Template for Industrial Ph.D. project description

Basic information

Project title	Extending the systemic half-life of peptide drugs
Industrial Ph.D. candidate	Esben Matzen Bech
Company	Gubra ApS
University, centre/institute	University of Copenhagen, Department of Chemistry
Any first third party	
Any second third party	

A. Objectives and success criteria (max. ½ page)

- The objectives of the project

Peptides often have short systemic half-lives and this limits their pharmaceutical applicability. The aim of this Ph.D. project is to improve the pharmacokinetics of peptides with pharmaceutical relevance, through the development of two novel methods for systemic half-life extension.
Promotion of non-covalent bindings between peptides and human serum albumin (HSA) is a well-known method for prolongation of peptide half-life.[1–8] Peptide lipidation has proven especially successful in this matter as it induce a strong peptide-HSA interaction with low immunogenicity and toxicity. Lipidation is, however, accompanied by a drastic reduction of plasma solubility making the strategy inapplicable for a broad set of peptide pharmaceuticals.[7] Thus, novel techniques mediating strong peptide-HSA interactions with negligible impact on plasma solubility are interesting. This project aims at developing a generally applicable method for promoting HSA interaction based on small molecules known to bind drug site 1 and drug site 2 of HSA. The second method developed during this Ph.D. project relies on the conjugation between a pharmaceutical peptide and a new class of peptides that we will refer to as beltides. Beltides have recently been shown to self-assemble with phospholipids to form high-density lipoprotein (HDL) nanodiscs large enough to avoid renal clearence.[9] By fusing beltides and pharmaceutically interesting peptides, we therefore have reason to believe that the systemic half-life of the peptide can be extended through an intrinsic self-assembly into nanodiscs.
The pharmaceuticals in focus will be peptide hormones regulating the energy metabolism, such as glucagon-like peptide 1 (GLP-1), amylin, peptide YY (PYY_{3-36}), and neuromedin U (NMU). The designed peptide conjugates will be tested by relevant *in vitro*-, and *in vivo* setups.

- Success criteria for the project

Non-covalent interaction to HSA is a previously proven method for peptide half-life extension. Thus, the HSA-binding strategy will be considered successful only if two or more pharmaceutically relevant peptides obtain significantly improved pharmacokinetics measurable *in vivo*. Furthermore, their water solubility should be higher than lipidated pharmaceuticals with similar systemic half-lives. The use of nanodiscs in this context is, to the best of our knowledge, unexplored. The beltide strategy will therefore be characterized as a success if the following is shown for a fusion peptide; nanodiscs self-assembly, retained pharmaceutical efficacy, and prolongation of peptide systemic half-life.

B. Commercial potential (max. 1 page)

- The project's commercial potential for the company

In 2012, the share of biologics in the overall pharmaceutical market was 18 % or $125 billion.[10] This number is expected to increase to ~20 % in 2017 with growth dominated by monoclonal antibodies and insulins.[10] Human insulin, as many other peptides, has a very short systemic half-life. Peptide modifications are therefore required to achieve long-acting analogues necessary for patient convenience and medicament compliance. From a commercial perspective, the advancement of techniques that extend the circulatory life of peptides are thus highly attractive. In this Ph.D. project, we aim to do just that by the development of two novel methods. The first, based on a finely tunable albumin binding mediated by small molecules could result in a significant improvement in respect to applicability and/or albumin affinity compared to currently applied HSA binders. The second method, based on the fusion of peptide pharmaceuticals and beltides, has a completely unexplored potential for the extension of peptide half-life. The techniques explored in this Ph.D. project therefore have the potential to become important factors in the expansion of the biopharmaceutical market share.

Concerning Gubra ApS, the use of small molecules or beltides for modulation of peptide pharmacokinetics could provide the company with a unique platform for optimization of biopharmaceuticals. Gubra is currently striving to develop peptide pharmaceuticals within the field of obesity and diabetes. The use of the proposed platforms, if successfully developed, are likely to aide this process, thus benefitting both company and patients suffering from diabetes or morbid obesity. Developing methods that improve the pharmacokinetics of biopharmaceuticals could also attract the attention of larger pharmaceutical companies, with whom Gubra could enter a mutually beneficial partnership. Gubra would provide the technology while large pharma would provide the resources to bring pharmaceuticals to the market. This would benefit all involved parties as well as the patients whose life quality the long-lived pharmaceuticals are meant to improve. Another approach to profit from this Ph.D. project, if the techniques prove effective, would be to obtain IPR rights and sell/lease these. The competition within the field of long-acting peptide pharmaceuticals is fierce, particularly within the fields of diabetes and obesity. It is therefore conceivable that some of the large pharmaceutical companies working within this area (Eli Lilly, Novo Nordisk, Sanofi etc.) would be interested in purchasing a novel technique for prolonging peptide circulatory half-life.
Finally, the project will provide information about peptide hormones used as models. This could e.g. be GLP-1 and amylin, which are important targets when working with obesity. The generation and exploration of new analogues should further the knowledge of these compounds, and the work could result in early drug candidates. This represents a significant value.

