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Microfluidic Peptide Synthesis on Magnetic Beads

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Abstract

A collaboration between the Becker Group of the Institute of Biological Chemistry of the University of Vienna and Efficient Robotics GmbH aims to design a microfluidic peptide synthesizer capable of massively parallel solid phase peptide synthesis (SPPS), for use in ultra-high-throughput screenings of peptides. The synthesizer would use magnetic beads as the solid phase for SPPS, enabling magnetic manipulation of the beads within the synthesizer, and droplet microfluidics for parallel synthesis and to elute the synthesized peptides directly into isolated droplets. Compared to previously existing microarrays and other in-situ synthesis strategies for high-throughput screening, which require additional steps for solution-based assays, this new method has the potential of rapidly synthesizing tens of thousands of peptides daily for direct use in solution-based assays.

For this master's thesis, three linkers HMBA, HMPB and HMPA were compared for SPPS of a test peptide on magnetic beads, in both manual synthesis and on a proof-of-concept microfluidic hand model. In addition, the viability of fluorescent markers FITC and NBD for labelling peptides synthesized on magnetic beads was determined. It was concluded that base-labile HMBA and FITC were most suitable candidates.

A synthesizer prototype from Efficient Robotics was also tested in Kornwestheim, revealing further challenges that need to be overcome.

Zusammenfassung

Die Becker Gruppe vom Institut für Biologische Chemie der Universität Wien und Efficient Robotics GmbH kollaborieren in einem Projekt mit der Zielsetzung einen mikrofluidischen Peptidsynthesizer für die hoch-parallele Festphasensynthese von Peptiden (SPPS), für Ultra-High-Throughput Screening von Peptiden zu entwickeln. Der Synthesizer soll magnetische Kugeln als feste Phase verwenden, damit diese im Synthesizer mittels Magnetfeldern kontrolliert werden können. Auch soll Tröpfchen-Mikrofluidik eingesetzt werden, um Parallelsynthese und die direkte Elution von Peptiden in Tröpfchen zu ermöglichen. Im Vergleich zu bestehende in-situ Methoden wie Microarrays, welche zusätzliche Schritte für Einsatz in löslichen Assays benötigen, soll diese neue Methode potenziell täglich mehrere zehntausende Peptide für den direkten Einsatz in homogenen Assays bereitstellen.

Für diese Masterarbeit wurden drei Linker (HMBA, HMPB und HMPA) für die SPPS eines Testpeptids auf magnetische Kugeln verglichen, sowohl manuell, als auch auf einem einfachen, handbetriebenen Modell des Synthesizers. Auch wurde die Nützlichkeit der Fluoreszenzmarker FITC und NBD für die Methodeetbalierung evaluiert. Der basenlabile Linker HMBA und der Fluorophor FITC waren die am besten geeigneten Kandidaten.

Ein Synthesizer-Prototyp vom Efficient Robotics wurde in Kornwestheim getestet, wobei weitere Herausforderungen entdeckt wurden, die im weiteren Entwicklungsprozess berücksichtigt werden müssen.

Abbreviations:

ACN	Acetonitrile
Boc	*tert*-Butoxycarbonyl
DCM	Dichlormethane
DIC	Diisopropylcarbodiimide
DIEA	Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	Dimethylformamid
ER	Efficient Robotics
FITC	Fluorescein-Isothiocyanate
FLD	Fluorescence Light Detector
Fmoc	9-fluorenylmethoxycarbonyl
HBTU	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMBA	4-(Hydroxymethyl)benzamide
HMPA	Hydroxymethylphenoxyacetic acid
HMPB	4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid
HPLC	High-pressure liquid chromatography
MS	Mass Spectrometry
NBD	7-Nitrobenzofurazan
PBS	Phosphate Buffer Solution
SPPS	Solid Phase Peptide Synthesis
tBut	*tert*-butyl
TFA	Trifluoracetic acid
TIPS	Triisopropylsilan

Introduction

Solid phase peptide synthesis (SPPS)

Peptides have long been a subject of interest in biology, medicine and other fields[3]. As ribosomal protein synthesis is limited to proteinogenic amino acids, chemical synthesis is thus necessary for peptides and proteins with non-proteinogenic amino acids, post-translational or other modifications.

Since Merrifield first developed solid phase peptide synthesis (SPPS) in 1963[1], it has rapidly become the dominating method for peptide synthesis[2]. By fixing the peptide chain to a solid phase during synthesis and elongation by addition of amino acid building blocks, application and removal of reagents in a liquid phase can be easily carried out via washing while the peptides are retained on the solid phase, greatly simplifying peptide synthesis compared to solution-based synthesis.

The synthesis direction in SPPS is from C- to N-terminus, attaching the carboxyl group to the solid phase, though it is possible to alternatively link an amine (or other functional groups) side chain to the solid phase for C-terminal modifications.

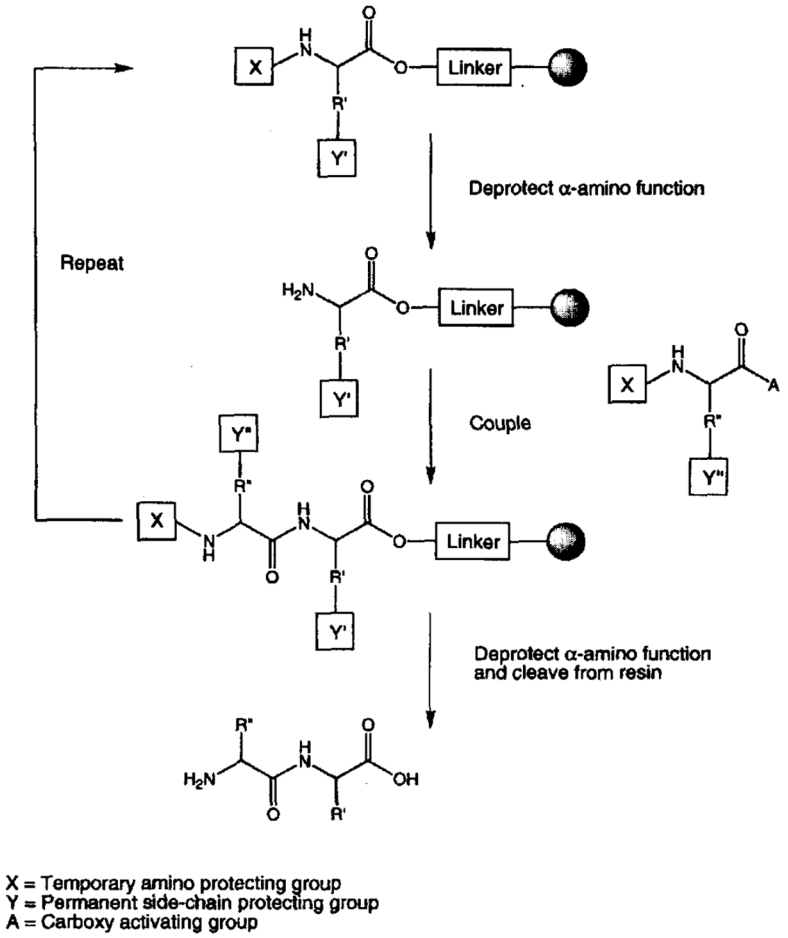


Figure 1 SPPS strategy[2]

As depicted in Figure 1, the SPPS cycle for each amino acid starts with deprotection of the peptide on the solid phase, followed by coupling of an activated amino acid, with washing off excess reagents in between. The α-amino groups of the newly added amino acids are blocked by a temporary protecting group that will be removed in the deprotection step of the next cycle, after excess amino acids have

been removed. The functional groups of amino acids are protected by permanent side protecting groups that will only be removed after all amino acids have been coupled.

The two most common SPPS strategies are Boc/Bz and Fmoc/tBu chemistry.

In the Boc/Bz strategy, acid-labile *tert.*-butoxycarbonyl (Boc) protects the α-amino group and more acid-resistant benzyl (Bz) several functional groups on the side chains. In the deprotection step, Boc is cleaved from the α-amino group with an acid such as trifluoracetic acid (TFA), which is unable to cleave the side protecting groups. Upon completion of peptide elongation, HF (or super acid mixtures) is used to deprotect the side groups. The use of toxic HF is a major safety concern and requires the use of HF-resistant lab equipment, and may cause unwanted reactions such as cleavage of (posttranslational) modifications or fluorophore damage.

The Fmoc/tBu method uses base-labile fluorenylmethoxycarbonyl (Fmoc) to protect the α-amino group and acid-labile tert-butyl (tBu) side protecting groups. Final deprotection of side chains is possible with milder acids such as TFA instead of HF, while Fmoc can be removed with weak organic bases, typically 20% piperidine in dimethylformamide (DMF). Fmoc removal leads to formation of dibenzofulvene, which can be quantified via UV absorption at 301nm to measure cleavage yield and efficiency.

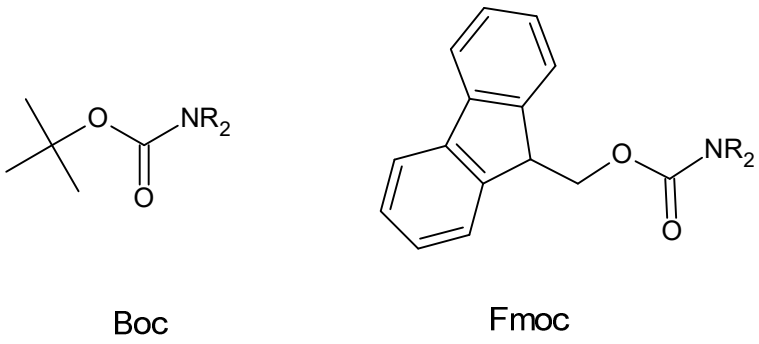


Figure 2 Boc and Fmoc

Alternative α-amino protecting groups are also possible, but often lead lead to lower yields compared to Boc and Fmoc, such as photosensitive amino protecting groups for photolithographic synthesis. Similarly, other side protecting groups requiring different deprotection conditions can be employed.

For the solid phase, several polymer resins with various properties are widely and commercially available, usually based on polystyrene, with amine groups or pre-attached linkers on the solid phase for coupling the peptide and substitution levels between 0.1-2 mmol/g.

A large variety of linkers are available for reversible coupling of the peptide chain to the solid phase. Acid-labile linkers such as HMPA enable simultaneous side chain deprotection and cleavage from solid support when using either Boc or Fmoc methods. Meanwhile, base-labile linkers such as HMBA[3] permit deprotection of the peptide with TFA while remaining on the resin, for instance for use in solution-based peptide assays (see below), after which the peptides can be cleaved from the resin using an aqueous base if necessary.

For amino acid coupling, the carboxylic group of the new amino acid is activated as an active ester with a suitable coupling reagent. Multiple different activators have been used in literature, such as carbodiimides or uronium salts[6]. The activated carboxylic group then reacts with the exposed amine of the preceding amino acid to form the peptide bond.

Chemical and post-translational modifications of amino acids may be present on the building blocks pre-coupling or can be introduced via an additional step following or during SPPS. For C-Terminal modifications, special resins, linkers and strategies have been developed to apply modifications directly during SPPS as well[3,5].

At room temperature, coupling times of around 20-30 min are required for optimal yields, depending on the amino acid. With microwave-assisted SPPS[7,8], reactions can be performed at higher temperatures up to 90°C and coupling times can be reduced down to less than 2 min and other steps can similarly be shortened.

Even with the high efficiency of SPPS, side products are not washed off and accumulate on the solid phase alongside the desired peptide chain, leading to decreasing product yield with increasing peptide length. Thus, while it is possible to synthesize longer peptide chains with over 100 amino acids, synthesis is normally limited to chains of up to around 50 amino acids length Longer peptide chains, such as for synthetic proteins, can instead be assembled with ligation of shorter peptide chains[9], common methods include native chemical ligation, coupling a cysteine N-Terminus with a thioester.

SPPS can be easily automated, with around 2-5 min per amino acid coupling when using microwave assisted coupling procedures. Both batchwise and continuous flow synthesis can be carried out. Common amino acids with standard protecting groups are commercially available at decent prices, permitting cheap and affordable synthesis of peptides, while uncommon ones (for instance with non-standard protecting groups or post-translational modifications) can be expensive or require synthesis in the lab.

Peptide-based Assays

A peptide assay screens a library of peptides against a target, usually a protein. The peptides are exposed to an excess of the target, whereupon peptides may successfully interact with the target. Successful interactions with the target are then detected to find hits for further investigation. Detection is commonly carried out with fluorescence markers attached to or reacting with the target, with high sensitivity and versatility. There are also other methods of detection such as electrochemical, or enzymatic. Immunoassays are a common example of a peptide/protein binding assay, taking advantage of the highly specific binding affinity of antibodies to antigens.

It is possible to distinguish between solid-based assays and solution-based assays. In solid-based assays, peptides are fixed on a surface and incubated with an excess of target molecules in solution. After washing to remove unbound molecules, the peptides can be screened for successful interactions. In solution-based assays, the peptide is in solution as well, useful for cellular bioassays.

Given the large number of possible amino acids combinations even in short peptides and without factoring in additional peptide modifications, it is not feasible to synthesize all possible peptides. Peptide libraries instead limit the number of peptide sequences based on either previous knowledge or combinatorial approaches[10]. For larger peptide libraries, high-throughput screening is thus a necessity to cover as much peptide space as possible in a reasonable amount of time. Ideally, only a minimum amount of each peptide is synthesized to reduce costs. Due to the high sensitivity of modern analytical instruments even small amounts are sufficient for screening approaches.

One option for proteinogenic peptides is phage display[11] or peptide display techniques such as mRNA display[12]. In phage display, a nucleotide sequence is inserted into a phage gene encoding a surface protein, expressing the peptide on the phage surface. It is possible to generate large phage libraries expressing different peptides for use in screening assays, with the genome of selected phages decoded

to determine the peptide sequence. Alternate vectors or mechanisms to identify the peptide can also be employed, such as in mRNA display, where the mRNA is modified to directly bind with the peptide during translation and used to identify the peptide after screening.

Microarrays are a common high-throughput method for assays, with peptides immobilised on discrete spots on a surface. Readily prepared peptides could be attached to the solid support, but this is unfeasible for synthesizing large numbers of different peptides, as would be required in high-throughput screening, and would require a spotting step. Since SPPS already binds the peptides to a solid phase, bypassing the spotting step, in-situ synthesis is generally more preferable. The synthesis needs to be clean and avoid side products accumulating alongside the desired peptide that would interfere with the assay[10]. Multiple methods have been developed, such as the SPOT array[16,17] on cellulose support, microwells, or photo-lithographic printing, capable of a million of spots per cm^2 [10,13]. New methods are being developed as well, including a combinatorial peptide microarray with microfluidic impact printing described by Li *et al.*[18,19].

Aside from large, flat surfaces, another method is the One Bead One Compound (OBOC) method[14,15]. Beads with peptide linkers are divided into separate reaction vessels and coupled with different amino acids, then combined and divided again into multiple reaction vessels for the next cycle of deprotection and coupling. As a result, each bead surface carries a single random peptide, and the beads can be rapidly screened in an assay. Selected beads with a positive signal can then be sequenced to identify the peptide. A large number of beads is needed for longer peptides, due to the random combinatorial nature of the peptide library and repeats for redundancy, increasing time needed for screening and sequencing.

It is possible to cleave peptide from solid phase, for solution-based assays[15,16]. This requires additional steps to prevent peptides from different synthesis sites from mixing and reduces the number of synthesized peptides. In SPOT, for instance, cellulose spots containing the peptides are removed from the cellulose sheet and transferred into microwells where the peptides are cleaved into solution, requiring larger spots compared to surface assays. Beads from OBOC will similarly require transfer into individual microwells before peptide cleavage. Despite the extra challenges, solution-based screening does confer advantages, as the peptides are able to adopt native conformations in solution, with all peptide orientations accessible for screening. The solvated peptides can also be more easily used for screening with cellular assays compared to fixed peptides[15].

Most in-situ methods require large and highly expensive automated robotic equipment to operate, limiting the rate of peptide assays that can be synthesized in a lab.

Microfluidic Systems

Microfluidic technology involves the behaviour and control of fluids at micrometre scale or below and has found a broad range of applications in diverse fields, particularly biotechnology. It can be split into continuous flow and droplet microfluidics.

Continuous flow microfluidics involves mixing continuous streams of fluid in microscale channels[20]. Mixing can be either slowly in laminar flows via diffusion, or quickly via inducing turbulence, usually passively with shape of the microfluidic channel but also actively through excitation with an external force such as ultrasonics. Immiscible fluids can also be mixed, and then separated into different channels, for instance for purification steps. Conditions in the microchannels can be precisely controlled, and multiple reaction steps can be performed in succession.[20]

In droplet microfluidics[24,25], one fluid is suspended as droplets or an emulsion in another fluid, with each droplet acting as a compartmentalised reaction space. Depending on the droplet generation method, droplets can be monodisperse or polydisperse, continuously generated or on-demand.

The separation fluid is usually an oil for biological applications, either hydrocarbon or fluorocarbon, with a surfactant added to stabilize droplets and modify properties of the separation fluid and phase interface[24]. Fluorocarbon oils are immiscible to many organic solvents, providing an option for organic solvent droplets. Depending on the separation fluid and surfactant, small molecules such as oxygen can diffuse through the separation fluid, which can be advantageous or cause issues.

The large area to volume for surface interactions between droplets and separation fluid are useful for catalytic or multiphase reactions but can cause issues such as accumulation of reagents on the surface instead of in droplet.

For biology, droplet microfluidics is especially advantageous due to being able to contain individual cells within each droplet, for example in directed evolution experiments, where millions of cells, each with a different random mutation, can be individually processed in a short span of time[24].

