margrét Thorsteinsdóttir, Torgny Fornstedt. **In** Journal of Chromatography A. In Press, Corrected Proof (available online 29 March). https://doi.org/10.1016/j.chroma.2019.03.043

A5) *Investigation of robustness for supercritical fluid chromatography separation of peptides: Isocratic vs gradient mode*. **By** Martin Enmark, Emelie Glenne, Marek Leśko, Annika Langborg Weinmann, Tomas Leek, Krzysztof Kaczmarski, Magnus Klarqvist, Jörgen Samuelsson, Torgny Fornstedt. **In** Journal of Chromatography A, 1568 (2018) 177–187. https://doi.org/10.1016/j.chroma.2018.07.029

A6) *Investigation of factors influencing the separation of diastereomers of phosphorothioated oligonucleotides*. **By** Martin Enmark, Maria Rova, Jörgen Samuelsson, Eivor Örnskov, Fritz Schweikart, Torgny Fornstedt. **In** Analytical and Bioanalytical Chemistry. Accepted for publishing [27-Mar-2019]. https://doi.org/10.1007/s00216-019-01813-2

Reviewed Lectures at international conferences made with the ÅForsk Foundation support by grant 17/500 and thus also thanked in Acknowledgements with grant number at last slide.

L1) **HPLC 2018 47th International Symposium & Exhibit on High Performance Liquid Phase Separations and Related Techniques**. July 29-August 2, Washington DC. Session: Oligomers, L-145) (Session FUN 11, Fun11O1-We) *On the Issue of Separating Diastereomers of Phosphorothioated Oligonucleotide*s. Martin Enmark, Jörgen Samuelsson, Maria Rova, Eivor Örnskov, Anders Karlsson, Torgny Fornstedt.

L2) **PREP 2018, 31st International Symposium on Preparative and Process Chromatography**. July 8-11, Baltimore, MD USA. (L-204) *Increasing the Robustness of SFC: Examples from Chiral and Peptide Separations*. Torgny Fornstedt, Martin

Enmark, Emelie Glenne, Marek Lésko, Annika Weinmann, Tomas Leek, Krzysztof Kaczmarski, Magnus Klarqvist, Jorgen Samuelsson.

L3) **SFC 2018 – Strasbourg, France, October 17-19 the 12th International Conference on Packed Column SFC**. *Investigation of robustness for supercritical fluid chromatography separation of peptides: Isocratic vs gradient mode*. Martin Enmark, Jörgen Samuelsson, Emelie Glenne, Marek Leśko, Annika Weinmann, Tomas Leek, Krzysztof Kaczmarski, Magnus Klarqvist, Torgny Fornstedt.

L4) **SPICA 2018 17th International Symposium on Preparative and Industrial Chromatography and Allied Techniques** Darmstadt, Germany October 7-10. (OC25) *Studies on Robustness of SFC: Examples from Chiral and Peptide Separations*. By Torgny Fornstedt, Jörgen Samuelsson, Emelie Glenne, Hanna Leek, Kristina Öhlén, Magnus Klarqvist, Patrik Forssén, Torgny Fornstedt.

L5) **Therapeutic proteins - focus on analysis and formulations, February 5-6, 2019, Stockholm, Sweden**. *Deeper numerical characterization of cell-based biosensor data*. By Torgny Fornstedt, Patrik Forssén, Evgen Multia, Jörgen Samuelsson, Thanaporn Liangsupree, Marja-Liisa-Riekkola, Teodor Aastrup.

L6) **PREP 2019, 32nd International Symposium on Preparative and Process Chromatography. July 7-10, Baltimore, MD, USA**. (9. Wednesday Keynote session, Peptides and Oligonucleotides, L-235). *Preparative Supercritical Fluid Chromatography Separation of Peptides: On the Issue of Solubility and Robustness.* Joakim Bagge, Martin Enmark, Marek Lesko, Emelie Glenne, Linda Thunberg, Annika Langborg Weinmann, Tomas Leek, Hanna Leek, Fredrik Limé, Jörgen Samuelsson, Torgny Fornstedt.

L7) **TIDES Europe: Oligonucleotides & Peptide Therapeutics 2019. November 12-15, Amsterdam, Netherlands**. Deeper understanding of separation of native and phosphorothioated oligonucleotides and its impurities using ion-pair reversed phase chromatography. Martin Enmark, Jörgen Samuelsson, Torgny Fornstedt.