C. State-of-the-art and theoretical background (max. 1 page excl. references)

- State-of-the-art and, if relevant, theoretical background for the Industrial Ph.D. project's field of research

Due to exquisite potency and positive safety profiles, peptide pharmaceuticals have received increasing attention the past three decades. More than 100 peptides and proteins are currently approved for clinical use.[11,12] However, most peptides have short half-lives *in vivo* due to renal filtration and degradation, and therefore require frequent administration for therapeutic efficiency.[13] To increase patient convenience and medicament compliance, methods that aim to prolong peptide half-life have been developed. Most such techniques are based on the conjugation between a peptide of interest and a molecular scaffold with a size above the glomerular filtration threshold of ~60 kDa.[14,15] This reduces renal filtration as well as extracellular enzymatic degradation. Scaffolds include polyethylene glycol (PEG),[16–18] large biodegradable polypeptides,[19,20] polysialic acid,[21,22] or naturally occurring plasma proteins like the Fc of IgG[23,24] and HSA[25,26].

Nanodisc self-assembly

Apolipoprotein A1 (ApoA1) is a 243 amino acid human protein that self-assembles into HDL nanodiscs in the presence of phospholipids (Figure 1A).[9,27] The ApoA1 nanodiscs are large enough to avoid glomerular filtration (approx. 10nm in diameter), and therefore have long circulatory half-lives (5 days).[28] Furthermore, ApoA1 nanodiscs are non-immunogenic and have shown no toxicity.[27] These properties makes ApoA1 highly interesting in respect to peptide half-life extension with the large molecular scaffold method. However, no investigations into this matter have been performed. Instead, ApoA1-like nanodiscs are currently used for stabilization of membrane proteins *in vitro* allowing for their study.[29–31] Also, their use in drug delivery systems are much anticipated as HDL nanodiscs can bind hydrophobic moieties inside their phospholipid bilayer and be modified to specifically locate to target tissue (e.g. cancer cells).[29,32,33] One such modification, made by Iovannisci and colleagues, was a single chain variable antibody (scFv)-ApoA1 chimera, which retained both the antigen-binding capability of the scFv and the nanodiscs forming ability of ApoA1.[33] With this chimera in mind, we find it plausible that a peptide pharmaceutical could be fused to ApoA1 for an increase in half-life without a dramatic loss of receptor potency. It is, however, tedious to chemically synthesize and purify proteins in the size of ApoA1. Furthermore, a compound with a smaller molecular mass will yield a better activity to mass ratio and, in general, a more stable pharmaceutical.[34] It is therefore interesting that an 18 amino acid peptide, a beltide, mimicking ApoA1 have been shown to form nanodiscs as well (Figure 1A).[9] We find it reasonable to believe that a chimera consisting of a pharmaceutical peptide and a beltide (or a dimer/trimer of beltides) can retain the capability to form nanodiscs in the bloodstream, thus extending the circulatory half-life of the pharmaceutical (Figure 1B).

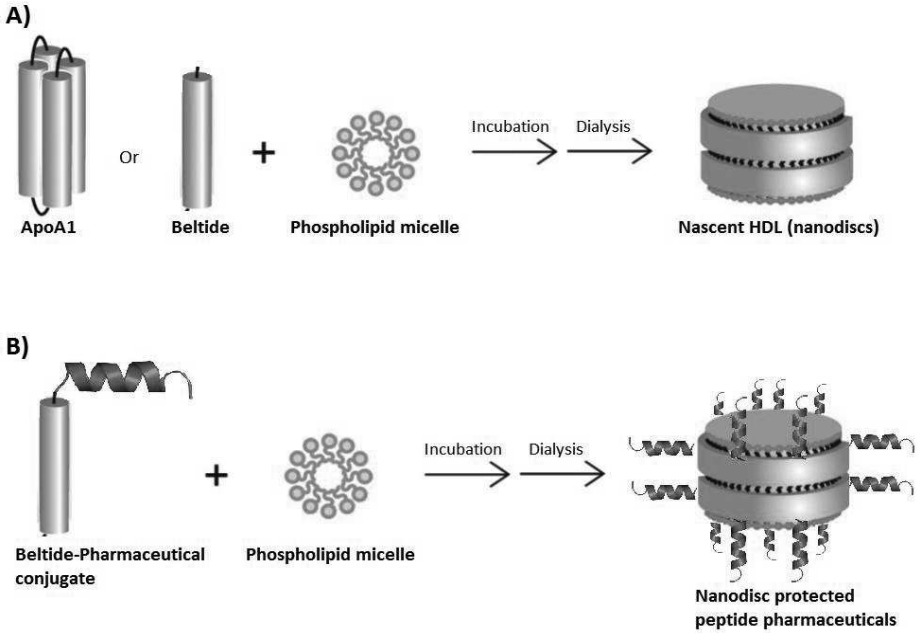


***Figure 1*:** A) Schematic illustration of the *in vitro* assembly of a high-density lipoprotein, a nanodisc, from ApoA1 or the ApoA1 mimicking beltide. Modified from Murakami, 2012.[27] B) Hypothesized formation of HDL nanodiscs from beltide-pharmaceutical chimer self-assembly.