Due to the small scale of the microfluidic channels, heat transfer and mass exchange can be fast and precisely controlled, allowing both faster and more specific reactions. In addition, the low reagent volumes reduce cost and produce less waste, increasing safety. Intermediate hazardous products can also be immediately used for the following reaction steps. The high surface-to-volume ratio in microfluidic systems is similarly advantageous for catalysts or phase-barrier reactions.

The most common materials for microfluidic systems are PDMS and glass, with surface modifications to change properties as needed. Other materials have also been successfully implemented, such as Teflon[28]. A variety of microfabrication techniques for designing and creating microfluidic systems are likewise available, such as soft lithography, often derived from other fields such as semiconductors or printing[23].

Additional functions can be incorporated into the microfluidic systems, such as in-line/online detection, instead of requiring external detection devices, and droplet selection. Microfluidic components can also be coupled with non-microfluidic external devices such as mass spectrometers.

Complexity rapidly increases as the microfluidic system is scaled up. Microfluidic components are designed for a specific application, and it is currently difficult to program them to be more versatile and flexible. There are also compatibility issues between different components. Many of these issues can be solved by integrating individual components onto a single device, such as a chip, leading to the concept of “Lab-on-a-chip”[20,27].

As an example, such integrated microfluidic systems can use microvalves to control a control layer of hydraulic microvalves that in turn regulate the flow of the actual microfluidic system[26]. The system is thus capable of using only a small number of externally controlled valves to control a large number of channels, especially for mass parallelisation of fluid channels.

Microfluidic and Parallel Synthesis

Microfluidic systems have been developed for synthesis of various nanomaterials, polymers, droplets, and other products. The microfluidic parameters, reagent flow rate (which determines reaction time in the microfluidic channel), reagent concentrations and reaction temperature, are used to adjust reaction conditions. Compared to macroscopic batch reactions, this offers better control over the reaction and product properties. It is possible to synthesize polymers such as polypeptides[21] with lower

polydispersity than batch reactions, as well as create nanostructures in particles, among other possibilities.

A major limitation of microfluidic synthesis is scaling up the synthesis for commercial and industrial applications. Either massive parallelisation or transitioning to macro-scale methods is required to produce significant quantities of product, both of which come with their own challenges[27]. For homogenous single-phase reactions where surface area is not a concern, microfluidic channels also do not necessarily improve the reaction compared to traditional macroscopic methods[22]. As such, macroscopic batch synthesis will not be fully replaced by microfluidic methods.

For ultra-high-throughput screening, microfluidic synthesis of the library would have several advantages. Mass parallelisation is useful to produce large number of different compounds in a short timeframe, while the low synthesis volumes reduce synthesis costs.

Peptide Synthesis in Microfluidic Systems

Cell-free protein synthesis has been successfully demonstrated in microfluidic systems, using ribosomes and reagents to synthesize a protein from a DNA template linked to microbeads in the microfluidic channel[28], with RNA-polymerase in the reaction mixture transcribing DNA to RNA for protein translation.

For synthetic peptides, SPPS using continuous flow microfluidic systems had been successfully demonstrated by Zheng et Wang in 2013[29], using a Teflon microfluidic chip with a reaction chamber where the solid phase, special rigid resin microbeads rather than conventional resin to prevent occlusion, was trapped. Coupling time was able to be reduced to 20 min compared to 1-2 h in conventional SPPS without heating.

For high-throughput synthesis requiring high-speed parallel synthesis of different peptides, continuous flow microfluidic systems face the challenge of selectively directing simultaneous flows of different amino acids with coupling reagents into their respective reaction chambers. While an alternative is to simply couple one specific amino acid at a time, it would greatly slow down the parallel synthesis.

In addition, the use of swelling resins requires diffusion of reagents into and out of the resin, limiting the minimum time required for amino acid coupling.

Magnetic Beads as Solid Phase

Magnetic beads are generally composed of a magnetic core protected by a resistant shell, whose surface often features functional groups or ligands, such as H_2N- or COOH-groups for covalently binding of cargo molecules such as peptides, proteins, DNA…. Their size is usually in the nanometre to micrometre range. Beads with superparamagnetic cores, which have no magnetic properties outside of an external magnetic field, have the advantage of behaving like non-magnetic beads when the external magnetic field is turned off, enabling easier bead manipulation and recovery[31].

As magnetic fields from permanent magnets or electromagnets can easily and selectively manipulate the magnetic beads, they have found numerous applications in biotechnology[30], for instance in detection and binding assays. Peptides or proteins can be fixed to the beads using standard ligation methods, alternatively beads precoated with common binding ligands such as streptavidin are commercially available as well. Target peptides or proteins interacting with ligands on the beads can then be bound to the beads, for detection, purification or enrichment as needed.

Magnetic beads have been successfully used in microfluidic analytical systems in various methods[31,32], as a purification and selection tool while also greatly increasing detection sensitivity by concentrating the signal onto the beads.

Little work has been found for peptide synthesis on such magnetic beads, as the beads are more expensive than conventional PEG resins, in addition to lower loading rates and handling issues, such as irreversible bead agglomeration when centrifuged or dried.

Nano-Titer-Pipe (NTP) High Throughput Screening

As an example of a commercial droplet microfluidics device, the company Efficient Robotics (https://www.efficient-robotics.com) successfully developed the Nano-Titer-Pipe (NTP) Screening Station, a fully automated system for high throughput screening for biotechnology, e.g. cell screening or directed evolution, capable of processing tens of millions of samples within 24 h.

The droplet microfluidic system uses fluid-fluid segmentation and compartmentalisation, with 2 immiscible phases (oil and water) as compartment and separation fluid. Using electronically controlled microvalves, fluid flow is pressure pulse-controlled, whereby the pulse length can vary from microsecond to second, allowing for injection of fluid volumes down to 1nL.[33]

The NTP Screening Station is capable of precise droplet mixing, as well as in-line fluorescence detection, with fluorescent detection sensitivity down to picomolar range, and droplet selection. Droplets with single cells can thus be mixed with a precise amount of reagents, incubated for a set amount of time before being analysed and sorted for successful hits in an automated workflow.

Collaboration with Efficient Robotics

A collaboration between ER and the Becker Group at the University of Vienna aims to develop a parallel microfluidic synthesizer using magnetic microbeads and droplet microfluidics for high-throughput peptide synthesis and in-solution screening. The final model is intended to synthesize medium-sized (8-20 aa) peptides in 1000 synthesis spots at a time and a daily synthesis rate of over 50,000 peptides. Following *in-situ* cleavage from the magnetic beads in the synthesis spots, the peptides can be stored or directly used in an assay without need for further processing, for instance with the NTP Screening Station.

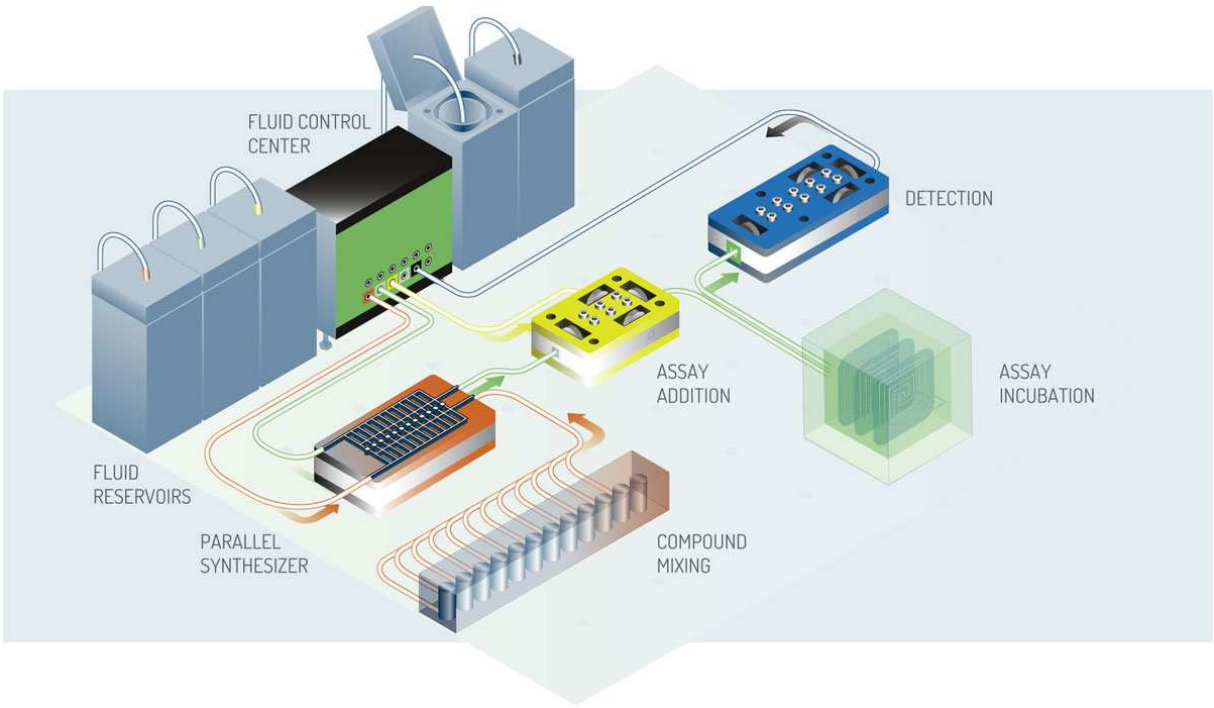


Figure 3 Concept of SPPS Parallel Microfluidic Synthesizer incorporated into NTP Workstation of Efficient Robotics

The use of magnetic beads as the solid phase rather than conventional resin will permit magnetic manipulation of the beads for fixing in and removal from synthesis spots before and after peptide synthesis and cleavage respectively. In addition, it is expected that SPPS on the surface of the magnetic beads, will avoid the need for reagent diffusion through the solid phase and thus significantly shorten reaction and washing times. The lower amount of peptides available from magnetic beads compared to conventional resin is expected to be still sufficient for ultra-high-throughput screening purposes.

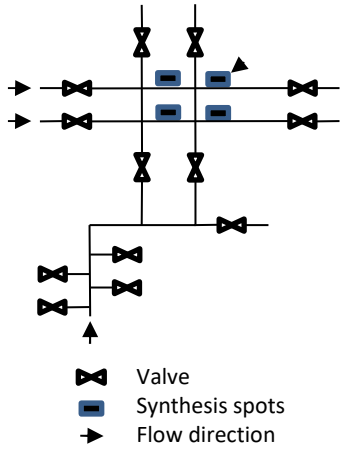


Figure 4 Example 2x2 Scheme for Parallel Peptide Synthesis (Source: ER)

Droplet microfluidics would enable simultaneous parallel couplings of different amino acids in each synthesis spot, as droplets containing the desired activated amino acid can be selectively directed to the individual synthesis spots without risk of cross-contamination. At the cleavage step, the lower volume of the droplets compared to continuous flow cleavage would also increase the peptide concentration, and the peptide-containing droplets simplify further use in solution-based assays.

For highly parallel peptide synthesis, to reduce the number of parts required, a proposed grid layout of synthesis spots would greatly reduce the number of valves per synthesis spot as the number of synthesis spots increases while still controlling individual spots. [26] As an example, Figure 4 shows a 2x2 layout with 8 valves controlling 4 synthesis spots. On the bottom of the figure, droplets are injected into the stream of separation fluid in the main channel from their respective inlets, where they are then directed towards the synthesis spot grid. By toggling the appropriate valves to enable and disable fluid flow, each droplet can be directed down the appropriate horizontal and vertical channel to the designated synthesis spots on the grid, then eluted from the system without passing through the other synthesis spots (for instance to the upper right spot by directing the droplet up along the right vertical channel, then redirecting it along the upper horizontal channel once it arrives at the intersection in a left-to-right flow of separation fluid). Scaling up, a 33x33 grid containing 1089

spots would require only 132 valves for the synthesis spot grid. This would require precise detection and direction of droplets into specific channels, based on technology already used in the NTP Screening Station from Efficient Robotics[33].

Previous work by Mag. Manuel Felkl, Ines Zettel and Sunil Kavunga has demonstrated the viability of SPPS synthesis on magnetic beads, both manually and using a proof-of-concept model. Test peptides 5-10 amino acids long were successfully synthesized.

This master's thesis continues optimising the method used. Three linkers HMPB, HMPA and HMBA were compared to determine the most suitable candidate and peptides were successfully labelled with fluorescent markers FITC and NBD. A recently developed prototype synthesizer from ER was also tested, providing valuable information for further development.

Experimental Protocol:

Materials and Equipment

The magnetic beads used were Magnabind beads (1-4 µm diameter, loading level 240 µmol/g). Unlike the resin used in SPPS, the beads agglomerate irreversibly when dried, frozen or centrifuged. To remove excess solution, a strong magnet was used to agglomerate the beads on the side of the Eppendorf tube. Depending on the solution, this could happen rapidly or require several minutes of sedimentation.

Figure 5 Magnabind beads aggregated in DMF solution

Linkers

HMPB and HMPA are similar Fmoc-linkers that can be cleaved using different concentrations of TFA. HMPB can be cleaved with dilute TFA/DCM, without deprotecting side chain functional groups. HMPA requires concentrated TFA for cleavage. Previous work used HMPA, which served as benchmark here. Cleavage was carried out with either concentrated TFA or TFA diluted in DCM.

HMBA linker is base-labile and stable in concentrated TFA. It is thus possible to remove the (acid-sensitive) protecting side groups on the peptide while it is still linked to the solid phase, followed by eluting the peptide directly into a basic aqueous solution. An aqueous solution would be useful for the synthesizer, as it can be neutralised with a buffer solution and used directly for screening. The peptide could be successfully cleaved using 0.1 M aqueous NaOH. Other bases are also effective, providing alternate C-terminal modifications if desired.[3]

Manual Synthesis

Coupling Linkers

All three linkers were coupled overnight using the same protocol. 50 mg beads (~12 µmol amine) stored in H_2O were washed four times with DMF/DCM 1:4 and suspended in 1 ml DMF/DCM 1:4. 16.7 equivalent (eq.) linker (200 µmol) and 16.7 eq. DIC (200 µmol, 31 µl) in DMF/DCM 1:4 were added to the beads and the suspension was stirred at least 18 h overnight. The beads were then washed four times with DMF/DCM 1:4.

Coupling Amino Acids to beads

The first coupling solution consisted of the first residue (Fmoc-Gly), DMAP and DIC in DMF/DCM 1:4. For 50 mg of beads in 1 ml DMF/DCM 1:4, 14.6 eq. Fmoc-Gly (175 µmol, 52 mg), 14.6 eq. DIC (175 µmol, 27.8 µl) and 2.5 mg DMAP in 100 µl DMF was added and the reaction mixture agitated for 2 h at RT. After washing the beads with DMF, uncoupled linkers were capped with an acetyl group using 1 ml 20% Ac_2O in DMF with 0.25 mg DMAP (dissolved in 10 µl DMF). The coupling of further residues was based on the standard Fmoc SPPS synthesis, with reduced reaction times: Fmoc was removed with two 20% piperidine in DMF solutions over 2 min and 4 min respectively. Following multiple washings with DMF, the amino acid coupling solution (per 50 mg beads 125 µmol Fmoc-aa, 0.5 M HBTU (2.38 eq.) in DMF, 5 eq. DIEA, 3 min reaction time for activation beforehand) was added, and the beads rotated for 15 min. The test peptide GNASG was synthesized and used for further steps. The beads were stored at 4°C in DMF.

Labelling with Fluorescent Markers

Two fluorescent markers, FITC and NBD, were used to label the test peptides.

Labelling with FITC: Prior to coupling FITC, an additional ß-Alanine (ßA) was coupled onto the test peptide. After Fmoc-deprotection, an excess of FITC solution (19.5 mg FITC (0.05 mmol) in 250 µl DMF, 171 µl DIEA) was then added to the beads. The FITC solution was removed and the beads washed with DMF. The initial reaction time was >8 h (overnight), which would be unfeasible for the desired screening rate of the synthesizer. Shorter reaction times and reduced FITC concentrations (to reduce material waste) up to 15 min were used to determine their feasibility.

Labelling with NBD: Following Fmoc-deprotection, 100 mg NBD-Cl and 10 µl DIEA in 1 ml DMF was added to 5 mg beads. After 2 h reaction time, the NBD-solution was removed and the beads washed with DMF.

Cleavage

Cleavage of peptides from HMPB/HMPA was carried out both with and without deprotection of side groups.

For full deprotection of the peptide, beads carrying the peptide were suspended in TFA/H_2O/TIPS 95/2.5/2.5 (95TFA) for 2 h. To retain side protecting groups during linker cleavage, 1%TFA in DCM without scavengers (1%TFA) was used as cleavage solution with 15 min cleavage time. Following linker cleavage, the beads were agglomerated using a magnet, followed by transfer of the mostly bead-free solution containing the peptides to a new Falcon Tube or Eppendorf tube depending on volume. The solution was diluted with H_2O or H_2O/ACN 50/50, then frozen in liquid nitrogen and lyophilised overnight. The dried residue containing peptide and leftover beads was resuspended in H_2O/ACN 50/50.

Cleavage of HMBA was carried out using 0.1 M aqueous NaOH for 30 min. After separation of beads and solution, the basic solution was neutralised with an equivalent amount of 0.1 M HCl. The peptide solution was used directly for analysis without any further preparation.

Analysis

The solution was centrifuged to remove the beads and transferred into sample tubes for further analysis. Peptides were analysed on the Waters LC-MS with both direct injection (DI) and liquid chromatography (LC) on C4/C18 Columns with a gradient from 5-65% ACN over 10 min.