Reviewed Posters at international conferences made with the ÅForsk Foundation support by grant 17/500 and thus also thanked in Acknowledgements with grant number.

P1) **HPLC 2018 47th International Symposium & Exhibit on High Performance Liquid Phase Separations and Related Techniques**. July 29-August 2, Washington DC. (P-T-1302) *Why Gradient Elution Lead to Increased Robustness of SFC Separations*. Martin Enmark, Emelie Glenne, Marek Lesko, Annika Langborg Weinmann, Tomas Leek, Krzysztof Kaczmarski, Magnus Klarqvist, Torgny Fornstedt, Jörgen Samuelsson.

P2) **HPLC 2018 47th International Symposium & Exhibit on High Performance Liquid Phase Separations and Related Techniques**. July 29-August 2, Washington DC. (P-T-1303) *Highlighting often Neglected Experimental Parameters in Analytical Supercritical Fluid Chromatography*. Martin Enmark, Jörgen Samuelsson, Anders Karlsson, Torgny Fornstedt.

P3) **PREP 2018, 31st International Symposium on Preparative and Process Chromatography.** July 8-11, Baltimore, MD USA. (P-M-138) *Proper Operational Conditions in Supercritical Fluid Chromatography of Complex Molecules, Set vs. Real Conditions*. Torgny Fornstedt, Martin Enmark, Jörgen Samuelsson, Anders Karlsson.

P4) **PREP 2018, 31st International Symposium on Preparative and Process Chromatography.** July 8-11, Baltimore, MD USA. (P-T-239*) Robust Operation of SFC using Peptide and Chiral Model Systems*. Torgny Fornstedt, Martin Enmark, Emelie

Glenne, Marek Lesko, Annika Langborg Weinmann, Tomas Leek, Krzysztof Kaczmarski, Magnus Klarqvist, Jörgen Samuelsson.

P5) **SPICA 2018 17th International Symposium on Preparative and Industrial Chromatography and Allied Techniques** Darmstadt, Germany October 7-10. *Investigations of robustness in SFC*. By Torgny Fornstedt, Martin Enmark, Emelie Glenne, Marek Leśko, Annika Langborg Weinmann, Tomas Leek, Krzysztof Kaczmarski, Magnus Klarqvist, Hanna Leek, Jörgen Samuelsson.

P6) **SPICA 2018 17th International Symposium on Preparative and Industrial Chromatography and Allied Techniques** Darmstadt, Germany October 7-10. *Evaluation of using Instrumental-Set Conditions vs. Real Operation Conditions in Supercritical Fluid Chromatography*. By Torgny Fornstedt, Martin Enmark, Jörgen Samuelsson, Anders Karlsson.

P7) **SFC 2018 – Strasbourg, France October 17-19, 2018, the 12th International Conference on Packed Column SFC**. *Investigation of robustness for supercritical fluid chromatography separation of peptides: Isocratic vs gradient mode*. Martin Enmark, Emelie Glenne, Marek Leśko, Annika Weinmann, Tomas Leek, Krzysztof Kaczmarski, Patrik Forssén, Magnus Klarqvist, Torgny Fornstedt and Jörgen Samuelsson.

Seminaries, Events, Popular Science articles & Web-based News

S1) **Forskning - en god affär för Sverige? Frukostseminarium på IVA dem 8 november 2018.** På seminariet diskuterades bland annat hur universiteten och högskolorna som ligger i framkant arbetar med kunskapsöverföring & vilka samarbetsformer som fungerar bäst. Medverkande: bl. a Teodor Aastrup, CEO på Attana AB, Ulf Hall, tillförordnad vd, KK-stiftelsen och Johan Sterte, Rektor, Karlstads universitet. https://www.iva.se/event/forskning-en-god-affar-for-sverige/

S2) **Tydliga mål viktiga för framgång i samarbetsprojekt.** Kontakter öga mot öga och en gemensam vision om målet. Det är förutsättningar för att samarbeten mellan forskare och företag ska bli en bra affär. (Publicerat på www.IVA.se) 15 november 2018; skribent Pär Rönnberg) https://www.iva.se/publicerat/tydliga-mal-viktiga-for-framgang-i-samarbetsprojekt/