Small molecule albumin binders

A peptide half-life extending strategy different from the attachment of large molecular scaffolds is to promote a reversible binding between the peptide of interest and HSA.[1–8] This strategy relies on the abundance of HSA in plasma, its wide tissue distribution, its promiscuous binding capabilities, and its extraordinarily long systemic half-life (~ 20 days).[35] Due to the non-covalent nature of the interaction, an equilibrium between free and HSA-bound peptide will arise. The bound fraction will be protected against glomerular filtration and enzymatic degradation while unbound peptides are capable of crossing the blood-brain barrier, which is important for many pharmaceuticals.[14,36] The most successful method for promoting albumin-peptide interactions is peptide lipidation. HSA is known to have three high affinity fatty acid binding sites,[5,37] resulting in an extensive interaction between lipidated peptides and albumin. Lipidation, however, drastically reduce water solubility of the peptide pharmaceutical making the strategy unfit for certain peptide drugs.[4,7]

Albumin is known to bind a variety of small molecule drugs with high affinity. Especially two sites, drug site 1 and drug site 2, are responsible for these non-covalent bindings. One example is diflunisal that is > 99 % albumin bound in plasma, which necessitates a dose of 500 mg per day for efficacy.[38] Recently, in a series of unpublished experiments, we found that diflunisal conjugated to GLP-1 promotes a strong, specific interaction to albumin with an equilibrium dissociation constant (K_D) of approx. 1 µM (measured using surface plasmon resonance, SPR). Moreover, we found that the interaction to HSA could be delicately adjusted by slight changes in the linker region or in the structure of the diflunisal moiety. Finally, small molecule drugs with high HSA affinity are not as hydrophobic as the fatty acids used for peptide lipidation. Compared to lipidation, the small molecule drugs can therefore be utilized with a low impact on peptide water solubility. We therefore find it interesting to investigate small molecule drugs in regard to a generally applicable technique for circulatory half-life extension of peptides (Figure 2).

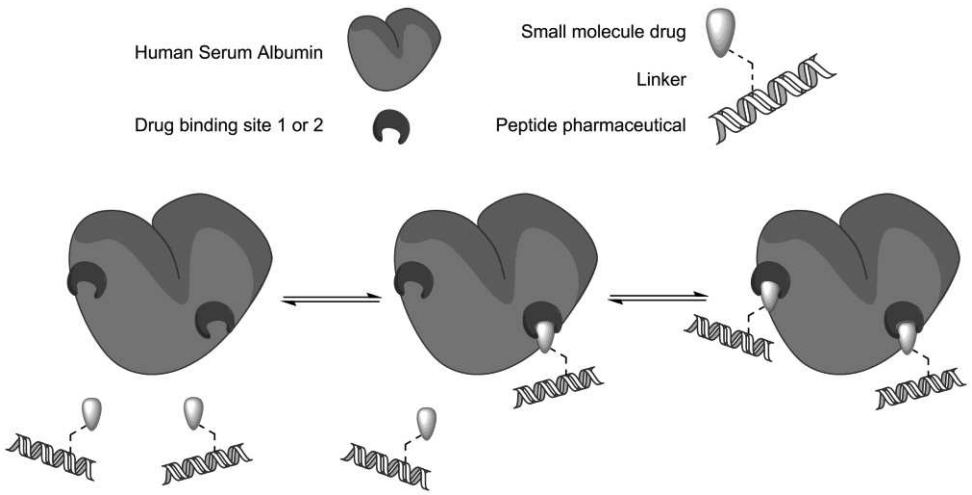


Figure 2: Small molecule drug promoted interaction between human serum albumin and a peptide pharmaceutical.

D. Project description (max. 4 pages)

- Project description

The experimental part of the Ph.D. will be divided into four phases starting with the design and synthesis of the peptides. Following synthesis, the peptides will be examined *in vitro* and candidates with promising properties will be submitted to *in vivo* validation. Finally, a pharmacokinetic analysis of selected peptides will be performed.

Phase 1: Design and synthesis of peptides

Model peptide: Glucagon-like peptide 1

GLP-1 is a glucoregulatory gut hormone, an incretin, derived from preproglucagon, which is the precursor of many glucagon related peptides.[39] In humans the peptide is found in two equipotent forms, GLP-1(7-36)-NH_2 and GLP-1(7-37)-OH.[39] Since the hormone was discovered in the early 1980s,[40] GLP-1 has attracted a significant amount of attention due to its antidiabetic properties.[41] GLP-1 stimulates insulin secretion and reduces glucagon secretion.[41,42] Furthermore, the peptide increases pancreatic β-cell longevity and proliferation and can therefore be used to protract the onset of diabetes.[42] GLP-1 is also known to prevent gastric emptying and induce satiety, which leads to a decrease in body weight highly desirable for treatment of morbid obesity.[39,41–43]

The downside of GLP-1 as a therapeutic is its short circulatory half-life of 2 min. However, several GLP-1 analogues with prolonged half-lives have been developed and are applied for the treatment of diabetes and, more recently, obesity.[41,44] A well-known, highly studied half-life extended analogue with a minimal alteration in peptide sequence is liraglutide (Victoza®, Novo Nordisk) (Figure 3). Arg34-GLP-1(7-37)-OH will be used as the model peptide of preference during this Ph.D., as it allows for selective Lys26 side-chain modifications and direct comparison to liraglutide in terms of, HSA affinity, pharmacokinetics, and potency. Furthermore, Gubra already have several ongoing projects with Arg34-GLP-1(7-37), and the group of Professor Knud J. Jensen have established functioning methods for measuring chemical and biophysical properties of the peptide. Both of which the experimental process of the Ph.D. will benefit from.