To determine optimal excitation and emission wavelengths for the fluorescence markers, a fluorescence spectrometer was used. Excitation and emission spectra were recorded for both FITC and NBD in PBS buffer, DCM and 1%TFA in DCM.

Fluorescently labelled peptides were analysed on using Dionex HPLC-FLD. As with the MS, C4/C18 columns were used with a gradient from 5-65% ACN over 40 min. As contaminants in the sample may degrade columns rapidly, lower quality purification columns were used instead of higher quality final analysis columns where possible.

Proof-of-Concept Model

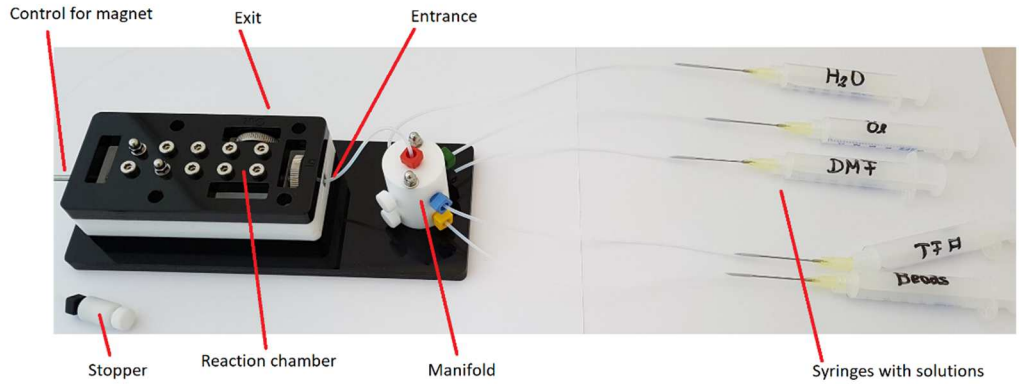


Figure 6 Proof of Concept Model

The proof-of-concept model, as shown in Figure 6, comprises a manifold with multiple inlets for injection of solutions, followed by a reaction chamber near a permanent magnet. By adjusting the distance of the permanent magnet to the reaction chamber with the use of a rod, a magnetic field in the reaction chamber can be activated or deactivated. Due to limits of manual control, the system is closer to a continuous flow system than droplet system.

Before starting synthesis, a suspension of beads was slowly injected into the reaction chamber with the magnet activated, where the beads are retained by the magnetic field. Excess beads were washed out with slow DMF flow after a few minutes. The eluted beads were collected and reused for later injections into the model.

Synthesis on the proof-of-concept model used the same solutions as manual synthesis. Both deprotection and coupling steps were shortened to 90 s each, with 45 s stationary without flow followed by 45 s slow flow for coupling. The reaction chamber was washed between steps with at least 90 s steady DMF flow. In order to reduce contamination, every entrance was briefly flushed with DMF to clean the dead volume. Due to concerns regarding TFA, peptide cleavage was done outside the proof-of-concept model. Upon completion of synthesis, the magnet in the reaction chamber was deactivated. After a few minutes to ensure demagnetisation of the beads, the beads were displaced from the reaction chamber with DMF at high pressure and collected in a 500 µl Eppendorf tube. Due to the small quantity of beads, 100 µl cleavage solution was used.

ER Synthesizer Prototype

In preparation for the experiments at Efficient Robotics in Kornwestheim, a number of preliminary tests were carried out in Vienna.

A short series of cleavages were carried out in the presence of the separation fluid used in the synthesizer. The goal was to determine the feasibility of using said fluid, as ER had previously used only aqueous solutions in combination with the separation fluid. The separation fluid was a Novec oil, with additional surfactants added to improve performance.

100 nM and 10 nM FITC in PBS, 1%TFA, and DCM were measured on the fluorescence spectrometer. The same measurements were carried out for NBD. A shifted emission maximum could be detected, and fluorescence detection was expected to work in the prototype as well.

Around 50 mg beads were used to synthesize FITC-labelled peptides for tests with the prototype. Another 50 mg beads were used to synthesize NBD-labelled peptides but were not used due to time constraints. Unused magnetic beads were sent to ahead to ER for use in the prototype.

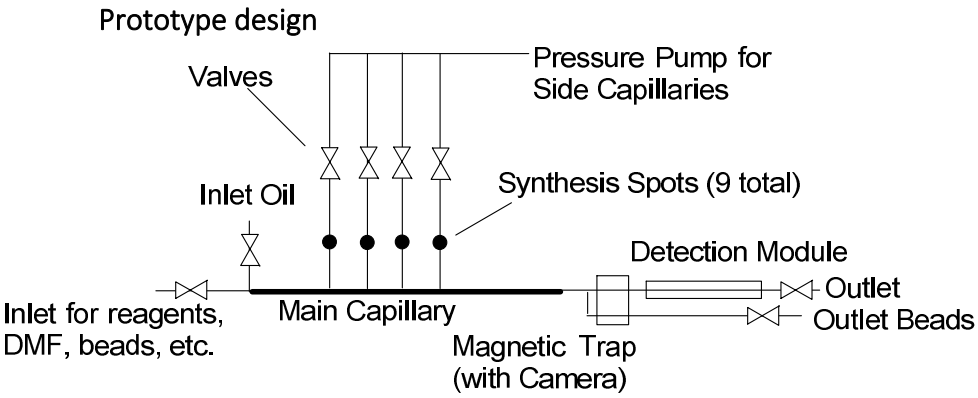


Figure 7 ER Prototype Layout

Figure 7 shows the basic layout of the ER prototype. Fluids would be pushed through the system by pressurizing the containers of the different reservoirs (inlet pressure, oil pressure, spot pressure of side capillaries). The flow was controlled by opening and closing the necessary valves. Individual valves could be controlled independently, and pressure could be set for each reservoir between 0.1 and 2.5 bar. Fluid separation was done using Novec oil from the *Inlet Oil*.

Other inlets were attached to reservoirs stored in a pressure container, using overpressure in the container to inject the fluids into the main capillary. They were initially mostly interchangeable. The exception was a designated inlet for the bead suspension, whose valve was able to transport beads. The remaining inlet valves were magnetic valves, which would likely be blocked by magnetic particles. Other inlets were intended for DMF, cleavage solution, and other reagents. The primary outlet was not designed for bead transport, and beads were to be flushed out through *Outlet Beads*.

Each side capillary contained a synthesis spot, where beads could be captured by a toggled magnet. The spots each have a spot valve, with a long tube length between synthesis spot and spot valve to act as a buffer for fluid in the side capillaries. The reservoir on the other side of the valves is filled with Novec oil, which is used to flush each spot.

The magnetic trap used an external magnet to capture magnetic particles passing through the tube, using a camera to observe bead accumulation. The detection module illuminated the fluid passing through using an LED lamp and measured the fluorescence. Excitation and emission wavelengths were set using available filters. For fluorescein in PBS, excitation filter 475/35 and emission filter 530/43 were used.

ER Experiments

The goal was to cleave peptides from beads in the synthesis spots, leading to quantification of cleavage yields and bead quantity in the synthesis spots.

Initial tests on the prototype used FITC in PBS buffer. The fluorescence detection module and control mechanisms functioned properly and gave good results. Problems arose during following tests with suspended beads, as well as the use of chemical solvents and TFA. Certain valves and the fluorescence detection module were damaged by the chemical solvents and TFA and had to be replaced. In addition, the magnetic beads tended to aggregate in the microfluidic tubes under certain conditions, eventually leading to irreversible clogging.

Due to these issues, the prototype required multiple redesigns to rectify the problems. In particular, the solvent-damaged fluorescence detection module was replaced with an improvised fluorescence detection module with lower sensitivity, which was unable to detect fluorescence from cleaved peptides. To estimate the quantity of beads captured in each synthesis spot, beads eluted from the

spot were captured in a magnetic trap positioned at the outlet, where the aggregation of captured beads was photographed using a mounted camera fixed to the magnetic trap. Using ImageJ software, the surface area and volume of beads could be measured, albeit with low precision. The magnetic trap was then deactivated, and the captured beads washed out with DMF.

Manual SPPS with HMBA linkers was carried out following the ER visit, with peptide synthesis of GNASG, and labelling with FITC and NBD.

Results:

Synthesis of Test Peptide with HMPA/HMPB Linkers

The test peptide sequence GNASG was first synthesized on 50 mg beads with the HMPB linker. 2x ~5 mg beads were used for peptide cleavage with 95% TFA and 1% TFA to validate the method. Following sample processing, the peptides were analysed with direct-injection mass spectrometry (DI-MS) followed by liquid chromatography mass spectrometry (LC-MS) on the C18 column. The peptide could be cleaved from the beads under both conditions. The protected peptide GNASG carrying trityl and t-butyl protecting groups as well as an Fmoc protecting group had a mass of 923 Da, while the deprotected peptide GNASG with Fmoc-on has a mass of 626 Da.

Table 1 Overview of peptide masses and retention times

Peptide			**Mass (Da)**	**LC-MS retention time (min)**	
				C18 Column	**C4 Column**
5mer GNASG	Fmoc	with protecting groups	925.1	14	NA
		without protecting groups	626.6	10	9.3
		Trityl protecting group	810	11.5	10.2
		t-butyl protecting group	682.7	NA	9.7
	No Fmoc	with protecting groups	702.8	11	10
		without protecting groups	404.5	NA	NA
		trityl protecting group	646.7	10	9
		t-butyl protecting group	460.5	NA	NA
6mer ßA-GNASG without Fmoc		with protecting groups	773.9	10.9	10
		Without protecting groups	475.5	NA	NA
NBD-ßG-NASG		with protecting groups	865.9	12.5	NA
		without protecting groups	567.6	6-7	NA
FITC-ßA-GNASG		with protecting groups	1163.3	12.2	10.9
		without protecting groups	864.9	8.5-9	NA

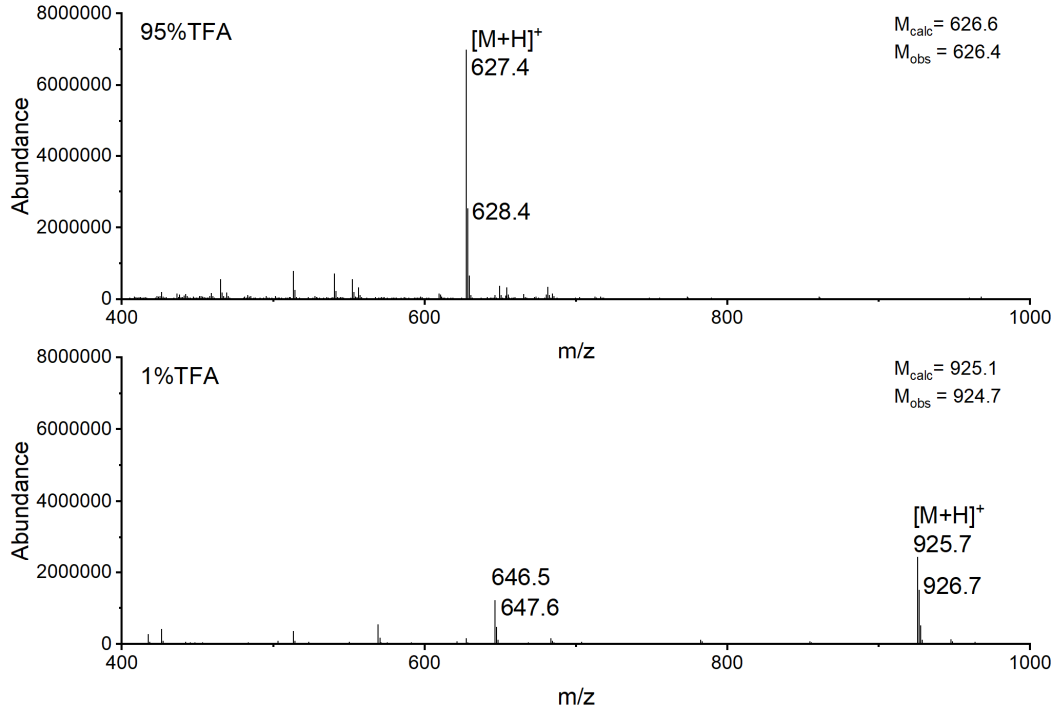


Figure 8 DI-MS of GNASG cleaved from HMPB linker with 95%TFA, deprotected peptide with m/z 627.4; 1%TFA, peptide with side protecting groups m/z 925.7

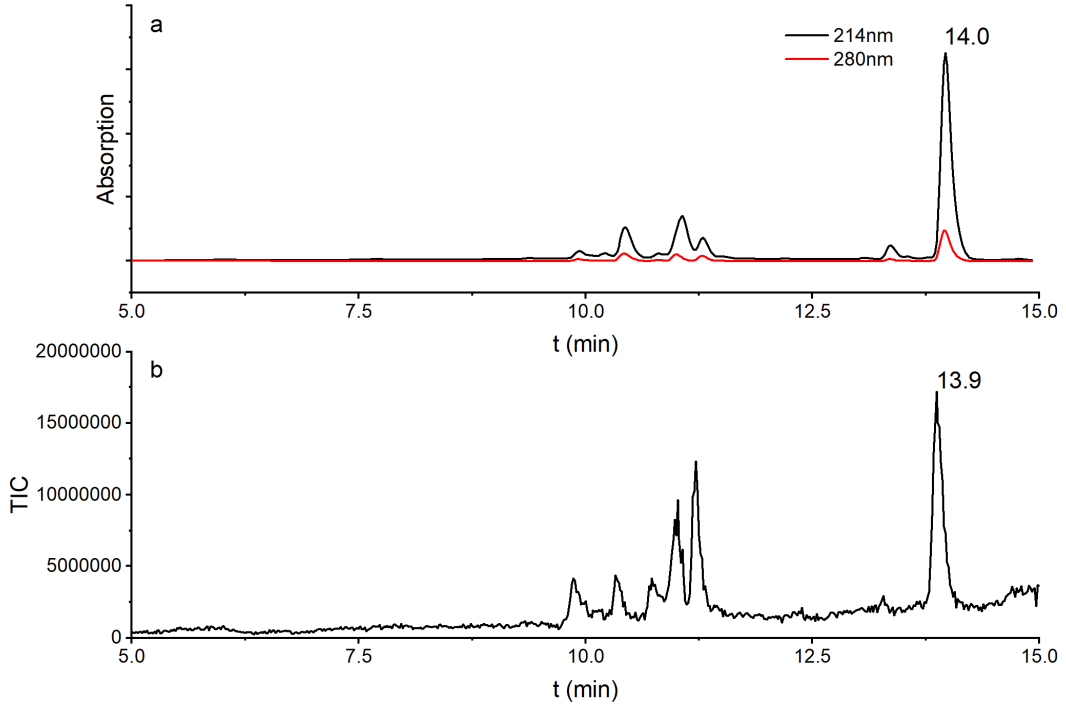


Figure 9 LC on C-18 column of GNASG cleaved from HMPB linker with 1%TFA, a) UV-Vis, 214 and 280nm; b) MS; GNASG with side protecting groups and Fmoc cap eluted at 14 min.

Beads were stored in DMF at 4 °C. Analysis of older bead samples revealed that Fmoc was cleaved from the peptide over time, due to the impurities in DMF such as dimethylamine acting as bases[34]. This

reduced the peptide mass by 221 Da, and decreased retention time. The peptide with side chain protecting groups had a mass of 702 Da, while the deprotected peptide without Fmoc had a mass of 405 Da and was eluted too quickly on the C4 and C18 columns for LC-MS analysis.

The above method was repeated using HMPA as the linker. Fully deprotected GNASG peptide could be detected when 95% TFA was used for peptide cleavage, but no peptide could be detected when 1% TFA was used.

To determine the completeness of 1%TFA cleavage, GNASG peptides on HMPB and HMPA linkers were cleaved first with 1%TFA, followed by 95% TFA. DI-MS results (Figure 10) indicated the HMPA-linker peptides, which were freshly synthesized, still had a Fmoc protecting group, while the HMPB-linker peptides, which had been in storage for a few weeks by then, were partially Fmoc-deprotected.

The processed samples from 1%TFA and 95TFA cleavage were analysed with LC-MS on the C-18 column (Figure 11). For HMPB, it was possible to conclude that most of the peptides were cleaved and eluted in the 1%TFA cleavage solution (703 Da peak at 11min, corresponding to GNASG with protecting groups and no Fmoc), with the remainder cleaved by the 95TFA solution afterwards (peak at 10min with a mass of 627 Da, matching the mass of the deprotected peptide with Fmoc).

For HMPA, in the 1%TFA cleavage solution, an unidentified peak at 11 min did not contain the product, (despite sharing a similar retention time to GNASG with protecting groups and no Fmoc), and only a small quantity of GNASG could be detected at 14 min (926 Da, corresponding to GNASG with protecting groups and Fmoc). The deprotected GNASG peptide (627 Da) could be detected in the 95TFA sample at 10 min, alongside other unidentified peaks.

As the results were sufficient for demonstrating the use of 1%TFA cleavage for HMPB and 95TFA cleavage for HMPA, 1%TFA cleavage followed by 95TFA cleavage was not repeated.