Besides GLP-1, at least one other peptide hormone will be included as a model peptide. This could e.g. be amylin or PYY_{3-36}, both of which are highly relevant for the development of future anti-obesity and/or anti-diabetic biopharmaceuticals due to their involvement in satiety, gastric emptying, weight loss etc.[45,46]

GLP-1:	H-H-A-E-G-T-F-T-S-D-V-S-S-Y-L-E-G-Q-A-A-**K**-E-F-I-A-W-L-V-**K**-G-R-G-OH
Model peptide:	H-H-A-E-G-T-F-T-S-D-V-S-S-Y-L-E-G-Q-A-A-**K**-E-F-I-A-W-L-V-R-G-R-G-OH

Figure 3: The sequence of human GLP-1(7-37) (Top) and the liraglutide backbone peptide (bottom). The substituted amino acid is colored blue and the lysines are in bold. In liraglutide, the ε-amine of Lys26 is functionalized with a γGlu spaced palmatic acid.

Furthermore the mentioned peptide hormones are relatively small (37 and 33 amino acids, respectively), and have very short systemic half-lives.[45,46] Finally, contrary to GLP-1, no long-acting analogues of these peptides are on the market.[10] For these reasons, we find it interesting to test our techniques on one of these peptides as well.

Design of small molecule albumin binders

Small molecule drugs known to bind HSA with high affinity will be chemically functionalized to allow selective incorporation into the GLP-1 peptides by solid-phase peptide synthesis (SPPS). To prevent the alterations from interfering with the albumin affinity of the small molecules, previous albumin studies will be meticulously examined. One example is an article by Mao *et al.*[38], which describes the impact of various diflunisal modifications on its affinity for both HSA and its target receptor cyclooxygenase-2. In recent, unpublished studies the information published by Mao[38] allowed us to conjugate diflunisal and GLP-1(7-37)-OH while retaining albumin affinity of the small molecule. Other examples of small molecule drugs with well-characterized HSA bindings include indomethacin, oxyphenbutazone, diazepam, salicylic acid, and many more.[47,48] Small molecule drugs with reduced target receptor potency and retained/increased HSA affinity

will also be designed and synthesized. One example of this could be the removal of one or both fluorines from diflunisal.[38]
The candidate will design all small molecule albumin binders, but the synthesis will be handled by an expert organic chemist from the group of Professor Knud J. Jensen at the University of Copenhagen (KU).

Synthesis of peptidic albumin binders
Model peptides conjugated to small molecule drugs for albumin binding properties, will be chemically synthesized in two steps. First, peptide precursors (the peptide backbone) will be synthesized by fully automated state-of-the-art solid-phase peptide synthesis (SPPS) according to the Fmoc strategy. Secondly, the modified small molecule albumin binders will be incorporated into the amino acid sequences at carefully selected side-chain functionalities chosen on the basis of structure-activity relationships available in the literature.[49–51]
For the peptide synthesis, the C-terminal amino acid will be anchored to an insoluble solid support (a resin), capable of swelling in certain solvents. The peptide will then be assembled in a series of repeated cycles containing steps of coupling, N^{α}-deprotection , and washing. The coupling steps include a coupling reagent, a non-nucleophilic base for reagent activation, an epimerization suppressant, and the relevant amino acid with N^{α}- and side-chain protecting groups. The N^{α}-deprotection will be performed under mild conditions by the addition of a secondary amine. Washing will be performed between each step to remove everything but the resin-bound peptides. Following the synthesis of peptidic albumin binders, the peptides will be fully deprotected and released from the resin. The crude peptide mix will then be subjected to reverse phase high-performance liquid chromatography (RP-HPLC) for purification, and the correct peptide will be characterized by liquid chromatography-mass spectrometry (LC-MS). Finally, solvents will be removed by lyophilization.

Beltide–pharmaceutical conjugate
A beltide consist of 18 amino acids (Figure 4) – approximately half the size of GLP-1(7-37)-OH. It is therefore plausible that nanodisc self-assembly of 1:1 beltide-pharmaceutical conjugates will be obstructed by steric hindrance of the larger pharmaceutical. If so, successful nanodisc self-assembly of beltide-pharmaceutical conjugates could require dimers or even trimers of beltides (2:1 or 3:1 beltide-pharmaceutical).
For the creation of the conjugates, linear synthesis by automated state-of-the-art SPPS according to the Fmoc strategy will be applied with GLP-1(7-37) positioned in the N-terminal of the chimer. Synthesis of large peptides is not trivial, and yields are often low. However, the group of Professor Knud J. Jensen are experts in the area, and routinely synthesize peptides ~60 amino acids and have previously synthesized peptides of >70 amino acids. Should linear synthesis fail, the peptides can be synthesized separately, purified by RP-HPLC, and conjugated by e.g. native chemical ligation (NCL). Native chemical ligation is the formation of a peptide bond by the reaction between an N-terminal cysteine of one peptide and a C-terminal thioester of another peptide.[52] The N-terminal cysteine is incorporated alongside the remaining amino acids during peptide synthesis, and the C-terminal thioester can be incorporated by usage of a thioester resin.[52] NCL is not necessarily straightforward, but the group of Professor Knud J. Jensen have experience in the field. Another option is to synthesize the pharmaceutical and the beltide monomer/dimer/trimer individually, and then create the chimer through a condensation reaction in solution.

H-D-W-L-K-A-F-Y-D-K-V-A-E-K-L-K-E-A-F-OH

Figure 4: The sequence of the proposed beltide

Phase 2: *In vitro* investigation

Estimation of water solubility
The impact of the small molecule drugs on peptide water solubility can be rapidly estimated by determination of the peptide concentration in a saturated solution using water or an aqueous buffer as solvent. The saturated solution will be centrifuged, and the peptide concentration of the supernatant estimated by UV-spectroscopy. The results will be compared to those of the naked peptides and lipidated analogues.

Human serum albumin binding

The half-life extension of peptides modified with small molecules will depend on their non-covalent binding to albumin. Thus, it is essential to determine the kinetic binding constants between HSA and the analogues. It is, however, notoriously hard to accurately determine a high affinity albumin binding of a peptide. A common issue is that a high affinity interaction with HSA will leave a very low concentration of unbound compound. Since many techniques for protein-ligand interaction determination rely on the measurement of the unbound fraction it will require highly sensitive instrumentation to accurately determine the kinetic parameters. This is e.g. true for equilibrium dialysis, ultrafiltration and ultracentrifugation which are among the most commonly used methods for determination of plasma protein binding.[53] Another classical method for determination of kinetic binding constants is isothermal titration calorimetry (ITC), which is based on the enthalpy change resulting from a complex formation. ITC, however, is of limited use if the peptide-of-interest is prone to aggregation, as this also induce an enthalpy change. ITC is therefore problematic when examining the interaction between e.g. HSA and peptides acylated with fatty acids (such as liraglutide). Fortunately, the Ph.D. candidate has recently, in a series of (yet) unpublished experiments, demonstrated an accurate method for determination of albumin binding for small molecule peptide conjugates using surface plasmon resonance (SPR). SPR biosensing is a highly sensitive, real-time method for studying the interaction between an immobilized compound and a molecule in solution, and the technique has been used extensively for albumin-small molecule ligand interaction studies.[54–56] HSA is immobilized to a gold surface in random orientation. The ligand of interest is then injected to the surface, and a response signal dependent on the ligands concentration, weight and HSA affinity is measured. From the SPR data, the equilibrium association and dissociation constants (K_A and K_D) can be accurately determined. Furthermore, the percentage of compound expected to bind HSA *in vivo* can be estimated.[54] In this Ph.D., determination of albumin binding for the small molecule peptide conjugates will be performed using SPR.

Nanodisc self-assembly

The ability to self-assemble into nanodiscs is crucial for prolonging the circulatory half-life of beltide-pharmaceutical conjugates. It is therefore essential to determine whether this beltide-feature remain in the conjugates.

In this Ph.D., the biophysical characteristics will be investigated by size-exclusion chromatography (SEC) and dynamic light scattering (DLS) available at KU. These techniques can detect the formation of higher order oligomers and can therefore confirm if the conjugates assemble into nanodiscs. Furthermore, small-angle X-ray scattering (SAXS) might be included in the project. SAXS measurements are interesting as it, in contrast to SEC and DLS, yields information on the shape of macromolecules. The candidate himself will perform the SEC and DLS experiments, while SAXS (if included) will be performed by a collaboration partner of Professor Knud J. Jensen.

Functional evaluation

Retaining the bioactivity of the pharmaceuticals is essential if the half-life extending methods are to be useful. The maximal efficacy and potency of the peptide analogues will therefore be evaluated by *in vitro* functional screening assays. Furthermore, the functional screening assays will be used to support the SPR data by indirect examination of albumin binding to peptides modified with small molecule drugs.

Receptor activation assays are typically performed on immortalized cell lines transformed to express a receptor in high density. When activated the target receptor will initiate a downstream cascade resulting in a measurable signal.[13] An example could be the activation of a G-protein coupled receptor (GPCR) like the GLP-1 receptor (GLP-1R). When activated the GPCR will initiate a signal cascade leading to the formation of cyclic adenosine monophosphate (cAMP) which is indirectly measured, preferably by fluorescence.[8] By a stepwise increase of agonist concentration the maximal response (E_{max}) inducible by the agonist can be found. This is equally true for the concentration of agonist required to reach 50% of the maximal response, the half maximal effective concentration (*EC50*) or potency.[13] The bioactivity of the modified pharmaceuticals can thus be evaluated. Albumin binding can also be investigated by functional screening as the peptides binds to HSA leads to a low activation of the receptor. Thus the functional assays are repeated with HSA present in first low, then high concentration. The HSA affinity of the peptides can then be indirectly estimated through a shift in the peptide receptor potency.[36] The functional HSA binding experiments can be compared with the SPR data.

Gubra are currently in the process of establishing an *in vitro* laboratory. The Ph.D. candidate has some experience with cell-based assays, and is interested in increasing his knowledge in the field. The candidate will cooperate with researchers at Gubra ApS for setting up cell-lines as well as the design and execution of experiments.