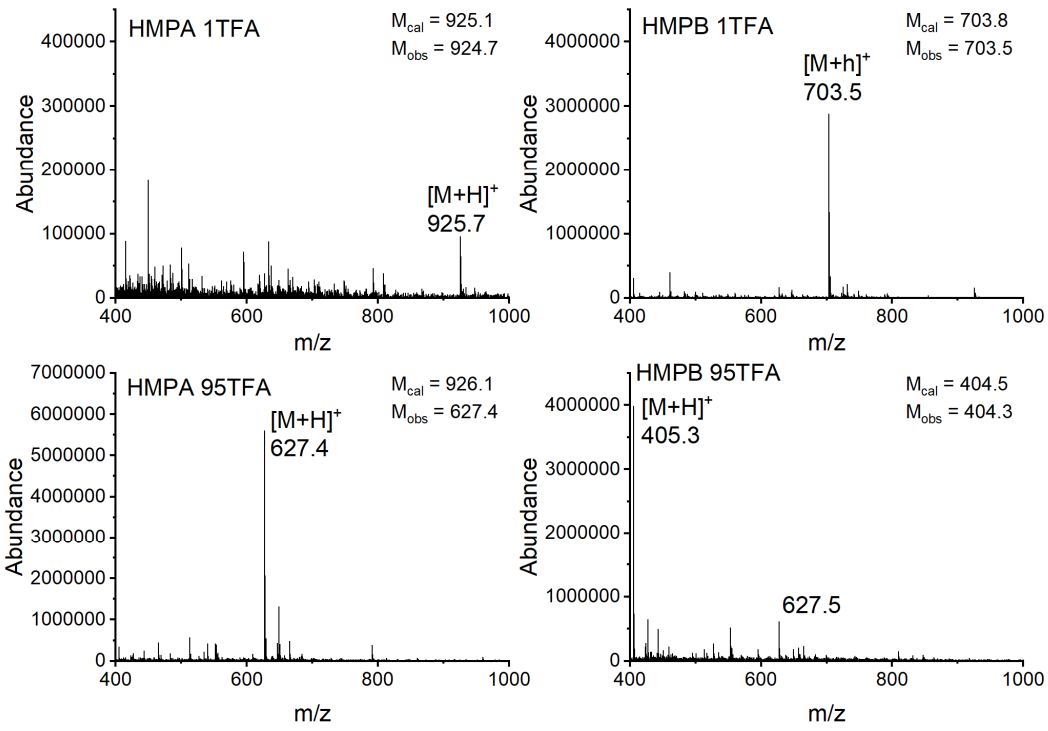


Figure 10 DI-Spectra of cleavage solutions for test peptide GNASG cleaved from HMPB and HMPA linkers using 1%TFA followed by 95TFA; Fmoc cleavage due to the age of HMPB linked GNASG peptides could be observed.

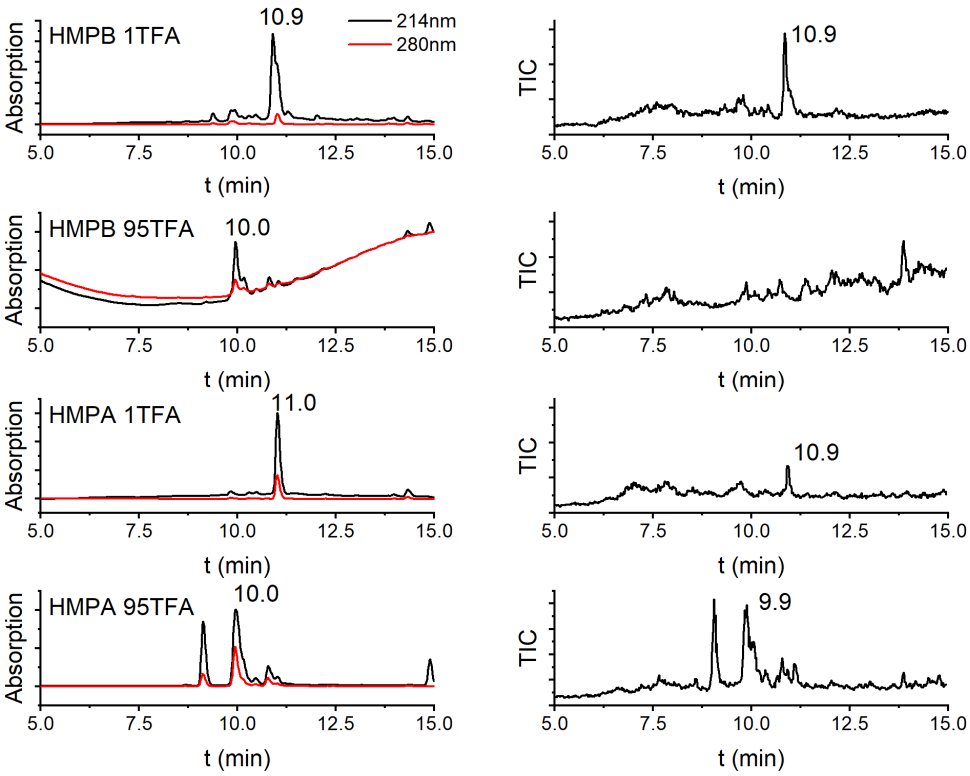


Figure 11 1%TFA cleavage followed by 95TFA cleavage of HMPA and HMPB linkers to determine completeness of cleavage, left LC-UV/vis 214nm 280nm, right LC-MS

Beads with the test peptide GNASG were prepared in batches between 50-250 mg multiple times. 2.5-5 mg samples were taken from each batch for quality controls. DI-MS and LC-MS results remained overall consistent between different batches. The masses and LC-MS retention times of the various products are summarised in Table 1.

Alternative cleavage conditions

Using the HMPB linker and 1%TFA, it was possible to obtain peptides with protected side groups. Cleavage with 50% TFA (TFA/DCM/H_2O/TIPS 50/45/2.5/2.5) and 60%TFA (TFA/DCM/H_2O/TIPS 60/35/2.5/2.5) and 1h cleavage time was carried out as possible alternatives for HMPA cleavage.

For cleavage with 50%TFA, the peptides were partially deprotected, retaining trityl or t-butyl side groups. Cleavage with 60%TFA was more effective at peptide deprotection, but a small amount of partially protected peptides was still present. It was decided to continue cleavage with 1%TFA and 95TFA.

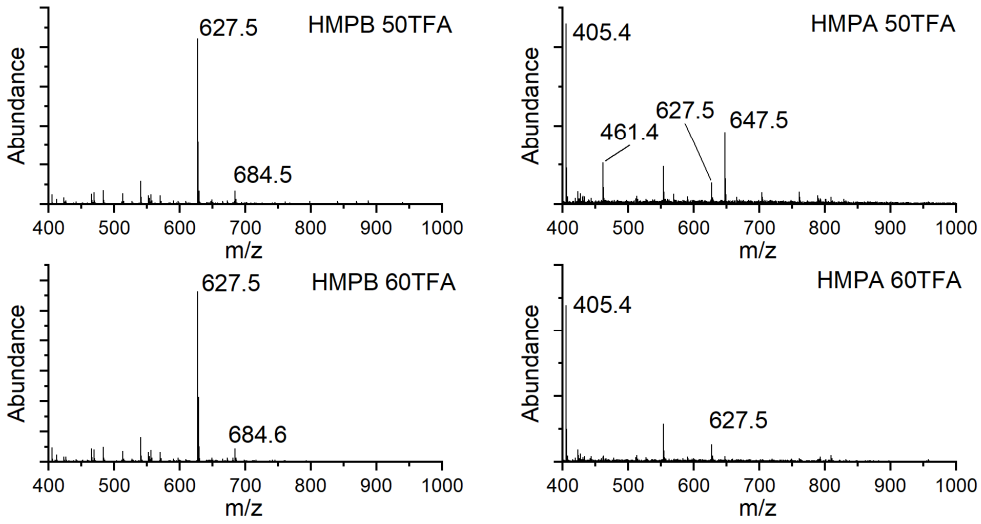


Figure 12 DI-MS of GNASG cleaved from HMPB and HMPA linkers with 50%TFA or 60%TFA, peptides lacking Fmoc in HMPA linker samples due to age of sample

As an alternative to DCM for diluting TFA, 2% and 50% TFA in MeOH was used to cleave the 6-mer ßA-GNASG (GNASG with an additionally coupled ß-Alanine) (as a batch of beads with ßA-GNASG had been synthesized for FITC-labelling, and no beads with GNASG were available at the time) from HMPB-linker beads. DCM is not miscible with water, which would be problematic in later stages of the synthesizer requiring buffering of the peptide with aqueous solution. LC-MS detected only the peptide with protected side groups. MeOH is therefore not a viable alternative to DCM.

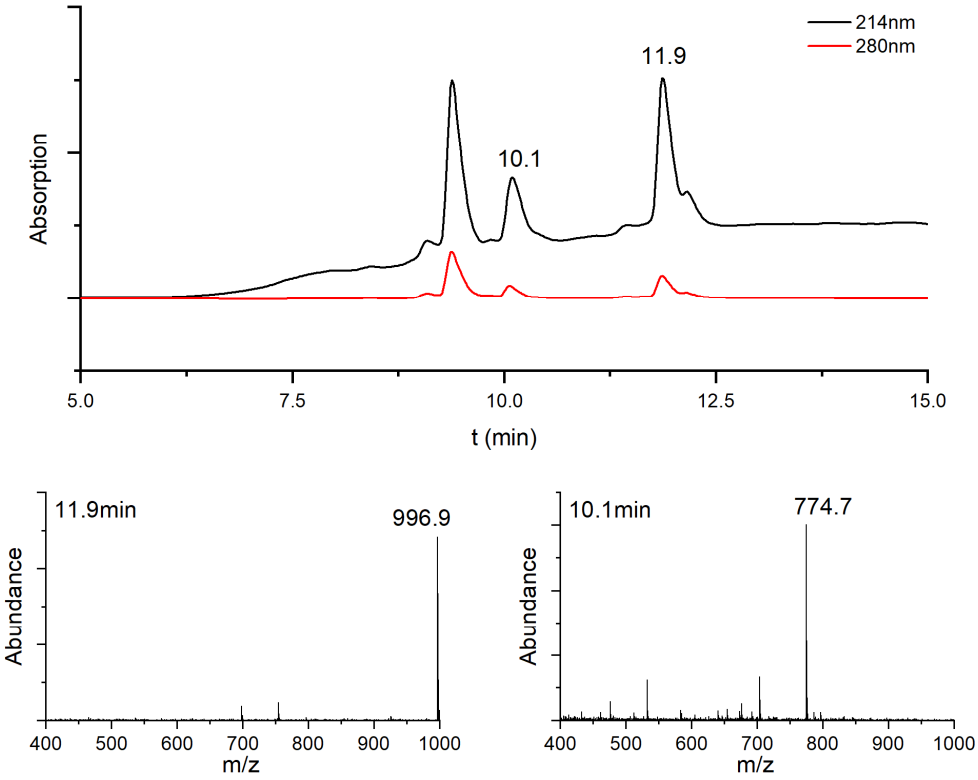


Figure 13 LCMS C4 Column, cleavage of test peptide ßA-GNASG from HMPB linker using 50% TFA in MeOH; Peptide with Fmoc and protecting side groups 11.9 min (expected mass 996), peptide with protecting side groups and without Fmoc at 10.1 min (expected mass 774), peak at 9.4 min unidentified, suspected to be GNASG with trityl protecting side group

Fluorescence Markers

A series of experiments were carried out to determine if FITC and/or NBD as fluorescent markers can be used for synthesis on magnetic beads and subsequently for peptide detection. GNASG continued to be used as the test peptide. For FITC labelling, ß-Alanine is additionally coupled to GNASG before FITC as a spacer, as FITC directly coupled to α-amino acids undergoes an unwanted reaction in acidic conditions such as is required for TFA cleavage, truncating the peptide and forming a fluorescein with the last α-amino acid[35].

Excitation and emission spectra of FITC and NBD-Cl in both PBS buffer and dilute TFA/DCM were measured. As FITC was known to lose fluorescence in acidic medium[36], and its spectra can be affected by the solvent[37], it was necessary to determine if the peptide could be detected in the cleavage solution of HMPA/HMPB. The results are shown in Table 2 and Figure 14.

For FITC, PBS results aligned with the values in literature for phosphate buffer solutions (Excitation and emission maxima 492nm and 519 nm)[37]. In dilute TFA/DCM, a weak signal was still detectable at 10 nM concentration, with shift of excitation and emission maxima to 450 and 513 nm respectively. No pH dependence in signal strength was observed, in contrast to literature. In addition, coupling FITC to peptides increases excitation peak to around 505 nm.

For NBD-Cl, only a weak signal was detected for NBD-Cl, with a strong shift of excitation and emission maxima from PBS to 1TFA. A later investigation of literature discovered NBD-Cl is not a strong fluorophore[38], and NBD coupled with an amide bond has an excitation and emission maxima of around 465nm and 535nm respectively in aqueous solutions[39].

Table 2 Measured Excitation and Emission maxima for FITC and NBD-Cl

	1%TFA		PBS	
	Excitation (nm)	Emission (nm)	Excitation (nm)	Emission (nm)
FITC	450	513	492	517
NBD	516	535	492	516

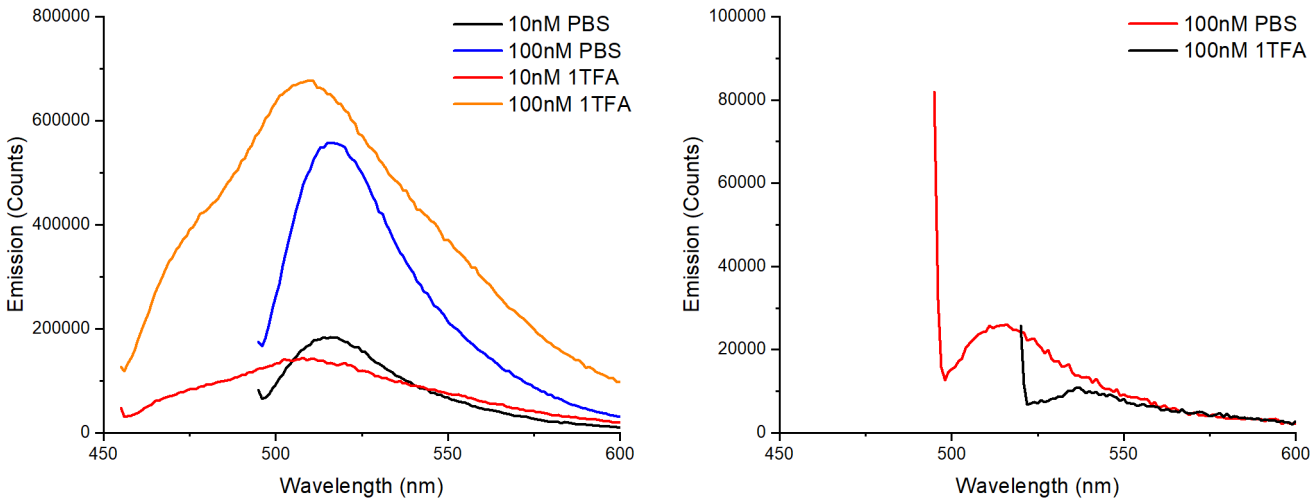


Figure 14 Emission Spectra of fluorescent markers in PBS and TFA, left: FITC; right: NBD-Cl

FITC

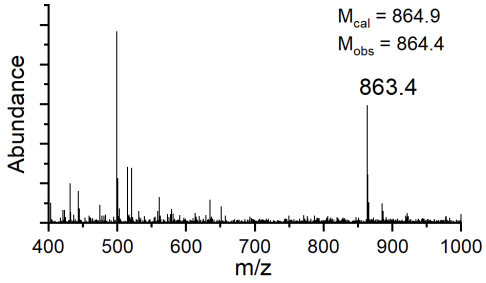


Figure 15 Neg. DI-MS of FITC-labelled test peptide ßA-GNASG cleaved with 95TFA

Initial synthesis of FITC-ßA-GNASG involved an overnight FITC coupling to ßA-GNASG. The peptide was then cleaved with 95% TFA. Standard DI-MS and LC-MS with positive ionisation gave a weak signal for m/z 865, the expected m/z of FITC-AGNASG. A DI-MS with negative ionisation (Figure 15) gave a clear signal of the product (m/z 863.4), confirming the positive MS. The weak signal with positive ionisation was due to poor ionisation of FITC-ßA-GNASG, with most of the peptides retaining a neutral net charge instead of being protonated, and thus not being detected by MS. Due to issues familiarizing with the HPLC equipment at the time, no FLD-chromatogram was available.

A second batch of FITC-ßA-GNASG was cleaved with 1%TFA and 95%TFA, LC-MS analysis of which detected both the protected and unprotected peptides (Figure 16). The protected FITC-labelled peptide had a mass of 1163 Da (with positive ionisation) and elution time of 12.1 min on the C18 column, the unprotected FITC-labelled peptide a mass of 865 Da and two peaks at 8.8 min and 9.4 min (presumably a partially deprotected peptide with t-butyl side group and mass 918 Da).

The 1%TFA sample was analysed on the LC-MS machine immediately following the 95%TFA sample, with the chromatogram of the 1%TFA sample containing a peak for deprotected FITC-ßA-GNASG. Previous results from non-fluorescently labelled peptides chromatogram indicate that the presence of the unprotected FITC-ßA-GNASG was due to contamination from the previous 95%TFA sample rather than undesirable deprotection reactions. This was confirmed with the corresponding HPLC-FLD of the 1%TFA and 95%TFA samples (Figure 18).

A major issue has been the poor solubility of the FITC-labelled peptides in aqueous solutions. When H_2O was added to the dried peptides following lyophilisation, they remained insoluble as a light yellow powder. This behaviour prevented LC-MS and HPLC-FLD/UV-vis analysis, and was suspected to be responsible for contamination in following measurements on the devices. Solubility was improved with TFA and addition of acetonitrile. (0.1%TFA in H_2O/ACN 50/50)

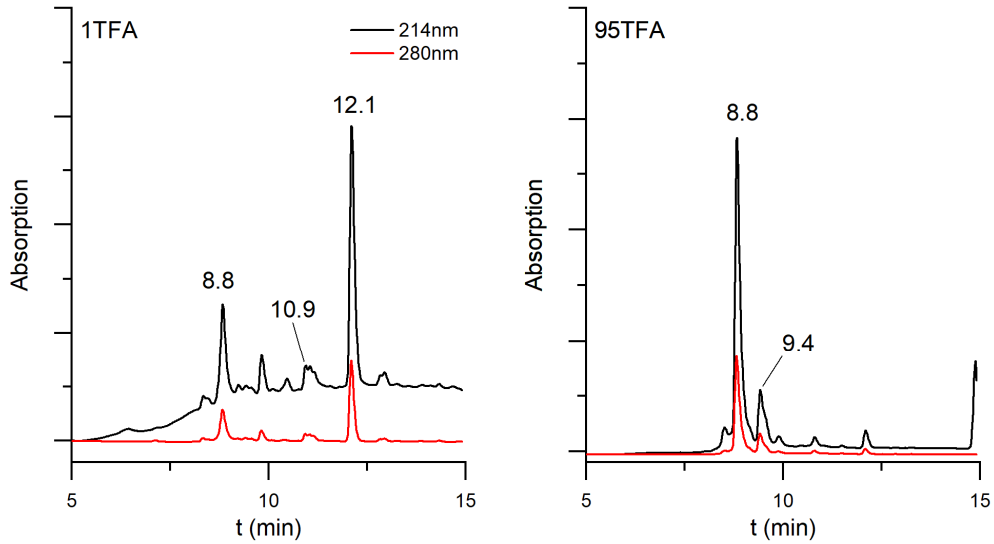


Figure 16 LC of FITC-ßA-GNASG peptide on C18 column, cleaved using 1%TFA and 95TFA. The 1%TFA analysis was contaminated by the preceding 95% TFA sample in LC, likely due to the low solubility.

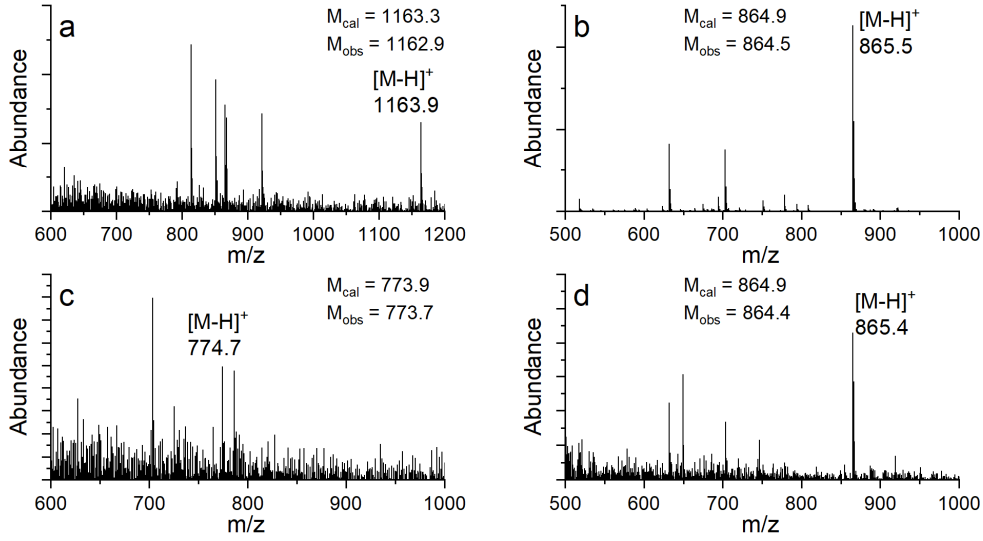


Figure 17 MS-spectra of peaks in Figure 16; a) 1%TFA, 12.1min, FITC-ßA-GNASG with protecting groups; b) 95%TFA, 8.8min; FITC-ßA-GNASG without protecting groups c) 1%TFA, 10.9min, ßA-GNASG with protecting groups; d) 95%TFA, 9.min, FITC-ßA-GNASG without protecting groups

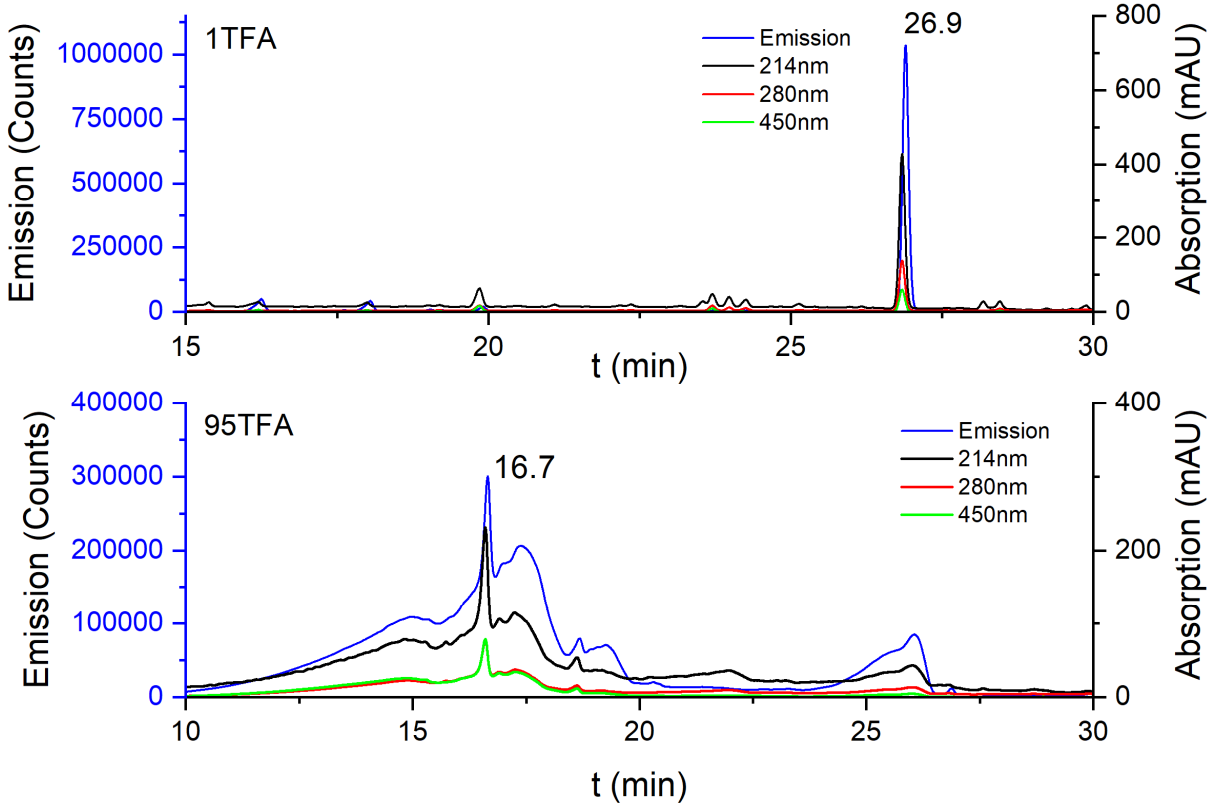
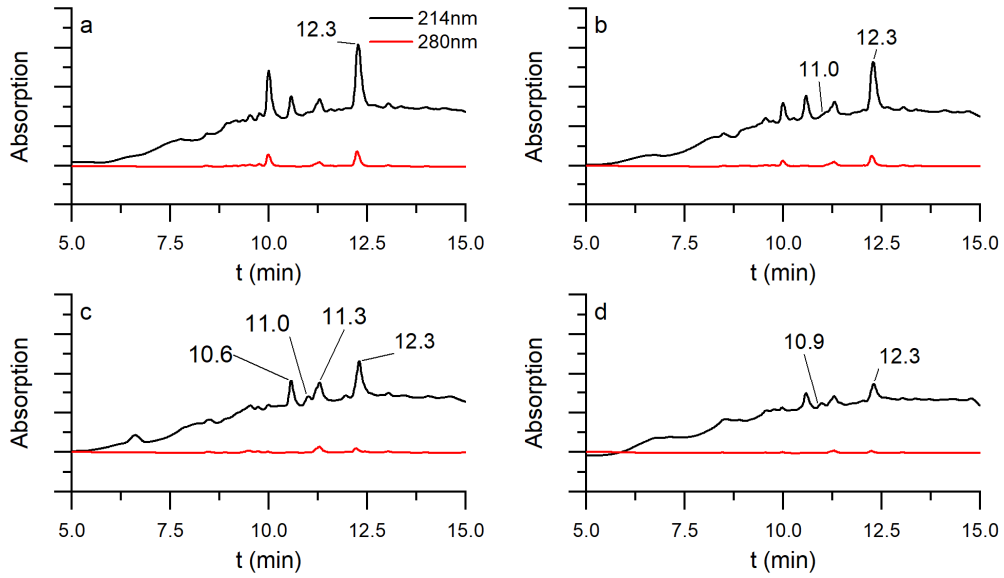


Figure 18 HPLC-FLD/UV-Vis Chromatogram on C18 Final Analysis Column FITC-ßA-GNASG cleaved with 1%TFA or 95%TFA

The original overnight reaction length of FITC-labelling was unsuitable for the goal of high throughput microfluidic synthesis. Four different conditions were tested, to determine if a shorter reaction time and reduced FITC concentration for the labelling reaction were suitable. FITC concentrations were reduced to 10 mg FITC in 250 µl DMF, 171.1 µl eq DIEA (~50 mM) and 1 mg 250 µl DMF, 171.1 µl eq DIEA (~5 mM), and reaction time was reduced to between 5 and 60 min. As the fully deprotected peptide would not be detected in LC-MS or HPLC-FLD, 1%TFA cleavage was carried out (Figure 19). With LC-UV-vis/MS, it was possible to determine only a small portion of the peptides remained unlabelled (11 min), even at 5mM FITC and 15 min reaction time, with FITC-ßA-GNASG detected at 12.3 min.



a: 50 mM FITC, 15min b: 50mM FITC, 5min
c: 5mM FITC, 60min d: 5mM FITC, 15min

Figure 19 LC-MS on C18 column of FITC-labelled peptides with different reaction conditions. 12.3 min: FITC-ßA-GNASG, 11.0 min: ßA-GNASG. Additional peaks at 10.0, 10.6, 11.3 min are not identified, but do not contain the peptide.

NBD

NBD-labelling of GNASG was successfully confirmed by LC-MS (Figure 21) and HPLC-FLD (Figure 22). DI-MS of the product produced m/z signals for both unlabelled and labelled peptide. In LC-MS, unlabelled and deprotected peptides in samples cleaved with 95% TFA could not be identified due to too rapid elution, leading to initial assumptions of high labelling efficiency. However, unlabelled peptides were detectable in DI-MS, while LC-MS of the peptide solutions cleaved with 1%TFA showed a strong signal for unlabelled peptides. Attempts to improve NBD-coupling, such as with adjustments of NBD and DIEA concentration, or the coupling of NBD with ß-Ala instead of glycine (Figure 22), did not improve the reaction efficiency.

As no method to improve NBD-labelling efficiency could be found, NBD was not considered a good candidate for further microfluidic SPPS development. However, it was decided to continue using NBD in further experiments as a comparison to FITC.

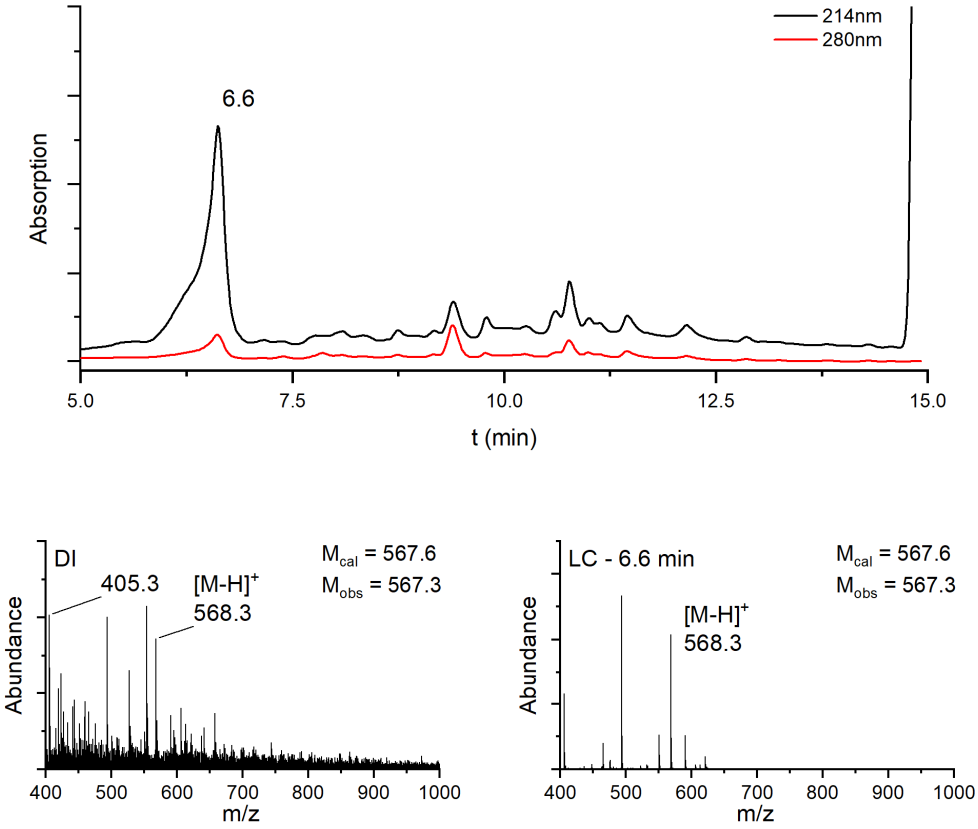


Figure 20 NBD-GNASG, cleaved with 95%TFA, above: LC-UV-vis on C18 column Absorption 214+280nm, NBD-GNASG at 6.6 min, bottom left: DI-MS, with m/z 405.3 of unlabelled peptide, bottom right: LC-MS peak of product (6.6min)

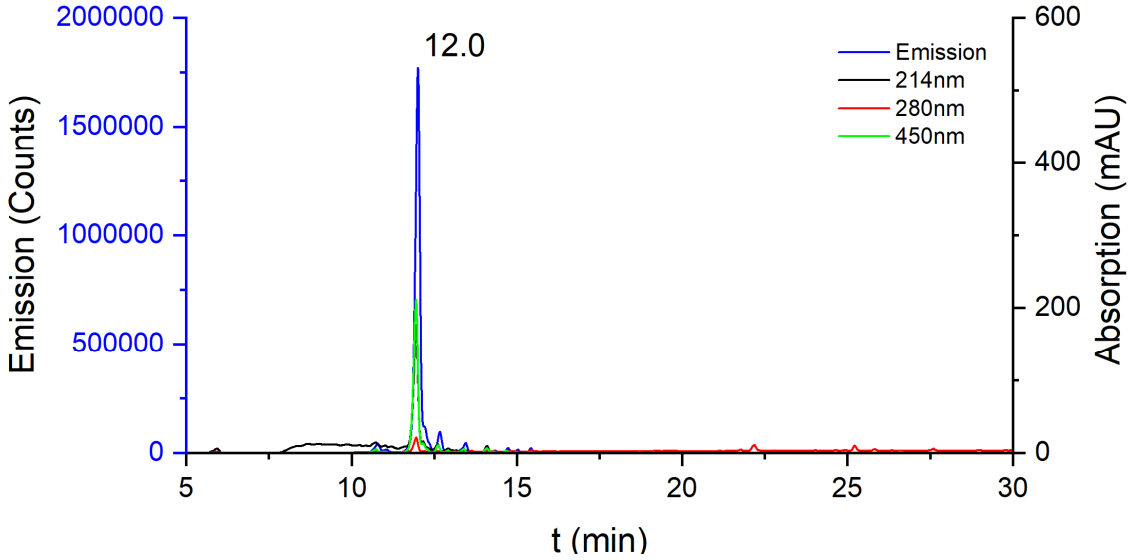
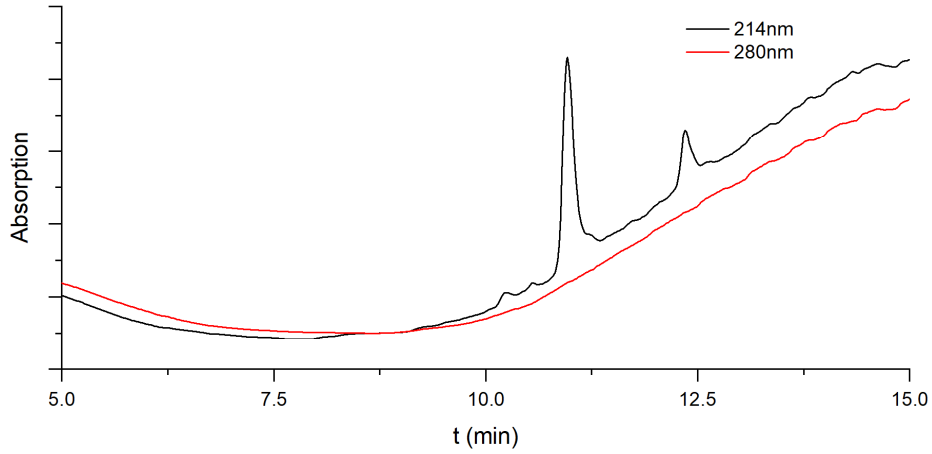


Figure 21 LC-FLD/UV-Vis of NBD-GNASG cleaved with 95%TFA



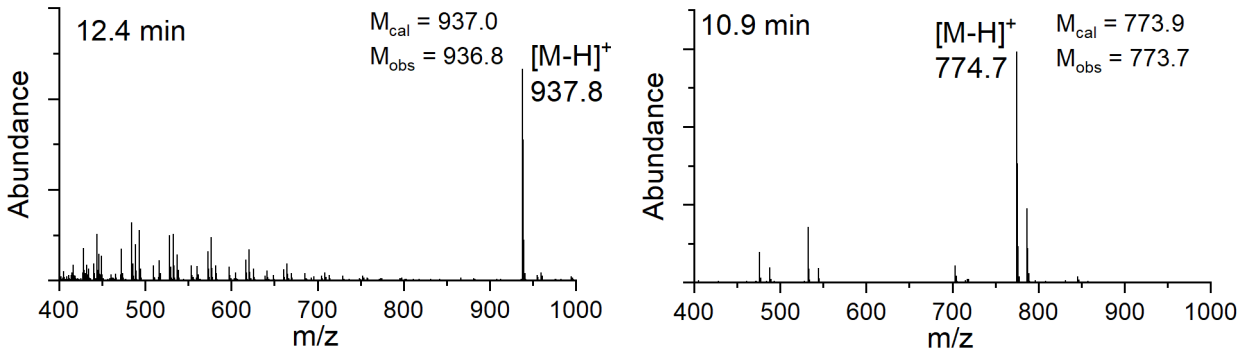


Figure 22 LC-MS of NBD-ß-A-GNASG cleaved with 1%TFA demonstrating incomplete labelling, with unlabelled peptide peak at 10.9 min and labelled peptide peak at 12.4 min

Model System for Proof-of-Concept Experiments

The proof-of-concept model from ER had previously been used to demonstrate the feasibility of microfluidic SPPS using the magnetic beads. As no labelling with a fluorescence marker had been carried out so far on the proof-of-concept model, fluorescence labelling in the microfluidic reaction chamber was to be demonstrated using FITC and NBD. While NBD did not couple efficiently with the peptides on magnetic, the fluorescent marker was used as a comparison to FITC.