Plasma stability

It is the hypothesis that the interaction to the large protein HSA or the self-assembly into nanodiscs will somewhat shield peptides from enzymatic degradation. Assessment of serum stability is included to investigate if the modifications have in fact conveyed a proteolytic protection of the model peptides.

The peptides will be incubated in human/murine plasma at 37°C for several days, and the plasma stability determined by measuring the relative concentration of intact peptide and major metabolites at fixed intervals. The determination of peptide concentration will be performed using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), which is an extremely sensitive instrument capable of detecting picomolar concentrations of e.g. peptides in complex mixtures like human serum. The UPLC-MS/MS achieves this high sensitivity through its tandem MS setup following the chromatographic separation. In MS1 an appropriate mother ion is selected. The mother ion is then fragmented and the resulting daughter ions analyzed in MS2. This two-step approach markedly improve the signal-to-noise ratio which improves the sensitivity.

Phase 3: *in vivo* validation

Acute feeding studies in lean mice

Based on the phase 2 experimental data, promising candidates of small molecule modified model peptides, as well as beltide conjugates will be selected for acute *in vivo* studies. The proposed model peptides are involved in satiety, glucose tolerance, and insulin sensitivity. Thus, these factors will be investigated. Each candidate drug will be tested for food intake in semi-acute (2.5 days) study design. Animals will be randomly separated into groups of 4 and subcutaneously (s.c.) dosed with vehicle, the peptide of interest, or the corresponding unmodified peptides (e.g. Arg34-GLP-1(7-37)-OH). The animals will be subjected to drug administration once daily during the study. Body weight will be monitored prior to each dosing, while the food intake will be monitored throughout the study by a fully automated system (HM-2 system) available at Gubra. To assess the effect on glucose tolerance and insulin sensitivity of the peptide conjugates, an oral glucose tolerance test (OGTT) will be conducted.

Phase 4: Pharmacokinetic analysis

Pharmacokinetic study in lean mice

The most promising albumin binding Arg34-GLP-1(7-37)-OH analogue and Arg34-GLP-1(7-37)-beltide conjugate, based on the data obtained *in vitro* and in the acute feeding study, will be subjected to a pharmacokinetic (PK) study to evaluate the impact on the pharmacokinetic properties by the modifications. Blood from the animals will be collected at relevant time points and stored for PK analysis.

Analysis of PK data

Serum isolated from the blood samples obtained in the PK study will be analyzed by UPLC-MS/MS to calculate the half-life of the model peptides with/without modification. Prior to the peptide concentration assessment, multiple transition ions (daughter ions) for each peptide-of-interest will be identified to optimize the procedure. Further optimization of LC and MS parameter such as the eluent gradient, cone voltage, and desolvation temperature will also be screened prior to the PK analysis to establish maximal sensitivity. The half-life will be calculated based on a standard curve from the pure peptide. To compensate for the matrix effect in serum samples, which decreases the ionization efficiency compared to that of pure peptide samples, stable isotopically labelled internal analogues of the peptides can be used.[57]

Roles allocated to involved participants

The laboratory of Professor Knud J. Jensen will provide facilities, equipment, reagents and expert aide for the synthesis, characterization and purification of the small molecule albumin binders. The same goes for solid-phase peptide synthesis and purification of peptide precursors. Furthermore, access to the instrumentation necessary for biophysical evaluation studies (DLS, SEC, and potentially SAXS) and peptide-HSA interaction studies (SPR) are available through the research collaborations of Professor Knud J. Jensen.

Functional screening, the assessment of plasma stability, as well as the *in vivo* validation of peptide effect will be performed at Gubra ApS, who will also provide facilities, equipment, reagents, animals and the necessary expertise. Likewise, Gubra ApS will provide facilities, equipment, reagents and the necessary expertise to perform PK studies and the following analysis by UPLC-MS/MS.

Other activities

Besides projected-related work, the candidate will participate in scientific exchange meetings, staff meetings, project-development meetings and meetings concerning intellectual property rights hosted by Gubra ApS. The candidate will also regularly participate in group meetings of the Professor Knud J. Jensen group at KU. Finally, the candidate will teach in a bachelor/master course (Bioorganic and Medicinal Chemistry) at KU available for a variety of students studying chemistry.

E. Publication plan

Proposed title and date of publication	Proposals for one or more acknowledged research journals as desired place of publication
Small molecule drugs promote peptide interaction with human serum albumin (01/12/2016)	Journal of Medicinal Chemistry Biochemical Journal
Small molecule drug modified glucagon-like peptide-1 (GLP-1) derivatives have pharmacokinetic properties suitable for once-daily administration (01/12/2018)	Journal of Medicinal Chemistry Biochemical Journal Journal of Pharmaceutical Sciences Diabetes, Obesity and Metabolism
Improving the pharmacokinetic properties of peptide pharmaceuticals by conjugation to short peptides (beltides) self-assembling into nanodiscs. (01/06/2018)	Soft Matter Journal of the American Chemical Society Science
A potent glucagon-like peptide-1 (GLP-1) beltide conjugate exhibit significantly improved pharmacokinetic properties (01/09/2018)	Journal of Medicinal Chemistry Biochemical Journal Journal of Pharmaceutical Sciences Diabetes, Obesity and Metabolism