After initial SPPS of fluorescent-labelled GNASG on the model was not successful, due to unknown reasons, individual steps of bead loading, amino acid coupling and fluorescent marker labelling were performed separately to confirm viability. De novo synthesis of the fluorescent-labelled peptide in the proof-of-concept model was then successfully carried out. Products could be detected with LC-MS and HPLC-FLD for linkers HMPB and HMPA and fluorescent markers FITC and NBD (Figure 23). Only a small quantity of beads were retained in the reaction chamber for each SPPS and eluted afterwards, leading to low but still detectable peptide quantities after cleavage. Several impurities were present in the crude cleavage mixture after synthesis on the proof-of-concept model. For NBD, a large quantity of unlabelled peptide GNASG was also detected during analysis.

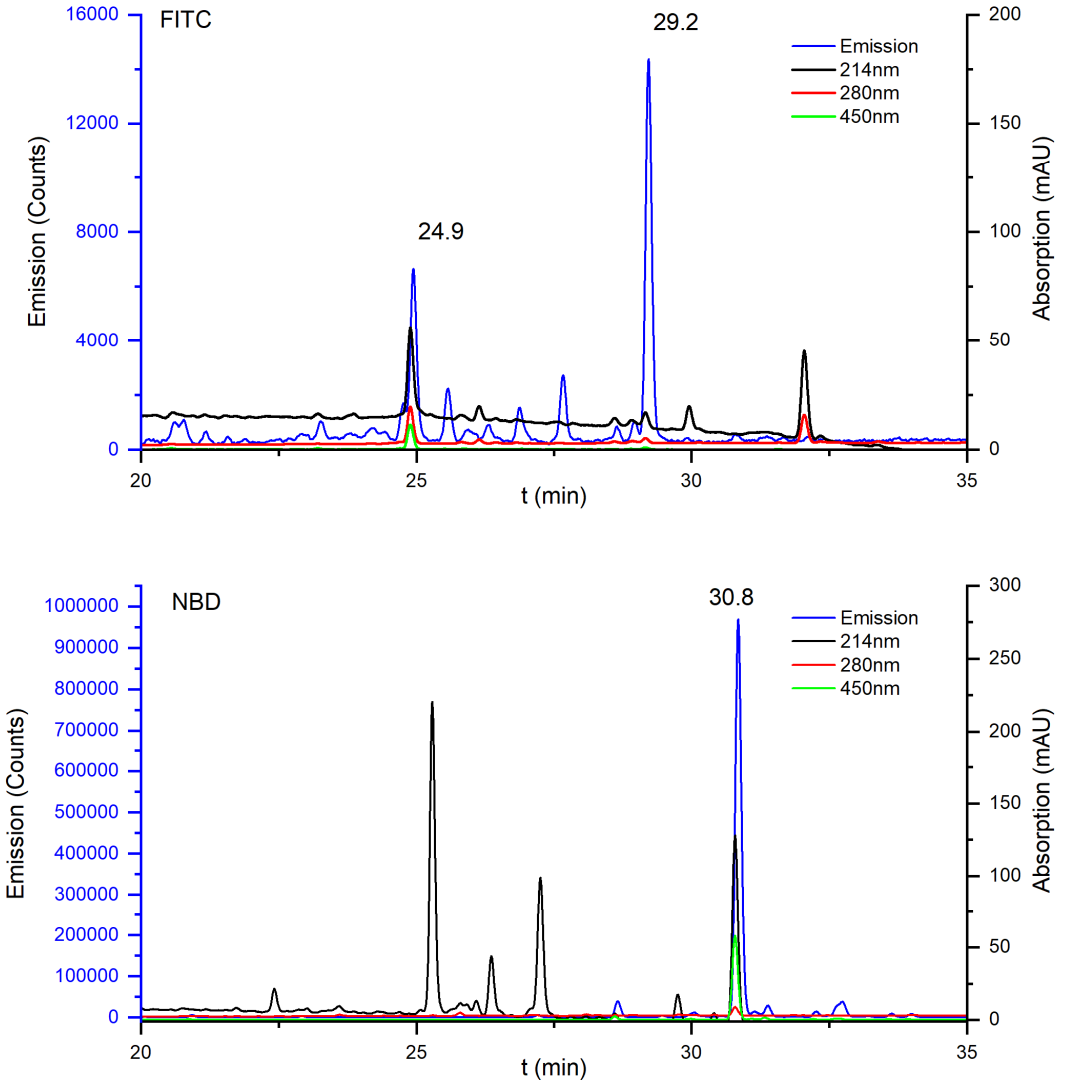


Figure 23 HPLC-FLD/UV-Vis of test peptide GNASG with fluorescent marker synthesized on proof-of-concept model, FITC: 24,9min, NBD: 30,8min.

Impact of Separation Fluid

The NTP systems from Efficient Robotics relies on Novec oil with surfactants as a separation fluid. It is thus necessary to determine if the separation fluid is compatible with all synthesis steps. The impact of the separation oil both with and without addition of the surfactant on peptide synthesis was analysed.

Initial tests showed DMF was not miscible with the separation fluid, both with and without surfactant. Beads with GNASG 5mer attached via HMPB-linker, suspended in DMF remained in the DMF phase following mixing with the separation fluid. Cleavage of the 5mer with both 95% TFA and 1% TFA afterwards was successful, whereby the addition of the surfactant reduced oil contamination in the product. As Fmoc-based synthesis uses DMF as solvent, the separation fluid should be useable for microfluidic peptide synthesis.

For determining the compatibility of separation fluid with cleavage of HMPB and HMPA linkers, 95% TFA and 1% TFA in DCM cleavage solutions containing cleaved GNASG 5mer test peptides were mixed with the separation fluid, followed by LC-MS of the cleavage solution. 1% TFA was not miscible with the separation fluid and retained the peptide, while 95% TFA was fully miscible, and the peptide was extracted from the separation fluid using ACN/H_2O 50:50 for LC-MS analysis.

The miscibility of 95% TFA with Novec oil is problematic if HMBA or HMPB linkers are used for peptide synthesis, likely requiring an alternative to the Novec oil as separation fluid. However, the HMBA linker (see below), with NaOH cleavage solution, would not be severely impacted, as 95% TFA would only be employed for peptide deprotection. By ensuring that, for the deprotection step, the Novec oil is fully displaced by 95% TFA in the peptide synthesis spots, and that TFA-contaminated Novec oil is fully flushed from the system following the deprotection step, the miscibility would not pose an issue.

In conclusion, the Novec oil is expected to be compatible with peptide synthesis.

Synthesizer Prototype

As part of the joint project to develop a massive parallel microfluidic peptide synthesizer, Efficient Robotics GmbH in Kornwestheim, Germany had created a prototype for testing. The prototype contained 10 synthesis spots, had a fluorescence detection module, and was designed to be capable of performing SPPS, though this had not been tested yet. Preliminary tests by *Efficient Robotics* with blank beads in PBS as well as fluorescein in PBS had yielded good results, and a series of tests were planned to validate peptide synthesis. For this purpose, blank beads were sent from Vienna to Efficient Robotics, along with beads containing FITC-ßA-GNASG and NBD-GNASG.

The original plan was to first calibrate detection of fluorescein in the 1%TFA cleavage solution, as well as validating the effectiveness of bead capture in the synthesis spots using an external magnetic trap. Due to peptide synthesis requirements, DMF was to replace PBS for suspending the peptides and washing the beads. Finally, peptides were to be cleaved from the beads captured in synthesis spots. The yield per synthesis spot was to be determined using fluorescence detection of the cleavage solution.

Over the course of the experiments at ER in Kornwestheim, multiple issues prevented the completion of the tests. Aside from technical problems, chemical stability of components was a major issue. DMF, DCM and TFA degraded several components of the prototype synthesizer, including the fluorescence detector, requiring modifications to the prototype as a result. Solutions were found for most issues, and the prototype synthesizer was able to eventually operate under the required conditions, however at reduced effectiveness.

A backup fluorescence detector with lower sensitivity was able to detect 100 nM fluorescein in PBS, but was unable to detect fluorescein in the 1% TFA cleavage solution, both due to lack of sensitivity and available excitation and emission filters being of sub-optimal wavelengths for FITC fluorescence in 1%TFA. As such, peptide cleavage from beads fixed in the synthesis spots of the synthesiser would be undetectable, due to the inability to detect FITC-ßA-GNASG in 1%TFA.

Another method was improvised for measurement of spot loading with visual quantification of beads. Beads eluted from the loading spots were captured in a magnet trap and the accumulated beads were photographed using a fixed camera. The images were analysed using the image-j program to measure the area of the bead aggregation and estimate the quantity of retained beads.

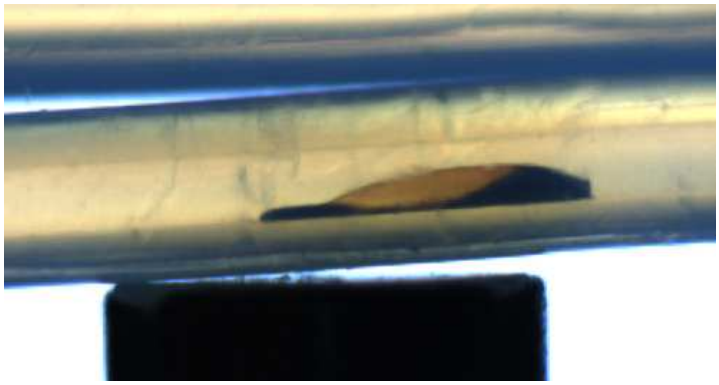


Figure 24 An example of beads captured in the magnetic trap (magnet below). The lighter area of the bead agglomerate appeared to be a thin bead film, and was not included in quantification.

Multiple series of spot loading were carried out with different loading times of beads into the synthesis spot in each side channel, DMF for eluting the beads and the flow pressure from inlet to outlet (Figure

25). The flow pressure controlled the flow rate, but due to other factors such as build-up of bead aggregations in the system, the flow rate fluctuated over time. Despite the low precision, a high variance in bead loading could be observed, which was also highly dependent on spot position.

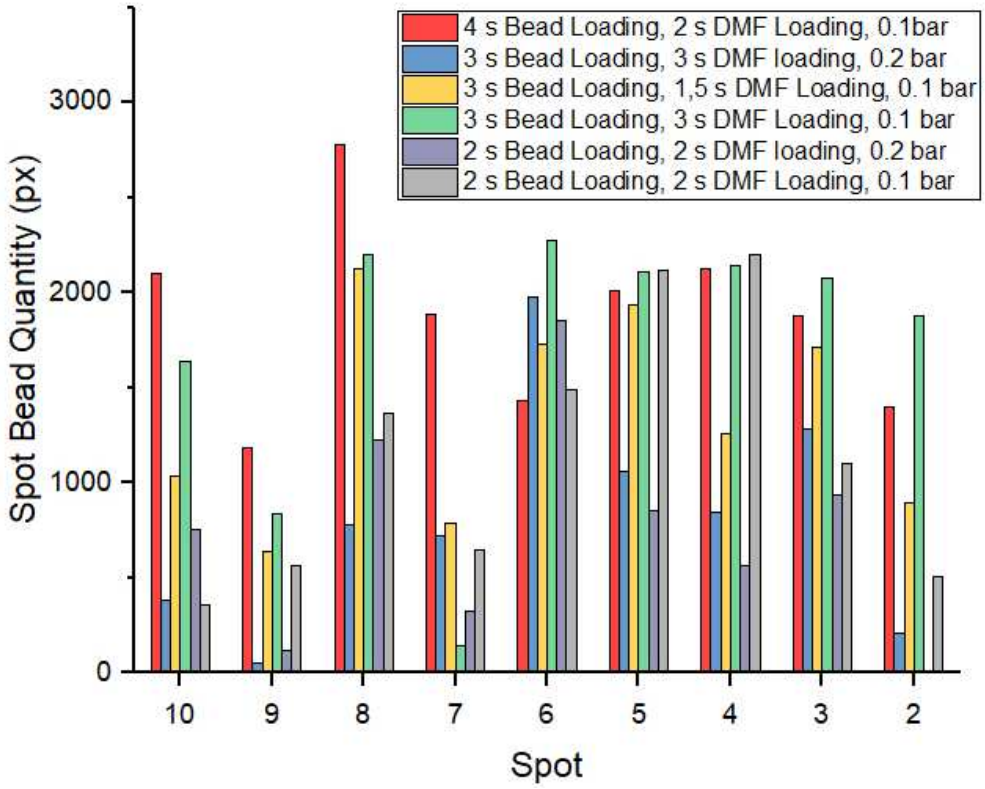


Figure 25 Impact of flow pressure and loading time on loading of synthesis spots with magnetic beads in synthesiser prototype. Bead quantity loaded on each spot was estimated with area of eluted beads captured by magnetic trap (measured by pixel count in photos taken by a mounted camera)

Despite the problems encountered, some useful information were obtained. The tests demonstrated that beads in DMF were fully compatible with the microfluidic system. Beads could be loaded into the synthesis spots and affixed using magnetic fields, then washed with separation fluid and DMF before being fully eluted from the spots. Good practices for avoiding bead aggregation in system and other issues could be determined.

Peptide Synthesis with HMBA Linker

Following the visit to Efficient Robotics, our focus shifted to the synthesis of peptides using HMBA linkers instead. 50 mg magnetic beads were used for a first synthesis. Binding of the HMBA linker to the solid phase was carried out using the same method as for HMPA and HMPB, as was the synthesis of the test peptide GNASG.

As an initial baseline, ~5mg beads were taken from the batch for the first peptide cleavage (HMBA-1). The beads were resuspended in 100 µl of 100 mM aqueous NaOH for 15 min**.** The cleavage solution was removed and neutralised with an equivalent volume of 100 mM HCl. The peptide solution was analysed using DI-MS and LC-MS (Figure 26), confirming successful synthesis and cleavage of GNASG. Given the problems posed by the high NaCl content for LC, DI-MS was not carried out for the remaining HMBA cleavages.