F. Courses, conferences and stays abroad (max. ½ page)

- Ph.D. courses

Course name	University	Time	Points
Peptide Chemistry	University of Copenhagen (KU)	Nov 2015 or 2017	7.5 ECTS
Analytical Chemistry	University of Copenhagen (KU)	Spring 2016	7.5 ECTS
Advanced Chromatography and Mass-Spectrometry	University of Copenhagen (KU)	Fall 2016	7.5 ECTS
Responsible Conduct of Research (mandatory for all Ph.D. students)	University of Copenhagen (KU)	Sep 2016	1 ECTS
Business Course for Industrial Ph.D. Students (mandatory for industrial Ph.D. students)	Technical University of Denmark (DTU)	Nov 2016	7.5 ECTS

- Conferences, seminars
  - Peptide Therapeutics (PEGS), 2016
  - European Peptide Symposium (EPS), 2016
  - Other relevant, available symposia related to the project if time permits

- Stays abroad

The candidate has expressed a wish to include a stay at a foreign university, which the supervisors fully support. Initial planning is currently ongoing and has not been finalized. The exact destination as well as the timing of the stay will depend on the progress of the project.

G. Dissemination plan (max. ½ page)

- Dissemination plan

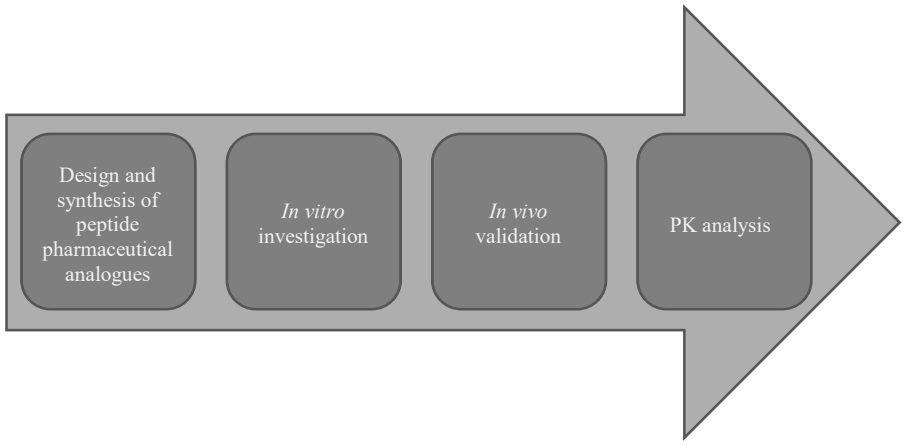
The candidate will communicate knowledge obtained during the project at internal company or KU meetings as well as international scientific symposia. Moreover, the most interesting discoveries of the project will be disclosed in international patent applications and peer-reviewed scientific journals. The time spent on dissemination will be distributed as follows:

- Regular project presentations at internal meetings (approx. 4 presentations a year; 7.5 hours each = 30 hours per year = 90 hours in total)
- Regular presentations of relevant scientific papers for internal journal clubs (approx. 4 presentations a year taking up 7.5 hours each = 30 hours per year = 90 hours in total)
- Communication of research results at international conferences and/or symposia (expect approx. 100 hours in total)
- Presentation of project results in international peer-reviewed journals and/or patent applications (approx. 2-4 papers; 1 month each = 300-600 hours total)
- Preparation of Ph.D. thesis and industrial Ph.D. report (approx. 3 month = 450 hours)
- **Total estimate = 1200 hours**

H. Structure and time schedule (max. 1 page)

- The structure of the education and project, divided into work packages with corresponding milestones and success criteria, and illustrated in a time schedule chart

Flowchart of project phases



Phase 1: Design and synthesis of peptides

The aim of phase 1 is to create peptide drug candidates, which exhibits prolonged systemic half-lives due to either a specific interaction to HSA or by self-assembly into nanodiscs. Phase 1 includes the design, synthesis and purification of peptides. The design, synthesis, and evaluation (phase 1 and 2) of the peptides should be regarded as an iterative process, and will most likely continue throughout large parts of the project. The candidate will perform all of the work required in phase 1 except the functionalization of small molecule drugs for SPPS, which will be performed by an expert in organic chemistry in the group of Professor Knud J. Jensen.

Phase 2: *In vitro* investigation

The aim of phase 2 is to investigate the influence of the modifications on peptide pharmacokinetics and bioactivity using relevant *in vitro* setups. This include the estimation of peptide HSA affinity, analysis of peptide self-assembly, assessment of serum stability, and the evaluation of peptide receptor potency. The

Ph.D. candidate will perform all *in vitro* experiments, except potential SAXS studies, which will be handled by external partners. The investigation of the first peptides is expected to be finalized ultimo Q1 2017. However, like phase 1, phase 2 will continue throughout the project.

Phase 3: *In vivo* validation

The aim of phase 3 is to validate the pharmacological potential of promising peptide candidates identified in phase 2. For this purpose, the acute effect of the compounds in lean mice will be evaluated in relation to food intake, body weight, insulin sensitivity and glucose metabolism. Regarding the work in phase 3, the candidate will provide the test compounds and be involved in the design of the studies, whereas the *in vivo* pharmacology team of Gubra will perform the *in vivo* experiments.