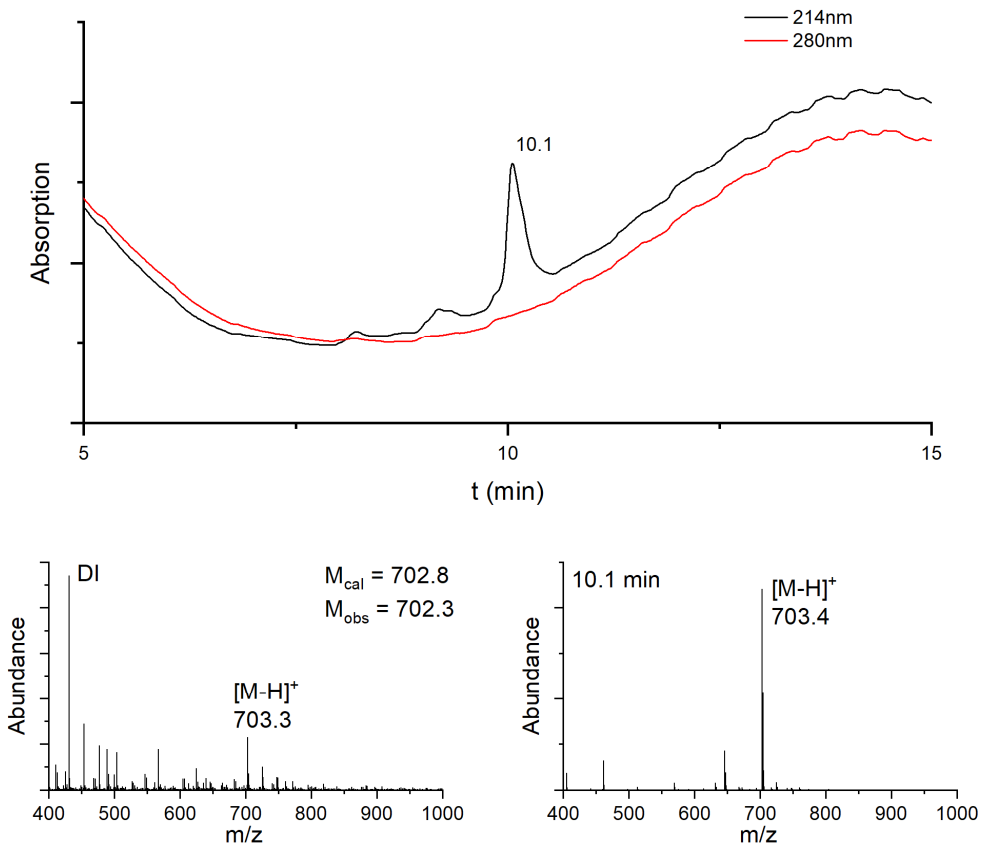


Figure 26 HMBA-1, above LC-UV-vis, below DI-MS and MS of LC peak

A second round of cleavage with ~5 mg beads from the HMBA-batch was carried out to determine if cleavage with 10 mM aq. NaOH was possible (HMBA-2). The beads were initially suspended in 10 mM aq. NaOH for 15 min, followed by collection of the liquid and full peptide cleavage with 100 mM aqueous NaOH. Both cleavage solutions were neutralised using 100 mM HCl. Analysis of the two solutions showed only a small portion of the GNASG test peptide was cleaved with 10 mM NaOH, with the majority of the peptides cleaved in the 100 mM solution (Figure 27). A concentration of 100 mM NaOH was therefore used for all remaining cleavages. As Efficient Robotics uses Novec routinely with aqueous solutions in their NTP-Workstation, compatibility and miscibility of Novec separation fluid with 100mM NaOH was not tested.

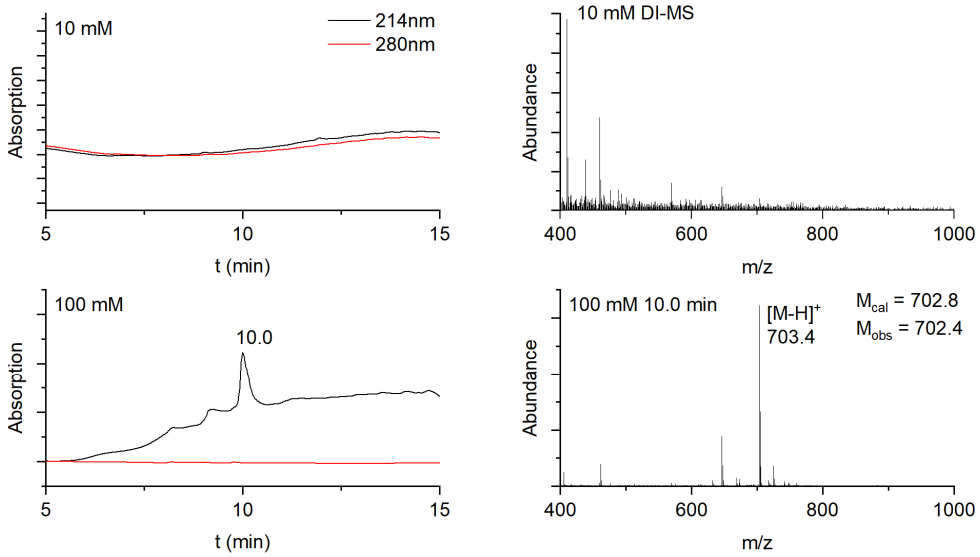


Figure 27 HMBA-2, peptide cleavage from HMBA linker with 10mM and 100mM aqueous NaOH

HMBA is acid-stable and base-labile, preventing linker cleavage even in concentrated TFA. It is thus possible to remove the protecting side groups on the peptide while it is still linked to the solid phase, followed by eluting the peptide directly into a basic aqueous solution, which can be neutralised with a buffer solution and used directly for screening. As an initial test, the peptides were deprotected for 30 min with 95 % TFA, which was collected and processed in the same manner as HMPB/HMBA cleavage solutions (HMBA-6). Following washing of TFA from the beads, the peptides were cleaved from the beads using 100 mM aqueous NaOH. LC-MS of the peptide solution yielded only trace amounts of protected and partially deprotected peptide (Figure 28), indicating most of the peptide had been deprotected and eluted too quickly through the C4 LC-column to be detected. However, it was not possible to rule out peptide loss during the TFA-deprotection step. This was resolved by carrying out TFA-deprotection of FITC-labelled peptides instead.

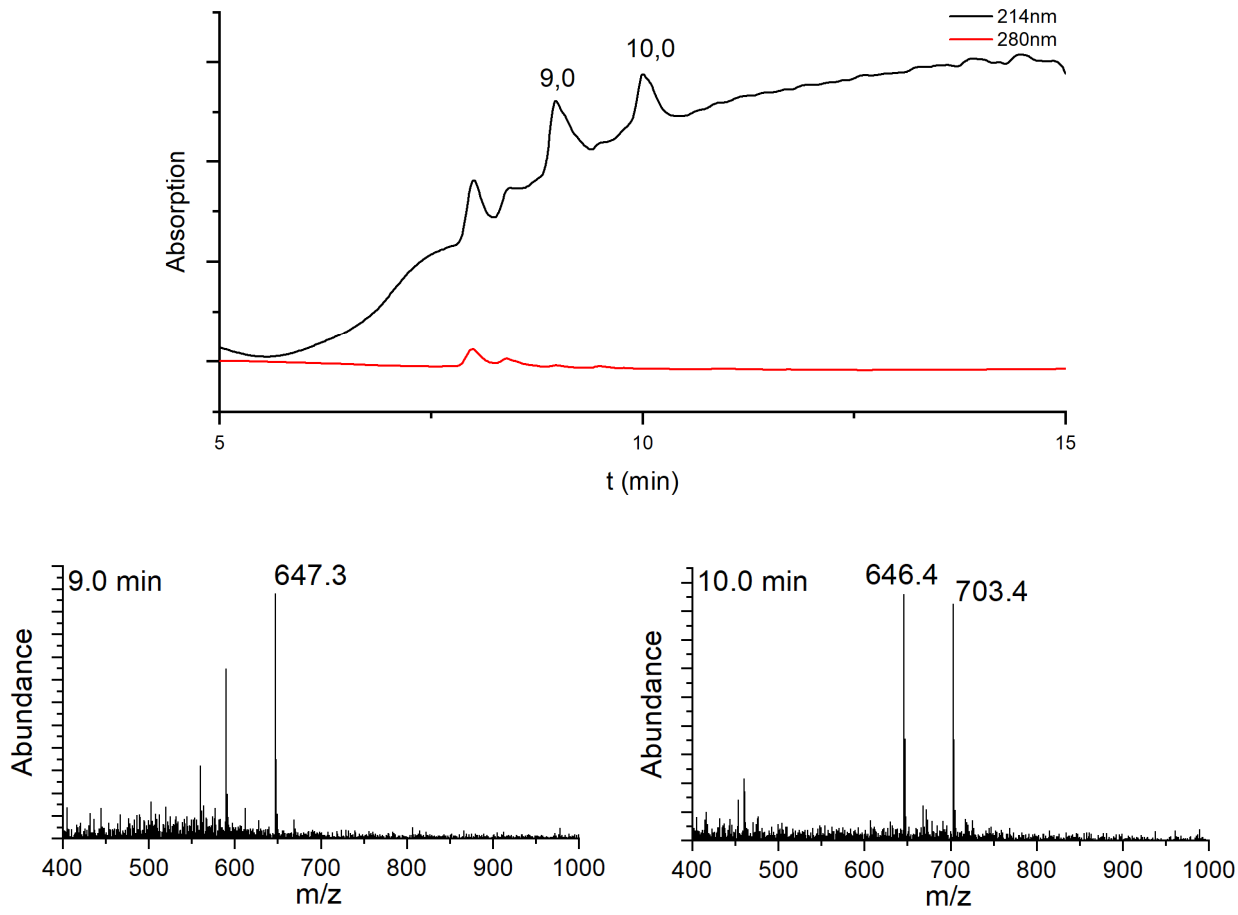


Figure 28 LC-UV/Vis of HMBA-6 on C4 Column, peaks at 9 and 10 min correspond to peptides with trityl or both protecting side groups

For the first FITC-coupling (HMBA-5), ~2 mg beads from the HMBA-batch were used. After coupling ß-Ala, the FITC reaction (2.2 mg FITC, 500 µl DMF, 160 µl DIEA, ~10 mM) was kept brief at only 15 min. Following coupling, cleavage was carried out without TFA-deprotection. The basic cleavage solution was strongly coloured, indicating the presence of FITC. During neutralisation with 100 mM HCl, yellow precipitation was observed, and the solution turned colourless. LC-MS and HPLC-FLD of the supernatant showed only the unlabelled peptide. The yellow precipitate was successfully dissolved in H_2O/ACN 50/50 0.1%TFA. Analysis of the second solution confirmed the presence of the FITC-labelled peptide via both LC-MS (Figure 29) and LC-FLD (Figure 30).

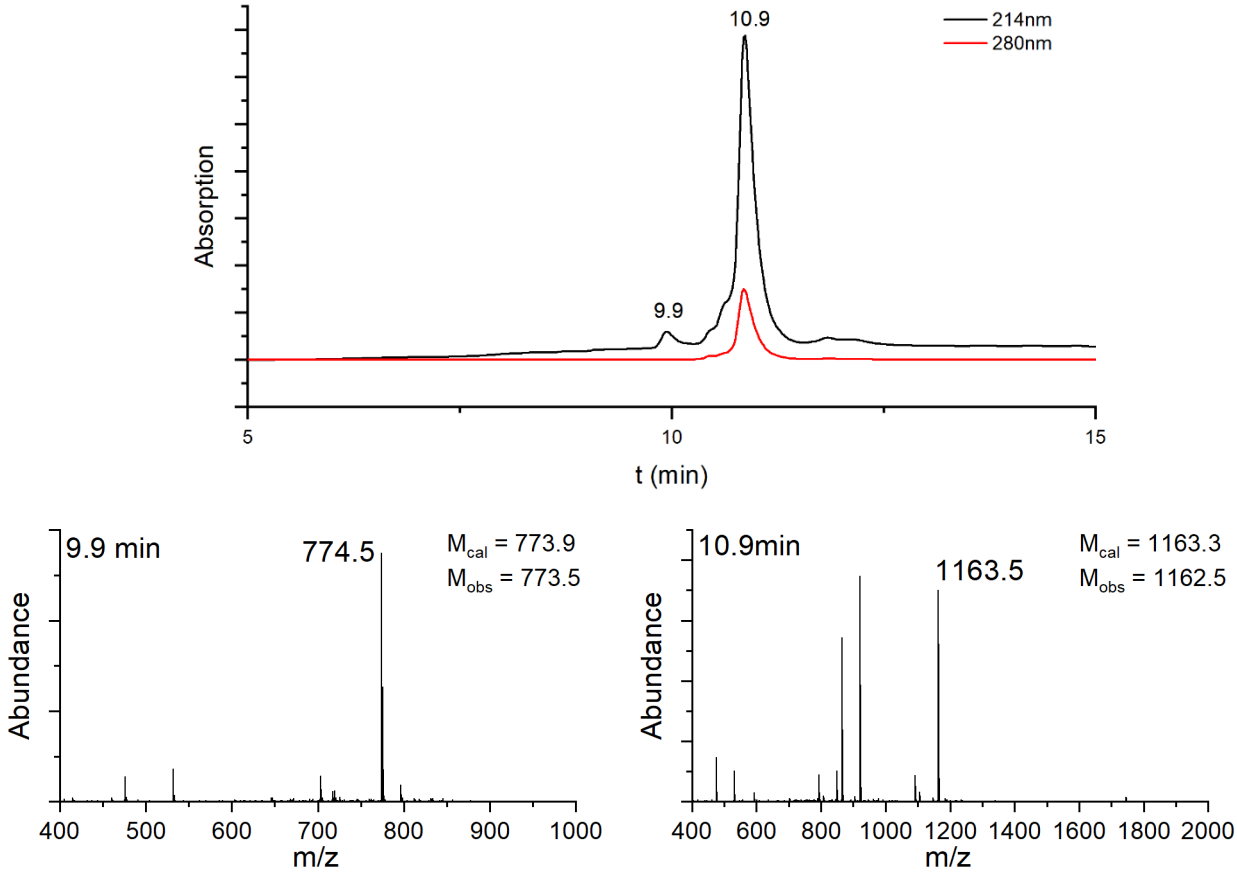


Figure 29 HMBA-5: LC on C4 column of H_2O/ACN 50/50 0.1%TFA solution, top LC-UV-vis, bottom MS-Spectra of peaks

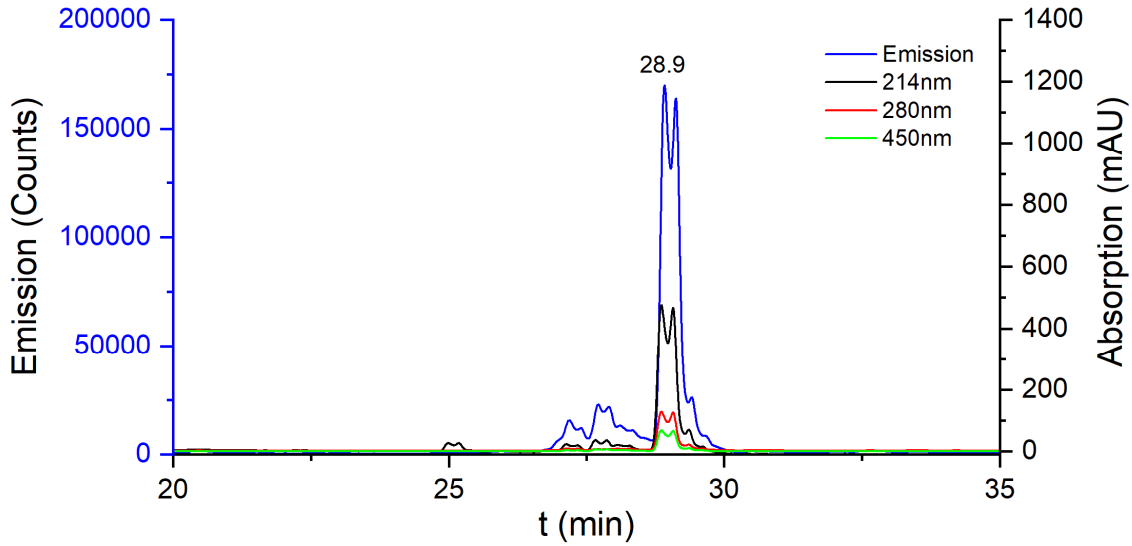


Figure 30 HPLC-FLD of HMBA-5 in H_2O/ACN 50/50 0.1%TFA

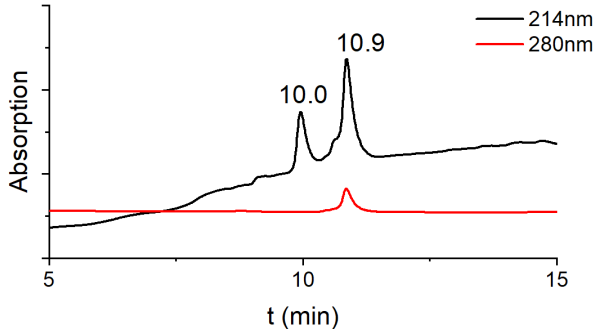


Figure 31 HMBA-9 - FITC-ßA-GNASG

For further analysis using the FITC-6mer, a 10mg batch of GNASG-coupled beads were used for a second FITC-coupling (HMBA-8). A test cleavage (HMBA-9) was performed. Analysis indicated that FITC-coupling was also incomplete (Figure 31), with a significant quantity of ßA-GNASG 6mer still present, likely due to too low FITC concentration and reaction time. Regardless, the 6mer was not expected to impact further experiments with HMBA-8.

Two 5mg aliquots of HMBA were deprotected, with 95% TFA (HMBA-10) and 75% TFA (HMBA-11) used as deprotection solutions. The beads were thoroughly washed with DMF and H_2O to remove TFA before peptide cleavage. In both cases, the peptide was fully deprotected, with only traces of partially protected peptides (Figure 32).

The TFA-solutions were processed using the same method as previously used for HMPB/HMPA cleavage solutions, and analysed to confirm HMBA stability in acidic medium. A peak at 17.1 min in HPLC-FLD (Figure 32) could be attributed to uncoupled FITC, no fluorescent-labelled product could otherwise be detected in either LC-MS or HPLC-FLD.