Phase 4: Pharmacokinetic analysis

The aim of phase 4 is to analyze the pharmacokinetic properties of promising peptide candidates based on phase 2 and 3. The PK study will be performed in lean mice and blood samples from the animals will be analyzed by UPLC-MS/MS. Prior to the study, the candidate, in collaboration with the MS/MS expert of Gubra, will develop the PK assay to ensure optimal conditions for peptide detection. The candidate will be involved in the design and planning of the PK study, but the experienced staff of Gubra ApS will perform most of the animal handling. Conversely, the candidate will carry out the following UPLC-MS/MS analysis.

Timetable	2015	2016				2017				2018		
	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3
Phase 1												
Synthesis of small molecule HSA-binders												
Synthesis of peptide analogues												
Estimation of water solubility												
Phase 2												
HSA interaction												
Nanodisc self-assembly												
Functional screening												
Plasma stability												
Phase 3												
In vivo efficacy												
Phase 4												
Development of PK assay												
PK analysis by UPLC-MS/MS												
Miscellaneous												
Ph.D. courses												
Teaching												
Article writing												
Finalization of Ph.D. thesis												

Project timetable: Time allocated to each task during the Ph.D. project presented in a Gantt Chart. Dark blue represent periods where task will be executed. Light blue indicate periods of time where tasks may be prepared for or extended to.

I. Time allocation

Allocation of the Industrial Ph.D. candidate's time	in months	in % of project time
In Danish division of host company	16	44 %
In non-Danish divisions of host company	0	0 %
At other companies or organizations	0	0 %
At the host university	15	42 %
At other universities and research institutions	5	14 %

J. Company (max. 2 pages)

- The company and its activities

Gubra ApS was founded in October 2008. Gubra is a privately held company that delivers services (consulting, pharmacology, histology) for the pharmaceutical industry within the main focus areas of Type 2 Diabetes and Obesity. Gubra has grown organically since 2008 and today the company consists of 45 full time employees. Since its inception Gubra has vested its revenues from its CRO activities into own research (target discovery and early peptide drug discovery projects). On average discovery activities take up approximately 25% of the resources at Gubra. Gubra currently employees 3 industrial Ph.D. students (two are scheduled to finish in 2016 and one in 2018).
Based on early target discovery activities Gubra has initiated a number of early drug discovery projects with the aim to use modified peptide based drugs for the treatment of type-2-diabetes, obesity and other related complications. In this respect, the potential of combining novel peptidomic targets with novel pharmacokinetic improving small molecule albumin binders or beltides are considered of high commercial value.

- The candidate's placement in the company

The candidate will initially reside at the University of Copenhagen, Frederiksberg Campus, Department of Chemistry in the group of Professor Knud J. Jensen. The initial work will involve automated peptide synthesis, manual peptide modifications, and purification of peptide products. These subjects are among the core competences of the Professor Knud J. Jensen group, and both equipment and expert aide are readily available. Furthermore, the equipment required for the biophysical evaluation planned for phase 2 is available at the Department of Chemistry. After phase 1 and the biophysical evaluation of phase 2, the Ph.D. candidate will relocate to Gubra ApS, Agern Allé 1, 2970 Hørsholm, Denmark. Gubra specialize in the fields' obesity and type 2 diabetes, and provide consultation, histology and pharmacology for customers. The company therefore employ personal extraordinarily qualified for performance of the *in vivo* validation of the peptide pharmaceuticals (phase 3), and the following analysis of their pharmacokinetics (phase 4). Furthermore, Gubra have their own animal facility, which facilitates access to the animals required in the phase 3 and 4 studies. The same is true for the UPLC-MS/MS essential for phase 4.

- Any exit strategy

Should Gubra ApS collapse during the course of the Ph.D., the candidate will continue at KU and seek partnership with other companies with focus on peptide pharmaceuticals. However, a bankruptcy is highly unlikely as Gubra have shown stable growth over the last seven years and have several long-term partnerships.

K. University (max. 1 page)

- Description of the university and centre / institute

The university connected to the Ph.D. project is the University of Copenhagen. More specifically the group of Professor Knud J. Jensen, Section for Chemical Biology and Nanobioscience, Department of Chemistry. The focus of the group is the interface between synthetic chemistry, biology, and biophysics, with a strong emphasis on peptide chemistry. The major subjects being researched in the group include the following:

- Usage of non-native ligands for self-assembly of peptides and control of their nanoscale properties.
- Peptide medicinal chemistry, with focus on hormones involved in energy metabolism such as GLP-1, amylin, and PYY_{3-36}.
- Design, synthesis, and utilization of new belt-peptides for the formation of nanodiscs.
- Development of new or improved techniques for solid-phase peptide synthesis.
- The incorporation of advanced biophysical methods (e.g. surface plasmon resonance biosensing and bio-layer interferometry) for the determination of peptide – protein interactions.

The group currently consist of 12 scientists, including four Ph.D. students. Several of these people are actively engaged in the field of medicinal chemistry. The Ph.D. students of the group engage in the daily work routines, as well as the weekly group meetings, which involves presentations of scientific articles, project updates, and conference reviews.

L. Third parties (max. 1 page per third party)

- Description of any third party

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