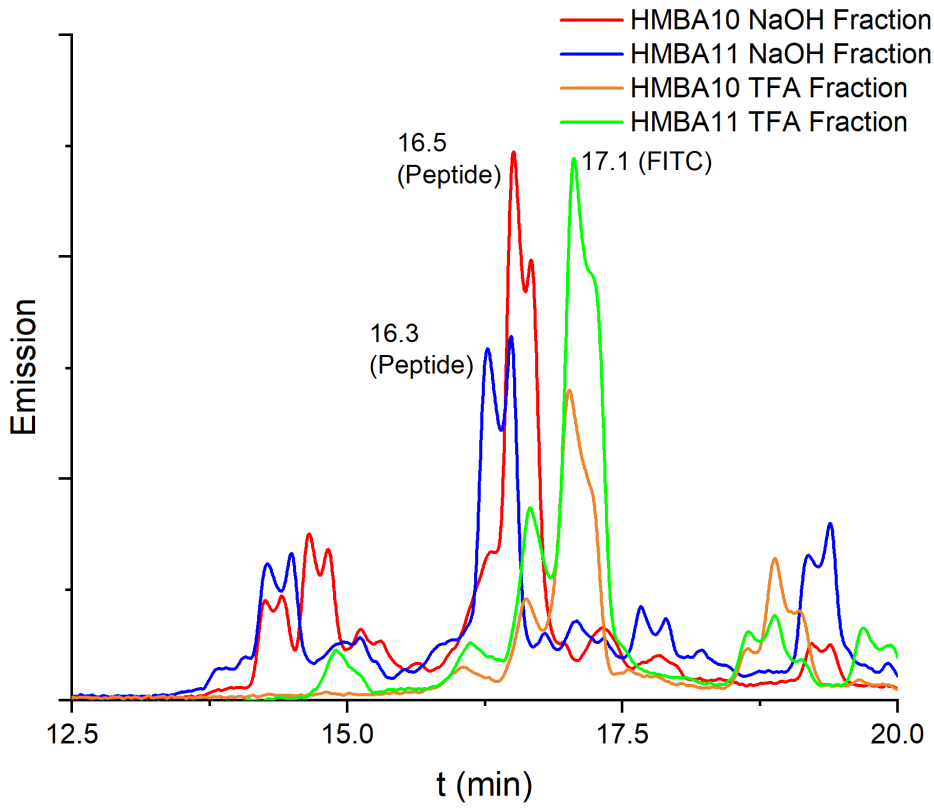


Figure 32 LC-FLD of deprotected FITC-labelled peptides (HMBA-10 – 95TFA, HMBA-11 – 75TFA)

Following coupling of the test peptide on the HMBA-linker, NBD was coupled to the HMBA-coupled peptides for comparison to FITC. 2 mg beads from HMBA-8 were taken for the labelling reaction (HMBA-12), using the conditions previously used for HMPB-linked beads. Following cleavage of the peptide, the 100mM NaOH cleavage solution had a strong yellow tint, indicating the presence of NBD. Neutralisation with 100 mM aq. HCl did not cause precipitation or loss of colour in solution. However,

LC-MS analysis detected only the unlabelled peptide (Figure 33). As previous NBD coupling had been partially successful, this result was unexpected. It is possible that the NBD fluorophore was base-labile and cleaved from the peptide by the cleavage solution, however due to time constraints, the hypothesis could not be confirmed.

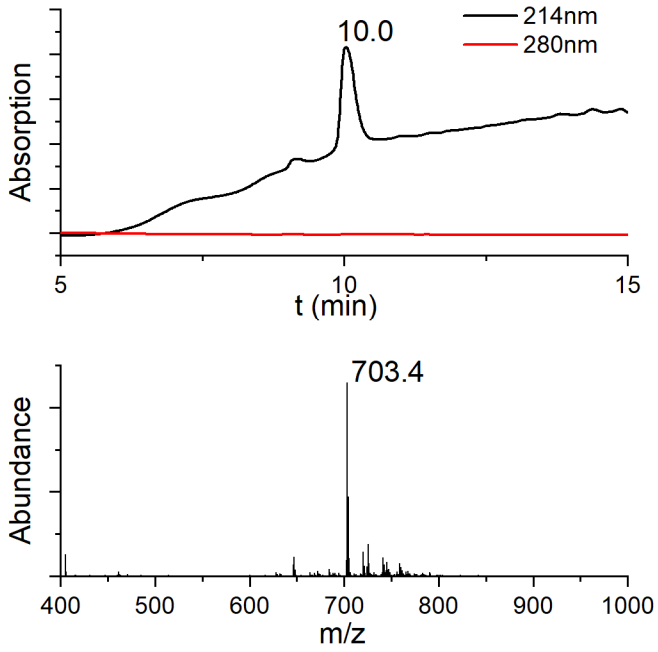


Figure 33 LC-MS on C4 column of HMBA-12 (NBD-labelling of GNASG on HMBA linker, followed by NaOH cleavage), absorption at 214+280nm, bottom MS-spectrum of peak

Synthesis yields

Precise determination of loading rates and synthesis yields require the dry mass of the solid phase. It was however not possible to directly measure the mass of beads used for each synthesis, as drying the magnetic beads resulted in irreversible agglomeration. Bead losses during manual synthesis could also be observed as brown discolouration of the liquid phase, discarded and replaced between synthesis steps, while the magnetic beads were agglomerated using a strong magnet. As such, only the initial bead density could be used for estimating yields.

Peptide yields were determined using Fmoc absorption at 301 nm. The initial loading rate for the first residue was around 160 µmol/g for HMPB. A similar loading rate was obtained for HMBA. With 250 µmol/g H_2N-density of the beads given by manufacturer, this equalled a ~60% loading rate. Further elongation had an efficiency between 80-90% (Figure 34). The lower efficiency compared to SPPS on PEG resin is likely attributable to bead loss, agglomeration and other factors. An automated synthesizer is expected to have a significantly higher step efficiency.

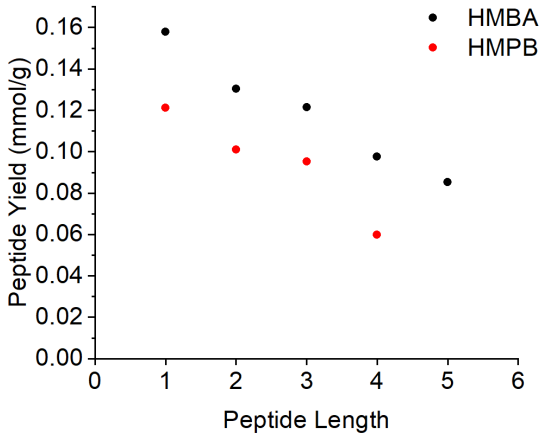


Figure 34 Synthesis efficiency based on Fmoc absorption

An attempt was made to determine the synthesis yield in the proof-of-concept model. Beads were injected into the reaction chamber at the start of synthesis, but most were eluted immediately afterwards, with only a small amount of beads retained in the reaction chamber. Fmoc cleavage solution from the first amino acid was collected and measured, giving around 20 nmol peptides in the reaction chamber. Assuming similar loading rates to manual synthesis, a rough estimate of 0.1-0.2 mg beads in the reaction chamber was obtained. Coupling efficiency of the following steps could not be determined, as contamination in the model prevented accurate Fmoc measurement. The most likely reason was that other reagents such as piperidine were not completely removed during washing steps, due to the large amount of dead volume in the system. Given the previously mentioned limitations of the proof-of-concept model, no further effort was dedicated to resolving the issue.

The above yields give a rough yield of at least 1-10 nmol peptide per synthesis spot in the synthesizer, which is expected to be sufficient for ultra high-throughput screening.

Discussion

Linkers and Fluorescent Markers

Previous tests with magnetic beads for SPPS have used the acid-labile linker HMPA, useful for simultaneous deprotection and cleavage with conc. TFA. HMPB was tested as an alternative acid-labile linker, with the advantage of being cleavable by weak acid 1%TFA, retaining the side chain protecting groups. The results of the practical work confirmed the viability of HMPA as well as the viability of HMPB for microfluidic SPPS on magnetic beads. However, the base-labile linker HMBA explored in this work, appears to be even more suited for the synthesis conditions required here. As the synthesized peptides are intended for use in assays immediately without further processing (purification), organic solvents or TFA (required for cleaving HMPA and HMPB) is problematic for biological assays with sensitive biomolecules or cells. In contrast, the basic aqueous cleavage solution for HMBA-linked peptides can be easily neutralised.

Of the two fluorescent markers used, NBD was initially more promising, with a strong fluorescence signal from NBD-labelled peptides cleaved from HMPB/HMPA. Unfortunately, NBD coupling was not efficient, with <50% of the fluorophore successfully coupled. Increases in reaction time and reagent concentration did not significantly affect the yield. The experiment was repeated with HMBA linker. Following cleavage with 0.1 M NaOH, a yellow colouration of the cleavage solution was observable. Only the unlabelled peptide could be detected using LC/MS, with no NBD-labelled peptide found. It is possible the NBD-label was unstable in 0.1 M NaOH, but due to time constraints this hypothesis was not confirmed. As such, NBD was not feasible for HMBA Fmoc-synthesis, and only of limited use with HMPA/HMPB linkers on magnetic beads.

FITC yielded overall satisfactory results. The primary challenge with FITC-labelled peptides was solubility, as FITC was poorly soluble in H_2O, and solubility of FITC-labelled peptides was significantly poorer compared to unlabelled peptides. The test peptide, GNASG, was by itself highly soluble in aqueous solutions, being short and possessing two hydrophilic side chains (serine and arginine) and only one hydrophobic side chain (alanine). This led to apparently poor yields for FITC-labelled peptides in initial experiments with HMPB and HMPA linkers. The TFA cleavage solutions were lyophilised, after which the dried peptide was supposed to be resuspended in 1:1 ACN/H_2O mixtures. The peptide remained undissolved, resulting in poor signal strength on LC/MS. Eventually, it was found that the labelled peptides could be dissolved in 70/30 ACN/H_2O, allowing for a strong signal with LC/MS or HPLC/FLD. When using the HMBA linker, the peptide was successfully dissolved in the 0.1 M NaOH cleavage solution. However, neutralising directly with 0,1M HCl resulted in precipitation of the peptide, requiring the addition of 0,1% TFA 50/50 ACN/H_2O to solvate the peptide for analysis. In the final synthesizer, ACN would be undesirable for peptide assays, requiring a different buffer capable of suspending FITC-labelled peptides.

Limits of manual synthesis

Due to limitations of manual synthesis, most reagents were still used in large excess. Reduction in reaction volume can be accomplished with automation of synthesis steps, ideally with volumes in the µl-scale. There might be a trade-off between reagent concentration and reaction speed, but far less material will be used for each synthesis when fully transitioning to microfluidic scales.

During the course of the practical work, the proof-of-concept model provided by ER was used to validate synthesis under microfluidic conditions. Short peptides could be successfully synthesized, although yield and purity fluctuated wildly. Limitations of the model included dead volumes, difficulty standardizing reaction times, flow rates and pressure, and contamination from previous runs. While

still useful as a proof-of-concept, there is likely little use for the model for further development. Manual injection with the syringes is too imprecise for optimising microfluidic synthesis conditions.

Impact of Separation Fluid of Synthesiser

The Novec oil to be used as separation fluid in the synthesizer is expected to be compatible with peptide synthesis. However, TFA used to deprotect peptide side groups is fully miscible with the separation fluid. With the use of HMBA as peptide linker, this would not pose an issue for peptide cleavage from beads, as simultaneous TFA deprotection of all synthesis spots is still possible. However, deprotection of side protecting groups in individual synthesis spots with 95%TFA is likely to be more problematic, due to diffusion and mixing of TFA with the separation fluid. A more thorough washing of the system will be needed as well to avoid TFA contamination after cleavage as well.

ER Synthesizer Prototype

Based on the previous proof-of-concept model, Efficient Robotics had developed a prototype synthesizer. It was designed with 10 parallel synthesis spots with the ability to couple amino acids. The goal was to test the final peptide cleavage step in the new synthesizer.

During the trip to Efficient Robotics, beads with HMPB-linked fluorescent-labelled peptides were intended to be loaded into the prototype synthesizer for peptide cleavage with dilute TFA/DCM. Unfortunately, several issues arose with the prototype synthesizer during testing. Most important were issues with bead sedimentation, chemical stability of synthesizer materials and fluorescence detection of dilute TFA/DCM. The majority of the issues could be at least partially resolved, and highlighted the need for a base-labile linker. Thus, after returning from ER, focus shifted away from HMPB towards the base-labile HMBA as linker.

A material stability test had been carried out in 2015. Silicon and epoxide were tested for stability in DMF, amine coupling solution (0.5M HBTU, DIEA in DMF), Fmoc cleavage solution and TFA. DCM was not tested. The ER-visit made it clear that the tests were insufficient, as problems arose with the organic solvents DMF and DCM, as well as dilute TFA/DCM. While using HMBA removes the need for DCM, more rigorous testing of chemical stability of materials will likely be needed to avoid a similar problem reoccurring.

The initial fluorescence detection module in the prototype synthesizer was not chemically stable under dilute TFA/DCM. A backup detection module was used, but was unable to detect a fluorescence signal from FITC in TFA/DCM. Measurement of peptide cleavage in the microfluidic synthesizer could not be performed. Multiple factors are suspected to have played a role: Fluorescein is known to lose fluorescence in acidic solutions. Using a fluorescence spectrometer, a weak fluorescence signal could be detected for FITC in TFA/DCM. The backup detection module was repurposed from a different device, and had a lower sensitivity than planned. In addition, fluorescence excitation and detection were performed with LED lamps (with narrow emission range) and filters with fixed wavelengths. The combination of a 470 nm LED lamp with 475/35 (475 nm with 43 nm bandwidth) excitation and 530/43 emission filters was suitable for fluorescein in aqueous buffer. None of the available lamps or filters were optimal for the FITC in TFA/DCM. It is possible that with a chemically resistant detection module and optimised wavelengths, FITC in TFA/DCM can still be detected. It would otherwise be more straightforward to use an aqueous, basic cleavage solution.

Bead sedimentation was an additional problem. When left stationary in the microfluidic channels, the beads quickly settle on the channel surface. Over time, this build-up hampers the flow in the system until the channel becomes fully clogged. This was exacerbated by the insolubility of the beads in oil, with the formation of large aggregates should the oil and beads come into direct contact. The issue

was resolved by extensive washing of channels with DMF preceding and following bead injection, to prevent oil and bead contact and remove leftover beads present in the system.

Sedimentation of beads in the reservoir over time would change the bead density pumped through the synthesizer and affect spot loading. Automated stirring of the bead suspension would be needed for the synthesizer. A large portion of the beads were lost while filling the synthesis spots, due to technical limitations of the prototype. In the final design, bead solutions would be delivered as droplets to the synthesis spots, minimising waste. Eventually, it may be possible to collect excess beads that were not fixed in the synthesis spots to be reused, but the bead density of the recollected beads would need to be determined, possibly with measurement of Fmoc-absorption to determine the new loading rate.

Bead loading in spots remained highly variable. In part, this was due to the intended fluorescence detection module not working as intended. Quantification of bead loading in spots therefore had to be done via visual measurement of magnetic beads caught in an improvised magnetic trap. While imprecise, it was still sufficient to determine that bead loading, and therefore the peptide yield of the synthesis spots fluctuated wildly based on flow rate and spot position. Flow rate was not set at a constant in the prototype synthesizer. An overpressure was set at inlet reservoirs to push the liquid through the system to various outlets. Due to the bead sedimentation mentioned above, as well as restructuring of the synthesizer to resolve blockage issues and different viscosities of the fluids used, the flow rate was highly unstable in the prototype.

DMF and DCM worked well with the separation oil currently in use for the synthesizer. There was no miscibility, and both solvents were rapidly displaced by the oil. Should HMBA be used as the standard linker for further development, DCM should not be a major problem for chemical stability of the synthesizer. While TFA is miscible with the separation fluid, this is not expected to hinder synthesis with HMBA linkers, as it will only be used for side group deprotection at the end of synthesis. TFA diffusion into separation oil is thus unlikely to lead to crosstalk and lower yields, provided the TFA is properly removed from the system following side group deprotection.

The most significant conclusion from the tests at Efficient Robotics was the necessity for more rigorous chemical stability testing. Chemical stability tests with materials and reagents were carried out in 2016 and were insufficient in scope. Valves and fluorescence detection module were degraded upon exposure to untested chemical solvents, impeding synthesizer testing. For further iterations of the synthesizer, materials and components should be tested for chemical stability against possible reagents before use in the synthesizer.

Conclusion

During the course of the practical work, three different linkers HMBA, HMPB and HMPA were used to link test peptides to the magnetic beads. HMBA has the most potential of the three, as its TFA stability and base lability allows side group deprotection with conc. TFA followed by peptide cleavage in 100 mM aqueous NaOH. The basic peptide solution can then be easily neutralised and buffered, allowing for easy use in biological assays, where organic solvents may be unfeasible.

Of the two fluorescent markers FITC and NBD used in this thesis, NBD coupling was highly inefficient. In addition, the NBD marker appears to be unstable in basic solutions, preventing use with HMBA linkers. FITC was much more promising, with short reaction times and good signal strength. The main difficulty with FITC is poor solubility in H_2O, including neutral NaCl solutions. An aqueous buffer will need to be determined in future studies to improve FITC solubility for proper quantification and to avoid peptide sedimentation in the system.

Following manual synthesis of fluorescent-labelled test peptides, the proof-of-concept model provided by ER was used to validate microfluidic synthesis. Small quantities of peptide with fluorescent markers could be successfully synthesized. Limitations of the model included dead volumes, manually controlled flow rates and pressure, and low purity of peptide products. Once a system with automated injection is available, validating methods with the proof-of-concept model should be unnecessary.

Testing of the prototype from Efficient Robotics revealed several issues, particularly pertaining to chemical stability of the microfluidic components and fluorescence sensitivity in the 1%TFA cleavage solution. These issues often also severely impacted flow stability of the prototype, preventing precise flow control and unpredictably interfering with experiments. ER is working to resolve the discovered technical issues of the design, while switching from acid-labile linkers to the base-sensitive HMBA linker will resolve the detection issues and alleviate several material concerns.

The next step in the development process would be the development of a synthesizer prototype with chemically stable materials and flow stability. Microfluidic synthesis with HMBA linkers can then be tested using the new synthesizer prototype. There remains some possible progress in manual synthesis, for instance validating new linkers and fluorescent markers, but there should be no further hurdles to moving on to automated synthesis instead.

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